

BIODEGRADABLE MAGNETO- LUMINESCENT MESOPOROUS NANOPARTICLES AS NEW NANOBIOMEDICAL TOOLS IN CANCER TREATMENT

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Biodegradable Magneto-Luminescent Mesoporous Nanoparticles As New Nanobiomedical Tools In Cancer Treatment

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To those that are,

and believed.

To those that have gone,

but never stopped believing.

With all my heart.

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for those that are and believed, and for those that are gone but never stopped believing.

"Nothing whets the intelligence more than a passionate suspicion, nothing develops all the faculties of an immature mind more than a trail running away into the dark." (Stefan Zweig).

ABSTRACT

Colorectal cancer (CRC) is the 3rd most common type of cancer worldwide and 2nd in mortality rate, accounting to 1.93 million cases and 916 000 deaths just in 2020. The development of CRC targeted therapies has been a hot topic in recent medical forums and among researchers, with the demand for non-invasive approaches, like oral ones, capable of preserving their therapeutic properties from administration to local action drastically increasing in the last decade. Its body location makes CRC a usually silent disease, whose commonly later detection and action usually lead to severe aggravations and even death. Targetability, drugs' resistance and toxicity of the said therapies are thus three of the key points for the development of effective therapies. On the other hand, the presence of cancer-associated bacterial infections and inflammations has been positively correlated with low treatment success rates and associated complications, with bacterial infections being particularly damaging in gastric and colorectal cancer. Some of the metabolites/toxins of these opportunistic bacteria have been linked to tumorigenesis and CRC drug-resistance. A conjoint therapy capable of addressing both CRC and associated bacteria, where drug preservation, null secondary toxicity and targetability are ensured.

In light of the above-mentioned, herein we cover the synthesis of a new magneto-fluorescent nanotherapy against both CRC and associated bacterial strains, going from the synthesis of sustainable, biocompatible, biodegradable and cheap nanocomposite particles (NANO3) to the testing of its dual drug cargo and delivery capacities, magneto-fluorescent properties and later assessment of its combined antibacterial and anti-tumor activity. The successful synthesis of new anti-CA IX targeting inhibitor as potential directing agent is also covered as a cheaper alternative to the commonly used and expensive antibodies. From an antimicrobial point of view, NANO3 showed significant activity against CRC-related bacterial strains, with their intrinsic magnetic properties potentiating a hyperthermia-assisted pH-

responsive release and antimicrobial therapy. The combined release of an antibiotic (i.e., ofloxacin) and an anti-tumor drug (i.e., doxorubicin) showed no hinderance on the activity of both active drugs. Accordingly, NANO3 anti-tumoral activity, arising solely from the loaded doxorubicin, was conserved and competitive to that of the free model drug. Targetability was conferred by successfully functionalizing of NANO3 with anti-EGFR and and/or anti-CA IX, the later favoring higher NANO3 uptake in HCT-116 cell line. A proof-of-concept oral delivery was also simulated, where a dual Eudragit® enteric coating successfully protected NANO3 formulations from stomachal and small intestine conditions, selectively dispensing it in colon environment and potentiating their use in a future anti-CRC oral therapy.

In this thesis an optimization of a luminescent antimicrobial activity assay specific for nanoparticles was also carried out along with the evaluation of other cyanine-based antimicrobial scaffolds for a potential biomedical and food-based application.

Keywords: CRC, opportunistic bacteria, magneto-fluorescence, biodegradable and biocompatible nanoparticles, sustainable.

RESUMO

O cancro colorrectal (CRC) é o 3º tipo de cancro mais comum a nível mundial e o 2º na taxa de mortalidade, contabilizando 1,93 milhões de casos e 916 000 mortes apenas em 2020. O desenvolvimento de terapias orientadas para CRC tem sido um tema recorrente no meio médico e entre investigadores, tendo a procura de abordagens não invasivas, tais como as orais, capazes de preservar as suas propriedades terapêuticas desde a administração até à acção local, aumentado drasticamente na última década. A sua localização corporal faz do CRC uma doença geralmente silenciosa, cuja detecção e acção tardias conduzem a agravamentos do quadro clínico ou mesmo à morte. A capacidade de carga, a resistência dos medicamentos e a toxicidade das referidas terapias são assim três dos pontos-chave para o desenvolvimento de terapias eficazes. Por outro lado, a presença de infecções e inflamações bacterianas associadas ao cancro têm sido positivamente correlacionadas com baixas taxas de sucesso de tratamento e ao aumento de complicações, com particular impacto prejudicial em situações de cancro gástrico e colorrectal. Alguns dos metabolitos/toxinas destas bactérias oportunistas têm sido ligados à tumorigenese e à resistência aos medicamentos em CRC. Uma terapia conjunta capaz de tratar tanto CRC como as bactérias associadas, onde a preservação do medicamento, a toxicidade secundária nula e a capacidade de carga são asseguradas.

Tendo em conta o acima mencionado, na presente tese detalha-se a síntese de uma nova nanoterapia magneto-fluorescente tanto contra CRC como contra estirpes bacterianas associadas. Indo assim, desde a síntese de partículas nanocompósitas sustentáveis, biocompatíveis, biodegradáveis e baratas (NANO3) até à dupla carga e entrega de medicamentos; bem como da avaliação das suas propriedades magneto-fluorescentes até à sua actividade antibacteriana e antitumoral combinada. A síntese bem-sucedida do novo inibidor de CA IX como potencial agente dirigente é também coberta como uma alternativa

mais barata aos anticorpos normalmente utilizados. De um ponto de vista antimicrobiano, NANO3 mostrou uma actividade significativa contra estirpes bacterianas relacionadas com CRC, com as suas propriedades magnéticas intrínsecas potenciando uma libertação pH-responsiva, assistida por hipertermia, e por fim a uma terapia antimicrobiana. A libertação combinada de um antibiótico (i.e., ofloxacina) e de um medicamento anti-tumoral (i.e., doxorubicina) não levou a qualquer redução da actividade de ambos os medicamentos. Assim, a actividade anti-tumoral de NANO3, proveniente unicamente da doxorubicina carregada, foi conservada e competitiva em relação à do medicamento livre. A capacidade de atuar especificamente sobre células de CRC foi conferida pela funcionalização das NANO3 com anti-EGFR e / ou anti-CA IX, o último dos quais favorecendo uma maior internalização de NANO3 na linha celular HCT-116. Foi também simulada uma prova de conceito de uma toma oral, onde um revestimento entérico duplo de Eudragit® protegeu com sucesso as formulações de NANO3 das condições estomacais e do intestino delgado, dispensando-o selectivamente em ambiente de cólon e potenciando a sua utilização numa futura terapia oral anti-CRC.

Na presente tese foi também realizada uma optimização de um ensaio de actividade antimicrobiana luminescente específico para nanopartículas, juntamente com a avaliação de outros substratos antimicrobianos à base de cianina para uma potencial aplicação biomédica e alimentar.

Palavas chave: CRC, bactérias oportunistas, magneto-fluorescência, nanopartículas biodegradáveis e biocompatíveis, sustentáveis.

CONTENTS

1	GENERAL INTRODUCTION - MAGNETIC, FLUORESCENT AND HYBRID NANOPARTICLES: FROM SYNTHESIS TO APPLICATION IN BIOSYSTEMS	1
1.1	Introduction.....	5
1.1.1	Quantum Dots.....	6
1.1.2	Superparamagnetic iron oxide nanoparticles	8
1.2	SPION nanoparticles: synthesis and applications	10
1.2.1	Physical routes	10
1.2.2	Chemical Routes.....	13
1.2.3	Biological Routes.....	22
1.2.4	SPIONs surface modifications and applications.....	23
1.3	SiQD nanoparticles: synthesis and applications	35
1.3.1	Top-down synthesis.....	38
1.3.2	Bottom-up approaches.....	42
1.3.3	Surface modifications, optical properties and applications.....	46
1.4	Magneto-fluorescent hybrids.....	57
1.5	Colorectal Cancer and Associated Opportunistic Microbiota.....	62
1.5.1	Colorectal Cancer	62
1.5.2	Existing chemo and targeted therapies.....	65
1.5.3	Opportunistic microbiota and infections.....	70
1.6	Conclusions and Future perspectives	74

1.7	Acknowledgments	75
1.8	References.....	75
2	OBJECTIVES	95
3	LUMINESCENT SILICON-BASED NANOCARRIER FOR DRUG DELIVERY IN COLORECTAL CANCER CELLS	97
3.1	Introduction.....	101
3.2	Experimental	102
3.2.1	Chemicals and starting materials.....	102
3.2.2	Synthetic procedures.....	103
3.2.3	Nanoparticles characterization	103
3.2.4	Doxorubicin loading and in vitro release studies	104
3.2.5	Cell culture.....	105
3.2.6	Cytotoxicity and intracellular accumulation of the nanoparticles	105
3.2.7	CLSM <i>in vitro</i> imaging of Nanoparticles-Treated cells	105
3.3	Results and Discussion.....	106
3.3.1	Synthesis and characterization	106
3.3.2	Doxorubicin loading/release.....	109
3.3.3	Dose-dependent of cytotoxicity of Nanoparticles	111
3.3.4	Cellular uptake of Doxorubicin-loaded Nanoparticles	112
3.4	Conclusions	114
3.5	Acknowledgments	115
3.6	References.....	115
4	A PROTEOMIC ANALYSIS OF COLORECTAL CANCER CELLS TREATED WITH A LUMINESCENT SILICON-BASED NANOCARRIER (<i>CHAPTER 3 CONTINUATION</i>).....	121
4.1	Introduction.....	123
4.2	Experimental Section	125
4.2.1	Materials and Instrumentation.....	125
4.2.1	Nanoparticles.....	126

4.2.2	Cell Culture and Pellets.....	126
4.2.3	Sample Treatment.....	127
4.2.4	Nano-LC-MS/MS.....	128
4.3	Results and Discussion.....	130
4.4	Conclusions	135
4.5	Acknowledgments	135
4.6	References.....	135
4.7	Supplementary Material	139
5	GOLD@MESOPOROUS SILICA NANOCARRIERS FOR THE EFFECTIVE DELIVERY OF ANTIBIOTICS AND BY-PASSING OF B-LACTAM RESISTANCE	141
5.1	Introduction.....	145
5.2	Materials and Methods.....	146
5.2.1	Instrumentation	147
5.2.2	Synthesis of gold-silica mesoporous nanoparticles (Au@MNs).....	148
5.2.3	Loading and Release trials.....	148
5.2.4	Antibacterial activity assays.....	149
5.3	Results and discussion	151
5.3.1	Synthesis of Gold @ mesoporous silica Nanoparticles and Characterisation ..	151
5.3.2	Antibiotics Loading and Release studies.....	155
5.3.3	Antibacterial activity assays.....	158
5.4	Conclusions	164
5.5	Aknowledgments	165
5.6	References.....	165
5.7	Supplementary Material	172
6	MAGNETO-FLUORESCENT MESOPOROUS NANOCARRIERS TO TACKLE OPPORTUNISTIC BACTERIAL INFECTIONS IN COLORECTAL CANCER	175
6.1	Introduction.....	179

6.2	Experimental Section	180
6.2.1	Materials.....	180
6.2.2	Instrumentation	181
6.2.3	Superparamagnetic iron oxide nanoparticles synthesis – NANO1	182
6.2.4	Mesoporous silica shell surface growth and surface modifications – NANO2.....	183
6.2.5	Silicon quantum dots (SiQDs) synthesis.....	183
6.2.6	Magneto-fluorescent nanoconjugates - NANO3	183
6.2.7	Loading and Release studies of anticancer-antimicrobial drugs.....	184
6.2.8	Magnetic characterization and hyperthermia studies.....	185
6.2.9	Antimicrobial activity assessment.....	185
6.3	Results and Discussion.....	186
6.3.1	Characterization of NANO2 and NANO3 systems	186
6.3.2	Loading, pH-mediated release and hyperthermia release assays.....	193
6.3.3	Antimicrobial activity	198
6.4	Conclusions	200
6.5	Acknowledgments	201
6.6	References.....	201
6.7	Supplementary Material	207
6.7.1	Tables.....	207
6.7.2	Figures.....	208
7	VALIDATION OF A STANDARD LUMINESCENCE METHOD FOR THE FAST DETERMINATION OF THE ANTIMICROBIAL ACTIVITY OF NANOPARTICLES IN <i>ESCHERICHIA COLI</i>	213
7.1	Introduction.....	217
7.2	Materials nad Methods	219
7.2.1	Synthesis of nanoparticles	221
7.2.2	Confirmation Assays	223
7.3	Results and Discussion.....	225

7.3.1	Bacterial transformation and validation	225
7.3.2	<i>E.coli</i> Lux susceptibility to ofloxacin.....	227
7.3.3	Nanoformulations synthesis	231
7.3.4	Nanoparticles antimicrobial activity against <i>E.coli</i> and <i>E.coli</i> Lux.....	234
7.4	Conclusions	241
7.5	Funding	242
7.6	Acknowledgements.....	242
7.7	References.....	243
7.8	Supplementary Materials	247
7.8.1	Experimental Procedures	247
7.8.2	Tables.....	249
7.8.3	Figures.....	252
8	A MAGNETO FLUORESCENT BIOCOMPATIBLE, COMBINATORY AND DUAL TARGETING NANOTHERAPY AGAINST COLORECTAL CANCER.....	259
8.1	Introduction.....	263
8.2	Materials and Methods	265
8.2.1	5-amino-1,3,4-thiadiazole-2-sulfonamide (AZM-NH ₂) synthesis	265
8.2.2	3-sulfanylpropanoyl chloride synthesis	266
8.2.3	N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)-3-sulfanylpropanamide (AZM-SH) synthesis.....	266
8.2.4	Modelling studies and molecular docking simulations.....	267
8.2.5	CA <i>in vitro</i> inhibition assay.....	268
8.2.6	Synthesis of NANO3-SH.....	268
8.2.7	NANO3 biofunctionalization with CTX-SH and AZM-SH	269
8.2.8	Drug loading and release assays.....	270
8.2.9	Enteric Coatings & Simulated Digestive Track Release	271
8.2.10	CA IX and EGFR expression in CRC cell lines	271

8.2.11	Cytotoxicity and Cellular Uptake.....	272
8.3	Results and Discussion.....	272
8.3.1	Synthesis of AZM-SH and anti-CAIX action.....	272
8.3.2	NANO3-SH characterization.....	278
8.3.3	Loading and Release assays.....	281
8.3.4	<i>Proof-of concept</i> : Enteric Coatings & Gastric Simulated Release	283
8.3.5	Biofunctionalization	286
8.3.6	Cytotoxicity and antitumor activity	287
8.3.7	Cellular uptake and targetability.....	289
8.4	Conclusions	291
8.5	Acknowledgments	292
8.6	References.....	292
8.7	Supplementary Materials	299
8.7.1	Materials and Methods.....	299
8.7.2	Tables.....	305
8.7.3	Figures.....	306
8.7.4	Bioscope Research Team Protocol.....	314
9	FUNCTIONAL CYANINE-BASED PVA:PVP POLYMERS AS ANTIMICROBIAL TOOLS TOWARDS FOOD AND HEALTH-CARE BACTERIAL INFECTIONS.....	317
9.1	Introduction.....	321
9.2	Results and Discussion.....	323
9.2.1	Synthesis.....	323
9.2.2	Photophysical characterization and sensorial ability to metal ions of dyes 1 to 3 324	
9.2.3	PVA:PVP and PVA:PVP:paper mockups supports for dyes 1 to 3 and Cu ²⁺ complexes	326
9.2.4	Antibacterial Assays	329

9.3	Conclusion	339
9.4	Experimental Section	340
9.5	Acknowledgements.....	342
9.6	References.....	343
9.7	Supporting Information.....	345
9.7.1	Figures.....	345
10	GENERAL CONCLUSIONS AND PERSPECTIVES	353

LIST OF FIGURES

Figure 1.1 - (A) Spectral range of emission for the most widely studied types of semiconductor nanocrystals. Reprinted with permission from [36]. Copyright 2016 American Chemical Society. (B) Normalized P of CdSe QDs under single wavelength excitation achieved by selectively varying the QDs size. (C) Pseudo colored image of human epithelial cells by staining with five different sizes of QDs. Actin filaments —Red (705 nm), Nucleus— Cyan (655 nm), Ki67 protein—Magenta (605 nm), Microtubules— Green (565 nm), Mitochondria— Orange (525 nm). Adapted with permission from [45]. Copyright 2015 Elsevier B.V.....	6
Figure 1.2 - Already available and studied Cd-free QDs and their representative applications in biophotonics and nanomedicine. Adapted with permission from [59]. Copyright 2016 American Chemical Society.	8
Figure 1.3 - Illustration of the nano-architecture of a therapeutic SPION, with specific surface coating and related therapies.....	9
Figure 1.4 - Comparison of published work on the synthesis of SPIONs by three main different routes.....	10
Figure 1.5 - The overall scheme for the ultra-large-scale synthesis of monodisperse nanocrystals: from metal–oleate precursors to their thermal decomposition in high boiling solvent to form monodisperse nanocrystals. Adapted with permission from [128]. Copyright 2004 Springer Nature.....	18
Figure 1.6 - Schematic illustration of the two w/o microemulsion synthetic approaches of SPIONs.....	20
Figure 1.7 - Schematic depicting SPIONs' available surface modification, numerous target receptors and respective feedbacks.....	24
Figure 1.8 - top: (A) TEM image of monodisperse superparamagnetic single-crystal magnetite NPs with mesoporous structure; bottom: respective saturation magnetization curve. Adapted	

and reproduced with permission from [172]. Copyright 2008 Elsevier B.V.. (B) TEM micrographs of core-shell SPIONs of 50 nm; bottom: field-dependent magnetization saturations of aqueous suspensions of Fe_3O_4 -CTAB and $\text{Fe}_3\text{O}_4@\text{mSiO}_2$ NPs. Adapted and reproduced with permission from [70]. Copyright 2012 WILEY-VCH Verlag GmbH & Co. KGaA. (C) top: TEM image of M-MSN samples synthesized with a TMB/CATB molar ratio of zero; bottom: Field-dependent magnetization curves at room temperature of M-MSN samples with varied pore swelling agent/CTAB ratios. Adapted and reproduced with permission from [173]. Copyright 2011 Elsevier B.V.. (D) top: TEM images of Fe_3O_4 NPs with sizes of 10.1 nm (scale bar = 20 nm); bottom: field-dependent magnetization to different shell thickness NPs. Adapted and reproduced with permission from [164]. Copyright 2012 American Chemical Society.27

Figure 1.9 - (A) Schematic illustration of the proposed mechanisms for the growth and pore modulation processes of silica/CTAB mesophase on SPIONs surface, in the presence of different pore swelling agents (TMB and decane). (B) La Mer-like diagram: hydrolyzed TEOS (monomers) concentration against time, on homogeneous nucleation and heterogeneous nucleation, and its role on the existence of $\text{Fe}_3\text{O}_4@\text{SiO}_2$ core/shell and core-free SiO_2 in the reaction at any moment. Adapted with permission from [164]. (C) Illustration of a o/w coating mechanism of SiO_2 on the surface of already synthesized SPIONs. Adapted with permission from [164]. Copyrights 2012 American Chemical Society.....28

Figure 1.10 - Illustration of ultra-large mesoporous silica@iron oxide nanovector for imaging (MRI), high protein loading, magnetothermal release and delivery in cancer cells (left). Detailed schematics on protein (mTFP-Ferritin) loading onto nanovectors' pores (right-a); and delivery function and mechanism of $\text{SiO}_2\text{-Fe}_2\text{O}_3$ mTFP-Ferritin nanovectors (right-b). Reproduced from [17]. Copyright 2016 Elsevier B.V.....29

Figure 1.11 - (A) Schematic depicting the binding of polyurethane carboxylic acid moieties onto SPIONs surface, with steric stabilization of the particles in the cell culture medium by means of PEO tail blocks (*left*); confirmed by microscopy images of the uptake of SPION-PEO-PU-PEO (2K-3-2K, 30%, 5 mg/mL) by C4-2 prostate cancer cells after 8h of incubation (*right*). Adapted and reprinted with permission from [75]. Copyright 2009 American Chemical Society. (B) TEM images of the hydrophobic inorganic core of SPIONs, with diameter of c.a. 27 ± 5 nm (*left*); and of antibody conjugated biocompatible water-soluble SPION (SPION-Ab), using phosphotungstic acid for background staining (*right*). Reproduced from [174]. Copyright 2011 Elsevier Ltd. (C) Schematic representation of pluronic F127-caped SPION formulation and drug loading. Reprinted with permission from [109]. Copyright 2005

American Chemical Society. (D) Structural characterization of three different self-assembly structures: STEM images (*top*) and illustrations (*bottom*) of magneto-core shell assemblies formed in (*from left to right*) DMF/THF (96.8% DMF), THF, dioxane/THF (96.8% dioxane), and (dioxane/THF (96.8% dioxane). The assemblies were prepared with PAA38-b-PS189 at the polymer concentration of 0.04 wt % and a nanoparticle mass percent of 27.1%. Adapted and reprinted with permission from [175]. Copyright 2011 American Chemical Society.....31

Figure 1.12 - (A) Schematics for the preparation of SPION, gold-deposited SPIONs, and gold-deposited SPION/glycol chitosan NPs with Heparin (composite NPs). (B) In vivo non-invasive NIR images and quantification analysis of Cy5.5/composite NPs with or without heparin. Adapted and reprinted with permission from [169]. Copyright 2011, American Chemical Society.33

Figure 1.13 - Schematic representation of core-shell nanoparticles with polymeric gaps: (A.a) Key steps involved in the synthesis of silver ring-coated SPIONs with a polymeric gap; (A.b) key steps involved in the synthesis of gold ring-coated SPIONs with a polymeric gap as the intermediate stage; and (A.c) surface treatment of gold core-shell nanoparticles with disulfide ethanedylbis(isonicotinate) followed by addition of silver ions. TEM panels are representative images showing the formation of monodispersed nanoparticles in various stages (scale bars 40 nm). Dual toxicology effect of multifunctional nanoparticles in bacteria and human cells: (B) Percentage of dead bacteria in 24 h staphylococcal biofilms, with and without external magnetic field, of *S. aureus* and *S. epidermidis* in the absence and presence of various particles. Experiments with kanamycin were carried out on bacteria for confirmation of strong antibacterial effects of multifunctional particles. (C) Toxicity evaluation of nanoparticles interacted with HepG2 cell lines for a period of 24 h. (D,E) TEM micrographs of HepG2 cells after 24 h of growth in tissue culture polystyrene wells in the presence of silver and silver-SPION core-shell particles (with the exactly the same concentration of nanoparticles). The electron microscopy images confirmed the highly toxic effects of silver nanoparticles on the cells, while there was no sign of toxicity for silver-SPION core-shell particles. Adapted and reprinted with permission from [183]. Copyright 2012, American Chemical Society.....34

Figure 1.14 - Summarizing illustration of SPIONs main fields of application and respective applied techniques.....35

Figure 1.15 - Formation of alkyl-passivated silicon quantum dots obtained by thermal decomposition of hydrogen silesquioxane, followed by etching and later surface modification by hydrosilylation39

Figure 1.16 - (A) 'Two-ring' configuration used for the synthesis of SiQDs: (*left*) Schematics of a microplasma reactor; (*center*) photo of the microplasma generated inside the quartz capillary; (*right*) TEM image of microplasma generated SiQDs. Reproduced with permission from [199]. Copyright 2014 AIP Publishing. (B) Schematics of the experimental setup for the synthesis of Si-NPs in a microwave plasma reactor. Reprinted with permission from [201]. Copyright 2009 WILEY-VCH Verlag GmbH & Co. KGaA.....41

Figure 1.17 - (A) Relationship between current density, catalyst and final products (*left*); SEM, TEM and HRTEM images of SiQDs/SiNPs and typical PL of different sized SiQDs (*right*). Adapted with permission from [204]. Copyright 2007 American Chemical Society. (B) H-SiQDs oxidation in EtOH/H₂O₂ and final emission color of oxidized SiQDs, ranging from salmon pink to blue. Adapted with permission from [190]. Copyright 2009 WILEY-VCH Verlag GmbH & Co. KGaA.....42

Figure 1.18 - Solution-phase reduction-based approaches for the synthesis of H-terminated SiQDs. Adapted with permission from [37]. Copyright 2009 WILEY-VCH Verlag GmbH & Co. KGaA.....43

Figure 1.19 - Summarizing schematics of zintl salt-mediated SiQD syntheses.....44

Figure 1.20 - Simplified schematic illustration of bottom-up one-pot microwave-assisted synthesis of SiQDs: from precursor reaction to nuclei formation and crystal growth (*left*). TEM / HRTEM images of as-prepared SiQDs (*right top*). Photostability comparison of FITC, CdTe QDs, CdSe/ZnS QDs, and SiNPs, and respective photographs under UV for time = 0 min (*right bottom*). Adapted and reprinted with permission from [58]. Copyright 2013 American Chemical Society.....45

Figure 1.21 - Schematic illustration of the formation of SiNPs using APTMS, DAMO, and AEEA molecules (top). Photographs of APTMS, DAMO, and AEEA SiQDs solutions under white light and UV (365 nm) irradiation (1 mg.mL⁻¹ at pH = 7.4), with respective QY; possible application as fluorescent inks (bottom). Adapted with permission from [57]. Copyright 2015, WILEY-VCH Verlag GmbH & Co. KGaA.....46

Figure 1.22 - (A) Schematic representation of surface passivation of H-SiQDs with N-containing species, under O₂ atmosphere, and consequent PL blueshift. Sequential photographs, under UV light, of H-SiQDs reacting with (B) TOAB and (C) NH₄Br. Adapted and reprinted with permission from [187]. Copyright 2013 American Chemical Society. (D) Illustration of halogenation and alkylation of H-SiQDs and emission spectra of hexyl functionalised SiQDs derived from Cl, I and Br modified surfaces ($\lambda_{\text{ex}} = 325 \text{ nm}$). Adapted and reprinted with permission from [214]. Copyright 2015 American Chemical Society.

Summarising table of all surface modifications of H-SiQDs surfaces, and corresponding optical properties; τ_1 or τ_2 – excite-state lifetimes. Adapted with permission from [186]. Copyright 2014 American Chemical Society.....47

Figure 1.23 - (A) Illustration of the overall synthetic procedure and SiQDs surface functionalisation with corresponding optical properties changes, under white and UV light. MTT reduction assay (B) and NR incorporation (C) after 2 and 24 h incubations of doxorubicin-loaded SiQDs@Isoc@MNs (50 $\mu\text{g/mL}$ –150 $\mu\text{g/mL}$) for HepG2 cell lines. Adapted with permission from [211].....50

Figure 1.24 - (A) Illustration of the one-pot synthesis of alkene-coated SiQDs and functionalization via thiol–ene click chemistry. (B) PL spectra of SiQDs with different surface groups (a–e) excited at 320 nm and (C) naked-eye fluorescence and hydrophobicity in hexene:water mixtures. Images were taken under UV light @ 365 nm. Reproduced with permission from [66] Copyright 2012 Royal Society of Chemistry. (D) Dispersity of SiQDs in polar and nonpolar solvents according to the different surface groups (a–d). (E) Fluorescence microscopy images depicting the presence of amine-functionalised SiQDs in HeLa cells under constant excitation at 405 nm. Scale bar = 10 μm . Upper panel: control group with no SiQDs added; bottom panel: cells incubated with SiQDs. Reprinted with permission from [43]. Copyright 2014 American Chemical Society.....51

Figure 1.25 - Laser scanning confocal microscopy photos of immunofluorescent cell imaging. (a) Left: microtubules of Hela cells are distinctively labeled by the SiQDs/protein bioconjugates. Middle: bright-field image. Right: superposition of fluorescence and transillumination images. (b–d) Stability comparison of fluorescence signals of Hela cells labeled by SiQDs (b), CdTe QDs (c), and FITC (d). Scale bar = 5 μm . Reprinted with permission from [68]. Copyright 2011 American Chemical Society.....52

Figure 1.26 - (1) Schematic of surface hydrosilylation of SiQDs, with R1 compounds, followed by micellar encapsulation commercially available on the phospholipids terminated with R2 functional groups. Reprinted with permission from [27]. Copyright 2008 American Chemical Society. (2) Ex vivo images (U,W) and luminescence images (V,X) of tumors harvested at 40 h post injection from mice treated with (U,V) MSiQD-cRGD or (W,X) MSiQD. (3) Illustration of the synthesis and surface functionalization of butenoic acid-capped SiQDs with DSPE-PEG(2000) phospholipids and derivatives. (4) In vivo multiplex NIR imaging of SiQDs subcutaneously injected in lower limbs of a mouse. (5) Sentinel lymph node imaging following localization of Si QDs in an axillary position. (6) Luminescence imaging of BALB/c mice injected with MSiQD. at different time points post injection. Note that autofluorescence

and unmixed SiQD signals are coded in green, red and/or yellow, for the above images. (2-7) were adapted and reprinted with permission from [26]. Copyright 2011 American Chemical Society.54

Figure 1.27 - (A) Synthetic approach for micellar hybrid nanoparticles encapsulating SPION and QDs within a single PEG-modified phospholipid micelle. Reproduced from [7]. Copyright 2008 Wiley-VCH Verlag GmbH & Co. KGaA. (B) Schematic Fe_2O_3 -CdSe MQDs and its silanization by reverse micro-emulsion, with further surface modulation with succinimide-PEG chains and oleyl groups (BAM). (C) TEM images of heterodimers of MQDs after magnetic harvesting (SPION, 8-10 nm; CdSe QD, 4-5 nm). (D) Magnetic and luminescence properties under UV (365 nm) of harvested red MQDs and of dispersed green and orange MQDs. Reprinted with permission from [11]. Copyright 2007 Wiley-VCH Verlag GmbH & Co. KGaA 58

Figure 1.28 - (A) Schematics of the formation of CdSe-CdS core-shell QDs with SPIONS (CS-SPs). (B-D) TEM images of CS-SPs at different magnifications. Scale bars: 500 nm, 100nm and 10nm, respectively. (E) Absorption spectra of CS-SPs (blue), SPIONs (green), QDs (purple) and PL spectra of CS-SPs (red). (F) Dark-field scanning TEM images and EDS elemental mapping of CS-SPs. Scale bars: 80 nm. Reproduced with permission from [226]. Copyright 2014 Springer Nature.....59

Figure 1.29 - (A) Illustration of the multistep sequential synthesis of magneto-fluorescent hybrid structures. (B-E) TEM micrographs: (B) CdSe@CdS fluorescent core, (C) intermediate step with metallic iron shell, (D,E) complete CdSe@CdS@hollow- Fe_2O_3 nanostructure. (F) Absorption spectra of hollow Fe_2O_3 (black), CdSe@CdS QD (red) and CdSe@CdS@hollow Fe_2O_3 (blue) along with the hybrid structure's photoluminescence (PL) spectra (purple). (G) Optical and magnetic features of iron oxide-QD core-shell, under ambient and UV light. (H-K) Microscopy images of a 3D cell culture, 13 days in vitro (DIV) and 6 days after introducing to the CdSe@CdS@ Fe_2O_3 : (H) Bright-field image displaying all cells, (I) QDs red fluorescence image, exclusively localized using a Rhodamine filter and (J,K) merged image and zoom. Adapted and reprinted with permission from [231]. Copyright 2018 American Chemical Society.....60

Figure 1.30 - (A) PL spectra of the acetonitrile-destabilized magnetic-fluorescent nanobeads (MFNBs) where QD:SPION ratio was varied from 0:1 to 1:1, 2:1, 6:1, 10:1, 20:1, and 40:1; with the amount of SPION constant at 0.1 nmol. (B) High magnification TEM images of MFNBs with QD:MNP ratios of 0:1 (*left*), 10:1 (*center*), and 40:1 nmol (*right*). (C) Confocal images of KB cells targeted and incubated with folic acid functionalized nanobeads (FA-MFNBs), for 1 h at 37 °C and a Fe concentration of 32 $\mu\text{g/mL}$ (*top panel*). The zeta stack analysis (5 μm slices),

confirming nanobeads internalization in cell cytoplasm (<i>bottom panel</i>). Adapted and reprinted with permission from [229]. Copyright 2011 American Chemical Society.....	61
Figure 1.31 - (A) Colon schematic representation with corresponding sections. (B- <i>top</i>) Cross-sectional and lateral representations of the colon wall; (B- <i>bottom</i>) Colon sections' characteristic pH-profiles, enzymatic secretions, microbiome, overall transit time, length and surface area.....	63
Figure 1.32 - CRC stage evolution and corresponding tissue perfusion. Reprinted with permission from [251] Copyright 2005-2022 American Society of Clinical Oncology (ASCO). 64	
Figure 1.33 - Potential biological pathways for targeted therapies against CRC - EGF/EGFR: epidermal growth factor/epidermal growth factor receptor; HGF: hepatocyte growth factor; VEGF/VEGFR: vascular endothelial growth factor/ vascular endothelial growth factor receptor; TGF: transforming growth factor; c-MET: mesenchymal–epithelial transition factor; IGF/ IGF-1R: insulin-like growth factor/ insulin-like growth factor 1 receptor. Adapted and reprinted with permission from [264]. Copyright 2022 Springer Nature Limited.....	66
Figure 1.34 - Known inflammatory mechanisms by which the microbiota contributes to CRC. Host–microbiota maladaptation in colorectal cancer. Reprinted with permission from [341]. Copyright 2022 Springer Nature Limited.....	71
Figure 1.35 - Possible roles of microbiota in CRC causation, through loss of epithelial barrier function, increased pathogenic abundance, microbial dysbiosis and altered metabolism Reprinted with permission from [342]. Copyright 2022 Springer Nature Switzerland AG.....	73
Figure 3.1 - (A) Emission and excitation spectra on SiQDs and solid-state emission spectrum of NANO1 nanoparticles ($\lambda_{exc} = 370$ nm, $\lambda_{em} = 510$ nm). (B) Naked-eye (I) and under UV lamp (II) ($\lambda_{exc} = 365$ nm) images of Silicon quantum dots in solution and of the NANO1 nanoparticles in solid state.....	107
Figure 3.2 - TEM images (A, B), EDX spectrum (C) and size distribution (D) of NANO1 nanoparticles. (E) Nitrogen adsorption –desorption isotherms of NANO1, SiQDsMNsSH, NANO2 and SiQDsMNs nanoparticles. (F) XDR spectra of NANO1, NANO2, and SiQDsMNsSH.	108
Figure 3.3 - (A) FTIR spectra of SiQDsMNsSH and NANO1 nanoparticles. (B) zeta potential, BET surface, pore volume and size data of the nanoparticles.....	109
Figure 3.4 - The release profile of doxorubicin loaded into the NANO1 and NANO2, at pH 7.4 and pH 5.0 PBS buffers. Chemical structure of doxorubicin.	110

Figure 3.5 - Effect of NANO1 and NANO2 in cell viability. Viability of HCT116 and HT29 cells was evaluated after 72h exposure to increasing doses of NANO1 and NANO2 NPs. Values are the mean +/- SEM from three independent experiments.....	111
Figure 3.6 - Effect of NANO1@DOX, NANO2@DOX and doxorubicin in cell viability. Viability of HCT116 and HT29 cells was evaluated after 72h exposure to increasing doses of NANO1@DOX, NANO2@DOX NPs and DOX. Values are the mean +/- SEM from three independent experiments.	112
Figure 3.7 - Laser Scanning Confocal Microscopy (LSCM) in vivo images of control cells (A) and internalized, NANO1@Dox (B and D), and NANO2@Dox (C and E) in HT29 cells. Confocal images obtained from single channels of nanoparticle with doxorubin fluorescence (B-D – red), nuclearStain (Hoeschst- Blue), and membrane stain (CellMask-green) are shown. Orthogonal sectioning and 3D reconstruction of the various z-stacks are shown in D and E images.....	113
Figure 4.1 - Venn's diagram summary of identified proteins from (A) CCD112-CoN cells (i.e., FB5, FB10, FB12) and (B) Caco-2 cells (i.e., Caco5, Caco10, Caco12 and Caco Control) samples.	130
Figure 4.2 - Correlation heatmap between each sample and respective technical (n = 2) and instrumental (n = 2) replicates. R1 / R2 - technical replicates and f1 / f2 - instrumental replicates.....	131
Figure 4.3 - Volcano plots of the differences between (<i>top</i>) FB# samples, (<i>middle</i>) Caco# samples and Caco Control, and (<i>bottom</i>) Caco# samples; where # = 5, 10, 12. Upregulated and downregulated genes are represented in red and blue, respectively, and their names highlighted. Student's t-test significance threshold for identification was set to p-value< 0.05.....	132
Figure 5.1 - <i>Top</i> Characterization of Au@MNs nanoparticles: (A) TEM image of Au@MNs–inset: close up on single Au@MNs (scale 69 nm). Size distribution histograms (for a population of N=50) of (B) Au@MNs and (C) Au-cores. Characteristic type-IV N ₂ isotherms of mesoporous (D) MNs and (E) Au@MNs. <i>Bottom</i> Summary of MNs and Au@MNs physical properties, obtained from the analysis of TEM images and BET and BJH models application in physisorption assays.....	153
Figure 5.2 - <i>Left</i> Au@MNs XRD pattern for 2θ angles between 2° and 80° - inset: close up on Au typical peaks for angles between 30°-80°. <i>Right</i> Summary on the registered peaks and associated intensities, 2θ angles, atomic planes, interplanar d-spacing (dhkl) and type of material.....	154

Figure 5.3 - (A) Absorption spectra of free antibiotics at approx. 10⁻⁵M. (B) Chemical structure of both amoxicillin and ofloxacin, and corresponding Loading Capacities (LC) of MNs and Au@MNs systems towards the same antibiotics. (C) Amoxicillin release profiles from MNs and Au@MNs, in DMSO at 37 °C, for 5 days – inset: first 6 hours of release. (D) Ofloxacin release profiles from MNs and Au@MNs, in DMSO at 37 °C, for 5 days – inset: first 4 hours of release..... 156

Figure 6.1 - Sequential representation of the step-by-step synthesis of NANO3, from the formation NANO1, and dispersion in CHCl₃, to the growth of a mesoporous silica shell (NANO2) in water, further surface modifications (NANO2-NH₂ and NANO2-COOH) and SiQD anchoring via amidation. Representations not at real scale. 187

Figure 6.2 - TGA weight loss curves for NANO2, NANO2-COOH and NANO3 (m_i = 5.0 mg) (a). N₂-isotherms (b) and pore size distribution (c) of NANO2 and NANO3. SEM micrograph of NANO2-COOH, *inset*: particle size distribution (d); and HRFSEM picture of NANO3, with distinguishable spherical shape and porosity (e). TEM images of NANO3, *inset*: particle size distribution (f, g); and brightfield STEM image of NANO3 (h)..... 190

Figure 6.3 - Absorbance and fluorescence spectra of SiQD, after excitation at 450 nm; *inset*: in-solution picture of SiQD dispersion under 365 nm irradiation (a). Fluorescence spectra of as-synthesized NANO3; *inset*: in-solution NANO3 under 365 nm irradiation (b). VSM magnetization loops of NANO1, NANO2 and NANO3 vs applied field at 273.15 K; *inset*: close-up of the same charts for hysteresis visualization (c). Hyperthermia (heat generation) capacity of NANO1, NANO2 and NANO3 in water, under a 274 kHz and 14 kA/m external AMF, for 1800 seconds, (environment at 19 °C) (d). Controlled temperature hyperthermia set-up and NANO3 hyperthermia heating profile with environment at 36.5 °C (e)..... 192

Figure 6.4 - . Ionization of DOX and OFLO in aqueous solutions (a); NANO3 systems loading capacities of DOX and OFLO (n=5), in single and dual combinatory formulations (b); DOX (*circles*) and OFLO (*squares*) release profiles from NANO3@D and NANO3@O (c), as well from NANO3@DO (d), under physiological and acidic conditions (n=3)..... 195

Figure 6.5 - Hyperthermia heating profiles of NANO3@D (a), NANO3@O (b) and NANO3@DO (c), at 37 °C, under an AMF at 274 kHz and 2880 W, for 1800 s (30 min). Maximum released drug from NANO3@D, NANO3@O and NANO3@DO, at 37 °C, under different conditions (n=3): blue = 0.01 M PBS pH 7.4, red = 0.01 M PBS pH 5.0 and grey = AMF at 274 kHz (d)..... 197

Figure 6.6 - Inhibitory activity, as bacterial growth (%), of NANO3, NANO3@O, NANO3@D and NANO3@DO against (*from top to bottom*) *E. coli*, *S. aureus*, MRSA, *P. aeruginosa* and *E.*

<i>faecalis</i> strains, by direct application (a) and after-hyperthermia (AMF of 274 kHz) application (b). Concentrations given as μg of nanoparticle per mL	199
Figure 7.1 - Bacterial transformants after electroporation; inset: <i>E. coli</i> Lux typical luminescence spectrum.....	226
Figure 7.2 - Temporal bacterial response of <i>E.coli</i> Lux to ofloxacin, under static conditions, for different concentrations. Responses were given in the form of LUX ₄₉₀ signal intensity and OD ₆₀₀	229
Figure 7.3 - Typical bacterial growth curve and its correlation with OD measurements (a); adapted from [51]. Obtained bacterial growth curve and correlation with the recorded luminescence, emitted by luminescent <i>E.coli</i> Lux (b).....	231
Figure 7.4 - Physical characterization of MNs and SPION@MNs: MNs TEM micrographs confirming the particles mesoporosity, at 100 nm scale (a) and close up at 20 nm scale (b); inset: particle size distribution (n=65). SPION@MNs SEM image, scale at 200 nm; inset: particle size distribution (n=50) (c). TEM micrograph of SPION@MNs depicting single Fe ₃ O ₄ magnetic cores encapsulated within silica mesoporous matrix, at 55 nm scale (d). 72 K N ₂ -isotherms of both MNs and SPION@MNs systems, again confirming its mesoporosity and availability for drug encapsulation (e). SPION@OA cores magnetization saturation curves (f). Summary of both MNs and SPION@MNs physical properties from surface area to pore diameter and volume, TEM size and surface zeta potential (g).....	232
Figure 7.5 - Antimicrobial activity of drug-loaded MNs on the parental <i>E. coli</i> strain, measured as the bacterial growth calculated by OD ₆₀₀ , for 24h assay.	236
Figure 7.6 - Antimicrobial activity of drug-loaded MNs on the mutant <i>E. coli</i> Lux strain, measured as bacterial growth calculated by OD ₆₀₀ and LUX ₄₉₀ , for 8h assay; raw LUX ₄₉₀ signal alone is depicted by the red dotted line and follows the same trend as the calculated bacterial growth.....	237
Figure 7.7 - Statistical comparison of the obtained relative growth reduction, via OD ₆₀₀ 24 h-assaying the parental <i>E.coli</i> and LUX ₄₉₀ 8 h-assaying <i>E. coli</i> Lux, for all MNs systems (concentrations related to nanoparticle); statistically significant levels represented as * $\equiv p \leq 0.05$, ** $\equiv p \leq 0.01$, *** $\equiv p \leq 0.001$	238
Figure 7.8 - Antimicrobial activity of drug-loaded SPION@MNs on the parental <i>E. coli</i> strain, measured as the bacterial growth calculated by OD ₆₀₀ , for 24 h assay.....	239
Figure 7.9 - Antimicrobial activity of drug-loaded SPION@MNs on the mutant <i>E. coli</i> Lux strain, measured as bacterial growth calculated by OD ₆₀₀ and LUX ₄₉₀ , for 8 h assay; raw LUX ₄₉₀	

signal alone is depicted by the red dotted line and follows the same trend as the calculated bacterial growth.....	240
Figure 8.1 - (a) Stacked ¹ H NMR spectra of AZM, AZM-NH ₂ AZM-SH (DMSO-d ₆ , 400 MHz), for δ between 6-14 ppm. (b) AZM-SH structure and atom number assignment. (c) Close-up of the ¹ H NMR spectrum of AZM-SH (CD ₃ CN, 400 MHz) and chemical shift assignment of sulfanylpropanamide C(2) and C(3) protons, seen as two crossed doublet-triplet (dt) signals.	274
Figure 8.2 - Suggested dimeric conformations of AZM-SH attained via hydrogen bonding.	275
Figure 8.3 - (a) Tetrahedral zinc geometry, with TZ pseudo-atom located at the unoccupied corner of the ideal coordination tetrahedral geometry. (b) AZM-SH zinc coordination via NA (nitrogen acceptor) atom type interaction with the attractive component (TZ pseudo-atom) of the zinc ion.....	276
Figure 8.4 - CAs relative activity in the presence of AZM-SH and respective Boltzmann regression for the determination of its IC ₅₀	278
Figure 8.5 - - Molecular interaction of Ellman's reagent with thiol moieties and corresponding spectroscopic response (i.e., absorbance band red shift to 412 nm) upon the formation of TNB free species; NANO3-SH systems spectroscopic signature upon thiol determination and activation.	280
Figure 8.6 - (left) DLS hydrodynamic diameter of NANO1 to NANO3-SH systems, and (right) respective surface ζ.....	280
Figure 8.7 - (a) Ofloxacin (OFLO) and (b) Doxorubicin (DOX) pH-stimulated release profiles from NANO@O-SH, NANO@DO-SH and NANO@D-SH, at pH 7.4 and 5.0.	282
Figure 8.8 - Simulated digestive track release from enteric-coated NANO3@ <i>drugs</i> systems (n=3), with medium pH and exposure time fitting the biological conditions of the selected sections of the digestive track (yellow – stomach, blue – small intestine, green – colon)	285
Figure 8.9 - Western blot for the overexpression levels of EGFR and CA IX in HCT-166 and HT-29 CRC cell lines.....	288
Figure 8.10 - LCSM images of HCT-116 and HT-29 cell lines treated for 3h with NANO3@D, NANO3@D-CTX and NANO3@D-AZM, according to their specific overexpressed targets (untreated cells were used as negative controls).....	290
Figure 9.1 - Absorption and emission spectra of dyes 1 to 3 in acetonitrile at 295K. Chemical structures of cyanine derivative 1 to 3 . [1] = [2] = [3] = 3 × 10 ⁻⁶ M.	324
Figure 9.2 - Spectrophotometric (A, B, C) and spectrofluorimetric (D) titrations of dyes 1 (A), 2 (B) and 3 (C, D) with the addition of Cu ²⁺ in acetonitrile. The inset represents the absorption	

(A, B, C) at 501 nm (A), 510 nm (B) and 624 nm (C); and the emission intensity (D) at 647 nm, as function of $[\text{Cu}^{2+}]/[\mathbf{1}]$ (A), $[\text{Cu}^{2+}]/[\mathbf{2}]$ (B) and $[\text{Cu}^{2+}]/[\mathbf{3}]$ (C, D). $[\mathbf{1}] = [\mathbf{2}] = [\mathbf{3}] = 3 \times 10^{-6} \text{ M}$, $\lambda_{\text{exc1}}=501 \text{ nm}$, $\lambda_{\text{exc2}}=510 \text{ nm}$, $\lambda_{\text{exc3}}=624 \text{ nm}$, $T = 295 \text{ K}$).....	325
Figure 9.3 - Schematic representation of PVA:PVP@1 constituents, synthetic steps and final product, under white and UV-light, with respective emission spectra after excitation at $\lambda = 501 \text{ nm}$	327
Figure 9.4 - (<i>top</i>) PVA:PVP@2, PVA:PVP@3, PVA:PVP:paper@2, PVA:PVP:paper@3, and their Cu^{2+} -complexed counterparts, under white and UV-light. (<i>bottom</i>) Emission spectra of each 2-and 3- containing material after excitation at $\lambda = 510 \text{ nm}$ and $\lambda = 624 \text{ nm}$, respectively..	328
Figure 9.5 - Obtained MICs for all cyanine derivatives (1 to 3 and respective copper complexes- 1:3 stoichiometry) and respective bacterial growth profiles, against Gram-negative (A) and Gram-positive (B) bacteria. Concentrations represented in μM and n/a = no activity.....	330
Figure 9.6 - Antibacterial assays of PVA:PVP and PVA:PVP:paper systems against S.aureus, MRSA and B. cereus. Blue doted squares delimitate 0.5 x 0.5 cm polymer pieces and blue arrows indicate red colored inhibition zones edges, due to migration of dye 2 from polymeric matrices.....	335

LIST OF TABLES

Table 1.1 - Summary of different SPION synthetic pathways and their related features.	11
Table 1.2 - Summarising table of all SPION synthetic routes and surface modifications, along with their magnetic properties and applications	25
Table 1.3 - Summarising table of all SiQDs synthetic pathways and reported works in literature, with respective reaction conditions and products optical properties.	36
Table 1.4 - Summarising table of some SiQDs surface modifications and respective optical properties and applications.	55
Table 3.1 - IC50 values of DOX and DOX-loaded NPs against HCT116 and HT29 cells. Depicted concentrations (in μM) correspond to that of free and loaded DOX.	112
Table 3.2 - Cellular quantification of nanoparticles uptake.	114
Table 4.1 - Total protein determination for each sample, obtained by tryptophan-based fluorescent assay (n = 3.)	127
Table 4.2 - Significantly altered (downregulated and upregulated) genes and proteins between tested fractions and their associated biological species and functions.	133
Table 5.1 - Bacterial strains specifications.	150
Table 5.2 - Summary of all tested nanoparticles, free antibiotics and controls, with their stock concentrations.	150
Table 5.3 - Comparison between in-work developed materials and other already published in literature.	157
Table 5.4 - Semi-empirical Korsmeyer-Peppas diffusion model parameters upon fitting to experimental amoxicillin and ofloxacin release data.	158
Table 5.5 - Summarising table with all growth inhibition zones' average diameters. Measured lengths represent the obtained means $\pm$ an associated absolute error of 0.5 mm	159

Table 5.6 - Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of DMSO, MNs, Au@MNs and the free antibiotics towards the selected bacterial strains.	161
Table 5.7 - Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for all amoxicillin-based tested formulations, against selected bacterial strains.	162
Table 5.8 - Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for all ofloxacin-based tested formulations, against selected bacterial strains.	163
Table 7.1 - MNs and SPION@MNs loading assay loading percentages and encapsulation efficiency (EE) for single and dual-combinatory formulations of EPI, DOX and OFLO.	233
Table 8.1 - Docking performances for similar conformational modulations, using AutoDock4 and AutoDock4 _{Zn} forcefields.	277
Table 8.2 - Summary of all final NANO3 formulations, with drug and targeting species content.	286
Table 8.3 - IC ₅₀ values of biofunctionalized NANO3 and NANO3@ <i>drugs</i> against HCT-116 and HT29 CRC cell lines, for 72h. Concentrations given as equivalent DOX concentrations (in μM).	289
Table 8.4 - Internalization rates of different NANO3@D systems, at different equivalent DOX concentrations (5, 10 and 25 μM) in HCT-116 and HT-29 CRC cell lines. Internalization rates given as the relative fluorescence of the samples against the fluorescence of the nanoparticles in medium before treatment (excitation at 480 nm, emission collected at 550 nm).	291
Table 9.1 - Results of antibacterial assays of PVA:PVP polymers against several bacterial strains. Obtained inhibition halos represented in cm^2 .	333
Table 9.2 - Results of antibacterial assays of PVA:PVP:paper mockups against several bacterial strains. Obtained inhibition halos represented in cm^2 .	338

PUBLICATIONS

Results of this PhD Thesis and other contributions have been disseminated in the following scientific publications and communications.

Total number of Journal Publications	14
Journal Publications as 1 st Author	11
Journal Publications derived from PhD thesis	7
Total number of citations	135*, 153 [†]
Total number of conference proceedings	19
Total number of oral communications	9
Total number of poster communications	19

* Scopus; [†] Google Scholar

Publications from PhD Thesis:

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ACRONYMS

AA	Ascorbic acid
AgNP	Silver Nanoparticles
AMF	Alternating magnetic field
Amox	Amoxicillin
APTES	3-aminopropyl triethoxysilane
APTMS	3-aminopropyl trimethoxysilane
ASAP	Accelerated Surface Area and Porosimetry
AuNPs	Gold Nanoparticles
AZM	Acetazolamide
BET	Brunauer-Emmet-Teller
BJH	Barret-Joyner-Halenda
CA	Carbonic Anhydrase
Caco-2	Human Colon Adenocarcinoma Cell Line Caco-2
CAE	Carcino Embryonic Antigen
CAPIRI	CAP+IRI (Capecitabine + Irinotecan)
CAPOX	CAP+OX (Capecitabine + oxaliplatin)
CCD-112Con	Normal Human Colon Fibroblast Cellosaurus Cell Line CCD-112Con
CCP	Copolymer Modified With Catechol

CFU	Colony-Forming Units
CTAB	Cetyltrimethylammonium bromide
CRC	Colorectal Cancer
CTX	Cetuximab
DAMO	<i>N</i> -[3-(trimethoxysilyl)propyl]ethylenediamine
DEG	Diethylene glycol
DEI	Disulfide ethanediylbis(isonicotinate)
DI	Deionized
DLS	Dynamic Light Scattering
DMF	<i>N,N</i> -dimethylformamide
DMSA	Dimercaptosuccinic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
ECDC	European Centre for Disease Prevention and Control
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDX	Energy dispersive x-ray spectroscopy
EFSA	European Food Safety Authority
EG / EtGly	Ethylene glycol
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EPI	Epirubicin
EPS	Extracellular polymeric substance
EtOH	Ethanol
FA	Folic acid
FASP	Filter Aided Sample Preparation

FBS	Foetal bovine serum
FDA	Food and Drug Administration
FGFs	Fibroblast Growth Factors
FI	Fluorescein iso-thiocyanate
FMN / FMNH₂	Flavin mononucleotide
FOLFOX	5-FU+OX (5-Fluorouracil + Oxaliplatin)
FOXFIRI	5-FU+IRI (5-Fluorouracil + Irinotecan)
FTIR	Fourier-transform infrared spectroscopy
HA	Hyaluronic acid
HCT116	Human Colorectal Carcinoma Cellosaurus Cell Line HCT116
hCA IX	Human Carbonic Anhydrase IX
HD	Hydrodynamic Diameter
HER	Human Epidermal Growth Factor Receptor
HGF	Hepatocyte Growth Factor
HRFESEM	High-resolution Field Emission Scanning Electron Microscope
HT29	Human Colorectal Adenocarcinoma Stable Cell Line HT29
IC50	Half Maximal Inhibitory Concentration
ICP-AES	Inductively Coupled Plasma—Atomic Emission Spectrometer
IGF	Insulin-Like Growth Factor
IGF-1R	Insulin-Like Growth Factor 1 Receptor
IPTES	3-isocyanatopropyltriethoxysilane
LFQ	Label-free Quantification
LSCM	Laser scanning confocal microscope
LUX₄₉₀	Luminescence
MBC	Minimum bactericidal concentration
MCM-41	Mobil Composition of Matter No. 41

mCRC	Metastatic Colorectal Cancer
MeOH	Methanol
MET	Mesenchymal–Epithelial Transition Factor
MHA	Muller Hinton Agar
MHB	Muller Hinton Broth
MIC	Minimum inhibitory concentration
MPTS	(3-mercaptopropyl)trimethoxysilane
MNs	Mesoporous silica Nanoparticles
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic acid
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MS	Mass Spectroscopy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MWCO	Molecular Weight Cut-Off
NHS	<i>N</i> -hydroxysuccinimide
NMR	Nuclear Magnetic Resonance
NP	Nanoparticle
OA	Oleic acid
OD / OD₆₀₀	Optical Density
OFLO / Oflox	Ofloxacin
OS	Overall Survival
PAA	Polyacrylic acid
PBS	Phosphate Buffered Saline
PDEA	2-(pyridyldisulfanyl) ethylamine
PDGF	Platelet-Derived Endothelial Cell Growth Factor
PDI	Polydispersity Index

PDT	Photodynamic Therapy
PEG	Polyethylene glycol
PEI	Polyethyleneimine
PEO	Poly(ethylene oxide)
PFS	Progression-free Survival
PL	Photoluminescence
PLGA	Poly(lactic-co-glycolic acid)
PLH	Poly-L-histidine
PMMA	Polymethyl Methacrylate
PSi	Porous silicon
PTX	Paclitaxel
QD	Quantum Dot
QY	Quantum Yield
ROS	Reactive Oxygen Species
SA	Sodium ascorbate
SDS	Sodium Dodecyl Sulphate
SCFAs	Short-Chain Fatty Acids
SEM	Scanning Electron Microscopy
SiNP	Silica Nanoparticles
SiNWs	Silicon Nanowires
SiQD	Silicon Quantum Dot
SLP	Specific Loss Power
SOC	Super Optimal broth with Catabolite repression medium
SPIONs	Superparamagnetic Iron-Oxide Nanoparticles
SPR	Surface Plasmon Resonance
STEM	Scanning Transmission Electron Microscopy

TEG	Tetraethylene glycol
TEM	Transmission Electron Microscopy
TEOS	Tetraethyl orthosilicate
TGA	Thermogravimetric analysis
TGF	Transforming Growth Factor
THF	Tetrahydrofuran
TMB	1,3,5-Trimethylbenzene
TOAB	Tetraoctylammonium bromide
TREG	Triethylene glycol
TSA	Trypto Casein-Soy Agar
TSB	Trypto Casein-Soy Broth
UA	Ultrasound Amplitude
UF	Ultrasound Frequency
UV-Vis	Ultraviolet-Visible
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VSM	Vibrating Sample Magnetometer
XRD	X-ray Diffraction
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
ZP	Zeta Potential

SYMBOLS

ζ Zeta Potential (symbol: ζ).

GENERAL INTRODUCTION - MAGNETIC, FLUORESCENT AND HYBRID NANOPARTICLES: FROM SYNTHESIS TO APPLICATION IN BIOSYSTEMS

Keywords: nanoparticles • SPIONs • fluorescence •
imaging • silicon quantum dots • multifunctionality

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Magnetic, fluorescent and hybrid nanoparticles: from synthesis to application in biosystems

Gonçalo A. Marcelo, Carlos Lodeiro, José Luis Capelo, Julia Lorenzo and Elisabete Oliveira.

Abstract: Multifunctional nanoparticles have emerged as an outstanding candidate for a new generation of biomedical applications, mainly due to their remarkable properties and biocompatibility. Individual reports on multi-metal, semiconducting and superparamagnetic nanoparticles (SPIONs), elucidating on each's unique intrinsic properties, have demonstrated that the biological application of such materials is highly dependent of their size, shape, surface nature and core nature. However, reviews combining nanoparticles with multiple properties, as fluorescence and paramagnetism, as well as biocompatibility, toxicology and biodegradability are yet seldom. This review highlights the highest output advances, of the last decade, on synthetic procedures for the design of multifunctional magneto-luminescent hybrid nanosystems based on quantum dots, SPIONs and mesoporous silica nanoparticles, as well as surface modifications and their role for biological applications. In here a detailed description of colorectal cancer (CRC) and its characteristics is also given, along with a review of some of the current therapeutic strategies to fight it, as well as in-development ones. Finally, the review covers an overview of the growing importance of intestinal and opportunistic microbiota in CRC growth and invasion, and highlights its importance in the success of anti-CRC therapies.

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1.1 Introduction

Theranostics is an ever-present concept that conjugates both therapeutic and diagnostic tools in the most various areas of medicine. In the last decades, the development of nanotechnology has attracted increasing scientific attention and has allowed the emergence of multifunctional nanocarriers and nanoconjugates integrating diagnostic and/or therapeutic functions [1-4]. Emerging fields of personalized medicine [5] molecular imaging [6-9], cellular labelling [8, 10-13], biological separation and purification [14-19] and targeted controlled drug delivery [7, 20-22], have worked as the main drives for such development and have promoted the preparation of a wide range of nanotechnological probes.

Nanoparticles with efficient fluorescence and strong magnetisation are examples of nanoprobes that have found high-technological applications [7, 10, 11, 23-30]. Already detailed in previous reviews, extensive individual studies on single or multi-metal nanoparticles (e.g. Au, Ag, Pt, Pd) [9, 31-35], semiconducting nanoparticles as quantum dots (e.g. CdTe, CdSe/ZnSe, CdS/ZnS, InP QD, Carbon QD, Silicon QD) [12, 36-40] and superparamagnetic nanoparticles of γ -Fe₂O₃ (maghemite), Fe₃O₄ (magnetite) or α -Fe₂O₃ (hematite) [8, 13, 41, 42] have elucidated on each's unique intrinsic properties and have demonstrated that the biological application of such materials is highly dependent on physicochemical characteristics as size, shape, surface nature and core nature [14, 25, 26, 43, 44].

Despite the reported individual studies in metal, paramagnetic nanoparticles, reviews combining nanoparticles with multiple properties, as fluorescence (silicon quantum dots) and paramagnetism (SPIONs), as well as biocompatibility studies in biological media are yet scarce.

Motivated by our research interests and goals in the development of luminescent, metal and magnetic nanoparticles, herein we present for the first time a critical review, covering the last ten years of the most relevant advances on the synthetic pathways of both biodegradable and biocompatible silicon QDs, and iron-based SPIONs nanoparticles, as well as that of magneto-luminescent hybrid nanosystems for biological applications. Surface modifications and their role for specific biological applications, prokaryotic or eukaryotic-based cells, will also be discussed.

1.1.1 Quantum Dots

Concerning fluorescent nanomaterials, quantum dots (QDs) comprised of inorganic elements of groups II–VI or III–V, are very promising, mainly due to their unique optical properties [36, 45] that arise from the confinement of charge carriers' energy states through the physical size reduction of these nanoparticles. Briefly, a decrease in the size, below the exciton Bohr's radius of the bulk material, leads to the quantisation of energy levels, now discrete when compared to the continuum energy levels observed in bulk materials. QDs size dependent quantisation of discrete energy levels - increasing with a decrease in the size - provides a tuneable grip to the optical properties of nanoparticles such as absorption and emission phenomena [46, 47]. For example, CdS and ZnSe QDs are known to emit from blue to near-UV light, and different sized CdSe QDs emission spans across the visible spectrum [48–50], while InP and InAs QDs emit in the far-red and near-infrared [51–53] (see fig. 1.1A).

This characteristic is utterly attractive for a large number of applications since that QDs of different sizes and/or arrangements can all be excited by a single light source, emitting distinct colours over a wide spectral range with minimal spectral overlap (see fig. 1.1B).

Size-dependent fluorescence, allied with QDs high quantum yields (QY) and photostability [46, 54–58], make them (particularly Cd-containing QDs) good alternatives to conventional dyes and fluorophores to which control over absorption and emission wavelengths would mean the expensive design and synthesis of new organic compounds. Besides, QDs high resistance to photobleaching allows them to be used for long-term *in vitro* and *in vivo* imaging [6, 12, 26, 40, 47, 59] (figure 1.1C).

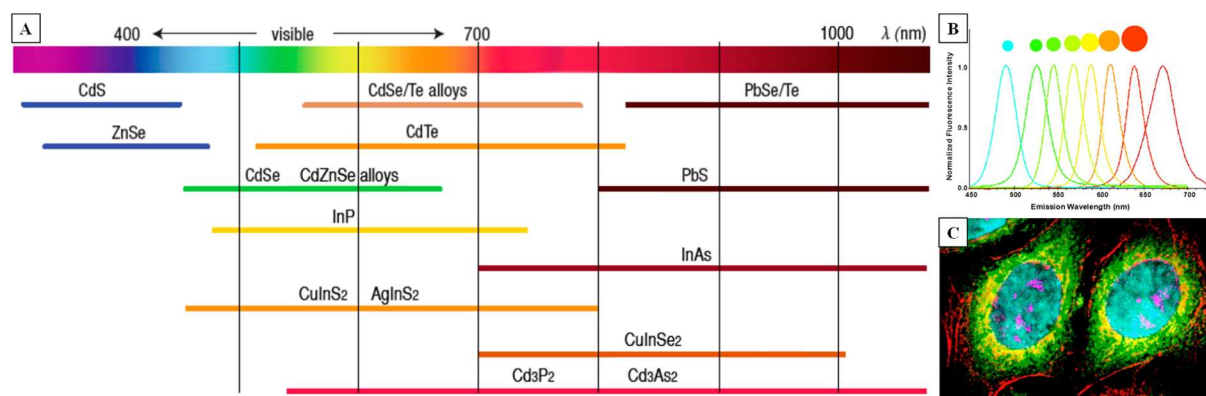


Figure 1.1 - (A) Spectral range of emission for the most widely studied types of semiconductor nanocrystals. Reprinted with permission from [36]. Copyright 2016 American Chemical Society. (B) Normalized photoluminescence of CdSe QDs under single wavelength excitation achieved by selectively varying the QDs size. (C) Pseudo colored image of human epithelial cells by staining with five different sizes of QDs. Actin filaments —

Red (705 nm), Nucleus— Cyan (655 nm), Ki67 protein—Magenta (605 nm), Microtubules— Green (565 nm), Mitochondria— Orange (525 nm). Adapted with permission from [45]. Copyright 2015 Elsevier B.V.

Although QDs are usually prepared as dispersions in organic media, for biological applications QDs must be water-dispersible. When initially synthesised in nonpolar organic media, surface modification is essential to transfer them to aqueous biological media. Ligand exchange and encapsulation of QDs using amphiphilic molecules, polymers or silica are common strategies to prepare water-dispersible QDs [11, 12, 44, 45, 60]. QDs large surface areas can then be conjugated with an assortment of biomolecules (e.g. proteins, peptides, amino acids, antibodies, DNA, enzymes or vitamins) by making use of their numerous available attachment sites.

However, with the rapid development of biological applications their potential toxicity became subject of debate, and accumulating evidence confirmed Cd-containing QDs toxicity/cytotoxicity when exposed to biological media, especially after oxidative and/or photolytic degradation [61, 62]. Notwithstanding, besides core nature, other properties as QD size, charge, concentration and outer coating bioactivity (capping material, functional groups) have each been shown to be determining factors in QD toxicity [63, 64].

Thus, at present, two tactics are being pursued to address these issues: 1) the use of one or more biocompatible and long-lasting polymeric coatings to enclose QDs and prevent degradation *in vivo* [23, 36, 45, 65]; and 2) the use of Cd-free QDs with performances comparable or superior to the existing Cd-containing QDs. Promising examples are Carbon-based (CQD) and Silicon-based (SiQD) QDs [24, 26, 37, 66-69] (see figure 1.2). The latter are considered as ideal biomedical probes, due to their known low-toxicity, biocompatibility, easy tuneability and photostability. They have, thus, been substituting high-grade II-VI QDs, and traditional fast-photobleaching dyes, on several biomedical applications, with silicon's extensive commercial use and availability making them outstandingly attractive cheap materials.

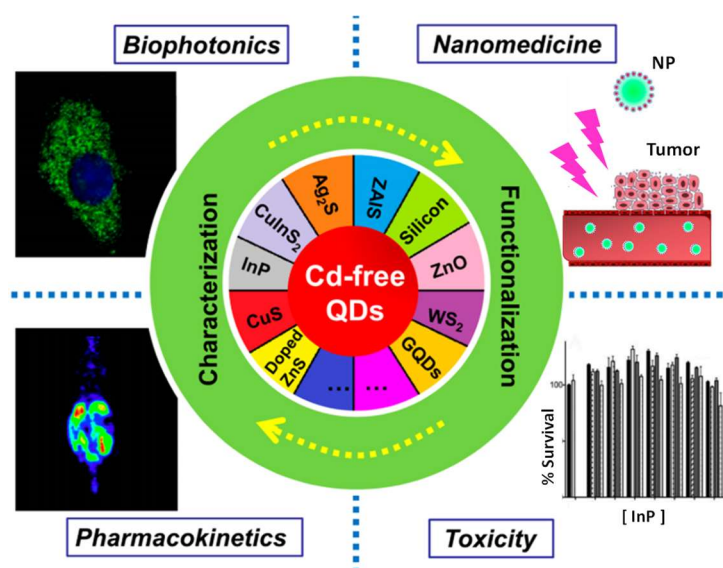


Figure 1.2 - Already available and studied Cd-free QDs and their representative applications in biophotonics and nanomedicine. Adapted with permission from [59]. Copyright 2016 American Chemical Society.

1.1.2 Superparamagnetic iron oxide nanoparticles

Superparamagnetic iron oxide nanoparticles (SPIONs) have emerged as one of the most appealing candidates for cancer diagnosis and therapy, among other well-known applications as contrast agents in medical techniques (e.g. magnetic resonance imaging) [13, 30, 70], treatment monitoring [71-74], drug delivery systems [17, 75-77] and absorbents [18, 19, 78]. Most synthetic magnetic materials and composites, comprising of manganese, niobium, gallium, copper [79] and magnetic ionic liquids [80, 81] γ - Fe_2O_3 (maghemite), α - Fe_2O_3 (hematite) or Fe_3O_4 (magnetite)-based materials have shown to be promising biocompatible magnetic nanoprobes.

SPIONs are recognized for their easily controllable sizes, from 1–100 nm, with dimensions smaller than or analogous to some biological systems such as cells, viruses, proteins and genes, with which they can directly interact.

SPIONs small size not only ensures their easy navigation in tissues, endocytosis, and intracellular trafficking in cancer cells but also their superparamagnetic properties. The miniaturisation of iron oxide from the bulk to the nanoscale results in the formation of single magnetic domains whose thermal energy is high enough to overcome the energy barrier of magnetic flipping (T_{block}) and confer them superparamagnetic characteristics [8, 82]. Meaning that, in the absence of an external field, whereas ferromagnetic NPs will maintain a net magnetisation, SPIONs will instead exhibit no net magnetisation due to the rapid flipping of their magnetic momentum. Additionally, due to single-domain magnetisation and fast

momentum flipping, SPIONs can, in the presence of an external AC magnetic field, undergo orientating thermal-based fluctuations, from either Brownian or Neel movement, and generate localised temperature gradients [17, 72, 73, 83].

Biodegradability and low toxicity are some of its main assets, as iron is known to enter the body's natural iron stores upon SPIONs degradation [23, 42, 84-86]. Thus, resulting in the approval of many formulations for clinical use, including Ferridex IV for liver and spleen imaging, Ferumoxytol for iron replacement therapy, and Combidex for imaging lymph node metastases. Similar to QDs, SPIONs life-span in biological systems is highly dependent on surface interactions. SPIONs can undergo a series of surface modifications with biocompatible coatings that allow the integration of multiple functionalities into a single particle from drug loading to membrane-permeating molecules and targeting ligands for specific cells lines (see figure 1.3).

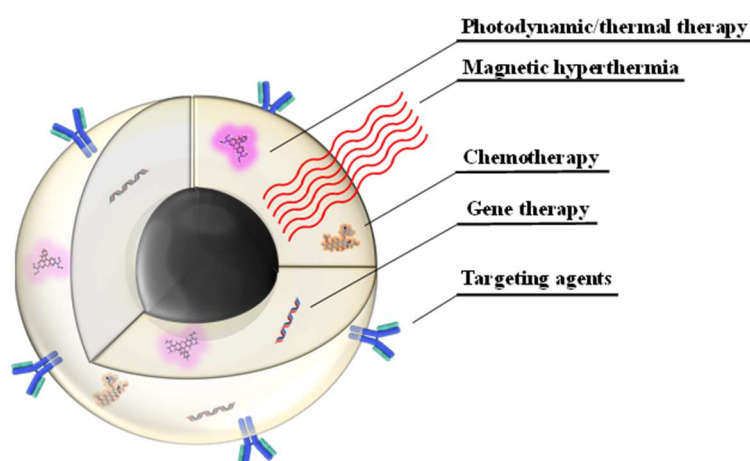


Figure 1.3 - Illustration of the nano-architecture of a therapeutic SPION, with specific surface coating and related therapies.

An active application of SPIONs rises from the interaction of ultra-small ($\leq 3\text{nm}$) SPIONs and its superparamagnetism with an external magnetic field, by creating inhomogeneities in the latter and triggering the dephasing of samples' protons. Thus, promoting the shortening of T_2 and T_2^* relaxation times of the neighbouring regions and producing a decreased signal intensity in T_2 - and T_2^* -weighted magnetic resonance imaging (MRI) [8, 70].

Combination of silicon QDs and iron-based SPIONs, particularly, provided the theragnostic field with the most versatile and biocompatible tools for both diagnosis and therapeutics. Interest on the conjugation of such systems, and their unique properties, has led some groups [7, 11, 23, 30, 87], although scarce, to pursue the elaboration of multi-

functional magneto-luminescent hybrids (e.g. Janus, strawberry / mulberry-like and core-shell nanoparticles), spanning from dye-doped Fe₃O₄ nanoparticles to magnetic-capped QDs. After that, the most recent advances in the synthesis of SPIONS, silicon quantum dots, and their applications in biological media will be discussed.

1.2 SPION nanoparticles: synthesis and applications

Extensive works have been dedicated to the synthesis and characterisation of diminutive SPIONs, resulting in various reported synthetic routes capable of producing appropriately sized, shaped, structured and stable SPION nanoparticles with high magnetic moments (see table 1.1). The synthetic routes can be of three main types: 1) physical, 2) chemical and 3) biological; with chemical routes being of predominant usage when developing biological probes, due to its easier manipulation and accessible and cheaper methodologies (see figure 1.4).

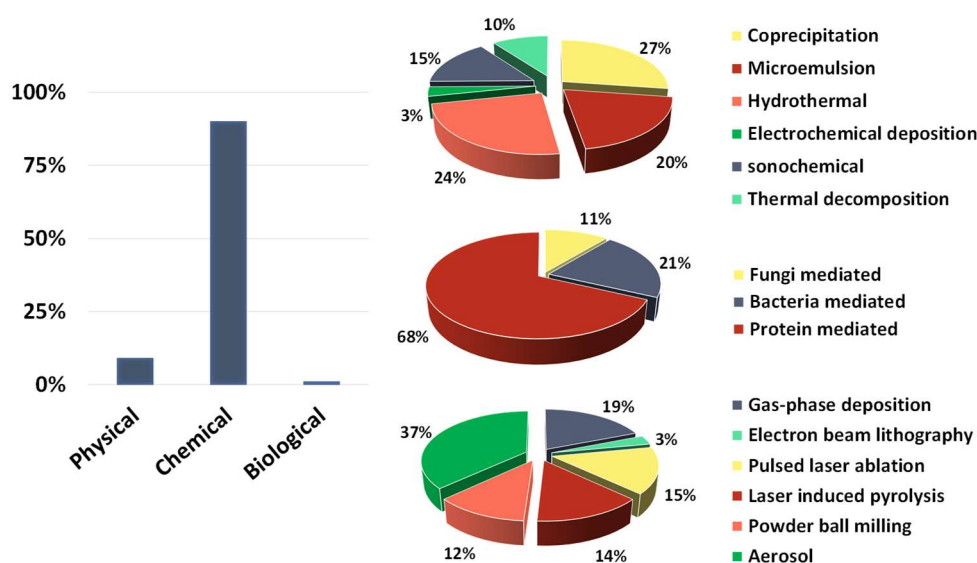


Figure 1.4 - Comparison of published work on the synthesis of SPIONs by three main different routes.

1.2.1 Physical routes

The synthesis of SPIONs through physical methods follows a top-down approach where bulk materials, or large particles, are broken into nanosized particles. Although suitable for large-scale productions, these methods, reported in detail elsewhere [88], are known to produce SPIONs of non-uniform size distributions and involve expensive and time-consuming technologies. Electron beam lithography (EBL) and Ball milling are examples of these approaches.

Table 1.1 - Summary of different SPION synthetic pathways and their related features.

Synthetic method	Reaction media & conditions	Reaction temperature [°C]	Reaction times	Advantages	Disadvantages	Yield
Co-precipitation	aqueous, inert or ambient atmosphere	20-150	minutes-hours	facile and controllable sizes with relative narrow distribution	agglomeration and low crystalline particles	high, scalable
Sol-Gel / Polyol	aqueous, ambient	20-200	hours	large-scale production and narrow distribution	agglomeration and large number of reaction variables	high, scalable
Thermal decomposition	organic, inert atmosphere	100-400	minutes-hours	highly crystalline and monodispersed SPION	high temperatures and organic-soluble SPIONs	high, scalable
Hydro or Solvothermal	Aqueous/Organic, high pressure	100-500	hours-days	highly crystalline and pure nanoparticles	Slow kinetics and non-monitorization of crystal growth, high pressures	high, scalable
Microemulsion	w/o or o/w, ambient	20-70	hours-days	controllable size and magnetic properties	agglomeration and complex equilibrium process	low
Sonochemical	aqueous, ambient	20-50	minutes	facile, fast and eco-friendly	amorphous SPION requesting annealing, uncontrollable shape	medium
Electrochemical	aqueous, ambient	20-60	hours	facile, high purity and eco-friendly	complex apparatus, energy consuming	medium
Biological	aqueous, ambient	r.t	days	extracts duality as reducing and capping agents, greens synthesis	complex and time-consuming process, extraction and purification techniques needed	low
Aerosol/Vapor	airborne, inert atmosphere	> 100	minutes-hours	relatively narrow distributions	agglomeration, uncontrollable size, complex and expensive procedure	high, scalable
Ball milling	solid (humid), ambient	r.t	minutes-hours	solvent free	uncontrollable size and agglomeration	high, scalable

1.2.1.1 Electron beam lithography (EBL)

Briefly, EBL can be described as the conversion of bulk high purity iron materials or films, deposited on the surface of a substrate, to iron oxide (Fe_3O_4) nanoparticles through the emission of an electron beam across the material [89, 90]. The electron beam causes the evaporation of the initial iron precursors and the formation of one-dimensional arrays of shape-controlled SPIONs. Two-dimensional arrangements have, however, been achieved by Krása et al. [91] through the copulation of EBL with radio-frequency sputtering.

1.2.1.2 Ball milling

Ball milling is a solid-state mechanical size reduction technique where iron precursors are converted to iron oxide nanoparticles. SPIONs are produced inside stainless steel cylindrical containers filled with grinding micron-sized spheres [92]. The addition of solvents (i.e. water) and/or excesses of salt species are known to promote effective milling and to work as anti-agglomeration agents [93, 94]. Additionally, the amount of solvent and the nature of grinding spheres, as well O_2 content, (e.g. iron, stainless steel or polymer) have been reported to directly affect the final phase of iron oxides (i.e. maghemite or magnetite) [93-95]. Milling time, in wet-milling procedures, has also been established, by Can et al. and Janot et al., as a determinant factor for the production of single-phased pure SPIONs [94, 96], with non-reduced Fe free SPIONs being produced for milling periods longer than 48h. Despite the usual broad size distributions, de Carvalho et al. reported a milling time-SPION size relationship, with magnetite nanoparticles of 20 nm (12h) being reduced to approx. 7 nm after 96h of continuous milling. All nanoparticles have shown high saturation magnetisations between 73-120 emu/g, close to that of bulk magnetite (92 emu/g) [95].

An unconventional mechanochemical methodology adopted by Lu et al., using ferric/ferrous hydrate chlorides salts as precursors allowed for the production of narrow sized SPIONs within short periods of milling (1h), although with saturation magnetisations lower than those of wet-milling nanoparticles (approx. 52 emu/g) [92]. Notwithstanding, the resulting nanoparticles showed good dispersibility and biocompatibility, due to the presence of surface hydroxyl groups that render them good water solubility and propensity to future surface modifications. Recently, Fe_3O_4 - SiO_2 nanocomposites, prepared by high energy ball milling, were reported for the first time by Piloni et al. for biological use [97]. The resulting nanocomposites, once stabilised with 3-aminopropyltriethoxysilane (APTES), were functionalized with poly(ethylene glycol) – PEGylation – to surpass the usual

mechanochemical agglomeration and dispersibility issues, while rendering them biocompatibility.

1.2.2 Chemical Routes

Chemical methods of producing SPIONs are considered bottom-up approaches where molecular entities containing iron condensate, in a series of conditions, to form full nanometric sized superparamagnetic nanoparticles. Condensation reactions have, however, been reported in the literature to be carried out in either solution/aqueous or gas phases. The former using solvents to mediate chemical reactions and the latter promoting precursors nucleation in the gas phase, usually requiring higher reaction energies.

1.2.2.1 Wet-chemical routes

Wet chemical approaches, comprising the well-known co-precipitation, sol-gel, microemulsion, hydrothermal / solvothermal, thermal decomposition, sonochemical and electrochemical techniques, are the most predominantly used and accessible synthetic pathways to obtain colloidal SPIONs. These have been reported to allow the control of numerous synthetic parameters during nucleation and growth and thus, the control over size, shape and surface chemistry.

1.2.2.1.1 Co-precipitation

Sugimoto et al. [98] describing a well-established synthesis of large, well-defined spherical magnetite NPs using ferrous salt in the presence of KNO_3 and KOH , Massart et al. was pioneer on the settlement of a facile co-precipitation approach to the synthesis of small-sized SPIONs in solution, being so far the most widely used and cited technique [99]. Briefly, it consists on the precipitation of an aqueous solution containing ferric (Fe^{3+}) and ferrous (Fe^{2+}) salts, at a 2:1 stoichiometric ratio, which undergoes subsequent nucleation and growth until the formation of full nanosized SPION, easily explained by the LaMer diagram.

The reaction occurs in the presence of a base, at room or elevated temperatures (70–90 °C), under oxygen-free atmospheres (i.e. N_2 or Ar). As reported in the literature, strong or weak bases can be used during the synthesis (e.g. NaOH , KOH , NH_4OH , Na_2CO_3) for effective precipitation of SPION, with the latter resulting in slower precipitation rates [98-101]. The addition of bases to precursor solutions has been reported as a critical step during synthesis, with Thanh et al. highlighting the impact of the addition speed on crystal phases transformations, the final phase and magnetic properties [102].

The size, shape and structure of SPIONs depend on experimental parameters as stirring rate, iron salts nature, $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratio, pH and ionic strength of the medium. Extensive studies by Sugimoto et al., and later by Vergés et al., on precipitation methods have demonstrated the influence of $[\text{OH}^-]$, or $[\text{Fe}^{2+}]$ excess over both size and morphology, as well as that of pH, with smaller and monodispersed sizes resulting of specific $[\text{OH}^-]:[\text{Fe}^{2+}]$ ratios [98, 103]. More recently, Blanco-Andujar et al. have synthesised naked-SPIONs using sodium carbonate as a co-precipitating agent, that promoted a slow evolution of the synthesis and enabled a detailed study of both the reaction and its products. An increase in the average particle size of about 10 nm per pH unit was confirmed for the magnetite phase, leading to 6.9 nm, 18 nm and 28 nm for pH 8, 9 and 10 respectively. Synthesis at pH 9 was also reported to promote a higher saturation magnetisation of 82 emu/g [100].

Aggregation and biocompatibility hinder SPIONs application in biomedical fields, and as a result, several surfactants and biomolecules have been introduced into co-precipitation formulations. As an example, Harris et al., and later Hafeli et al., have reported the use of polyethylene oxide (PEO) triblock copolymers as stabilising surfactants on the synthesis of biocompatible narrow sized SPIONs (8.8 nm) with saturation magnetisations of 34 emu/g [104]. This work has also demonstrated the influence of PEO chain-length, with PEO tails of 15K monomers reducing saturation magnetisation to only 5 emu/g and magnetite content to approx. 7% (wt%) [75]. Other stabilising or peptizing additives such as citric acid, perchloric acid, nitric acid and PEG have also been reported to highly increase SPIONs stability, monodispersity and magnetic properties [105-108]. It should be noted that saturation magnetisations obtained through co-precipitation tend to decrease with the reduction of particles size.

Additionally, the precipitation of sized controlled and monodispersed iron oxide nanoparticles in the presence of polymeric stabilisers is responsible for the separation of nucleation and growth phases, as coatings tend to inhibit the extensive growth of crystal on small SPION-seeds, after nucleation. Jain et al. proved such dependency with the synthesis of oleic acid-capped SPIONs, where higher amounts of stabiliser led to a decrease in size from 400 to approx. 30 nm, along with an increase in SPIONs stability [109].

Notwithstanding, alternative co-precipitation approaches have been recently reported. Wu et al. described ultrasound-assisted precipitation of Fe_3O_4 -based $\text{C}_{12}\text{H}_{25}\text{OSO}_3\text{Na}$ -capped SPIONs of approx. 15 nm and 75 emu/g, by an open-air acidic leaching method [110]. Attempting to avoid the usage of strong bases, Pereira et al. have recently reported a novel one-step synthesis of superparamagnetic MFe_2O_4 (where $\text{M}=\text{Fe}$, Co and Mn) using alkanolamines

bases, (i.e. isopropanolamine and diisopropanolamine). Remarkably, the resulting monodispersed SPIONs presented up to 6 times smaller particle sizes (approx. 5 nm) and 1.3 times enhanced saturation magnetisations (approx. 80 emu/g) when compared to those conventionally prepared with NaOH [111].

Although the co-precipitation method is one of the successful and classical techniques for synthesising SPIONs with high saturation magnetisation, more attention should be paid to overcoming the shortcomings of this method, such as the broad particle size distribution of products, and the utilisation of a strong base in the reaction process.

1.2.2.1.2 Sol-gel / Polyol

Based on the hydrolysis and polycondensation of molecular precursors in solution, the *Sol-Gel* method is a well-established approach for the synthesis of functional SPIONs. Briefly, it involves the formation of a stable colloidal solution, by the hydrolysis of precursors, that transmutes from discrete particles (sol) to a three-dimensional polymeric network (gel), through electrostatic interactions, hydrogen bonds or even covalent bonds. The gelation process is thus determined as being reversible, or irreversible, by the type of interactions between colloidal particles.

Similarly to co-precipitation approaches, typical iron-precursors include iron alkoxides and iron salts, as chlorides, nitrates, sulphates and acetates) that undergo hydrolysis followed by polycondensation. Although these reactions can be run at room temperature, further heat treatment is essential to render SPIONs with a crystalline phase. Generally, this method follows a two-step phase transformation, $\text{Fe}(\text{OH})_3 \rightarrow \text{FeOOH} \rightarrow \text{Fe}_2\text{O}_3$ [103, 112, 113].

As described by Sugimoto et al., resulting SPIONs are highly dependent on the structure created during the sol stage. Large magnetite, maghemite and hematite SPIONs were synthesised from a $\text{FeOOH-PO}_4^{3-}/\text{SO}_4^{2-}$ suspension, whose ratio was proven to modulate both shape (spherical or ellipsoidal) and size, in the presence of $\alpha\text{-Fe}_2\text{O}_3$ seeds. Phase transmutation, to magnetite or maghemite, was achieved by further ageing in either open or inert atmosphere [113].

However, sol-gel formulations are known as synthesising aggregated nanoparticles, an issue only surpassed by surface functionalisation. Dong et al. were the first to propose a modified sol-gel approach, by using ethylene glycol during sol formation with FeCl_3 , with whom HCl (a by-product of iron precursor hydrolysis) reacts to form PEG. The resulting PEG acts here as capping and stabilising agent of the as-synthesised SPION [112]. Woo et al. have also described a water/oleic acid sol-gel mediated method for the synthesis of Fe_2O_3 nanorods. The diameter and length of the nanorods were controlled by the H_2O :oleic-acid

ratio in the gelation process and the phase of the nanorods could be controlled by the temperature, atmosphere, and hydrous state of the gels during crystallisation [114].

Recently, Zhang et al. have reported a general ultra large-scale sol-gel method for the synthesis of monodispersed metal ions-doped (e.g. La^{3+} , Ti^{4+} and Y^{3+}) $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles, at low temperatures. Here, iron precursors were previously boiled in ethanol to achieve full hydrolysis, followed by the addition of metal ion precursors and propylene oxide, as a gelling agent and latter stabiliser. Metal ions inclusion allowed for the modulation of $\text{Fe}_3\text{O}_4 \rightarrow \gamma\text{-Fe}_2\text{O}_3$ phase transformation. SPIONs with an average size of 5 nm with narrow distributions were obtained, yielding in a single reaction of as much as 60 g [115].

Also recognised as an inversed sol-gel method, the polyol method uses reduction reactions, rather than oxidation reactions typical of sol-gel approaches. Polyols have a dual functionality during the reaction, serving as both reducing and stabilising agents, controlling particle growth and preventing aggregation phenomena. As detailed by Cai et al., iron precursors (e.g. $\text{Fe}(\text{acac})_3$) are suspended in a liquid polyol, such as ethylene glycol (EG), diethylene glycol (DEG), triethylene glycol (TREG) and tetraethylene glycol (TEG). Reduction of iron precursors occurs during the boiling of the polyol solution, to later form monodispersed Fe_3O_4 nanoparticles of narrow distribution, with average sizes of 7.2 nm, saturation magnetisations of 69 emu/g and good aqueous dispersibility [116]. The process occurs at argon atmosphere.

Conversely, Hachani et al. have recently reported the synthesis of biocompatible SPION through high pressure and temperature *via* TREG-mediated polyol route. Size and morphology were modulated by varying solvents, the amount of iron precursor and reaction times. This process yields a narrow small particle size distribution (average of approx. 8 nm), with particles of good crystallinity and with a high saturation magnetisation of 85.4 emu/g. Water stability and dispersibility were achieved by polyol displacement for 3,4-dihydroxyhydrocinnamic acid (DHCA) and tartaric acid (TA) [117].

Despite late improvements and high water dispersibility in either aqueous or polar solvents, allied to high crystallinity and saturation magnetisation, sol-gel and polyol methods still use high energy processes that sum to the numerous control variables.

1.2.2.1.3 Thermal decomposition

Also known as pyrolysis, thermal decomposition is an extremely accurate technique responsible for the formation of highly crystalline and monodisperse SPIONs, with controllable size and shape. Against the traditional co-precipitation method, the decomposition of organometallic iron precursors at high temperatures (i.e. approx. 200-300

°C) and in organic solvents promotes proper separation of nucleation and growth phases, while avoiding complex hydrolysis reactions [118]. Reported iron precursors include $\text{Fe}(\text{CO})_5$ [119, 120], $\text{Fe}(\text{acac})_3$ (i.e. acetylacetonate) [121-123], $\text{Fe}(\text{Cup})_3$ (i.e. N-nitrosophenyl hydroxylamine) [124], Prussian blue ($\text{Fe}_4[\text{Fe}(\text{CN})_6 \cdot 14\text{H}_2\text{O}]$) [125], iron-oleate [70], iron-urea complex ($[\text{Fe}(\text{CON}_2\text{H}_4)_6](\text{NO}_3)_3$) [126] and ferrocene ($\text{Fe}(\text{C}_5\text{H}_5)_2$) [127]. Monodispersibility is obtained through the use of many organic molecules as oleic acid, 1-octadecene, 1-hexadecene, 1-tetradecene, octyl ether, trioctylamine and oleyamine, that act as both solvents and stabilisers. Such stabilisers have been reported to delay both nucleation and growth steps by hindering the adsorption of iron atoms to the seeds and favour the formation of small SPIONs (i.e. < 30 nm).

Hyeon et al. reported the synthesis of monodispersed $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles using a $\text{Fe}(\text{CO})_5$ -oleate complex, whose size was successfully tuned by increases in the $\text{Fe}(\text{CO})_5$:oleate ratio [119]. This pathway was later improved by Park et al. when using iron chloride as a non-toxic precursor for the synthesis of monodispersed Fe_3O_4 nanocrystals with sizes of 5-22 nm. Size control was achieved by changing reaction solvents, and consequently ageing temperatures, with higher temperatures resulting in larger nanoparticles [128] (see figure 1.5). Sun et al. have also exposed the importance and role of stabilisers as possible reducing agents during thermal decomposition, allowing this process to happen at relatively milder conditions. With oleyamine acting as both strong reducing agent and stabiliser, $\text{Fe}(\text{acac})_3$ was reductively decomposed, in the presence benzyl ether, and particle size controlled by varying volumetric ratios of oleyamine and benzyl ether [123].

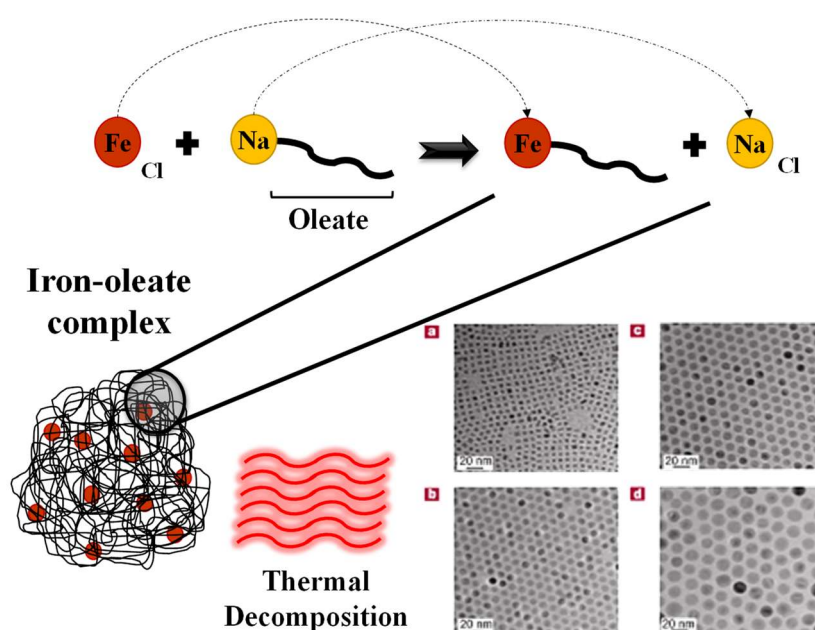


Figure 1.5 - The overall scheme for the ultra-large-scale synthesis of monodisperse nanocrystals: from metal-oleate precursors to their thermal decomposition in high boiling solvent to form monodisperse nanocrystals. Adapted with permission from [128]. Copyright 2004 Springer Nature.

The preparation of different shaped SPIONs has also been reported to strongly depend on the addition of stabilisers, as well as to precursors' and solvents' nature. Shavel and Liz-Marzán overview on the effect of specific additives (i.e. pure oleic acid, sodium oleate and tetraoctylammonium bromide - TOAB) on an iron-oleate mediated decomposition has confirmed the preparation of faceted iron oxide nanoparticles, with cubic or octahedral shapes. The use of squalene and octadecene as solvents was also reported to induce the reduction of iron precursors and lead to the formation of core-shell nanocubes, or island-like octahedrons, of metallic iron-iron oxide composite [129]. Most recently, the solventless thermal decomposition of various mixtures of ferrocene and poly(vinylpyrrolidone) (PVP) was reported by Amara et al. for the synthesis of magnetite nanocubes and nanospheres, with PVP: ferrocene weight ratio and ferrocene decomposition reaction time as determinant variables. However, the resulting nanocrystals showed low saturation magnetisations (8.2-29.7 emu/g) [127]

Although of high interest to various applications due to their strong monodispersity and reproducibility, SPIONs obtained through thermal decomposition are preferably dissolved in non-polar solvents. Posing an impediment to biomedical applications, time-consuming ligand exchanges and surface functionalisation is then indispensable to render some solubility in aqueous and biological media.

Notwithstanding, Dai et al. have recently attempted to thermally decompose $\text{Fe}(\text{acac})_3$ in tetraethylene glycol (TREG/TEG), in the presence of poly(ethylene glycol) bis(carboxymethyl) ether as a stabiliser. The resulting PEG-coated SPIONs, once treated with sodium citrate, were stable in aqueous media and relatively monodispersed with sizes of 11-15 nm and magnetic saturation of 30 emu/g, ready for biological application [121]. Sancho et al. have also reported the synthesis of SPION using TREG to avoid agglomeration problems, as well as the functionalisation by ligand-exchange with dimercaptosuccinic acid (DMSA) to render them aqueous dispersibility and biocompatibility [130].

1.2.2.1.4 Hydrothermal/Solvothermal

The hydrothermal approach is a high-temperature and/or high-pressure process, where iron precursors are dissolved in aqueous media and whose conditions are favourable for the formation of high crystalline hydrophilic SPIONs. This process is carried inside a Teflon-sealed stainless-steel autoclave, where water is in supercritical/sub-supercritical state, and Fe^{2+} ions undergo hydrolysis, forming hydroxide intermediates (FeOH_n), followed by dehydration to form the desired final iron oxide phase [131].

The solubility of iron precursors in the aqueous media, and thus their concentration, has been reported to play an essential role in seed formation and consequently on particle size. Ge et al. have described that more concentrated precursors usually lead to smaller particles due to an increased in seed formation [132]. Additionally, it has been repeatedly reported that the addition of reducing agents (e.g. ammonia), as well as the mixing of surfactant/organic molecules with the reaction media, can outstandingly improve the quality and dispersibility of the resulting SPIONs [122, 132, 133]. This approach has also allowed for in-situ SPIONs surface functionalization with the most varied type of reactive groups, such as amines, carboxylic acids, alcohols, thiols or glucose [122, 134-136]. Briefly, the control over parameters as pressure, temperature, reaction time [137] and precursor-to-surface coating ratios allow for the production of biocompatible SPIONs with various sizes, shapes and magnetic properties. Hydrothermal intrinsic long reaction times, related to slow kinetics, have however set some limitations on its usage. As a result, a microwave-assisted hydrothermal method was proposed by a few groups as a rapid, economical and improved alternative route [138, 139].

An alternative solvothermal synthesis had methanol, isopropanol, toluene and ethylene glycol reported as some alternative reactive media [131, 140-142] for the production of SPION nanoparticles. However, with poor water-dispersibility.

1.2.2.1.5 Microemulsion

Clear and stable mixtures of two immiscible phases, (i.e. water and oil) with the presence of surfactants and co-surfactants, microemulsions are one of the possible pathways for the synthesis of SPIONs. Surfactant molecules are known to form a monolayer at the water-oil interface and promote a defined limit between the aqueous phase, containing metals, salts and hydrophilic compounds, and the oil phase, containing hydrocarbons and olefins.

Microemulsions can be of two types: direct (oil dispersed in water, o/w) and reversed (water dispersed in oil, w/o); with both being used for the synthesis of SPIONs and other types of nanoparticles [143]. Commonly used surfactants include cetyltrimethylammonium bromide (CTAB), sodium dodecyl sulphate (SDS) and PVP, which are known to play significant roles in reaction dynamics, particle stabilisation and size control. Control over sizes and shapes is achieved by varying not only the iron precursor concentration but also the surfactants and solvents [143-146].

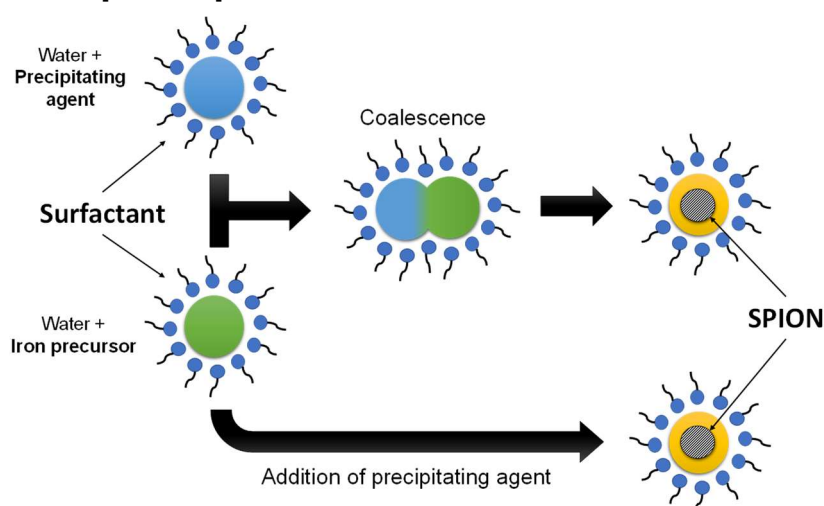


Figure 1.6 - Schematic illustration of the two w/o microemulsion synthetic approaches of SPIONs.

As demonstrated by Okoli et al., a facile and fast CTAB-mediated w/o microemulsion synthesis of spherical 7-10 nm SPIONs can follow either a two-microemulsion pathway, by the coalescence of both reactants microemulsions, or a single-microemulsion approach, by the addition of NH_3 as precipitating agent [146] (figure 1.6). Okoli et al. have also evaluated the influence of CTAB-based w/o and synperonic 10/6-based o/w microemulsions over size and shape. Thus reporting w/o SPIONs to be less monodispersed, with rod-like particles (width: 5-10 nm, length: 20-50 nm) co-existing with spherical particles (5-8 nm) when compared to o/w spherical SPIONs of an average diameter of 3 nm [143]. Magnetisation properties have also been reported to decrease with SPIONs size, with o/w particles having

saturation magnetisations of approx. 10 emu/g, smaller than those of larger w/o particles of approx. 35 emu/g.

Despite microemulsions easy control over the size and magnetic properties, as well as the presence of surfactants, aggregation is still a barrier to monodispersity, usually overcome by several washing processes and further stabilisation treatments for later biomedical application.

1.2.2.1.6 Sonochemical

A non-conventional synthesis, the sonochemical route can be considered an adaptation of the co-precipitation process, where ultrasounds are used to create cavitation bubbles to produce SPIONs. The use of cavitation-generated energy (i.e. local 5000 K, 2000 atm and 10^6 K/s) is responsible for the decomposition of iron precursors, allowing this type of reactions to be carried out at room temperatures in very short periods (approx. 30-60 min), without the use of reducing agents. However, due to such extreme local conditions, produced SPIONs are usually in the amorphous form with crystallisation only achieved by posterior sintering [147, 148]. Despite their relative low mono-dispersibility, size and shape of SPIONs can be controlled by varying refluxing and irradiation times. Well-dispersed SPIONs have, although, been reported in literature through the direct use of suitable stabilisers during synthesis [149]. Dolores et al. have described the generation of monodispersed (24–26 nm) Fe_3O_4 nanoparticles in an aqueous ethylene glycol solution, through the sole use of ferrous salts as iron sources, where Fe^{2+} oxidation to Fe^{3+} is regulated by the production of hydroxyl radicals through cavitation phenomena [150]. Particle size has also been confirmed to be frequency independent, with Fe^{3+} reduction not affecting particle formation. Most recently, a one-pot synthesis detailed by Stephen et al. was able to produce stable and biological active SPIONs, with Fe^{2+} and Fe^{3+} co-precipitating in the presence of chitosan and poly(ethylene glycol) (PEG) copolymer modified with catechol (CCP), and polyethyleneimine (PEI) modified CCP (CCP-PEI) and later binding of DNA stands for gene transfection therapy [108].

1.2.2.1.7 Electrochemical

The electrochemical pathway to synthesise SPIONs is based on the reduction and/or oxidation of an iron-based sacrificial electrode, immersed in an electrolyte. Upon the application of current, the anode is oxidised to metal ion species that are further reduced to its metal form at the cathode, where it grows until the formation SPIONs, with the assistance of stabilisers [151]. Me_4NCl and TOAB have been reported as usable electrolytes in aqueous mixtures, in ethanol or *N,N*-dimethylformamide (DMF), with the use of amine and other surfactants as stabilising species [152, 153]. As described by Cabrera et al., while particles size

is voltage-dependent, where higher voltages corresponding to smaller sizes, size distributions tend to be narrow with higher current intensities. SPIONs (Fe_3O_4) purity although can be only maintained for potentials below 6V, as high values lead to the formation of Fe impurities [152]. SPIONs obtained through this method are usually obtained in the form of a coating or thin film, both of high interest for coatings, electronics and materials industries.

1.2.2.2 Aerosol/vapour phase routes

Gas phased pathways are based on the evaporation of large-sized grained iron containing FeO , Fe_2O or Fe_2O_3 , for the formation of nanosized particles through supersaturation phenomena. As described in various protocols, precursors decomposition is carried in proper reactors using different energy sources, varying from flame to laser or plasma. Thus, resulting in distinct technologies [154].

Phase, size, shape, purity and magnetic properties of as-synthesized SPIONs can be regulated by varying a series of parameters, such as O_2 content, temperature and pressure for flame reactors [155, 156]; aerosol conditions and oxidizers/stabilisers proportion for laser reactors [157]; and the injection point and plasma gas for plasma reactors [158]. Pure and mixed $\gamma\text{-Fe}_2\text{O}_3$, $\alpha\text{-Fe}_2\text{O}_3$ and Fe_3O_4 have all been reported in the literature. However, highly aggregated SPION have been obtained by any of the techniques, with usually lower saturation magnetisations (approx. 40 emu/g), than those obtained by chemical approaches.

1.2.3 Biological Routes

To avoid the aforementioned issues that arise from both physical and chemical methodologies, as high production costs, use of toxic reagents and production of hazardous by-products, several reports have been recently published with the development of novel, greener and sustainable synthetic pathways; reviewed and thoroughly described elsewhere [159].

These green syntheses bring numerous benefits, such as simple, fast and straightforward manufacturing procedures, as well as economic and less waste productive technologies.

Green biosynthesis of nanoparticles employs bottom-up approaches where metal atoms, from metal precursors, assemble to form nanoparticles in green substrates. The biological compounds present in green substrates are expected to act as both reducing and covering agents that can stabilise nanoparticles during the synthesis process. Thus, controlling the

final size, shape, dispersibility and biocompatibility of nanoparticles, when conjugated with a series of controlled parameters as temperature, pressure, pH and incubation times. So far, green biosynthesis of SPIONs has been reported to span from microbial-based [160] to plant-based procedures (fungi included) [159].

Bacteria-mediated synthetic pathways have been using magnetotactic and iron reducing bacteria to produce, intracellularly or extracellularly, single domain iron oxide nanoparticles under anaerobic conditions. With Bharde et al. reporting the use of *Actinobacter* sp. for the extracellular tuneable synthesis of maghemite and iron sulphide nanoparticles [161] and, most recently, Abdeen et al. successfully synthesising Fe₃O₄ nanoparticles using *Aspergillus niger* YESM 1 in supercritical ethanol, with saturation magnetisations of ~ 68 emu/g [162]. It should be noted, however, that intracellular techniques usually require posterior treatments, as calcination, sonication and detergent usage, to eliminate the excess of capping biomolecules.

Plant-based methods have, however, used a series of different substrates, from callus to leaf to fruit and even seed extracts, with the successful production of a series of Fe₃O₄ nanoparticles with saturation magnetizations between 13–70 emu/g (at room temperature) and average particle sizes of but tens of nanometres, entirely suitable for future bio applications as drug delivery and cancer cell targeting [159].

1.2.4 SPIONs surface modifications and applications

It has been long perceived that a successful application of SPIONs' full properties in biological media is directly dependent on SPIONs surface conditions (i.e. capping agent), despite their natural appetite for aqueous media, biocompatibility and biodegradability. Several of the above-mentioned synthetic routes, be it for good dispersibility or uniform transformation of iron oxides phases, have introduced numerous surface modifications on their formulations [28, 82]. Notwithstanding, additional surface functionalisation have later been proposed with several materials, spanning from silica shells (e.g. non- or mesoporous silicas) [163-165], to polymers [72, 134, 166], small molecules (e.g. dyes) [76, 167, 168], polysaccharides [108, 122, 169], drugs [77, 166], biomolecules (e.g. proteins, enzymes and DNA) [101, 130] and metals (e.g. Au and Ag) [101, 170] (figure 1.7; table 1.2).

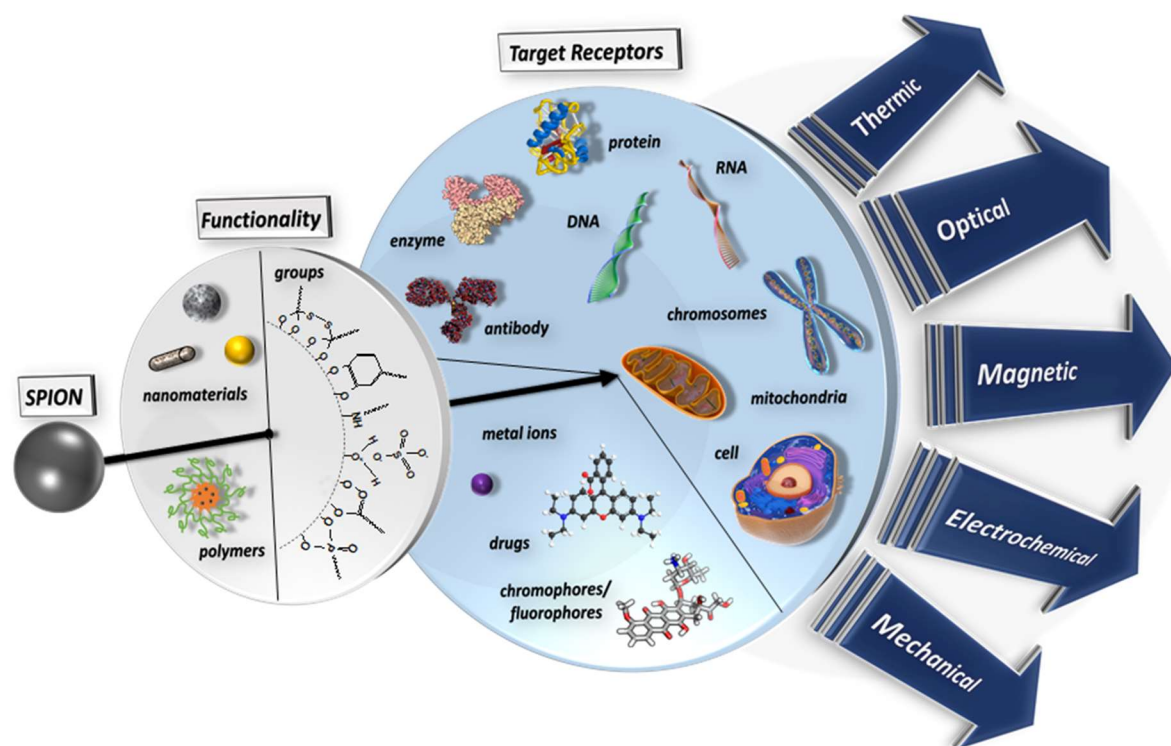


Figure 1.7 - Schematic depicting SPIONs' available surface modification, numerous target receptors and respective feedbacks.

1.2.4.1 Silica-based coatings

Silica has been reported as an outstanding inert material for the encapsulation of numerous types of nanoparticles due to its insulating properties and optical transparency, as well as its biocompatibility, biodegradability and appetency to form both compact or porous structures [171].

For magnetic nanoclusters or individual particles, the encapsulation can prevent SPIONs agglomerating into large chunks, while providing a useful platform for further surface chemistry and further introduction of polymeric or biological active capping layers. Several works have been reported to use such approach on the development of various magnetic nanosystems, from drug delivery [76, 167, 172], to targeted cancer therapy [71, 77, 163, 165], MRI (i.e. cancer or stem cells signalling) [13, 70, 173] and heavy ions removal [18]. With the resulting materials ranging from mesoporous to compact silica shells and possessing saturation magnetisations spanning from c.a. 2 to 60 emu/g (see figure 1.8).

Table 1.2 - Summarising table of all SPION synthetic routes and surface modifications, along with their magnetic properties and applications

Core synthesis	Core / shape	Coatings	Size [nm]	Sat. Mag. [emu/g]	Application / Aim	Reference
Sol-Gel/Polyol	Fe ₃ O ₄ / spherical	SiO ₂ (TEOS)@APTES@FA-PEG	13	15.1	Carboxylate Quercetin targeted drug delivery to HeLa, MCF-7 and A549 cancer cells	[71, 163]
		Diethylene glycol (DEG)	5.8	68		
	Fe ₃ O ₄ , γ-Fe ₂ O ₃ / spherical	Triethylene glycol (TEG)	9.1	84.5	MRI contrast agents for applications such as stem cell tracking or cancer cell-targeted imaging	[117]
		Tetraethylene glycol (TREG)	13.9	79.1		
		Dihydroxyhydrocinnamic acid	154 [†]	84.1		
		Tartaric acid	193 [†]	85.4		
Thermal decomposition	Fe ₃ O ₄ / spherical	SiO ₂ (TEOS)	8.8-12	2-30	-	[164]
	Fe ₃ O ₄ / cubic	PEG(300)-galloI	-	-	Magnetic hyperthermia and photothermal bimodal treatment of SKOV3 (ovarian cancer), PC3 (prostate cancer) and A431 epidermoid cancer) cells	[72]
	Fe ₃ O ₄ / cubic	poly-(maleic anhydride alt-1-octadecene)	19	78-95		[73]
		PEG	(12-30)		Magnetic hyperthermia	
	Fe ₃ O ₄ / spherical	Uncoated	10	58.6	Treatment of Antibiotic-Resistant Biofilms	[170]
		Dimercaptosuccinic acid@(Ag, Zn, Fe)		(91.5, 22.6, 23.9)		
	Fe ₃ O ₄ / spherical	TREG@COOH	15.7		Test immunoreactivity of antibody particle conjugates by an enzyme-linked immunosorbent assay (ELISA)	[130]
		TREG@COOH@As143/As167	-	64		
		DMSA	13.5			
		DMSA@As143/ As167	-			
	Fe ₃ O ₄ / spherical	Dimercaptosuccinic acid	15	63.3	<i>In vitro</i> and <i>in vivo</i> bioimaging of human myoblasts	[84]
	Fe ₃ O ₄ / spherical	Mesoporous silica	25-95	48.3-47.1	Highly efficient, nontoxic MRI T ₂ contrast agent with tuneable proton relaxivities	[70]
	Fe ₃ O ₄ / spherical	Amphiphilic polymer	30	-	Cancer cell separation in fresh whole blood	[174]
		Amphiphilic polymer@anti-HER2	40	-		
	Fe ₃ O ₄ / spherical	Uncoated	12	32	Antibacterial activity in bacterial biofilms through deep penetration nanoparticles and upregulation of ROS species,	[183]
		Dextran-Eb (isocyanate)@Ag	18	24		
		Dextran-Poly-L-histidine (PHL)@Au	27	15		
		Dextran-PLH@Au@DEI@Ag	21	16		
Co-precipitation	Fe ₃ O ₄ / -	PEO-polyurethane-PEO triblock copolymer (0.75K-3-0.75K to 15K-3-15K)	8-10	5-34	Uptake and <i>in-vivo</i> toxicity assessment in PC3 and C4-2 cancer cells, human umbilical vein endothelial cells (HUVECs), and human retinal pigment epithelial cells (HRPEs)	[75]
	Fe ₃ O ₄ / spherical	APTMS@Rhodamine 610	13	71.2-67.2	Drug delivery	[76, 167]
		SiO ₂ @coumarin-chlorambucil conjugate (7-HC-Cbl)	9	-	Controlled delivery of anticancer drug chlorambucil and cell luminescence imaging	
	Fe ₃ O ₄ / spherical	SiO ₂ @APTES@Poly(2-Vinyl-4,4-dimethylazlactone)@L-asparaginase	12	73.1-46.4	Preparation of L-asparaginase reactor for acute lymphoblastic leukaemia treatment	[14]
	Fe ₃ O ₄ , γ-Fe ₂ O ₃ / spherical	Uncoated	10-20	-	Antimicrobial activity against <i>Bacillus subtilis</i> (Gram-positive) and <i>Escherichia coli</i> (Gram negative)	[178]
	Fe ₃ O ₄ / spherical	Chitosan				
	Fe ₃ O ₄ / spherical	Tetramethylammonium hydroxide	15-30	25	Aqueous removal of Cr ⁶⁺ and Pb ²⁺ ions	[78]

Adapted from GA. Marcelo et al.

Mat. Sci and Eng: C 106, 2020, 110104

	Fe ₃ O ₄ / spherical	APTES	20-25	59	Temperature and pH dual responsive core-shell nanoparticles, comprising smart polymer shell-coated on magnetic nanoparticles, as an anti-cancer drug carrier and cancer cell-specific targeting agent	[77]
	Fe ₃ O ₄ / spherical	APTES@PNIPA-b-PAA	240 / 280	48	Dual cancer therapy and magnetic resonance imaging	[166]
	Fe ₃ O ₄ / spherical	Oleic acid@PLGA@PEG@DOX/PTX	35	63.1	Resonance imaging (MRI) contrast enhancement and efficient gene transfection	[108]
	Fe ₃ O ₄ / spherical	Chitosan@CCP	72	51.7		
	Fe ₃ O ₄ / spherical	Chitosan@CCP-PEI	54	-		
	Fe ₃ O ₄ / spherical	Chitosan@CCP-PEI@DNA(10:1)	20	71	Fluorescence/magnetic resonance bimodal imaging of tumour <i>in vivo</i>	[30]
	Fe ₃ O ₄ / spherical	Undecenoic acid	200	141		
	Fe ₃ O ₄ / spherical	Undecenoic acid@PSi	18.5	-	Lung cancer imaging	[168]
	Fe ₃ O ₄ / spherical	PEG@FA@Cy5.5	40-70	2.0-2.7	Magnetic resonance imaging or biomolecules encapsulation	[173]
	Fe ₃ O ₄ / spherical	Mesoporous silica	400-30	86.1	Drug carrier system for systemic administration of water-insoluble drugs while simultaneously allowing magnetic targeting and/or imaging	[109]
	Fe ₃ O ₄ / spherical	Oleic acid	200	88.8		
	Fe ₃ O ₄ / non-spherical, composite	Oleic acid@Pluronic F127@DOX	20.000-300.000	5-42	Magnetic probe for the immobilization of biomolecules	[185]
		PEG				
		PAA				
Sonochemical	Fe ₃ O ₄ / spherical	Ethylene Glycol (EG)	21-23	65-81	Study Fe ²⁺ oxidation via sonochemical method and development of green emitting scavenger/probe for biotechnological application	[150]
		EG@folate	24-26	60-93		
		EG@folate@cisplatin	29-31	62-69		
Microemulsion	Fe ₃ O ₄ / hollow spheres	DOX	300	-	AC Magnetic field responsive drug release	[184]
	Fe ₃ O ₄ / spherical	(w/o) CTAB	7-10	30	Protein purification	[143, 146]
	Fe ₃ O ₄ / spherical	(o/w) Synperonic 10/6	2-3	10		
	Fe ₃ O ₄ / spherical	SiO ₂	91	-	Targeted chemotherapy	[165]
Hydrothermal	Fe ₃ O ₄ / spherical	SiO ₂ @Y ₂ O ₃ /Yb, Er@DOX	115	1.28		
	Fe ₃ O ₄ / spherical	Au@DNA	150	-	Simultaneous Detection of Ag ⁺ and Hg ⁺ ions	[101]
	Fe ₃ O ₄ / spherical	PEI	21.8 [†] , 310 [†]	-	Development of hemocompatibility and rich amine conjugation, for various biomedical applications, especially for magnetic resonance imaging and therapy.	[134]
	Fe ₃ O ₄ / spherical	PEI@PEG	18.7 [†] , 162 [‡]	-		
	Fe ₃ O ₄ / spherical	PEI@Acetyl anhydride	161.8 [‡]	-		
	Fe ₃ O ₄ / spherical	PEI@Succinic anhydride	217.6 [‡]	-	Targeted tumour MR imaging	[135]
	Fe ₃ O ₄ / spherical	PEI@FI@HA _{6K/13K}	15-16	-		
	Fe ₃ O ₄ / spherical	Glucose/Gluconic acid	4-16	14.8-29.6		
Biological	Fe ₃ O ₄ / spherical	Uncoated	15-31	53.3-97.4	-	[122]
	Fe ₃ O ₄ / spherical	Uncoated	15-31	53.3-97.4	-	[132]
Biological	Fe ₃ O ₄ / spherical	EPS of <i>Klebsiella</i> sp. J1	50	79	Simultaneous redox sorption and recovery of silver ions as silver nanoparticles	[176]

* TEOS – tetraethyl orthosilicate; DEI - disulfide ethanediybis-(isonicotinate); FA – folic acid; APTES - aminopropyl triethoxysilane; APTMS - 3-aminopropyl trimethoxysilane; PNIPA-b-PAA - poly(N-isopropylacrylamide)-block-poly(acrylic acid); PEG – polyethylene glycol; PAA – polyacrylic acid; DOX – doxorubicin; PTX – paclitaxel; CCP – PEG@catechol copolymer; CCP-PEI – PEG-catechol@polyethylenimine; PSi – porous silicon; FI - fluorescein iso-thiocyanate; HA - hyaluronic acid; EPS – Extracellular polymeric substance. † size obtained by TEM; ‡ size obtained by DLS – hydrodynamic size.

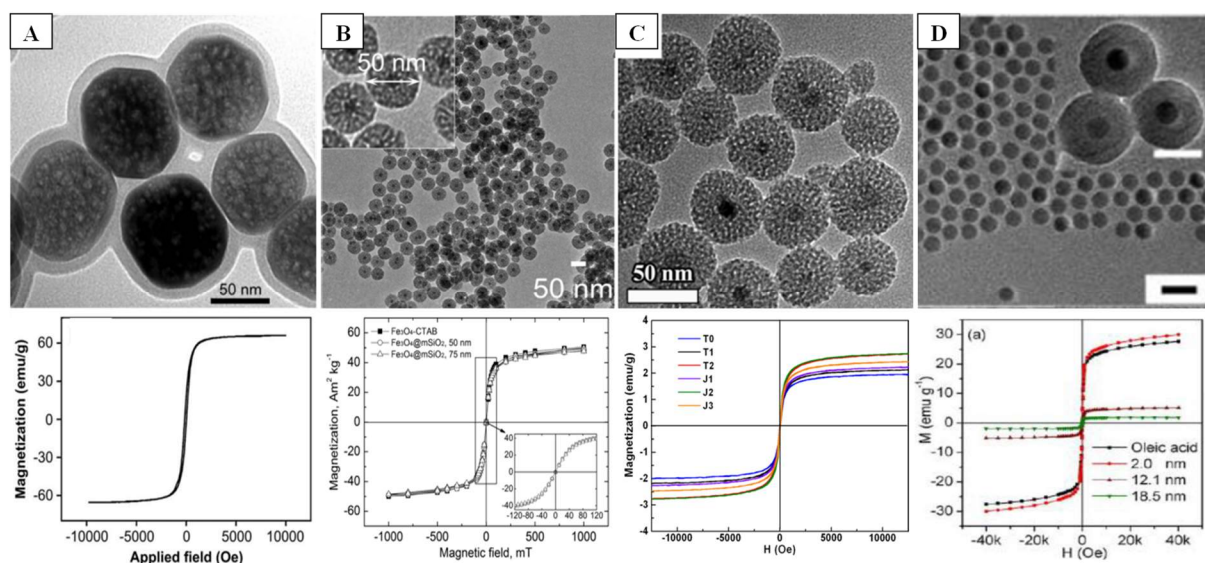


Figure 1.8 - top: (A) TEM image of monodisperse superparamagnetic single-crystal magnetite NPs with mesoporous structure; bottom: respective saturation magnetization curve. Adapted and reproduced with permission from [172]. Copyright 2008 Elsevier B.V.. (B) TEM micrographs of core-shell SPIONs of 50 nm; bottom: field-dependent magnetization saturations of aqueous suspensions of Fe₃O₄-CTAB and Fe₃O₄@mSiO₂ NPs. Adapted and reproduced with permission from [70]. Copyright 2012 WILEY-VCH Verlag GmbH & Co. KGaA. (C) top: TEM image of M-MSN samples synthesized with a TMB/CATB molar ratio of zero; bottom: Field-dependent magnetization curves at room temperature of M-MSN samples with varied pore swelling agent/CTAB ratios. Adapted and reproduced with permission from [173]. Copyright 2011 Elsevier B.V.. (D) top: TEM images of Fe₃O₄ NPs with sizes of 10.1 nm (scale bar = 20 nm); bottom: field-dependent magnetization to different shell thickness NPs. Adapted and reproduced with permission from [164]. Copyright 2012 American Chemical Society.

The growth of silica shells can happen either during SPION synthesis or after, over as-synthesised SPIONs. The latter being by far the most commonly used, with an initial full growth and formation of SPIONs, and prior surface modification and silica growth. As an example, SPIONs obtained by thermal decomposition have been functionalised by a silica-shell through the well-known Stöber method, after phase transfer, as described by Akal et al. [71]. In this work, tetraethyl orthosilicate (TEOS) was used as a silica source, and silanization occurred under ultrasonic conditions. Further surface modification by 3-aminopropyltriethoxysilane (APTES), rendered the system conjugation capacity with a folic acid-modified PEG for better cell internalisation and drug delivery (quercetin) [71, 163].

The former, however, has been reported for microemulsion or micelle-based SPION synthetic routes, where iron oxide crystal grows inside micellar structures that serve as a template for further silica deposition and shell growth. Zhang et al. have reported the synthesis and in-situ encapsulation of Fe₃O₄ SPIONs with a silica shell through a modified w/o microemulsion method, where CTAB was used as surfactant and TEOS as a silica source [173]. 1,3,5-trimethylbenzene (TMB) and decane were used as swelling agents, allowing pore

size modulation and ethylene glycol as TEOS hydrolysis retardant (see figure 1.9A). Resulting silica-coated SPIONs presented small particle sizes (40-70 nm), tunable pore sizes (3.8-6.1 nm) optimal for the loading of biomolecules and/or active drugs. Unfortunately, low saturation magnetisations were obtained with values of 2.0-2.7 emu/g.

Due to the high interest in such structures, Ding et al. reported a detailed study on coating regulations of Fe_3O_4 nanoparticles, by reverse microemulsion. In their work, they reveal that the ammonia to Igepal CO-520 (surfactant) ratio determines the size and number of aqueous domains and consequently, the size of the resulting $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles (figure 1.9B). Silica shell thickness was also described to be controlled by either the fractioned increment of TEOS content or by the increase of ammonia accompanied by a decrease of TEOS amount. The authors also emphasise that to ensure the inexistence of core-free SiO_2 particles, TEOS concentration must fulfil the condition $C_s < C < C_{\text{homo}}$ throughout the reaction process by fractioned drop addition [164] (figure 1.9C).

Silane species (e.g. APTMS, APTES, etc.) have also been widely reported in the literature to render SPIONs stability, water-dispersibility and functionality [71, 76, 163, 167].

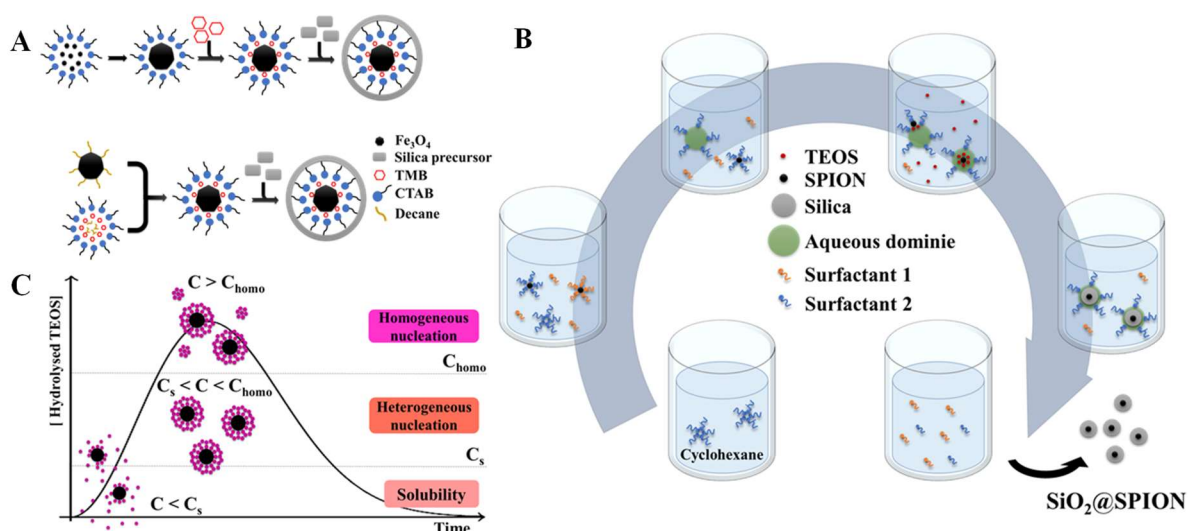


Figure 1.9 - (A) Schematic illustration of the proposed mechanisms for the growth and pore modulation processes of silica/CTAB mesophase on SPIONs surface, in the presence of different pore swelling agents (TMB and decane). (B) La Mer-like diagram: hydrolyzed TEOS (monomers) concentration against time, on homogeneous nucleation and heterogeneous nucleation, and its role on the existence of $\text{Fe}_3\text{O}_4@\text{SiO}_2$ core/shell and core-free SiO_2 in the reaction at any moment. Adapted with permission from [164]. (C) Illustration of a o/w coating mechanism of SiO_2 on the surface of already synthesized SPIONs. Adapted with permission from [164]. Copyrights 2012 American Chemical Society.

Recently, however, more exotic nanocomposites have been developed, with Omar et al. reporting the synthesis of ultra-large mesoporous silica@iron oxide nanovector for imaging (MRI), high protein loading, magnetothermal release and delivery in cancer cells. The

resulting materials had diameters of about 130 nm, with pores from 20-60 nm, ideal for the loading of large amounts of biomolecules (figure 1.10). Water stability and biodegradability were also studied, with nanoparticles showing good to medium stability in aqueous media, with consequent visible swelling effect (i.e. size increase from as-synthesised 129 nm to 270 nm). Immersion in protein-containing media led to the natural degradation of the nanovectors, supporting the non-toxicity and further use in *in vitro* and *in vivo* systems [17].

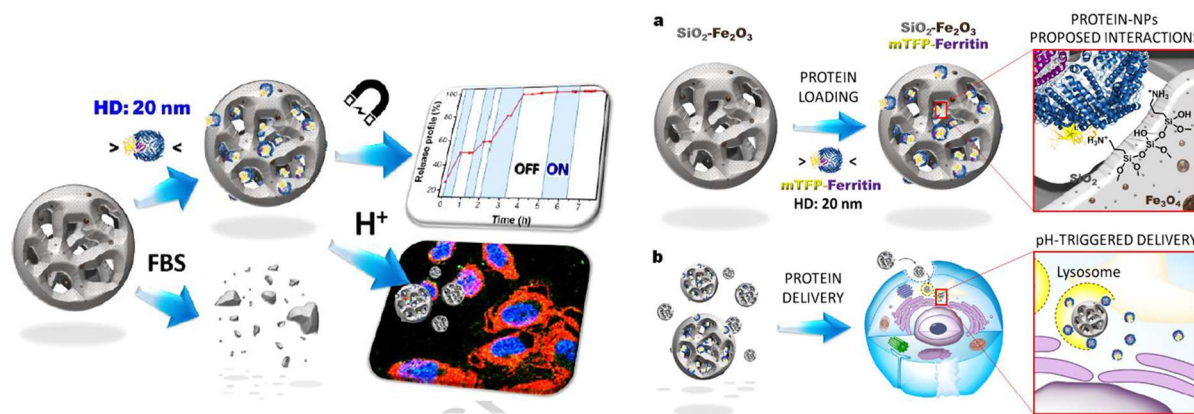


Figure 1.10 - Illustration of ultra-large mesoporous silica@iron oxide nanovector for imaging (MRI), high protein loading, magnetothermal release and delivery in cancer cells (left). Detailed schematics on protein (mTFP-Ferritin) loading onto nanovectors' pores (right-a); and delivery function and mechanism of SiO₂-Fe₂O₃ mTFP-Ferritin nanovectors (right-b). Reproduced from [17]. Copyright 2016 Elsevier B.V.

1.2.4.2 Polymeric coatings

Along with silica shells, the polymeric functionalisation of SPION surfaces has been one of the most used coatings approaches rendering monodisperse SPIONs. Multiple formulations have either seen as-synthesised modified SPIONs or silica shells modified by polymers. Polymers introductions have also worked itself as a support for later functionalization, or substitution, with active biomolecules for numerous biomedical applications [123, 136, 168, 174].

Again, SPIONs modification with can be obtained in two ways: 1) in-situ, with polymers being added during SPION synthesis; or 2) post-modification of as synthesised SPIONs.

For instance, a sol-gel/polyol approach reported by Hachani et al. produced DEG, TEG and TREG-capped SPIONs with outstanding magnetic properties for MRI stem cell tracking or cancer cell-targeted imaging. The same authors also reported the fast and facile ligand exchange of TEG-coated SPIONs with DMSA and tartaric acid [117]. A similar approach, following a thermal decomposition synthetic route of TREG-coated SPIONs, was detailed by Miguel-Sancho et al.; with further ligand exchange for DMSA [130].

Conversely, Guardia et al. have described a post-synthesis modification of the cubic dodecane-SPIONs surface with a first poly (maleic anhydride-alt-1-octadecene) polymeric coat (PC), followed by a second PEG-shell, in water. Resulting SPIONs, with hydrodynamic sizes spanning from 40-60 nm, presented high saturation magnetisations of 80 emu/g and outstanding cell-internalisation and effective hyperthermal and photothermal behaviour. The authors have also reported the intrinsic cytotoxicity of the systems, with PC-SPIONs showing higher cytotoxicity attributed to the partial flocculation in the cellular medium and PEG-SPIONs presenting none or irrelevant cytotoxicity [73]. Folic acid (FA)-PEG-coated SPIONs have also been described to provide the optimal linkage of labelling-agent Cy5.5, for lung cancer therapy [168], and of hyaluronic acid (HA), for targeted tumour MR imaging [135]. Some groups also reported the use of polyvinyl acid (PVA) [136] and polymethyl methacrylate (PMMA) [15] as suitable coatings for the biological application of SPIONs.

The use of block-polymers, or copolymers, has also been reported in the literature. Polyethylene oxide (PEO) tail block copolymers (PEO-polyurethane-PEO) have been used to the development of magnetic probes for the uptake and *in vivo* toxicity assessment in PC3 and C4-2 cancer cells, human umbilical vein endothelial cells (HUVECs), and human retinal pigment epithelial cells (HRPEs) [75] (see figure 1.11A). The authors report the binding of the carboxylic acid moieties onto the iron oxide nanoparticle surface and steric stabilisation of the particles in cellular media through the extension of the hydrophilic PEO tail blocks. The extension of such PEO blocks has likewise been correlated to SPION systems cytotoxicity, with longer PEO tails (15K-3-15K) presenting lower toxicity and higher cell internalisation. However, saturation magnetisations are severely affected, dropping to only 5 emu/g. Amphiphilic copolymers have gained huge attention on the development of hydrophilic SPIONs, with Xu et al. reporting a highly efficient process for a SPION-based immunomagnetic separation of tumour cells from fresh whole blood. It involved the functionalization of oleic acid-capped SPIONs with pluronic F-127, later modified with anti-HER2 or anti-HER2/neu antibodies [174] (figure 1.11B). The same approach was taken by Jain et al., by developing F-127-capped SPIONs for the delivery of anticancer drugs (e.g. doxorubicin) [109] (figure 1.11C). Co-polymers were also used by Hickey et al., who demonstrated how poly(acrylic acid)-polystyrene block-copolymer (PAA-b-PS) can be used to modulate the shape of self-assembling magnetic nanoparticles, ranging from micelles to vesicles [175] (figure 1.11D).

Most recently, the pursuit for greener and straight-forward approaches has produced a series of natural polymer-coated SPIONs, of cellular and microbial origin [159, 162, 176].

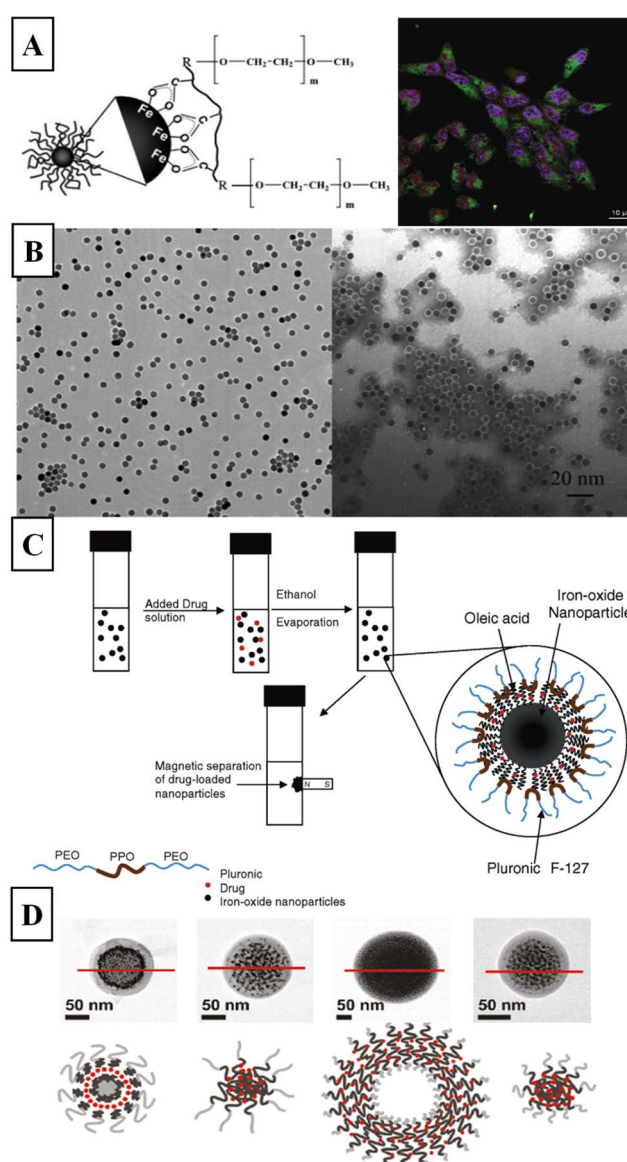


Figure 1.11 - (A) Schematic depicting the binding of polyurethane carboxylic acid moieties onto SPIONs surface, with steric stabilization of the particles in the cell culture medium by means of PEO tail blocks (*left*); confirmed by microscopy images of the uptake of SPION-PEO-PU-PEO (2K-3-2K, 30%, 5 mg/mL) by C4-2 prostate cancer cells after 8h of incubation (*right*). Adapted and reprinted with permission from [75]. Copyright 2009 American Chemical Society. (B) TEM images of the hydrophobic inorganic core of SPIONs, with diameter of c.a. 27 ± 5 nm (*left*); and of antibody conjugated biocompatible water-soluble SPION (SPION-Ab), using phosphotungstic acid for background staining (*right*). Reproduced from [174]. Copyright 2011 Elsevier Ltd. (C) Schematic representation of pluronic F127-capped SPION formulation and drug loading. Reprinted with permission from [109]. Copyright 2005 American Chemical Society. (D) Structural characterization of three different self-assembly structures: STEM images (*top*) and illustrations (*bottom*) of magneto-core shell assemblies formed in (*from left to right*) DMF/THF (96.8% DMF), THF, dioxane/THF (96.8% dioxane), and (dioxane/THF (96.8% dioxane)). The assemblies were prepared with PAA38-b-PS189 at the polymer concentration of 0.04 wt % and a nanoparticle mass percent of 27.1%. Adapted and reprinted with permission from [175]. Copyright 2011 American Chemical Society.

1.2.4.3 Polysaccharides and other molecules

Beyond, polymeric and silica coating, polysaccharides have likely gained importance on SPIONs coating due to their natural resemblance to natural sugars, and thus easier integration in biological media. Chitosan, heparin and dextran are but examples of currently used polysaccharides for SPIONs coating.

Chitosan, also known by [poly(1,4- β -D-glucopyranosamine)], is a biodegradable natural polymer, derived by the deacetylation of chitin, that due to its rich -OH and NH₂ groups structure has seen itself widely used for the modification of SPIONs surfaces. Its protective properties have been used on the development of stable MR I probes, with Shi et al. reporting a (carboxymethyl)chitosan-modified nanosystem with properties compared to the commercial Resovist®. These nanoparticles were internalised into human mesenchymal stem cells (hMSCs) via endocytosis, not showing significant cytotoxicity by MTT assay. The in vitro detection threshold of cells after incubation with 0.05 mg/mL of CMCS-SPIONs for 24 h was estimated to be about 40 cells [13, 177]. Later, chitosan was as well used by Stephen et al., as a binding site for a catechol-modified poly(ethylene glycol) (CCP-PEG) and catechol-modified cationic polyethyleneimine (CCP-PEI), for facile binding and delivery of DNA in gene therapy. The same materials were confirmed to exhibit improved magnetic resonance imaging (MRI) contrast [108]. Additionally, a new study has evaluated the use of chitosan-modified SPIONs for antimicrobial activity against Gram-positive (*Bacillus subtilis*) and Gram-negative (*Escherichia coli*) bacteria. Chitosan was confirmed to enhance ROS production on the microbial environment, hence the final antimicrobial activity [178].

Heparin is a highly sulphated, anionic glycosaminoglycan, whose anticoagulant properties and strong interactions with various proteins containing heparin-binding domains, within extracellular matrix environments. As a result, it has been widely employed in a series of engineering applications [179], with SPION-mediated drug delivery systems [180] and anticoagulant activity [181] included. Most recently, a glycol chitosan/heparin-SPION-gold nanocomposite with high tumour-targeting appetency for MRI was reported by Yuk et al. [169] (figure 1.12).

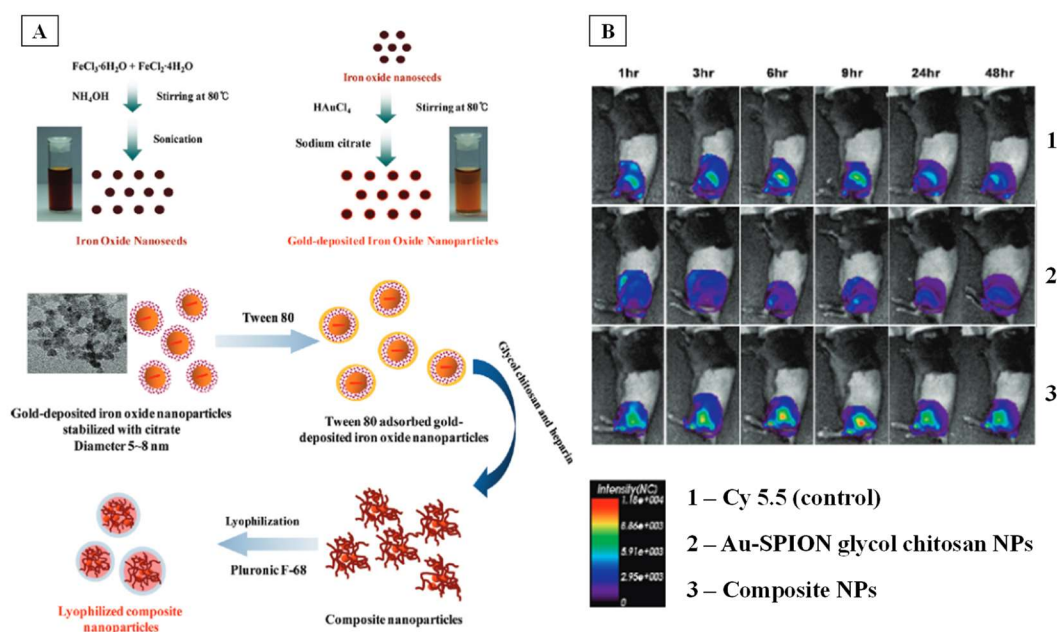


Figure 1.12 - (A) Schematics for the preparation of SPION, gold-deposited SPIONs, and gold-deposited SPION/glycol chitosan NPs with Heparin (composite NPs). (B) In vivo non-invasive NIR images and quantification analysis of Cy5.5/composite NPs with or without heparin. Adapted and reprinted with permission from [169]. Copyright 2011, American Chemical Society.

Dextran-coated SPIONs are well-established platforms for the synthesis of multifunctional imaging agents, where dextran constitutes a versatile platform for the conjugation to targeting ligands. Due to the non-covalent nature of dextran-SPION binding interactions, dissociation under specific biological conditions presents a significant drawback, only surpassed by treatment with epichlorohydrin, and later with ammonia, to crosslink the dextran coating [182]. Recently, dextran has been included on a SPION@Au@Ag nanocomposite for the development of promising easily controllable antibacterial resistance agents [183]. In a layer-by-layer approach, dextran was used as a spacer between Au and Ag layers, rendering the resulting nanoparticles with the capacity to selectively eradicate resistant bacteria, while cohabiting with healthy cells (figure 1.13).

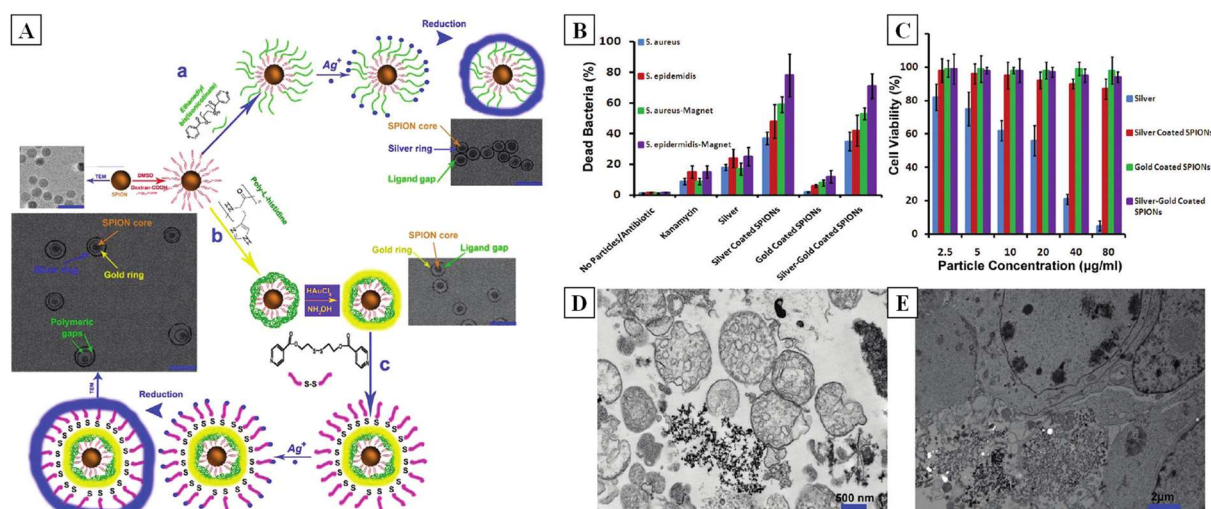


Figure 1.13 - Schematic representation of core-shell nanoparticles with polymeric gaps: (A.a) Key steps involved in the synthesis of silver ring-coated SPIONs with a polymeric gap; (A.b) key steps involved in the synthesis of gold ring-coated SPIONs with a polymeric gap as the intermediate stage; and (A.c) surface treatment of gold core-shell nanoparticles with disulfide ethanedithylbis(isonicotinate) followed by addition of silver ions. TEM panels are representative images showing the formation of monodispersed nanoparticles in various stages (scale bars 40 nm). Dual toxicology effect of multifunctional nanoparticles in bacteria and human cells: (B) Percentage of dead bacteria in 24 h staphylococcal biofilms, with and without external magnetic field, of *S. aureus* and *S. epidermidis* in the absence and presence of various particles. Experiments with kanamycin were carried out on bacteria for confirmation of strong antibacterial effects of multifunctional particles. (C) Toxicity evaluation of nanoparticles interacted with HepG2 cell lines for a period of 24 h. (D,E) TEM micrographs of HepG2 cells after 24 h of growth in tissue culture polystyrene wells in the presence of silver and silver-SPION core-shell particles (with the exactly the same concentration of nanoparticles). The electron microscopy images confirmed the highly toxic effects of silver nanoparticles on the cells, while there was no sign of toxicity for silver-SPION core-shell particles. Adapted and reprinted with permission from [183]. Copyright 2012, American Chemical Society.

Small glucose and gluconic acid molecules have also been reported on the surface functionalization of SPIONs as stabilising agents, introduced during SPIONs synthesis [122]. Reports on additional SPIONs functionalization with small molecules [78, 84] and/or biomolecules have also been found in literature, spanning from surfactants [143], to anticancer drugs [184], metallic coatings [101, 165], DNA, proteins and enzymes [101]. As well as dyes and metal atoms, which render SPION nanosystems with photothermal therapeutic properties. The vast universe of possible conjugations and modifications of SPIONs surfaces, allied to their intrinsic properties, makes this type materials one of the most studied and developed branches of nanotechnology, with numerous applications throughout diagnosis, therapeutics and theranostics (figure 1.14). Notwithstanding continued efforts should be made to keep SPIONs up to date with the developments of other scientific areas, as well as their hybridisation with other nanosystems, rendering them greener characteristics and sustainability.

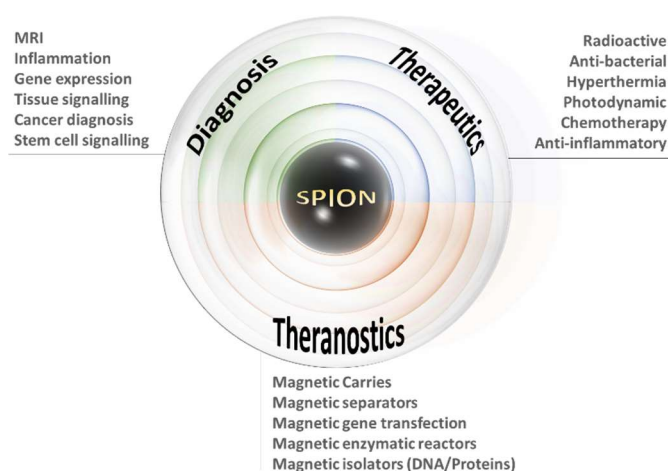


Figure 1.14 - Summarizing illustration of SPIONs main fields of application and respective applied techniques.

1.3 SiQD nanoparticles: synthesis and applications

Among all available QDs, fluorescent silicon nanocrystals, also denominated as silicon quantum dots (SiQDs), are regarded as an ideal biomedical probe. Due to its no- or low-toxicity, biocompatibility, and photostability, they have seen themselves substituting high-quality II-VI semiconductor quantum dots, and traditional fast-photobleaching dyes, on several biomedical applications. It should also be noted that silicon's extensive commercial usage and availability in nature makes it an outstandingly attractive cheap material.

Although initially thought to be solely dependent on quantum-confinement effect, with smaller sizes leading to blue-shifted emitting nanocrystals similarly to, recent theoretical studies revealed that SiQDs optical properties are significantly influenced by surface condition [186, 187]. Particularly, oxygen-mediated oxidation of the SiQDs surface often reduces the band gap and consequently limits luminescent emission, resulting in low quantum yields. As a result, numerous synthetic routes to prepare free-standing, or coated, high-quality tuneable SiQDs have been reported, with them were classified into two categories: 1) top-down approaches, including electrochemical etching of bulk silicon or silicon nanowires (SiNWs) [188], thermal decomposition of silicon-rich oxides (SiO_x) [68, 189], precursors decomposition and reassembly by laser-assisted pyrolysis and plasma-based synthesis [26, 190, 191]; and 2) bottom-up approaches, including wet-chemical reduction of silicon precursors, Zintl salts metathesis [56, 192] and microemulsion or micelle-based approaches [193, 194]. The routes mentioned above can also be divided into two main groups based on reaction temperatures, with resulting properties differing between high and low temperature prepared SiQDs (table 1.3).

Table 1.3 - Summarising table of all SiQDs synthetic pathways and reported works in literature, with respective reaction conditions and products optical properties.

Method	Si precursor	Reagents	Ligands	Solvent(s)	Temp. (°C)	Avg. size (nm)	QY [%]	$\lambda_{PL,max}$ [nm]	Ref.
MW decomposition	SiNW	N ₂	glutaric acid	water	180	3.1	15	660	[68]
laser decomposition	Si	SSO solution	DNA/RNA	water	-	3.5	0.4	450	[198]
	SiH ₄	HF ⁺ , HNO ₃ ⁺	H	N ₂	-	1-8	15	450-900	[25, 26]
aqueous reduction	APTES	N ₂ , Na ₃ (citrate)	NH ₂ C ₃ H ₆	water	m.w, 160	2.2	20-25	460	[58]
	APTES	SA	alkylamines	water	r.t.	2.0	21	530	[212]
	APTES	SA	IPTES	water	40	3.6	13	467	[211]
	APTES	AA	alkylamines	water	40	1.6	8.2	520	[10]
	APTES DAMO AEEA	N ₂ , Sodium citrate	alkylamines	water	m.w, 160	2.2	23.5	445	[57]
						3.4	82.5	450	
						3.5	6.4	440	
metathesis	NaSi	NH ₄ Br	H	DMF	r.t.	3.9	28	438	[209]
microemulsion	SiCl ₄	LiAlH ₄ , TOAB, CuSO ₄	H	toluene	r.t.	1.8 [†]	-	420, 522	[194]
	SiCl ₄	LiAlH ₄ , allyl SiCl ₃ , sonication	allyl	toluene	r.t.	3.7	2.5	375	[43, 66]
	SiBr ₄	LiAlH ₄ , TOAB	H	N ₂ , toluene	r.t.	3.0	-	-	[206]
	SiCl ₄	Na(naphthalide)	Cl	glyme	r.t.	2.0	8	405	[56]
plasma	SiH ₄	-	H	Ar	20k-50k	2-8	-	700- >800	[200]

	SiH ₄	HF [‡] , HNO ₃ [‡]	H	Ar, H ₂ , methanol	m.w., r.t.	≤ 13	-	450-735	[201]
	Si	HF [‡]	H O C	Ar	1100	- - -	- - -	800 800 740	[213]
	SiH ₄	HF [‡] , H ₂ O ₂ * Heat [†]	H	Ar, ethanol*	-	5-10	-	490*, 430 [†]	[67]
electrochemical	Si wafer	HF	H	ethanol	r.t.	~ 126	-	~ 800	[6]
	Si wafer	HF, H ₂ O ₂	H	ethanol	r.t.	2.6	-	450	[54]
	Si wafer	HF	H	methanol	r.t.	-	45-55	621	[12]

DMF - *N,N*-dimethylformamide; APTES – 3-aminopropyl trimethoxysilane; SA- sodium ascorbate; AA – ascorbic acid; IPTES – 3-isocyanatopropyltriethoxysilane; DAMO - N -[3-(trimethoxysilyl)propyl]ethylenediamine; AEEA - 3-[2-(2-aminoethylamino)ethylamino]propyl-trimethoxysilane; naphthalene – NaC₁₀H₈; TOAB – tetraoctylammonium bromide; m.w. – microwave assisted; sso – single stranded oligonucleotides; ‡ Etching of as-synthesized SiNP. * Chemical oxidation. † Thermal oxidation.

1.3.1 Top-down synthesis

In a general manner top-down synthesis depart from large-size silicon materials as crystalline silicon particles, SiO_x powders, bulk silicon or silicon nanowires (SiNWs), to produce well-defined and high photoluminescent hydrophobic SiQDs [12]. Resultants SiQDs are usually high crystalline, exhibiting quantum-confined, size-dependent photoluminescence (PL) analogous to that reported for their II-IV-based counterparts. However, besides their high temperatures and tedious procedures, most of the as-synthesised materials lack water dispersibility and stability, requiring further surface modifications with hydrophilic ligands. Notwithstanding, several improvements have been made towards aqueous top-down approaches, as is the case of electrochemical routes.

1.3.1.1 Thermal decomposition

The thermal decomposition of silica species (e.g. pure silanes, silica and organosilanes) is a common strategy for the synthesis of SiQDs. However, it integrates a more significant type of reactions, that of sol-gel. As above mentioned, sol-gel approaches have four main stages, of which thermal decomposition (also known as “ageing”) plays a crucial role in the breakdown of a jellified sol onto small particles. The adaptation of such approach to the development of a top-down route has thus been widely applied in the synthesis of SiQD.

Hessel et al. have introduced the commercially available hydrogen silesquioxane (HSQ) as a molecular precursor for the synthesis of Si-SiO₂ nanocomposites and H-capped SiQDs. The method relied on the annealing of HSQ, at high temperatures (c.a. 1100 °C) and under a reductive H₂/N₂ gas flow, followed by chemical etching of the resultant Si/SiO₂ composite, in an HF/EtOH solution, to render photoluminescence to the resulting SiQDs (see figure 1.15). The obtained SiQDs showed good monodispersibility and crystallinity, with PL spanning across the whole visible range due to confining effect. The authors also showed PL dependency on both annealing temperature and etching time [189, 195]. Water stability showed to be a major drawback, with most as-synthesised SiQDs being stabilised by organic molecules. Despite prone to properties alterations, Dasog et al. have reported a series of surface modifications over thermal decomposition obtained SiQDs to render them water solubility [186].

More recently, He et al. developed a one-pot aqueous microwave-assisted thermal decomposition methodology, where silicon nanowires, obtained by silica etching in HF solution, were decomposed in the presence glutaric acid, for 15 min at 160°C, to form 3.1 nm

ready-to-use red-PL SiQDs [68]. Addressing, thus, the water solubility issue, while giving the resulting SiQDs appetency for future bioconjugation. It should be noted that the microwave introduction allowed for shorter reaction times as well as for a more simple and straight forward methodology to control crystal growth.

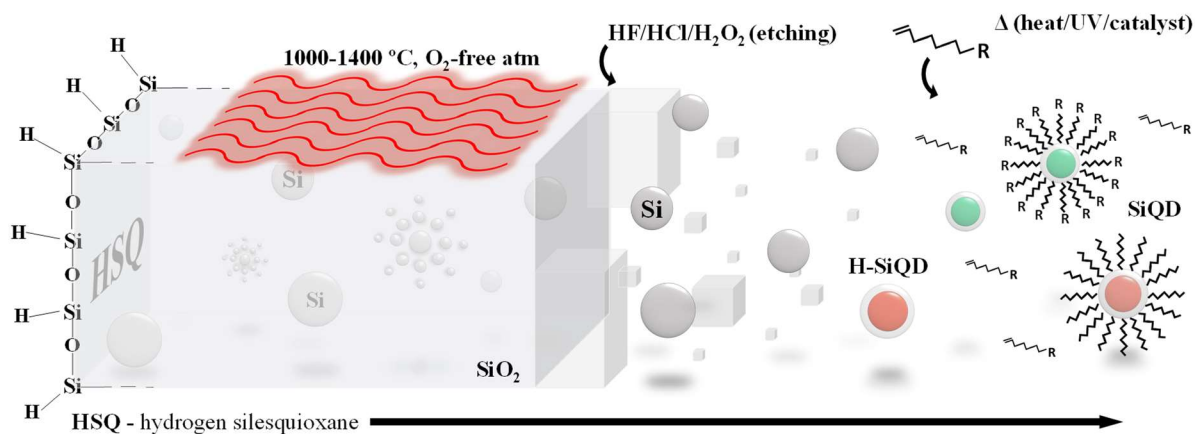


Figure 1.15 - Formation of alkyl-passivated silicon quantum dots obtained by thermal decomposition of hydrogen silesquioxane, followed by etching and later surface modification by hydrosilylation

1.3.1.2 Laser pyrolysis/ablation

Laser pyrolysis has emerged as an alternative to the conventional thermal decomposition of silica sols to the formation of freestanding silicon nanocrystals. As detailed by Li et al. [196], the nanocrystals formed through laser irradiation of silane gas undergo later etching in an HF (48%) and HNO₃ solution, in the absence of oxygen, to render the resulting SiQDs luminescence. This method has been repeatedly reported to produce H-SiQDs with luminescence spanning through the whole visible spectrum and QY of about 2-15%. Erogbogbo et al. have reported the production of SiQDs, on a scale of 30-300 mg, with sizes ranging from 2-8 nm according to determined etching times [25-27]. Most recently, as reported by Intartaglia, quantum confined SiQDs were prepared by ultrafast laser ablation of a silicon target in deionised water. The absence of reducing agents led to the production of SiQDs with sizes between 2.5-60 nm solely varying accordingly to laser pulse energies (i.e. 0.40 mJ to 0.16 mJ, respectively) [197]. Pulsed laser ablation synthesis of SiQDs, in liquid, was also reported by the same group to be carried in the presence of oligonucleotides solutions to render them excellent water stability and biocompatibility. DNA and RNA presence was confirmed to directly influence both size and size distribution (3.5 nm), as well as PL (QY of 0.4%) [198].

Despite the recent advances and ability to produce SiQDs in the mg-g scale, agglomeration still poses a strong drawback, along with the need for expensive, specific and high energy-requiring equipment.

1.3.1.3 Plasma/microplasma-based synthesis

A pure gas-phase approach, the use of plasmas for the synthesis of SiQD from Si-gaseous precursors has been thoroughly investigated and described in detail elsewhere [191, 199]. Briefly, SiH₄ gas, in an inert atmosphere, undergoes pyrolysis, with further nucleation and growth of purely crystalline silicon (see figure 1.16A). Photoluminescence is rendered by consequent etching under acidic environment, usually using hydrofluoric acid (HF) and/or H₂O₂ and HNO₃ as oxidising agents. This approach has been reported by Mangolini et al., using radio-frequency based plasmas on the synthesis of red emitting SiQDs with sizes in the range of 2-8 nm [200]. Gupta et al. have however reported the use of microwave- induced plasmas for the fast synthesis of H-passivated spherical SiQDs, in low-pressure flow reactors (figure 1.16B). Their method allows for direct sampling of as-synthesised silicon nanoparticles and tailoring of SiQDs, by controlling the total gas pressure, SiH₄ concentration and microwave power. Despite their single and high crystallinity, the resulting quantum confined SiQDs presented a 1-2 nm SiO₂ amorphous shell and relatively agglomerated clusters. Emissions were reported as etching time-dependent, spanning from blue (456 nm) to red (735 nm) emitting SiQDs. The authors have also confirmed the influence of O₂ exposure over emission, inducing a total blue shift between 4-24 hours of exposure, accompanied by the increased formation of SiO₂ [201]. More recently, Fan et al. have reported the use of atmospheric plasmas on the synthesis of water stable blue and green emitting SiQDs, thermally etched in the presence of HF and with the aid of ultrasounds [67].

Despite the straight-forward and reproducible synthesis [202], optimal for industry and electronics [191], plasma-induced synthesis still stands as an expensive route, synthesising relatively agglomerated SiQDs.

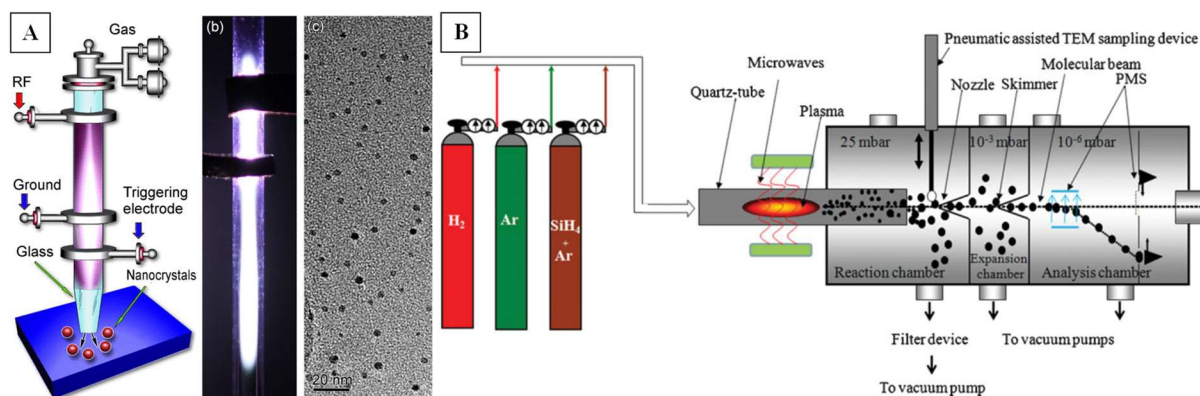


Figure 1.16 - (A) 'Two-ring' configuration used for the synthesis of SiQDs: (*left*) Schematics of a microplasma reactor; (*center*) photo of the microplasma generated inside the quartz capillary; (*right*) TEM image of microplasma generated SiQDs. Reproduced with permission from [199]. Copyright 2014 AIP Publishing. (B) Schematics of the experimental setup for the synthesis of Si-NPs in a microwave plasma reactor. Reprinted with permission from [201]. Copyright 2009 WILEY-VCH Verlag GmbH & Co. KGaA.

1.3.1.4 Electrochemical etching

Electrochemical etching is a conventional top-down methodology for the synthesis of SiQDs, although separated from the afore-mentioned synthetic routes for running in an aqueous medium.

Initially reported by Belomoin and co-workers, the electrochemical synthesis of SiQDs by silicon wafers etching in aqueous or ethanolic hydrofluoric acid (HF), successfully produced H-capped silicon nanocrystals (H-SiQD) of small sizes (1.0, 1.7, 2.15, 2.9, and 3.8 nm). The resulting mix sized SiQDs solution had, however, to undergo time-consuming separation techniques before blue, green, yellow and red PL could be attributed to obtained sizes, respectively [203]. Later Kang et al., following the same procedure, directly synthesised monodisperse and narrow distributed SiQDs (1-4 nm), without further separation steps, with the introduction of polyoxometalates (POMs) as reaction catalysts. The combined action of $\text{H}_3\text{PMo}_{12}\text{O}_{40}$ and H_2O_2 , in ethanolic HF solution, promoted the production of 1 ± 0.1 , 2 ± 0.1 , 3 ± 0.2 and 4 ± 0.2 nm SiQDs with monodispersions of about 90%, 85%, 75%, and 65%. In the same work, the authors highlight shape and size dependency on current density and POM/ H_2O_2 ratio [204]. Further etching of as-synthesized 3 nm SiQDs, in an ethanol- H_2O_2 solution, was reported by the same group to render SiQDs water solubility through the formation of a $\text{Si}/\text{SiO}_x\text{H}_y$ core-shell structure (figure 1.17A). Surface time-dependent oxidation was also reported to tune SiQDs photoluminescence, a consequence of core reduction, without PL compromise [190] (figure 1.17B). A POM-free approach was reported by Park et al. on the synthesis of luminescent porous silicon nanoparticles (PSiNPs) for anticancer drug (DOX) delivery. The resulting particles were obtained with a spherical shape, of the average

hydrodynamic size of 126 nm, with good uniformity, well-defined mesoporous and quantum yields (QY) of approx. 10.2% [6].

Despite POM expensive character, electrochemical etching has not only been one of the most facile, fastest and greenest synthetic routes but has also displayed significant efforts towards the development of tailored and directed SiQDs architectures [188].

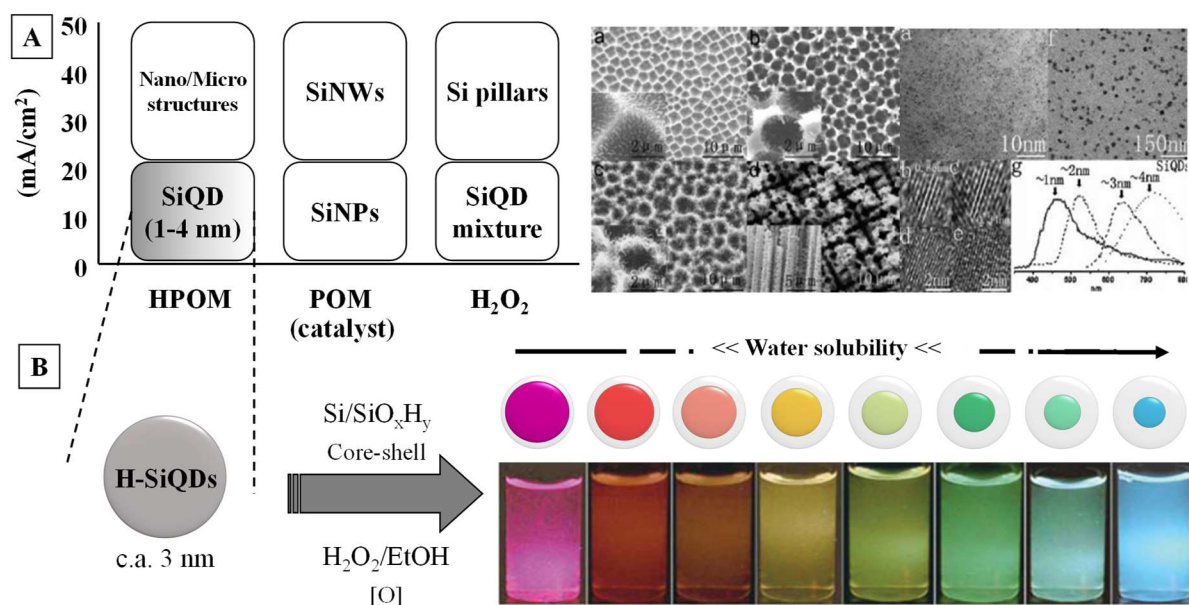


Figure 1.17 - A) Relationship between current density, catalyst and final products (*left*); SEM, TEM and HRTEM images of SiQDs/SiNPs and typical PL of different sized SiQDs (*right*). Adapted with permission from [204]. Copyright 2007 American Chemical Society. (B) H-SiQDs oxidation in EtOH/H₂O₂ and final emission color of oxidized SiQDs, ranging from salmon pink to blue. Adapted with permission from [190]. Copyright 2009 WILEY-VCH Verlag GmbH & Co. KGaA.

1.3.2 Bottom-up approaches

Surface or ligand modifications, as well as multi-step reactions, have proven to have a strong impact on SiQDs final photoluminescent (PL). With the former invariably degrading optical and surface properties of SiQDs. Thus, to avoid such drawbacks, as well as the use of expensive equipment and high temperatures, bottom-up approaches have been thoroughly investigated, bringing numerous *in situ* synthetic approaches for the synthesis of surface-tailored SiQDs, spanning from microemulsions to full aqueous synthetic routes.

1.3.2.1 Solution based reduction

One bottom-up procedure for the synthesis of SiQDs uses reducing agents in the presence of silane precursors in solution. SiCl₄ and TEOS have been widely reported to undergo a reduction in the presence of various reducing agents as sodium (Na) and LiAlH₄, to form blue photoluminescent SiQDs [66, 205]. These routes, however, yielded polydispersed

colloid solutions with diminished control over particle size and monodispersity. To overcome such drawback, Tilley et al. have reported an improvement on the afore-mentioned approaches, through the introduction of tetraoctylammonium bromide (TOAB) as a phase-transfer agent, surfactant and stabiliser [193]. The formation of reverse micelles, in toluene, allowed for the desirable control over silicon precursors precipitation, crystal growth and final particle size. Generated SiQDs showed narrow size distribution, with sizes ranging 2-3 nm, with the usually invariable blue PL and appetency for later facile surface functionalization. Dung et al. have lately reported the use of a modified protocol, by introducing CuSO_4 as quenching agent, known to improve the functionalization of H-SiQDs with acetylene derivates [194]. SiQDs purification and separation from surfactants has, however, proven itself a non-trivial task [206]. The use of alkyl/allyl- SiCl_3 species was reported as an alternative micelle-mediated route, with the development of ready-to-functionalized SiQDs without additional purification procedures [43, 207]. Sodium naphthalide as a reducing agent, in glyme, was reported in an additional approach, with Li et al. being able to synthesise high QY (i.e. 50% and 75%) and water dispersible SiQDs. As-synthesised Cl-capped SiQDs of 2 nm were further modified with diphenylamine and carbazole, rendering them high PL and water stability [56]. All the mentioned approaches are summarised in figure 1.18.

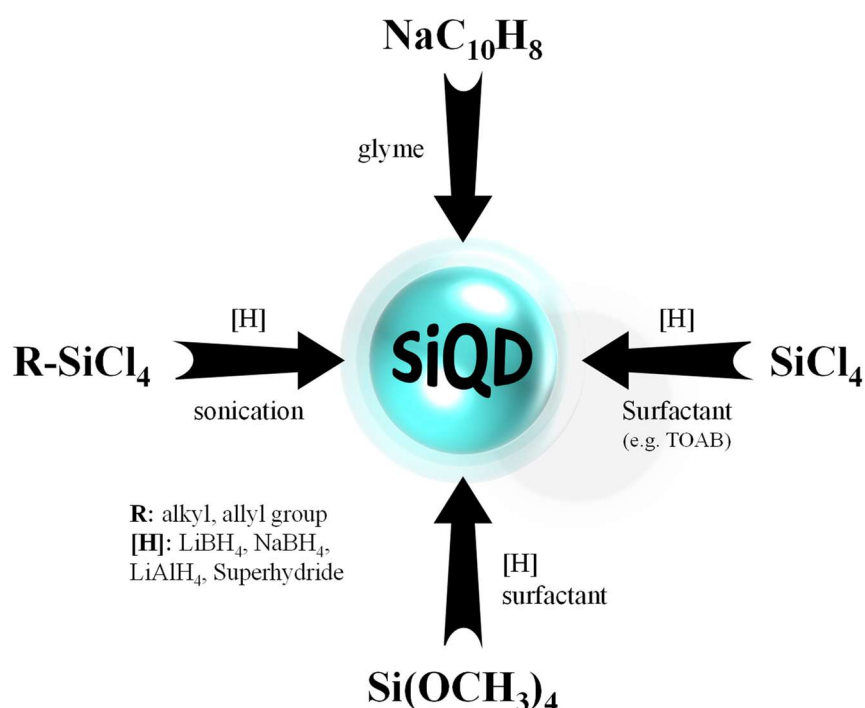


Figure 1.18 - Solution-phase reduction-based approaches for the synthesis of H-terminated SiQDs. Adapted with permission from [37]. Copyright 2009 WILEY-VCH Verlag GmbH & Co. KGaA.

1.3.2.2 Zintl salt metathesis

Silicon Zintl salts (XSi_n , $\text{X}=\text{Na}$, K , Mg , etc.) are ionic compounds, with unusual bonding, structural configuration and oxidation that undergo disproportion reactions in the presence of SiCl_4 or SiBr_2 to form SiQDs (see figure 1.19). Firstly reported by Kauzlarich et al., Cl-capped SiQDs were produced by metathesis reaction of KSi , NaSi , or MgSi_2 in the presence of SiCl_4 , glyme and under inert atmosphere [192, 208, 209]. The resulting nanoparticles showed relative good water dispersibility and disposition for surface modification. A comparative method, however, was developed using NaSi and ammonium bromide (NH_4Br), in excess glutaric acid [210]. The resulting H-capped SiQDs with blue luminescence had an average size of 3.9 nm and QY of 32% in hexane and 18% in water [209].

Such reactions have proved useful solution routes due to their capacity to produce differently capped SiQDs with high QY, at low temperatures and pressures. Despite their easy control, metathesis reactions still rely on two-step syntheses for development of water stable SiQDs.

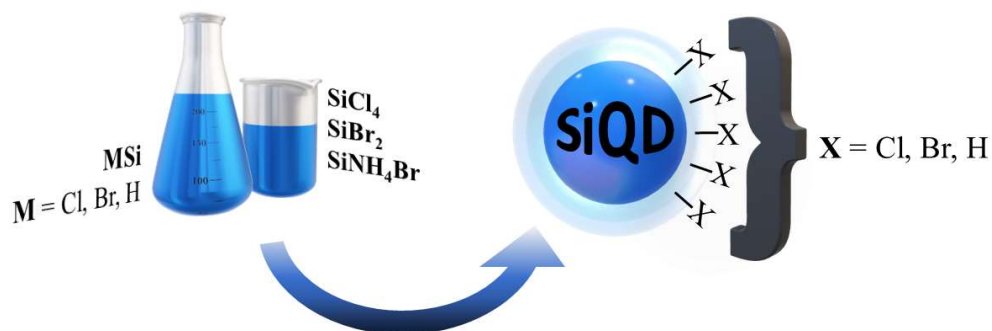


Figure 1.19 - Summarizing schematics of zintl salt-mediated SiQD syntheses.

1.3.2.3 Aqueous reduction

The development of water-based synthetic strategies for the synthesis of water-stable, high luminescent SiQDs has been long sought as the optimal approach for the development of biocompatible probes for biomedical applications. Despite their importance, reports on such synthesis are yet scarce and very recent, most using alkyl methoxysilanes as silica sources. Zhong et al. have reported the development of a facile one-pot microwave-assisted aqueous synthesis of SiQDs, yielding up to 100 mg, by the reduction of 3-aminopropyl trimethoxysilane (APTES) in the presence of trisodium citrate. 2.2 nm SiQDs were obtained in an N_2 -saturated environment within rapid reaction times (c.a. 10 min), with blue stable PL and QYs comparable and even higher (20-25%) to some top-down synthesis based SiQDs (figure 1.20).

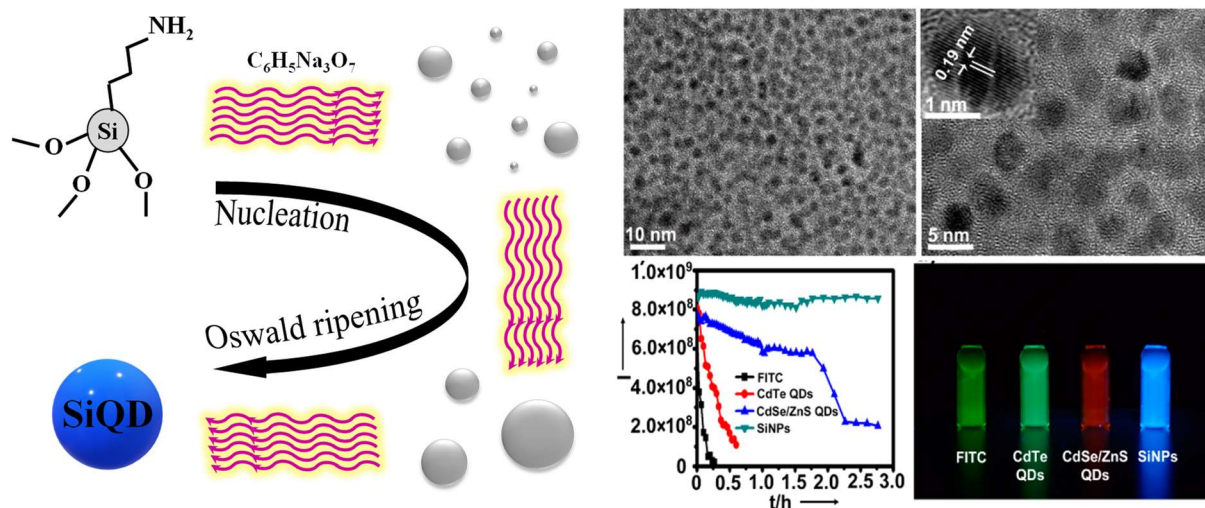


Figure 1.20 - Simplified schematic illustration of bottom-up one-pot microwave-assisted synthesis of SiQDs: from precursor reaction to nuclei formation and crystal growth (*left*). TEM / HRTEM images of as-prepared SiQDs (*right top*). Photostability comparison of FITC, CdTe QDs, CdSe/ZnS QDs, and SiNPs, and respective photographs under UV for time = 0 min (*right bottom*). Adapted and reprinted with permission from [58]. Copyright 2013 American Chemical Society.

The nanoparticles high appetency for bioconjugation, deriving from surface alkylamine groups, allowed for the linkage IgG-COOH molecules for further immunofluorescence assays, with little influence over the original PL [58]. Using the same protocol, Wu et al. were able to report the influence of different silica precursors, as well as nitrogen content, over the resulting SiQDs. Accounting with promising QYs of 23.5%, 82.4% and 6.4% for APTES, N-[3-(trimethoxysilyl) propyl]ethylenediamine (DAMO) and 3-[2-(2-aminoethylamino) ethylamino]propyl-trimethoxysilane (AE EA) as silica precursors, respectively [57] (see figure 1.21). Non-microwave-assisted approaches have also been reported by several groups using sodium ascorbate and ascorbic acid as reducing agents, at room or low temperatures (e.g. 40 °C) and open atmospheres [10, 211, 212]. Resulting SiQDs showed narrow-sized distributions and good dispersibility, however with lower QY values than those reported by Wu et al. It should be noted that all detailed approaches are made in oxygenated environments and in the presence of amine compounds, thus their characteristic blue PL [187].

Although scarce, one-pot aqueous reduction routes represent the next class of green, cheap and facile syntheses of SiQDs, working as promising take-off platforms for future biological conjugations and biomedical applications.

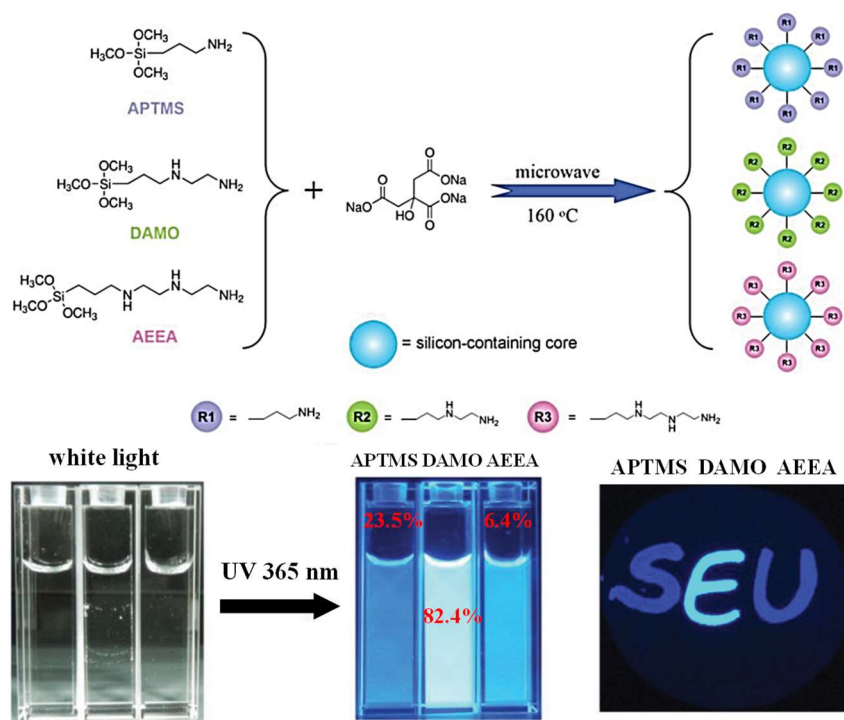


Figure 1.21 - Schematic illustration of the formation of SiNPs using APTMS, DAMO, and AEEA molecules (top). Photographs of APTMS, DAMO, and AEEA SiQDs solutions under white light and UV (365 nm) irradiation (1 mg.mL⁻¹ at pH = 7.4), with respective QY; possible application as fluorescent inks (bottom). Adapted with permission from [57]. Copyright 2015, WILEY-VCH Verlag GmbH & Co. KGaA.

1.3.3 Surface modifications, optical properties and applications

While SPIONs' surface modification presented itself essential for their further bio application, SiQDs functionalisation not only is critical for their protection from external conditions but crucial on the determination of their luminescent properties and colour.

Although not fully understood, SiQDs photoluminescence has been hypothesised to be both size and surface-states/impurities dependent. Wen et al. have shown that SiQDs follow a two-regime PL, where: 1) diameters greater than 3 nm follow a normal quantum confinement regime, with PL spanning from near-infrared to a yellow 590 nm critical wavelength; and 2) diameters smaller than 2 nm have a non-radiative dominant regime, with blue and green PL arising from the presence of defects/surface states [46]. The influence of such defects was also studied by Dasog et al. when trying to understand why high-temperature methods (e.g. thermolysis of hydrogen silsesquioxane - HSQ) were able to produce SiQDs with a quantum confined-typical red PL, while SiQDs from the reduction of SiCl₄ or obtained by Na₄Si₄/NH₄Br reaction consistently exhibited blue PL [187]. The group confirmed, through the exposure of red-emissive hydride SiQDs (H-SiQDs) to N-containing species (i.e. TOAB and NH₄Br) under oxygenated atmosphere, the induction of blue PL and

short excited state lifetimes upon the formation of both Si-O and Si-N bonds; even at low N concentrations (c.a. < 0.2 N %). TEM analyses confirmed that the consequent PL blueshift was not accompanied by a decrease of SiQDs size, but rather enhanced with increasing concentrations of N containing species (see figure 1.22 A-C).

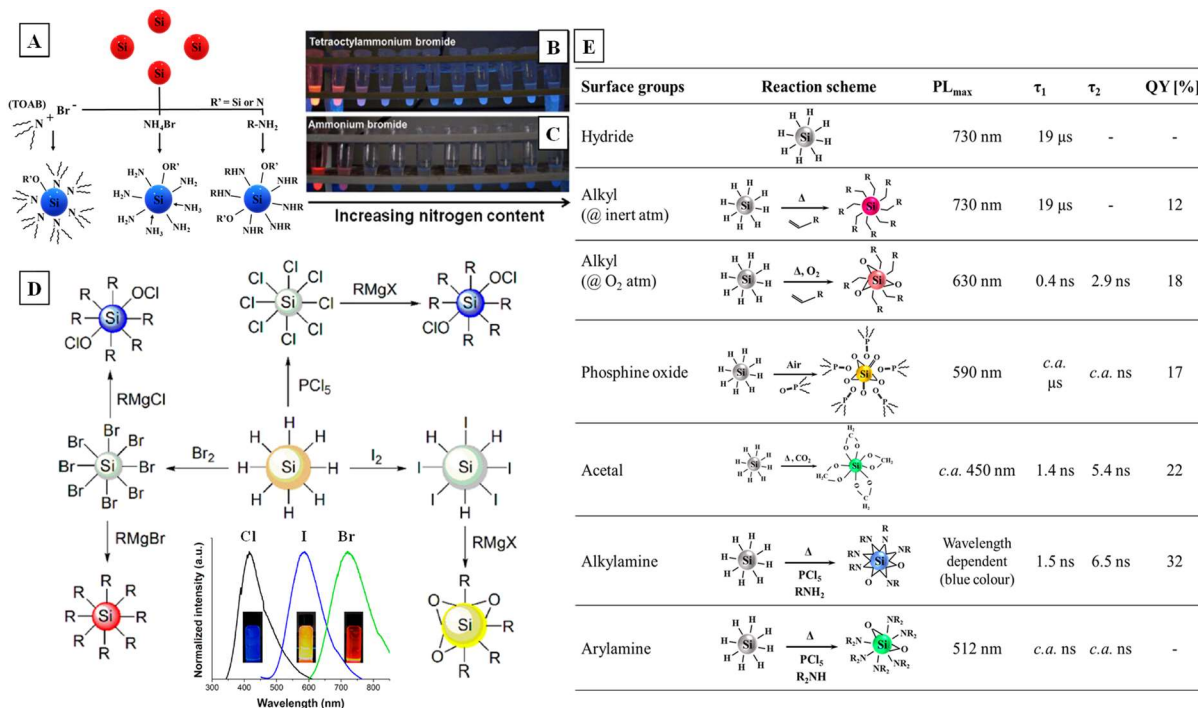


Figure 1.22 - (A) Schematic representation of surface passivation of H-SiQDs with N-containing species, under O₂ atmosphere, and consequent PL blueshift. Sequential photographs, under UV light, of H-SiQDs reacting with (B) TOAB and (C) NH₄Br. Adapted and reprinted with permission from [187]. Copyright 2013 American Chemical Society. (D) Illustration of halogenation and alkylation of H-SiQDs and emission spectra of hexyl functionalised SiQDs derived from Cl, I and Br modified surfaces ($\lambda_{ex} = 325$ nm). Adapted and reprinted with permission from [214]. Copyright 2015 American Chemical Society. Summarising table of all surface modifications of H-SiQDs surfaces, and corresponding optical properties; τ_1 or τ_2 – excite-state lifetimes. Adapted with permission from [186]. Copyright 2014 American Chemical Society.

Extensive studies have also been developed by Dasog et al. on the tuning of freestanding SiQDs PL across the visible spectra via surface groups, in variable conditions [186, 214]. Halides role on the optical properties of SiQDs were firstly studied, with the authors reporting the chlorination (Cl-SiQD), bromination (Br-SiQD) and iodination (I-SiQD) of bare SiQDs, using PCl₅, Br₂ and I₂, and later alkylation using Grinard agents (figure 1.22 D). Herein, trace halide impurities were confirmed to play an essential role in the final SiQDs PL. SiQDs derived from Br doped surfaces showed quantum confinement-dominated PL (λ_{em} c.a. 750 nm), while I and Cl-doped surfaces led to yellow and blue emission, believed to arise from oxide defects at the surface. The impact of surface halide species was also investigated by changing the employed Grinard reagents, with Br-SiQDs exhibiting red emission when

reacting with RMgBr and blue emission with RMgCl. Leading the authors to support such results with a possible halide exchange during alkylation, which was later confirmed by the presence of surface oxide/oxychloride emissions, in the Si 2p region, (c.a. 103 eV) on SiQD surfaces [214]; similarly to the abovementioned oxynitride defect states.

Additional functionalization of SiQDs surfaces with dodecyl (in an inert or O₂ atmosphere), phosphine oxides, acetal, alkylamine and arylamine groups were also reported by the same group and are summarised in figure 1.22 E [186]. Briefly, all surface modifications induced Si oxidation and the formation of Si-O, Si-O-Si, Si-N-Si and Si-N-O bonds, responsible for PL blueshift.

A typical analysis of all abovementioned studies states that quantum confined SiQDs exhibit wavelength-dependent μ s lifetimes, whether blue emitting nanocrystals are entirely dependent on the presence of surface defects with PL ns lifetimes. Thus, correlating microseconds PL lifetimes with band-gap phenomena and nanoseconds PL lifetimes with surface defects.

Therefore, rendering stability and bio applicability to SiQDs, while tuning and maintaining optical properties has shown itself to be a challenging goal. Notwithstanding, different approaches have been developed by several groups, such as O₂ surface oxidation (also known as thermally induced hydrosilylation), EDS/NHS mediated linkage, silanization, click reactions, condensation reactions, physisorption and UV or Pt catalyst-mediated hydrosilylation.

1.3.3.1 Hydrosilylation

By far one of the most used and extensively described techniques when modifying SiQDs surfaces [215], hydrosilylation can occur via three distinct pathways that might, or not, involve different catalysts: O₂, UV and Pt-based catalysts.

The first and most conservative approach was extensively used by Dasog et al. in the aforementioned studies, where thermal induced hydrosilylations were done in either inert atmosphere (at 190 °C for 15h) or open atmosphere, with O₂ acting as radical initiator and allowing the reaction to proceed readily at approx. 100 °C for 12h. The latter, however, is known to prone surface oxidation and promote optical properties changes. The same approach is used by Li et al. when modifying the surface of Cl-capped SiQDs with diphenylamine, carbazole, N-methyl aniline and propylamine; promoting the formation of water-dispersible green and blue emissive SiQDs with quantum yields of 50% and 75%, respectively [56]. Interestingly the group has concluded that the N-linkage of the phenyl ring to the Si surface is essential for the suppression of molecular-motion-based nonradiative

relaxations and the induction of single radiative channels with high excitation-independent PL.

Pt-catalysed surface reactions on SiQDs have been repeatedly used on the binding of alkyl molecules, with Speier's catalyst (H_2PtCl_6) and Karstedt's catalysts as main initiators. Zhang et al. have confirmed to be able to produce sufficiently stable octyl-capped SiQD using H_2PtCl_6 catalyst at room temperature and anaerobic conditions [209]. However, such approach usually requires further purification of SiQDs and extraction of the Pt catalyst from the reacting solution. Notwithstanding, water stable and biocompatible SiQD-based nanosystems were produced upon the growth of poly(acrylic acid) chains around octyl terminal groups, without further adverse effects on PL and rendering them with quantum yields of about 18%. A similar approach was also used by Dung et al. on the production of octyl-capped (Oct), (trimethylsilyl)vinyl-capped (TMS) and styryl-capped (Phe) SiQDs [194]. Ligand influence over PL was also investigated, with the authors pointing out an increasing delocalisation of electronic wavelengths of electron-hole pairs, the formation of additional states within the band gap of SiQDs and a decrease in PL photon energy, from Oct-SiQD to Phe-SiQDs. The existence of π - σ surface states was confirmed to highly increase decay rates of excitons and enhance the probability of radiative transitions, thus being responsible for higher PL quantum yields. This led Abdelhameed et al. to report the usage of Pt-catalysed hydrosilylation for the coupling of fluorophore species, as phenanthrene, pyrene and perylene, to the surface of SiQDs for the development of bioimaging nanosystems of HeLa cancer cells.

Despite both approaches above' relatively straightforwardness and successful results, associated high energy inputs, time-consuming reactions and process/reagent costs (e.g. the use of high temperatures and Pt catalysts), have made UV hydrosilylation a more attractive, direct and clean alternative. Hua et al. have successfully demonstrated the attachment of numerous terminal double bond alkenes to H-SiQD surfaces via UV irradiation ($\lambda_{\text{ex}} = 254 \text{ nm}$); with 1-pentene, 1-hexene, 1-octene, 1-dodecene, 1-octadecene, styrene vinyl acetate, ethyl undecylenate, 1-undecanol and 5-hexen-1-ol photoinitiated grafting happening at much shorter reaction times, within 30 to 180 min, when compared to those needed for thermal and Pt-catalysed hydrosilylation [216]. Buriak et al. have also investigated UV-mediated hydrosilylation, stating that the lack of necessity of additional reagents or inputs of thermal energy may lead to reduced numbers of defects and surface contaminations [217]. Moreover, the authors also discussed the unique size-dependent functionalization, also confirmed by Kelly et al. [218], where SiQDs with too large band gaps (i.e. small diameters) are not prone to react, while smaller band gaps can absorb UV photons and undergo surface hydrosilylation.

Notwithstanding, numerous works developed by Erogbogbo and co-workers have used UV hydrosilylation to render bare SiQDs high stability through styrene, octadecene and ethyl undecylenate [27] or biocompatibility through undecylenic acid [25] and butanoic acid [26]; all working as optimal platforms for latter functionalization and conjugation with other molecules through other techniques.

1.3.3.2 Silanization

Direct silanization of SiQDs surfaces usually happens through the oxidation of as-synthesised SiQDs by a variety of techniques, as described above. However, aqueous reduction synthetic routes for SiQDs have been described to result in non-pure silicon nanoparticles, containing crystalline silicon cores enveloped in a Si-O-Si network, whose surface contains hydrocarbon chains and polar groups that render them bio applicability, stability and availability for further surface modifications [10, 57, 212]. Interestingly, Marcelo et al. have recently reported a one-pot synthesis of (3-isocyanatopropyl)triethoxysilane-coated SiQDs (isoc-SiQDs), whose stability allowed for the growth of an additional mesoporous silica shell and the production of an emissive pH-responsive biodegradable and non-toxic nanocarrier for the treatment of HepG2 and Caco-2 cancer cell lines [211]. PL blueshift was registered for both surface functionalisations, with PL maxima shifting from the as-synthesised maximum at 510 nm to 467 nm and 450 nm, with a final quantum yield of about 13% (figure 1.23).

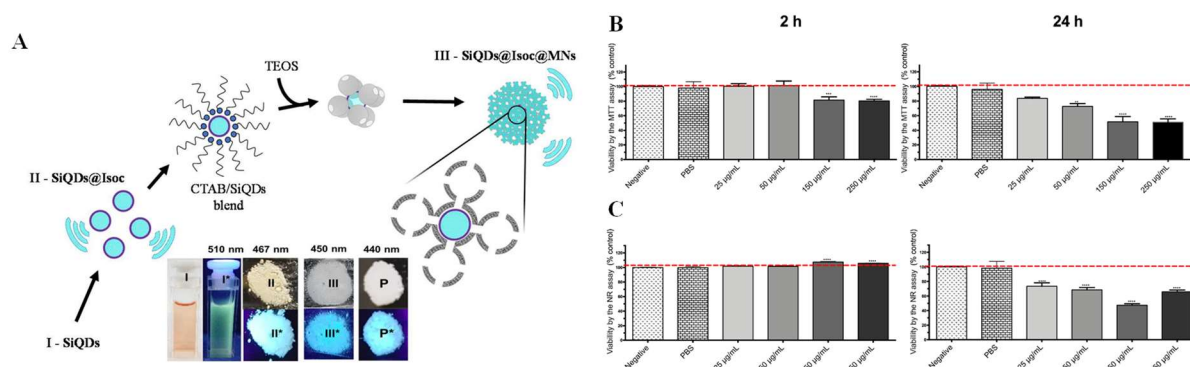


Figure 1.23 - (A) Illustration of the overall synthetic procedure and SiQDs surface functionalisation with corresponding optical properties changes, under white and UV light. MTT reduction assay (B) and NR incorporation (C) after 2 and 24 h incubations of doxorubicin-loaded SiQDs@Isoc@MNs (50 µg/mL–150 µg/mL) for HepG2 cell lines. Adapted with permission from [211].

The same nanoparticles were also functionalised, through silanization, with polyethylene glycol (PEG)-silane derivatives (e.g. p-toluenesulfonyl chloride-PEG₈₀₀₀-silane), to render them longer circulation lifetimes, and loaded with doxorubicin, with significant toxic effects after 24h against HepG2 cancer cell lines (figure 1.23 B-C).

1.3.3.3 Other modifications and conjugated surface structures

The most complex and more bio-adjoining conjugations of SiQDs, reported in the literature, are based on SiQDs that had already undergone hydrosilylation modifications.

Examples of such complex nanocomposites are those reported by Cheng et al. [43, 66] where “click” chemistry reactions were used to modify alkene coated SiQDs and introduce specific functionalities as -NH_2 , -COOH , -SO_3^- and alkane. In a first approach, thiol-ene mediated reactions occurred under UV irradiation (254 nm), rendering SiQDs with biocompatible functional groups and allowing the authors to switch SiQDs solubility, from hexane-soluble to fully water-soluble [66]. Thiol groups attachment is however accompanied by QY decrease, with the most significant drop registered in -COOH terminated particles of only 0.9%, and by PL emission maxima redshifts (figure 1.24 A-C). A second development involved the copper-catalysed alkyne-azide cycloaddition (CuAAC) “click” reaction to uniformly functionalised 1,8-nonadiyne-capped SiQDs with azide groups, for effective cell imaging [43]. Solubility was, again, totally controlled according to terminal groups, with amine-functionalised SiQDs displaying optimal *in vitro* internalisation and stable PL labelling of HeLa cells (figure 1.24 D-E).

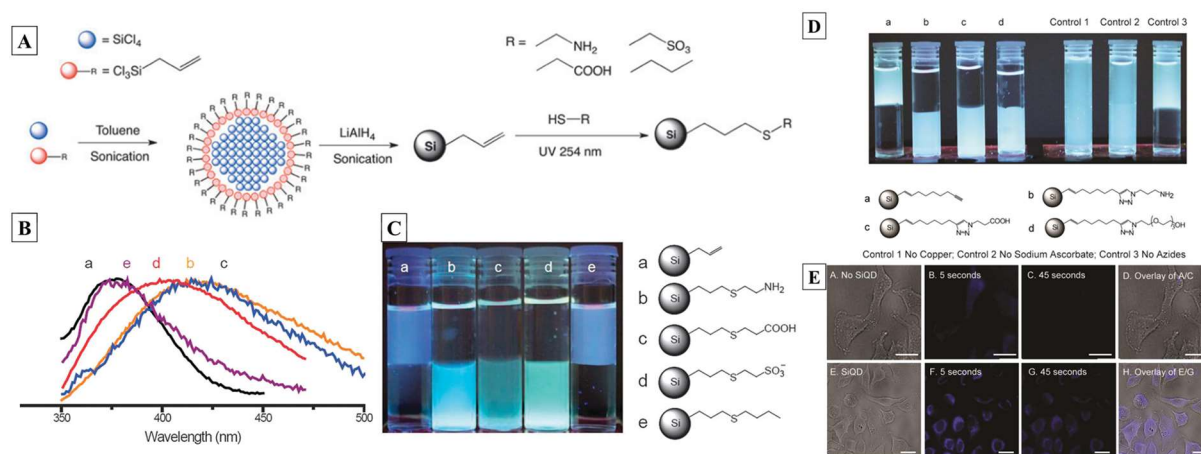


Figure 1.24 - (A) Illustration of the one-pot synthesis of alkene-coated SiQDs and functionalization via thiol-ene click chemistry. (B) PL spectra of SiQDs with different surface groups (a-e) excited at 320 nm and (C) naked-eye fluorescence and hydrophobicity in hexene:water mixtures. Images were taken under UV light @ 365 nm. Reproduced with permission from [66] Copyright 2012 Royal Society of Chemistry. (D) Dispersibility of SiQDs in polar and nonpolar solvents according to the different surface groups (a-d). (E) Fluorescence microscopy images depicting the presence of amine-functionalised SiQDs in HeLa cells under constant excitation at 405 nm. Scale bar = 10 μm . Upper panel: control group with no SiQDs added; bottom panel: cells incubated with SiQDs. Reprinted with permission from [43]. Copyright 2014 American Chemical Society.

Another class of modifications is based on EDC/NHS reactions, useful when envisioning the binding of biomolecules through amide bonds to render nanoparticles direct biological functions or bioactivity. Small water-dispersible green SiQDs were reported by

Erogbogbo et al. to have active bio application after EDC/NHS-mediated coupling of lysine, transferrin or folic acid, at room temperature. Briefly, undecylenic acid-capped SiQDs were reacted with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to form an unstable intermediate, later reacted with N-hydroxysulfo succinimide (Sulfo-NHS) to give place to a semi-stable amine-reactive NHS-ester. This intermediate, once in the presence of a primary aminated molecule promotes amide bond formation and solid bioconjugation of nanoparticles. The authors report a decrease in QY from $15 \pm 5\%$, before bioconjugation, to $5 \pm 3\%$, after bioconjugation, and a successful anti-mesothelin and folic acid-conjugated SiQDs application in fluorescence imaging of Panc-1 pancreatic cancer cells.

Conversely, He et al. have investigated the use of red emissive glutaric acid-capped SiQDs, with outstanding hydrophilicity, in the direct conjugation with a goat anti-mouse IgG antibody via traditional EDC/NHS cross-linking reaction [68]. Produced antibody-SiQDs showed exceptional specificity towards HeLa microtubules and ultrabright photostability (with SiQDs-labelled microtubules yielding stable fluorescent signals for 120 min continuous observation), particularly suitable for long-term and real-time cellular imaging (figure 1.25). A similar approach was also used by Nakahara et al. on the modification of blue emissive SiQDs surface with monosaccharides, as D-glucosamine and D-galactosamine.

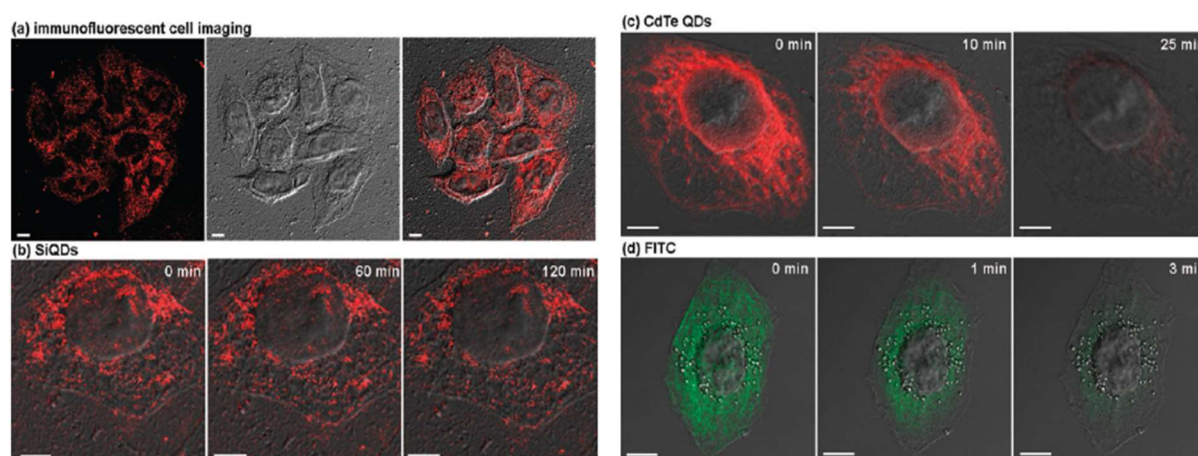


Figure 1.25 - Laser scanning confocal microscopy photos of immunofluorescent cell imaging. (a) Left: microtubules of HeLa cells are distinctively labeled by the SiQDs/protein bioconjugates. Middle: bright-field image. Right: superposition of fluorescence and transillumination images. (b-d) Stability comparison of fluorescence signals of HeLa cells labeled by SiQDs (b), CdTe QDs (c), and FITC (d). Scale bar = $5 \mu\text{m}$. Reprinted with permission from [68]. Copyright 2011 American Chemical Society.

The last tactic on the modification and biopreparation of SiQDs for biological applications involves the physisorption of amphiphilic compounds on the surface of bare or already modified SiQDs. Such examples are those extensively reported by Erogbogbo et al., where the non-toxic micellar encapsulation of multiple hydrophobic SiQDs is achieved by the use of 1) phospholipids [27] (see figure 1.26.1-2) DSPE-PEG(2000)-NH₂, -COOH and -folate

modified phospholipids [26] (see figure 1.26.3). In both works the authors report the multiple encapsulations of stable single-sized or multiple-sized SiQDs onto 50-120 nm spherical micelles (MSiQDs), that allow for single or multiple *in vivo* labelling, spanning from blue PL (~450 nm) to NIR PL (~900 nm). Moreover, through an additional EDC/NHS modification of -COOH terminated micelles, bioconjugation and high specificity can be easily achieved when linking active biomolecules (e.g. proteins, enzymes or antibodies). Such asset proved essential on the targeted *in vivo* labelling and imaging of subcutaneously implanted Panc1-tumors in nude mice when compared to non-bioconjugated MSiQDs. Toxicity, lifetime circulation and biodistribution were also extensively studied for bioconjugated MSiQDs, that revealed not only longer circulation times (due to their confinement in the oil-like interior of phospholipid-PEG micelles that prevents their rapid degradation), but also minor spleen and liver uptake levels. Spleen and liver MSiQDs degradation were confirmed by continuous monitorization of the decreasing PL signals up to 2 months after MSiQDs injection. Remarkably, Sentinel Lymph Node (SLN) mapping was also possible by using 20 nm tailored MSiQDs that, after subcutaneous injection, travelled through the lymphatic system and migrated to axillary locations (figure 1.26.2-7).

A similar protocol was developed by Pi et al., this time, encapsulating red emissive dodecane-capped SiQDs within block-co-polymeric micelles of pluronic F127, that showed good dispersibility and stable PL in water [44]. Lastly, Park et al. proposed the co-encapsulation of NIR emissive SiQDs with doxorubicin, within a dextran-containing micelle for *in vivo* and *ex vivo* imaging of cancerous HeLa cells treated with subcutaneous, intramuscular and intravenous injections. Stable PL and allowed for the signals of all injections to be clearly observed without skin autofluorescence, confirming biodistribution and localized antitumoral effect [6].

These last approaches open the possibility for the development of promising multifunctional biocompatible luminescent probes through the co-encapsulation of SiQDs with additional nanoparticles, drugs, pro-drugs or contrast agents.

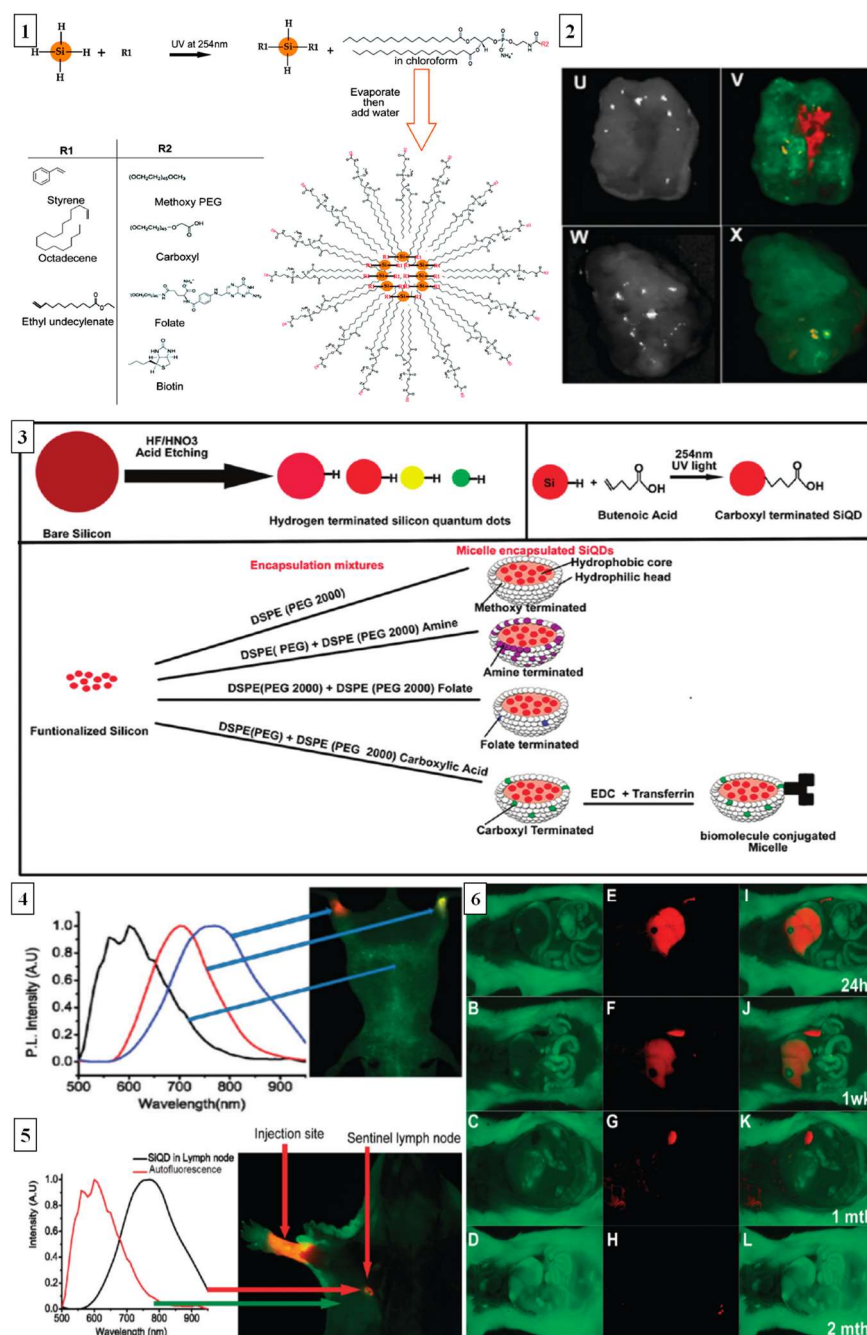


Figure 1.26 - (1) Schematic of surface hydrosilylation of SiQDs, with R1 compounds, followed by micellar encapsulation commercially available on the phospholipids terminated with R2 functional groups. Reprinted with permission from [27]. Copyright 2008 American Chemical Society. (2) Ex vivo images (U,W) and luminescence images (V,X) of tumors harvested at 40 h post injection from mice treated with (U,V) MSiQD-cRGD or (W,X) MSiQD. (3) Illustration of the synthesis and surface functionalization of butenoic acid-capped SiQDs with DSPE-PEG(2000) phospholipids and derivatives. (4) In vivo multiplex NIR imaging of SiQDs subcutaneously injected in lower limbs of a mouse. (5) Sentinel lymph node imaging following localization of Si QDs in an axillary position. (6) Luminescence imaging of BALB/c mice injected with MSiQD. at different time points post injection. Note that autofluorescence and unmixed SiQD signals are coded in green, red and/or yellow, for the above images. (2-7) were adapted and reprinted with permission from [26]. Copyright 2011 American Chemical Society.

Table 1.4 - Summarising table of some SiQDs surface modifications and respective optical properties and applications.

Method	Core avg. size [nm]	QY _{as-syn.} [%]	Surface modification/Coating	QY _{final} [%]	$\lambda_{PL,max}$ [nm]	Objective / Obs.	Reference
O₂ oxidation	2.0	8; 405 nm	diphenylamine	50	500	Development of nanothermometer through long fluorescence lifetime (FLT) imaging, by surface modification of SiQDs	[56]
			carbazole	75	480		
			N-methylaniline	10	500		
			propylamine	<10	-		
EDC/NHS^a	3.1	15; 660 nm	goat anti-mouse IgG	-	-	Targeted immunological labelling of Hela cells	[68]
	4.8	3.8; 450 nm	D-glucosamine ^c D-galactosamine ^c	-	450	Biological fluorescent imaging	[219]
physisorption^b	126	-, ~ 800 nm	DOX@dextran	-	810-875	Long-time circulation nanocarriers for drug delivery in cancer treatment	[6]
	3.3	26; 683nm	dodecyl@pluronic F127 ^c	15-24	690	Water-dispersible SiQD micelles for bioimaging	[44]
silanization	3.6	13; 467 nm	mSiO ₂ @DOX	13	450	Controlled drug delivery to HepG2, hCMEC/D3 and Caco-2 cell lines	[211]
			mSiO ₂ @silane-PEG(200)TsO	13	440		
UV-mediated hydrosilylation^c	≤13	-; 700 nm	hexene	-	650	Resolve agglomeration of Si-NPs while maintaining their crystallinity	[201]
			dodecene	-	658		
			octadecene	-	625		
			ethyl undecylenate	-	640		
					-		
	2-8	15; 450-900 nm	und-folate		645	<i>In vivo</i> targeted cancer imaging, sentinel lymph node mapping and multi-channel imaging	[25, 26]
			und-lysine ^a		-		
			BA		-		
			DSPE-PEG ^b	5-10	-		
			DSPE-PEG@DSPE-PEG amine ^b		-		
			DSPE-PEG@DSPE-PEG folate ^b		-		
			DSPE-PEG@ DSPE-PEG-COOH ^b		-		
			DSPE-PEG@ DSPE-PEG-cRGD ^a		-		

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Mat. Sci and Eng: C 106, 2020, 110104

	~ 5 / 2.0	-, ~ 450 nm	PAA	-	-	Development of stable <i>in vitro</i> and <i>in vivo</i> bioimaging probes.	[67, 220]
Pt-catalysed hydrosilylation (H ₂ PtCl ₂)	3.9	28; 438 nm	octyl octyl-PAA	18	483	Synthesis of water soluble SiQDs	[209]
	1.8	- ; 420 nm	octyl (trimethylsilyl)vinyl styryl	8 5.5 10	410	Increase SiQDs stability and monodispersity, study capping influence on photoluminescence	[194]
			alkanethiol-NH ₂	1.2	430		
click chemistry	3.7	2.8/5.2, 375 nm	alkanethiol-COOH	0.9	435		
			alkanethiols-SO ₃ -	1.6	405		
			alkanethiols-butene	1.2	380	Prepare water soluble and biocompatible SiQD	[43, 66]
			azido-propylamine	1.4	425		
			azido-propionic-acid	-	455		
			(EO) ₃ -azide	3.3	425		

PAA – poly(acrylic acid); mSiO₂ – mesoporous silica; silane-PEG(200)-TsO – poly(ethylene glycol)-p-toluenesulfonylsilane oxide; HepG2 - human hepatoma; hCMEC/D3 - human brain endothelial; Caco-2 - human epithelial colorectal adenocarcinoma; und -undecylenic acid; BA – butanoic acid; DSPE-PEG - 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (ammonium salt);PAA -poly(acrylic) acid.

1.4 Magneto-fluorescent hybrids

Despite the growing interest and importance of SPIONs and SiQDs in the development of biocompatible and biodegradable nanoprobe for biomedical application, efforts on the creation of hybrid systems are but scarce. Few works have been reported on the conjugation of both systems, as well as on their conjugated properties, but challenges like charge or energy transfer phenomena and wide the iron oxide broad absorption on the visible spectra significantly hamper their trivial production by quenching QD/SiQDs PL [221-224]. To overcome such drawbacks and ensure the thrive of both luminescent and magnetic properties, long interparticular spacers (i.e. ionic surfactants, long aliphatic carbon chains, polymers and silica shells), either covalently or electrostatically bind [222, 225, 226], are commonly used along with a balancing on SPION:SiQD/QD molar ratio, with SiQD/QDs usually in excess [87, 227-229].

For instance, Park et al. have reported the development of micellar hybrid nanoparticles, through the conjugation of magnetic nanoparticles and quantum dots in PEG-modified phospholipid for the delivery of anticancer drugs, such as doxorubicin, and imaging [7] (see figure 1.28A). Parallely, Selvan et al. developed janus-like nanoparticles, containing Fe_2O_3 -CdSe nanocomposites, stabilised by both a SiO_2 shell and Oley-PEG-succinimidyl-modified ligands [11] (figure 1.27B-D). The resulting nanocomposites presented excellent dispersibility, PL (QY 8-18%) and magnetic properties. QD-SPION nanoconjugation was also reported by You et al., with the development of high-luminescent yellow magnetic nanocomposites through the flocculation of CdTe QDs in the presence of Fe_3O_4 SPIONs. Maximised luminescent-magnetic properties were achieved for a QD: SPIONs molar ratio of 2.0, with saturation magnetisations of about 6.24 emu/g [227]. The electrostatic conjugation of thiol-capped CdTe QDs with CTAB-passivated SPION was also reported by Ahmed et al. on the development of probes for specific cancer cells imaging, with CTAB working as stabilizing spacer to ensure QDs better PL [225].

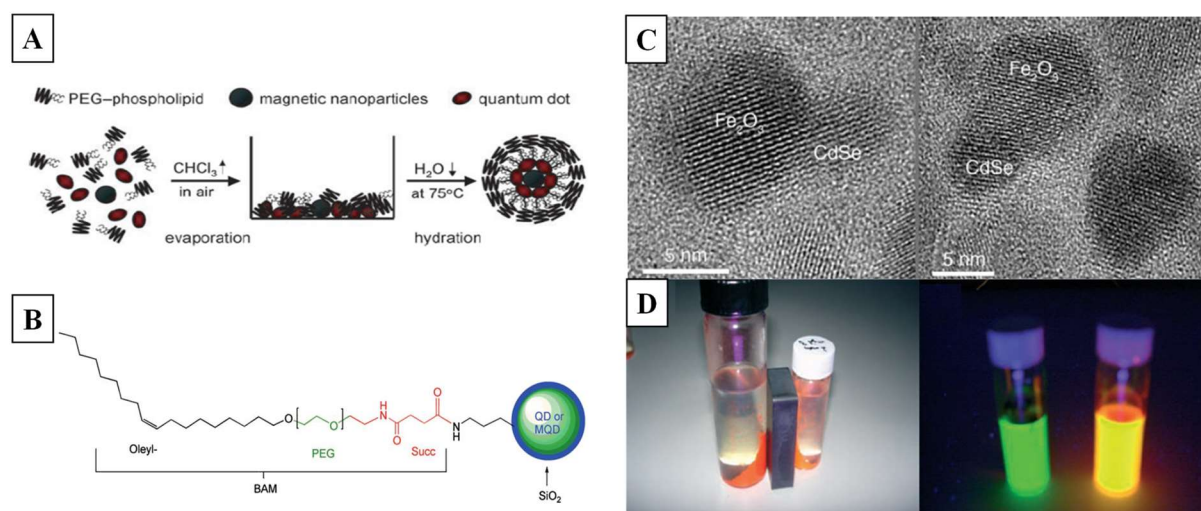


Figure 1.27 - (A) Synthetic approach for micellar hybrid nanoparticles encapsulating SPION and QDs within a single PEG-modified phospholipid micelle. Reproduced from [7]. Copyright 2008 Wiley-VCH Verlag GmbH & Co. KGaA. (B) Schematic Fe_2O_3 -CdSe MQDs and its silanization by reverse micro-emulsion, with further surface modulation with succinimide-PEG chains and oleyl groups (BAM). (C) TEM images of heterodimers of MQDs after magnetic harvesting (SPION, 8–10 nm; CdSe QD, 4–5 nm). (D) Magnetic and luminescence properties under UV (365 nm) of harvested red MQDs and of dispersed green and orange MQDs. Reprinted with permission from [11]. Copyright 2007 Wiley-VCH Verlag GmbH & Co. KGaA

A different approach was detailed by Li et al., on the development of mesoporous silica micro-nanobeads functionalized with both QDs and SPIONs. Despite their strong red luminescence, ensured by the loss of SPIONs absorption by virtue of the spatial separation, the low saturation magnetisation of 0.6 emu/g and low water solubility make them inapt for biological applications [226]. Multimodal core-shell imaging probes were also developed Chen et al., using PVP as micellation agent for the encapsulation of hydrophobic dodecyl trimethylammonium bromide (DTAB)-functionalized CdS QDs and SPIONs (figure 1.28). Great colloidal stability and biocompatibility were rendered by an additional thin PEGylated silica layer [228s]. The conjugation of citrate-capped SPIONs and mercaptopropionic acid-capped CdTe QDs was reported by de Melo et al. through electrostatic interactions, with CTAB working as linker and steric spacer. With such approach, the authors report a minimization of the Förster resonance energy transfer (FRET) restriction to maintain QDs' strong emission properties and overcome the critical limitations of FRET mechanisms [222]. Recently, Filho et al. were able to covalently bind, by EDC/NHS reactions, CdTe QDs and SPIONs. The resulting nanoplatforms aimed for a targeted interaction with transferrin receptors in cancer cells, for specific multimodal fluorescent analysis and T_2 -weighted imaging [230].

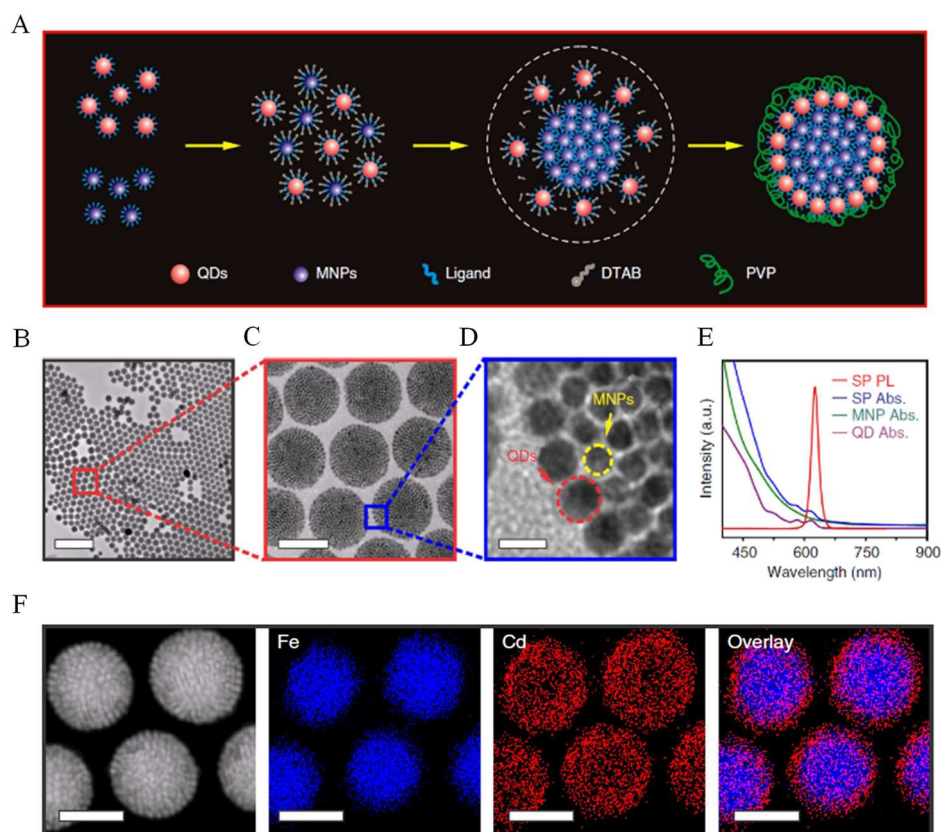


Figure 1.28 - (A) Schematics of the formation of CdSe-CdS core-shell QDs with SPIONs (CS-SPs). (B–D) TEM images of CS-SPs at different magnifications. Scale bars: 500 nm, 100 nm and 10 nm, respectively. (E) Absorption spectra of CS-SPs (blue), SPIONs (green), QDs (purple) and PL spectra of CS-SPs (red). (F) Dark-field scanning TEM images and EDS elemental mapping of CS-SPs. Scale bars: 80 nm. Reproduced with permission from [226]. Copyright 2014 Springer Nature

An innovative technique for the growth of stable CdSe QDs on the surface of SPIONs was developed by Cho et al., where both magnetic and size-dependent optical properties, with PL QYs of 1–5%, of each component were retained. In their study, some variables were analysed, as well as their influence on the number of CdSe cores around SPION seeds and PL properties [223].

More recently, “yolk-shell” nanoparticles were reported by Pajari et al. where CdSe@CdS@hollow-Fe₂O₃ nanocomposites served as exemplary multimodal imaging sensors at cellular and intracellular levels [231] (figure 1.29).

Polymeric shells have also been reported as useful encapsulating media. Feld et al. have studied the use of polystyrene-based polymers on a layer-by-layer encapsulation and hybridisation of QD/QRs and SPIONs in single existing entities of approx. 100 nm. The nanocomposites retained the original components’ magnetic and fluorescent properties [232]. Recently, a polymeric encapsulation approach was also detailed by Guo et al., where

oleic acid-capped SPIONs and ocatadecylamine-coated CdSe/ZnS QDs were encapsulated into a poly(maleic anhydride-alt-1-octadecene (PMAO) and PMMA composite. The resulting materials showed a ca. 226-fold stronger PL than that of individual QDs and retained 45.4% of the original SPIONs saturation magnetization. The authors report their application as not only a good purifying and enriching tool for aflatoxin B1 from dark soy sauce, but also as an outstanding fluorescent membrane-based immunochromatographic assay identifier of aflatoxin B1 with detection limits of ca. 3 pg/mL [233].

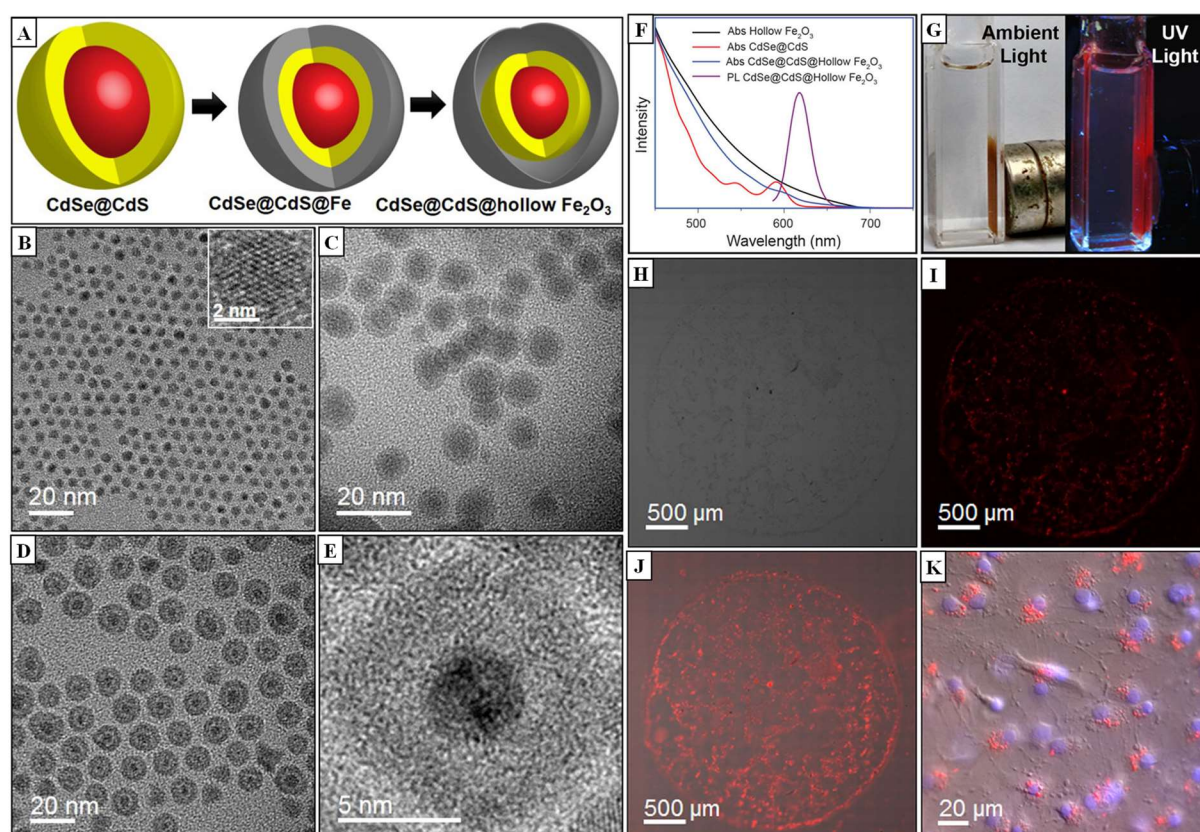


Figure 1.29 - (A) Illustration of the multistep sequential synthesis of magneto-fluorescent hybrid structures. (B-E) TEM micrographs: (B) CdSe@CdS fluorescent core, (C) intermediate step with metallic iron shell, (D,E) complete CdSe@CdS@hollow-Fe₂O₃ nanostructure. (F) Absorption spectra of hollow Fe₂O₃ (black), CdSe@CdS QD (red) and CdSe@CdS@hollow Fe₂O₃ (blue) along with the hybrid structure's photoluminescence (PL) spectra (purple). (G) Optical and magnetic features of iron oxide-QD core-shell, under ambient and UV light. (H-K) Microscopy images of a 3D cell culture, 13 days in vitro (DIV) and 6 days after introducing to the CdSe@CdS@Fe₂O₃: (H) Bright-field image displaying all cells, (I) QDs red fluorescence image, exclusively localized using a Rhodamine filter and (J,K) merged image and zoom. Adapted and reprinted with permission from [231]. Copyright 2018 American Chemical Society.

Di Corato et al. have recently reported the successful development of multifunctional polymer nanobeads, containing SPIONs and SiQDs and an amphiphilic polymer, for bioimaging. The conjugation mechanism was confirmed to follow a SPION aggregation →

SiQD condensation → polymerisation chain of events, to give place to tuneable beads with sizes between 60-80 nm. Strong red-PL was confirmed to be maximum at a 40:1 SiQD:SPIONs ratio, with SiQD-SPIONs interactions been inexistent or negligible [229] (figure 1.30). Additionally, the authors have also confirmed the existence of FRET process from the blue-emitting polymer to the QDs, increasing the latter PL. Erogbogbo et al. have also described the production of biocompatible magneto-fluorescent probes through the co-encapsulation of multiple hydrophobic SiQDs and SPIONs within phospholipid-PEG micelles. SiQDs red-PL was confirmed to be SPION-amount and temperature dependent, with increases in temperature up to 70 °C and SiQD:SPIONs=15 leading to PL decreases in 40 and 50%, respectively [87] (figure 1.31). A recent report by Sato et al. discussed the synthesis of size tuneable janus-like SiQD/Fe₃O₄/γ-Fe₂O₃ nanocomposites for *in vivo* diagnosis imaging. Fe-content was confirmed to induce a blueshift and quenching on SiQDs PL (QY of 1.3-5%), the latter being total for Fe amounts above 8.3%. Magnetic properties were also correlated with Fe-content, with hybrid systems exhibiting a maximum saturation magnetisation of 20 emu/g for Fe content at 5.3% and ferromagnetic behaviour [42]. Covalent binding of SiQDs and SPIONs has also been studied by Javadi et al., via dicyclohexylcarbodiimide (DDC) coupling of APTES-modified Fe₃O₄ nanoparticles and undecenoic acid-passivated SiQDs [234].

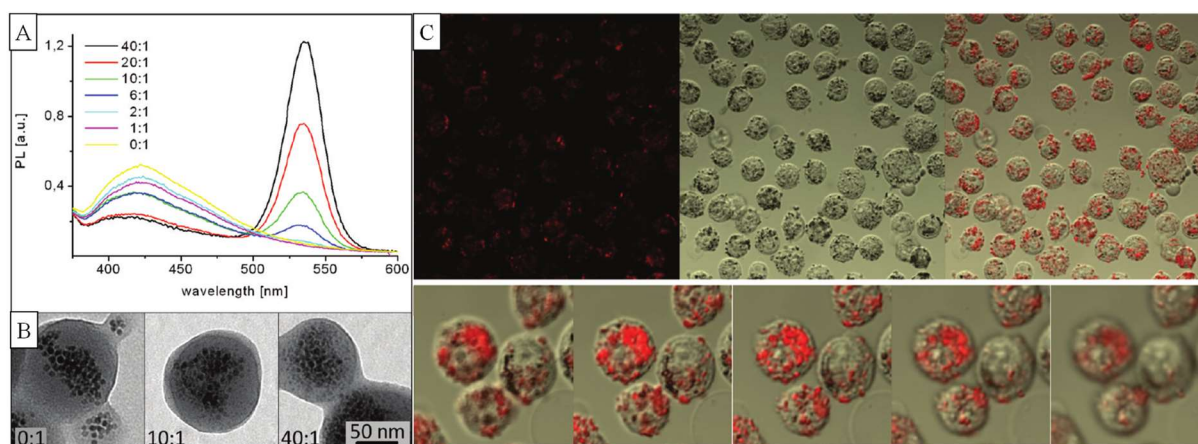


Figure 1.30 - (A) PL spectra of the acetonitrile-destabilized magnetic-fluorescent nanobeads (MFNBs) where QD:SPION ratio was varied from 0:1 to 1:1, 2:1, 6:1, 10:1, 20:1, and 40:1; with the amount of SPION constant at 0.1 nmol. (B) High magnification TEM images of MFNBs with QD:MNP ratios of 0:1 (*left*), 10:1 (*center*), and 40:1 nmol (*right*). (C) Confocal images of KB cells targeted and incubated with folic acid functionalized nanobeads (FA-MFNBs), for 1 h at 37 °C and a Fe concentration of 32 μg/mL (*top panel*). The zeta stack analysis (5 μm slices), confirming nanobeads internalization in cell cytoplasm (*bottom panel*). Adapted and reprinted with permission from [229]. Copyright 2011 American Chemical Society.

Luminescent porous silicon was also used on the development of magneto-luminescent probes, with ω-alkene terminated SPIONs passivating H-capped PSiNPs by

microwave-induced hydrosilylation, as detailed by Xia et al. [30]. Despite their study in xylene, the resulting nanosystems showed strong NIR-PL and outstanding saturation magnetisation, of c.a. 150 emu/g.

Alternative tactics have also been reported using Mn/MnO₂ doped SiQDs as magneto-fluorescent probes for dual fluorescence-magnetic resonance imaging and photodynamic therapy [235-237]. A similar approach was detailed by Singh et al. using Fe-doped SiQDs [238].

Despite the low number of publications on QD/SPION nanosystems, various alternative magneto-luminescent hybrids have been developed and reported in the literature, as SPION-dye systems [239], SPION-metal nanocomposites [237, 240-242] and SiO₂@SPION-dye/drug [243-245].

1.5 Colorectal Cancer and Associated Opportunistic Microbiota

1.5.1 Colorectal Cancer

Colorectal cancer (CRC) [246] is defined as any cancer case that appears and develops in either the colon or the rectum. CRC has been classified as the 3rd most common and 2nd deadliest type of cancer, with 1.93 million cases and 916 000 deaths, just in 2020 [247-249]. CRCs distinguish themselves into several types, identified as adenocarcinoma, Gastrointestinal Stromal Tumors (GIST), lymphoma, carcinoids, Turcot Syndrome, Peutz-Jeghers Syndrome (PJS), Familial Colorectal Cancer (FCC), Juvenile Polyposis Coli, depending on their physiological and genetic origin. Moreover, CRCs can be classified as proximal/right-sided CRC or distal/left-sided CRC, with the first being identified as all CRC cases located in the upper part of the colon track, at both the ascending colon and transverse colon, and the latter as all cases occurring in the descending and sigmoid colon, as well as in the rectum [250] (Figure 1.31)

Though not true in all cases, the majority of CRCs generally develop over time from adenomatous polyps. Polyps can change after a series of mutations in their cellular DNA, differentiating into: (i) adenomatous polyps (tubular, villous, and tubulovillous); (ii) hyperplastic polyps and inflammatory polyps; and (iii) sessile serrated polyps (SSP) and traditional serrated adenomas (TSA). Special attention is given to the last ones, often treated like adenomas due to their higher risk of developing into cancer.

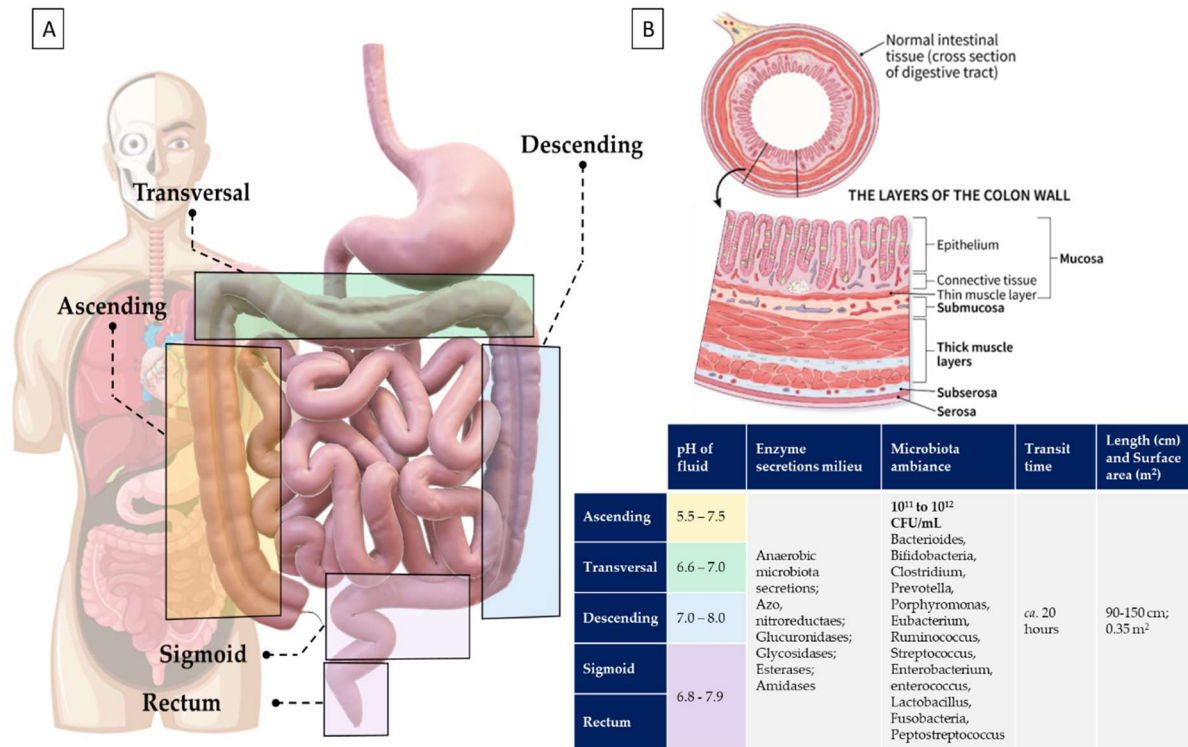


Figure 1.31 - (A) Colon schematic representation with corresponding sections. (B-*top*) Cross-sectional and lateral representations of the colon wall; (B-*bottom*) Colon sections' characteristic pH-profiles, enzymatic secretions, microbiome, overall transit time, length and surface area.

Like other types of cancers, CRC stages are classified by how deeply the tumor has grown into the wall of the colon and how much it has spread outside the colon or rectum (Figure 1.32) [251, 252].

CRC is considered a silent cancer as most cases are only diagnosed in an advanced stage at which conventional primary chemotherapies no longer prove useful and where the only fast alternative is either the sole shrinkage and suppression of the tumor, or the removal of invaded colon sections by surgery. Colon surgery in these cases entails the partial or total removal of the colon, in two possible ways: (i) via open colectomy if a long incision in the abdomen is at play, or (ii) via laparoscopic-assisted colectomy [253, 254]. Moreover, colon blockage due to large sized cancer masses also poses a serious issue in patients' health and survival, with the only alternative being surgery to either insert a stent to by-pass the blockage, or a colectomy followed by colostomy/ileostomy, where the top end of the colon is connected to a stoma in the abdomen and then to a bag [255, 256].

Despite being the best alternatives so far, these approaches do not bring any comfort and safety to patients and are often prone to serious aggravations and development of

further complications. The risks associated with such invasive surgeries go from the inefficient removal of all tumor sites to the patient's body inability to adapt and accept external bodies, as well as severe pain, colon ileus (i.e., delayed function) and bowel adhesions. Severe risks also include colon puncture and consequent stool leakage, which can develop into acute bacterial spread and infections, and lastly, if not treated, septicemia [257, 258].

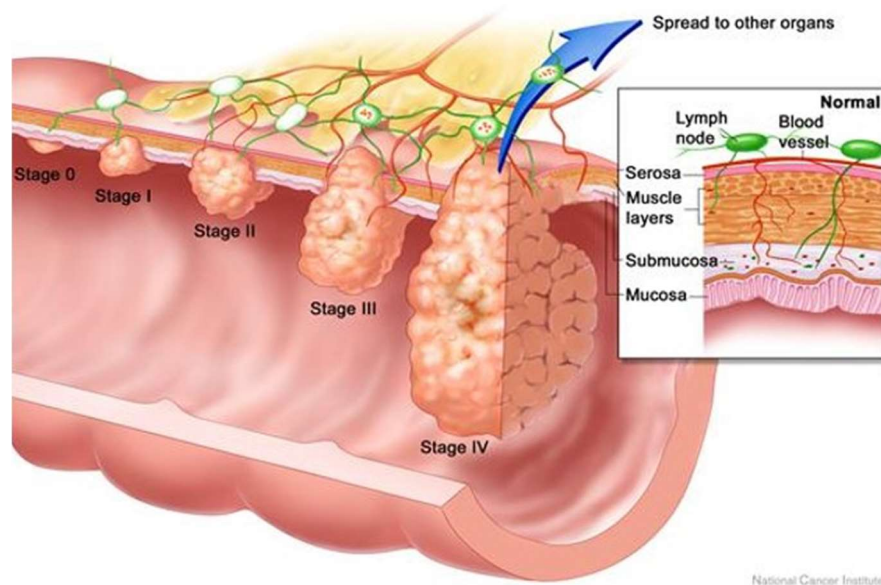


Figure 1.32 - CRC stage evolution and corresponding tissue perfusion. Reprinted with permission from [251] Copyright 2005-2022 American Society of Clinical Oncology (ASCO).

The early detection of CRC is thus of utter importance, as it could prevent advanced cases, allow earlier and easier treatments, and avoid severe cases and surgery. Yet, often this is not the case as CRC detection techniques are most of the time invasive (e.g., colonoscopies and double contrast barium enema) and inadequate to reach tumor site. This is aggravated by the lack of recurrent screening and associated stigma [259]. Due to its length (*ca.* 1.5 m colon alone), sinuous disposition and location at the end of the gut system, CRC detection approaches frequently focus on the distal part of the colon that is more easily accessible via rectum. This convention has demonstrated to be very successful in the discovery of distal CRC cancers, like sigmoid and rectal carcinomas, leading several professional to defend the hypothesis of a prevalence of distal CRC incidence over its proximal counterpart. Paralerelly, proximal CRC cases were branded as more invasive and acute. However, recent studies have proven that this is not the case, for proximal CRC incidence was only less reported due to its difficult access [260, 261]. This extends itself to the successful rates of involved therapies, with proximal CRC therapeutics having lower survival rates and being only detected when in advanced stages, most of the time from their advanced secondary ill effects [262, 263].

1.5.2 Existing chemo and targeted therapies

All above stated brings to the attention the current need for more effective chemotherapies to increase therapeutic success rates, even against more evolved tumors, as well as preventive measures to advanced stages of CRC. Current chemotherapies exist in the form of either intravenous, oral or rectal therapies that rely on the administration of high amounts of anticancer drugs, with moderate targeting capacities. Meaning, that from most administered formulations, only a small part reaches the targeted region, be it a tumor or infection.

A paraphernalia of available chemotherapies exists that attempt to tackle not only the issue of targeting, but also that of growing resistance on CRC lines to some of the available therapies [264]. Current commercial chemotherapy includes both single-agent therapy, mainly fluoropyrimidine (5-FU)-based, and multiple-agent regimens containing one or several drugs, as oxaliplatin (OX), irinotecan (IRI), and capecitabine (CAP). Although studies have argued that first-line single-agent therapy is not inferior to combined treatments regarding overall survival (OS),^{13,14} the combined therapy FOLFOX (5-FU+OX), FOXFIRI (5-FU+IRI), CAPOX (CAP+OX), and CAPIRI (CAP+IRI) remain the mainstream approaches in first-line treatment, with single-agent therapy being recommended for patients with poor performance or at low risk of deterioration.

Targeted therapies work on cancer cells by direct inhibition of tumor cell proliferation, differentiation, and migration. Moreover, the tumor microenvironment, including local vasculature and immune cells, can also be targeted to hinder tumor progression and enact greater immune surveillance and attack. Small molecules, like monoclonal antibodies and other molecular antigens, are major players in targeted therapies, working either as therapeutic or/and directing agents [264]. While some of these small molecules work within cells to inhibit mostly enzymatic activity, there are those that seek to target cell surface receptors or membrane-bound domains. This is the case of monoclonal antibodies or therapeutic antibodies that can recognize and bind them to directly regulate downstream cell cycle progression, cell death and cell adhesion [265]. Interestingly, certain monoclonal antibodies can also work on immune cells, helping to modulate the immune system to attack human tumors (Figure 1.33).

Aberrant expression levels of epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor (HER) have been identified in a group of cancers, including gastrointestinal and colorectal tumors, with the overexpression of EGFR and HER2 being observed in 25–77% and 1.3–47.7% of CRCs, respectively [266, 267]. Their overexpression triggers numerous downstream signalling pathways that mediate cell proliferation and metabolism, both vital in cancer initiation and progression. Targeting EGFR and EGFR-related pathways is a known strategy in current first-line chemotherapies against CRC, with cetuximab and panitumumab as preferred monoclonal EGFR-targeting antibodies [268]. Moreover, combinatory formulations with both antibodies and typical chemotherapies have displayed promising results, revealing better progression control, OS and progression-free survival (PFS) [264]. This approach however is side-dependent, as left-sided CRC tends to have higher EGFR expression than right-sided CRC, leading to different clinical outcomes [269]. Notwithstanding, new evidence suggests that triple formulations of encorafenib, binimetinib, and cetuximab, or of dabrafenid, trametinib and panitumumab offered significantly better survival indexes for patients with BRAF mutated metastatic CRC than previous therapies, with an equivalent rate of adverse events [270, 271]. Hence, second- or third-line treatments for BRAF-V600E-mutated mCRC may now include the “revitalized” anti-EGFR agents, by combining them with dabrafenib + trametinib, vemurafenib + IRI or encorafenib + binimetinib.

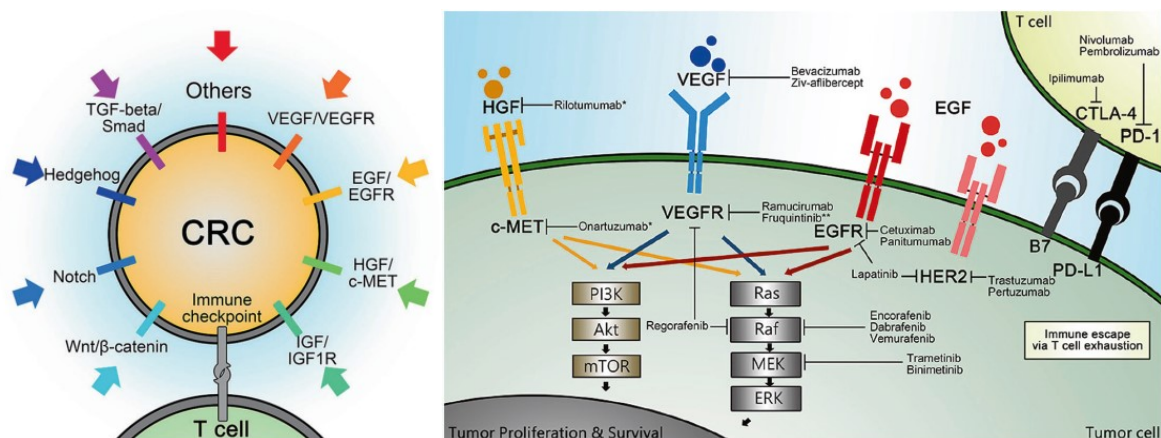


Figure 1.33 - Potential biological pathways for targeted therapies against CRC - EGF/EGFR: epidermal growth factor/epidermal growth factor receptor; HGF: hepatocyte growth factor; VEGF/VEGFR: vascular endothelial growth factor/ vascular endothelial growth factor receptor; TGF: transforming growth factor; c-MET: mesenchymal–epithelial transition factor; IGF/ IGF-1R: insulin-like growth factor/ insulin-like growth factor 1 receptor. Adapted and reprinted with permission from [264]. Copyright 2022 Springer Nature Limited.

With many shared downstream pathways, HER2 inhibitors work similarly to EGFR and are, thus, used in CRC therapeutics [272]. Studies on the combinatory targeting of HER2 and

EGFR revealed that the former's amplification might compensate for the latter's blockage, and enhance the inhibition of tumor cell proliferation, when compared to that achieved using a single agent [273]. Dual-targeted HER2 therapies have proven very promising in their clinical trials, as is the case of the double treatments with trastuzumab (classic HER2 inhibitor) and pertuzumab (HER2 dimerization inhibitor), trastuzumab and lapatinib (tyrosine kinase inhibitor targeting both HER2 and EGFR, working against HER2-positive CRC and metastatic CRC [274, 275].

Angiogenesis is a physiological process at which new vessels form, playing a vital role in tumor initiation, growth, and metastasis. Angiogenesis is regulated by various proangiogenic and antiangiogenic factors, such as VEGF, fibroblast growth factors (FGFs), TGF- α , TGF- β , platelet-derived endothelial cell growth factor (PDGF), to name a few. Overexpressed VEGFR-2 contributes to the differentiation, proliferation, migration, and apoptosis resistance of epithelial cells, thus increasing vascular tubulogenesis and permeability, both important for cancer angiogenesis and progression. [276]. Bevacizumab is a humanized IgG monoclonal antibody targeted to VEGF-A and was the first approved VEGF-targeted agent for metastatic CRC, when used either alone or with FOLFOX/FOXIRI [277]. This approach, unlike EGFR therapy, not only is side independent with both left and right-sided tumors responding well to it, but also has been validated as an efficient second-line treatment [264]. VEGFR-1 and VEGFR-2 extracellular domain recombinant fusion proteins, like Aflibercept, are an alternative when targeting VEGF-A and VEGF-B. Although its single use has its limits, when combined with other chemotherapies like FOXIRI increased beneficial responses were acquired in metastatic CRC patients [278]. Ramucirumab, a fully humanized monoclonal VEGFR-2-targeted IgG antibody, is another approved second-line treatment for metastatic CRC, whose combination with FOLFIRI significantly prolonged PFS and OS [279].

The hepatocyte growth factor (HGF) and the mesenchymal-epithelial transition factor (MET) play essential roles in tumor proliferation, survival, metastasis, and acquired drug resistance, CRC included [280-282]. This is particularly true for CRC cases where patients with advanced CRC display elevated serum HGF levels at diagnosis as well as increased mRNA and proteins levels of MET in CRC tissues, connecting it to tumor progression and metastasis [283-285]. By taking advantage of the crosstalk between MET/HGF and other factors (like EGFR) and their simultaneous overexpression, MET/HGF has been identified as the first factor responsible for EGFR and VEGFR inhibitor resistance, even in the absence of related mutations. Hence, agents targeting HGF/MET could help overcome resistance against EGFR or VEGFR inhibitors, showing improved PFS in patients MET overexpression [286, 287].

Tumors harboring numerous genetic and epigenetic alterations can be detected and eliminated by the host immune system through the expression of abnormal antigens. However, the secretion of immunosuppressive factors, like TGF- β and IL-6, the enrollment of immunosuppressive cells, or the loss of immunogenicity by downregulation of the major histocompatibility complex (MHC) molecule MHC-1, held by antigen-presenting cells (APC), can contribute to immune escape [288, 289]. Tumor-related T cell inactivation and collapse via activation of coinhibitory immune checkpoint receptors, include programmed death-1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA-4). While PD-1 is a peripheral immune checkpoint on tumor, stromal, and immune cells, CTLA-4 is a central checkpoint targeting B7-1/B7-2 or CD80/CD86 on APCs [290]. The overexpression of such ligands in metastatic CRC makes them interesting key points of interaction, and thus possible immune checkpoint targeted therapeutics capable of enhancing immune surveillance and suppression against cancer. The first PD-1 blocker with good efficacy against MMR-deficient CRC was the humanized IgG4 antibody, pembrolizumab, that started a new chapter in CRC immunotherapy when approved by FDA for metastatic CRC treatment in 2017 [291]. Parallely, in the same year, nivolumab, another humanized monoclonal IgG4-based PD-1 antibody, also gained FDA approval for dMMR or MSI-H metastatic CRC [292]. Interestingly, a combination of nivolumab with the CTLA-4 inhibitor ipilimumab was reported to surpassed single-agent immune checkpoint blocking with an acceptable rate of adverse events. This combined therapy helped patients with these types of CRC, and who had previously received chemotherapy, to reach a PFS of 71% and an OS of 85% in one year [293, 294]. For that, the combined formulation of nivolumab and ipilimumab-gained FDA approval as a first-line therapy for patients with chemotherapy-refractory metastatic CRC.

Hypoxia and acidic extracellular pH are also common features of many solid tumors, whose uncontrolled growth often results in improperly structured and functioning vasculature, low oxygenation, and upregulation of homeostasis-associated enzymes, of which carbonic anhydrases are part of [295]. The occurrence of an elevated number of CA isoforms at the cellular membranes of numerous organs and the association of their overexpressed activity to some types of tumors, have gain CAs special interest as potential targets for tumor diagnosis and therapy [296, 297]. The human (h) CA IX is an example of a tumor associated CA isoform, with several groups associating it to tumor activity, cell proliferation, cell adhesion, and malignant cell invasion [298, 299] and describing it as potential biomarkers for tumor diagnosis [265]. More specifically, hCA IX's levels and its overexpressed activity have been recently associated with higher rates of hypoxia survival and cell invasion in tumors

[300, 301]. In a potential anti-tumoral therapies, specific CA and hCA IX inhibitors like acetazolamide and derivatives, have also been reported as prospective low cost, hypoxia-activable inhibitors and targeting agents [296, 302-304]. The inhibition of CAs has been increasingly reported as a possible pathway for cancer therapy, by using CA-directed pharmacological agents as CAs inhibitors capable of imparting CAs activity and normal cell mechanisms [305, 306]. The development of novel inhibitors capable of targeting CAs, specifically hCA IX, is thus of high interest, especially given their potential conjugation with pro-drugs or with nanometric devices, rendering them targetability to hCA IX overexpressing tumors like CRC [265].

The use of nanosized vectors in the formulation of anti-CRC therapies and diagnosis has been the most recent attempt to combine targeted therapies with lower dose chemotherapies, as well as make use of additional useful properties of their constituents to better tackle CRC progression and invasiveness [307-309]. Moreover, the incorporation of nanoparticles in conventional therapies has been reported to resuscitate some outdated or inefficient drugs, by by-passing existing resistances [310]. This owns it to their capacity of masking their cargo or induce the activation of other uptake and metabolic pathways

Beyond the typical drug delivery systems that use nanoparticles as delivery vectors for conventional anti-CRC/cancer agents, like fluorouracil and doxorubicin, these can also serve as solid-state supports for targeted therapies [311, 312]. Of these, anti-EGFR systems are the most disseminated of all, with Cetuximab-conjugated nanoparticles behaving particularly well against CRC [313, 314]. Anti-CAE nanotherapies, for CRC therapies, have also been reported as an efficient targeting approach [315, 316]. Mesoporous silica nanoparticles and lipid nanoparticles have been increasingly used in such systems since both have their external surface and internal hollow structures available for both the loading of drugs and the anchoring of targeting agents. In these cases, the systems' cytotoxicity arises solely from the additive and biological active APIs, as both nanovectors' structures have repeatedly been reported to hold virtually no toxicity [317-319]. Interestingly, and with this in mind, the overall cytotoxicity of the final products can then be modulated by varying their drug content, as well as by controlling their delivery mechanisms and kinetics. The availability of their surface for further modifications paves way to the introduction of chemical gates capable of controlling the release of drugs to certain external stimuli. Thus, managing, locally and timewisely, the desired toxicity [320, 321].

Polymeric nanoparticles (e.g., PEG/PGLA and Eudragit) [322-325] and chitosan nanoparticles [326] are also common examples of inert/non-toxic solid matrices for the

development nano-based therapies, whose surface is similarly available to further targeting modifications. The former are also known to have stimuli responsive behaving matrices, modulating drug release and/or anticancer activity according to the microenvironment pH and/or temperature, to name a few.

Gold nanoparticles (AuNPs) have also been widely reported as ideal solid-stated supports for targeted therapy, owing it to gold's biocompatibility and zero toxicity to living organisms. Several are the works that use gold nanoparticles conjugated with antibodies and/or proteins in a targeting-like therapy [327, 328]. However, AuNPs are also characterized by peculiar surface electron oscillations known as surface plasmon resonance (SPR). This resonance occurs only at specific light wavelengths and can be fine-tuned by nanoparticle size, shape, as well as by their proximity to each other [329]. Owing to this property, AuNPs have also been reported as ideal agents in combined photodynamic therapies (PDT), where its high light absorbance is used to achieve light-induced ablation of tumorous cells through the generation of reactive oxygen species (ROSs) [329]. The modulation of particles shape in gold nanorods, nanoshells, or nanocages is particularly interesting as it enables near-infrared light absorption, which is optimal for deep tissue penetration applications.

Much similarly, and as afore-mentioned, SPION-based nanosystems have been reported to play a central role in hyperthermia applications, using its magnetic constituents (e.g., SPIONs) to generate heat via resonance with external alternating magnetic fields (AMF). Such applications have been reported to bring interestingly results and enhancements in anticancer therapies, be it by the generation of sub-febrile local temperatures that can stimulate immune system, by thermal ablation of tumors, or by working as local stimulus for a controlled drug-release [330]. Moreover, their use has been identified as a potential tool to overcome existing resistances to conventional anti-cancer drugs [321]

Lastly, silver nanoparticles (AgNPs) have gained increased interest due to their antiproliferative and apoptotic potentials, when obtained from *Olea chrysophylla* and *Lavandula dentata* leaf extracts. Olea-AgNPs have shown significantly antitumoral potential in the late stage of apoptotic and necrotic cells in comparison to Lav-AgNPs-treated cells [331].

1.5.3 Opportunistic microbiota and infections

Despite the available therapeutics, especially immune checkpoint targeted ones, the overall aggressiveness is accompanied by significant exhausted immune systems and consequently an increase in opportunistic bacterial infections.

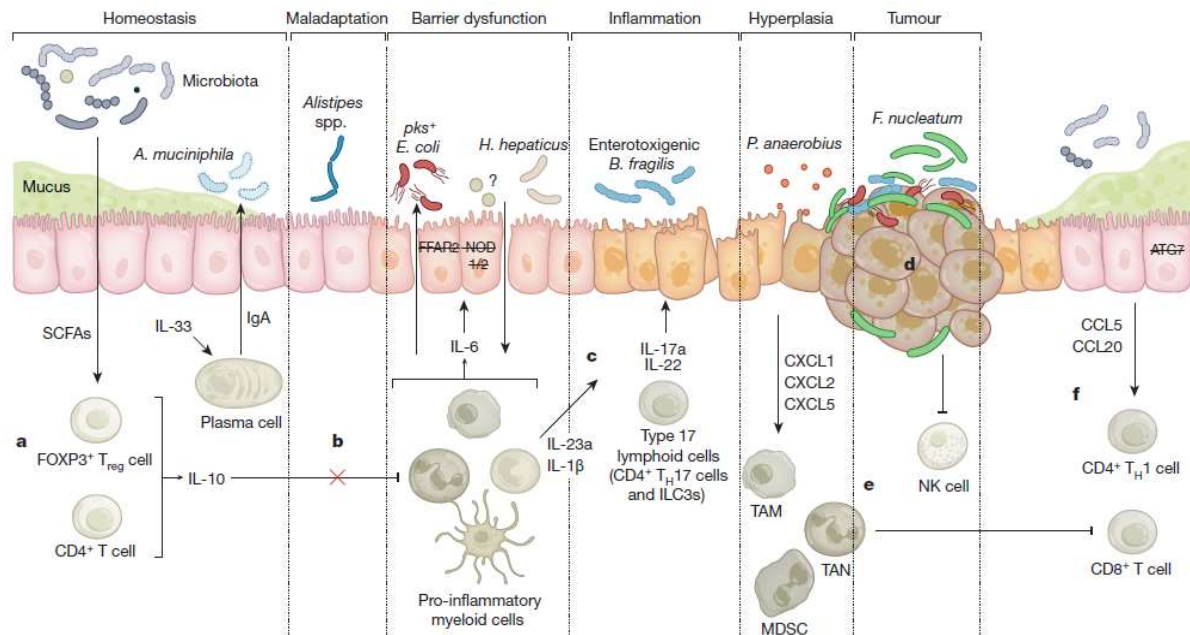


Figure 1.34 - Known inflammatory mechanisms by which the microbiota contributes to CRC. Host-microbiota maladaptation in colorectal cancer. Reprinted with permission from [341]. Copyright 2022 Springer Nature Limited.

Cancer-associated bacterial infections and inflammations have been proposed to be positively correlated with low treatment success rates and treatment associated complications [332-334]. Bacterial infections are particularly damaging in gastric and colorectal cancer (CRC) cases, with strains like *Helicobacter pylori*, *Enterococcus faecalis* and *Escherichia coli*, among others, being recurrently linked to cancer invasiveness, and progression, as well as angiogenesis [335-339].

CRC angiogenesis and progression have been reported to arise from a variety of factors, with chronic inflammations and gut microbiota dysbiosis being pointed out as interconnected and dependent first-line responsible factors in the creation of pro-tumorigenic microenvironments. An imbalanced microbiota allied an increased intestinal permeability lead lipopolysaccharides of the outer membranes of some types of bacteria to penetrate the host's intestinal cells, and consequently induce the immune system to secrete pro-tumorigenic cytokines and start a cascade of reactions that act as growth factors and promote angiogenesis [338]. Inflammation has also been reported to play a pivotal role in the formation of polyps, particularly after the bacterial invasion of specific strains that induce the proliferation of epithelial cells, producing damage in the epithelial barrier, causing inflammation and increasing the expression of several inflammatory factors like *Cxcl2*, *Tnf-α*, and IL-1/IL-6/IL-17/IL-23 [340, 341] (Figure 1.34).

In addition, the invasion of bacterial strains is not the sole factor contributing to angiogenesis and cancer proliferation, with different secreted toxins producing significant DNA damaging and tumorigenic mutations. To name a few, while *E. coli* colibactin toxin is known to cause DNA crosslinks and double strand DNA breaks, Salmonella AvrA protein has recently been revealed to induce β -catenin signalling and enhance colonic tumorigenesis through the activation of the STAT3 pathway in colon cancer models. *Fusobacterium nucleatum* has also been identified, among others, to have a CRC predisposition, by binding to E-cadherins on the surface of colon cells, via FadA adhesion, and activating Wnt/B-catenin signalling, an inflammatory and oncogenic response [338].

Bacterial metabolites are also included in the list of tumor-promoting toxins, with short-chain fatty acids (SCFAs), bile acids, trimethylamine and its *N*-oxide counterpart (TMA/TMAO), polyamines, *N*-acyl amides and hydrogen sulfide being recurrently identified in many reports as core CRC drivers [342-345]. Most of their action goes through the generation of significant oxidative stress/damage through reactive oxygen species and, thus, DNA damage (Figure 1.35).

Lastly, the occurrence of mucus-invasive bacterial biofilms in the colon mucosa of both CRC and healthy patients has also had its role in angiogenesis under debate, with several studies strongly reporting it as a potential tumorigenesis driver [344, 346, 347]. Despite the lack of a full understanding of its action mechanisms, it is suggested that biofilms take part in the evolution of tumors by disruption of homeostasis through dysbiosis.

All above-mentioned factors, when allied to an increasing occurrence of antimicrobial resistant bacteria, pose a severe threat to not only current cancer therapies successful rates, but most importantly to patients' health and survival rates [348].

Consequently, the search for new strategies to battle bacterial infections and outstrip existing resistances is a current hot topic, with several groups exhaustively using their resources on either the production of new antibiotic [349], or the use of alternative nature-occurring APIs [350]. Equally, the combinatory use of conventional antibiotics and adjuvants [351], or the use of engineered nanomaterials [352] have also been addressed as novel tactics to resurrect or enhance the activity of conventional antimicrobial agents.

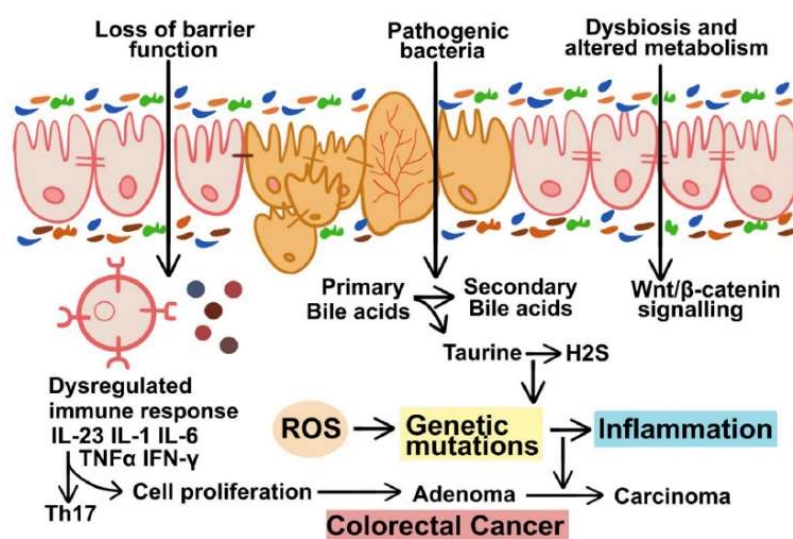


Figure 1.35 - Possible roles of microbiota in CRC causation, through loss of epithelial barrier function, increased pathogenic abundance, microbial dysbiosis and altered metabolism Reprinted with permission from [342]. Copyright 2022 Springer Nature Switzerland AG.

Much like its role in anti-tumor therapies, nanotechnology has gained especial attention throughout the last decades, due to their intrinsic properties (i.e., antimicrobial activity), tunability and easier manipulation. Among other metallic systems, silver and copper nanoparticles are successfully commercial examples of nanoparticle systems where the very material has antimicrobial properties, due to the continuous release of metallic ionic species. Iron oxide nanoparticles have also piqued the interest of researchers since they not only provide greater control and maneuverability over therapies due to their magnetic properties, but they have also been shown to have antibacterial and anti-biofilm qualities. [353, 354]. Again, the employment of magnetic iron oxide nanoparticles as approved contrast agents, as well as heat producing and delivery probes in a variety of diagnostic and therapeutic approaches (as above-mentioned), proves their crucial biocompatibility and safe use in humans. [355]. Moreover, and as above-mentioned, the stimulation of the immune system by the generation of subfebrile temperatures, as well as the capacity to tackle biofilm formation make them ideal for antimicrobial applications [330]. Non-antimicrobial nanoparticles have also been used in the making of antimicrobial solutions through the combination of a non-toxic matrix with bacteriostatic or bactericidal chemicals. This conjugation has been shown to have synergistic therapeutic benefits between its parts, whether by a combined action or the masking and effective delivery of the antimicrobial agent to its target, by the nanomaterial. [319]. Much similarly to their application in anti-cancer therapeutics, gold and mesoporous silica nanoparticles are examples of functional non-toxic solid matrices, whose surfaces can not only be easily functionalized but also work as good anchoring sites for bioactive

molecules, namely antibiotics to render them with antimicrobial properties [356, 357]. Beyond their additional surface functionalization, the combination of multiple particle types renders them with extra simultaneous properties, like stimuli-responsive and targeted drug delivery, long-term blood circulation, higher cellular up-take, magnetic/photothermal hyperthermia and fluorescence, that can be used to expand their antimicrobial properties [358].

All things considered, the development of tailored nanotherapies capable of combating both cancer and bacterial infections is critical for the future development of effective therapies. [359]. This is supported by the recent, yet scarce, reports on the interconvertible use of antibiotics as anticancer targets and of anticancer drugs as antimicrobial agents, as well as their combinatory, synergic therapeutic effects [360-362]. A preliminary study of the combined properties of a nanosystem with a combinatory anticancer-antibiotic formulation against several cancer-related strains has shown us the potential of such strategies for efficient therapeutics against targeted strains, as well as resistant strains, as methicillin-resistant *Staphylococcus aureus* (MRSA) [319].

1.6 Conclusions and Future perspectives

From this close review on synthesis, post-synthesis modifications and arising applications of both SiQDs and SPIONs, the importance of their use and improvement has been highlighted. Both systems have shown promising results due to their unique intrinsic properties, stability, biocompatibility and, most important, tuneability.

Despite their outstanding performances and benefits as individual systems, efforts on the development of hybrid systems and on the comprehension and optimisation of its underlying mechanisms are yet scarce. With little to no perception on SPIONs shape influence, SPION-QD/SiQD optimal distances, SPION-QD/SiQD charge transfer mechanisms, linking-molecules' or loaded drugs' influence on magneto-fluorescent properties, as well as additional surface modifications as mesoporous silica or metallic mesostructures, to name a few. Thus, leaving a gap in knowledge (and also an opportunity) which, through few *state-of-the-art* approaches, has started to prove to be as tuneable, effective and applicable as their individual counterparts, while allowing for outstanding multi-tasking and multi-targeting applications in areas as imaging/mapping, drug delivery, diagnosis, theragnostic and remediation for the same, or even less, amount of resources.

The various synthetic methods herein addressed for SPIONs, SiQDs and magneto-fluorescent hybrids have allowed for the creation of nanoparticles with different sizes,

structures, dispersions and surface modifications. Indeed, surface modifications of these nanoparticles with other molecules, polymers and silica lead to a stabilisation of the nanosystems, as well as, to a decrease in toxicity allowing then biological applications. Despite the toxicological studies of some of these nanoparticles, the exact mechanism of nanoparticles toxicity remains unclear, being necessary a deeper study on the toxicity and biocompatibility of the fluorescent-magneto hybrid nanoparticles and their individual constituents.

Notable advances in the synthesis of nanoparticles with flexible designs for smart delivery and personalised treatment have been made. Multifunctional nanoparticles, as magnetic-fluorescent nanoparticles, are with no doubt the most useful tools. For target magnetic-fluorescent nanoparticles have tremendous potential for diagnosis and medical applications, although some improvements and insights are urgently required regarding their construction and toxicity.

1.7 Acknowledgments

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1.8 References

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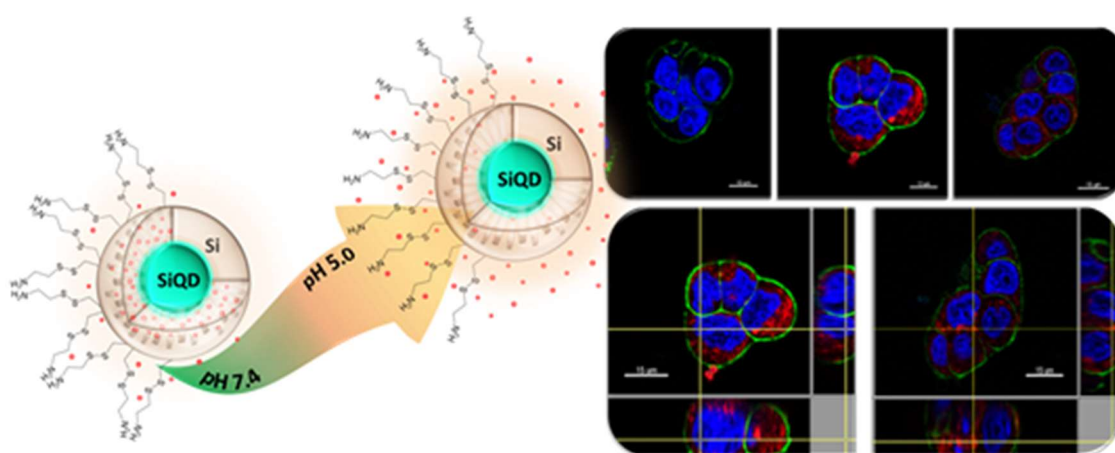
OBJECTIVES

Given the urgent need for non-evasive. CRC specific therapies, capable of simultaneously eradicate opportunistic bacterial infections, and the relevance of nanoparticles for the construction of maneuverable and stimuli-responsive drug-delivery vectors, the present PhD thesis aims to contribute to the field with the design, synthesis, characterization and *in vitro* evaluation of biocompatible and biodegradable magneto-fluorescent nanoparticles, for the drug delivery of anticancer (e.g., doxorubicin) and antimicrobial (e.g., ofloxacin) drugs, in a new oral nanotherapy against CRC and associated bacterial strains.

The specific objectives referred to each chapter are:

- The development of a non-toxic, pH-responsive, SiQD-based fluorescent nanocarrier for the delivery of doxorubicin as model anticancer drug to CRC cell lines.
- The acquisition of a proteomic insight in the effects of different surface modified nanoparticles on CRC cells proteome and membrane proteins.
- The production of core-shell-like mesoporous Au@Si nanoparticles and assessment of their potential antimicrobial activity against common antimicrobial resistant bacterial strains, via integration of amoxicillin and ofloxacin as model antibiotics.
- The synthesis of core-shell fluorescent superparamagnetic silica nanoparticles, from sustainable materials, with pH- and hyperthermia-enhanced cargo delivery capacity of a dual doxorubicin-ofloxacin formulation, in a potential dual therapy against cancer and cancer associated opportunist bacterial Infections.
- The development, validation and optimization of a new and more sustainable bioluminescent-based method for the assessment of the antimicrobial activity of nanoparticles against bacteria, by an in-house modification of a commercial *E. coli* strain.
- The synthesis of a hCA IX-targeting acetazolamide derivative and its use on the optimization and surface co-biofunctionalization, with the EGFR-specific Cetuximab, of the previously produced magneto-fluorescent silica nanoparticles. The assessment of its combined doxorubicin and ofloxacin delivery capacity, *in vitro* anticancer and antimicrobial activity and biodegradability, for a future oral CRC-specific therapy.
- The production of PVA:PVP biocompatible polymeric scaffolds doped with alternative cyanine derivatives as antimicrobial active agents, for potential inclusion in medical surfaces as an extra layer of security against antibiotic-resistant bacterial strains.

LUMINESCENT SILICON-BASED NANOCARRIER FOR DRUG DELIVERY IN COLORECTAL CANCER CELLS



Keywords: Luminescence • silicon quantum dots • mesoporous silica nanoparticles • drug delivery • colorectal cancer cells • doxorubicin

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Luminescent Silicon-Based Nanocarrier For Drug Delivery In Colorectal Cancer Cells

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Abstract: Nanocarriers sensitive to exogenous or endogenous stimuli emerged as an attractive alternative to target drug delivery, with inorganic silica mesoporous nanoparticles (MNs) playing a core role in the development of a new generation of non-toxic and tuneable nanocarriers. A sensitive nanovector (NANO1) comprising luminescent silicon quantum dots (SiQDs) and functionalised with MNs was synthesised and loaded with doxorubicin (DOX). NANO1 nanoparticles have a size of 74 ± 10 nm and DOX loading percentages of ca. 43%. As a control sample, a similar nanocarrier (NANO2), without SiQDs, was also synthesised and loaded with DOX. Release profile studies, in PBS, revealed the strong NANO1@DOX pH-dependant behaviour, with a pH 5.0 favouring the release of DOX to percentages of ca. 70%. Cytotoxicity assessments of both free and DOX-loaded nanocarriers were evaluated in human cell lines of colon, revealing both free drug and drug-loaded nanoparticles to be concentration-dependent.

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3.1 Introduction

Colorectal cancer (CRC) is the second most common cancer and the fourth cause of cancer deaths in the world, with nearly 1.4 million new cases diagnosed in 2012 [1]. The development of new nanotech-based carriers to increase (i) specificity to target tumour cells, (ii) delivery capacity to the tumour site, therefore maximising the efficacy of the therapy and (iii) minimising undesirable side effects, is crucial [2, 3]. Nanocarriers sensitive to exogenous or endogenous stimuli emerged as an attractive alternative to target drug delivery, but only a few stimuli-responsive nanosystems have reached clinical stage due to their complex design and challenging synthetic scale-up [4].

From all nanoparticles systems that have been used as drug delivery agents, luminescent inorganic silica mesoporous nanoparticles (SiQDs MNs) emerged as a promising multifunctional new generation of nanocarriers. Their use as drug delivery devices is due to their low toxicity, high specific surface area, large pore volume, tunable pore structures, and size [5-7]. Usually, to facilitate their tracking in biological systems, nanocarriers are labelled with a luminescent dye or, in this case, a luminescent inorganic quantum dot. Based on previous studies, silicon quantum dots are non-toxic biocompatible emissive materials, with some *in vivo* experiments revealing that silicon degrades to silicic acid that is excreted in urine [8-11].

The first reports on silicon nanocrystals were published two decades ago [12, 13]. However, challenges still remain on the working procedures for this type of material [14], as i) the preparation of high-quality colloidal silicon nanocrystal in a feasible manner, ii) the easy control of their size and optical properties and iii) the avoidance of surface oxidation on silicon quantum dots. Regarding the available synthetic methods for SiQDs, most methods reported to date show related harsh conditions (strong acids, toxic reagents, organic solvents, high temperature, inert atmosphere), specialised equipment, complex experimental procedures, not easily approachable by non-experts, and with scaling up problems [14]. For a successful application in biological systems, SiQDs must be water-soluble, avoiding then any post-treatment and expensive processes. Recently, Y. Zhong and co-workers reported an aqueous synthetic method to prepare fluorescent SiQDs under microwave irradiation at 160°C [15]. Later, the same group developed a photochemical strategy towards the synthesis of large-quantity of SiQDs under UV irradiation [16]. Fabrication of ultrabright water-dispersible SiQDs through a designed chemical surface modification [17], as well as, one-step

synthesis of water-dispersible SiQDs for time-resolved imaging of living cells [18] and for selective sensing of heparin and cell imaging [19] were already reported by other authors. Based on these previous studies, in this work, SiQDs were performed in a one-step synthesis in water using (3-aminopropyl) trimethoxysilane (APTMS) and sodium ascorbate (SA) [18].

Despite all reported synthetic methods of silicon quantum dots in water [15-19], nanocarriers resulting from the combination of silicon quantum dots and silica mesoporous nanoparticles are yet scarce [20, 21]. Taking into account that all nanocarriers developed to date are under the preclinical stage for CRC [22-35], and none of them combines luminescent quantum dots (silicon quantum dots) and mesoporous silica nanoparticles. Herein, it is presented for the first time the synthesis of a sensitive nanocarrier combining silicon quantum dots and silica mesoporous nanoparticles and consequent surface functionalization, as well as its cytotoxic evaluation in colorectal cancer cells and drug delivery capacity (via pH stimuli) in saline buffer (at pH 7.4 and 5.0).

To surmise, the delivery of anti-cancer drugs in fully non-toxic materials (non-use of toxic luminescent QDs) comprising SiQDs and mesoporous matrixes, has, according to literature [36, 37], never been reported. Thus, the novelty of the work stands, despite a recent report by the authors on the sole toxicity of mesoporous SiQD-based systems [21], on the foremost development of similar nanosystems in the controlled delivery of DOX in specific cancer colorectal cell lines, for future applications as building blocks in targeted nanotherapies.

3.2 Experimental

3.2.1 Chemicals and starting materials

3-aminopropyl)triethoxysilane (APTES, $\text{H}_2\text{N}(\text{CH}_2)_3\text{Si}(\text{OC}_2\text{H}_5)_3$, 99%), (+)-sodium, L-ascorbate (SA, $\text{C}_6\text{H}_7\text{NaO}_6$, $\geq 98\%$), tetraethyl orthosilicate (TEOS, $\text{SiC}_8\text{H}_{20}\text{O}_4$, 98%), ammonia (NH_3 , $\geq 99.9\%$), cetyltrimethylammonium bromide (CTAB, $\text{C}_{19}\text{H}_{42}\text{BrN}$, $\geq 98\%$), (3-mercaptopropyl)trimethoxysilane (MPTS, 95%), aldrithiol-2 (98%) (2,2'-Dipyridyl disulfide), cysteamine ($> 98\%$), were purchased from Sigma-Aldrich. Ammonium nitrate (NH_4NO_3 , 99.9%) was purchased from Alfa Aesar.

Methanol (MeOH) and ethanol (EtOH) were produced by Carlo Erba Reagents. All solvents and reagents were of analytical reagent grade and were used as received.

3.2.2 Synthetic procedures

Silicon quantum dots (SiQDs) were prepared by adding 2 mL of 3-aminopropyltriethoxysilane (APTES) to 4 mL of miliQ water, followed by the addition of 4 mL of an aqueous solution of sodium ascorbate (0.1 M). The final mixture was stirred for 2 hours at 50°C, leading to the formation of green emissive SiQDs, as reported in literature [18]. To the previous mixture (SiQDs) was added 10 mL of an aqueous solution of CTAB (0.1 M) and kept under stirring for 30 minutes at 50°C. Ammonia (500 μ L) was added and the mixture was stirred for a further 10 minutes. Then, 240 μ L of TEOS was added dropwise and the final mixture left under stirring 2 hours at 70°C. After 2 hours, 3-Mercaptopropyltrimethoxysilane (1 mL) was added and after 30 minutes of stirring the resulting nanoparticles were washed with water and centrifuged, resulting in the nanoparticles SiQDsMNsSH. The SiQDsMNsSH nanomaterial (200 mg) was treated with a methanol solution containing the precursor PDEA (2-(pyridyldisulfanyl) ethylamine) (200 mg), which was previously synthesised accordingly to references [38, 39], for 24 hours under stirring. The resulting nanomaterial SiQDsMNsS-S-NH₂ (NANO1) containing the 2-(propyldisulfanyl) ethylamine linker was centrifuged and washed several times with methanol and dried. Template removal was achieved by solvent extraction by resuspending the nanoparticles in a 20 mL methanolic solution containing 60 mg ammonium nitrate, that was stirred for 2 h at 60 °C. The procedure was repeated twice and the nanoparticles centrifuged and washed in water.

As a control sample the same synthesis was repeated but without the addition of silicon quantum dots (SiQDs), resulting in MNsS-S-NH₂ (NANO2) nanoparticles.

3.2.3 Nanoparticles characterization

UV-Vis absorption spectra were recorded on a *Jasco V-650* spectrophotometer and a NANODROP ND-1000; and Fluorescence emission in a HORIBA Scientific FLUOROMAX-4 spectrofluorimeter from Proteomass-Bioscope facility (Portugal).

Infrared spectra were acquired in a FTIR Spectrometer Spectrum two, UATR TWO PERKIN ELMER.

Hydrodynamic size and zeta potential were obtained by dynamic light scattering, by dispersion of the nanoparticles in miliQ water, in a Malvern Nano ZS Zetasizer instrument with a 633 nm laser diode, from Proteomass – Bioscope facility (Portugal).

Transmission electron microscopy (TEM) images were obtained in a JEOL JEM 2010F operating at 200 kV, TEM images were collected using a multiscan camera and Digital Micrograph software from Gatan (CCD Gatan895 USC4000).

Surface area and pore size distribution were obtained from N₂ adsorption/desorption isotherms at 77 K, in a Micromeritics ASAP 2010 (Accelerated Surface Area and Porosimetry), at the Laboratory of Analysis from FCT - UNL. Specific surface areas (S_{BET}) were estimated with the BET method [40].

XRD spectra were acquired with RIGAKU MiniFlex II X-ray diffractometer equipped with a Cu-K α source (30 KV/15 mA) for 2 θ angles ranging from 3° to 80°.

Fluorescence quantum yield of SiQDs was measured using as standard fluorescein ($\phi=0.79$) [41] in ethanol. The value of fluorescence quantum yield was corrected accordingly with the different refraction indexes.

3.2.4 Doxorubicin loading and in vitro release studies

To a solution of doxorubicin (DOX) (1 mg/mL), previously prepared in phosphate buffer (PBS) pH 7.4, were added 24 mg of NANO1 (SiQDsMNsS-S-NH₂) or NANO2 (MNsS-S-NH₂) nanoparticles and kept under stirring for 24 hours at room temperature. After that, the sample was centrifuged and washed four times with PBS 7.4. The supernatants were quantified by absorption in the NANODROP ND-1000. From which, was obtained the encapsulation efficiency (EE%) and the loading capacity (mg/g) were determined by the following equations (t_{DOX} : the total amount of DOX, f_{DOX} : the amount of free DOX in solution) [42]:

$$EE (\%) = \frac{t_{\text{DOX}} - f_{\text{DOX}}}{t_{\text{DOX}}} \times 100\% \quad (\text{Equation 1})$$

$$\text{loading capacity (mg/g)} = \frac{t_{\text{DOX (mg)}} - f_{\text{DOX (mg)}}}{\text{amount of nanoparticles (g)}} \quad (\text{Equation 2})$$

The *in vitro* doxorubicin release of the encapsulated NANO1@DOX and NANO2@DOX was performed by suspending the nanoparticles (10 mg) in a 2 mL solution of PBS 7.4 and PBS 5.0. All suspensions were stirred and kept at 37 °C. Aliquots were collected at different times and the released doxorubicin quantified by absorption ($\lambda = 480 \text{ nm}$) in the NANODROP ND-1000.

3.2.5 Cell culture

Colon cancer cell lines HT29 and HCT116 were obtained from the ATCC (American Type Culture Collection). All cell cultures were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) in standard growth conditions (37°C, 10% CO₂).

3.2.6 Cytotoxicity and intracellular accumulation of the nanoparticles

The cytotoxicity of free nanoparticles NANO1 and NANO2, as well as loaded with doxorubicin (NANO1@DOX; NANO2@DOX) was tested in human cancer colon cells using the PrestoBlue assay. Cells were seeded into a 96-well plate at a cell density of 3.0×10^3 cell/well and incubated for 24 h before nanoparticles were added at a concentration from 1 to 300 µg/ml. After 72 h, 20 µL of prestoBlue reagent were added to each well and the plates were further incubated for 4 h at 37°C. The effect on cell viability was measured using a multilabel plate reader (Victor3 PerkinElmer). Its fluorescence was quantified after excitation at $\lambda = 531$ nm and collection at $\lambda = 615$ nm, while its absorbance was monitored at $\lambda = 570$ nm. Cell cytotoxicity was evaluated in terms of cell growth inhibition in treated cultures and expressed as a percentage of the control conditions. Each experiment was repeated at least three times, and each concentration was tested in at least three replicates.

For cellular accumulation determination, 10^5 HT29 cells were plated in 35 mm-cell culture dishes and were allowed to grow overnight. Cells in exponential growth were incubated, for 24 h, with 50 µg/ml of each nanoparticle (untreated cells and cells treated with 1.75 µM of doxorubicin were used as a control). After incubation, cells were washed twice with PBS and lysed with PBS containing 1% SDS. Fluorescence intensity of the lysates was measured in a Jasco FP-8200 spectrofluorometer at $\lambda_{\text{ex}} = 370$ nm/ $\lambda_{\text{em}} = 499$ nm and $\lambda_{\text{ex}} = 470$ nm/ $\lambda_{\text{em}} = 589$ nm. Initial fluorescence intensity was measured from 50 µg/ml of each nanoparticle in PBS containing 1% SDS (Sodium Dodecyl Sulphate), and the uptake ratio was calculated for every fluorescence-emitting nanoparticle.

3.2.7 CLSM *in vitro* imaging of Nanoparticles-Treated cells

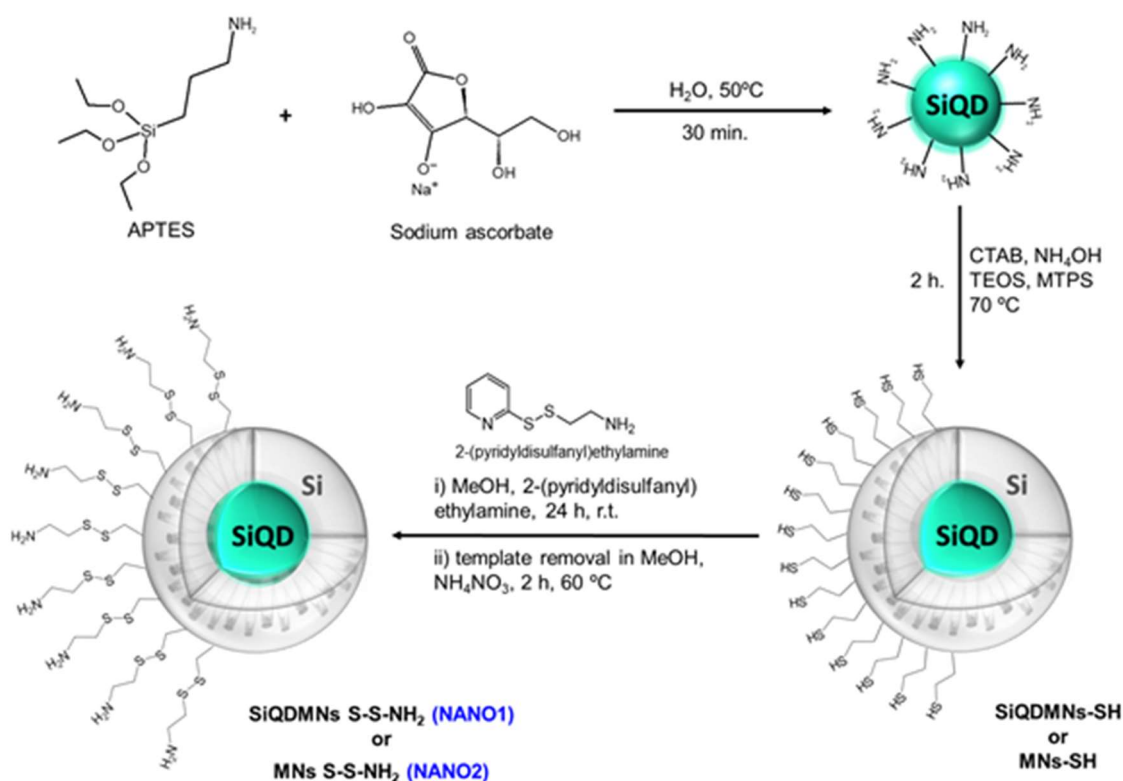
Cellular uptake experiments were performed on HT29 cell line. Cells were seeded at a density of about 2×10^4 cells per plate on 14 mm glass-bottom microwell dishes (MatTek, corp). After 24 h, the cells were treated with 25 µg/ml of NANO1@DOX and NANO2@DOX, and incubated for additional 24 h. Cells were washed three times with cell media, and cell

nuclei and membrane were stained with Hoechst or CellMask respectively for 15 min. Cell images were taken using a laser scanning confocal microscope (LSCM) SP5 (Leica).

3.3 Results and Discussion

3.3.1 Synthesis and characterization

Luminescent Silicon quantum dots (SiQDs) were synthesised in water and in open-air conditions at 50°C for 2 hours. As silica source and as stabilizer/reducer APTES and sodium ascorbate were used. Green luminescent SiQDs show excitation and emission maximum bands centred at ca. 370 nm and ca. 510 nm, respectively (Figure 3.1). Additionally, the SiQDs presented a hydrodynamic size of 3.6 nm, a zeta potential of -3.6 mV and a fluorescence quantum yield of 13%. The resulting SiQDs were covered with a layer of mesoporous silica nanoparticles (SiQDsMNs), and further functionalized with a layer of MPTS (SiQDsMNsSH) and finally the 2-(propyldisulfanyl) ethylamine linker was added to the nanoparticles, leading the final nanoparticles NANO1 (SiQDsMNsS-S-NH₂), depicted in Scheme 3.1 and Figure 3.1.



Scheme 3.1 - General synthetic pathway of the NANO1 and NANO2 nanoparticles.

As control sample for the *in vitro* assays in colon cancer cell lines, the same nanocarrier without silicon quantum dots was also synthesized (NANO2). The final

nanocarrier NANO1 was characterized by solid-state emission, TEM, FTIR, zeta potential, N_2 isotherms and XRD. Moreover, the different coatings were confirmed by zeta potential, FTIR and N_2 isotherms. As can be seen in figure 2.1B, NANO1 was obtained as a white blue emissive powder with a maximum emission band centred at 475 nm. As reported by Dasog and co-workers [37, 43, 44], the emission shift from green to blue upon functionalization of the SiQDs with mesoporous silica nanoparticles is attributed to the presence of nitrogen and oxygen sources leading the formation of silicon oxynitride type species.

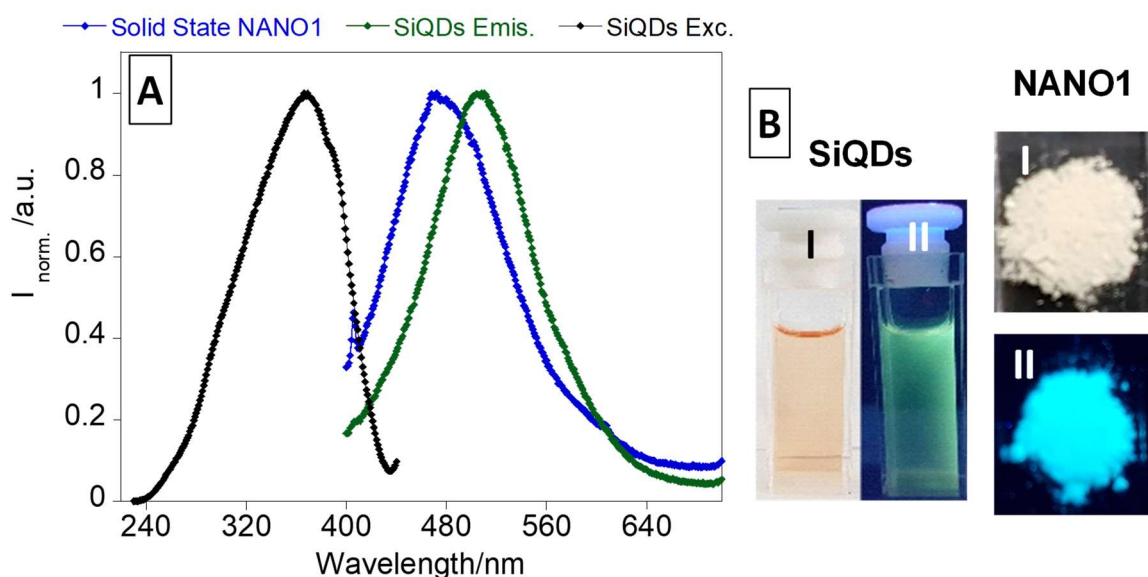


Figure 3.1 - (A) Emission and excitation spectra on SiQDs and solid-state emission spectrum of NANO1 nanoparticles ($\lambda_{\text{exc}} = 370$ nm, $\lambda_{\text{em}} = 510$ nm). (B) Naked-eye (I) and under UV lamp (II) ($\lambda_{\text{exc}} = 365$ nm) images of Silicon quantum dots in solution and of the NANO1 nanoparticles in solid state.

Based on TEM images NANO1 present a spherical shape with a size of 74 ± 10 nm (see Figure 3.2A-B). The presence of silica element was also demonstrated in the EDX spectra in Figure 3.2C.

To investigate the porosity the nitrogen adsorption/desorption isotherms were performed (Figure 3.2E). The luminescent un-functionalized, SiQDs MNs, showed a BET surface area of $677.7 \text{ m}^2/\text{g}$ and pore size of 4.1 nm (BJH model). The isotherm can be classified as a type IV isotherm with an adsorption step behaviour at P/P_0 around $0.3\text{-}0.35$, characteristic of mesoporous materials [45]. With the addition of further coatings, it is notable a decrease of surface area, with values of 96.9 and $13.5 \text{ m}^2/\text{g}$ for the nanocarriers of SiQDs MNsSH and SiQDs MNs-S-S- NH_2 (NANO1), respectively. Due to the decrease of surface area, and so of the mesoporosity of the system, the consequent release through the pores of the silicon quantum dots is prevented, and the luminescent characteristics of the nanocarrier

maintained. The XRD spectra (Figure 3.2F) showed a broad peak at ca. 22° (SiQDsMNsSH, NANO1, NANO2), which corresponds to the amorphous peak of silica oxide.

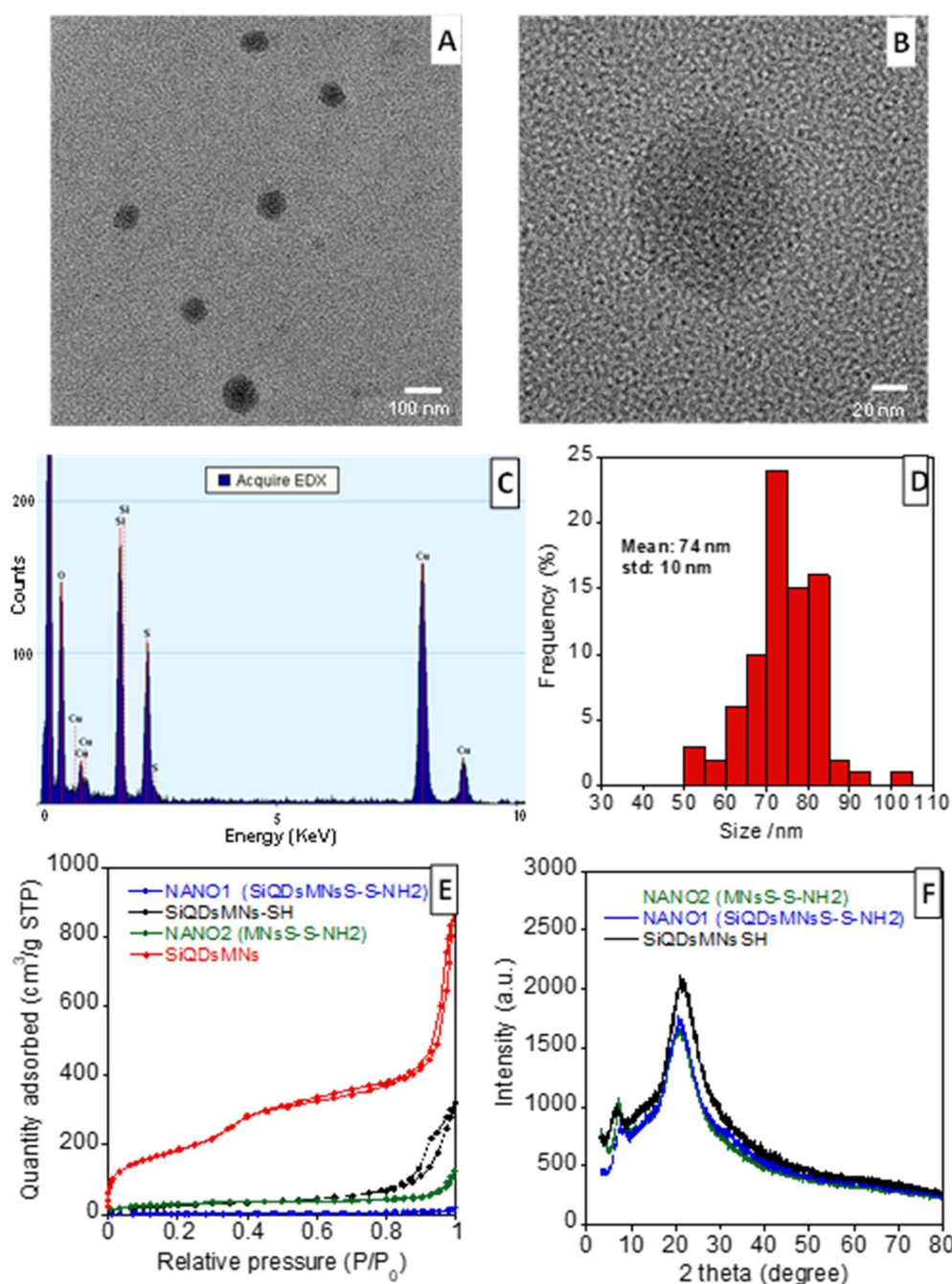


Figure 3.2 - TEM images (A, B), EDX spectrum (C) and size distribution (D) of NANO1 nanoparticles. (E) Nitrogen adsorption –desorption isotherms of NANO1, SiQDsMNsSH, NANO2 and SiQDsMNs nanoparticles. (F) XDR spectra of NANO1, NANO2, and SiQDsMNsSH.

Zeta potential showed that the NANO1 had a surface charge of 44.8 ± 0.8 mV, while that of SiQDsMNs and SiQDsMNsSH was of 28.6 ± 0.6 mV and 40.6 ± 0.7 mV, respectively (Figure 3.3). The above described values were obtained in miliQ water and at neutral pH of

7.0. From the general point of view the increase of coatings, and so, surface functionalization stabilises the nanocarrier, turning it more positive. The different coatings were also verified by infrared spectroscopy. The FTIR spectra (Figure 3.3) of SiQDsMNsSH and NANO1 show the characteristic bands of silica structure with a peak at 1028 cm^{-1} corresponded to the vibrations Si-O-Si (asymmetric stretching). The absence of a peak at ca. 965 cm^{-1} of the SiQDsMNsSH nanoparticles shows that -OH on the nanosilica surface has reacted completely with MPTS. Covering by MPTS can also be confirmed by the peaks at 1117 cm^{-1} (-Si-O-C-stretching), 1237 cm^{-1} (Si-CH₂-R stretch) and 2932 cm^{-1} (CH stretch). Moreover, the amino linker was confirmed by the peak at 1521 cm^{-1} , 1627 cm^{-1} corresponded to the groups (C-N stretch) and (R-NH₂ in-plane bend), respectively.

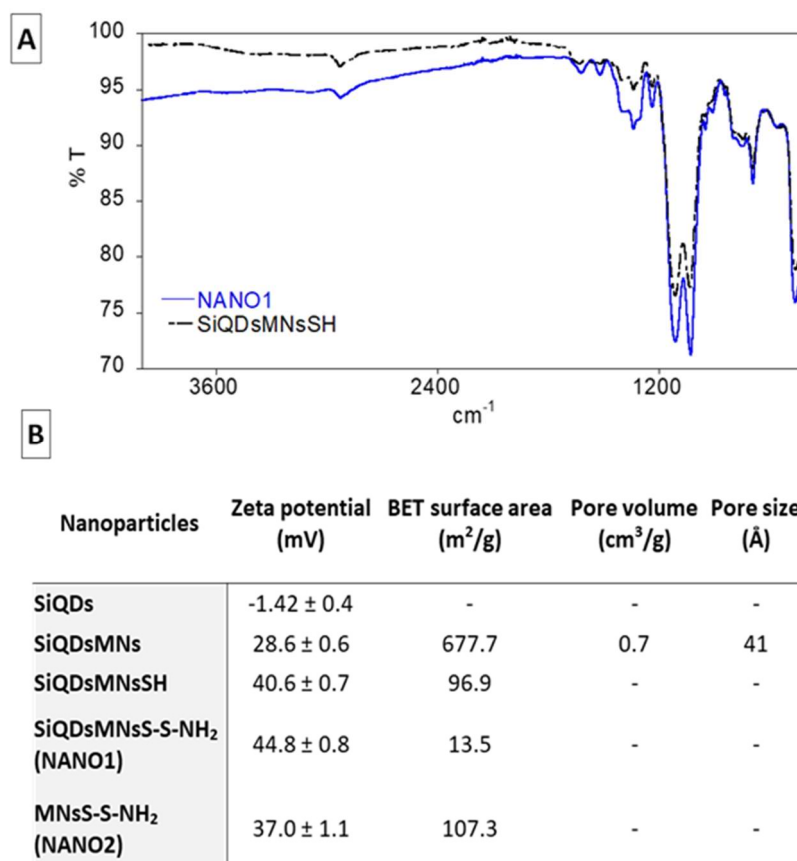


Figure 3.3 - (A) FTIR spectra of SiQDsMNsSH and NANO1 nanoparticles. (B) zeta potential, BET surface, pore volume and size data of the nanoparticles

3.3.2 Doxorubicin loading/release

Doxorubicin, a common drug used in cancer treatment, was chosen as a drug model to investigate the potential application of the luminescent nanocarrier NANO1. Doxorubicin loading into NANO1 and NANO2 was performed according to section 3.2.4, and from

equations (1) and (2) were obtained an encapsulation efficiency of 41%-43% and a loading capacity of 18 mg and 20 mg of doxorubicin per gram for NANO1 and NANO2. According to the obtained results, the total covering the NANO1 surface and the core of SiQDs, led to a reduction in the system's mesoporosity, implying that DOX interactions and loadings occur mostly at nanoparticles surfaces *via* strong electrostatic and hydrogen bonding. In NANO2 despite the surface coverage, the absence of a SiQD core, lead to a slightly higher surface area, with some DOX adsorbing as well onto the pore's walls, thus justifying the marginally superior loading ratio. Drug release assays were performed in PBS at two different conditions: pH 7.4 and pH 5.0 (Figure 3.4). Whilst at pH 7.4 no significant drug release was observed, for NANO1 (ca. 5%), this phenomenon was favoured at pH 5.0 where release percentages of ca. 70% were obtained. As previously reported for mesoporous silica nanoparticles [46], a burst of DOX release (5 minutes) was observed for NANO1 nanocarrier. In contrast, NANO2 nanocarrier release percentages of ca. 20% and 50%, lower than those of NANO1, were obtained at pH 7.4 and pH 5.0 only after 30 minutes of continuous release. The observed release behaviour is partly attributed to DOX solubility differences, and the attractive and repelling forces developed between the drug and the modified nanoparticles at the respective pH values. The nanoparticles have a positive charge, while DOX is positively charged at low pH and partly neutral at physiological pH conditions ($pK_a = 8.25$). The repulsion between DOX and the organic coating and DOX with the pores' wall is different. The repulsion at acidic pH is higher between positive charged DOX and positively charged surface coating, which justifies the higher percentage release in NANO1 at pH 5.0. Thus, the DOX release NANO1 nanocarrier is favoured by acidic conditions (Figure 3.4).

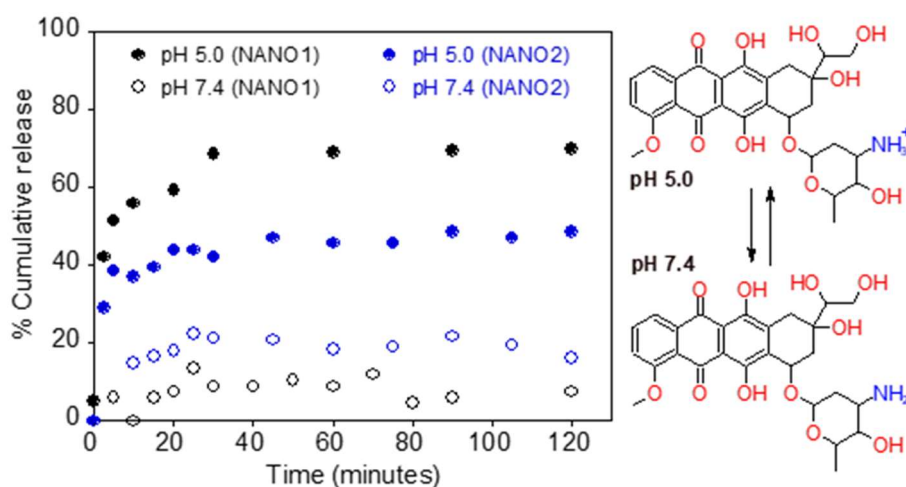


Figure 3.4 - The release profile of doxorubicin loaded into the NANO1 and NANO2, at pH 7.4 and pH 5.0 PBS buffers. Chemical structure of doxorubicin.

3.3.3 Dose-dependent of cytotoxicity of Nanoparticles

The viability of cell cultures, after exposure to free doxorubicin and the different DOX-loaded, or non-loaded, nanoparticles, was examined using PrestoBlue (Cell viability reagent [47]). The assay measures the amount of resazurin reduction by mitochondrial dehydrogenase and assumes that cell viability (corresponding to reductive activity) is proportional to the production of resorufin which is measured spectrophotometrically (570nm), or fluorescently, by exciting at 531 nm and collecting the emission at 615 nm. Exponentially dividing cells were treated with the different concentrations (from 1 to 300 $\mu\text{g}/\text{ml}$) of each nanoparticle for 72 h, as described in the experimental section. Control DOX-treated cells were exposed to 1.75 μM of doxorubicin to simulate the equivalent concentration of the drug contained inside the tested nanoparticles. The absence of the cytotoxicity of NANO1 and NANO2 can be observed in Figure 3.5. No significant effect on cell viability was detected at concentrations up to 75 $\mu\text{g}/\text{mL}$ and 150 $\mu\text{g}/\text{mL}$, in tumoral cell lines HCT116 and HT29, respectively.

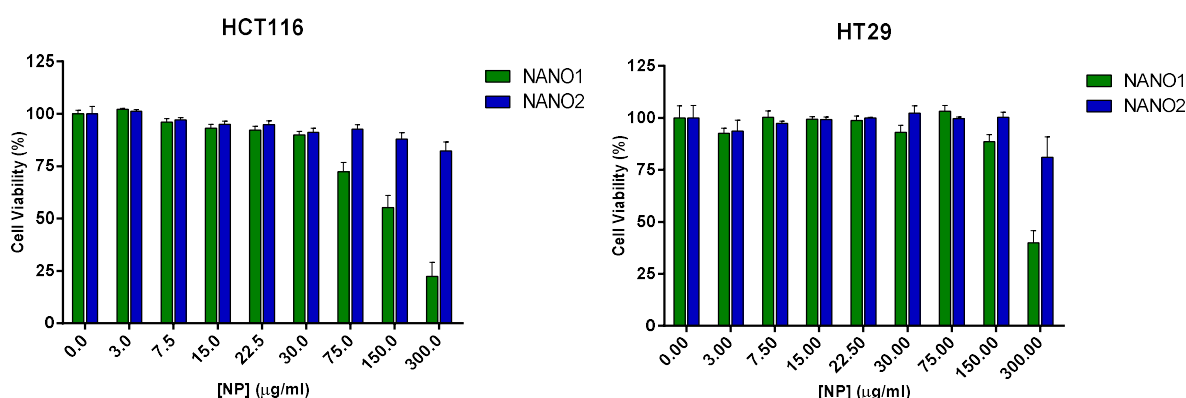


Figure 3.5 - Effect of NANO1 and NANO2 in cell viability. Viability of HCT116 and HT29 cells was evaluated after 72h exposure to increasing doses of NANO1 and NANO2 NPs. Values are the mean $\pm$ std from three independent experiments.

Figure 3.6 shows the dose-response effect of the two cancer cells lines against free DOX, NANO1@DOX and NANO2@DOX nanoparticles (NPs). As expected for all cell lines, we can confirm that as DOX concentration increased cell survival decreased. Indicating, thus, that the effect of both free drug and drug-loaded NPs is concentration-dependent.

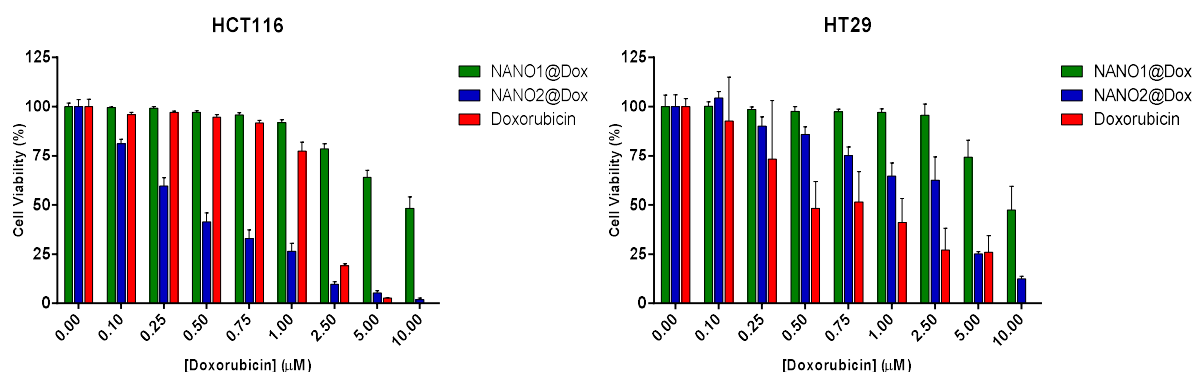


Figure 3.6 - Effect of NANO1@DOX, NANO2@DOX and doxorubicin in cell viability. Viability of HCT116 and HT29 cells was evaluated after 72h exposure to increasing doses of NANO1@DOX. NANO2@DOX NPs and DOX. Values are the mean $\pm$ std from three independent experiments.

From these results, for both cancer cell lines, we can observe that NANO2@DOX and DOX show similar cytotoxic behaviours. With, IC50 values of free DOX and NANO2@DOX NPs being very similar: 1.54 ± 0.13 and 0.75 ± 0.28 for DOX; and 0.37 ± 0.02 and 2.36 ± 0.02 μ M for NANO2@DOX. Conversely, NANO1@DOX showed less cytotoxicity, with IC50 values of 8.92 ± 0.87 and 9.35 ± 0.84 μ M, respectively (Table 3.1).

Table 3.1 - IC50 values of DOX and DOX-loaded NPs against HCT116 and HT29 cells. Depicted concentrations (in μ M) correspond to that of free and loaded DOX.

72 h		
Nanoparticles / Drugs	IC50 (μ M) HCT116	IC50 (μ M) HT29
NANO1@DOX	8.92 ± 0.87	9.35 ± 0.84
NANO2@DOX	0.37 ± 0.02	2.36 ± 0.81
DOX	1.54 ± 0.13	0.75 ± 0.28

From these values, we conclude that DOX-loaded nanoparticles significantly decreased IC50 values for both HTC116 and HT29, with NANO2@DOX specifically performing 5 to 8 times better than NANO1@DOX. NANO2@DOX was also confirmed to have a better performance against HTC116 than that of free DOX, and similar behavior against HT29. The low IC50 value obtained in NANO1@DOX could be related to an incomplete total release of DOX.

3.3.4 Cellular uptake of Doxorubicin-loaded Nanoparticles

After 24h of exposure to nanoparticles, cells were stained and imaged by LSCM. Analysis of z-stack images (Figure 3.7. B and C) and their orthogonal sectioning

demonstrated that both NANO1@DOX and NANO2@DOX are internalized by HT29 cells (Figure 3.7. D and E). The red fluorescence from doxorubicin is mostly localized in the cytoplasm, between the blue fluorescence of Hoechst, that signals nuclei position in cells and the green fluorescence of cellular membrane staining. From these images, we can see that DOX was internalised by the cells, arriving at the nuclei of some of them. The fluorescence of SiQDs contained within NANO1 and NANO1@DOX, as well as DOX fluorescence from NANO1@DOX and NANO2@DOX, inside the cells, were quantified by analysing cell lysates in a spectrofluorometer (excitation wavelengths: 370 and 490 nm; emission wavelengths: 499 and 589 nm respectively for SiQDs and DOX).

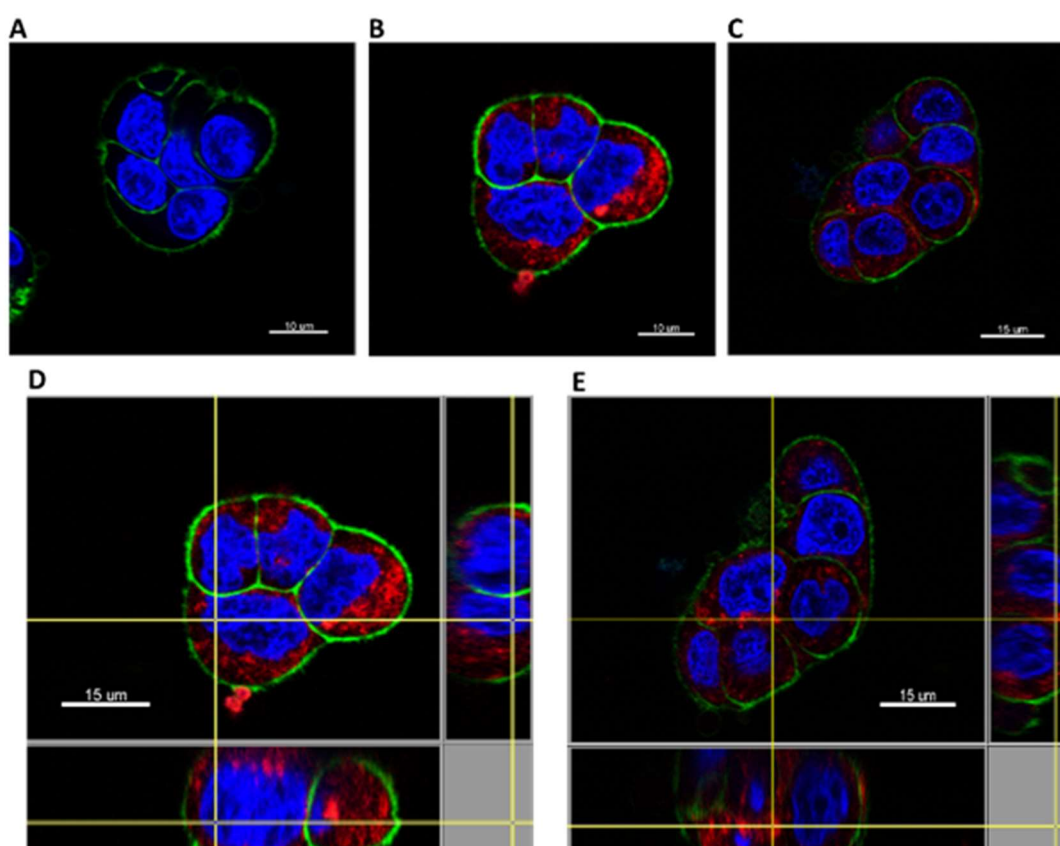


Figure 3.7 - Laser Scanning Confocal Microscopy (LSCM) in vivo images of control cells (A) and internalized, NANO1@Dox (B and D), and NANO2@Dox (C and E) in HT29 cells. Confocal images obtained from single channels of nanoparticle with doxorubicin fluorescence (B-D – red), nuclearStain (Hoeschst- Blue), and membrane stain (CellMask-green) are shown. Orthogonal sectioning and 3D reconstruction of the various z-stacks are shown in D and E images.

Table 3.2 displays the relative cellular internalisation, in percentage, of added nanoparticles and DOX to the cell culture medium. It is interesting to notice that the magnitude of detected fluorescence for DOX, in the case of both nanoparticles, is higher than that of free drug. The relative cellular internalisation of NANO1 and NANO1@DOX was

around 30%, with values for the calculated uptake, at 370 nm for the SiQDs and 470 nm for the DOX, being very similar.

Table 3.2 - Cellular quantification of nanoparticles uptake.

Nanoformulation / Drug	Uptake	Uptake
	($\lambda_{\text{ex}} = 370 \text{ nm}$)	($\lambda_{\text{ex}} = 470 \text{ nm}$)
NANO1	29,94% $\pm$ 1,20%	N/A
NANO1@DOX	32,33% $\pm$ 12,28%	34,50% $\pm$ 2,28%
NANO2	N/A	N/A
NANO2@DOX	N/A	24,06% $\pm$ 0,39%
DOX	N/A	14,04% $\pm$ 0,79%

The percentage of uptake obtained for NANO2@DOX was around 24%, which is slightly lower than the uptake obtained for NANO1@DOX.

In the attempt to correlate the obtained uptakes and cytotoxicity, we can affirm that, although, with the lowest uptake percentages, DOX and NANO2@DOX have the most significant cytotoxic effects against cancer cell lines, with small differences in terms of IC50. While DOX, with the lowest uptake (ca. 14%), has the best IC50 for HT29 cells, on the other hand, NANO2@DOX with a higher uptake showed outstanding and unmatched cytotoxicity against HCT116 cells.

Considering NANO1@DOX high uptake but low cytotoxicity, and according to a fluorescence evaluation-based uptake, it is possible that for NANO1@DOX we are detecting fluorescence of DOX that is attached to the nanoparticles. Not being a free drug, DOX-NP complexes lack the capacity to arrive at its target and induce cellular death, within the *in vitro* tested timescale. Suggesting a possible NANO1@DOX controlled release of DOX, overtime (for $t > 72 \text{ h}$).

3.4 Conclusions

In this study, we successfully synthesised DOX-loaded nanoparticles based on silica, with sizes around 70-80 nm, with pH-dependent behaviours. The *in vitro* biological effects of the DOX loaded nanoparticles were evaluated on human cancer cell lines of colon. The results indicate that doxorubicin-loaded NPs induced noticeably cytotoxicity and cellular uptake on cancer cells with cytotoxic effects comparable to that of free drugs. The luminescent nanocarrier herein presented (NANO1) showed low toxicity and very promising

results on the load and release of DOX, while keeping its fluorescent in cellular environment. Based on these results we envisage the addition of a targeting molecule at the surface of this nanocarrier, in order to make it more selective for a specific type of cell, increasing its range of applications and specificity, as well as promoting a decrease in prescribed DOX, and anti-cancer drugs, in cancer therapies.

3.5 Acknowledgments

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A PROTEOMIC ANALYSIS OF COLORECTAL CANCER
CELLS TREATED WITH A LUMINESCENT SILICON-
BASED NANOCARRIER (*CHAPTER 3 CONTINUATION*)

Keywords: proteomics • silicon quantum dots • mesoporous
silica nanoparticles • toxicity • colorectal cancer cells •

*A Proteomic Analysis Of Colorectal Cancer Cells
Treated With A Luminescent Silicon-Based Nanocarrier*

4.1 Introduction

A distinct, yet complementary, approach to understand the possible effects that the nanocarriers tested in Chapter 3 might have on both healthy and cancer colon cell lines, namely in their metabolism and transmembranar transport, goes through the analysis of the latter's' proteome after contact with the formers.

Proteomics is a fast and powerful discipline aimed at the study of the whole proteome, or the sum of all proteins from an organism, tissue, cell, biofluid, or subfraction, from which results an information-rich landscape of expressed proteins and their variations under specific conditions. Most up to date proteomic discoveries up to dte have focused on specific areas as cancer research, drug and drug-target discovery, biomarker discovery, as well as the analysis of any alterations or modifications produced in native protein when organisms are subjected to a plethora of changes [1, 2].

These developments have included advances in mass spectrometry (MS) technology, protein fractionation techniques, bioinformatics and genomic-proteomic-metabolomic data integration strategies. Proteomics relies on three basic technological keystones that include a method to fractionate complex protein or peptide mixtures, MS to acquire the data necessary to identify individual proteins, and bioinformatics to analyze and assemble the MS data. No area of proteomics has evolved faster than bioinformatics. Proteomics is critically dependent on bioinformatics to process the raw mass spectral data into protein data. While routinely used by every laboratory, the most critical software programs are those that take peptide mapping and/or tandem MS results and determine the protein or peptide sequence that most closely matches the experimental data [3].

One other area that has boosted technical advances and has supported the versatility of proteomics, together with genomics and metabolomics, is the study of nano-bio interactions. This area has permitted several groups to correlate the physiochemical properties of nanomaterials with specific protein expression levels, cellular uptake, cellular response/behavior, antibacterial and antitumoral activity and thus new therapeutic pathways [4, 5]. More importantly, the study of these interactions has paved way to yet another important field, that of nanotoxicity. Proteomics has recently been seen as a high quality and important tool, working as a real-time window to the realm of proteins and peptides and by

linking their expression and modifications to the responses of organisms to tested nanomaterials and their bio-reactivity [6, 7].

The enlightenment on the influence of certain properties of nanoparticles on their final toxicity and bio-interaction, has highlighted the type of core, particle size and surface modifications as main driving factors [8]. Driessen et al. have extensively studied diverse types of nanoparticles and modifications, stating that a qualitative and quantitative description of protein carbonylation is a versatile and sensitive method to describe NP-induced oxidative stress and, therefore, can be used to identify NPs of concern [9]. Increased oxidative stress is closely connected to various cellular responses like DNA damage, endoplasmic reticulum stress, lipid peroxidation, mitochondrial dysfunction, immune dysfunction, and apoptosis, to name a few, all mechanism that have a significant proteomic signature and can be used to assess and follow bio-nano interactions [10]. Omics experimental strategies have been used to explained the toxicity of titanium oxide nanoparticles in several animal cell lines, which was correlated to impacts on the abundance of 30 protein species that correspond to 22 gene products related to the some of the above-mentioned biological functions [11, 12]. Similar results have been reported for silver nanoparticles (AgNP), whose inclusion in new antibacterial materials and consumer products make it mandatory that their whole toxic profile should be urgently known [10]. Interestingly, a proteomic approach by Gioria et al. using label-free MS, studied the effect of AgNPs in colorectal cancer Caco-2 cells. The authors pointed out a total of 291 altered proteins in just 24h, corresponding to biological processes like cell cycle, cell morphology, cellular function and maintenance [13]. Similar works on the toxicity of AgNPs towards colorectal Caco-2 and HT29 cell lines, have also highlighted alterations in ubiquitination, glutathione biosynthesis, metabolism, immune response, and again oxidative stress [14, 15].

Silica and silica-based nanoparticles (SiNP) have gained significant interest in the pharmaceutical and nanomedicine areas, owing their inclusion in many novel therapies to their biocompatibility, biodegradability, drug delivery capacity and easy modulation. However, research into overall proteomic changes occurring during initial exposure and correlated nanotoxicity is limited, particularly in healthy or cancerous intestinal cell lines. Of the few proteomic studies on this type of particles, only plasma, hepatic, and skin cell models have been reported in literature. Dalzon et al stated that, at a plasma level, SiNPs induced cellular cytoskeleton and phagocytic capacity alterations, as well as a greater sensitivity of macrophages to DNA alkylating agents, such as styrene oxide [16]. Regarding hepatic cell lines treated with SiNPs, proteomic analyses performed after 4 h of incubation suggested

changes in the expression of 47 proteins, that fell into four functional groups: apoptosis regulation, structural reorganisation and regulation of actin cytoskeleton, unfolded protein response, and protein synthesis [17]. Lee et al. also described the expression of proteins involved in mRNA splicing, cell cycle, and mitochondrial function to be significantly downregulated; while, with the increase of ROS after 10h of exposure, expression of proteins associated with oxidative phosphorylation, as well as the immune system, was upregulated [18]. Moreover, and as to consolidate the previous results, critical altered proteins (including RPL3, HSP90AA1, SOD, PGK1, GOT1, and PNP), identified abnormal protein synthesis, protein misfolding, oxidative stress, and metabolic dysfunction [19]. Interestingly, Lorscheidt et al found that free standing SiNPs in cytoplasm caused extensive changes in the proteome, whereas when inside liposomes seemed unproblematic accounting for minimal changes in the protein profile [20]. Lastly, for skin cells, it was observed that 16 differentially expressed proteins were induced by SiNP exposure, and that expression levels were associated with the particle size. These proteins were identified as oxidative stress-associated proteins, cytoskeleton-associated proteins, molecular chaperones, energy metabolism-associated proteins, and apoptosis and tumor-associated proteins [21].

All considered, and despite the yet scarce number of reports on SiNPs and proteomics, there is evidence that supports the somewhat disturbing effect of the former on the whole proteome. This and the lack of reports on the effects of SiNPs in intestinal cell models (healthy and cancerous), justifies the herein aimed goal of getting some insight, via a proteomic analysis, on the influence of SiNPs' presence in colon model cell lines.

4.2 Experimental Section

4.2.1 Materials and Instrumentation

Ammonium bicarbonate (AmBic, BioUltra $\geq 99.5\%$ (T)), 1,4-Dithio-DL-threitol (DTT, $\geq 98\%$ - HPLC, $\geq 99.0\%$ - titration), iodoacetamide ($\geq 99\%$ - NMR, crystalline), sodium dodecyl sulphate (SDS, BioXtra $\geq 99\%$), Tris base (99.9%), formic acid and urea were acquired from Sigma-Aldrich. Hydrochloric acid solution 37% (HCl) was purchased from Fluka. L-Tryptophane (W, 99%) was obtained from AlphaAesar and acetonitrile was purchased from Panreac. Pierce™ trypsin protease (100 $\mu\text{g/mL}$, MS Grade) and Pierce™ quantitative colorimetric peptide assay were bought from Thermo Fisher Scientific. Minimum Essential Medium with Earle's salts (MEM), fetal bovine serum (FBS), Non-Essential Amino Acids, antibiotic-antimycotic (100X)

solution, sodium pyruvate (100 mM), phosphate buffered saline (PBS 1X solution, pH 7.4) and trypsin-EDTA (0.05%, phenol red), were bought from Gibco™, Thermo Fisher Scientific. Human colon adenocarcinoma cells (Caco-2, HTB-37™) and normal human colon fibroblasts (CCD112-CoN, CRL-1541™) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All solvents and reagents were of analytical reagent grade and were used as received. Unless otherwise mentioned, all solutions were prepared in DI MiliQ water.

All centrifugations were carried out on a Montreal Biotech MBI refrigerated centrifuge (Montreal, Canada), at 20 °C. Homogenization was achieved using a Hielscher UP100H Lab Homogenizer with MS1 Sonotrode, from Hielscher Ultrasonics GmbH (Teltow, Germany). A mini incubator from Labnet (New Jersey, USA) was used for cell lysis/denaturation, protein reduction and digestion in the classic filter aided sample preparation (FASP) protocol.

Sample preparation was performed via classic FASP in 10 KDa MWCO Vivacon® 500 filters from Sartorius Lab Instruments (Goettingen, Germany). CLARIOstar® High Performance Monochromator Multimode from BMG LABTECH (Germany) was used for tryptophan-based total peptide assay. Mass Spectrometry analyses were carried out in the Laboratory for Biological Mass Spectrometry – Isabel Moura (Proteomass Scientific Society, Portugal), using UHR-QqTOF IMPACT HD from Bruker-Daltonics (Bremen, Germany). Chromatographic separation of peptides was carried out using an Ultimate 3000 nLC nano-system equipped with a trap-column Acclaim™ PepMap™ 100, 5 µm, 100 Å, 300 µm i.d. × 5 mm (Thermo Fisher Scientific) and an analytical column Acclaim™ PepMap™ 100 C18, 2 µm, 0.075mm i.d × 150mm (Thermo Fisher Scientific). Data analysis was carried out using Compass DataAnalysis V4.2 from Bruker-Daltonics.

4.2.1 Nanoparticles

All tested nanoparticles were obtained as described in Chapter 3. For further assaying, SiQDMNs, MNs and MNsS-S-NH₂ were denominated as C5, C10 and C12. Their corresponding Caco-2 and CCD112-CoN fractions were equally identified, according to the number of the nanomaterial sample.

4.2.2 Cell Culture and Pellets

Caco-2 cells were routinely cultured in MEM containing 20% heat-inactivated fetal bovine serum and 1% antibiotic-antimycotic solution), at 37°C in a humidified 10% CO₂ atmosphere. Healthy fibroblast CCD112-CoN cells were equally maintained in MEM,

containing 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, non-essential amino acids and 1% (v/v) antibiotic-antimycotic solution, in the same conditions.

Caco-2 and CCD-112Con cells in exponential growth were seeded in 100 mm dishes and incubated overnight. Working solutions of all the tested nanoparticles (see section 4.2.1) were prepared at 10 µg/ml in medium and the cells were treated for 3 hours. Culture medium without nanoparticles was used as a negative control. After incubation, all cells were washed twice with PBS pH 7.4 and incubated for 5 minutes with trypsin-EDTA 0.05%, to break cell adherence to the plate. After incubation, cells were collected, centrifuged at 300 g for 5 minutes and the supernatant discarded. Pelleted cells were stored at -80°C for further analysis. Yielded pelleted cells were denominated as Caco5, Caco10, Caco12, Caco Control, FB5, FB10 and FB12, according to the type of nanoparticle that were treated with and the cell line. No healthy CCD-112Con cells sample was collected to further analysis as it was deemed unviable due to a culture contamination.

4.2.3 Sample Treatment

All samples (see section 4.2.2) were treated accordingly, starting by the lysis/denaturation of cells, followed by a classic FASP protocol, with a workflow comprising of sample wash, reduction, alkylation, protein digestion and peptide collection [22].

Initially, all pelleted cells were thawed to room temperature before being resuspended in 600 µL of lysis buffer and homogenized. Lysis buffer was prepared in 100 mM aqueous TrisHCl (pH 8.5), containing 2% (w/v) SDS and 50 mM DTT. The suspensions were denatured at 100 °C for 5 - 10 min, homogenized with an ultrasonic homogenizer (MS1 sonotrode) during 3.33 min (10 cycles: 10 s/10 s on/off UT, 100% UA, 20 kHz UF), followed by another 5 min at 100 °C and cooled to room temperature. Total protein determination was achieved via a tryptophan-based assay (Table 4.1), as described by Wisniewski and Gaugaz [23].

Table 4.1 - Total protein determination for each sample, obtained by tryptophan-based fluorescent assay (n = 3.)

Sample ID	[protein] (µg/µL)
Caco Control	0.48 ± 0.00
Caco5	0.50 ± 0.01
Caco10	0.48 ± 0.02
Caco12	0.55 ± 0.03
FB5	0.19 ± 0.01
FB10	0.17 ± 0.02
FB12	0.15 ± 0.01

After an initial filter washing with 100 μ L of MiliQ water for 15 min at 10,000 x g, 50 μ g and 25 μ g of protein from Caco-2 cells (i.e., Caco# and Caco Control) and CCD112-CoN cells (i.e., FB#) samples, respectively, were loaded in the membrane, washed twice with 200 μ L of a 8 M urea 25 mM AmBic solution and centrifuged for 15 min at 10,000 x g. Note that the reduction step was skipped as the lysis buffer already incorporated DTT. All samples were alkylated during 45 min in the dark at 20 °C, by adding 100 μ L of 50 mM iodoacetamide in an 8 M urea 25 mM AmBic solution. Subsequently, the samples were centrifuged for 15 min at 10,000 x g and washed four times with 200 μ L of a 25 mM AmBic solution. Finally, for protein digestion 100 μ L of 1:30 trypsin in a 12.5 mM AmBic solution were added to each sample. Protein digestion progressed overnight (approximately 16 hours) at 37 °C and the resulting peptides were collected with a 15 min centrifugation at 10,000 x g, after the addition of another 50 μ L 12.5 mM AmBic solution. Finally, 50 μ L of 3% (v/v) acetonitrile and 0.1% (v/v) formic acid were added to each sample, followed by a centrifugation of 10 minutes at 10,000 x g. This step was repeated twice. Peptides were transferred to a 500 μ L microtube, vacuum-dried and stored at -20 °C until further peptide quantification and analysis by Nano-LC-MS/MS. The whole procedure was prepared for two technical replicates ($n_t = 2$).

After digestion, peptides were quantified using the Thermo Fisher Scientific pierce™ quantitative colorimetric peptide assay following manufacturer's instructions.

4.2.4 Nano-LC-MS/MS

LC-MS/MS analysis was carried out using a Ultimate 3000 nano LC system coupled to an Impact HD (Bruker Daltonics) with a CaptiveSpray nanoBooster using acetonitrile as dopant. Peptides were resuspended in 100 μ L of 3% (v/v) acetonitrile containing 0.1% (v/v) aqueous formic acid. Then, samples were homogenized by 5 min on vortex followed by 10 min on an ultrasonic bath at 100% UA, 35 kHz UF. Subsequently, samples were quantified using a Pierce™ quantitative colorimetric peptide assay. Then, 5 μ L containing 750 ± 5 ng of peptides were loaded onto a trap column (Acclaim PepMap100, 5 μ m, 100 Å, 300 μ m i.d. x 5 mm) and desalted for 5 min from 3% to 5% with B (where, B: 90% acetonitrile 0.08% formic acid) at a flow rate of 15 μ L/min. Then the peptides were separated using an analytical column (Acclaim™ PepMap™ 100C18, 2 μ m, 0.075mm i.d x 150 mm) with a linear gradient at 300 nL/min (mobile phase A: aqueous 0.1% (v/v) formic acid; mobile phase B 90% (v/v) acetonitrile and 0.08% (v/v) formic acid) for 5 - 90 min from 5% to 35% of mobile phase B, 90-100min linear gradient from 35% to 95% of mobile phase B, 100-110 95% B.

Chromatographic separation was carried out at 35 °C. MS acquisition was set to cycles of MS (2 Hz), followed by MS/MS (8-32 Hz), cycle time 3.0 s, with active exclusion (precursors were excluded from precursor selection for 0.5 min after acquisition of one MS/MS spectrum, intensity threshold for fragmentation of 2500 counts). Together with active exclusion set to 1, reconsider precursor if the intensity of a precursor increases by a factor of 3, this mass was taken from temporarily exclusion list and fragmented again, ensuring that fragment spectra were taken near to the peak maximum. All spectra were acquired in the range 150-2200 m/z [22]. It should be noted that for each technical replicate, two instrumental replicates were analyzed ($n_i = 2$, $n_{\text{total replicates}} = 4$).

MS/MS peak labeling was done using the MaxQuant platform (Max-Planck-Institute of Biochemistry) [24]. Perseus (version 1.6.0.16) was used to process the data with the default settings. Protein group LFQ intensities were log₂-transformed, and the quantitative profiles were filtered for missing values with the following settings: minimum valid percentage of 70% in at least one group and values greater than 0. Missing LFQ values were imputed using the parameters, width = 0.5 and down shift = 1.8. Log ratios were calculated as the difference in average log₂ LFQ intensity values between the tested conditions (two-tailed, Student's t-test, permutation-based FDR = 0.05 and $S_0 = 0.1$) [22, 25].

4.3 Results and Discussion

From the proteomic analysis, a total of 1016, 1087, 976 proteins were identified in FB12, FB10 and FB5 samples, and a total of 1319, 1303, 1329 and 1328 in Caco12, Caco10, Caco5 and Caco Control, respectively. As depicted in Figure 4.1, CCD-112Con (FB#) and Caco-2 (Caco#) cell samples have a significant percentage of common proteins within each cell type, with the first sharing 75.4% of their whole proteome between samples, and the last sharing 87.4%. Interestingly, all Caco numbered samples share *ca.* 90% of their proteome with Caco Control (i.e. untreated with nanoparticles). These results point out the high similarity between samples, regardless of the treatment with or without nanoparticles.

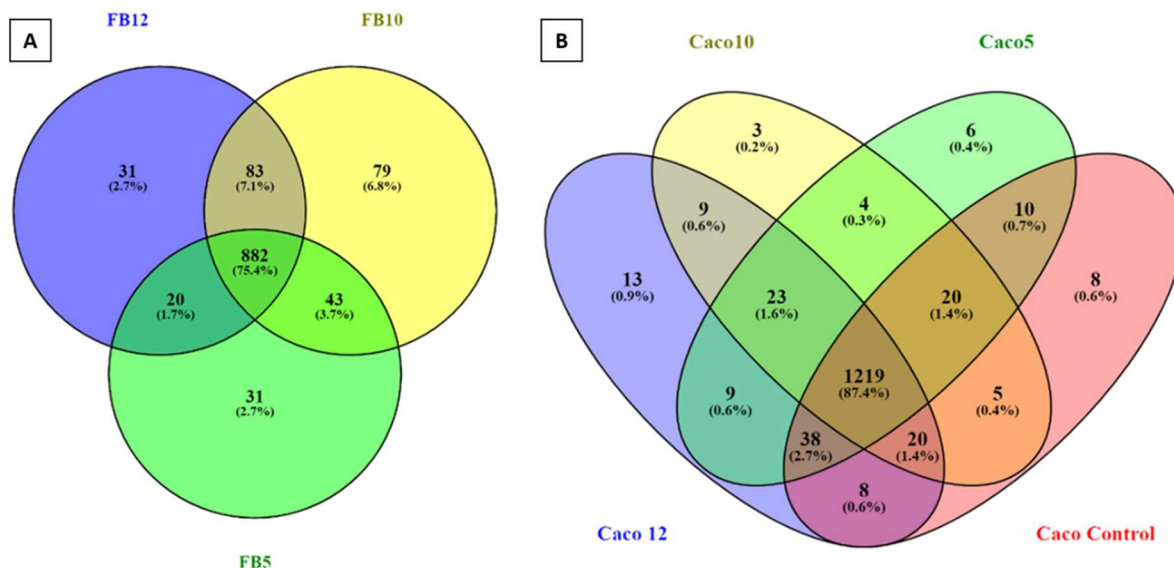


Figure 4.1 - Venn's diagram summary of identified proteins from (A) CCD112-CoN cells (i.e., FB5, FB10, FB12) and (B) Caco-2 cells (i.e., Caco5, Caco10, Caco12 and Caco Control) samples.

A correlation analysis of the same samples emphasizes the above-stated similarity inside each type of cell line, for all tested replicates ($n = 4$), which is translated by the visible strong correlation (above 0.9) between samples of the same line (Figure 4.2). This is an indicator of the high reproducibility of the method and samples, supporting the robustness of the selected sample treatment and proteomics analysis. A relative correlation, with a minimum value of 0.616, is visible however between Caco-2 and CCD112-CoN cells, owing it to its similar origin regardless of the former tumorigenicity.

Despite the small variability observed in the previous data analysis, by statistically analyzing the difference of expressed proteins/genes between each 2 samples of a cell type line, for a significance of $p < 0.05$, we were able to identify altered expressed proteins/genes. The selected genes/proteins' expression was labeled as upregulated or downregulated

according to their position against the Student's t-test threshold, for the difference of mean of each of the samples.

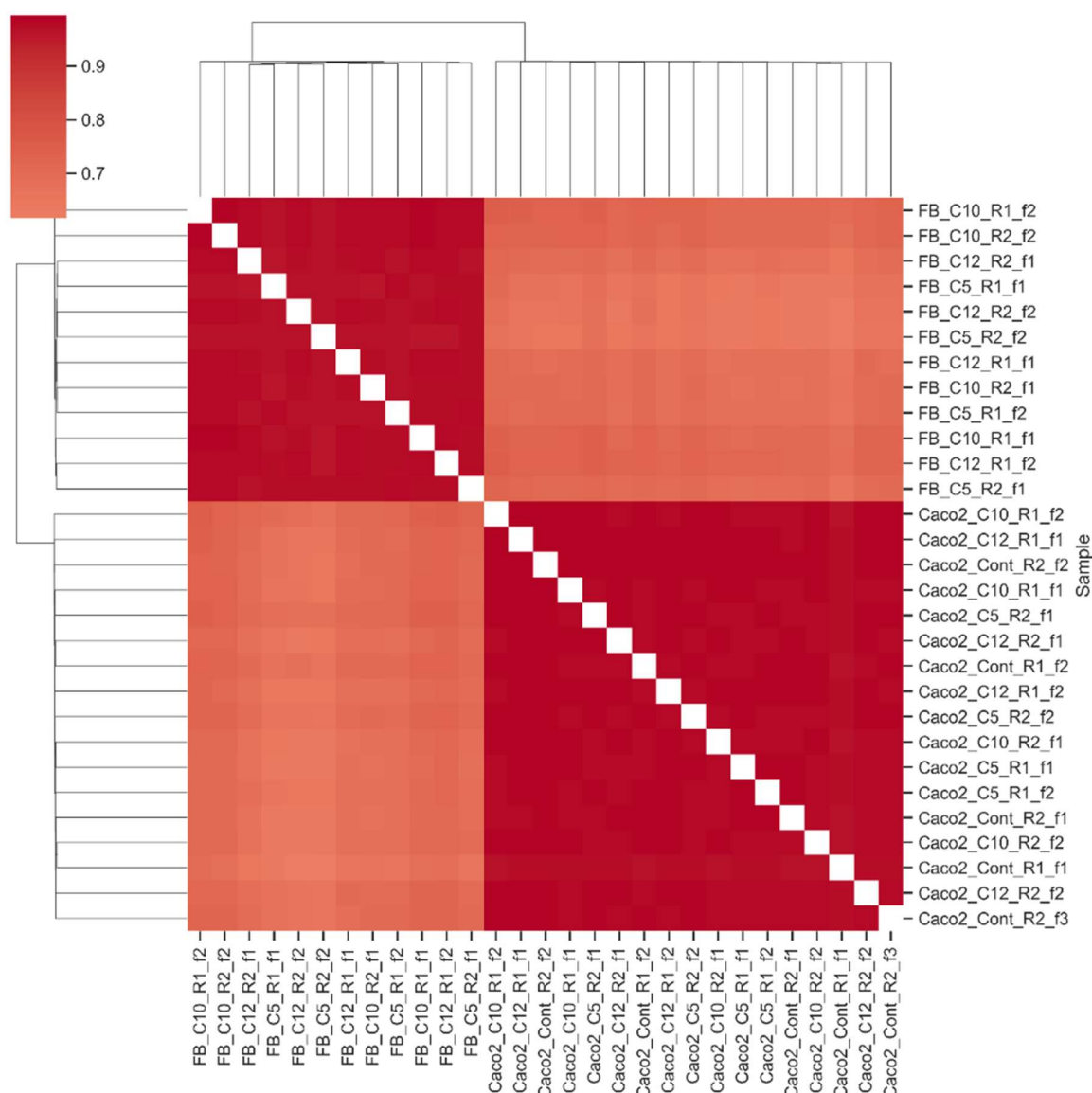


Figure 4.2 - Correlation heatmap between each sample and respective technical (n = 2) and instrumental (n = 2) replicates. R1 / R2 - technical replicates and f1 / f2 - instrumental replicates.

By comparing FB samples' proteomes (Figure 4.3.A), it is clear that almost no alterations are seen between samples, except between FB12 and FB5. In this case, FB12 presents two altered gene expressions in regard to FB5, with HSPH1 downregulated and EEF1E1/EEF1E1-BLOC1S5 upregulated. As detailed in Table 4.2, HSPH1 gene regulates the expression of heat shock proteins that prevent the aggregation of denatured proteins in cells under severe stress, while EEF1E1/EEF1E1-BLOC1S5 regulate eukaryotic translation elongation factor 1 epsilon which is a positive modulator of ataxia telangiectasia mutant (ATM) response to DNA

damage. This might be correlated with the higher uptake that surface modified particles have, when compared with naked ones, particularly those with silicon quantum dots (SiQD), that creates some stress on the membrane and on the homeostasis of the cells. Moreover, the upregulation of a positive modulator of ATM as response to DNA damage and regulation of apoptotic signals [26] might indicate some cytotoxic action of C12 nanoparticles' surface moiety (i.e., MNsS-S-NH₂), containing thiol/disulfide and amine groups, that might have play some role to DNA breaks [27, 28]. Interestingly, this is in accordance with the registered cytotoxicity of the surface modified nanoparticles when tested against CCD112-CoN cells, in the conditions described in Chapter 3, sections 3.2.6 and 3.3.3 (see Figure S - 4.1).

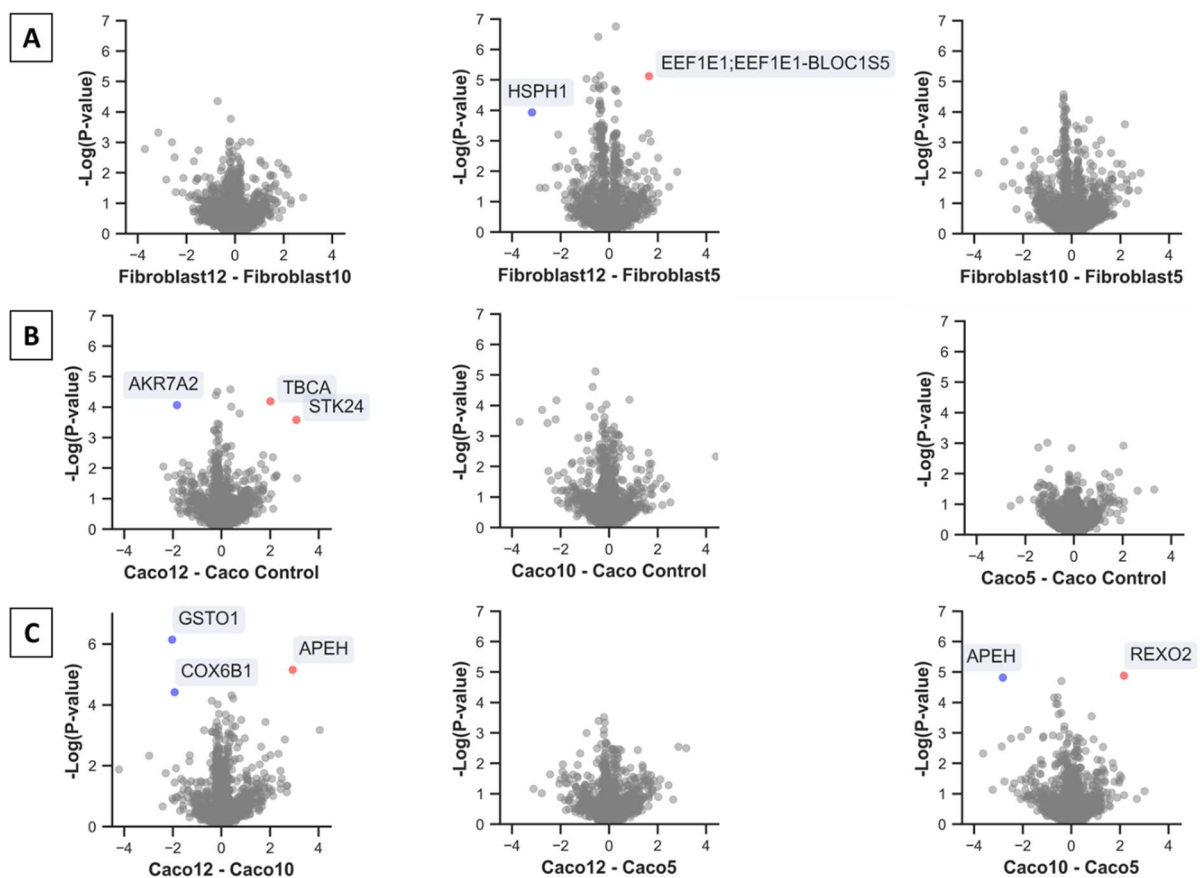


Figure 4.3 - Volcano plots of the differences between (*top*) FB# samples, (*middle*) Caco# samples and Caco Control, and (*bottom*) Caco# samples; where # = 5, 10, 12. Upregulated and downregulated genes are represented in red and blue, respectively, and their names highlighted. Student's t-test significance threshold for identification was set to $p\text{-value} < 0.05$.

The influence of C12 nanoparticles' surface moieties might have also been highlighted in the Caco-2 cell samples, with Caco12 samples being the only ones showing significantly altered expressions against the control and other samples (Figure 4.3.B-C).

Table 4.2 - Significantly altered (downregulated and upregulated) genes and proteins between tested fractions and their associated biological species and functions.

Interaction	Gene	Expression	Related Proteins	Biological Species	Biological Function *
FB12 - FB10	-	-	-	-	-
FB12 - FB5	HSPH1	Downregulated	Q92598-2; Q92598; Q92598-3; R4GN69; Q5TBM3	Heat shock protein 105 kDa	Acts as a nucleotide-exchange factor (NEF) for chaperone proteins HSPA1A and HSPA1B, promoting the release of ADP from HSPA1A/B thereby triggering client/substrate protein release. Prevents the aggregation of denatured proteins in cells under severe stress, on which the ATP levels decrease markedly.
	EEF1E1; EEF1E1-BLOC1S5	Upregulated	D6RBD7; C9J1V9; O43324-2; O43324; H0YAL7; D6RCQ0	Eukaryotic translation elongation factor 1 epsilon-1.	Positive modulator of ATM response to DNA damage.
FB10 - FB5	-	-	-	-	-
Caco12 - Caco Control	AKR7A2	Downregulated	H3BLU7; O43488; A0A499FJT0; Q8NHP1	Aflatoxin B1 aldehyde reductase member 2	Catalyzes the NADPH-dependent reduction of succinic semialdehyde to gamma-hydroxybutyrate.
	TBCA	Upregulated	E5RIW3; E5RJD8; O75347; E5RHG6; O75347-2	Tubulin-specific chaperone A	Tubulin-folding protein involved in the early step of the tubulin folding pathway.
	STK24	Upregulated	H0Y630; B4DR80; Q9Y6E0-2; Q9Y6E0; Q5JV98; Q5JV99; C9JDH9; C9J6L2; C9JCC0; C9J232; H7C279; O00506-3; O00506-2; O00506	Serine/threonine-protein kinase 24; Serine/threonine-protein kinase 24 36 kDa subunit	Acts on both serine and threonine residues and promotes apoptosis in response to stress stimuli and caspase activation. Mediates oxidative-stress-induced cell death by modulating phosphorylation during oxidative stress. Plays a role in a staurosporine-induced caspase-independent apoptotic pathway by regulating the nuclear translocation of AIFM1 and ENDOG and the DNase activity associated with ENDOG.
				Serine/threonine-protein kinase 24 12 kDa subunit	
Caco10 - Caco Control	-	-	-	-	-
Caco5 - Caco Control	-	-	-	-	-
Caco12 - Caco10	GSTO1	Downregulated	P78417; Q5TA02; P78417-2; P78417-3; Q5TA01	Glutathione S-transferase omega-1	Exhibits glutathione-dependent thiol transferase and dehydroascorbate reductase activities. Has S-(phenacyl)glutathione reductase activity. Has also glutathione S-transferase activity. Involved in the metabolism of xenobiotics and carcinogens.
	COX6B1	Downregulated	P14854; A0A494C160; K7EQD3	Cytochrome c oxidase subunit 6B1	Component of the cytochrome c oxidase, the last enzyme in the mitochondrial electron transport chain which drives oxidative phosphorylation. Component of the respiratory chain that catalyzes the reduction of oxygen to water.
	APEH	Upregulated	C9JIF9; P13798; H7C1U0; H7C393; C9JLK2; F8WEH5; H0YFE5	Acylamino-acid-releasing enzyme	This enzyme catalyzes the hydrolysis of the N-terminal peptide bond of an N-acetylated peptide to generate an N-acetylated amino acid and a peptide with a free N-terminus.
Caco12 - Caco5	-	-	-	-	-
Caco10 - Caco5	APEH	Downregulated	C9JIF9; P13798; H7C1U0; H7C393; C9JLK2; F8WEH5; H0YFE5	Acylamino-acid-releasing enzyme	This enzyme catalyzes the hydrolysis of the N-terminal peptide bond of an N-acetylated peptide to generate an N-acetylated amino acid and a peptide with a free N-terminus.
	REXO2	Upregulated	Q9Y3B8-3; H0YG54; H0YGR4; Q9Y3B8-2; Q9Y3B8; F5GYG5	Oligoribonuclease, mitochondrial	3'-to-5'exoribonuclease that preferentially degrades DNA and RNA oligonucleotides composed of only two nucleotides. Plays dual roles in mitochondria, scavenging nanoRNAs produced by the degradosome and clearing short RNAs that are generated by RNA processing. Essential for correct initiation of mitochondrial transcription, degrading mitochondrial RNA dinucleotides to prevent RNA-primed transcription at non-canonical sites in the mitochondrial genome. Essential for embryonic development.

* Information from UniProt [29].

On the first situation, AKR7A2, TBCA and STK24 expression is significantly altered, with the first downregulated and the last two upregulated. Of these, STK24 is of especial interest due to its association with, first the mediation of oxidative-stress-induced cell death during oxidative stress, and second the promotion of apoptosis under stress stimuli and caspase activation [30]. Curiously, SiNPs have already been signaled as enhancers of p53 phosphorylation (in p53-caspase pathway), with decreasing particles sizes elevating the activation of downstream caspase-9, -7 and -3, which work as the main way of SiNP-mediated apoptosis [31]. The functionalization of C12 particles was confirmed to decrease the final polydispersity of the particles, increase its colloidal stability and circulation as sole-particle systems and, thus avoid aggregation (see Chapter 3, section 3.3.1) [32]. This contributes to a global discretization of the particles and a decrease in size, which in turn might promote a certain level of cytotoxicity.

When compared with its non-modified counterpart (i.e., samples C10), C12-treated Caco-2 cells showed a deficient expression of cytochrome c oxidase subunit 6B1 (COX6B1), which drives terminal oxidative phosphorylation in the mitochondrial electron transport chain and catalysis the reduction of oxygen to water [33]. Its downregulation blocks an important cellular mechanism of elimination of excessive intracellular oxygen species and, thus, paves way to the increased generation of ROS. This might explain the upregulation of STK24 in the previous comparison, which is connected to oxidative stress conditions [30]. The simultaneous downregulation of glutathione S-transferase omega-1 (GSTO1), demonstrates that thiol+amine moieties at the surface of C12 particles somehow block the role of this protein, whose overexpression has been reported as an important inhibitor of lipid peroxidation in SiNPs-induced cell death [34]. Glutathione S-transferases (GST), GSTO1 included, have also been identified as important antioxidant enzymes that regulate stress-induced signaling pathways and protect against irreversible oxidation, particularly in cancers [35, 36]. Consequently, its downregulation in Caco-2, when in presence of C12 particles, justifies the growth of oxidative stress conditions inside cell and the above-observed STK24 upregulation and COX6B1 downregulation in Caco12 samples. Lastly, the upregulation of Acylamino-acid-releasing enzyme (APEH) acts as compensatory process to the downregulation of GSTO1, by making use of its capacity of sustaining the homeostasis of the cytoplasmic antioxidative system [37, 38]. One more time, all evidence points to SiQDMNsS-S-NH₂ (C12 samples) and its moieties as having a certain degree of cytotoxicity against both healthy and cancer colon cell lines, through the induction of increased oxidative stress conditions and eventual ROS generation and cell death.

The lack of differences in the expression of proteins between Caco12 and Caco5, lead us to conclude that part of the effect seen with C12 particles also occurs with SiQDMMNs particles. This is confirmed by obtained low levels of expression of APEH in Caco10, when compared with Caco5 (which in conversely is upregulated in relation to Caco10).

4.4 Conclusions

We can conclude that a proteomic analysis of the nano-bio interaction is an important tool in the assessment of any nanomaterial's cytotoxicity, by highlighting the levels of expression of certain genes/proteins in the targeted cells' proteome. Regarding the tested nanoparticles we confirmed that surface functionalized nanoparticles, particularly those containing thiol/disulfide and amine moieties like C12 mesoporous particles (i.e., SiQDMMNs-S-NH₂), can be used in future targeted therapies to induce significant alterations in key proteins. These alterations not only disrupt the normal function and control of the cells, but also amplify the effect of potential therapeutic cargo (e.g., anticancer drugs) by simultaneously deliver it inside the cells but also by weakening its defense mechanisms.

4.5 Acknowledgments

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4.7 Supplementary Material

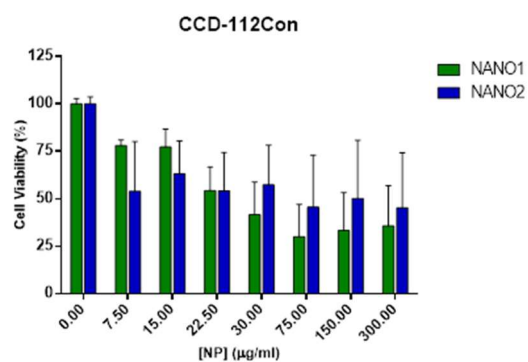
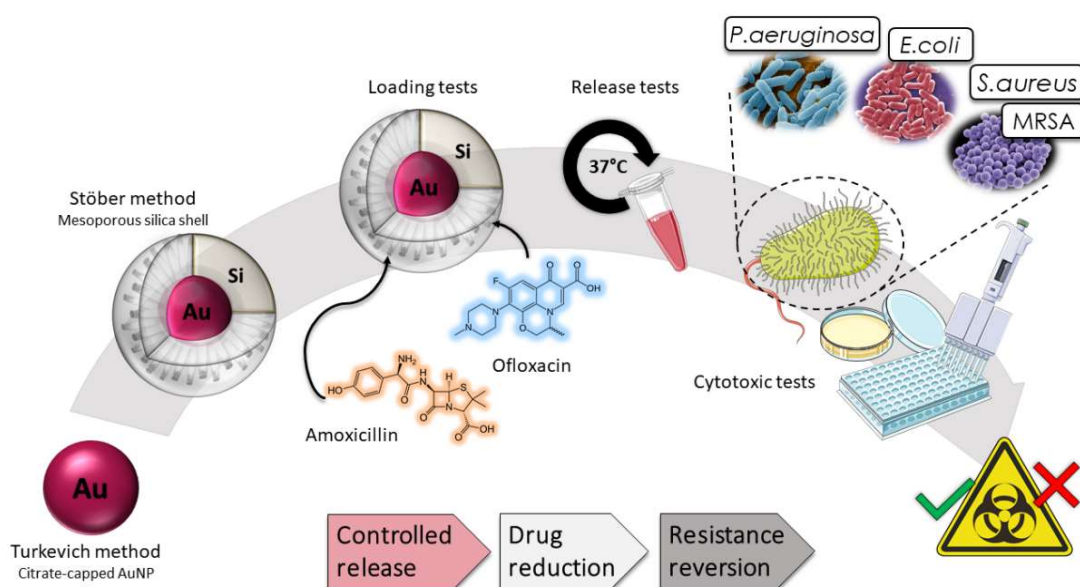


Figure S - 4.1 - Dose-dependent cytotoxicity of empty. nanoparticles against CCD112-CoN cells; where NANO1 = SiQDsMNsS-S-NH₂ and NANO2 = MNsS-S-NH₂.

GOLD@MESOPOROUS SILICA NANOCARRIERS FOR THE EFFECTIVE DELIVERY OF ANTIBIOTICS AND BY-PASSING OF B-LACTAM RESISTANCE



Keywords: mesoporous silica nanoparticles • amoxicillin • ofloxacin • antimicrobial resistance • bacteria • gold nanoparticles

Adapted from GA Marcelo et al.

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Gold@mesoporous silica nanocarriers for the effective delivery of antibiotics and by-passing of β -lactam resistance

Gonalo A. Marcelo, Maria P. Duarte and Elisabete Oliveira

Abstract: Current antibiotics effectiveness relies on higher doses and administration frequency, which are responsible for the growth of antimicrobial resistance (AMR). AMR is one of the major threatening issues of the century with last-line antibiotics already failing. To overcome such problems associated with bacterial infections, nanoparticles combined with antibiotics emerged as a promising strategy. In this work, nanocarriers comprising of gold-silica core-shell mesoporous nanoparticles (Au@MNs) and silica mesoporous nanoparticles (MNs) were synthesized, loaded with amoxicillin (Amox) and ofloxacin (Oflox) and investigated regarding its antibacterial activity towards *S. aureus*, methicillin-resistant *S. aureus* (MRSA), *E. coli* and *P. aeruginosa*. Both nanocarriers showed a beneficial role in the effective delivery of amoxicillin against MRSA and the well-known β -lactam resistant *P. aeruginosa*. Reductions of 10-fold (Amox@MNs) and 20-fold (Amox@Au@MNs) in the amount of antibiotic to treat *P. aeruginosa*; and a reduction of 20-fold (Amox@MNs) towards MRSA allied to a full reversion of resistance, strongly supports the promising potential of these nanocarriers to tackle antibiotics resistance.

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5.1 Introduction

The inappropriate administration of antibiotics and their relative non-specificity are responsible for bacterial infections treatment's ineffectiveness and for the growth of antimicrobial resistance [1,2]. Recent studies presented by the European Food Safety Authority (EFSA) and by the European Centre for Disease Prevention and Control (ECDC) conducted in the 28 European member states revealed an increased resistance to antibiotics in the *Salmonella*, *Escherichia coli* and *Staphylococcus aureus* bacteria strains [3][2]. As a consequence, current antibiotics effectiveness relies on higher drug doses and administration frequency, which in turn favour the emergence of new resistant bacteria [3]. To overcome this issue, the production of novel antibiotics has been proposed as a solution. However, with time these drugs will cease to be effective, as well. The resolution should focus then on the increase of the durability and effectiveness of existing drugs and not in the design of new ones [3].

The implementation of nanoparticles as antibacterial agents appears to be a very promising strategy to overcome such problems associated with bacterial infections. Toxicity reduction, drug solubility enhancement, controlled release of multiple drugs, lower antibiotic dosage, resistance overcoming and lower costs are some of the advantages reported by the use of the nanomaterials, compared with conventional antibiotics [4,5]. Regarding antibacterial nanomaterials, inorganic nanoparticles containing silver, gold, copper oxide, titanium oxide, and cerium oxide nanoparticles have been found to have potent antibacterial effects [6-9]. It has been proposed that these materials can exert antibacterial activity through multiple modes of action, such as metal ions release, non-oxidative and oxidative stress, leading to multiple gene mutations in the bacterial cell and consequently inhibiting bacteria from acquiring resistance against nanoparticles [10].

Among all nanomaterials, mesoporous silica nanoparticles (MNs), are very promising for pharmaceutical use, mainly due to their multiple design options, providing different strategies to combat bacterial infections [11]. MNs have a high surface area and pore volume with a large loading capacity to combat bacterial infection in all stages (prevention, detection and treatment). Several studies showed that loading antibiotics onto MNs induces an improvement in the effectiveness of the drug [3,12,13]. Moreover, the combination of MNs with other metals (copper, zinc, silver, gold) can bring an added value in bacterial eradication [14,15].

As an example, Zink et al. [16] reported the antibacterial effect of silver nanocrystal into MNs against *E. coli* and *Bacillus anthracis*, showing efficiency against *B. anthracis* in the smallest tested concentration (20 µg/mL). A more sophisticated system was published by Zhou et al. [17] where chlorhexidine (CHX) was loaded into silver@MNs and tested against *S. aureus* and *E. coli*. In both bacteria, the CHX@silver@MNs system showed a synergistic bactericidal effect higher than AgNO₃ and CHX separately, with a minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 25 µg/mL and 12.5 µg/mL for *S. aureus* and *E. coli*, respectively.

Silver core-mesoporous silica nanoparticles loaded with ofloxacin (AgMNs@oflox) were also tested with *S. aureus* and *E. coli*. A synergetic effect was observed, being the best antibacterial results obtained for AgMNs@oflox for the strain *S. aureus* ATCC 25923, with MIC and MIB values of 5 µg/mL and 25 µg/mL [18].

Other metal ions, especially the mesoporous silica copper and nickel-supported nanoparticles, MNs-Schiff base (SB)-Cu and MNs-SB-Ni, presented a bactericidal and bacteriostatic effect against *S. aureus* and *E. coli* [19]. However, gold nanoparticles' inexistence towards living organisms (prokaryotic and eukaryotic), as well as their known photothermal behaviour, make gold-based systems viable and biocompatible building blocks for complex nanostructures of biological application, namely their possible conjugation with antibacterial agents for the application in living eukaryotic systems.

Thus, in this work we present the synthesis of core-shell gold-silica based mesoporous silica nanoparticles for the effective delivery of ofloxacin and amoxicillin towards *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli* and *Pseudomonas aeruginosa*. Synergetic effects and drug delivery efficiency between the antibiotics and nanoparticles will also be studied, aiming for a significant decrease of administered antibiotic, while maintaining bactericidal performance.

5.2 Materials and Methods

Hydrogen tetrachloroaurate(III) hydrate (HAuCl₄, 99%), sodium citrate tribasic, ethylene glycol (EG) hexadecyltrimethylammonium bromide (CTAB, ≥98%), ammonium nitrate, amoxicillin, ofloxacin, ethanol (EtOH) and methanol (MeOH) were used as purchased from Sigma-Aldrich. Tetraethyl orthosilicate (TEOS, 99.999%), sodium hydroxide (NaOH) and sodium chloride (NaCl, 99%) were bought from Alfa Aesar and PanReact, respectively. Dimethyl sulfoxide (DMSO) was acquired from CarloErba. All reagents were used as bought

and all solutions unless otherwise indicated, were prepared in deionised (DI) MiliQ water. Antibacterial assays were performed against gram-negative *Escherichia coli* ATCC® 8739™ (*E. coli*) and *Pseudomonas aeruginosa* ATCC® 9027™ (*P. aeruginosa*), and gram-positive bacteria *Staphylococcus aureus* ATCC® 6538™ (*S. aureus*), methicillin-resistant *Staphylococcus aureus* ATCC® 33591™ (MRSA). Muller Hinton Agar (MHA), Muller Hinton Broth (MHB) and Trypto Casein-Soy Agar (TSA) were obtained from Biokar Diagnostics (Allonne, France)

5.2.1 Instrumentation

Gold concentration was obtained, at 267 nm, by Inductively Coupled Plasma - Atomic Emission Spectrometer (ICP-AES, Horiba Jobin-Yvon), equipped with a 40,68 MHz RF generator, Czerny-Turner monochromator with 1,00 m (sequential), autosampler AS500 and CMA (Concomitant Metals Analyzer), from REQUIMTE Laboratory of Analysis from FCT-UNL (Portugal). UV-Vis absorption spectra were acquired on a Jasco V-650 spectrophotometer (Jasco Corporation, Tokyo, Japan) and Nanodrop 1000 Spectrophotometer (ThermoFisher); nanoparticle size distributions and zeta potential were measured using a dynamic light scattering Malvern Nano Zetasizer, with a 633 nm laser diode, from Proteomass-Bioscope facility (Caparica, Portugal). Pore size distribution and surface areas were determined by N₂ adsorption/desorption at 77 K, in a Micromeritics ASAP 2010 (Micromeritics Instrument Corporation, Norcross, USA) (Accelerated Surface Area and Porosimetry), at the Laboratory of Analysis from FCT—UNL. Specific surface areas (S_{BET}) pore volume were estimated using Brunauer Emmett and Teller (BET) and Barrett-Joyner-Halenda (BJH) methods. Infrared spectra (IR) were collected on a PerkinElmer Spectrum Two spectrometer, FTIR-ATR (Perkin Elmer Inc., Llantrisant, UK) and X-ray Diffraction (XRD) spectra on a RIGAKU MiniFlex II X-ray diffractometer, equipped with a Cu-K α source at 30 KV/15 mA, between 2° - 80°, at the Laboratory of Analysis from FCT-UNL.

Bacterial suspensions' turbidity was adjusted with the aid of a DEN-1B McFarland Densitometer (Grant-bio) and quantified in a UV-Vis CLARIOstar spectrophotometer (BMG Labtech). During antibacterial assays, asepsis was ensured using a laminar flux chamber Steril – VBH. Incubations were performed within a Mermmet Incubator B50. Diffusion assays were performed in sterile paper disks with a diameter of 6 mm, bought at Bacto disks, and microdilution assays in sterile 96-well plates, bought at Greiner Bio-One.

SEM images were obtained in a Quanta 650 FEG operating between 5-30 kV and 3.6×10^{-4} Pa of vacuum in the chamber. Transmission electron microscopy (TEM) images were

obtained in a JEOL JEM-2100-HT operating at 200 kV, TEM images were collected using a "OneView" 4k x 4k CCD camera.

5.2.2 Synthesis of gold-silica mesoporous nanoparticles (Au@MNs)

Gold nanoparticles (AuNPs) were synthesised following the traditional Turkevich method for the production of 20 nm AuNPs [20,21]. 4.9 mg of HAuCl_4 were dissolved in DI MiliQ water and placed under reflux for approx. 30 min. Separately, 1.25 mL of a 10 mg/mL sodium citrate tribasic aqueous solution was prepared and poured onto the reaction pot and left to stir for an additional 15 minutes under reflux. Heating was kept until a deep wine-red colour was obtained. Citrate-capped AuNPs were cooled at room temperature and stored at 4 °C. For the growth of a mesoporous silica shell [22,18], 6.25 mL of AuNP colloid (9.2×10^{11} particles/mL) were mixed with 10 mL of a 15 mg/mL CTAB aqueous solution and stirred for 30 min at 50 °C. In this order, 30 mL of DI MiliQ water, 10 mL of Ethylene Glycol and 0.7 mL of a 1 M NaOH aqueous solution were poured into the reaction pot and vigorously stirred for 30 min at 80 °C. Then, 750 μL of TEOS were dropwisely added to the reaction mixture and stirred for 2 hours at 80 °C. The resulting Au@MNs material was centrifuged (8.000 rpm, 10 min), washed three times with MeOH and dried at open-air conditions.

Blank mesoporous silica nanoparticles (MNs) were obtained following the same protocol, without the addition of citrate-capped AuNPs. For template removal, the Au@MNs and MNs were resuspended in 20 mL of a 30 mg/mL ammonium nitrate methanolic solution and stirred for 1 hour at 60 °C, followed by centrifugation and washing in MeOH. This process was repeated twice and confirmed by IR spectroscopy for both materials.

5.2.3 Loading and Release trials

Amoxicillin and ofloxacin were loaded, separately, into the pores of MNs and Au@MNs. Briefly, for ofloxacin, 50 mg of each material were resuspended in 1 mL of DMSO and mixed with 1 mL of a suspension of the antibiotic (10 mg/mL), in DMSO. Amoxiciillin loading followed the same procedure but in water. Reaction mixtures were let to stir for 24 hours at 20 °C. After 24 hours, 3 mL of EtOH were added to the reaction pots to stop the loading process and the product centrifuged (8.000 rpm, 5 min). The loaded NPs (Amox@MNs, Oflox@MNs, Amox@Au@MNs and Oflox@Au@MNs) were washed several times with EtOH. Loading yields (L%) and loading capacities (LC) were determined by UV-vis spectroscopic quantification and mass balances (Equations 5.1 and 5.2), in triplicates, at 271

nm ($\epsilon = 871.9 \text{ M}^{-1}\text{cm}^{-1}$) and 292 nm ($\epsilon = 36543.6 \text{ M}^{-1}\text{cm}^{-1}$) for amoxicillin and ofloxacin, respectively.

$$L\% = \frac{ma_i - ma_e}{ma_i} \times 100 \quad \text{Equation 5.1}$$

$$LC(\text{mg/g}) = \frac{ma_i - ma_e}{m_{NPs}} \quad \text{Equation 5.2}$$

Where, ma_i (mg) is the initial amount of the antibiotic, ma_e (mg) the amount of antibiotic in the supernatant, and m_{NPs} (mg) the mass of MNs or Au@MNs.

Release profiles were performed for all materials and measured in a Nanodrop 1000 Spectrophotometer. Briefly, 4 mg of each material were resuspended in 2 mL of DMSO and stirred at 37 °C. 0.5 mL aliquots of each reaction pot were collected after 30 min, 1h, 2h, 4h, 6h, 12h, 24h, 48h, 72h and 96h (4 days), centrifuged and quantified by UV-vis spectroscopy. Amoxicillin was quantified at 276 nm ($\epsilon = 141.7 \text{ M}^{-1}\text{cm}^{-1}$) and ofloxacin at 292 nm ($\epsilon = 2825.8 \text{ M}^{-1}\text{cm}^{-1}$). The same volume was, then, poured back into the reaction pot. The total amount of released antibiotics was determined by mass balance and their transport profiles estimated by the semi-empirical Korsmeyer-Peppas model (Equation 5.3).

$$\frac{M_t}{M_\infty} = k \times t^n \quad \text{Equation 5.3}$$

Where, M_t is the absolute cumulative amount of released antibiotic at time t , M_∞ is the total amount of antibiotic in MNs or Au@MNs at equilibrium, k is the diffusion coefficient that incorporates structural and geometric characteristics of each material, and n is the release parameter that determines the specific transport mechanism [23].

5.2.4 Antibacterial activity assays

The antibacterial activity of the synthesised nanoparticles was assayed against both Gram-negative bacteria - *Escherichia coli* (ATCC® 8739TM) and *Pseudomonas aeruginosa* (ATCC® 9027TM) - and Gram-positive bacteria - *Staphylococcus aureus* (ATCC® 6538TM), methicillin-resistant *Staphylococcus aureus* (ATCC® 33591TM) (MRSA) (see Table 5.1). Bacteria were kept frozen at -70 °C in broth containing glycerol (15% v/v).

Table 5.1 - Bacterial strains specifications.

Strain	Relevant phenotype	Ref.
<i>E. coli</i> ATCC 8739	Gram-negative; ofloxacin sensitive	[24,25]
<i>P. aeruginosa</i> ATCC 9027	Gram-negative; amoxicillin resistant, ofloxacin sensitive	[26,27]
<i>S. aureus</i> ATCC 6538	Gram-positive, methicillin sensitive, ofloxacin sensitive	[28,29]
<i>S. aureus</i> ATCC 33591 (MRSA)	Gram-positive, methicillin/amoxicillin resistant, ofloxacin sensitive	[30,31]

ATCC – American Type Culture Collection

All nanoparticle powders and antibiotics were suspended in DMSO to concentrations of 8 mg/mL and 1 mg/mL, respectively (table 5.2).

Table 5.2 - Summary of all tested nanoparticles, free antibiotics and controls, with their stock concentrations.

Particles	ID	m (mg)	V _{DMSO} (mL)	[] _{stock} (mg/mL)	m _{Drug} (ug)	[] _{Drug} (mg/mL)
MNs	1	4.00	0.5	8.00	-	-
Au@MNs	2	4.00	0.5	8.00	-	-
Oflox@MNs	3	4.00	0.5	8.00	221.9	0.4
Amox@MNs	4	4.00	0.5	8.00	491.0	1.0
Oflox@Au@MNs	5	4.00	0.5	8.00	165.7	0.3
Amox@Au@MNs	6	4.00	0.5	8.00	563.4	1.1
Amox free	7	1.00	1.0	1.00	-	-
Oflox free	8	1.00	1.0	1.00	-	-
DMSO	9	-	1.0	-	-	-

Initially, antibacterial activity was assayed by the agar diffusion method proposed by Kirby-Bauer [32]. Frozen microorganisms were initially inoculated in TSA and incubated overnight at 35 ± 2 °C. Isolated colonies were transferred to saline medium (NaCl, 0.85% m/v) and the turbidity of the suspension was adjusted to 0.5 on the McFarland scale (approx. 1.5 × 10⁸ CFU/mL) [19]. Then, with a sterile swab, bacteria suspensions were spread on the surface of MHA *Petri* dishes, onto which sterile paper disks were set. Each disk was carefully loaded with 10 µL of each sample suspension and the plates were incubated, in the dark, for 24 h at 35 ± 2 °C. Antibacterial activity was evaluated, in duplicates, by measuring the

diameter of the growth inhibition zone around the sterile paper disks, compared with those of controls. Solvent-loaded sterile paper disks were used as negative control.

To evaluate and quantify the bactericidal and bacteriostatic effects of the tested nanocomposites, all samples were assayed by the broth microdilution method in 96-well microplate. Briefly, two-fold serial dilutions of each sample and controls were prepared in sterile MHB to a final volume of 100 μ L per well. Then, each well was inoculated with the previously prepared bacteria suspensions. The microplates were incubated, in the dark, at 35 ± 2 °C for 24 h. Free antibiotics, MNs and Au@MNs incubated in the presence of bacteria were used as positive controls, while bacteria incubated in the absence of NPs were used as negative controls. NPs and antibiotics incubated in the absence of bacteria were used as sterile controls. Each sample was tested in duplicate.

Upon the appointed time, an aliquot of each well was spread onto TSA *Petri* dishes and incubated in the dark, for an additional 24 h at 35 ± 2 °C. All 96-well plates were then analysed, with each well's turbidity being observed at both naked-eye and in a 96-well plate ClarioStar spectrophotometer, at 600 nm. The obtained results were compared with those obtained after the 24 h-incubated TSA *Petri* dishes. A minimum inhibitory concentration (MIC) was considered for a reduction in turbidity/viability of approx. 50%, and a minimum bactericidal concentration (MBC) for a reduction in 99.9%, when compared to that of the controls [33].

5.3 Results and discussion

5.3.1 Synthesis of Gold @ mesoporous silica Nanoparticles and Characterisation

Gold nanoparticles (AuNP) were obtained by the Turkevich method, leading to the synthesis of citrate-capped AuNPs, that were initially characterised by UV-Vis absorption spectroscopy and Dynamic Light Scattering (DLS) techniques for the determination of shape, size and zeta potential. The as-synthesised deep-red coloured AuNPs (50 μ L) were diluted in 2.95 mL of MiliQ water (pH = 7.0), giving place to a light-red dispersion with a typical plasmonic band in its absorption spectra at *ca.* 525 nm, characteristic of sphere-like dispersed AuNPs (Figure S-5.1) [34,21]. Their average hydrodynamic diameter (HD), polydispersion index (PDI) and zeta potential (ζ) were of 27 ± 5 nm, 0.28 ± 0.06 and -30 ± 4 mV (for n replicates, with $n = 8$), confirming not only their high stability but also the effective capping

of AuNPs surface with citrate molecules. Obtained HDs are also according to those stated in literature for the same synthetic methodology [21,35]. The final gold concentration in AuNPs purified dispersions was obtained by ICP, with $[Au] = 8.24 \times 10^{-4}$ M corresponding to an average AuNPs concentration of 9.2×10^{11} particles/mL.

Gold mesoporous nanoparticles (Au@MNs) were obtained through the growth of a silica shell by a well-established and reported Stöber method for the synthesis of MCM-41 [18,36,37]. Here, TEOS was used as silica source, CTAB as a cationic templating surfactant, ethylene glycol as stabiliser and NaOH as reducing and morphological agent. MCM-41 mesoporous silica nanoparticles (MNs) produced in the absence of gold cores were used as control for all experiments. Both nanosystems were obtained in the form of dry powders and were characterised by DLS, FTIR, XRD TEM, SEM, ICP and N_2 adsorption-desorption isotherms (Figure 5.1 and 5.2)

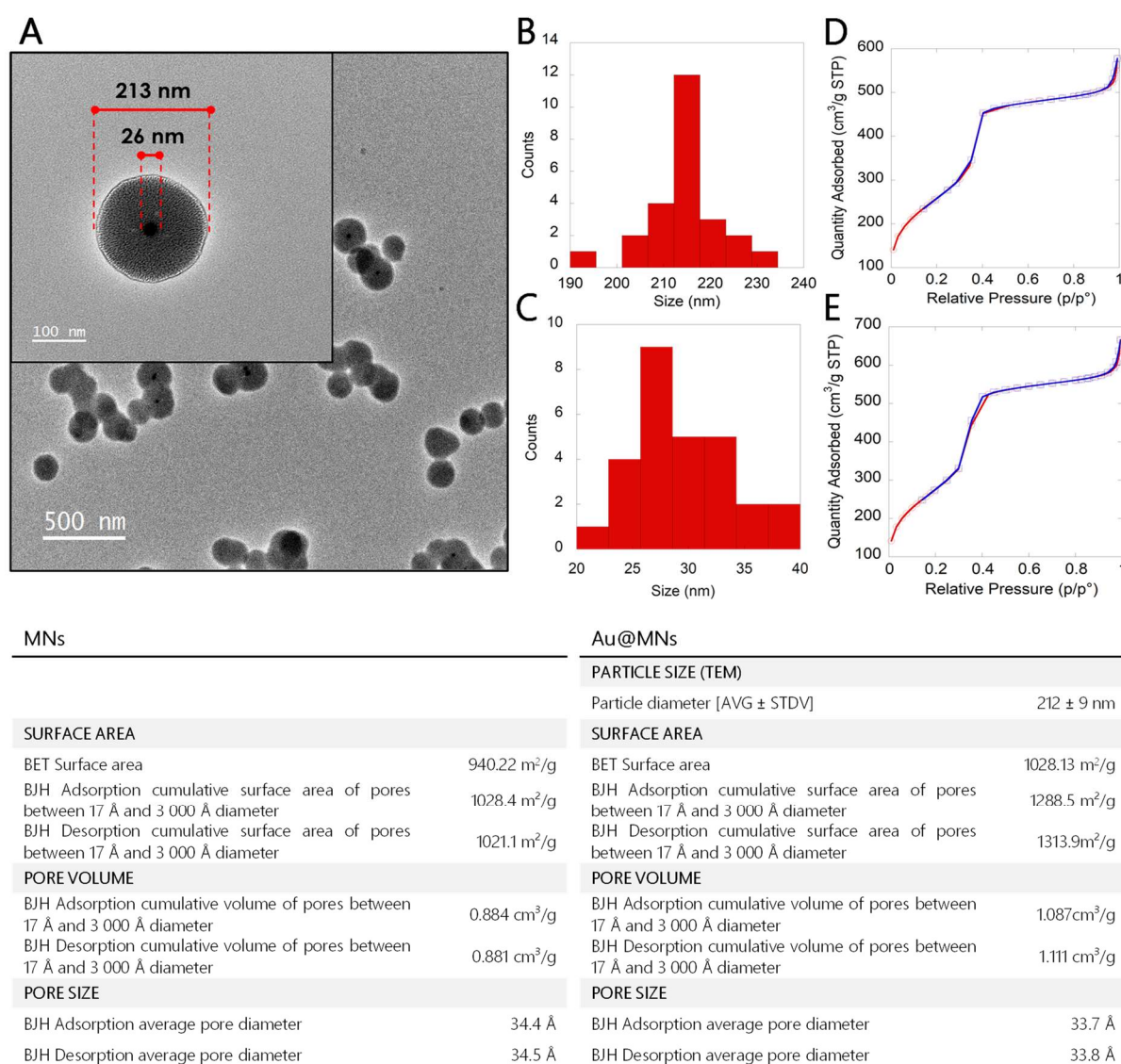


Figure 5.1 - Top | Characterization of Au@MNs nanoparticles: (A) TEM image of Au@MNs– inset: close up on single Au@MNs (scale 69 nm). Size distribution histograms (for a population of N=50) of (B) Au@MNs and (C) Au-cores. Characteristic type-IV N₂ isotherms of mesoporous (D) MNs and (E) Au@MNs. **Bottom** | Summary of MNs and Au@MNs physical properties, obtained from the analysis of TEM images and BET and BJH models application in physisorption assays

MNs nanoparticles had their HD and water stability determined by DLS assays (HD = 443 ± 19 nm, PDI = 0.45 ± 0.09 and $\zeta = -21 \pm 0.2$ mV), showing similar results to those already reported for the same system, in the same conditions [37]. The successful production of silica nanoparticles was validated by their FTIR and XRD spectra. The presence of characteristic Si-O-Si (1225 cm^{-1} , 1065 cm^{-1} , 445 cm^{-1}), Si-O (965 cm^{-1} , 795 cm^{-1}) and Si-OH (3388 cm^{-1} , 1645 cm^{-1}) vibrations in the obtained FTIR spectra confirm the presence of a silica matrix with surface H-bonded silanols (Figure S-5.2). Its corresponding XRD pattern revealed

a broad wave packet at ca. 20°-30°, arising from the formation of a predominant amorphous silica phase in MNs nanoparticles, and well-defined diffraction peaks at 2.30°, 3.86°, 4.42° and 5.86°, corresponding to (100), (110), (200), (210) diffraction planes, respectively (Figure S-5.3). The presence of these planes is typically associated with highly-ordered hexagonal mesoporous MCM-41 structures [38-40].

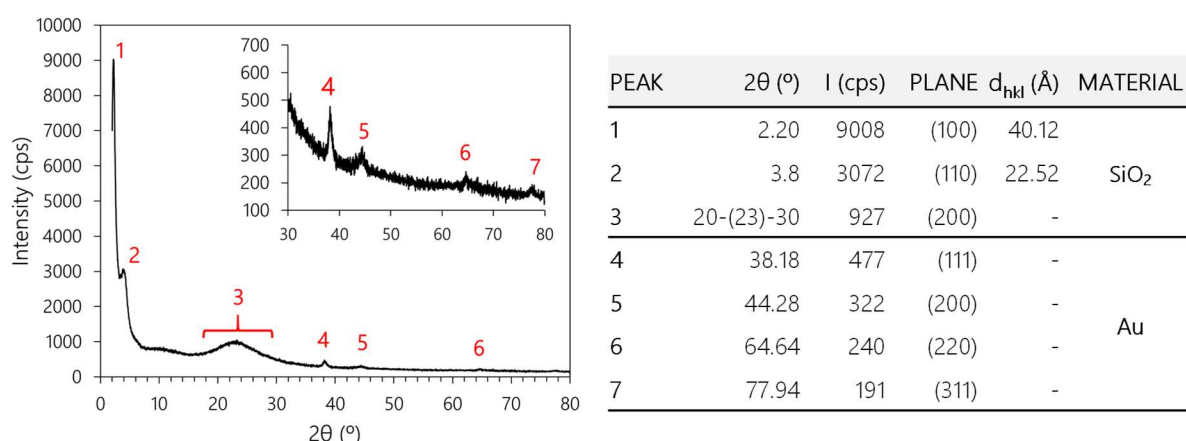


Figure 5.2 - *Left* | Au@MNs XRD pattern for 2θ angles between 2° and 80° - inset: close up on Au typical peaks for angles between 30°-80°. *Right* | Summary on the registered peaks and associated intensities, 2θ angles, atomic planes, interplanar d-spacing (d_{hkl}) and type of material

A high mesoporosity was confirmed by N₂ adsorption-desorption type IV isotherms, with an accentuated adsorption step behaviour at p/p₀ ca. 0.30–0.35 (figure 5.1.E). Surface area, porous volume and pore diameter were assessed through Brunauer Emmett and Teller (BET) and Barrett-Joyner-Halenda (BJH) methods, with values of 940.22 m²/g, 0.884 cm³/g and 34.5 Å, respectively.

Au@MNs nanoparticles were successfully obtained and showed excellent water dispersibility and stability, with HDs of about 333 ± 4 nm, for PDI = 0.16 ± 0.01 and ζ = -39 ± 1 mV. Total template removal was confirmed by FTIR spectra of the nanocomposite through the loss of the typical strong C-H stretching between 2900-3000 cm⁻¹ (Figure S-5.2). However, it should be noted that citrate distinct peaks at ca. 2970 cm⁻¹, 1730 cm⁻¹, 1390 cm⁻¹, 1230 cm⁻¹ and 880 cm⁻¹ are yet visible, hinting the existence of sodium citrate anhydrous-like species and consequently an effective encapsulation of citrate-capped AuNPs. Silica shell growth was also confirmed through FTIR spectroscopy, with characteristic Si-O-Si (1068 cm⁻¹, 445 cm⁻¹), Si-O (965 cm⁻¹, 799 cm⁻¹) and Si-OH (3337 cm⁻¹, 1641 cm⁻¹) stretchings and bendings displayed in Au@MNs FTIR spectra. Similar to MNs, and again confirming the presence of an ordered mesoporous and amorphous silica shell, Au@MNs' XRD pattern showed a broad signal at ca. 20°-30° along with well-defined peaks 2.20° and 3.80°, corresponding to Si(100)

and Si(110) planes, respectively. Interestingly, weak but defined peaks at 38.18°, 44.28°, 64.64° and 77.94° match those previously reported in literature for Au-silica nanocomposites [41,42], corresponding to Au(111), Au(200), Au(220) and Au(311) planes, respectively (figure 5.2).

TEM images confirmed what above-mentioned techniques already hinted for, showing highly porous well-defined spherical nanoparticles centred by a single AuNP core, with average diameters of 213 ± 5 nm and 26 ± 3 nm, respectively (Figure 5.1.A-C and Figure S-5.4). N₂ adsorption-desorption assays developed type IV isotherms, typical of mesoporous materials, with surface area, porous volume and pore size of 1028.13 m²/g, 1.111 cm³/g and 33.8 Å, respectively (figure 5.1.D). Note that, the increase in surface area and porous volume is consequence of Au@MNs larger particle diameter. Au content in Au@MNs was determined by ICP, amounting to 6.83 mg of Au per gram of nanoparticles. SEM micrographs of MNs and Au@MNs showed a particle size of 193 ± 27 nm and 194 ± 23 nm, respectively.

5.3.2 Antibiotics Loading and Release studies

The physical adsorption of amoxicillin and ofloxacin into nanoparticles (i.e. MNs and Au@MNs) pores was achieved in MiliQ water (pH 7), or DMSO, respectively. The loading solvents were selected to ensure good solubility and homogeneity of each antibiotic, and nanoparticles, during the loading process. Both antibiotic loadings were evaluated after 24h incubations, at 20 °C, and upon each wash's supernatant quantification by UV-vis absorbance spectroscopy. Amoxicillin and ofloxacin solutions of 1×10^{-5} M, prepared in MiliQ water and DMSO respectively, were used as standards. Their spectra were collected at 20 °C, with absorption maxima centred at 271 nm, for amoxicillin, and 292 nm, for ofloxacin. The latter also presenting a shoulder at 330 nm (Figure 5.3.A). Maxima were used to quantify supernatants, with amoxicillin encapsulation reaching L% values of 62% (i.e. LC = 122.75 mg/g) and 70% (i.e. LC = 140.85 mg/g), for MNs and Au@MNs respectively. Conversely, ofloxacin loadings reached LC values of 62.32 mg/g and 42.18 mg/g, for MNs and Au@MNs respectively (Figure 5.3.B).

The systems displayed great loading capacity matching already published silica-based materials for the same antibiotics, some of which had to undergo a previous organofunctionalisation process of their pores and surfaces to attain their loading levels (Table 5.3). Amox@Au@MNs showed L% and LC values competitive with those obtained for surface-modified mesoporous silicas; while their Au-free counterpart (Amox@MNs) bested, by far, any as-synthesised mesoporous silica material reported in literature. Here in produced

ofloxacin-loaded materials, exhibited great encapsulation aptency, reproducing similar results to those of Nuti et al. [18]. The scarceness of reported ofloxacin-loaded mesoporous silica nanomaterials, represents an opportunity for the introduction of such materials in bacteria eradication while aiming for a reduction in prescribed ofloxacin quantities.

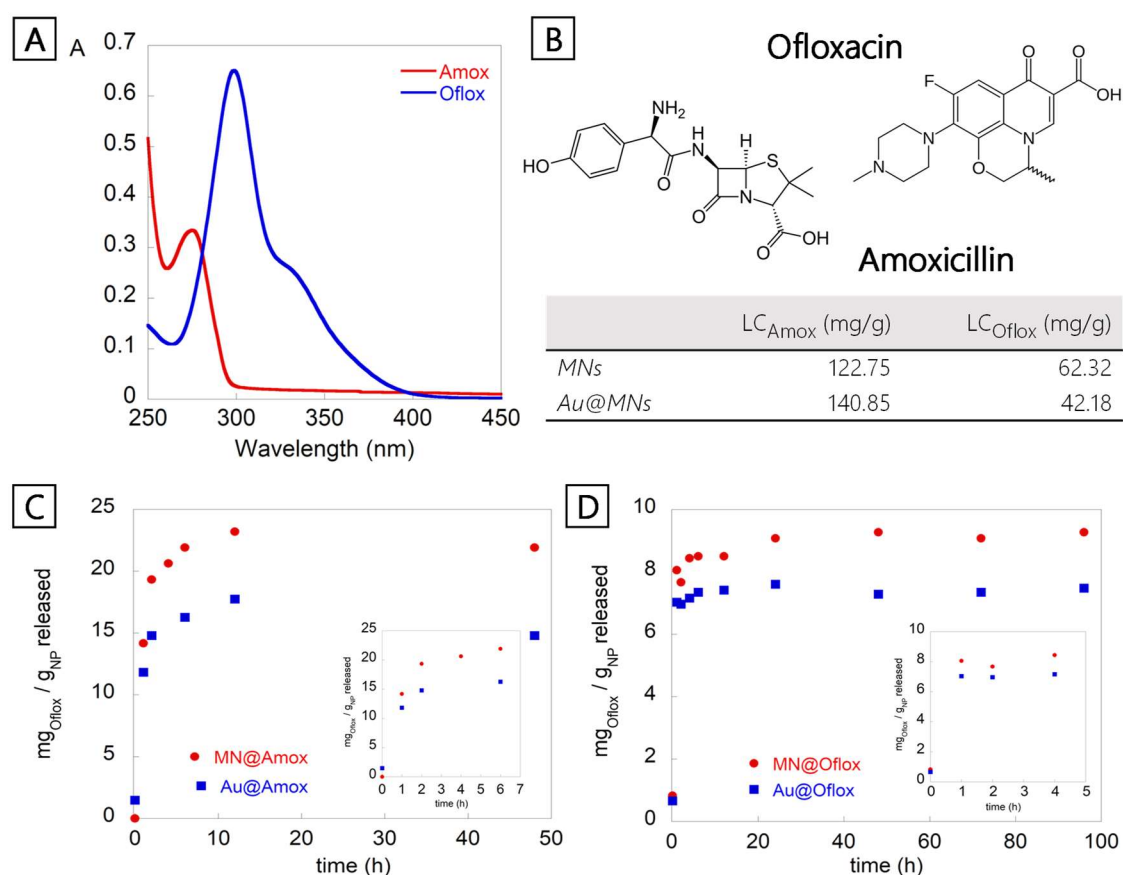


Figure 5.3 - (A) Absorption spectra of free antibiotics at approx. 10–5M. (B) Chemical structure of both amoxicillin and ofloxacin, and corresponding Loading Capacities (LC) of MNs and Au@MNs systems towards the same antibiotics. (C) Amoxicillin release profiles from MNs and Au@MNs, in DMSO at 37 °C, for 5 days – inset: first 6 hours of release. (D) Ofloxacin release profiles from MNs and Au@MNs, in DMSO at 37 °C, for 5 days – inset: first 4 hours of release

Amoxicillin and ofloxacin loaded materials were also characterised by DLS measurement of their size and zeta potential. Amox@Au@MNs and Oflox@Au@MNs showing larger HDs (454 ± 24 nm and 557 ± 10 nm, respectively) and lower ζ (-17 ± 1 mV and -19 ± 1 mV, respectively) can be attributed to an efficient functionalisation of pores and surfaces, although with a destabilising effect.

Table 5.3 - Comparison between in-work developed materials and other already published in literature.

Antibiotic	Supporting material	Material ID	Type of interaction	% L (%)	LC (mg/g)	Ref.
Amoxicillin	SBA-15	SBA-15-x	adsorption	-	-	[43]
		SBA 15-xA		19	-	
		SBA-15-xM		25	-	
		SBA-15-xT		-	-	
	SBA-16	SBA-16	covalent	3.2	13	[44]
		SBA-16IPD		51	204	
		SBA-16IPDB		51	204	
	MCM-41	SiMCM-41	adsorption	91	300	[45]
		PCiMCM-41		91	300	
		PNH ₂ MCM-41		91	300	
		CiMCM-41		91	300	
		CNH ₂ MCM-41		91	300	
		TPCiMCM-41		82	270	
		TPCCiMCM-41		89	290	
		TPNH ₂ MCM-41		89	290	
		TCNH ₂ MCM-41		91	300	
	MCM-41	Fe ₃ O ₄ /SiO ₂ /CTAB-SiO ₂	adsorption	-	363	[46]
	MCM-41	Pure MCM-41	adsorption	-	8	[47]
		Si _{Micro}		-	1	
		Si _{Micro} /20Fe150		-	3	
		5Fe150		-	15	
		10Fe150		-	20	
		20Fe150		-	25	
		50Fe150		-	20	
		5Fe200		-	10	
		10Fe200		-	15	
		20Fe200		-	18	
		50Fe200		-	12	
	MCM-41	MNs	adsorption	62	123	This work
		Au@MNs		70	141	
Ofloxacin	MCM-41	MSNPs	covalent	30	-	[18]
		AgMSNPs (DMSO)		35	-	
		(PBS 7.4)		84	-	
	MCM-41	Si-P ₇₀₀	adsorption	80	-	[48]
		Si-NP ₈		67	-	
	MCM-41	MNs	adsorption	31	62	This work
		Au@MNs		35	42	

Release assays were performed in DMSO at 37 °C, as all samples were dispersed in DMSO for 1 h, prior to its application in antimicrobial activity assays. The release assay was followed by absorbance measurements of all supernatants, at each drug maximum, for 5 days. Amoxicillin release maxima for MNs and Au@MNs were of ca. 15% and 10% (i.e. 19 mg/g and 15 mg/g), respectively (Figure 5.3.C), while ofloxacin released maxima were of ca. 14% and 12% (i.e. 9 mg/g and 7 mg/g) (Figure 5.3.D). Such results indicate a delaying effect of Au-cores presence over antibiotics release, which was confirmed by fitting the collected data to the semi-empirical Korsmeyer-Peppas. Through a linearization of Equation 5.3 we have,

$$\log \frac{M_t}{M_\infty} = \log k + n \cdot \log t \quad \text{Equation 5.4}$$

And fitting to the already treated and logarithmised data, it was possible to determine both n and k (h^{-n}) for all antibiotic-nanoparticles systems, summarised in Table 5.4 (see Figure S-5.5). With all systems presenting a spherical shape and having values of $n < 0.43$, it can be concluded that, as expected, we are working with non-swelling matrixes where diffusion phenomena rule antibiotics mass transfer, from nanoparticles to the surrounding medium. In these cases, diffusion follows a quasi-fickian model with diffusion rates as functions of time, for k values defined by t^{-n} [49-51].

Table 5.4 - Semi-empirical Korsmeyer-Peppas diffusion model parameters upon fitting to experimental amoxicillin and ofloxacin release data.

NP sample	R ²	n	k (t^{-n})
Amox@MNs	0.9227	0.19	0.705
Amox@Au@MNs	0.9983	0.11	0.636
Oflox@MNs	0.9987	0.11	0.794
Oflox@Au@MNs	0.9989	0.13	0.847

5.3.3 Antibacterial activity assays

The antibacterial activity of each system was initially tested in sterile paper disks, which were placed in contact with *E. coli*, *P. aeruginosa*, *S. aureus* and MRSA cultures. All nanoparticles were loaded at a concentration of 8.0 mg_{NP}/mL, corresponding to: 1.0 mg_{Amox}/mL and 1.1 mg_{Amox}/mL for Amox@MNs and Amox@Au@MNs, respectively; and 0.4 mg_{Oflox}/mL and 0.3 mg_{Oflox}/mL for Oflox@MNs and Oflox@Au@MNs. The formation of growth inhibition zones and their diameters are evidence of each formulation's antibacterial activity, when compared to those formed, or not, in the presence of free amoxicillin, free ofloxacin and their blank MNs and Au@MNS counterparts.

From the results displayed in Table 5.5, it is evident that most strains have a high susceptibility to ofloxacin, with all systems showing significant growth inhibition zones after incubations. This is in accordance with the information detailed in previous reports [52,53], as well as ATCC and BacDive database [24-31,54], where no ofloxacin resistance is indicated for any of the studied strains.

Regarding amoxicillin, the methicillin-susceptible *S. aureus* strain exhibited susceptibility to all formulations (i.e. free antibiotics and amoxicillin-loaded NPs), confirming not only the presence of the antibiotic in the nanosystems, but also its effective delivery from the nanoparticles to the bacterial medium. For *E. coli*, an inhibition zone of 13 mm was recorded around the free amoxicillin disk. However, with loaded-MNs and Au@MNs no measurable growth inhibition zones were detected, suggesting an inability of these nanosystems to deliver the necessary amount of antibiotic.

Table 5.5 - Summarising table with all growth inhibition zones' average diameters. Measured lengths represent the obtained means $\pm$ an associated absolute error of 0.5 mm

NP sample	[] _{Drug} (ug/disk)	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>	Methicillin-resistant <i>S. aureus</i>	<i>Pseudomonas aeruginosa</i>
		$\bar{d}$ Inhibition zone (mm)	$\bar{d}$ Inhibition zone (mm)	$\bar{d}$ Inhibition zone (mm)	$\bar{d}$ Inhibition zone (mm)
MNs	-	0	0	0	0
Au@MNs	-	0	7	0	7
Free Amox	10.0	13	45	7	0
Amox@MNs	9.8	0	19	0	0
Amox@Au@MNs	11.3	0	15	0	0
Free Oflox	10.0	38	31	32	25
Oflox@MNs	4.4	20	19	17	9
Oflox@Au@MNs	3.3	17	17	14	8
DMSO	-	0	0	0	0

Additionally, and as expected, both MRSA and *P. aeruginosa* showed strong resistance towards amoxicillin, as no zones of inhibition were observed for any formulation. Amoxicillin is a β -lactam antibiotic that binds to transpeptidase domain of penicillin-binding proteins (PBP) preventing peptidoglycan biosynthesis [55]. MRSA methicillin-resistance[3] confers them resistance to other β -lactam antibiotics, such as oxacillin, penicillin and amoxicillin [56]. This resistance results from expression of β -lactamases and of an altered form of PBP (encoded by *mecA* gene) with low affinity to β -lactam and higher rates of release of the bound drug [57,58] On the other hand, the interaction between the inducible β -

lactamase AmpC and the *P. aeruginosa* resistance nodulation cell division multidrug efflux systems, lists this strain as one towards which amoxicillin is not effective [59].

Despite all registered zones of inhibition for antibiotic-loaded MNs and Au@MNs systems, none exhibited diameters larger than those obtained for the free antibiotics. This fact may derive from nanoparticles dispersibility issues in the sterile paper disk matrix, that did not allow for a total dispersion and transport of the antibiotics to bacterial media.

Broth microdilution assays were used to overcome dispersion issues, with each formulation (i.e. free antibiotics, MNs, Au@MNs, Amox@MNs, Amox@Au@MNs, Oflox@MNs and Oflox@Au@MNs) being resuspended in DMSO to attain good dispersibility and homogeneity. This method allows not only for direct contact between nanoparticles and bacteria but also for greater sensibility. A gradient of concentrations for each formulation, ranging between ca. $10 - 0.006 \mu\text{g}_{\text{Antibiotic}}/\text{mL}$ ($765 - 1 \mu\text{g}_{\text{NPs}}/\text{mL}$), was tested to assess both MIC and MBC by absorbance spectroscopic techniques, against *E. coli* ATCC 8739TM, the susceptible *S. aureus* ATCC 6538TM, the resistant MRSA ATCC 33591TM and *P. aeruginosa* ATCC 9027TM. MIC and MBC validation was attained by plating on TSA plates.

No antibacterial activity was registered with MNs, Au@MNs and DMSO, that served as controls along the whole experiment. It should be noted that antibiotic concentration is variable, depending on each nanoparticle loading capacity (see section 5.3.2. Antibiotics Loading and Release studies). Free antibiotics were also tested within a $95 - 0.015 \mu\text{g}_{\text{Antibiotic}}/\text{mL}$ concentration range and used as standards (Table 5.6).

Overall, amoxicillin-loaded systems followed a similar tendency to that observed in disk diffusion assay, having a more effective activity against the susceptible *S. aureus* strain, than for other bacteria. Amox@MNs and Amox@Au@MNs MICs towards *S. aureus* matched their own MBCs, that were confirmed for concentrations of $1 \mu\text{g}_{\text{Amox}}/\text{mL}$ ($80 \mu\text{g}_{\text{NPs}}/\text{mL}$) and $3 \mu\text{g}_{\text{Amox}}/\text{mL}$ ($360 \mu\text{g}_{\text{NPs}}/\text{mL}$), respectively. However, when compared to the obtained viability of the free amoxicillin formulation (Table 4.6), which led to inhibition at $0.015 \mu\text{g}_{\text{Amox}}/\text{mL}$ and death at $0.07 \mu\text{g}_{\text{Amox}}/\text{mL}$, no significant beneficial role can be assigned to the antibiotic decorated nanoparticles.

Table 5.6 - Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of DMSO, MNs, Au@MNs and the free antibiotics towards the selected bacterial strains.

Formulation description	Strain	MIC ($\mu\text{g/mL}$) <i>Antibiotic (NP)</i>	MBC ($\mu\text{g/mL}$) <i>Antibiotic (NP)</i>
DMSO	<i>E. coli</i> ATCC 8739	N/D	N/D
	<i>S. aureus</i> ATCC 6538	N/D	N/D
	MRSA ATCC 33591	N/D	N/D
	<i>P. aeruginosa</i> ATCC 9027	N/D	N/D
MNs	<i>E. coli</i> ATCC 8739	N/D	N/D
	<i>S. aureus</i> ATCC 6538	N/D	N/D
	MRSA ATCC 33591	N/D	N/D
	<i>P. aeruginosa</i> ATCC 9027	N/D	N/D
Au@MNs	<i>E. coli</i> ATCC 8739	N/D	N/D
	<i>S. aureus</i> ATCC 6538	N/D	N/D
	MRSA ATCC 33591	N/D	N/D
	<i>P. aeruginosa</i> ATCC 9027	N/D	N/D
Free amoxicillin	<i>E. coli</i> ATCC 8739	5 (-)	5 (-)
	<i>S. aureus</i> ATCC 6538	0.015 (-)	0.07 (-)
	MRSA ATCC 33591	N/D	N/D
	<i>P. aeruginosa</i> ATCC 9027	95 (-)	N/D
Free ofloxacin	<i>E. coli</i> ATCC 8739	0.03 (-)	0.03 (-)
	<i>S. aureus</i> ATCC 6538	0.15 (-)	0.3 (-)
	MRSA ATCC 33591	0.3 (-)	0.6 (-)
	<i>P. aeruginosa</i> ATCC 9027	0.6 (-)	0.6 (-)

Within the range of tested concentrations, amoxicillin-nanosystems were only able to inhibit *E. coli* and *P. aeruginosa* growth. All assayed concentrations of Amox@MNs and Amox@Au@MNs failed to reduce both bacteria viabilities by 99%, pointing to MBCs at values $> 765 \mu\text{g}_{\text{NPs}}/\text{mL}$. The MIC of Amox@MNs, towards *E. coli*, was determined for a concentration of $10 \mu\text{g}_{\text{Amox}}/\text{mL}$ ($765 \mu\text{g}_{\text{NPs}}/\text{mL}$), whereas no MIC was registered for Amox@Au@MNs (MBC and MIC $> 765 \mu\text{g}_{\text{NPs}}/\text{mL}$). Again, when compared to those obtained for the free antibiotic (Table 5.6) no beneficial role is evident. Regarding *P. aeruginosa*, whilst free amoxicillin only had a MIC at $95 \mu\text{g}_{\text{Amox}}/\text{mL}$, Amox@MNs and Amox@Au@MNs displayed MICs at lower antibiotic concentrations of $10 \mu\text{g}_{\text{Amox}}/\text{mL}$ ($765 \mu\text{g}_{\text{NPs}}/\text{mL}$) and $5 \mu\text{g}_{\text{Amox}}/\text{mL}$ ($765 \mu\text{g}_{\text{NPs}}/\text{mL}$), respectively. This result gives our nanosystems a beneficial role in the reduction of prescribed antibiotics, with Amox@MNs and Amox@Au@MNs allowing for a 10-fold and a 20-fold reduction, respectively, while maintaining its bacteriostatic activity.

Concerning MRSA, concentrations up to $95 \mu\text{g}_{\text{Amox}}/\text{mL}$ of free amoxicillin failed to reduce bacteria viabilities by 50% (MIC and MBC $> 95 \mu\text{g}_{\text{Amox}}/\text{mL}$) (Table 5.6). However,

prominent bacteriostatic and bactericidal activity was observed for Amox@MNs that exhibited a MIC of 5 $\mu\text{g}_{\text{Amox}}/\text{mL}$ (i.e. for 360 $\mu\text{g}_{\text{NPs}}/\text{mL}$) and a MBC of 10 $\mu\text{g}_{\text{Amox}}/\text{mL}$ (765 $\mu\text{g}_{\text{NPs}}/\text{mL}$). Thus, leading to not only an higher than 20-fold decrease in needed antibiotic, but also allowing a circumvention of the already existing resistance. Amox@Au@MNs, however, showed no MIC and MBC within the tested concentration range. All amoxicillin-based formulation results are summarised in Table 5.7.

Table 5.7 - Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for all amoxicillin-based tested formulations, against selected bacterial strains.

Formulation description	Strain	MIC ($\mu\text{g}/\text{mL}$)	MBC ($\mu\text{g}/\text{mL}$)
		<i>Antibiotic (NP)</i>	<i>Antibiotic (NP)</i>
Amox@MNs Amox c.a. 122.75 mg/g	<i>E. coli</i> ATCC 8739	10 (765)	N/D
	<i>S. aureus</i> ATCC 6538	1 (80)	1 (80)
	MRSA ATCC 33591	5 (360)	10 (765)
	<i>P. aeruginosa</i> ATCC 9027	10 (765)	N/D
Amox@Au@MNs Amox c.a. 140.85 mg/g	<i>E. coli</i> ATCC 8739	N/D	N/D
	<i>S. aureus</i> ATCC 6538	3 (360)	5 (765)
	MRSA ATCC 33591	N/D	N/D
	<i>P. aeruginosa</i> ATCC 9027	5 (765)	N/D

As observed with free ofloxacin, both ofloxacin nanosystems showed strong effectiveness against all bacterial strains, for most tested concentrations. In general, Oflox@MNs performance was more stressed than that of its Au-based counterpart, being in line with the above mentioned release profiles of ofloxacin-decorated nanoparticles, where Oflox@MNs showed higher appetency to dispense the antibiotic than Oflox@Au@MNs.

The determined MIC and MBC of Oflox@MNs towards *E. coli* were both of 0.03 $\mu\text{g}_{\text{Oflox}}/\text{mL}$ (4 $\mu\text{g}_{\text{NPs}}/\text{mL}$), and identical to those obtained for free ofloxacin (Table 5.6). Oflox@Au@MNs however, were able to reduce the MIC by half, inducing a 50% reduction in bacteria viability at 0.015 $\mu\text{g}_{\text{Oflox}}/\text{mL}$ (4 $\mu\text{g}_{\text{NPs}}/\text{mL}$). A similar tendency was observed towards the susceptible *S. aureus* strain, with Oflox@MNs having a MIC (i.e. 0.15 $\mu\text{g}_{\text{Oflox}}/\text{mL}$ for 20 $\mu\text{g}_{\text{NPs}}/\text{mL}$) and a MBC (i.e. 0.3 $\mu\text{g}_{\text{Oflox}}/\text{mL}$ for 40 $\mu\text{g}_{\text{NPs}}/\text{mL}$) equal to those of the free antibiotic. The presence of Au cores played no beneficial role, leading instead to an increase of the MBC to 0.6 $\mu\text{g}_{\text{Oflox}}/\text{mL}$ (170 $\mu\text{g}_{\text{NP}}/\text{mL}$). Regarding MRSA, whilst Oflox@Au@MNs were capable of decreasing the MIC to half of that obtained for free ofloxacin (i.e. 0.15 $\mu\text{g}_{\text{Oflox}}/\text{mL}$ for 40 $\mu\text{g}_{\text{NPs}}/\text{mL}$), only Oflox@MNs were able to reduce in half the MBC to 0.3 $\mu\text{g}_{\text{Oflox}}/\text{mL}$ (40 $\mu\text{g}_{\text{NP}}/\text{mL}$). Lastly, both Oflox@MNs and Oflox@Au@MNs MBCs showed no significant

differences towards *P. aeruginosa*, when compared to that of the free antibiotic. The two formulations were able to induce a 2-fold reduction in the MIC, to concentrations of 0.3 $\mu\text{g}_{\text{Oflox}}/\text{mL}$ (40 $\mu\text{g}_{\text{NP}}/\text{mL}$ and 80 $\mu\text{g}_{\text{NP}}/\text{mL}$ for Oflox@MNs and Oflox@Au@MNs, respectively). A compilation of all ofloxacin-based formulation is displayed in Table 5.8.

Table 5.8 - Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for all ofloxacin-based tested formulations, against selected bacterial strains.

Formulation description	Strain	MIC ($\mu\text{g/mL}$) <i>Antibiotic (NP)</i>	MBC ($\mu\text{g/mL}$) <i>Antibiotic (NP)</i>
Oflox@MNs Oflox c.a. 62.32 mg/g	<i>E. coli</i> ATCC 8739	0.03 (4)	0.03 (4)
	<i>S. aureus</i> ATCC 6538	0.15 (20)	0.3 (40)
	MRSA ATCC 33591	0.3 (40)	0.3 (40)
	<i>P. aeruginosa</i> ATCC 9027	0.3 (40)	0.6 (80)
Oflox@Au@MNs Oflox c.a. 42.18 mg/g	<i>E. coli</i> ATCC 8739	0.015 (4)	0.03 (10)
	<i>S. aureus</i> ATCC 6538	0.15 (40)	0.6 (170)
	MRSA ATCC 33591	0.15 (40)	0.6 (170)
	<i>P. aeruginosa</i> ATCC 9027	0.3 (80)	0.6 (170)

The significant decrease in needed antibiotic is hypothesised to be the result of a mixed effect between the already reported protective role of the mesoporous structures over loaded drugs and antibiotics [60,10], and the attaching and stressing power of MNs shells onto the surface of bacteria [61,62].

It should be noted that, tested concentrations (of NPs and antibiotics) are three to four orders of magnitude lower than those tested in a sterile paper disk, highlighting again the importance of direct contact between nanoparticles and bacteria.

Silica-based nanoparticles that have been reported to efficiently bind to both Gram-negative and Gram-positive bacteria, as all herein tested bacteria [63], and have already been reported to change surface roughness and stability, while inducing membrane stress and membrane disruption (namely by the silanol groups that have appetency for phospholipid membrane destruction) [64]. Thus, an increase in membrane permeabilization could expedite antibiotic access and, consequently, their action inside bacterial cells [65].

Particularly, in what concerns β -lactam antibiotics, high antibiotic concentrations could be dispensed in bacterial cells by nanoparticle/antibiotic combinations, allowing the antibiotic to act before being degraded by bacterial β -lactamases [66,67]. Additionally, antibiotic nanosystems could inhibit the transmembrane pump that catalyses drug efflux from *P. aeruginosa* cells [67].

Moreover, the induction of MRSA resistance reversion by Amox@MNs could be related to MNs perturbations in cell envelope upon interaction with the bacteria. As hypothesised by Kong et al. [68], the resulting perturbations in cell envelope could affect the expression of resistance-responsible genes, by disrupting the activity of sensor-inducer proteins located in cell membrane, which are responsible for the expression of resistance proteins.

Briefly, the herein synthesised formulations had a considerable beneficial role on the effective delivery of amoxicillin to the selected strains; namely, against the resistant *S. aureus* mutant, MRSA, and the well-known β -lactam resistant *P. aeruginosa*. Among its more promising results, are the significant reduction of amoxicillin in future prescriptions, reducing the possibility of development of resistance, and Amox@MNs' surprising circumvention of MRSA resistance. Ofloxacin loaded nanoparticles did not show as outstanding performances as those of their amoxicillin-loaded counterparts, being only able to slightly reduce ofloxacin amounts for both MIC and MBC.

5.4 Conclusions

To summarise, nanocarriers comprising of core-shell gold-silica based mesoporous nanoparticles (Au@MNs) and silica mesoporous nanoparticles (MNs) were successfully synthesised and fully characterised. Both nanocarriers revealed a spherical shape and high porosity with surface areas of 1028.13 m²/g and 940.22 m²/g for Au@MNs and MNs, respectively. The loading ability of these nanosystems was evaluated towards amoxicillin and ofloxacin antibiotics, where high encapsulation yields of 62% (MNs) and 70% (Au@MNs) were achieved for amoxicillin. Amox@MNs, Amox@Au@MNs, Oflox@MNs and Oflox@Au@MNs nanoformulations were tested against *S. aureus*, methicillin-resistant *S. aureus* (MRSA), *E. coli* and *P. aeruginosa* in sterile paper disks and by broth microdilution assays. The best results were achieved by broth microdilutions assays, since this method allows a direct contact between nanoparticles and bacteria. Which was confirmed as crucial variable. Within the range of tested concentrations, amoxicillin-nanosystems were able to inhibit *E. coli* and *P. aeruginosa* growth. From the obtained results, it is clear their significant and beneficial role, by promoting a 10-fold (Amox@MNs) and a 20-fold (Amox@Au@MNs) reduction of needed amoxicillin to inhibit *P. aeruginosa*. Regarding MRSA, not only a 20-fold decrease in amoxicillin MIC was obtained for Amox@MNs, but also a full circumvention of the resistance was possible at a MBC of 5 μ Amoxg/mL. Ofloxacin loaded

nanoparticles were also able to reduce ofloxacin amounts in both MIC and MBC, although less significantly. The above obtained results, aligned with the straightforwardness and cheapness of the reported base materials, may place these novel formulation as a possible and determining therapeutic tools to tackle AMR and its development.

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5.7 Supplementary Material

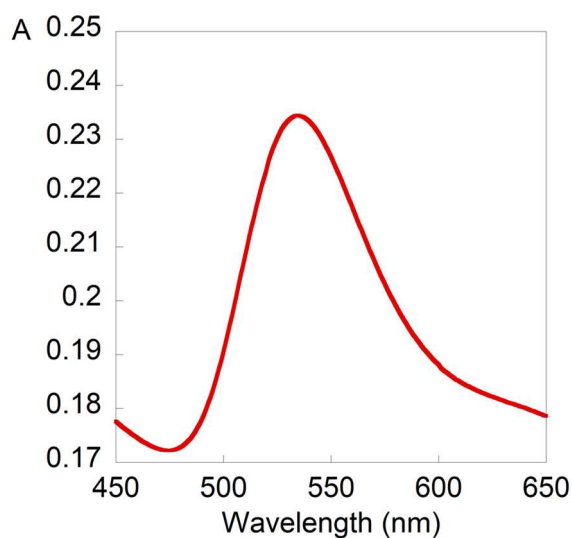


Figure S - 5.1 - Absorption spectra of as-synthesised citrate-capped AuNPs. Obtained after proper dilution, in MilliQ water, of the initial deep-red colour dispersion in a factor of 1:60 ($V_t = 3$ mL). Absorption maximum centered at $\lambda_{\text{max}} = 525$ nm.

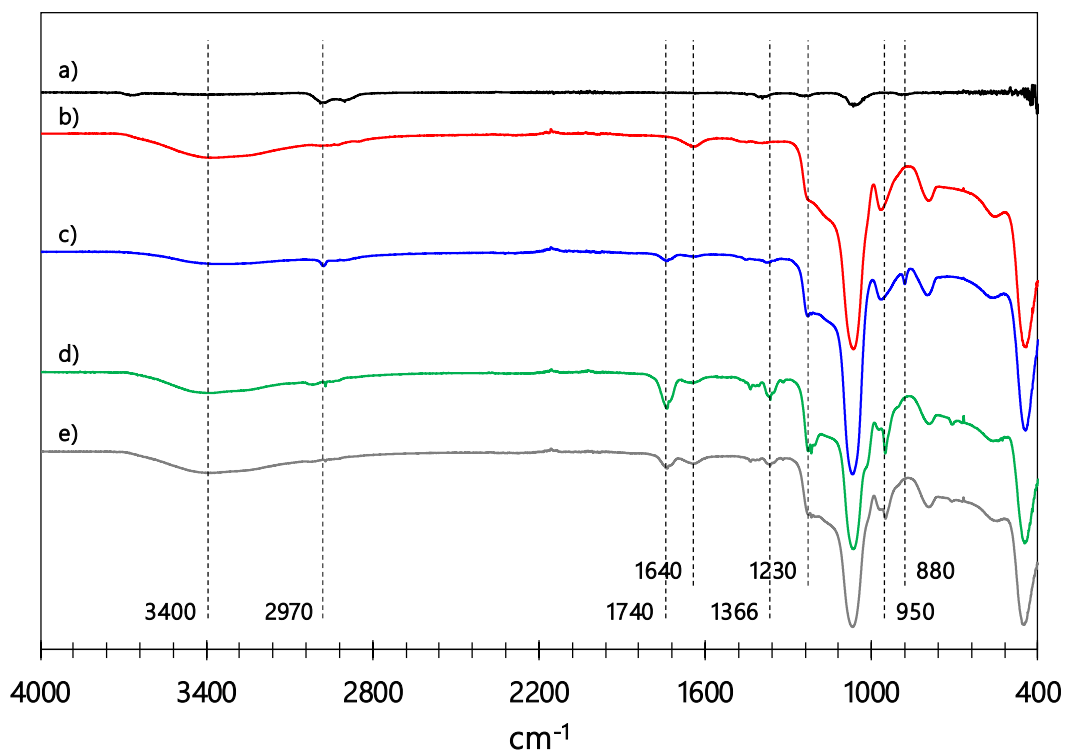


Figure S - 5.2 - FT-IR ATR spectra of a) citrate-AuNPs, b) MNs, c) Au@MNs, d) Oflox@Au@MNs and e) Amox@Au@MNs, with their characteristic cm^{-1} stretchings. All samples containing a mesoporous silica shell had their template removed before analysis.

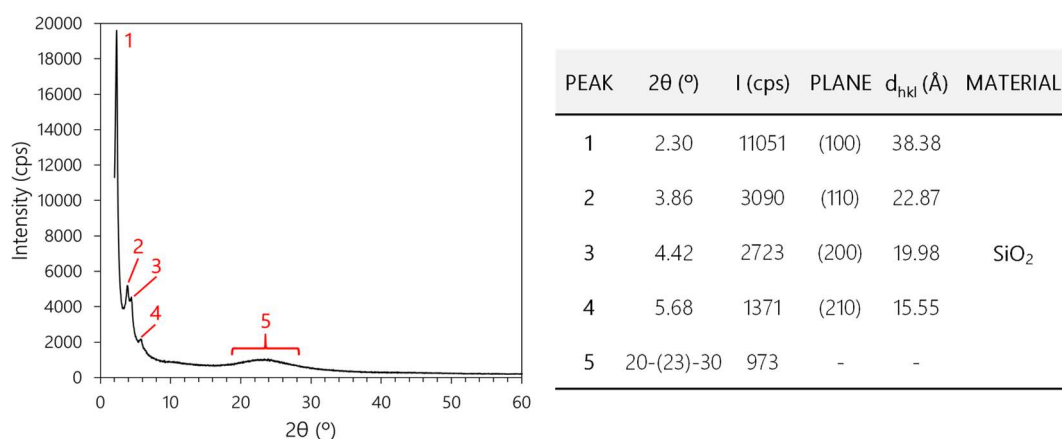


Figure S - 5.3 - *Left* | XRD pattern of MNs and its respective peaks collected for 2θ angles between 2° and 80°. *Right* | Peaks summary and corresponding atomic planes, interplanar d-spacing (d_{hkl}) and type of material.

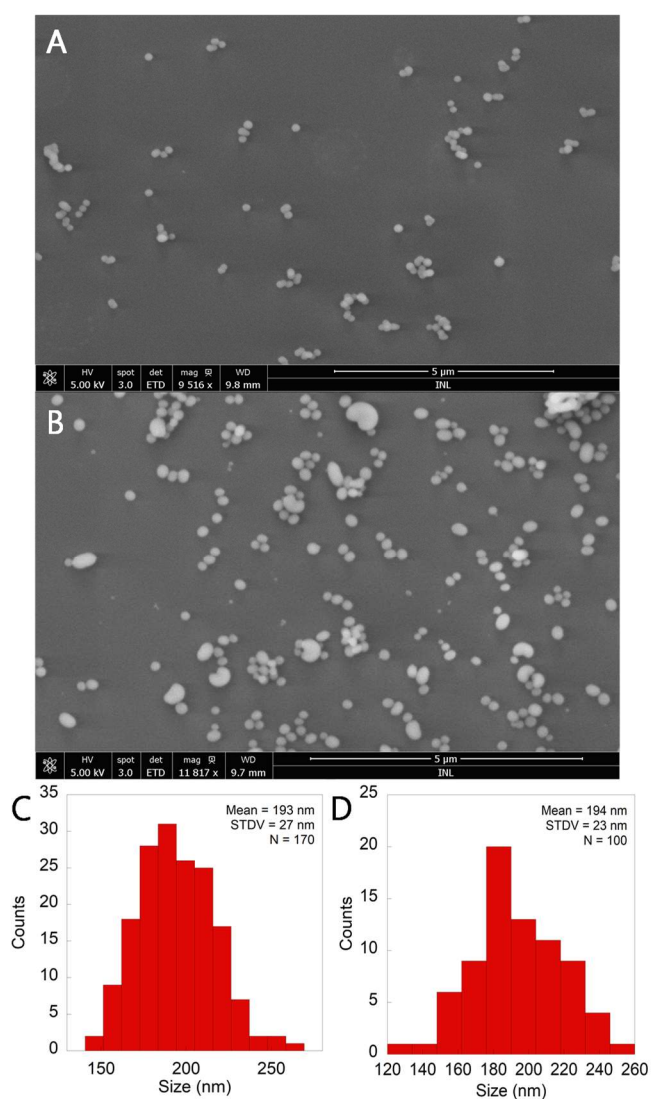


Figure S - 5.4 - SEM micrographs of A) MNs and B) Au@MNs, with their correspondent histograms where: C) MNs and D) Au@MNs average sizes were estimated to be of 193 ± 27 nm (for $N = 170$) and 194 ± 23 nm ($N = 100$), respectively.

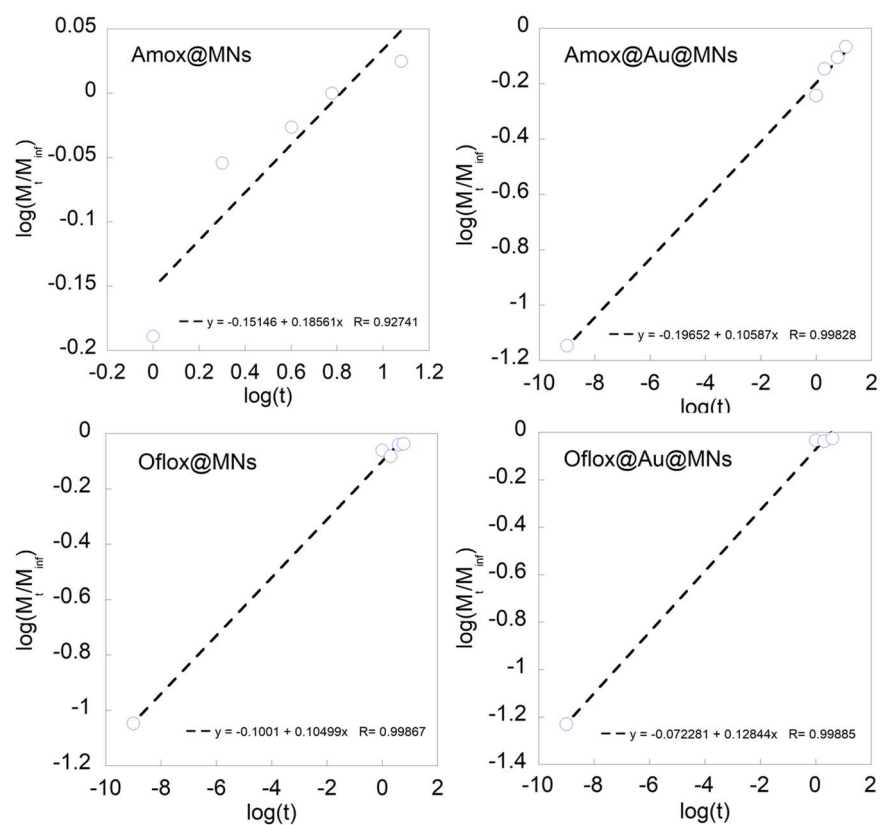
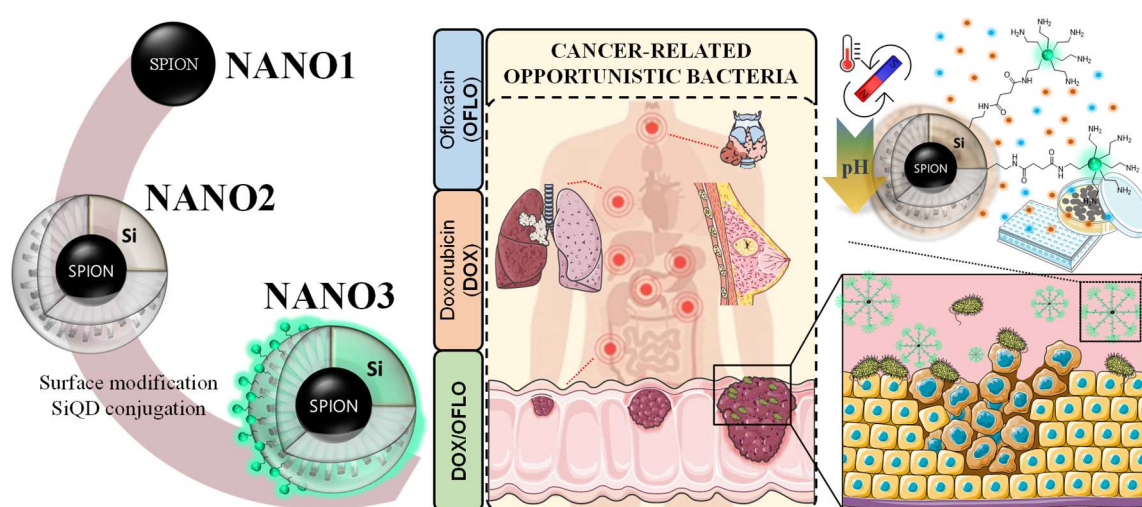


Figure S - 5.5 - Amoxicillin and ofloxacin release data ($M_t/M_\infty < 0.6$) fitting to the linearized semi-empirical Korsmeyer-Peppas model.

MAGNETO-FLUORESCENT MESOPOROUS NANOCARRIERS FOR THE DUAL-DELIVERY OF OFLOXACIN AND DOXORUBICIN TO TACKLE OPPORTUNISTIC BACTERIAL INFECTIONS IN COLORECTAL CANCER



Keywords: opportunistic bacterial infections • cancer treatment • magneto-fluorescent nanoparticles • antimicrobial activity

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Magneto-fluorescent mesoporous nanocarriers for the dual-delivery of ofloxacin and doxorubicin to tackle opportunistic bacterial infections in colorectal cancer

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Abstract: Cancer-related opportunistic bacterial infections are one major barrier for successful therapies, often correlated to the production of genotoxic factors and higher cancer incidence. Although dual anticancer and antimicrobial therapies are a growing therapeutic fashion, they still fall short in specific delivery and local action in *in vivo* systems. Nanoparticles are seen as potential therapeutic vectors, be it by their intrinsic antibacterial properties and effective delivery capacity, but also by their repeatedly reported modulation and maneuverability. Herein we report the production of a biocompatible, antimicrobial magneto-fluorescent nanosystem (NANO3) for the delivery of a dual doxorubicin-ofloxacin formulation against cancer-related bacterial infections. Drug delivery capacity, rendered by its mesoporous silica matrix, is confirmed by the high loading and stimuli-driven release of both drugs, with preference for tumor-like acidic media. The pH-dependent emission of its surface fluorescent SiQDs, provide an insight of NANO3 surface behaviour and pore availability, with SiQD working as pore gates. Hyperthermia induces heat generation to febrile temperatures, doubling drugs release. NANO3 loaded systems demonstrate significant antimicrobial activity, specifically after the application of hyperthermia conditions. NANO3 structure and antimicrobial properties confirm their potential use in a future dual anticancer and antimicrobial therapeutical vector, due to their drug loading capacity and their surface availability to further modification with bioactive, targeting species.

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6.1 Introduction

Cancer-associated bacterial infections and inflammations have been pointed as one of the main causes of low treatment success rates and treatment associated complications¹⁻³. Bacterial infections are particularly damaging in gastric and colorectal cancer (CRC) cases, with strains like *Helicobacter pylori* and *Escherichia coli* being linked to cancer invasiveness and progression^{2, 4-7}. This, allied to the increased occurrence of antimicrobial resistant bacteria, poses a severe threat to current cancer therapies successful rates, but most importantly to patients' health and survival⁸⁻¹⁰.

Strategies to fight bacterial growth and surpass existing resistances is a current hot topic, with several worldwide research groups and institutions developing distinct approaches. Be them, the production of new antibiotics¹¹, the use of nature occurring molecules of animal or plant origin^{12, 13}, the combinatory use of conventional antibiotics and adjuvants^{14, 15}, or the use of engineered nanomaterials^{16, 17}. In particular, nanomaterials have gained especial attention throughout the last decades, particularly due to their intrinsic properties, tunability and easier manipulation. For instance, silver and copper nanoparticles, among others, are examples of nanoparticle systems whose constituents have antimicrobial properties¹⁸⁻²⁰. Iron oxide nanoparticles, for example, have been of great interest as they not only bring greater control and manoeuvrability over therapies, due to their magnetic properties, but have also been reported to have antimicrobial and anti-biofilm properties²¹⁻²³. Moreover, the use of magnetic iron oxide nanoparticles as approved contrasting agents, heat generating and delivery probe in several diagnostic and therapeutic approaches, particularly in cases of cancer, demonstrates its important biocompatibility and safe use in humans²⁴⁻²⁶. Non-antimicrobial nanoparticles have also been the stage for the production of antimicrobial solutions, by conjugating an initial non-toxic matrix with conventional bacteriostatic or bactericidal agents. This conjugation has been reported to produce synergic therapeutic effects between its constituents, be it through a joint action, or by the masking and effective delivery of the antimicrobial agent, by the second material, to its target²⁷⁻²⁹. A wide variety of non-toxic matrices has been reported in literature, as is the case of gold and mesoporous silica nanoparticles to name a few, whose surfaces can not only be easily functionalized but also work as good anchoring sites for bioactive molecules^{28, 30-33}. Moreover, additional functionalization of nanoparticles' surface^{34, 35} and particles combination^{36, 37} render them with extra properties, from controlled and targeted drug delivery to stimuli responsive behaviour, long-term blood circulation, higher cellular take-up,

magnetic- or light-induced heat generation and fluorescence, to name a few, that can be used to improve their antimicrobial properties.

All considered, the production of adapted nanotherapies, capable of tackling both cancer and bacterial infections is thus of utter relevance ³⁸. This is supported by the recent, yet scarce, reports on the use of antibiotics as anticancer targets ³⁹ and of anticancer drugs as potential antimicrobial agents ⁴⁰, as well as their synergic effects in a combined therapy ⁴¹. In a first step to address this, in our group we have recently reported the use of mesoporous silica nanoparticles for the combined therapy against several cancer-related bacterial strains, using doxorubicin, epirubicin and ofloxacin as model anticancer and antimicrobial drugs ²⁷. The combination of these two classes of drugs allowed us to produce an efficient nano-based therapy, with better activity than their constituents alone and the capacity to target resistant strains, as methicillin-resistant *Staphylococcus aureus* (MRSA).

In an attempt to improve the system and prepare it to a further application against cancer and related bacterial infections, in this work we report the synthesis of magneto-fluorescent mesoporous nanosystem for the combinatory delivery of doxorubicin and ofloxacin, as model anticancer drug and antibiotic. With the produced nanosystems, comprising of a superparamagnetic iron oxide core, a mesoporous silica matrix and fluorescent silicon quantum dots (SiQD), we seek to bring together the hyperthermia properties of the magnetic core ^{22, 26}, the cargo capacity of the mesoporous silica and the trackable fluorescence of SiQDs ⁴², while using sustainable, biocompatible, and biodegradable materials. Their antimicrobial activity will be tested against a set of common bacterial strains, usually associated with cancer, to validate its application in a combined therapy.

6.2 Experimental Section

6.2.1 Materials

Iron(III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 97.0-102.0%), iron(II) chloride hydrate ($\text{FeCl}_2 \cdot \text{H}_2\text{O}$, 99%), ammonium nitrate (NH_4NO_3 , 99.999%), N-hydroxysuccinimide (NHS, 98+%), ethylenediaminetetraacetic acid tetrasodium salt hydrate (EDTA, 98%) and tetraethyl orthosilicate (TEOS, $\geq 99.999\%$ metals basis) were bought at AlfaAesar. *n*-Cetyltrimethylammonium bromide (CTAB), (3-aminopropyl) triethoxysilane (APTES, $\geq 98\%$), thioglycolic acid ($\geq 99.9\%$), succinic anhydride ($\geq 99\%$ GC), (+)-sodium L-ascorbate (crystalline,

≥98%), N-3-dimethylaminopropyl-*N*-ethylcarbodiimide hydrochloride (EDC, ≥97%), acetone (≥99.5% GC), 4-morpholineethanesulfonic acid (MES, ≥99%), TRIS (hydroxymethyl)aminomethane hydrochloride (TRIS HCl) and phosphate-buffered saline (PBS, tablets) were purchased from Sigma Aldrich. Ethanol absolute (EtOH, extra pure), hydrochloric acid (HCl, 37%) and sodium hydroxide (NaOH) were bought at Scharlab, SL. Oleic acid (OA, 65.0 - 88.0 %) was acquired from Honeywell Fluka and sodium chloride (NaCl, 99.0-100.5%) at PanReac AppliChem. Bacto™ Glycerol was bought from Becton Dickinson&Co (Sparks, MD, USA). Muller Hinton Broth (MHB) and Trypto Casein-Soy Broth (TSB) were obtained from Biokar Diagnostics. All reagents were used as acquired, without any further purification, and all solutions, unless otherwise indicated, were prepared with deionized Millipore miliQ water.

6.2.2 Instrumentation

Temperature controlled incubations and release studies were performed in a HC24 PCMT Thermo-shaker (Grant Instruments, Cambridge, UK). Magnetization studies were performed in a Vibrating Sample Magnetometer (VSM) Lakeshore 7304 (Lake Shore Cryotronics, Ohio, USA), with a maximum applied magnetic field of 1.45 T. HRFSEM (and EDS analysis) and SEM images were taken with a GeminiSEM 500 Fiel Emission Scanning Electron Microscope (ZEISS Oxford Instruments) and a FEI Helios NanoLab 450S DualBeam – FIB with UHREM FEG-SEM. TEM images and EDS analysis were acquired in a 200 kV JEM 2100F JEOL–GATAN Oxford Nanomegas, with X-Ray Detector (EDX) DIGISTAR/ADT 3D/ASTAR, from Universitat Politècnica de València (UPV). DLS experiments were performed in a Malvern Nano ZS Zetasizer (633 nm laser diode). UV–Vis absorption spectra were obtained in a Jasco V-650 spectrophotometer and fluorescence spectroscopy measurements taken in a HORIBA Scientific FLUOROMAX-4 spectrofluorometer, from the Bioscope Group LAQV- FCT NOVA, and a JASCO FP-8300 spectrophotometer, from UPV. Powder X-ray diffraction (PXRD) measurements were taken on a D8 Advance diffractometer (Bruker, Massachusetts, United States). Thermogravimetric analyses were carried out on a TGA/SDTA 851e balance (Mettler Toledo, Columbus, Ohio, USA) in an oxidizing atmosphere (air, 80 mL min⁻¹). Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was performed in an ICP Ultima model (Horiba Jobin-Yvon, France). N₂ adsorption-desorption isotherms were recorded in a Tristar II Plus automated analyzer (Micromeritics, Norcross, GA, USA). The samples were degassed at 90 or 120 °C under vacuum overnight. ATR-infrared spectra were collected in a Tensor 27 (Bruker, Massachusetts, United States), and ¹H / ¹³C nuclear magnetic

resonance spectroscopy (NMR) performed in a Bruker FT-NMR Avance 400 (Ettlingen, Germany) spectrometer.

Hyperthermia studies were conducted under an AC magnetic field, obtained with a water cooled two-spire Helmholtz coil, operated by an Ambrell EASYHeat 0224 power generator. Samples were placed in a thermostatic chamber with temperature controlled by the flow of heated air between the sample container and the cork-insulator that isolates the system from the coils. The whole ensemble is isolated from external air convection by an acrylic chamber.

Transparent flat-bottom sterile 96-well plates were purchased from Greiner Bio-One and U-bottom opaque black 96-well plates from RatioLab (Dreieich-Buchschlag, Germany). Aseptic bacterial assays and modifications were handled in a Steril-VBH laminar flux chamber. Turbidity for each assayed bacterial suspension was adjusted in a DEN-1B McFarland Densitometer (Grant-Bio). Incubations were conducted in a Mermmet Incubator B10, at 37 °C. Optical density (OD, 600 nm) measurements were conducted in a UV-Vis CLARIOstar® Plus spectrophotometer (BMG Labtech).

6.2.3 Superparamagnetic iron oxide nanoparticles synthesis – NANO1

The synthesis of well dispersed superparamagnetic iron oxide (Fe_3O_4) nanoparticles (NANO1) was achieved by a modification of the co-precipitation approach detailed by Zhang et al.⁴³. Briefly, 2.4 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 1.0 g of $\text{FeCl}_2 \cdot \text{H}_2\text{O}$ were stirred until total dissolution in 10 mL of miliQ H_2O , at 80 °C, under continuous Ar bubbling. 5 mL of NH_4OH (25 wt.%) were then added, and the resulting black solution stirred for an additional 1h, at 80 °C under continuous Argon (Ar) bubbling. After, 425 μL of oleic acid were injected into the mixture, which was bubbled for an additional 5 min and then sealed in Ar atm, to stir for 1.5h at 80 °C. The OA- coated NANO1 were magnetically decanted with the aid of a neodymium magnet, washed 10 times with miliQ H_2O to remove OA excess and transferred to 20 mL of chloroform. NANO1 concentration was estimated through the total content of Fe, determined by ICP-AES; the size of NANO1, determined by TEM; and its crystalline lattice structure⁴⁴, yielding *ca.* 6.8×10^{15} particles/mL (2.4×10^{17} particles/g).

6.2.4 Mesoporous silica shell surface growth and surface modifications – NANO2

In a typical approach, 150 mg of CTAB were dissolved in 10 mL of miliQ H₂O, mixed with 0.74 mL of NANO1 and sonicated for 1h, in an ultrasound bath at 35 KHz and 50 °C. The resulting emulsion was then heated to 70 °C and stirred for 30 min to evaporate the residual chloroform. Then, 30 mL of miliQ H₂O, 10 mL of ethylene glycol and 0.7 mL of NH₄OH (25 wt.%), were added, in this order, to the mixture which was stirred for at 70 °C. After 30 min of homogenization, 750 µL of TEOS were introduced dropwise to the reaction which was stirred for 3h at 70 °C. The as-prepared sample - NANO2 - was cooled to room temperature, centrifuged, washed in MeOH 3 times and left to dry at 70 °C.

For the modification of NANO2 surfaces, 50 mg of NANO2 were resuspended in 12 mL of miliQ H₂O and mixed with 1.2 mL of a THF solution containing 350 µL of APTES, for 24h at room temperature. The resulting NANO2-NH₂ were collected by centrifugation and washed with H₂O and acetone, before being resuspended in 5 mL of acetone and mixed with 3 mL of a 1.5 M succinic anhydride solution in acetone. The mix was stirred for another 24 h, at room temperature, and the samples collected by centrifugation and washed in MeOH. CTAB removal from the pores of the resulting NANO2-COOH samples was achieved by resuspension in 20 mL of 3 mg/mL methanolic solution of NH₄NO₃, for 1h at 60 °C (3 times).

6.2.5 Silicon quantum dots (SiQDs) synthesis

In an adaption of the synthesis detailed by Marcelo et al ⁴², green emissive silicon quantum dots (SiQDs) were prepared by dissolving 50 mg of sodium ascorbate in 10 mL miliQ H₂O and mixing with 2 mL of APTES. The reaction was initially stirred for 40 min at 50 °C and aged for 24h at room temperature.

6.2.6 Magneto-fluorescent nanoconjugates - NANO3

Magneto-fluorescent nanoconjugates (NANO3) were obtained by conjugation of SiQDs' amine terminal moieties and NANO2-COOH, via a two-step EDC/NHS crosslinking reaction in a 1:3:3 COOH:EDC:NHS molecular ratio. Briefly, 20 mg of NANO2-COOH were resuspended in 4 mL MES Buffer 0.1 M (pH 6.0), containing 19.2 mg EDC and 14.4 mg NHS, and stirred for 30 min at room temperature. To the resulting mixture, 4 mL of SiQD were added and stirred for 24h at room temperature, in dark. The SiQDs' basicity was brought to pH 8.0 before conjugation with the modified NANO2-COOH, to ensure optimal reaction

conditions. The resulting nanoparticles – NANO3 – were collected (10,000 rpm for 10 min) and washed with miliQ H₂O and MeOH.

6.2.7 Loading and Release studies of anticancer-antimicrobial drugs

For drug loaded nanoparticles, as-synthesised NANO2-COOH had first their pores emptied via NH₄NO₃ solution route, as detailed above. Ofloxacin (OFLO) and doxorubicin (DOX) were used as antimicrobial and anti-tumoral model drugs and were loaded either alone or conjugated in a 1:1 wt. ratio. A 2 mg/mL doxorubicin stock solution was prepared in miliQ H₂O and a 2 mg/mL ofloxacin stock solution in PBS 0.01 M (pH 7.0). Loadings were performed in batches of 20 mg of template-free NANO2-COOH. Briefly, 20 mg of NANO2 were resuspended in 3.2 mL of the corresponding drug stock solution and diluted in the same volume of the complementary solvent (Table S-6.1). Co-loading of OFLO and DOX was performed similarly with 20 mg of NANO2-COOH being resuspended in 3.2 mL of OFLO and 3.2 mL of DOX stock solutions.

Each sample was collected by centrifugation (10,000 rpm for 10 min) and washed 4 times with 1 mL of PBS 0.01M (pH 7.0). All supernatants were isolated and used to estimate, by mass balance, the loading capacity of NANO2-COOH for each drug, *via* UV-vis spectroscopy at 330 (OFLO) and 480 (DOX) nm. The loaded nanoparticles were then submitted to the same surface modifications described above, yielding NANO3@D, NANO3@O and NANO3@DO and their DOX and OFLO content assessed by the same procedure.

pH-dependent release studies were conducted in PBS 0.01M, with pH of 7.4 and pH 5.0, at 37 °C. Briefly, 1 mg of each loaded NANO3 was resuspended in 2 mL of buffer solution and shaken for up to 72h. After centrifugation (12,000 rpm for 5 min), the released drug content in the supernatant was measured for the 5, 15, 30, 45, 60, 90 min and 2, 3, 4, 24, 72 h time points, by fluorescence: OFLO at pH 7.4 (λ_{exc} = 300 nm, λ_{exc} = 460 nm), OFLO at pH 5.0 (λ_{exc} = 300 nm, λ_{exc} = 490 nm) and DOX (λ_{exc} = 480 nm, λ_{exc} = 560 nm). The drug release mechanism of each loaded NANO3 system was studied assuming the Korsmeyer-Peppas approach (Equation 6.1) ⁴⁵.

$$\frac{M_t}{M_\infty} = K_m t^n \quad (6.1)$$

Where, M_t and M_∞ stand for the released and initially loaded mass of drug, K_m for the kinetic constant and n the release exponent, from which the type of release is determined.

6.2.8 Magnetic characterization and hyperthermia studies

Magnetization hysteresis loops of NANO1, NANO2 and NANO3 were obtained by vibrating sample magnetometer (VSM), and mass saturation magnetizations and coercivity calculated from them accordingly. For that 24.4 mg, 9.5 mg and 13.7 mg of each dry sample, respectively, were submitted to a saturating magnetic field of 1 T.

Induction heating of the nanoparticles was achieved using the experimental set-up (Ambrell based) previously described^{26, 46}, for AC magnetic field with 14 kAm⁻¹ amplitude and 274 kHz frequency. NANO1 were prepared in chloroform to yield a final concentration of 5.1x10¹³ particles/mL, by dispersing 75 µL of the stock dispersion in a total volume of 3 mL, to equal the estimated number of cores in the tested amounts of NANO2 and NANO3 (4 mg). The two last were dispersed in 2 mL of water (2 mg/mL) prior to testing, sonicated till full homogeneity and immediately subjected to the alternating magnetic field (AMF). Each assay run for 1800s (30 min) and the dispersions temperature were registered continuously by an optical fiber probe (0.1 °C accuracy).

Specific loss power (SLP) was determined from the measured temperature increase for each sample, assuming a constant dissipated power H for the nanoparticles under ac magnetic field and linear losses to the thermostatic chamber⁴⁷. The SLP is normalized by the magnetic nanoparticles mass, m , according to equation 2, assuming no physical and chemical changes⁴⁸:

$$SLP = \frac{H}{m} \quad (6.2)$$

Since NANO2 and NANO3 comprise a non-magnetic layer, normalization was also done per mass of iron.

Magnetic-mediated release was performed alongside hyperthermia studies of drug-loaded NANO3 systems. Similarly, to the above described 4 mg of each drug-loaded NANO3 system were resuspended in 2 mL (2 mg/mL), sonicated, heated to *ca.* 37 °C and placed under an AMF with the same specifications, for 1800 seconds (30 min), with the thermostatic environment at 36.5 ± 0.5 °C. Each sample was recovered by centrifugation at 10.000 rpm, for 5 min, and their supernatants quantified. Both pellet and supernatants were stored and used in further antimicrobial assays.

6.2.9 Antimicrobial activity assessment

The antimicrobial activity of the synthesised nanoparticles was assayed against gram-negative *Escherichia coli* (ATCC[®] 8739[™]) and *Pseudomonas aeruginosa* (ATCC[®] 9027[™]) as well as gram-positive *Staphylococcus aureus* (ATCC[®] 6538[™]), methicillin resistant

Staphylococcus aureus (ATCC[®] 33591[™], MRSA) and *Enterococcus faecalis* (ATCC[®] 29212[™]). Bacteria were kept frozen at – 70 °C in broth containing glycerol (15% v/v). 2.5 mg/mL suspensions of all selected nanomaterials (NANO3@D, NANO3@O, NANO3@DO) were prepared in H₂O and used for all assays. Drug solutions containing the same concentration of the drugs loaded into each material were also prepared, in H₂O. Bacterial suspensions for all strains were prepared from the subcultured TSA plates, in 3 mL of sterile 0.85% NaCl solution. The turbidity of the suspensions was adjusted to 0.5 on the McFarland scale (*ca.* 10⁸ CFU/mL) and diluted with 0.85% NaCl to about 10⁷ CFU/mL.

To evaluate and quantify the bactericidal and bacteriostatic effects of the tested nanocomposites, all samples were assayed by the broth microdilution method in a 96-well microplate. Briefly, two-fold serial dilutions of each sample, and controls, were prepared in sterile MHB to a final volume of 100 µL per well. Then, each well was inoculated with 10 µL of each previously prepared bacterial suspension (to achieve a concentration of 10⁶ CFU/mL in each well). Drug solutions containing the same concentration of the drugs loaded into each material were used as positive controls. Each material in the absence of bacteria and the bacteria without samples, were used as negative controls for the experiments.

Plates were left to incubate for 18 hours, at 37°C. After the appointed time, an aliquot of each well was subcultured on the surface of TSA plates and incubated for an additional 18 hours, at 37 °C, for a naked-eye assessment of the MBC. Magnetic hyperthermia samples were used as above-mentioned, with both pellets and supernatants being mixed and homogenised before being applied to bacteria in the antimicrobial assay.

OD₆₀₀ measurements of the 96-well plates were conducted in a plate reader at 600 nm. Bacterial growth and MIC₅₀ were calculated from OD₆₀₀ (Equation 6.3).

$$\text{Bacterial Growth} = \frac{\text{Sample OD} - \text{NANO OD}}{\text{Bacteria control OD}} \quad (6.3)$$

Where, *Sample OD* and *NANO OD* stand for the incubated sample OD₆₀₀ signal and the correspondent NANOs suspension OD₆₀₀ control signal.

6.3 Results and Discussion

6.3.1 Characterization of NANO2 and NANO3 systems

Magneto-fluorescent mesoporous nanoparticles were successfully obtained in a step-by-step synthetic approach, from NANO1 to NANO3, using iron oxide, mesoporous silica, and silicon as main build-block reagents (Figure 6.1).

The initial production of the NANO1 magnetic Fe_3O_4 cores followed a typical co-precipitation method of Fe^{2+} and Fe^{3+} salts, in the presence of oleic acid as surface stabilizer and modulator⁴³. NANO1 were obtained as homogeneous and small size spherical nanoparticles (Figure S-6.1 with an average diameter of 11.73 ± 1.47 nm, typical of ultra-small superparamagnetic nanoparticles (SPIONs)³⁶. The superparamagnetism of NANO1 arises from its small dimensions that ensure the existence of a single reverse spinel phase iron oxide crystalline structure and thus a homogeneous and fast response to an external magnetic field⁴⁹. This was confirmed even after the grafting of the mesoporous silica shell, by XRD and TEM analysis of NANO2, that showed not only a typical SiO_2 XRD pattern and Fe_3O_4 2 θ peaks at 30.26° , 35.60° , 43.36° , 53.72° , 57.16° and 62.65° (hkl planes: $\langle 220 \rangle$, $\langle 311 \rangle$, $\langle 400 \rangle$, $\langle 422 \rangle$, $\langle 511 \rangle$ and $\langle 400 \rangle$, respectively)⁵⁰, but also single oriented core crystalline structures, with calculated interplanar distances matching those obtained by XRD (Figure S-6.2).

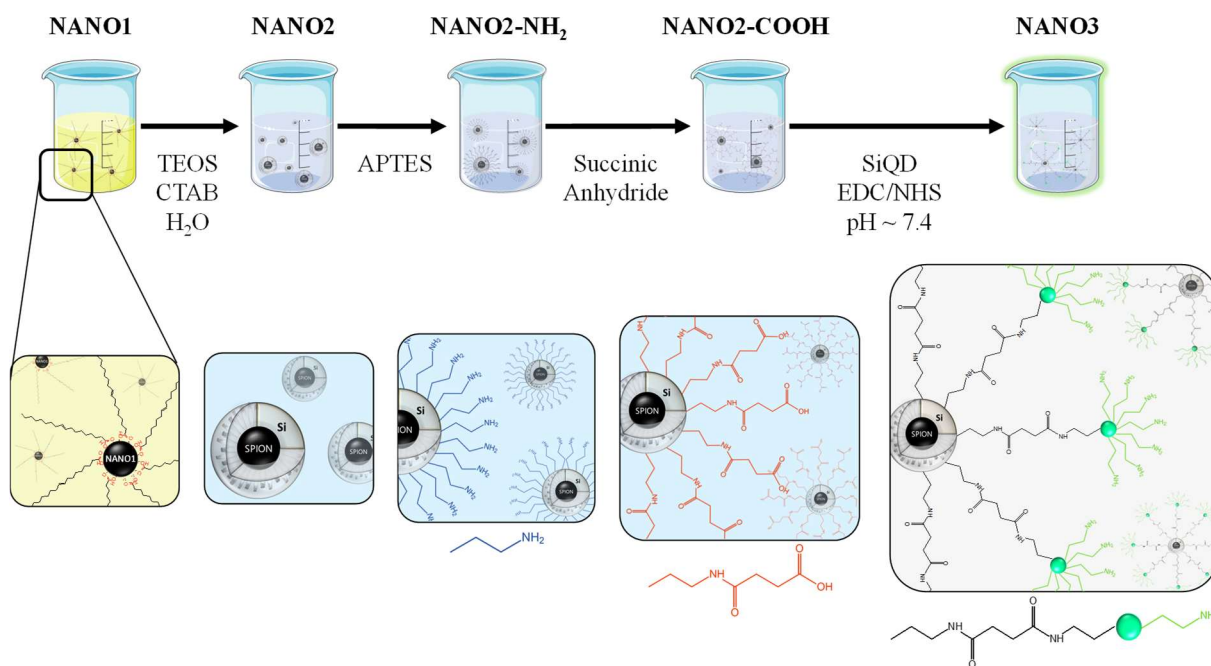


Figure 6.1 - Sequential representation of the step-by-step synthesis of NANO3, from the formation NANO1, and dispersion in CHCl_3 , to the growth of a mesoporous silica shell (NANO2) in water, further surface modifications (NANO2- NH_2 and NANO2- COOH) and SiQD anchoring via amidation. Representations not at real scale.

The growth of the mesoporous silica shell onto NANO1's surface was obtained by an initial surface co-stabilization with oleic acid and CTAB, where the alkyl chains of OA and CTA^+ are interdigitated. The shifting of NANO1 to the aqueous phase is then followed by the well-established Stöber method for the synthesis of MCM-41. Here, TEOS was used as silica source, ethylene glycol as surface stabilizer and NH_4OH as reducing and morphological agent. The mesoporosity of the matrix is ensured by the presence of CTAB, that works as a

cationic templating surfactant. NANO2 were obtained in the form of a brown, magnetic powder and in a concentration of *ca.* 2.0×10^{13} nanoparticles/mg. Further surface modifications, first with APTES and then with succinic anhydride to yield NANO2-COOH, and with SiQDs (via EDC/NHS cross-linking) to yield NANO3, were continuously followed by DLS measurements of their hydrodynamic diameter (HD) and zeta potential (ζ) in water (Figure S-6.3). The sequential surface modifications are complemented by appropriate changes in surface charge, with bare NANO2 having an initial net negative charge (-27.7 ± 0.4 mV) typical of non-templated mesoporous silicas⁵¹, which changes into $+50.8 \pm 0.7$ mV, after the grafting of APTES and its $-NH_2$ terminal groups, and later reverts to -39.2 ± 1.3 mV with the addition of succinic anhydride, that undergoes a ring opening amidation reaction to form terminal succinamic acid moieties. The successful addition of SiQDs to NANO2-COOH, by EDC/NHS mediated amidation with amine surface groups of the former, is translated by an inversion of ζ to $+12.4 \pm 1.3$ mV in NANO3. All registered changes in ζ influence the final HD of each NANO system, with NANO2- NH_2 and NANO2-COOH highest surface charges corresponding to the smallest HDs (203.3 ± 2.9 nm and 115.5 ± 4.7 nm, respectively) and, thus, increased dispersibility and stability in water. The addition of the nanometric SiQDs to its surface, as pointed by NANO3's ζ , destabilizes the system and results in a large HD of 1076.0 ± 159.9 nm. This is consequence of the hair-like distribution of the surface chains of NANO3, whose fluctuations make it non-homogeneous overtime, and the weak surface charge that arises from the low protonation of surface $-NH_2$ moieties from the anchored SiQDs (pK_a immobilized-APTES = 7.6^{52, 53}) in the aqueous medium (pH = 6.8). The overall positive charge of the final NANO3 poses as a potential asset in antimicrobial applications, as recent studies have pointed positively charged particles as the most effective against bacterial strains^{54, 55}. SiQD concentration during the cross-linking reaction was adjusted to ensure NANO2-COOH surface saturation and avoid interparticle cross-linkage.

Surface modifications were also confirmed by FTIR spectroscopy, with carboxylic acid (strong-broad O-H stretch, 3000 cm^{-1} ; strong C=O stretch, 1715 cm^{-1} ; medium O-H bend, 1400 cm^{-1} ; strong C-O stretch, 1320 cm^{-1}) and secondary amide peaks (weak C=O stretch, 1635 cm^{-1} ; medium N-H bend, 1545 cm^{-1}) emerging in NANO2-COOH spectrum, and later turning into solely secondary amide (weak C=O stretch, 1635 cm^{-1} ; medium N-H bend, 1545 cm^{-1}) and primary amine (weak-broad N-H stretch, 3350 cm^{-1}) signals in NANO3 (Figure S-6.4). Moreover, successful template removal is translated by the absence of the typical strong C-H stretching between 2900 and 3000 cm^{-1} , in NANO2 spectrum. Conversely, the increase of

the same signals in NANO2-COOH and NANO3 confirm the presence of the aliphatic linking chains at their surfaces.

The presence of the very same aliphatic chains is evident in TGA, with NANO2-COOH and NANO3 having weight losses of ca. 20% and 30%, respectively (Figure 6.2.a), where the 10% difference between them corresponds to the 3-aminopropyl groups at the surface of SiQDs. Again, this confirms the successful functionalization of NANO2 surface towards NANO3. The mesoporosity of the systems, before and after surface modifications, was verified by the N₂ adsorption–desorption type IV isotherms of NANO2 and NANO3, both showing a typical, accentuated adsorption step gradient at P/P₀ of ca. 0.35–0.40 (Figure 6.2.b). Surface area, porous volume and pore diameter were assessed by applying Brunauer Emmett and Teller (BET) and Barrett-Joyner-Halenda (BJH) methods, with NANO2 having 912.43 m²/g, 0.862 cm³/g and 30.1 Å, and NANO3 having 580.33 m²/g, 0.443 cm³/g and 23.8 Å, respectively (Figure 6.2.c-d). The decrease in porosity and pore diameter is indicative of an efficient surface functionalization, where the aliphatic chains and SiQDs partially cap the pores of the silica mesoporous matrix. Notwithstanding, the high porosity of the systems

confirms their availability as cargo delivery vectors, permitting the loading of drugs of interest.

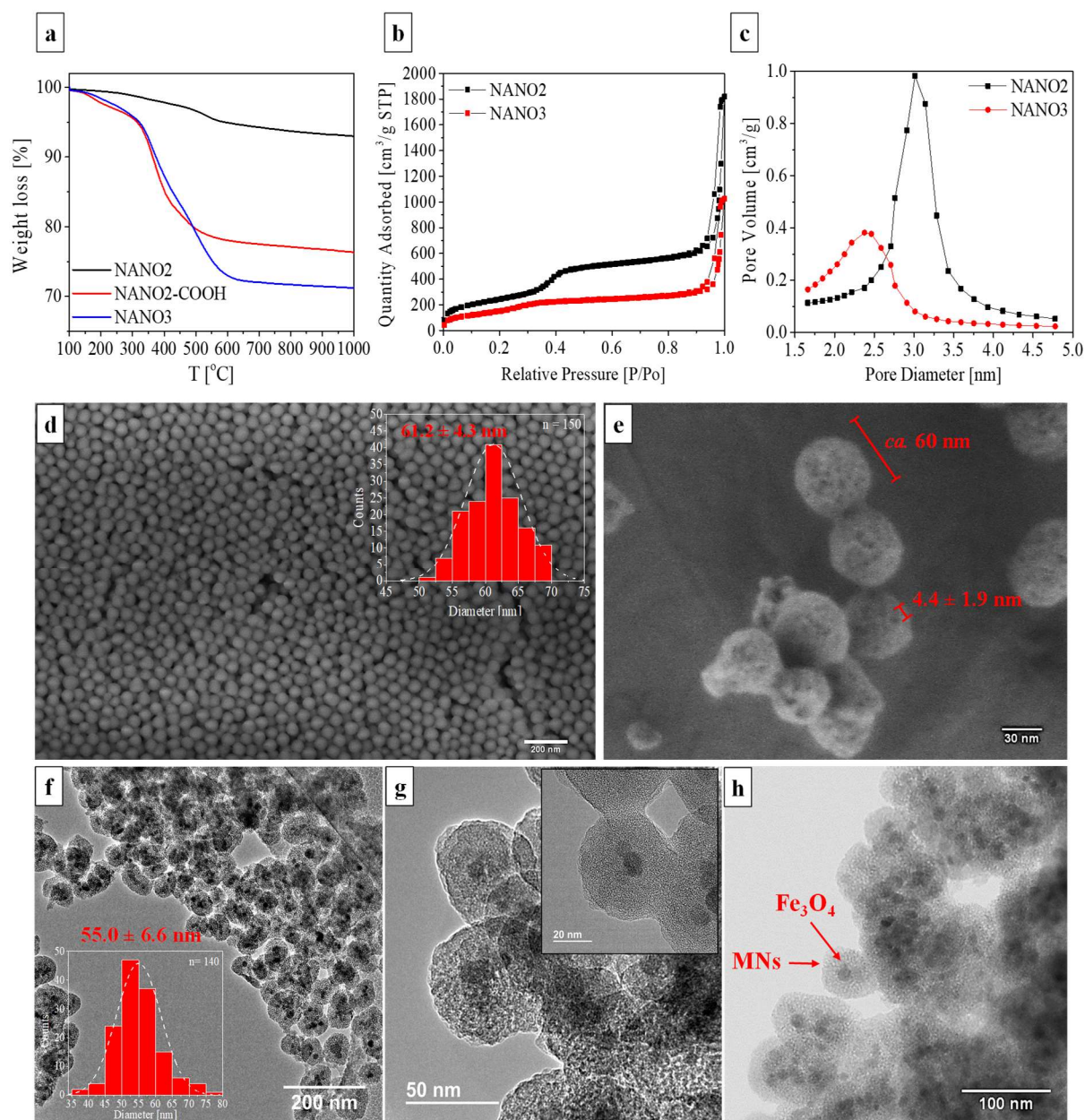


Figure 6.2 - TGA weight loss curves for NANO2, NANO2-COOH and NANO3 ($m_i = 5.0$ mg) (a). N₂-isotherms (b) and pore size distribution (c) of NANO2 and NANO3. SEM micrograph of NANO2-COOH, *inset*: particle size distribution (d); and HRFESEM picture of NANO3, with distinguishable spherical shape and porosity (e). TEM images of NANO3, *inset*: particle size distribution (f, g); and brightfield STEM image of NANO3 (h).

The morphology and size of NANO2-COOH and NANO3 was confirmed by SEM and TEM analysis. Both systems have an unquestionable spherical shape, as depicted in Figure 6.2.d and Figure 6.2.e, and an average size of about 60 nm. Spherical particles and sizes between 1 – 100 nm have been repeatedly reported to be responsible for longer circulation times, higher internalization, stability, and interaction with cellular walls, all necessary for biological applications and an effective drug delivery⁵⁶, specifically to target in-body, tumor-

associated bacterial infections. Moreover, the porosity of the system is also evidently depicted in the HRFSEM picture of NANO3 (Figure 6.2.e).

From TEM (Figure 6.2.f-g) and brightfield STEM (Figure 6.2.h) images, and as hinted by the above characterization techniques, it can be concluded that the herein synthesized systems are mainly comprised of single iron oxide cores, successfully functionalized with a silica mesoporous matrix of well-defined limits. Due to their small size and similar nature to the silica mesoporous layer, no SiQDs can be directly distinguished, being only perceived as the loss of the smooth surface of the particles in detriment of an embossed surface with blob-like structures of ca. 3.6 nm (Figure 6.2.g) ⁴². Elemental analysis on NANO3 was assessed via dark-field STEM analysis, that confirmed Si, O, N and Fe as main constituents (Figure S-6.5), with iron atoms being confined to the core of the particles and the remaining elements to the outer silica layer.

Beyond their physiochemical properties, and as NANO3 synthesis sought to produce hybrid magneto-fluorescent materials, the assessment of their spectral properties (i.e., fluorescence and magnetism) is also of utter importance. As-synthesized aqueous SiQDs, as previously reported ⁴², due to their incorporation of N and O atoms have a typical green emission, with a maximum emission band centered at 530 nm (Figure 6.3.a). The anchoring of SiQDs on the surface of NANO2-COOH render the final NANO3 with a similar, although slightly blue shifted, bluish-green emission, with an emission maximum centered at 515 nm (Figure 6.3.b). This blue shift, as hinted in previous reports ^{36, 57}, arises from the change in SiQD surface groups, as part of the original -NH₂ moieties changed into amide >NH groups during the cross-linking with NANO2-COOH, being responsible for its bluish tonality. Additionally, this also indicates that the final fluorescence is not affected by the presence of a magnetic core, with the spacing materials (i.e., silica shells and aliphatic chains) effectively protecting the former from the latter.

The retainment of the original NANO1 paramagnetic properties through all synthetic steps towards NANO3, was also confirmed by the VSM analysis of magnetization loops of NANO1, NANO2, NANO3 (Figure 6.3.c). While bare NANO1 (in chloroform) showed a high saturation magnetization of 56.8 emu/g – typical of small SPIONs ⁵⁸ –, for NANO2 and NANO3 the increase in their non-magnetic particulate masses was accompanied by a significant decrease in saturation magnetization to 9.8 and 6.2 emu/g, respectively. The lack of significant hysteresis in the magnetization loops of all particles corroborates the above statements on the single domain crystallinity of NANO1 lattice. Moreover, the propagation of this pattern to NANO2 and NANO3 show that, despite all modifications, both the core lattice single domain and its capacity to instantly orient and magnetize itself to an external field

remain unchanged. This is also pointed out by their near zero coercivity, with only NANO1 having a small coercivity of 3 Oe. The decrease in both magnetization and coercivity have been attributed to surface spin effects and to changes in the anisotropy constant after modifications⁵⁹.

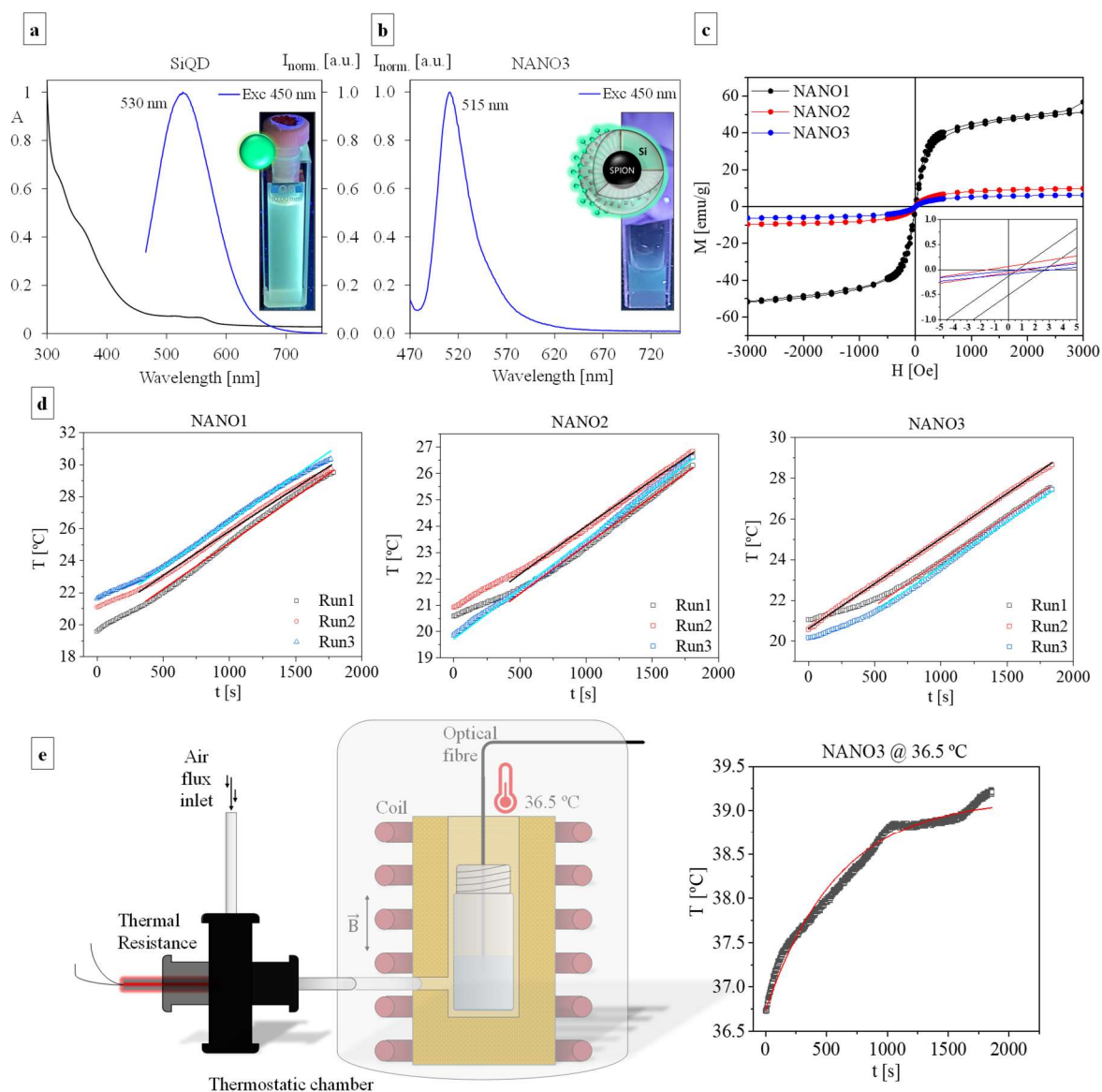


Figure 6.3 - Absorbance and fluorescence spectra of SiQD, after excitation at 450 nm; inset: in-solution picture of SiQD dispersion under 365 nm irradiation (a). Fluorescence spectra of as-synthesized NANO3; *inset*: in-solution NANO3 under 365 nm irradiation (b). VSM magnetization loops of NANO1, NANO2 and NANO3 vs applied field at 273.15 K; *inset*: close-up of the same charts for hysteresis visualization (c). Hyperthermia (heat generation) capacity of NANO1, NANO2 and NANO3 in water, under a 274 kHz and 14 kA/m external AMF, for 1800 seconds, (environment at 19 °C) (d). Controlled temperature hyperthermia set-up and NANO3 hyperthermia heating profile with environment at 36.5 °C (e).

These results reflect themselves in the hyperthermia profiles of all NANO1, NANO2 and NANO3, with all three being capable of efficiently generate heat and induce temperature

risers of *ca.* 10 °C when submitted to a 274 kHz AMF and 2280 W, for 1800 s (Figure 6.3.d). The overall heating for each sample shows a linear relationship with time and corresponds to approximately similar power losses of 0.0367, 0.0588 and 0.0503 W for NANO1, NANO2 and NANO3, that were used, along with their masses, to estimate each sample SLP⁴⁸. NANO2 (4.0 mg) and NANO3 (4.0 mg) showed SLPs of 11 ± 1 and 13 ± 3 W/g_{NANO}, respectively, that have no significant since a large fraction of their mass comes from their non-magnetic silica shells. The magnetic cores in these samples are similar to the ones in sample NANO1 (0.22 mg) and are characterized by an SLP of 170 ± 17 W/g_{NANO} (235 ± 24 W/g_{Fe}), typical of small SPIONs with diameters between 11 – 14 nm and suitable for hyperthermia applications^{59, 60}. This result, allied to the lack of hysteresis losses and a particle size of about 12 nm, indicates that NANO1 (cores) heating arises from Neel and Brownian relaxations, with a predominance of the former^{61, 62}. NANO2 and NANO3 can also have an increased contribution of Brownian relaxation due to the enlargement of the coated nanoparticles. Normalizing the SLPs values for NANO2 and NANO3 by the mass of iron, the values obtained are of 270 ± 35 and 295 ± 47 W/g_{Fe}, respectively, in agreement with the value of NANO1. Thus, defending the hypothesis that both these systems can also be used for hyperthermia. To mimic an *in-body* application, NANO3 hyperthermia heating capacity was tested under a controlled temperature environment of 36.5 ± 1.0 °C (Figure 6.3.e). The assay yielded similar results to those at room temperature, with the system having the capacity to increase the medium temperature up to 39.5 °C and having an overall power loss of 0.0577 W corresponding to a SLP value of 14 ± 2 W/g_{NANO} (339 ± 52 W/g_{Fe}). The maximum reached temperature, although below that usually used in therapeutic hyperthermia approaches, has been demonstrated to be sufficient to not only induce mild cell wall disturbances, biofilm detachment and immune system stimulation (inducing febrile temperatures)⁶³, but also increased drug delivery, without their own deactivation⁶⁴⁻⁶⁶.

With these results, we can state that we were able, for the first time, to successfully synthesize hybrid magneto-fluorescent nanoparticles for drug delivery, comprising sustainable, biocompatible and biodegradable materials as iron oxide for the magnetic core, silicon for the fluorescent SiQD probes and silica for the mesoporous matrix.

6.3.2 Loading, pH-mediated release and hyperthermia release assays

To render the synthesized nanocarriers with antimicrobial properties, single and loaded combinatory formulations of OFLO and DOX were physically adsorbed into NANO2-COOH empty mesoporous matrix, in PBS 0.01 M (pH 7.4). The efficient pore loading was confirmed by DLS, with NANO2@D, NANO2@O and NANO2@DO showing more negative ζ s

(-33.0 ± 0.7 mV, -35.9 ± 0.4 mV, -35.6 ± 2.7 mV respectively), pointing towards a negligible binding interaction with surface carboxyl groups and a preference for the empty pores. This also translated into smaller HD (126.1 ± 8.8 nm, 121.8 ± 1.4 nm and 148.7 ± 22.0 nm, respectively) and a higher stability (PDI of 0.155 ± 0.013 , 0.207 ± 0.004 and 0.275 ± 0.033 , respectively), with the slight positive drugs within the pores inducing a higher polarisation of the surface.

The drug content of the loaded systems was evaluated at each surface modification step, rendering the final NANO3@D with 98.4 ± 7.7 $\mu\text{g}_{\text{DOX}}/\text{mg}_{\text{NANO3}}$, NANO3@DO with 111.3 ± 3.0 $\mu\text{g}_{\text{DOX}}/\text{mg}_{\text{NANO3}}$ and 69.7 ± 9.3 $\mu\text{g}_{\text{OFLO}}/\text{mg}_{\text{NANO3}}$, and NANO3@O with 94.9 ± 6.7 $\mu\text{g}_{\text{OFLO}}/\text{mg}_{\text{NANO3}}$ (Figure 6.4). The high encapsulation capacities are in accordance with those reported in other studies for similar mesoporous systems⁶⁷, with OFLO successfully replicating the results obtained in other works of our group^{28, 33}. These own it to the correct selection of loading media's pH (pH7.4) during NANO2-COOH loading, with both DOX ($\text{pK}_{\text{a-NH3}^+} = 8.2$, $\text{pK}_{\text{a-phenol}} = 9.5$) and OFLO ($\text{pK}_{\text{a-COOH}} = 6.1$, $\text{pK}_{\text{a(piperiziny ring)}} = 8.2$) being partly, positively charged at physiological pH (Figure 4.a)⁶⁸⁻⁷² and having preferential, however not permanent, attractive electrostatic interaction with NANO2-COOH, whose surface carboxylic groups and pore silanol groups render it a net negative charge. Regarding the combinatory drug formulation, the relative reduced DOX solubility in the buffered media contributes to the preferential loading of DOX within the pores of NANO systems (Figure 6.4.b), in detriment of OFLO encapsulation⁷³.

Interestingly, the fluorescent profiles of the loaded NANO3 systems (Figure S-6.6) also reflect the incorporation of both OFLO and DOX, by having their respective 450 nm and 590 nm emission peaks alongside that of grafted SiQDs ($\lambda = 515$ nm), after excitation at 330 nm, 450 nm and 480 nm.

Release assays were performed over the final NANO3 loaded systems, in both physiological (pH 7.4) and acidic (pH 5.0) conditions (Figure 6.4.c and 6.4.d) for 72 h, to simulate a pH-responsive drug delivery of each drug and facilitate the understanding of its drug efficiency enhancement and toxic side effect inhibitions in a future dual application, when targeting cancer and associated opportunistic bacterial infections. The DOX delivery capacity, both from NANO3@D and NANO3@DO, is higher at pH 5.0 (i.e., of 14.2 % wt. and 19.7 % wt., respectively) than at pH 7.4 (i.e., of *ca.* 5.0 % wt. and *ca.* 3.0 % wt., respectively), which is in accordance with the already reported tendency for aminated DOX-containing delivery nanocarriers^{34, 42}, as is the case of all NANO3 systems. Here, there is a compromise between DOX solubility at different pHs and the surface steric hinderance created by the

aminated SiQD, with acidic pHs not only increasing DOX solubility in aqueous media, but also the protonation of surface NH_2 groups and the creation an electrostatic repulsion between surface SiQDs. A spectroscopic analysis of SiQDs in both pHs confirm this protonation phenomena, with SiQD shifting their emission from green to blue when lowering medium pH from 12 to 4.0 (Figure S-6.7). This last phenomenon leaves the pores open enough to release entrapped DOX molecules, even if slowly due to the partly positively charged DOX ⁷⁴. It should also be noted that at pH 5.0, DOX delivery from NANO3@DO is more time-paced, contrary to the initial burst release from NANO3@D at the same pH, which might be due to the simultaneous presence of positive charged OFLO molecules.

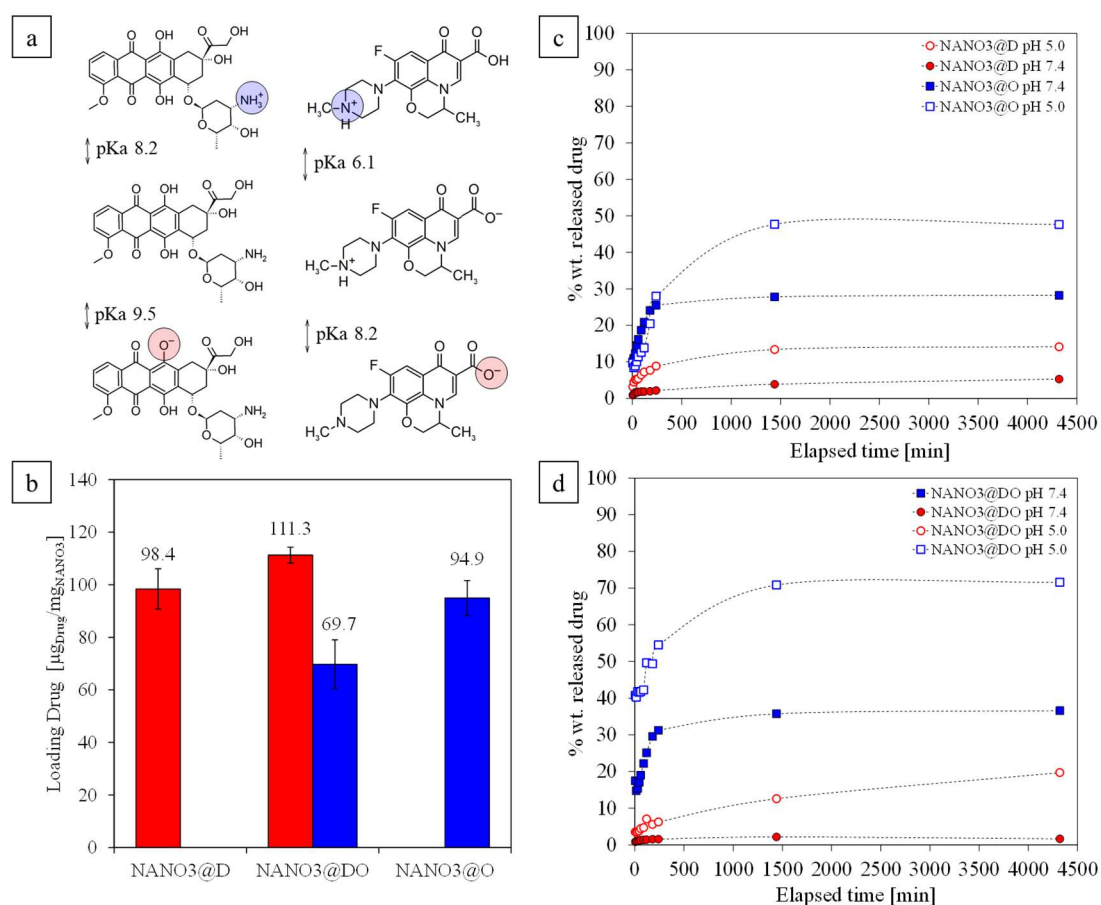


Figure 6.4 - . Ionization of DOX and OFLO in aqueous solutions (a); NANO3 systems loading capacities of DOX and OFLO (n=5), in single and dual combinatory formulations (b); DOX (*circles*) and OFLO (*squares*) release profiles from NANO3@D and NANO3@O (c), as well from NANO3@DO (d), under physiological and acidic conditions (n=3).

Similarly, OFLO delivery has also shown to be highly dependent on pH variations, nearly doubling its maximum release from *ca.* 30% wt. to 47.6% wt. and 71.6% wt. for NANO3@O and NANO3@DO, respectively, when decreasing the pH from 7.4 and 5.0. The increased release of OFLO from NANO3@DO at pH 5.0, when compared to that of NANO3@O, is accompanied by the highest delivery of DOX. Again, this suggests that when

in a combinatory formulation, both positively charged OFLO and DOX catalyse each other's delivery, through their increased electrostatic repulsive interaction within NANO3 systems' pores.

From a mechanistic point of view, by fitting the collected data to the semi-empirical Korsmeyer-Peppas model, and its further linearization (Equation 6.4) for the determination of both n and K_m parameters, we can conclude that for all loaded NANO3 delivery is ruled by quasi-Fickian diffusion mechanisms (Table S-6.2). This is given by an $n < 0.43$, for spherical particles, which indicates there is no interference from matrix swelling or erosion phenomena in the diffusion of drugs ⁴⁵.

$$\ln \frac{M_t}{M_\infty} = \ln K_m + n \cdot \ln t \quad (6.4)$$

Briefly, this preference for acidic media makes NANO3 optimal to actuate in cancer environments where pH is generally lower, releasing both anticancer and antibiotic in higher quantities and thus addressing both cancer growth and opportunistic bacterial infections.

The incorporation of the magnetic NANO1 core within the structure of NANO3 renders it with the capacity to not only generate heat under an alternating external magnetic field through synchronous vibration, but also, as previously reported, increase drug delivery and therapeutics performance ^{75, 76}. Thus, the use of NANO3 systems' magnetic core to promote a hyperthermia-mediated delivery was also studied for each drug formulation. For this, the resuspended NANO3@D, NANO3@O and NANO3@DO, in a concentration of 2 mg/mL, were submitted to an external AMF of 274 kHz and 2880 W, for 1800 s (30 min) at 37 °C. The analysis of the obtained hyperthermia heating profiles shows that all can heat the media to final temperatures of 39.0 °C (NANO3@O and NANO3@DO) and 40 °C (NANO3@D) (Figure 6.5.a-c). Moreover, while DOX encapsulation leads to no changes in NANO3@D hyperthermia capacity, with a SLP of 14 W/g_{NANO} (322 W/g_{Fe}) similar to that of empty NANO3, OFLO presence in NANO3@O and NANO3@DO produces higher SLPs of 21 and 24 W/g_{NANO} (506 and 557 W/g_{Fe}). This increase for OFLO-containing formulations is due to the observable increased viscosity of the suspensions with OFLO, which is directly related with Brownian relaxation times and, thus, with increases in SLPs ⁵⁹.

Regarding the release of the encapsulated drugs, the obtained released amounts were compared with those obtained from the releases in PBS at pH 7.4 and pH 5.0, for the same time point (i.e., 30 min). As depicted in Figure 6.5.d the use of hyperthermia as stimuli significantly boosts the release of OFLO and DOX, when compared to the release amounts of each at pH 7.4 and 5.0. This is evident for NANO3@O, where the release of OFLO under AMF

influence is 3 times more than at both tested pHs; for NANO3@D, where the release of DOX is ca. 3.5 times more than at physiologic pH; and lastly, for NANO3@DO, from which the release of OFLO and DOX under AMF is 5% more than that at pH 7.4 and 3 times more than any of tested pH, respectively. Remarkably, the release profiles after this short time are comparable to those obtained after 3 to 4 h of incubation at pH release, and thus supports the idea that AMF employment boost drugs release by inducing extra particulate vibrations, local heat and higher permeability^{77, 78}. Like in pH release assays, for AMF hyperthermia assays DOX release is higher when in a dual formulation with OFLO, again supporting the idea that both catalyse each other's delivery, through their increased electrostatic repulsive interactions within NANO3 systems' pores.

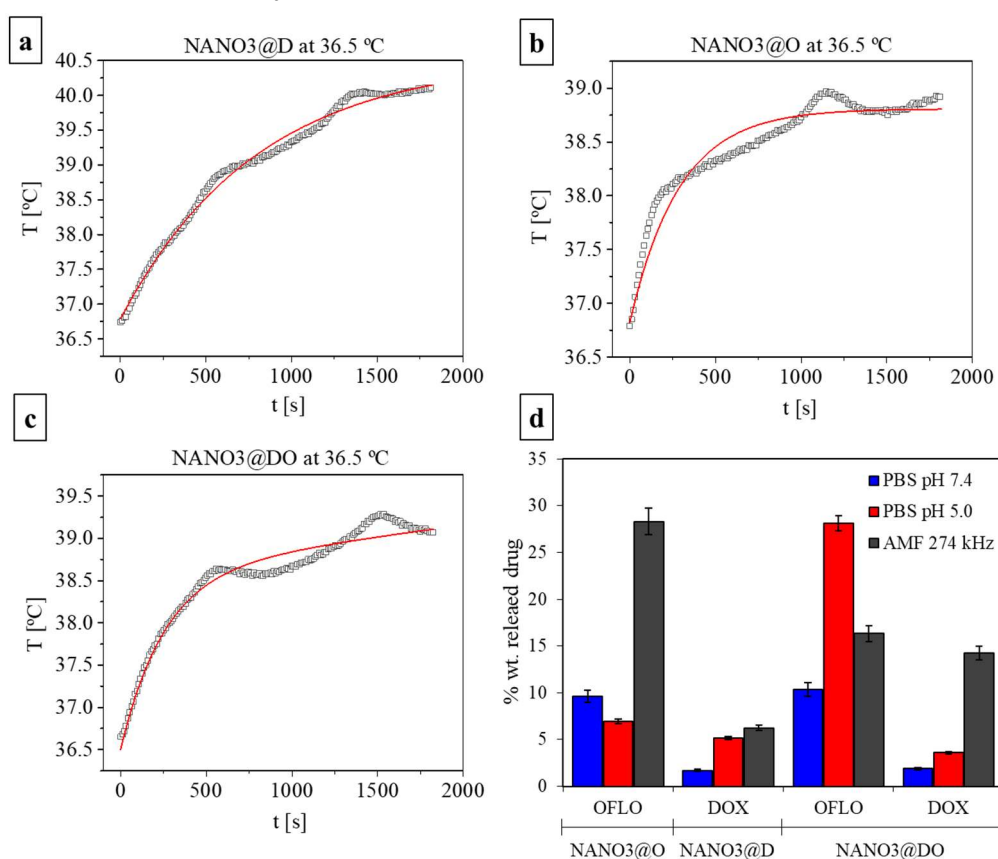


Figure 6.5 - Hyperthermia heating profiles of NANO3@D (a), NANO3@O (b) and NANO3@DO (c), at 37 °C, under an AMF at 274 kHz and 2880 W, for 1800 s (30 min). Maximum released drug from NANO3@D, NANO3@O and NANO3@DO, at 37 °C, under different conditions (n=3): blue = 0.01 M PBS pH 7.4, red = 0.01 M PBS pH 5.0 and grey = AMF at 274 kHz (d)

In general, the cumulative OFLO release from either single or dual combinatory formulations is more intense than that of DOX, regardless the selected pH condition or if under the influence of an external AMF. From the obtained results it can be concluded that in a possible combinatory treatment, there is an initial burst release of OFLO into the medium,

regardless of the pH, killing most local opportunistic bacteria, followed by a paced and controlled release of DOX to treat the associated cancer cells.

6.3.3 Antimicrobial activity

The potential use of the produced nanoparticles as antimicrobial materials against cancer-related, opportunistic bacterial strains was finally tested for a battery of five standard strains. *E. coli*⁷⁹, *S. aureus*⁸⁰, MRSA⁸¹, *P. aeruginosa*⁸² and *E. faecalis*⁸³ were selected due to their identified relationship, and locality, to some types of tumors, as well as their known response to the selected drugs²⁸. With the assay we aimed to determine of the minimum inhibitory concentration of NANO3, NANO3@O, NANO3@D and NANO3@DO, given as the lowest concentrations at which bacterial growth was $\leq 50\%$ (MIC₅₀) and $\leq 10\%$ (MIC₉₀)⁸⁴. All samples were resuspended in water and diluted in growth medium.

As is perceived in Figure 6.6.a, for the direct application of suspensions, the inhibitory activity of the NANO3 systems against most strains arises from the loaded drugs, specifically ofloxacin. This is exempted only by *E. faecalis* where there is significant inhibitory activity from the nanoparticles at the higher concentrations, with only NANO3@O having a slightly higher activity than NANO3. Similar to other reported assessments^{27, 28}, *E. coli* is the most susceptible strain to this combination of drugs and, whereas a distinguishable MIC₅₀ and MIC₉₀ was registered for NANO3@O, at 170.1 and 375.1 $\mu\text{g/mL}$, for NANO3@DO a stronger inhibitory activity is perceived as the overlap of both MIC₅₀ and MIC₉₀ at 81 $\mu\text{g/mL}$. Their action against *S. aureus*, MRSA and *P. aeruginosa* is, however, reduced when compared with that seen for *E. coli*. For *S. aureus*, NANO3@O and NANO3@DO only show, respectively, MIC₅₀ and MIC₉₀ at the highest concentration of 357.1 $\mu\text{g/mL}$. Conversely, for MRSA only NANO3@O keeps its MIC₅₀ at 357.1 $\mu\text{g/mL}$, with NANO3@DO no longer having the same activity and only being capable of reducing bacterial growth in 30%. Lastly, against *P. aeruginosa* both NANO3@O and NANO3@DO show a MIC₅₀ at the highest concentration 357.1 $\mu\text{g/mL}$. It should noted, that this small activity against *P. aeruginosa* might rise from an increased biofilm production due to the presence of Fe species from the core of NANO3 systems, as all samples had a strong green coloration and high viscosity after the assay.

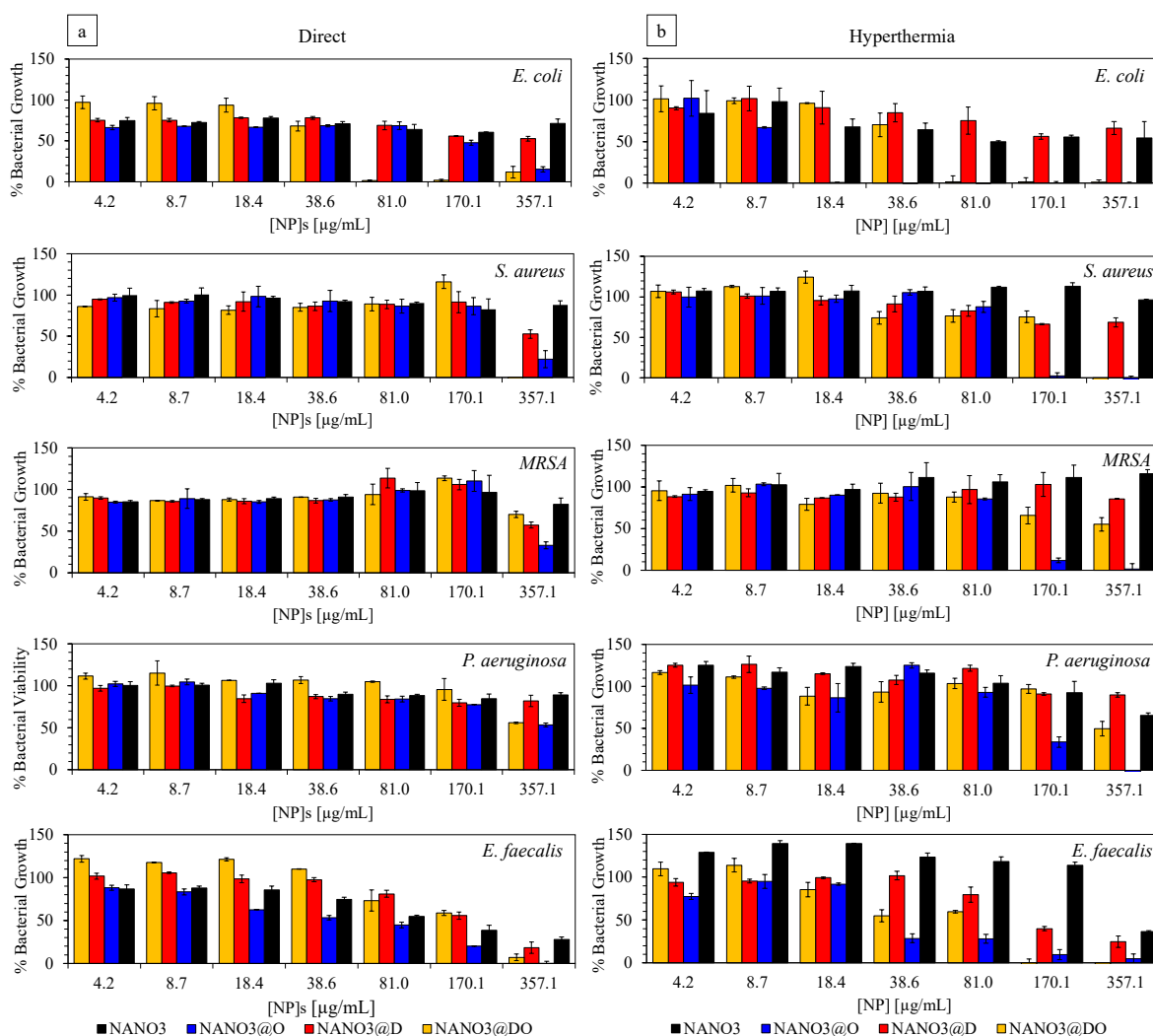


Figure 6.6 - Inhibitory activity, as bacterial growth (%), of NANO3, NANO3@O, NANO3@D and NANO3@DO against (from top to bottom) *E. coli*, *S. aureus*, MRSA, *P. aeruginosa* and *E. faecalis* strains, by direct application (a) and after-hyperthermia (AMF of 274 kHz) application (b). Concentrations given as μg of nanoparticle per mL.

The use of hyperthermia before the application of nanoparticles has been confirmed to increase the release of the loaded drugs, and this is also reflected in the antimicrobial activity of the NANO3 systems (Figure 6.6.b). In *E. coli*, the determined MIC₅₀ and MIC₉₀ of NANO3@O are reduced to a particle concentration of 18.4 $\mu\text{g/mL}$. This approximately 90% reduction in the inhibitory concentration is supported by the higher OFLO release after the application of AMF and thus higher availability in the medium. The use of an AMF also benefits the antimicrobial activity of NANO3@O and NANO3@DO against *S. aureus* and MRSA, reducing the first's MIC₉₀ to 170.1 $\mu\text{g/mL}$, for both bacteria, and the second's MIC₅₀ to 38.6 $\mu\text{g/mL}$ and 170.1 $\mu\text{g/mL}$, against *S. aureus* and MRSA respectively. Similarly, against *P. aeruginosa*, NANO3@O sees its MIC₅₀ reduced by half to a concentration of 170.1 $\mu\text{g/mL}$ after the application of an AMF. Lastly, regarding *E. faecalis* susceptibility to NANO3@O and NANO3@DO, it can be seen that after AMF-induced hyperthermia the MIC₅₀ of both

NANO3@O and NANO3@DO are reduced by a 4.5 factor to a concentration of 38.6 µg/mL. Interestingly, in this case the inhibitory activity of these two loaded systems no longer follows the tendency of the empty NANO3, that only have its MIC₅₀ at 357.1 µg/mL. The antimicrobial activity of NANO3@D is similar to that of empty NANO3, as expected, showing only some activity against *E. faecalis*.

The obtained results are in accordance with the release profiles after AMF hyperthermia, where OFLO delivery, especially in its single formulation. Overall, we can conclude that upon application of an external AMF, NANO3@O and NANO3@DO drug delivery capacity and antimicrobial activity are enhanced, promoting the dispensing of both antibiotic and anti-cancer drug in a future combinatory AMF-mediated therapy to tackle opportunistic cancer-related bacterial infections.

6.4 Conclusions

Engineered nanomaterials have shown a significant role in the regulation of microbial growth, exhibiting bactericidal and bacteriostatic effects. The regulation of opportunistic bacterial infections during cancer treatments is an important factor in patients' survival and therapies success. The herein reported nanoparticles seek to address this issue by incorporating multiple parts that render them with simultaneous magnetic and fluorescent properties, as well as cargo delivery capacity of single and combinatory formulations of anticancer and antimicrobial drugs. NANO3 were successfully synthesized with biocompatible and biodegradable materials, comprising of a superparamagnetic Fe₃O₄ core, a highly mesoporous silica shell and surface fluorescent SiQDs. The system showed a high predisposition to simultaneously incorporate and deliver OFLO and DOX as model anticancer drug and antibiotic, with a preferable release in acidic media like that of invasive tumors and bacterial infections. pH dependent release is mediated by surface SiQDs whose amine terminal groups are sensible to proton medium concentration and control pore access. Hyperthermia-mediated release and heat generation under an external AMF have been confirmed for all NANO3 systems, with all achieving febrile temperatures capable of stimulating the immune system and enhanced drug release. Finally, NANO3@O and NANO3@DO the materials showed effective antimicrobial activity against *E. coli* and *E. faecalis* strains, increased only when submitting them to an external AMF and NANO3@DO against *S. aureus*. AMF application increases the previously detected antibacterial activity and also extends NANO3 systems inhibitory activity to otherwise non-susceptible strains like *S.*

aureus, MRSA and *P. aeruginosa*. The successful antimicrobial activity of the system was confirmed and future studies will seek to apply it in a combined antimicrobial-anticancer therapy, thoroughly screening its cytotoxic and antitumoral activities.

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6.7 Supplementary Material

6.7.1 Tables

Table S - 6.1 - Reaction conditions for the loading assays of NANO2-COOH with DOX, OFLO and a combination of both.

	m_{NANO2} (mg)	$V_{\text{drug stock}}$ (mL)	V_{solvent} (mL)	V_{total} (mL)
OFLO	20	3.2 ¹	3.2 (miliQ H ₂ O)	6.4
DOX	20	3.2 ²	3.2 (PBS 0.01M)	6.4
DOX + OFLO	20	3.2 + 3.2	-	6.4

¹ OFLO stock (2 mg/mL) prepared in PBS 0.01M (pH 7.0)

² DOX stock (2 mg/mL) prepared in miliQ H₂O

Table S - 6.2 - Korsmeyer-Peppas calculated parameters for all pH related assays.

	pH 7.4				pH 5.0			
	NANO3@D	NANO3@O	NANO3@DO	NANO3@DO	NANO3@D	NANO3@O	NANO3@DO	NANO3@DO
	DOX	OFLO	DOX	OFLO	DOX	OFLO	DOX	OFLO
K_m	0.681	0.043	0.006	0.058	0.007	0.040	0.012	0.071
n	0.25	0.33	0.17	0.30	0.21	0.25	0.32	0.26

6.7.2 Figures

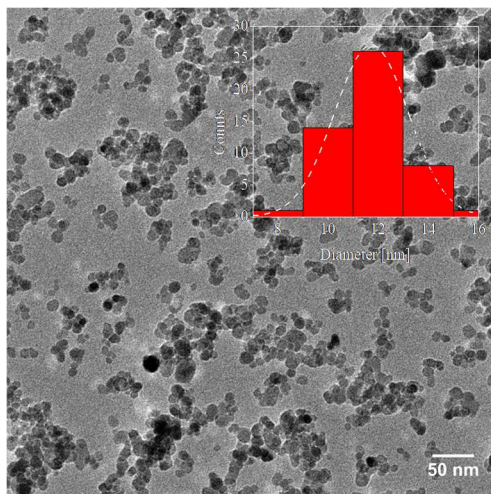


Figure S - 6.1 - TEM micrograph of as-synthesized NANO1; *inset*: particle size distribution, with $d = 11.73 \pm 1.47$ nm ($n = 50$). Scale bar of 50 nm.

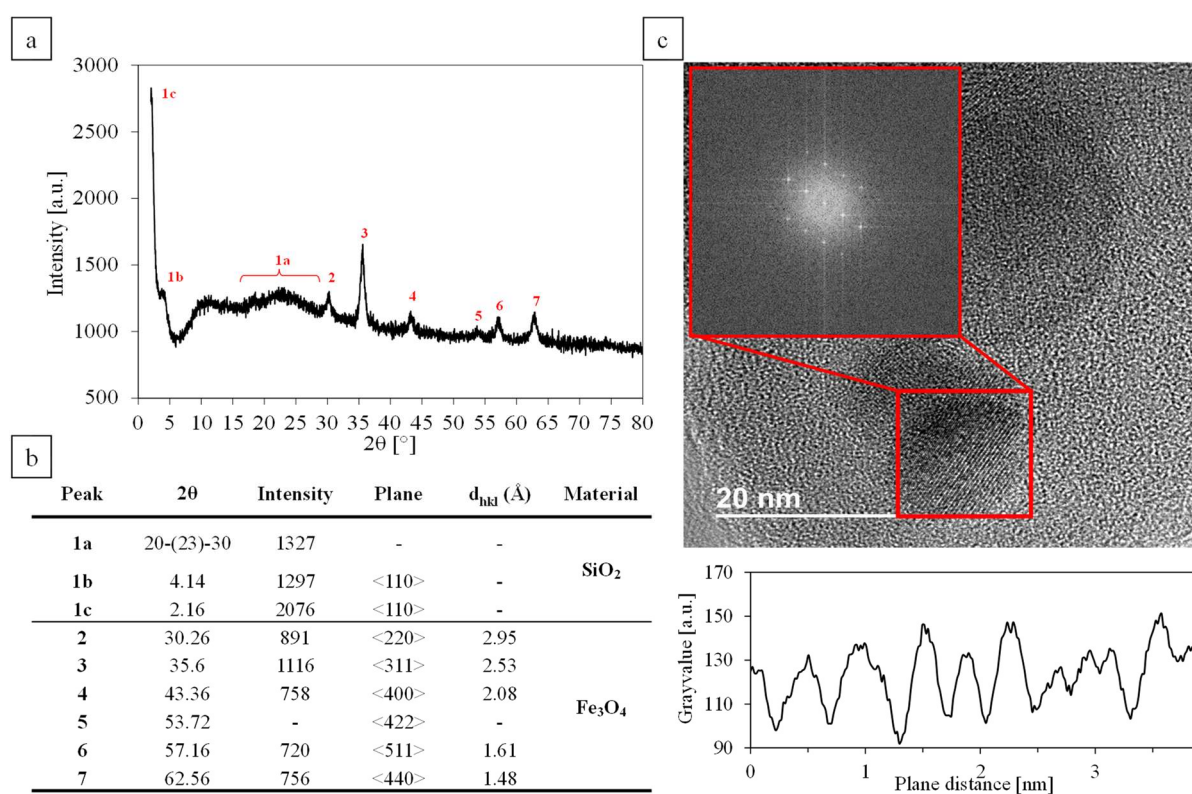


Figure S - 6.2 - XRD spectrum of NANO2 (a), and 2θ peaks' information and attribution to silica (SiO₂) and magnetite (Fe₃O₄) hkl lattice distances (b). TEM micrograph NANO2 and respective ImageJ generated FFT measurement of the core lattice d-spacing of 2.50 Å, assigned to the <311> plane of Fe₃O₄; assessment via grayscale profile plot, by counting the cycles (10) and dividing by total distance (4 nm)¹ (c).

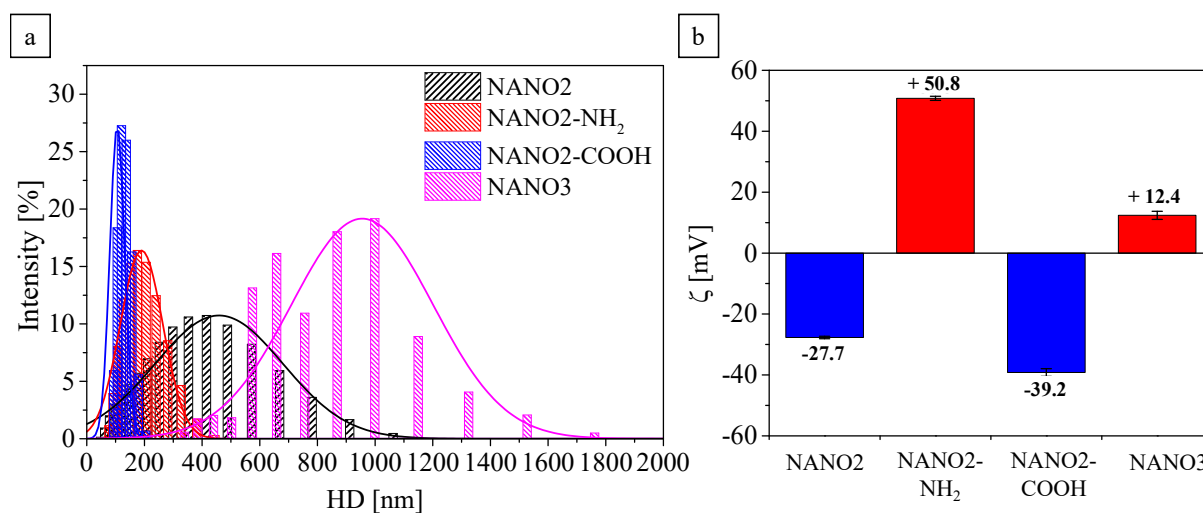


Figure S - 6.3 - DLS intensity-determined average hydrodynamic diameter (HD) of each synthetic product (a), and correspondent average zeta potential (ζ).

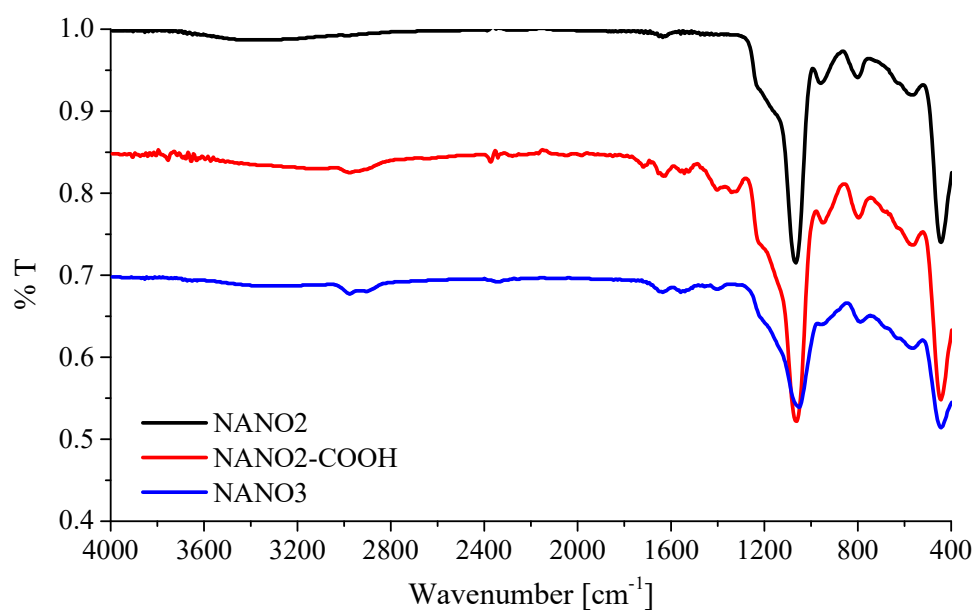


Figure S - 6.4 - ATR-FTIR spectra of as-synthesized NANO2, NANO2-COOH and NANO3.

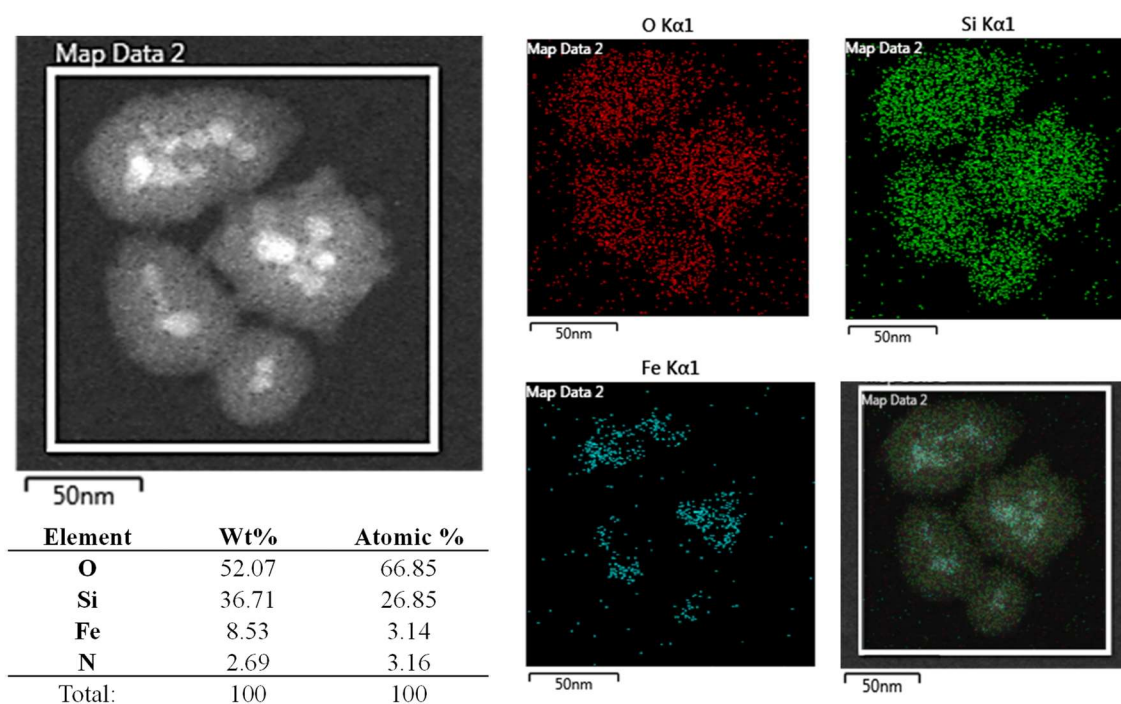


Figure S - 6.5 - Dark-field STEM image of NANO3 and respective elemental analysis and distribution.

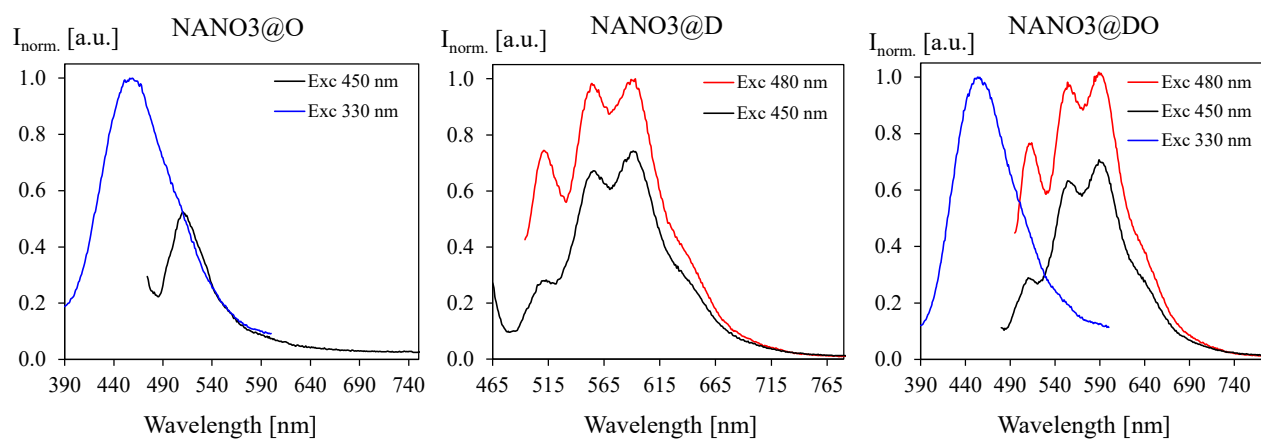


Figure S - 6.6 - Fluorescence spectra of conjugated SiQD, DOX and OFLO in NANO3@O, NANO3@D and NANO3@DO systems, in water.

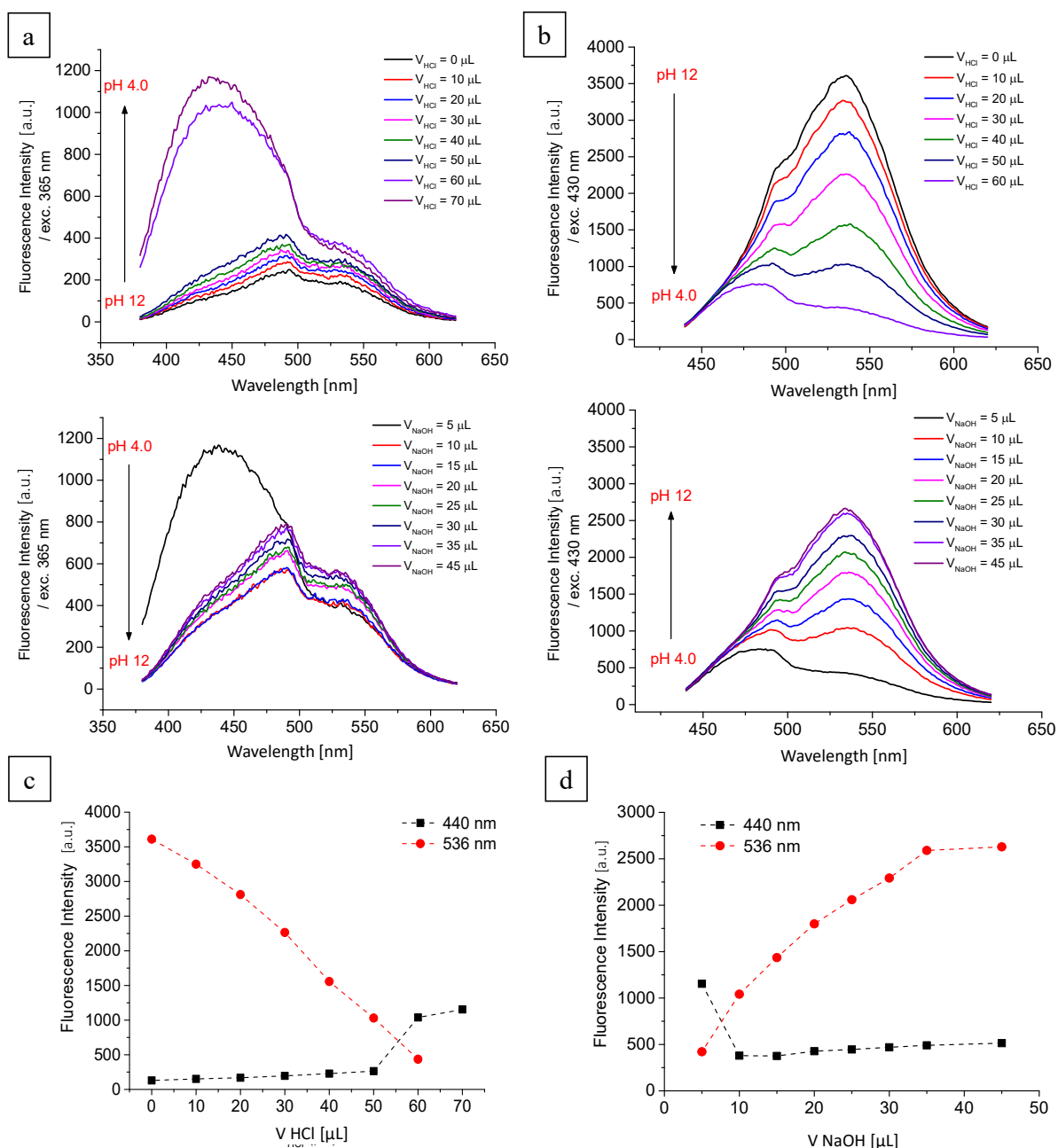
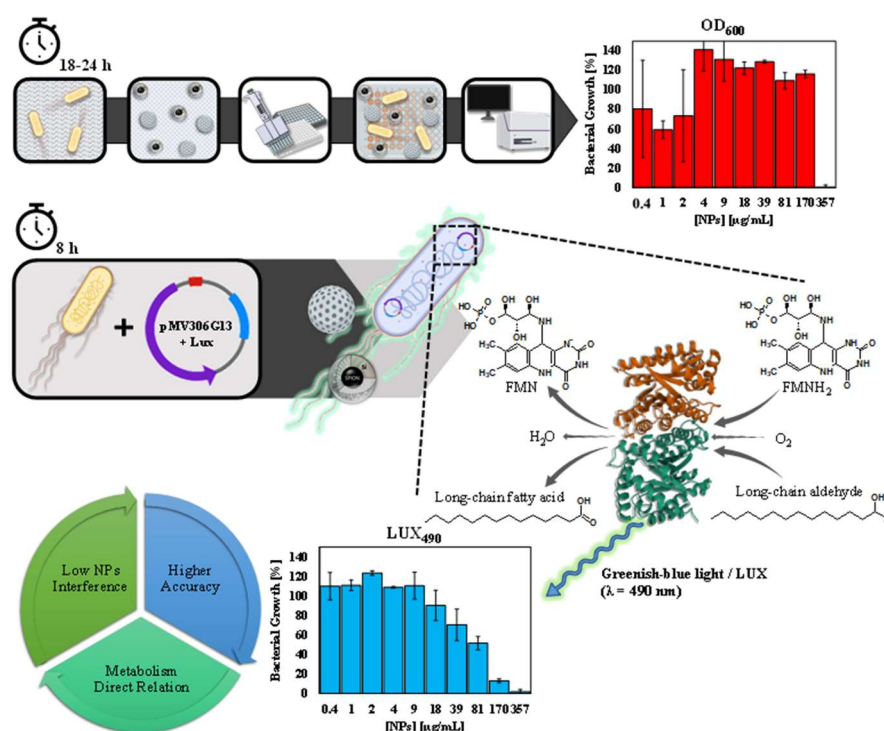


Figure S - 6.7 - SiQD fluorescence spectra variations by sequential addition of HCl and NaOH 10 and 5 μ L, respectively, after excitation at 365 nm (a) and 430 nm (b). Emission maxima (440 and 536 nm) variations with HCl (c) and NaOH (b) added volumes.

1. ImageJ, FFT Measurements, <https://imagej.nih.gov/ij/docs/examples/tem/>, (accessed 29/03/2022, 2022).

VALIDATION OF A STANDARD LUMINESCENCE METHOD FOR THE FAST DETERMINATION OF THE ANTIMICROBIAL ACTIVITY OF NANOPARTICLES IN *ESCHERICHIA COLI*



Keywords: nanoparticles • antimicrobial activity assessment • minimum inhibitory concentration • luminescence • *E. coli*

Adapted from GA Marcelo et al.
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Validation Of A Standard Luminescence Method For The Fast Determination Of The Antimicrobial Activity Of Nanoparticles In *Escherichia Coli*

Gonçalo A. Marcelo, Joana Galhano, Maria Paula Duarte, Jose Luis Capelo-Martínez, Carlos Lodeiro, and Elisabete Oliveira

Abstract: The use of nanoparticles in multiple industries has risen concerned voices about the assessment of their toxicity/antimicrobial activity and the development of standardized handling protocols. Issues emerge during the antimicrobial assaying of multiple cargo, colorimetric, colloidal nanoformulations, as standard protocols often rely on visual evaluations, or optical density (OD) measurements, leading to high variance inhibitory concentrations (MIC). Thus, a fast, luminescence-based assay for the effective assessment of the antimicrobial activity of nanoparticles is herein reported, using bioluminescence of an in-house *E.coli* ATCC® 8739™ construct with the pMV306G13+Lux plasmid (*E.coli* Lux). The new strain's sensitivity to ofloxacin as standard antibiotic was confirmed and the methodology robustness verified against multiple nanoparticles and colorimetric drugs. The reduction of incubation from 24 to only 8 hours, and the sole use of luminescence (LUX₄₉₀) to accurately determine and distinguish MIC₅₀ and MIC₉₀, are two main advantages of the method. By discarding OD measurements one can avoid turbidity and color interferences in when calculating bacterial growth. This approach is an important tool that contributes to the standardization of methods, reducing samples' background interference and focusing on luminescence as a direct probe for bacterial metabolic activity, growth and, most importantly, the correct assessment of nanomaterials antimicrobial activity.

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7.1 Introduction

The growing use of nanomaterials and nanoparticles in multiple industries, from cosmetics to healthcare and environmental sectors, has risen concerned voices on the assessment of their toxicologic properties and the development of standardized and optimized experimental and clinical protocols when handling such materials [1-3]. One major player in the development of new nanoparticle-based applications is the pharmaceutical industry. This sector benefits from the former's known high surface areas, carrier capacities, synergic properties, and antimicrobial activities to enhance the effectiveness of in-market drugs or develop completely new formulations capable of by-passing existing resistances, physical hindrances, and target specific targets in complex *in vivo* systems [4, 5].

Notwithstanding, some issues emerge when nanometric colloidal formulations are used for such applications, specifically, during toxicological and antimicrobial assays. The standard protocols used to assess the toxicity and antimicrobial activity of a specific drug, or material, often rely on either the visual evaluation of the results, or on an optical density (OD)-dependent assessment of the samples [6], for the determination of the Minimum Inhibitory Concentration (MIC), illustrating the concentration values at which $\geq 50\%$ (MIC₅₀) and $\geq 90\%$ (MIC₉₀) of the isolates in the test population are inhibited, respectively [7-10].

This is the case of the diffusion disk (agar dilution) technique or the Muller-Hinton microdilution broth approach [6], commonly used in antimicrobial activity assays, which are prone to errors and high variability, especially when colloidal nanoparticles are involved. The fact that the first technique relies on the migration of target compounds from disks to the solid medium is itself a problem when working with nanoparticles, whose migration is often non-uniform, affinity- and size-dependent, as well as contact-dependent [11]. Moreover, the visual measurement of the resulting inhibition zones (halo diameters) is another factor accounting for the possible high variability of the results.

Regarding the second liquid-based approach, although solving all migration and material-bacteria contact problems, the fact that most protocols point to a visual determination of the MIC₅₀ and the MIC₉₀ encourages an erratic determination of both [12]. OD-mediated bacterial growth measurements (usually at 600 nm and 630 nm), although more sensible and, in theory capable of more easily distinguish between MIC₅₀ and the MIC₉₀ [11], are particularly hindered when nanoparticles are involved. The latter usually accounts for an increase in the turbidity of the evaluated medium, be it by their dispersion in it or for a

time-promoted deposition at the bottom of the plate's wells. This phenomenon results in erratic OD values with high standard deviations, decreasing the degree of certainty at which MIC₅₀ and MIC₉₀ are determined [13]. To add up, none of the above-mentioned methods is capable of providing direct information on bacteria metabolism and activity, with growth only being obtained by comparison with controls and further additional assays [14].

Whether on one hand alternative colorimetric approaches have been developed to address these issues, as is the case of the well-established MTT/XTT reduction and Neutral Red uptake assays for cell toxicological/antimicrobial assays [15, 16] or other reported bacterial staining-based assays [7, 13, 17]; on the other, their use with colorimetric nanoparticles (e.g., AuNPs, AgNPs or iron oxide NPs) or cargo-filled porous nanoparticles (e.g., MCM-41 silica NPs) with colorimetric molecules/dyes, can still lead to baseline related aberrations and the wrong determination of both MIC₅₀ and MIC₉₀ [18]. In these cases, the colorimetric nanosystems can not only contribute to an increase in the baseline of the acquired absorbance spectra, but also interfere with the specific spectral maxima of each assay, thus producing inconsistent results.

This problem is especially aggravated when, for example, drug-delivery nanoparticles with multiple cargo, and multiple colorimetric profiles, are used, and the employment of a single assay is not sufficient. The development of multiple drug delivery nanoformulations has seen a consistent growth in the last decade, with particular interest in the production of multiple stimuli-responsive and of multiple therapeutic materials for some of the most important health-related industries [19, 20].

Bioluminescence is a widely known phenomenon that occurs in specific species and bacterial strains (e.g. *Mycobacterium marinum* and *Photobacterium luminescens*). It results from the expression of luminescence responsible genes, without the need for external radiating or exciting sources [21]. The production of light by action of luciferase enzymes on their cognate substrates can be directly correlated with the being's metabolism, thus working as an immediate representative of its growth [22]. Bioluminescence-based assays to determine cell metabolic activity and/or growth have also been reported as highly sensitive, easy and rapid methods [23-26]. The use of bioluminescence as a preferable tag in disease progression follow up, as well as ecotoxicological, antimicrobial, and kinetic assays of molecular probes, has been reported in the literature to work as an excellent real-time probing mechanism with no disposition to interference [27-32]. Such example is the use of luminescent *E. coli* for the detection of β -lactams [33] or as *light-on/light-off* sensors for bioavailable heavy metals, like copper and mercury [34]. Interestingly, little effort has been done in the establishment of

straightforward, standardized approaches fully devoted to the determination of the antimicrobial activity of nanoparticles and nanoformulations, where *in-lab* pre-modified strains or special genetic constructs are needed [35, 36]. An example of such is the one-time reported use of luminescent *E.coli* strains on the investigation of copper nanoparticles possible antibacterial mechanisms [37].

Considering this, in this work we report a real-time, fast, luminescence-based antimicrobial assay for the effective assessment of the antimicrobial activity of multiple nanoformulations, and with no interference from the latter. The herein developed *state-of-art* approach uses the natural bioluminescence of an *in-house* constructed *Escherichia coli* strain (*E.coli* Lux), by insertion of the pMV306G13+Lux plasmid in its parent commercial *Escherichia coli* ATCC® 8739™. This strain was tested for its sensitivity after the modification and the methodology's robustness was verified against multiple silica mesoporous nanoparticles, either bare or comprising a magnetic iron oxide core. A set of three loaded drugs was also used for its verification, using them in either single or dual formulations. Assay timings were also optimized by direct follow-up of the assayed bacteria. With this work, we seek to take a first step towards the establishment of a viable and standard solution for future clinical testing and potential industrial applications of nanomaterials.

7.2 Materials nad Methods

Iron(III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 97.0-102.0%), iron(II) chloride hydrate ($\text{FeCl}_2 \cdot \text{H}_2\text{O}$, 99%), ammonium nitrate (NH_4NO_3 , 99.999%) and tetraethyl orthosilicate (TEOS, $\geq 99.999\%$ metals basis) were bought at AlfaAesar. *n*-Cetyltrimethylammonium bromide (CTAB), anhydrous N,N-dimethylformamide (DMF, 99.8%), anhydrous tetrahydrofuran (THF, 99.9%), acetone ($\geq 99.5\%$ GC), glucose monohydrate and phosphate-buffered saline (PBS, tablets) were purchased from Sigma Aldrich. Ethanol absolute (EtOH, extra pure), dichloromethane (DCM, extra pure) and hydrochloric acid (HCl, 37%) were bought at Scharlab, SL. Dimethyl sulfoxide (DMSO) and methanol (MeOH) and sodium acetate anhydrous were acquired from CarloErba Reagents. Oleic acid (OA, 65.0 - 88.0 %), ethanol 96%, sodium chloride (NaCl) and potassium chloride (KCl) were obtained from Honeywell Fluka. Sodium hydroxide (NaOH, tablets) was bought from Panreac AppliChem. Doxorubicin hydrochloride (DOX, $>99\%$) and Epirubicin hydrochloride (EPI, $>99\%$) were bought at LC Laboratories, while Ofloxacin (OFLO, $\geq 99\%$) and Kanamycin sulfate were purchased from Sigma-Aldrich.

Bacto™ Glycerol was bought from Becton Dickinson&Co (Sparks, MD, USA) and magnesium chloride (MgCl_2) was from LabChem Inc. Muller Hinton Agar (MHA), Muller Hinton Broth (MHB), Trypto Casein-Soy Agar (TSA) Trypto Casein-Soy Broth (TSB), tryptone and yeast extract were all obtained from Biokar Diagnostics. All reagents were used as acquired, without any further purification, and all solutions, unless otherwise indicated, were prepared with deionized Millipore miliQ water.

Antibacterial assays were performed against Gram-negative *Escherichia coli* ATCC® 8739™ (*E. coli*). Transparent flat-bottom sterile 96-well plates were purchased from Greiner Bio-One and U-bottom opaque black 96-well plates from RatioLab (Dreieich-Buchschlag, Germany). pMV306G13+Lux was a gift from Brian Robertson & Siouxsie Wiles (Addgene plasmid # 26160; <http://n2t.net/addgene:26160>; RRID:Addgene_26160). GeneJET Plasmid Miniprep Kit #K0502 and all its respective solutions were used as bought from Thermo Scientific.

DLS experiments were performed in a Malvern Nano ZS Zetasizer (633 nm laser diode), from the PROTEOMASS-BIOSCOPE Group LAQV-NOVAFCT. N_2 adsorption-desorption isotherms were recorded in an ASAP 2010 automated analyzer (Micromeritics, Norcross, GA, USA), also from the Analysis Laboratory at LAQV-NOVAFCT. Specific surface areas were calculated from the adsorption data within the low-pressure range using the Brunauer-Emmett-Teller (BET) model. Pore size was determined following the Barrett-Joyner-Halenda (BJH) method. SEM images were obtained in a Quanta 650 FEG operating between 5-30 kV and 3.6×10^{-4} Pa of vacuum in the chamber. Transmission electron microscopy (TEM) images were obtained in a JEOL JEM-2100-HT operating at 200 kV, TEM images were collected using an "OneView" 4k x 4k CCD camera, from INL-Braga.

UV-Vis absorption spectra were collected in a Jasco V-650 spectrophotometer and fluorescence spectroscopy measurements were taken in a HORIBA Scientific FLUOROMAX-4 spectrofluorometer, from the PROTEOMASS-BIOSCOPE Group LAQV-NOVAFCT. ATR-infrared spectra were collected in an Alpha II FT-IR spectrophotometer (Bruker, Massachusetts, United States), from the PROTEOMASS-BIOSCOPE Group LAQV-NOVAFCT. Magnetization studies were achieved in a Vibrating Sample Magnetometer (VSM) Lakeshore 7304 (Lake Shore Cryotronics, Ohio, USA), with an applied magnetic field of 1.45 T, from the ISOM-Universidad Politécnica de Madrid.

Aseptic bacterial assays and modifications were handled in a Steril-VBH laminar flux chamber. Turbidity for each assayed bacterial suspension was adjusted in a DEN-1B McFarland Densimeter, from FCTNOVA (Grant-Bio). Incubations were conducted in a

Mermmet Incubator B10, at 37 °C. Optical density (OD₆₀₀, 600 nm) and luminescence (LUX₄₉₀, 490 nm) measurements were conducted in a UV-Vis CLARIOstar® Plus spectrophotometer, from the PROTEOMASS-BIOSCOPE Group LAQV-NOVAFCT (BMG Labtech). Plasmid quantification was performed in a Nanodrop 1000 Spectrophotometer (ThermoFisher). Electroporation protocols were performed in a Bio-Rad Gene Pulser® from Laboratory of Genetics of NOVA Medical School, Centro de Estudos de Doenças Crónicas (CEDOC).

7.2.1 Synthesis of nanoparticles

7.2.1.1 Mesoporous silica nanoparticles synthesis

In a typical procedure, MCM-41 mesoporous silica nanoparticles (**MNs**) were synthesized via Stöber method as reported in literature [38, 39]. Briefly, CTAB (180 mg) was dissolved in Milli-Q H₂O (10 mL), at 50°C. After 10 minutes, 20 mL of Milli-Q water (20 mL), ethylene glycol (10 mL) and NaOH 1M (700 µL) were sequentially added, and the mixture stirred for 30 min, at the same temperature. Afterwards, TEOS (750 µL) was added dropwise to the reactional mix and stirred for 2 h, at 70°C. The material was collected by centrifugation and washed twice with MeOH, prior to dryness.

7.2.1.2 Oleic acid stabilized SPION synthesis

Oleic acid stabilized super paramagnetic Fe₃O₄ magnetic nanoparticles (**SPION@OA**) were prepared by an adaptation of the co-precipitation method reported by Zhang et al [40]. Briefly, FeCl₃·6H₂O (2.4 g) and FeCl₂·4H₂O (0.98 g) were vigorously dissolved in MiliQ water (10 mL), under continuous Ar bubbling at 80 °C. After full dissolution, 5.0 mL of NH₄OH (25 wt.%) were injected into the solution, which was stirred for 1 h at 80 °C, under Ar bubbling. Then, 425 µL of oleic acid (OA) were added and the reaction bubbled for an additional 5 min, before being sealed and stirred at 80 °C, for another 1.5 h. The resulting SPION-OA cores were magnetically decanted using a neodymium magnet and thoroughly washed with MiliQ water until neutral. Excess OA was controlled by thin-layer chromatography. Later, SPION-OA were transferred *in situ* into chloroform (20 mL) to achieve a final particle concentration of 6.8x10¹⁵ particles/mL. SPION concentration was calculated according to the total Fe content by ICP-AES and sizing by TEM analysis.

7.2.1.3 Single-layer mesoporous silica surface modification

SPIONs' surface modification with single-layer mesoporous silica (**SPION@MNs**) was achieved in adept approach to that reported by Zhang et al [40]. In brief, CTAB (150 mg) was

dissolved in MiliQ H₂O (10 mL), mixed with SPION (0.74 mL) and sonicated for 1h at 50 °C (35 kHz, degas mode). The resulting emulsion was then heated to 70 °C and stirred for 30 min to evaporate the residual chloroform. Then, MiliQ H₂O (30 mL), ethylene glycol (10 mL) and NH₄OH (25 wt.%, 0.7 mL) were sequentially added to the mixture, which was stirred for another 30 min at 70 °C. After, TEOS (750 µL) was introduced dropwise to the reaction and stirred for 3h, at 70 °C. The as-prepared sample was cooled to room temperature, recovered and washed in MeOH 3 times and left to dry at 60 °C. SPION@MNs were structurally characterized by DLS, XRD, FTIR, N₂ sorption and SEM/TEM and magnetization properties confirmed by VSM.

7.2.1.4 Template removal

The removal of pores template, from both MNs and SPION@MNs, was achieved after resuspension in a 30 mg/mL NH₄NO₃ methanolic solution (50 mL) and stirring for 1h at 60 °C. This process was repeated twice over, and the products obtained in the form of white and light brown magnetic powders, respectively, after washing in methanol and drainage at air [39].

7.2.1.5 Drugs loading and release assays

MNs and SPION@MNs materials were both single-loaded with OFLO, DOX and EPI drugs, as well as with 1:1 dual combinatory cocktails of the same drugs (i.e., DOX+OFLO and EPI+OFLO). For single loadings, 5 mg of each material were resuspended in 2 mL of PBS buffer solution (pH 7.4, 0.01M) with a drug concentration of 62.5 µg/mL. 1:1 combinatory drug loadings were achieved by resuspending 5 mg of each material in 2 mL of each drug solution (62.5 µg/mL), in PBS buffer, to a total volume of 4 mL. The resulting suspensions were stirred for 24 h at room temperature, in the dark. Each loaded material was recovered by centrifugation (10 000 rpm, 10 min) and washed 5 times with PBS buffer solution. All supernatants were collected and quantified by UV-Vis spectroscopy, in triplicates, at 330 nm (OFLO, $\epsilon = 12806 \text{ M}^{-1}\text{cm}^{-1}$), 480 nm (EPI, $\epsilon = 12958 \text{ M}^{-1}\text{cm}^{-1}$) and 480 nm (DOX, $\epsilon = 10096 \text{ M}^{-1}\text{cm}^{-1}$). Loading efficiency (%E) and loading capacities (mg/g) were calculated by Equations 7.1 and 7.2.

$$\%E = \frac{t_{drug} - f_{drug}}{t_{drug}} \times 100 \quad (7.1)$$

$$\text{Loading capacity} \left(\frac{\text{mg}}{\text{g}} \right) = \frac{t_{drug}(\text{mg}) - f_{drug}(\text{mg})}{m_{NP}(\text{mg})} \quad (7.2)$$

Where, t_{drug} corresponds to the initial total mass of drug, f_{drug} the final mass of drug in the supernatants, and m_{NP} the mass of nanoparticles per loading batch of each material.

In vitro drug release from loaded MNs and SPION@MNs was performed by suspending 0.5 mg of each material in 1.5 mL PBS pH 7.4 and PBS pH 4.0 solutions, accordingly. All suspensions were stirred at 37 °C, for 24 h, in the dark. Again, the suspensions were centrifuged, and the supernatants were collected and quantified by the same approach.

7.2.2 Confirmation Assays

7.2.2.1 CFU Counting

E. coli Lux was subcultured on TSA plates and incubated overnight at 37 °C. Isolated colonies were transferred to 0.85% NaCl, and the turbidity of the suspension was adjusted to 0.5 in the McFarland scale. From this suspension, serial decimal dilutions in 0.85% NaCl were performed and plated in TSA plates, that were incubated at 37°C overnight. Finally, the number of visible colonies (CFU) present on the TSA plates was determined and multiplied by the dilution factor providing the CFU/mL of the initial suspension (Fig. S-6.2).

7.2.2.2 Luminescence Assay

E. coli Lux was subcultured on TSA plates and incubated overnight at 37 °C. Isolated colonies were suspended in 100 μ L 0.85% sterile NaCl. This suspension was transferred to a flat-bottom transparent 96-well plate, as well as to a U-bottom black opaque 96-well plate, for the acquisition of its OD₆₀₀ intensity and LUX spectra, respectively. LUX maximum intensity was determined for a 490 nm wavelength (LUX₄₉₀).

7.2.2.3 Optimization of antimicrobial assay conditions with *E. coli* Lux

Bacterial suspensions of *E. coli* Lux and of the parental strain (*E. coli* ATCC® 8739™) were prepared as previously described (Section 7.2.2.1). Then, in steril 96-well microplates, two-fold serial dilutions of ofloxacin (120 to 7.5 ng/mL) were prepared in sterile MHB to a final volume of 100 μ L per well. Each well was inoculated with a 1:10 dilution of the previously prepared bacterial suspensions (to achieve a concentration of 10⁶ CFU/mL in each well). Negative controls (without ofloxacin) were also prepared. Plates were left to incubate at 37 °C, for appropriate timepoints, under both static and stirring (250 rpm) conditions. At 0, 1, 2, 3, 4, 6, 8 and 24 hours, the OD₆₀₀ was measured in a plate reader. The suspensions were then transferred to a black non-sterile 96-well plate and their LUX₄₉₀ collected.

The susceptibility of *E. coli*-Lux to OFLO was compared with that of its parental *E. coli* strain (**Figure. S-3**). An 8-hour optimal assay time for the luminescent *E. coli*-Lux transformant was determined by comparing OD₆₀₀ and LUX₄₉₀ vs incubation time, with LUX₄₉₀ being a direct measurement of metabolic activity and bacterial growth.

7.2.2.4 Antimicrobial activity assessment

The antimicrobial activity of the different synthesized material was assessed against the parental *E. coli* strain and the new *E.coli*Lux strain.

In all assays, 2.5 mg/mL suspensions of all selected nanomaterials (MNS, SPION@MNs, MNs-drugs and SPION@MNs-drugs) were prepared in H₂O. Drug solutions containing the same concentration of the drugs loaded into each material were also prepared, in water. Bacterial suspensions (0.5 on the McFarland scale) were prepared as previously described (Section 7.1.2.2) and diluted with NaCl 0.85% to about 10⁷ CFU/mL.

To evaluate and quantify the bacteriostatic effects of the tested nanocomposites, all samples were assayed by the broth microdilution method in a 96-well microplate. Briefly, two-fold serial dilutions of each sample, and controls, were prepared in sterile MHB to a final volume of 100 µL per well. Then, each well was inoculated with 10 µL of each previously prepared bacterial suspension (to achieve a concentration of 10⁶ CFU/mL in each well). Drug solutions containing the same concentration of the drugs loaded into each material were used as positive controls. Each material in the absence of bacteria and the bacteria without samples, were used as negative controls for the experiments. Plates regarding the *E. coli* Lux strain were left to incubate for 8 h, at 37 °C, whereas those of the parental *E. coli* were left to incubate for 24 hours, at 37°C.

OD₆₀₀ and LUX₄₉₀ measurements of the 96-well plates were conducted in a plate reader at 600 nm and 490 nm, respectively. Luminescence monitorisation was achieved after transferring all suspensions into U-bottom opaque black 96-well plates. Bacterial growth and the MIC were calculated from luminescence data (Equation 3), in the case of *E. coli* Lux, and from OD (Equation 7.4), for the remaining bacteria.

$$\text{Bacterial Growth} = \frac{\text{Sample LUX}_{490}}{\text{Bacteria control LUX}_{490}} \quad (7.3)$$

$$\text{Bacterial Growth} = \frac{\text{Sample OD}_{600} - \text{NP OD}_{600}}{\text{Bacteria control OD}_{600}} \quad (7.4)$$

Where, sample OD₆₀₀ and NP OD₆₀₀ stand for the incubated sample OD₆₀₀ signal and the correspondent NP suspension OD₆₀₀ control signal.

7.2.2.5 Statistical Analysis

Data presented as mean $\pm$ standard deviation, with n indicating the number of independent experiments. Student's 2-tailed t-test to compare two groups with unequal variances, for $\alpha = 0.05$. Statistical significance was shown as **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

7.3 Results and Discussion

Bioluminescent bacteria transformants and an adapted Muller-Hinton microdilution broth protocol were readily produced and validated, with reduced interference from the tested nanoformulations and increased sensitivity towards bacteria growth profiles, when compared to common protocols.

7.3.1 Bacterial transformation and validation

A parental commercially available *Escherichia coli* ATCC® 8739TM, widely used to test the efficiency of antimicrobial agents, was selected as a *state-of-art* strain for bacterial transformation with the pMV306G13+Lux plasmid (Figure S-7.1) [28]. This plasmid, from *E. coli* DH5 α , was selected as it contains the *Mycobacterium marinum* + *Photobacterium luminescens* lux (luxABCDE) operon, capable of conferring constitutive bioluminescence to bacteria. The luxAB genes encode the production of the luciferase enzyme, while the luxCDE genes encode proteins that produce long-chain aldehydes, substrates for this bacterial luciferase [41]. Light production originates from a biochemical mechanism, catalyzed by the luciferase enzyme, in which FMNH₂, the reduced form of the flavin mononucleotide, and an aliphatic aldehyde react in the presence of oxygen [42]. The transformation of the aldehyde into a long-chain fatty acid is accompanied by the production of flavin mononucleotide (FMN) and energy in the form of a photon of greenish-blue light, with an emission maximum at *ca.* 490 nm. The emission is readily detectable at naked-eye in a dark environment or by spectroscopic techniques [43]. The selected plasmid also contains a kanamycin resistance cassette that works as a selection marker for the new transformants of interest.

Plasmid purification, after extraction, is of extreme importance as extraction media high ionic strength can interfere with electroporation mechanisms [44]. The purification step yielded the plasmid at a concentration of 17.8 ng/mL and moderate purity. Purity was estimated according to the 260/280 nm ratio, with a ratio of *ca.* 1.8 being accepted as pure (obtained: 1.5) [45].

After competence induction and electroporation, the obtained transformants were left to recover and stabilize in SOC medium and were then plated onto TSA-kanamycin plates. Kanamycin presence allowed for an appropriate selection of the transformants from the non-transformed kanamycin-susceptible parent *E.coli* strain. After 24 hours of incubation, the plates presented several well-structured colonies, that when in the dark showed the expected intense greenish-blue luminescence, easily detected at naked-eye. Luminescence spectra of four random isolated colonies suspensions were spectroscopically scanned, with all showing a maximum of luminescence at 490 nm (LUX₄₉₀) and high intensities of ca. 3000 relative luminescence units (rlu) (Figure 7.1). Thus, confirming a successful transformation and production of a luminescent *E.coli* Lux strain.

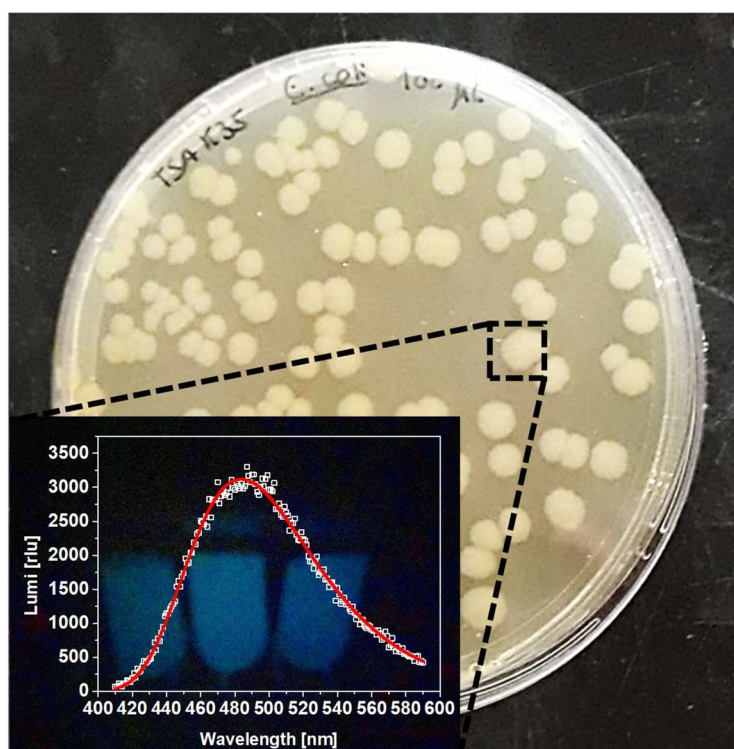


Figure 7.1 - Bacterial transformants after electroporation; inset: *E. coli* Lux typical luminescence spectrum.

The relationship between the turbidity of the new *E. coli* Lux suspensions and cell concentration in CFU/mL was verified (Figure S-7.2). This relationship is essential for antibacterial assays since cell concentration is regulated by adjusting the turbidity of bacterial suspensions according to the McFarland scale, with a turbidity of 0.5 corresponding to a cell concentration of *ca.* 10^8 CFU/mL [46, 47]. As *E.coli* Lux transformants show intrinsic luminescence, it was necessary to verify if this phenomenon had no interference with the measurement of the turbidity of the sample, and subsequently with cell concentration. The preparation of successive decimal dilutions from an initial 0.5 McFarland suspension and

further 24h incubation, in TSA plates, confirmed the correct concentration of the initial suspension (*ca.* 10^8 CFU/mL), proving that the bioluminescence of *E. coli* Lux has no interference in the turbidity of the suspensions and further adjustment of bacterial concentration.

7.3.2 *E.coli* Lux susceptibility to ofloxacin

Ofloxacin is a well-known second-generation fluoroquinolone with a wide antibiotic spectrum, used for the treatment of several types of infections in the respiratory tract, kidney, skin, soft tissue, and urinary tract [48]. This antibiotic is active against many Gram-positive bacteria and most Gram-negative bacteria, *E.coli* included, via its inhibitory action against DNA gyrases and type II bacterial topoisomerases [49].

The assessment of *E. coli* Lux susceptibility to ofloxacin, as a model antibiotic, is thus an essential hallmark to the validation of the new strain, as a similar response to that obtained for the parent *E. coli* is pursued. In this assay, *E. coli* Lux were incubated with a range of ofloxacin concentrations from 120 to 7.5 ng/mL, and its response (in OD₆₀₀ and LUX₄₉₀) was evaluated at different timepoints, under both static and orbital shaking incubation conditions. The assaying ofloxacin concentration range was set after an initial assay with the non-luminescent parent *E. coli* strain (OD₆₀₀) to determine both MIC₅₀ and MIC₉₀ of ofloxacin (Figure S-7.3). From the initial static and shaking datasets, it was found that a more reliable positive correlation between OD₆₀₀ and LUX₄₉₀, and consequently a better estimation of bacterial metabolic activity and thus, growth, is obtained under static conditions (Figure S-7.4). For this, static growth was deemed as the optimal mechanical condition for the method, and the data obtained under static growth was considered for further analysis (Figure 7.2).

During the first 4 hours, the lack of significative bioluminescence variations and its overall low intensity, between different ofloxacin concentrations, following a parallel trend to that represented by OD₆₀₀ measurements, confirms that this period corresponds to the lag phase of bacterial growth, where no significant growth is evident, and no significant bacterial activity occurs [50]. However, after 6 hours of incubation, and now in the exponential phase of bacterial growth, characterized by an exponential increase in bacterial growth and metabolic activity, an increase in the LUX₄₉₀ of the bacteria exposed to 15 and 7.5 ng/mL ofloxacin points toward a less effective action of the drug against *E. coli* Lux in this range. This is further corroborated by the low LUX₄₉₀ and OD₆₀₀ measurements obtained for higher concentrations (120, 60 and 30 ng/mL), where ofloxacin action is evident and intensity of bioluminescence and OD₆₀₀ is kept minimal. The trend continues and is more evident after 8

hours, where the LUX₄₉₀ signal is maximum at an ofloxacin concentration of 7.5 ng/mL (ca. 890 rlu). At this point, the LUX₄₉₀ signal tightly mimics the behavior of OD₆₀₀ measurements, allowing for the estimation of both inhibitory concentrations (MIC₅₀ and MIC₉₀) through a direct correlation between bacterial metabolic activity and LUX₄₉₀ intensity and its fluctuations in response to several antibiotic concentrations. A direct look at the luminescence curve highlights the disparity and abrupt decrease in the LUX₄₉₀ signal between ofloxacin concentrations of 15 and 30 ng/mL, from 668 rlu to 170 rlu, representing a 75% reduction from one point to another. This reduction, supported by similar profiles obtained through OD₆₀₀ measurements allow us to determine with certainty the MIC₅₀ of ofloxacin for this bacterial strain.

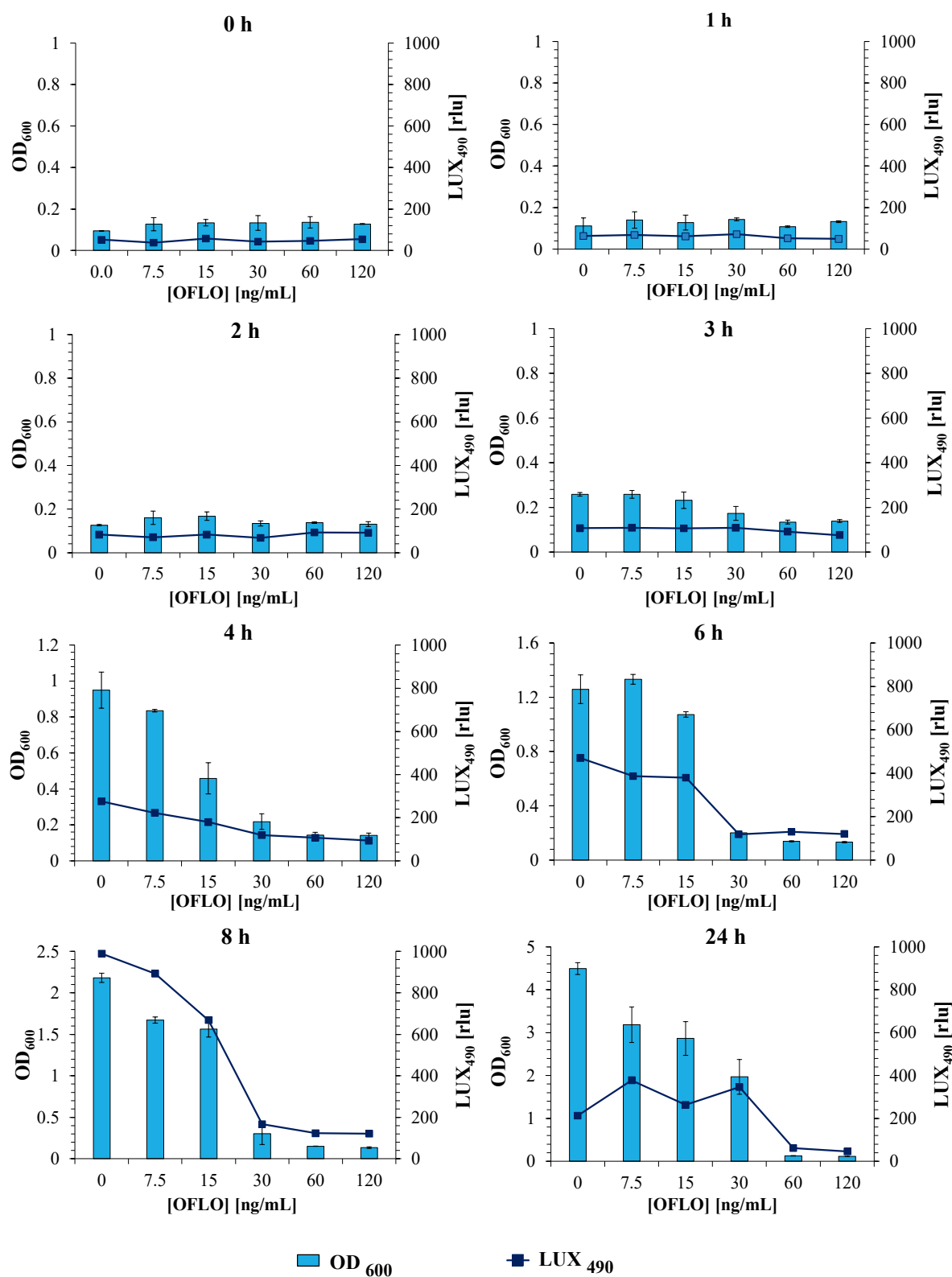


Figure 7.2 - Temporal bacterial response of *E. coli* Lux to ofloxacin, under static conditions, for different concentrations. Responses were given in the form of LUX₄₉₀ signal intensity and OD₆₀₀.

Interestingly, the LUX₄₉₀-determined MIC₅₀ (between 15 – 30 ng/mL), at 8 h, is the same as that determined for the parent *E. coli* strain, via OD₆₀₀ at 24h (30 ng/mL of ofloxacin) (Figure 7.3) [11], verifying three major points. First, that the response of *E.coli* Lux to ofloxacin is similar to that of the wild-type parent *E. coli* strain; second, that it is possible to accurately determine inhibition concentrations, equivalent to those obtained by the standardized OD₆₀₀ measurements after 18-24 hours of incubation, through luminescence measurements after just 8 hours of incubation; and lastly, that the results obtained for LUX₄₉₀ are a reliable source for the estimation of bacterial metabolic activity and growth, in spite of OD₆₀₀ measurements.

These last two points are of utter interest, as OD₆₀₀ measurements solely rely on the turbidity of the samples, which is directly correlated to the quantity of cells in the suspension, regardless of their growth phase at the moment of the measurement. This means that dead bacterial cells continue to contribute to the full turbidity of the samples, even in the death stage of bacterial growth (Figure 7.3.A), leading to erratic conclusions on bacterial growth estimation and MIC₅₀ determination, as do samples that contribute to the overall turbidity of the suspensions, such as nanomaterials. On the contrary, luminescence measurements (i.e., LUX₄₉₀) not only are not hampered by the turbidity of the suspensions, which can suffer fluctuations due to the presence of samples under analysis but are also thoroughly connected with the metabolism of the bacteria, allowing for a more reliable determination of the response of the bacteria to the tested compounds, and thus of their growth status at the time of the measurement.

Unlike the other timestamps, after 24 hours of incubation, bioluminescence intensity decreases exponentially, while OD₆₀₀ measurements are maintained at higher values, since bacteria might be present in their stationary stage of growth. In this phase, no further growth occurs, stabilizing OD₆₀₀ measurements. Furthermore, a decrease in metabolic activity is verified by the abrupt decrease on LUX₄₉₀ intensity (Figure 7.3.B). An estimation of bacterial growth using this data, after an incubation period of 24 hours, might thus prove unreliable due to the reduced activity of the bacteria. The time growth curve of *E. coli* Lux without exposure to ofloxacin was thus evaluated to confirm the origin of such intense decrease in LUX₄₉₀. Whereas the OD₆₀₀ profile through time follows an upward trend, reaching its maximum plateau at > 24 hours, LUX₄₉₀ profile only presents this uphill tendency for the first 8 hours of the assay, falling abruptly after. This proves again the correlation between bioluminescence intensity and bacterial metabolic activity at this specific stage of bacterial growth, where both a higher metabolic activity and growth occur. Once again, at this stage of

development is possible to estimate bacterial growth in response to stimuli, through its tight relationship with metabolic activity, [22].

As a last validation assay to the proposed approach, the new *E. coli* Lux has yet to be tested against the target of the work, this is, drug-delivery nanoparticles that can work as optical and colorimetric interferents in the evaluation of their own antimicrobial activity.

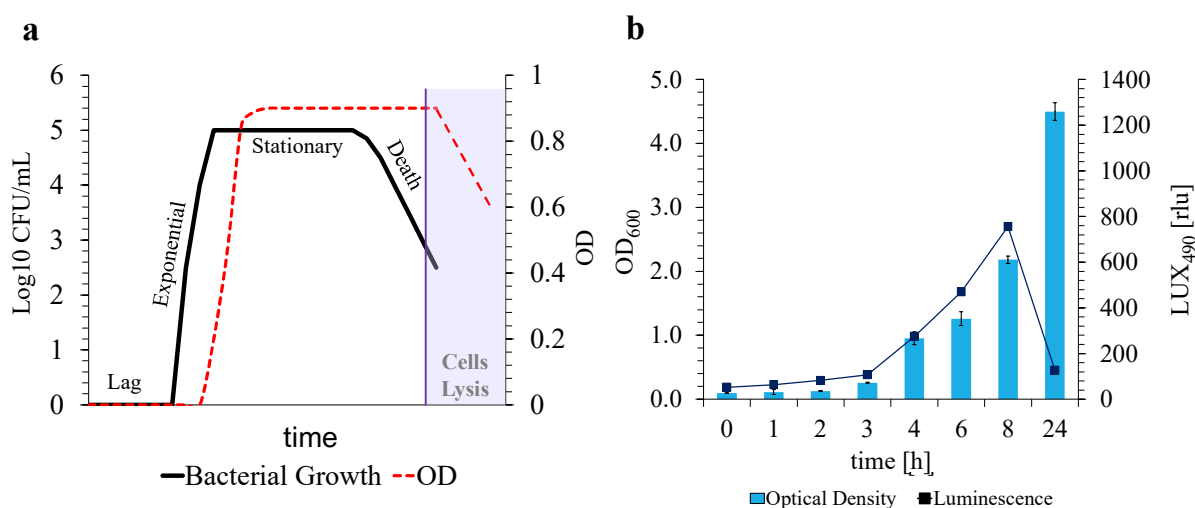


Figure 7.3 - Typical bacterial growth curve and its correlation with OD measurements (a); adapted from [51]. Obtained bacterial growth curve and correlation with the recorded luminescence, emitted by luminescent *E.coli* Lux (b).

7.3.3 Nanoformulations synthesis

To describe a reliable and accurate bioluminescent antimicrobial activity assay, two distinct types of nanoparticles, loaded with a set of three drugs, were used as possible interferents to test the method's robustness when compared to the performance of the commonly used OD-mediated approach. For that, MNs and SPION@MNs were successfully synthesized and obtained in the form of dry white and dark brown powders, respectively. This is evident in the spectroscopic analysis of the suspensions of the bare nanoparticles in water, with both having a significant wide light absorption, ranging from 650 to 200 nm (Figure S-7.5). Both MNs and SPION@MNs showed a spherical conformation, with diameters of several tens of nanometers, and a mesoporous silica matrix, with pores of *ca.* 2.7 and 2.9 nm and available high surface areas of *ca.* 800 and 900 m²/g, respectively, typical of this type of mesoporous particles [11, 38, 39]. The slight larger pore size of SPION@MNs own it to the presence of the SPION stabilizer, oleic acid, that contributes to a certain degree of pore size modulation (Figure. 7.4).

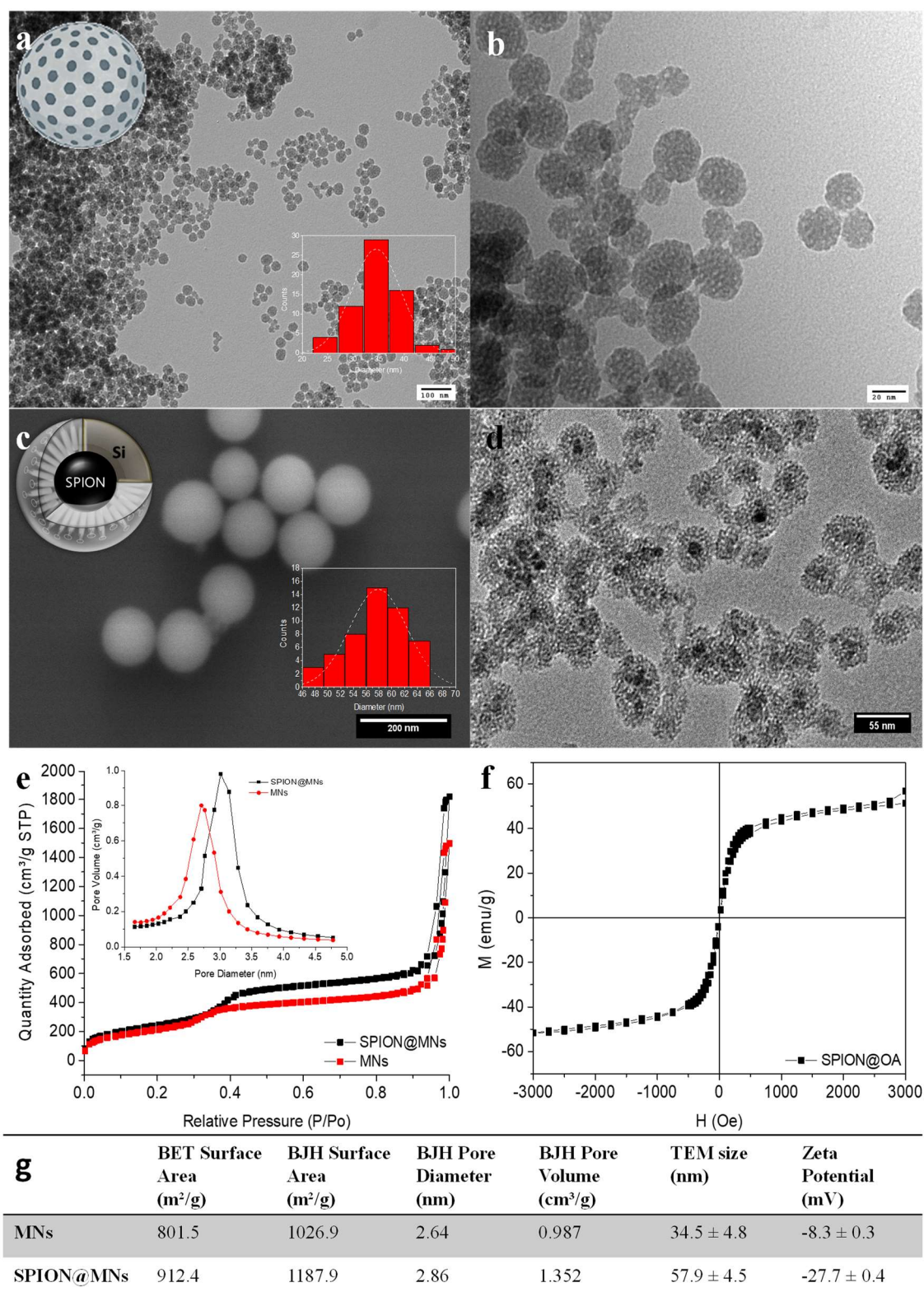


Figure 7.4 - Physical characterization of MNs and SPION@MNs: MNs TEM micrographs confirming the particles mesoporosity, at 100 nm scale (a) and close up at 20 nm scale (b); inset: particle size distribution (n=65). SPION@MNs SEM image, scale at 200 nm; inset: particle size distribution (n=50) (c). TEM micrograph of SPION@MNs depicting single Fe₃O₄ magnetic cores encapsulated within silica mesoporous matrix, at 55 nm scale (d). 72 K N₂-isotherms of both MNs and SPION@MNs systems, again confirming its mesoporosity and availability

for drug encapsulation (e). SPION@OA cores magnetization saturation curves (f). Summary of both MNs and SPION@MNs physical properties from surface area to pore diameter and volume, TEM size and surface zeta potential (g).

The successful incorporation of the magnetic oleic acid-coated iron-oxide nanocores within the mesoporous silica shell was not only confirmed by TEM imaging of SPION@MNs where clear core-shell SPION-MNs systems are present, but also by its magnetic susceptibility that arises from the magnetic core. Both type of particles showed the typical negative zeta potential (ZP), in water, of non-templated, bare mesoporous silica particles/shells that arise from their surface -OH groups. From their ZP results we can predict possible the role of each nanoparticle as interferent in conventional OD assays, with more neutral ZPs like that of MNs leading to higher particle aggregation and higher interference with OD signal and its reproducibility.

Following the synthesis of the nanomaterials, loading assays were conducted using epirubicin (EPI), doxorubicin (DOX) and ofloxacin (OFLO) as three model drugs (Figure S-7.6) and both MNs and SPION@MNs were successfully loaded with either single-drug or 1:1 two-drugs formulations (i.e., DOX+OFLO or EPI+OFLO) (Table 7.1). This was also confirmed through spectroscopic analysis of the loaded nanoparticles where each drug absorbance profile overlapped with that of each nanoparticle (Figure S-7.5). The loaded nanoparticles were also used as testing samples against the robustness of the luminescence-based assay. The particles' capacity to release the loaded drugs was also assessed at pH = 7.4 and pH = 4.0, with both loaded MNs and SPION@MNs systems showing significant release, similar to previously reported MNs systems [38, 39], and thus an additional potential antimicrobial activity (Table S-7.1).

Table 7.1 - MNs and SPION@MNs loading assay loading percentages and encapsulation efficiency (EE) for single and dual-combinatory formulations of EPI, DOX and OFLO.

Nanomaterial	Drug	Loading %	EE (mg/g)
MNs	EPI	87.1 ± 9.6	21.0 ± 3.9
	DOX	72.4 ± 4.7	19.1 ± 0.1
	OFLO	38.8 ± 12.9	9.8 ± 3.0
	EPI + OFLO	<i>Epirubicin</i>	90.9 ± 6.7
		<i>Ofloxacin</i>	58.1 ± 8.3
	DOX + OFLO	<i>Doxorubicin</i>	83.7 ± 14.1
		<i>Ofloxacin</i>	57.8 ± 21.2
			14.5 ± 3.3

SPION@MNs	EPI		100.0 ± 0.0	25.0 ± 0.0
	DOX		100.0 ± 0.0	25.0 ± 0.0
	OFLO		99.7 ± 0.2	24.9 ± 0.1
	EPI + OFLO	<i>Epirubicin</i>	88.3 ± 9.1	22.1 ± 2.3
		<i>Ofloxacin</i>	47.3 ± 8.4	11.8 ± 2.1
	DOX + OFLO	<i>Doxorubicin</i>	80.5 ± 18.7	20.1 ± 4.7
		<i>Ofloxacin</i>	47.2 ± 10.0	11.8 ± 2.5

7.3.4 Nanoparticles antimicrobial activity against *E.coli* and *E.coli* Lux

The final assessment of the method's robustness and usefulness is achieved by incubating *E. coli* Lux bacteria with the selected nanosystems (i.e., MNs and SPION@MNs-based nanoparticles), for a period of incubation of 8h, previously determined as the optimal incubation period for bioluminescence measurements, with further determination of their MIC₅₀ and/or MIC₉₀. The assays were paralleled by incubating the same nanosystems against the parental *E. coli* strain, for 24h, for comparison. According to what was stated in other sections, *E. coli* Lux and its parental *E. coli* strain were considered equivalents and thus equally susceptible to the selected antibiotic. While MNs systems were tested for concentrations of 362 to 0.5 µg/mL, via sequential dilutions, SPION@MNs systems were tested for 357 to 0.5 µg/mL. Each material was incubated without bacteria and used as negative controls and background signals for the calculation of bacterial growth. Similarly, bacteria were incubated in the same conditions, without the presence of any nanosystem, and taken as bacteria positive controls. The action of each free drug formulation was also assessed and used as drug positive controls (Figure S-7.7).

From the incubation of MNs against both the parental *E. coli* strain (Figure 7.5) and the *E. coli* Lux transformant (Figure 7.6) it is clear that only those containing OFLO have significant inhibitory activity, as expected. Be it in a single or dual-combinatory formulations (MNs@OFLO, MNs@EPI-OFLO and MNs@DOX-OFLO), the obtained MIC₅₀ was of ca. 1 µg/mL. Despite its undoubtful strong activity, the determination of a correct MIC₅₀, via OD₆₀₀ signal after 24h, is hampered by the visible large associated errors, as is the case of MNs@OFLO and MNs@DOX with errors of about ca. 50% and 20%, respectively. This issue persists for the other MNs, whose associated errors account for a high variability (ca. 20%) of the calculated value (Figure 7.5). The calculated value is entirely dependent on the turbidity of the incubated suspension (OD₆₀₀ signal), comprised of the turbidity of the bacteria and that of the nanomaterial, as well as on the latter's homogeneity in the suspension, inducing

the propagation of errors during MIC calculation and significant fluctuations in the obtained bacterial growth trend of *E. coli*. The vulnerability of OD₆₀₀-based results to colorimetric samples (i.e., EPI and DOX) is perceived when looking at the obtained bacterial growth for each free drug formulation (Figure S-7.7), with EPI and DOX having a fluctuating trend along the tested drug concentration gradient, despite their lower associated errors due to the lack of nanomaterials.

The introduction of LUX₄₉₀ signal as a probe for the calculation of bacterial growth, and consequently of each nanosystem MIC₅₀, favors the elimination of high standard deviations and signal fluctuation, by discarding any interference caused by nanoparticles. The MIC₅₀ obtained by the 8h incubating *E. coli* Lux method, although similar for MNs@DOX-OFLO and MNs, differ from those obtained via 24h OD₆₀₀ for the rest of the systems. Whereas with 24h OD₆₀₀ signal, no decrease in bacterial growth was registered for both 362 µg/mL MNs@EPI and MNs@DOX, with LUX₄₉₀ growth percentages relative to the control were of $38.73 \pm 9.71\%$ and $57.68 \pm 8.26\%$ for 362 µg/mL, respectively. While in the first approach the results point towards a non-existence of a MIC, with the luminescence method it is clear that the effect of both MNs@EPI and MNs@DOX at a concentration of 362 µg/mL is rather that of a MIC₅₀, with a *ca.* 50% reduction of the metabolic activity, LUX₄₉₀ signal and consequently of bacterial growth. A clear case of variability reduction is that of MNs@DOX+OFLO, for which the 8h LUX₄₉₀-calculated growth is $55.87 \pm 7.62\%$, rather than the $77.64 \pm 21.35\%$ calculated from the 24h OD₆₀₀ signal, where no sensible determination of the MIC₅₀ was possible. The same was obtained for MNs@EPI+OFLO, with LUX₄₉₀ signal pointing towards a growth of $0.39 \pm 0.88\%$ at a concentration of 1 µg/mL, and thus a possible MIC₉₀ rather than a MIC₅₀, as suggested by the results calculated from OD₆₀₀.

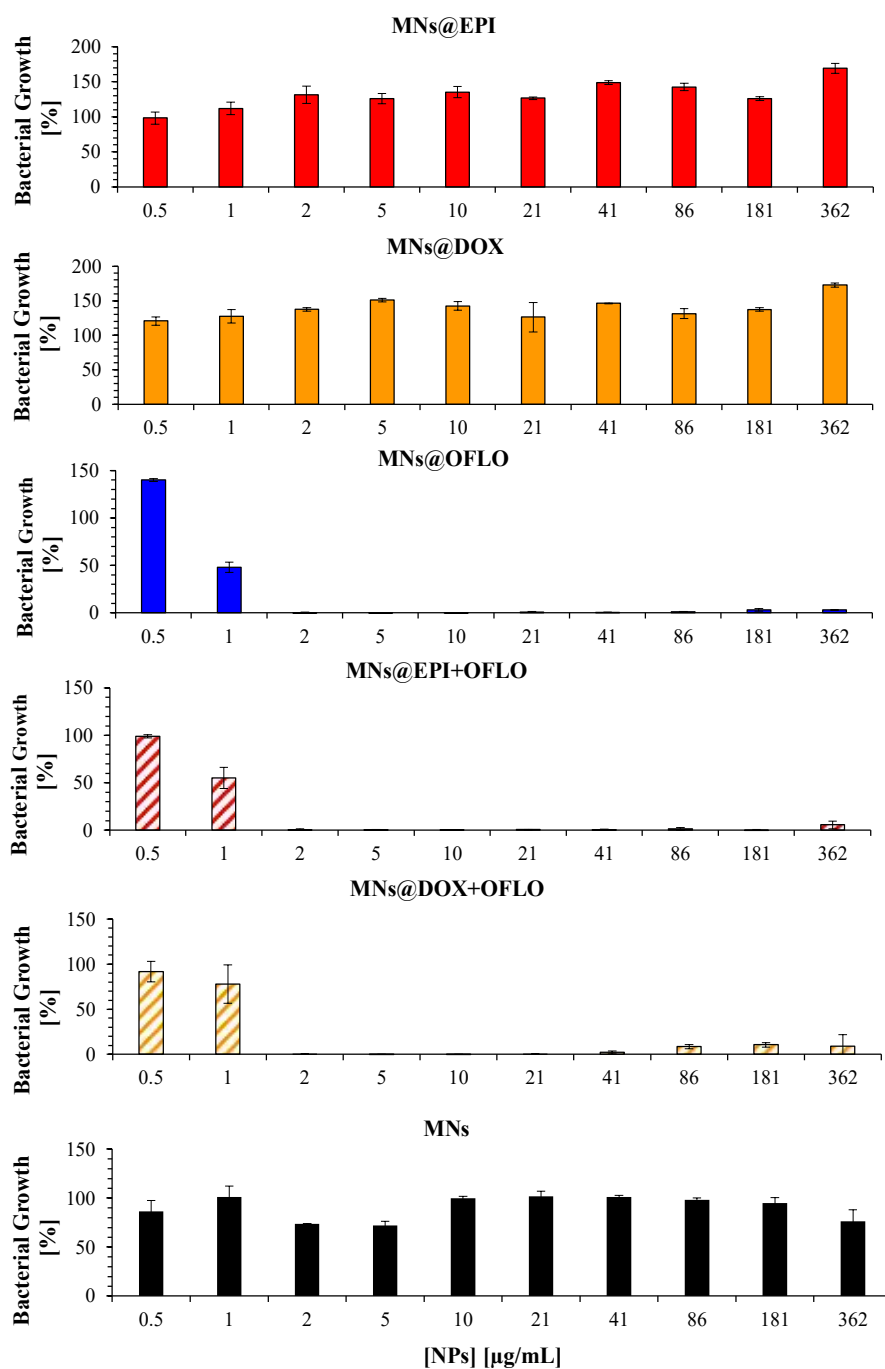


Figure 7.5 - Antimicrobial activity of drug-loaded MNs on the parental *E. coli* strain, measured as the bacterial growth calculated by OD₆₀₀, for 24h assay.

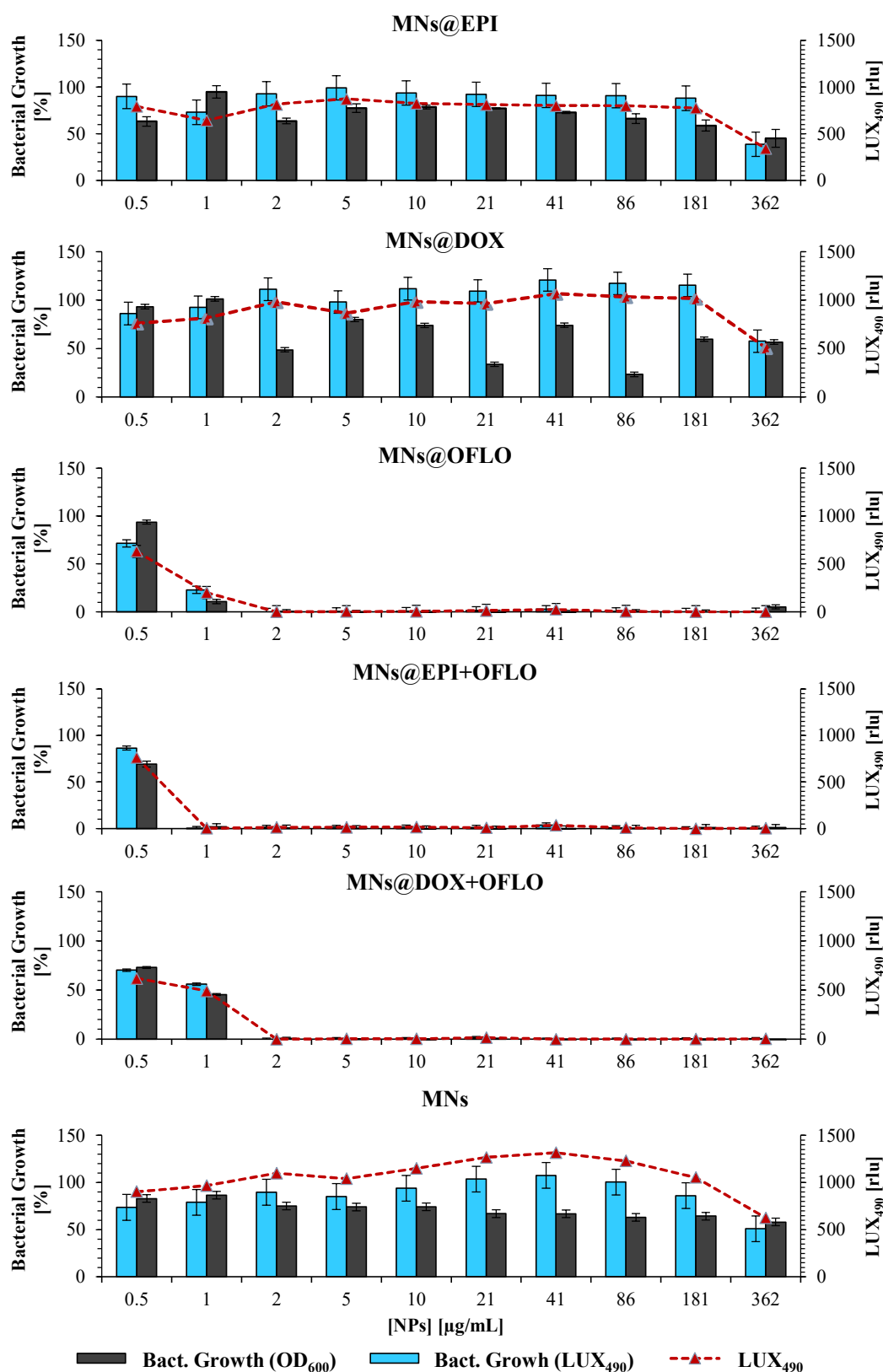


Figure 7.6 - Antimicrobial activity of drug-loaded MNs on the mutant *E. coli* Lux strain, measured as bacterial growth calculated by OD₆₀₀ and LUX₄₉₀, for 8h assay; raw LUX₄₉₀ signal alone is depicted by the red dotted line and follows the same trend as the calculated bacterial growth.

The statistical analysis (t -test, $\alpha = 0.05$, $n = 4$) of the differences between the obtained MIC₅₀ and MIC₉₀ from both methods (Table S-7.2), and for each MNs nanosystem, showed that, even though similar in some cases, the introduction of LUX₄₉₀ gives significantly different growths from those obtained from OD₆₀₀ (Figure 7.7 and Table S-7.3). Justifying, thus, a correction of the initially OD perceived MIC₅₀ to the new LUX-related and metabolism-related MIC₅₀.

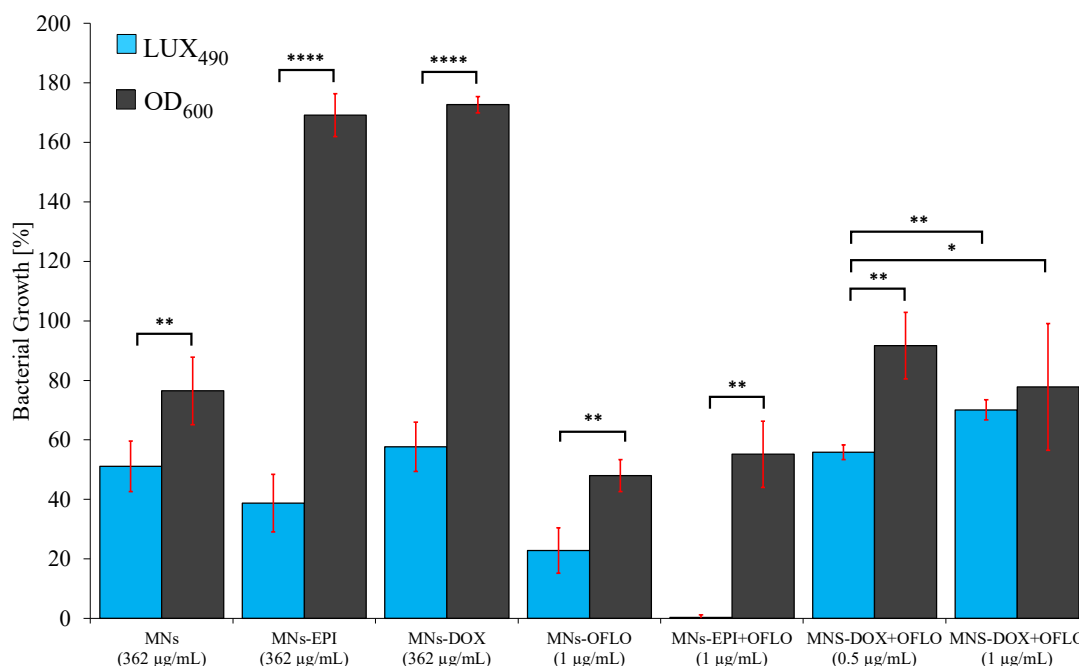


Figure 7.7 - Statistical comparison of the obtained relative growth reduction, via OD₆₀₀ 24 h-assaying the parental *E.coli* and LUX₄₉₀ 8 h-assaying *E. coli* Lux, for all MNs systems (concentrations related to nanoparticle); statistically significant levels represented as * $\equiv p \leq 0.05$, ** $\equiv p \leq 0.01$, *** $\equiv p \leq 0.001$.

A similar assessment was done for the SPION@MNs, by comparing the bacterial growth obtained from 24h OD₆₀₀ (Figure 7.8) and 8h LUX₄₉₀ (Figure 7.9) signals. Regarding SPION@MNs, SPION@MNs-EPI and SPION@MNs-DOX nanosystems, whereas the 24h OD₆₀₀-calculated MIC₅₀ stay between a concentration range of 357 - 170 µg/mL, a shift in the obtained MICs to smaller concentrations of 170 to 81 µg/mL is seen when the 8h LUX₄₉₀ signal is used. In this last case, a clear decreasing growth gradient is seen as particle concentration increases, without the abrupt decreases that are registered for 24h OD₆₀₀-calculated growth, as well as for 8h OD₆₀₀ growth in *E. coli* Lux. The possible MIC₉₀ of these nanosystems however is the same, with growth of *ca.* 0% for a concentration of 357 µg/mL, calculated for both LUX₄₉₀ and OD₆₀₀. In brief, by using the LUX₄₉₀ signal after 8h of incubation, fluctuations in the growth gradient can be eliminated and a more sensible determination and distinction of MIC₅₀ and MIC₉₀ is possible.

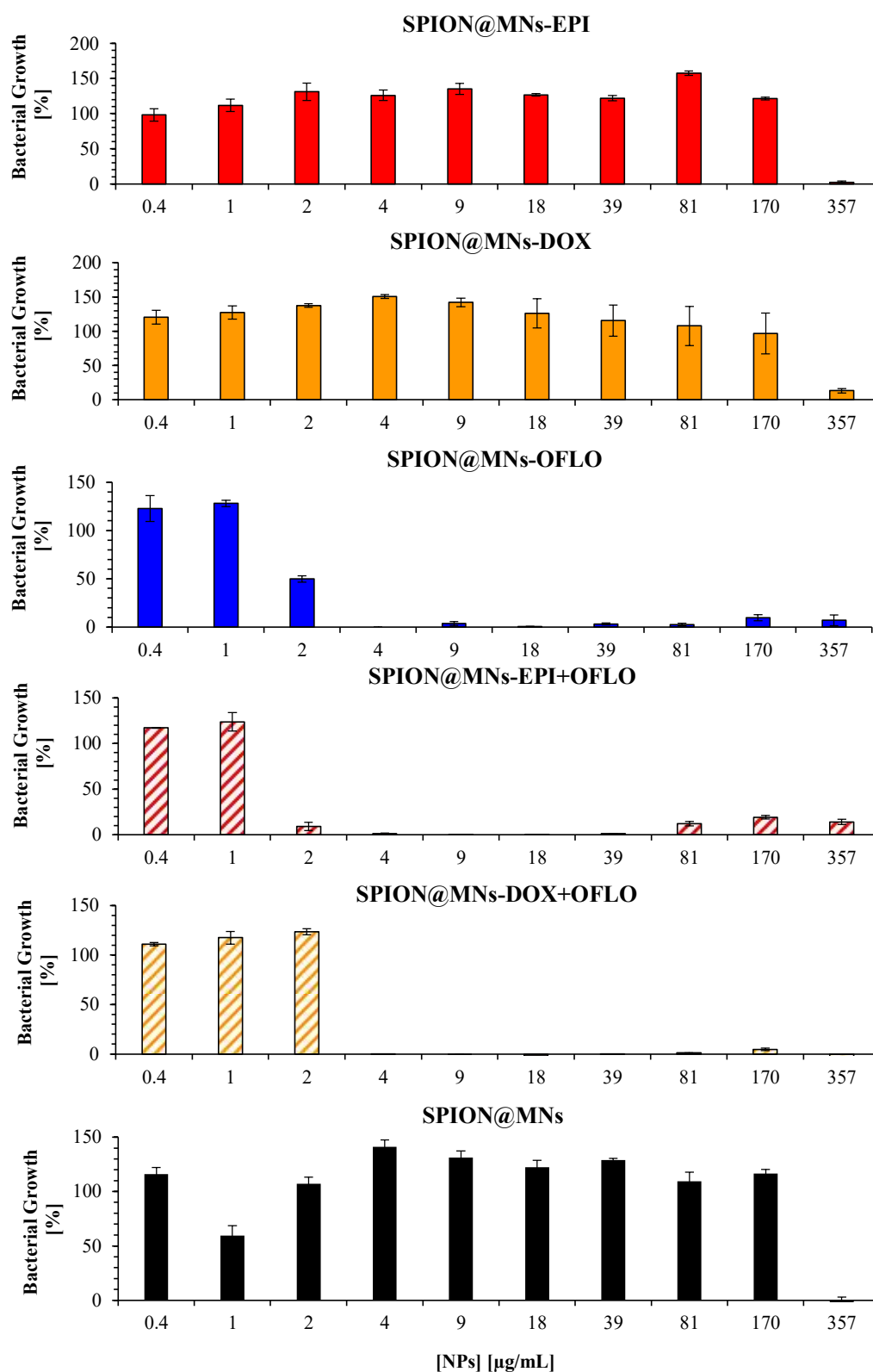


Figure 7.8 - Antimicrobial activity of drug-loaded SPION@MNs on the parental *E. coli* strain, measured as the bacterial growth calculated by OD₆₀₀, for 24 h assay.

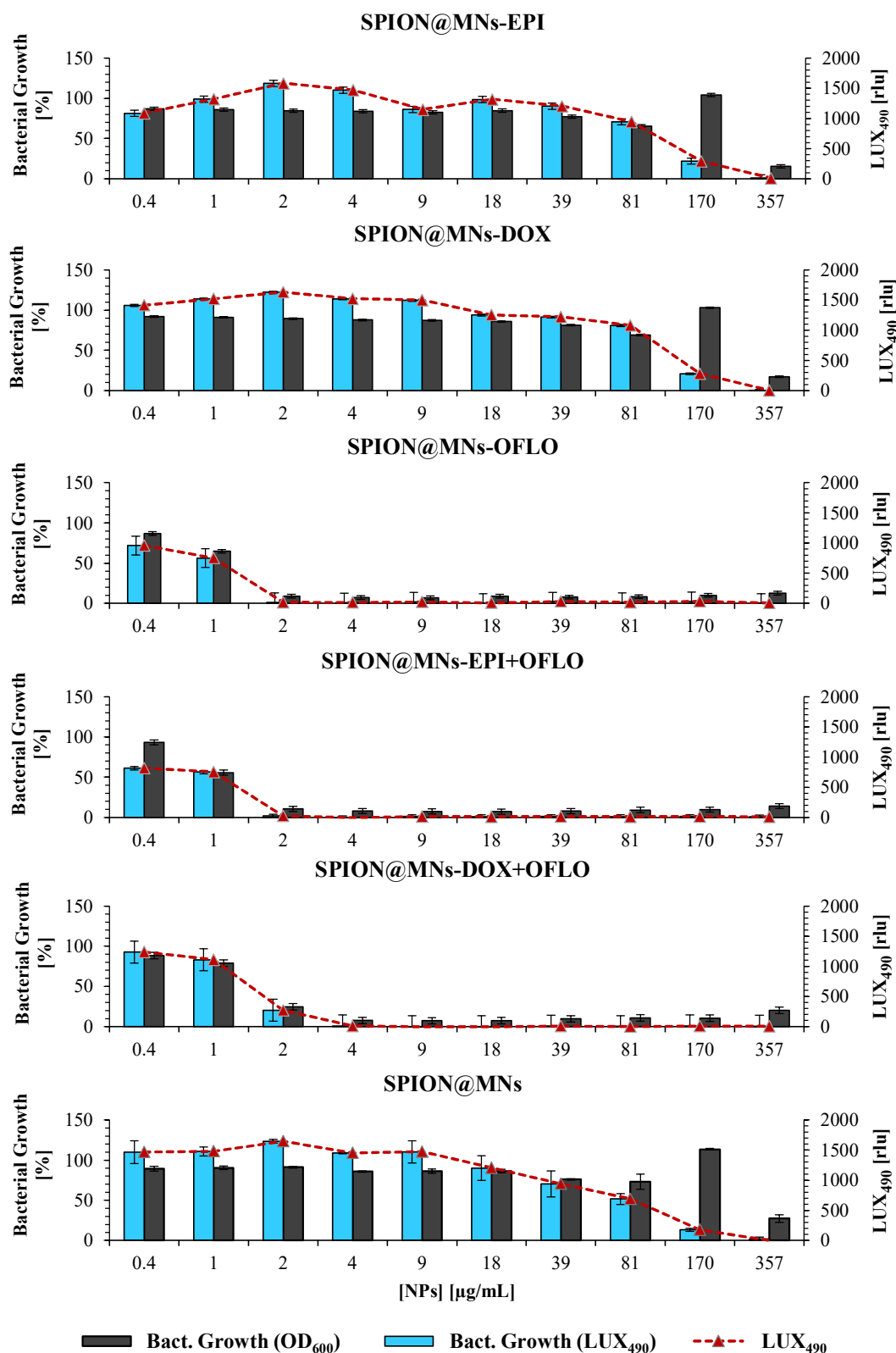


Figure 7.9 - Antimicrobial activity of drug-loaded SPION@MNs on the mutant *E. coli* Lux strain, measured as bacterial growth calculated by OD₆₀₀ and LUX₄₉₀, for 8 h assay; raw LUX₄₉₀ signal alone is depicted by the red dotted line and follows the same trend as the calculated bacterial growth.

When comparing the results obtained, through both methods, for SPION@MNs-OFLO and SPION@MNs-EPI+OFLO nanosystems, the same trend is seen with 8h LUX₄₉₀-calculated growth that produce a smoother decreasing gradient, with less variability, as particle concentration increases. In these cases, a correct determination of the MIC₅₀ is possible, with SPION@MNs-OFLO and SPION@MNs-EPI+OFLO having significant inhibitory activity at concentrations of 1 and 0.4 µg/mL, respectively, and a common MIC₉₀ at 2 µg/mL. Lastly, a more sensible determination of both MIC₅₀ and MIC₉₀ of SPION@MNs-DOX+OFLO nanoparticles is also possible, with 8h LUX₄₉₀ signal pointing to a 50% inhibitory concentration at 2 µg/mL, contrary to those obtained from the 24h OD₆₀₀ signal, and a common 90% inhibitory concentration at 4 µg/mL.

The substitution of OD₆₀₀ signal for that of LUX₄₉₀ as a more sensible probe for bacterial growth calculation, and the correct determination of MIC₅₀ and MIC₉₀, is again supported by the statistical analysis of growth differences between the obtained MIC₅₀ and MIC₉₀ for both methods (Tables S-7.4 and S-7.5). As can be seen in Figure S-7.8, the obtained growth from LUX₄₉₀ not only are significantly different (for $\alpha = 0.05$, $n = 4$) between adjacent concentrations, but also between those obtained from 24h OD₆₀₀ signal.

Lastly, a test to the new method's reproducibility was achieved by repeatedly assaying *E. coli* Lux with all OFLO-containing drug combinations, in different days and with different operators. The statistical analysis between the obtained MIC₅₀ and MIC₉₀ of both assays (2-tailed *t*-test to the means, for $\alpha = 0.05$, $n = 2$) confirmed that there is no significant statistical difference between the results of each assay and thus that the method is reproducible (Figure S-7.9).

7.4 Conclusions

The herein detailed luminescence-based antimicrobial activity assay presents several advantages when compared to the traditional OD₆₀₀-based assays. The first main advantage is the reduction of incubation time from 24 to only 8 hours. This is related to the time at which optimal luminescence is obtained after incubation and at which bacterial growth is in its exponential phase. This shorter incubation time allows for the acquisition of results concerning bacterial growth in the same day the assay is conducted.

The second main advantage relates to the type of measurements. Unlike OD₆₀₀-based assays, by measuring LUX₄₉₀ is possible to not only calculate definite percentages of bacterial growth, but also determine and more easily distinguish between MIC₅₀ and MIC₉₀

concentrations by the direct analysis of the LUX₄₉₀ raw data. Moreover, it allowed for a reduction in the overall variability and uncertainty of the obtained results. This is of extreme importance when working with nanoparticle suspensions and colorimetric compounds, whose turbidity and colour (usual interferents in OD₆₀₀ calculations) no longer need to be taken into consideration in the overall MIC calculation. Such is the case of MNs and SPION@MNs colorimetric formulations (i.e., with DOX and EPI) where otherwise determined MIC₅₀ were correctly assessed as rather MIC₉₀, moving the real assessment of MIC₅₀ to lower concentrations. This is possible since luminescence is directly correlated with the metabolic response of the bacteria to the provided stimulus, and not to the physical behaviour of the suspension, providing more accurate results.

In light of current worldwide efforts to find standard and reliable toxicological procedures for the determination of nanoparticles toxicity and antimicrobial activities, we believe that this approach is an important tool that contributes to the standardization of methods in potential industrial applications of nanoparticles, as it reduces samples background interference and focuses on luminescence as a direct probe for bacterial metabolic activity and growth, as well as a the correct determination of the antimicrobial activity of the said nanomaterials.

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7.8 Supplementary Materials

7.8.1 Experimental Procedures

7.8.1.1 Plasmid Extraction and Purification

For the extraction of the pMV306G13+Lux plasmid [1], the acquired plasmid-containing growth strain, *E. coli* DH5 α , was incubated in TSB for 16 h, at 37 °C, 210 rpm, and the plasmid was extracted by applying the protocol detailed in the Thermo Scientific™ GeneJET Plasmid Miniprep Kit #K0502, yielding a concentration of 27 ng/mL.

Subsequently, the pDNA solution was de-salted to remove salts that could interfere with the electroporation procedure. Briefly, to precipitate the pDNA, 5 μ L of a 3 M sodium acetate solution (pH 5.2) and 100 μ L of ethanol 96% were added to 50 μ L of the plasmid solution. The mixture was left at -70 °C for 15 minutes and then, centrifuged (12 000 x g, 15 min) at 4 °C. The pellet was later washed with ethanol 70% and left to dry at 37 °C. Finally, the dry pellet was resuspended in 50 μ L of sterile deionized (DI) Milli-Q water and stored at -70 °C. The resulting plasmid solution was quantified in a Nanodrop 1000 spectrophotometer, in Nucleic Acids mode. Purity was accessed according to the 260/280 nm ratio.

7.8.1.2 Development of the new *E. coli* Lux strain

7.8.1.2.1 Competence induction

Competent bacteria were obtained following an adapted protocol from the MicroPulser™ Electroporation Apparatus Operating Instructions and Applications Guide from BioRad126 [2]. In detail, *Escherichia coli* ATCC® 8739™ was grown in L-broth (10 g tryptone, 5 g yeast extract and 5 g NaCl in 1 L Milli-Q water) overnight, at 37 °C and 210 rpm. Then, 500 mL of L-broth were inoculated with 1/100 volume of the bacterial culture. The suspensions were incubated at 37 °C and 250 rpm, until an OD 600 nm of 0.5 - 0.7 was achieved. The cells were cooled on ice for 20 min and centrifuged (4000 x g, 15 min) at 4 °C. All subsequent centrifugations were conducted with the same parameters. Supernatants were discarded and the pellet resuspended in 500 mL of cold 10% glycerol, followed by another centrifugation

cycle. This step was repeated 2 additional times, decreasing the resuspending volume of 10% glycerol to 250 and 20 mL, respectively. Following this centrifugation step, the supernatant was again discarded, and the pellet resuspended in 2 mL of cold 10% glycerol. From this suspension, 150 μ L aliquots were prepared and stored at -70 °C.

7.8.1.2.2 Bacterial Transformation by Electroporation

Electroporation protocols were performed in the Laboratory of Genetics of NOVA Medical School Centro de Estudos de Doenças Crônicas (CEDOC). 8 μ L of the purified pMV306G13+Lux plasmid was added to a 150 μ L competent bacterial aliquot, followed by a gentle homogenization. The suspension was incubated for a brief period on ice and was then transferred to a cold electroporation cuvette. The electroporation parameters were of 2.1 kV, 100 Ω and 25 μ F. After the pulse, a τ = 2.4 was obtained.

The sample was retrieved, and 1 mL of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM monohydrated glucose) was added. Bacterial suspensions were incubated at 37 °C and 210 rpm, for *ca.* 1 h, and then plated in kanamycin-TSA (35 μ g/mL kanamycin).

Plates were incubated at 37 °C, overnight. Transformed *E. coli* cells (*E. coli* Lux strain) were streaked in TSA plates, supplemented with 35 μ g/mL of kanamycin, and incubated at 37 °C overnight. Finally, isolated luminescent colonies were inoculated into TSB (35 μ g/mL) and incubated at 37 °C and 210 rpm. Glycerol stocks (15%) were prepared and stored at -70°C.

7.8.2 Tables

Table S - 7.1 - Loaded MNs and SPION@MNs cumulative release assay results, in PBS 0.01M pH 7.4 and pH 4.0, at 24h.

Nanomaterial	Drug		Release % (pH 7.4)	Release % (pH 4.0)
MNs	EPI		12.7 ± 3.2	45.8 ± 2.2
	DOX		8.4 ± 0.2	15.3 ± 0.02
	OFLO		41.5 ± 1.5	52.1 ± 1.3
	EPI + OFLO	<i>Epirubicin</i>	12.7 ± 0.7	57.3 ± 0.5
	(EO)	<i>Ofloxacin</i>	46.7 ± 2.2	67.4 ± 0.8
	DOX + OFLO	<i>Doxorubicin</i>	25.0 ± 0.3	62.1 ± 4.3
	(DO)	<i>Ofloxacin</i>	38.9 ± 1.4	43.5 ± 2.3
SPION@MNs	EPI		8.3 ± 2.0	100.0 ± 57.0
	DOX		10.7 ± 4.3	44.6 ± 23.9
	OFLO		23.7 ± 0.9	20.8 ± 0.5
	EPI + OFLO	<i>Epirubicin</i>	14.5 ± 1.3	80.7 ± 39.3
	(EO)	<i>Ofloxacin</i>	38.4 ± 9.8	43.3 ± 7.6
	DOX + OFLO	<i>Doxorubicin</i>	23.1 ± 9.4	63.0 ± 21.6
	(DO)	<i>Ofloxacin</i>	28.8 ± 6.4	58.7 ± 10.4

Table S - 7.2 - Inhibitory concentrations of all SPION@MNs systems against *E.coli* and *E.coli* Lux, for 24h and 8h assays, with respective bacterial growth (average ± standard deviation).

Nanosystem	[NP] [µg/mL]	LUX ₄₉₀		OD ₆₀₀	
		Bacterial growth [%]		Bacterial growth [%]	
		Average (n=4)	Standard deviation	Average (n=4)	Standard deviation
MNs	362	51.11	± 8.5	76.51	± 11.32
MNs-EPI	362	38.73	± 9.71	169.13	± 7.19
MNs-DOX	362	57.68	± 8.26	172.68	± 2.73
MNs-OFLO	1	22.85	± 7.62	48.01	± 5.38
MNs-EPI+OFLO	1	0.39	± 0.88	55.20	± 11.10
MNS-DOX+OFLO	0.5	55.87	± 2.49	77.80	± 21.30
MNS-DOX+OFLO	1	70.10	± 3.37	91.70	± 11.20

Table S - 7.3 - Statistical analysis of the bacterial growth averages obtained from LUX₄₉₀ (8h) and OD₆₀₀ (24h), for all inhibitory concentrations of MNs systems against *E.coli* Lux and *E.coli*, respectively; *t*-test (two-tailed) for $n = 4$, $\alpha = 0.05$, $t(3, 0.05/2) = 3.128$ ($H_0: \bar{X}_1 = \bar{X}_2$, $H_1: \bar{X}_1 \neq \bar{X}_2$, where 1 and 2 stand for LUX or OD depending on the type of interaction).

Nanosystem	[NP] interaction [$\mu\text{g/mL}$]	<i>t</i> -score	Result	<i>p</i> -value	Classification	Type of interaction
MNs	362 x 362	-6.80	Reject H_0	0.00650	**	LUX x OD
MNs-EPI	362 x 362	-39.97	Reject H_0	0.00003	****	LUX x OD
MNs-DOX	362 x 362	-29.49	Reject H_0	0.00009	****	LUX x OD
MNs-OFLO	1 x 1	-9.32	Reject H_0	0.00261	**	LUX x OD
MNs-EPI+OFLO	1 x 1	-9.91	Reject H_0	0.00219	**	LUX x OD
MNS-DOX+OFLO	0.5 x 0.5	-6.56	Reject H_0	0.00720	**	LUX x OD
	1 x 1	-0.73	Accept H_0	0.51823	ns	LUX x OD
	0.5 x 1	-4.04	Reject H_0	0.02722	*	LUX x OD
	0.5 x 1	-2.07	Accept H_0	0.12987	ns	OD x LUX
	0.5 x 1	-12.52	Reject H_0	0.00109	**	LUX x LUX
	0.5 x 1	0.02	Accept H_0	0.98529	ns	OD x OD

ns $\equiv p > 0.05$, * $\equiv p \leq 0.05$, ** $\equiv p \leq 0.01$, *** $\equiv p \leq 0.001$, **** $\equiv p \leq 0.0001$

Table S - 7.4 - Inhibitory concentrations of all SPION@MNs systems against *E.coli* and *E.coli* Lux, for 24h and 8h assays, with respective bacterial growth (average $\pm$ standard deviation).

Nanosystem	[NP] [$\mu\text{g/mL}$]	LUX ₄₉₀		OD ₆₀₀	
		Bacterial growth [%]		Bacterial growth [%]	
		Average ($n=4$)	Standard deviation	Average ($n=4$)	Standard deviation
SPION@MNs	81	51.60	± 6.66	109.52	± 8.33
SPION@MNs	357	1.83	± 2.28	-4.81	± 7.69
SPION@MNs-EPI	170	21.91	± 0.95	121.48	± 1.99
SPION@MNs-EPI	357	0.93	± 1.11	2.37	± 1.77
SPION@MNs-DOX	170	20.86	± 2.27	96.93	± 29.68
SPION@MNs-DOX	357	0.40	± 1.85	13.27	± 3.28
SPION@MNs-OFLO	1	56.28	± 3.81	128.00	± 3.31
SPION@MNs-OFLO	2	1.30	± 0.58	49.90	± 3.30
SPION@MNs-EPI+OFLO	1	56.24	± 6.135	123.72	± 10.03
SPION@MNs-EPI+OFLO	2	1.94	± 0.85	9.06	± 4.71
SPION@MNS-DOX+OFLO	1	83.01	± 1.79	117.47	± 6.49
SPION@MNS-DOX+OFLO	2	20.30	± 7.25	123.56	± 3.14
SPION@MNS-DOX+OFLO	4	0.78	± 1.22	0.21	± 0.05

Table S - 7.5 - Statistical analysis of the bacterial growth averages obtained from LUX₄₉₀ (8h) and OD₆₀₀ (24h), for all inhibitory concentrations of SPION@MNs systems against *E.coli* Lux and *E.coli*, respectively; t-test (two-tailed) for $n = 4$, $\alpha = 0.05$, $t(3, 0.05/2) = 3.128$ ($H_0: \bar{X}_1 = \bar{X}_2$, $H_1: \bar{X}_1 \neq \bar{X}_2$, where 1 and 2 stand for LUX or OD depending on the type of interaction).

Nanosystem	[NP] interaction [$\mu\text{g/mL}$]	<i>t</i> -score	Result	<i>p</i> -value	Classification	Type of interaction
SPION@MNs	81 x 81	-23.18	Reject H_0	0.00018	***	LUX x OD
	357 x 357	1.81	Accept H_0	0.16858	ns	LUX x OD
	81 x 357	29.34	Reject H_0	0.00009	****	LUX x OD
	81 x 357	-26.89	Reject H_0	0.00011	***	OD x LUX
	81 x 357	15.90	Reject H_0	0.00054	***	LUX x LUX
	81 x 357	71.66	Reject H_0	< 0.00001	****	OD x OD
SPION@MNs EPI	170 x 170	-113.81	Reject H_0	< 0.00001	****	LUX x OD
	357 x 357	-2.09	Accept H_0	0.12753	ns	LUX x OD
	170 x 357	26.21	Reject H_0	0.00012	***	LUX x OD
	170 x 357	-145.79	Reject H_0	< 0.00001	****	OD x LUX
	170 x 357	73.35	Reject H_0	< 0.00001	****	LUX x LUX
	170 x 357	259.98	Reject H_0	< 0.00001	****	OD x OD
SPION@MNs DOX	170 x 170	-5.14	Reject H_0	0.01426	*	LUX x OD
	357 x 357	-9.50	Reject H_0	0.00247	**	LUX x OD
	170 x 357	6.42	Reject H_0	0.00766	**	LUX x OD
	170 x 357	-6.52	Reject H_0	0.00734	**	OD x LUX
	170 x 357	30.97	Reject H_0	0.00007	****	LUX x LUX
	170 x 357	5.67	Reject H_0	0.01085	*	OD x OD
SPION@MNs OFLO	1 x 1	-76.20	Reject H_0	0.00334	**	LUX x OD
	2 x 2	-29.92	Reject H_0	0.00008	****	LUX x OD
	1 x 2	6.71	Reject H_0	0.00675	**	LUX x OD
	1 x 2	-77.77	Reject H_0	0.00083	***	OD x LUX
	1 x 2	29.22	Reject H_0	0.00009	****	LUX x LUX
	1 x 2	607.55	Reject H_0	< 0.00001	****	OD x OD
SPION@MNs EPI-OFLO	1 x 1	-17.01	Reject H_0	0.00044	***	LUX x OD
	2 x 2	-3.08	Accept H_0	0.05426	ns	LUX x OD
	1 x 2	23.97	Reject H_0	0.00016	***	LUX x OD
	1 x 2	-24.37	Reject H_0	0.00015	***	OD x LUX
	1 x 2	17.87	Reject H_0	0.00038	***	LUX x LUX
	1 x 2	25.89	Reject H_0	0.00013	***	OD x OD
SPION@MNs DOX+OFLO	1 x 1	-11.05	Reject H_0	0.00159	**	LUX x OD
	2 x 2	-31.63	Reject H_0	0.00007	****	LUX x OD
	4 x 4	0.94	Accept H_0	0.41643	ns	LUX x OD
	1 x 2	-31.46	Reject H_0	0.00007	****	LUX x OD

1 x 4	92.14	Reject H ₀	< 0.00001	****	LUX x OD
2 x 4	5.55	Reject H ₀	0.01156	*	LUX x OD
1 x 2	60.38	Reject H ₀	0.00001	****	OD x LUX
1 x 4	36.60	Reject H ₀	0.00004	****	OD x LUX
2 x 4	84.74	Reject H ₀	< 0.00001	****	OD x LUX
1 x 2	17.87	Reject H ₀	0.00038	***	LUX x LUX
1 x 4	124.20	Reject H ₀	< 0.00001	****	LUX x LUX
2 x 4	5.47	Reject H ₀	0.01203	*	LUX x LUX
1 x 2	-2.14	Accept H ₀	0.12162	ns	OD x OD
1 x 4	36.13	Reject H ₀	0.00005	****	OD x OD
2 x 4	78.51	Reject H ₀	< 0.00001	****	OD x OD

ns $\equiv p > 0.05$, * $\equiv p \leq 0.05$, ** $\equiv p \leq 0.01$, *** $\equiv p \leq 0.001$, **** $\equiv p \leq 0.0001$

7.8.3 Figures

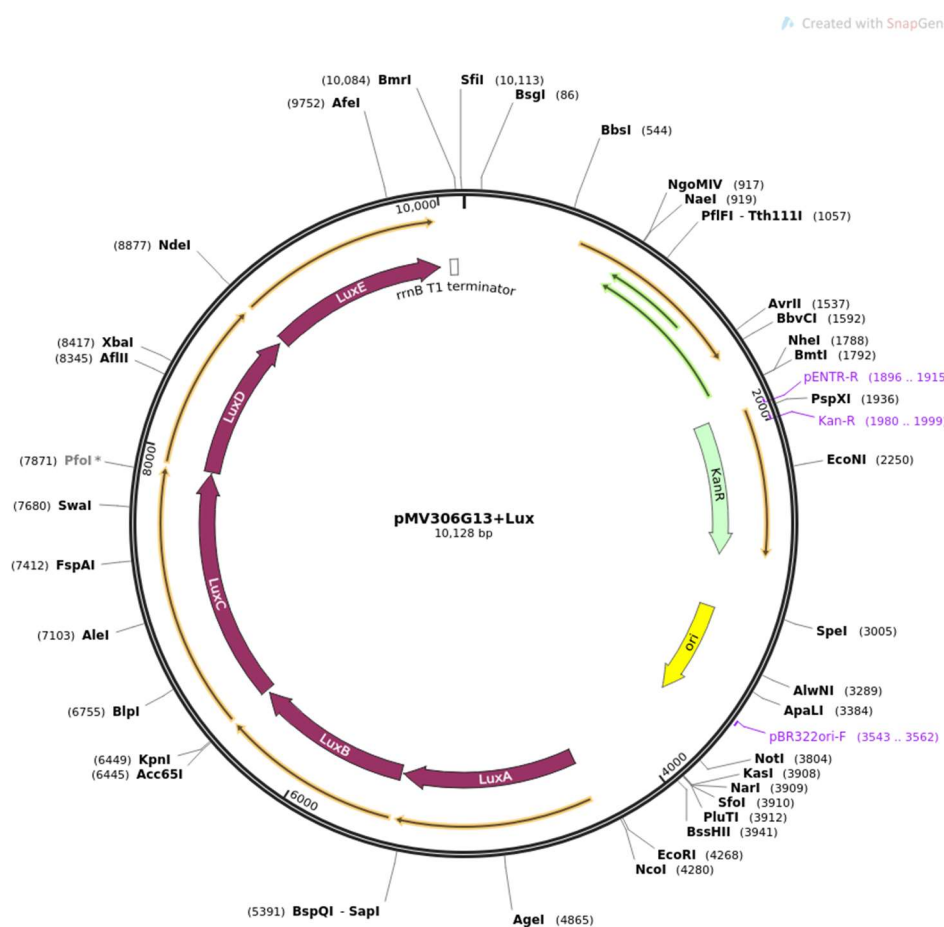


Figure S - 7.1 - pMV306G13+Lux plasmid constitution. Reproduced from [3].



Plate	Expected CFUs	Counted CFUs
TSA Lux3 (100 μ L of 10^{-4} dilution)	1000	>300
TSA Lux4 (100 μ L of 10^{-5} dilution)	100	115
TSA Lux5 (100 μ L of 10^{-6} dilution)	10	10

Figure S - 7.2 - Plates with the dilutions 10^{-4} (TSA Lux3), 10^{-5} (TSA Lux 4) and 10^{-6} CFU (TSA Lux5) for CFU counting of luminescent *E. coli* Lux suspension.

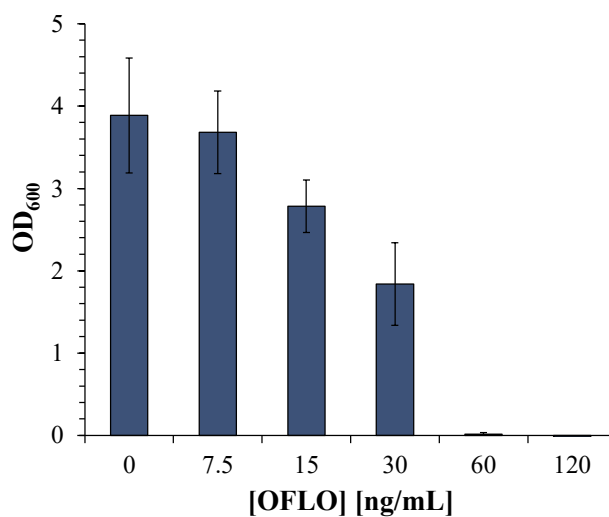


Figure S - 7.3 - The susceptibility of the wild-type parent *E. coli* (ATCC® 8739™) to ofloxacin, given as raw OD₆₀₀ vs concentration of OFLO.

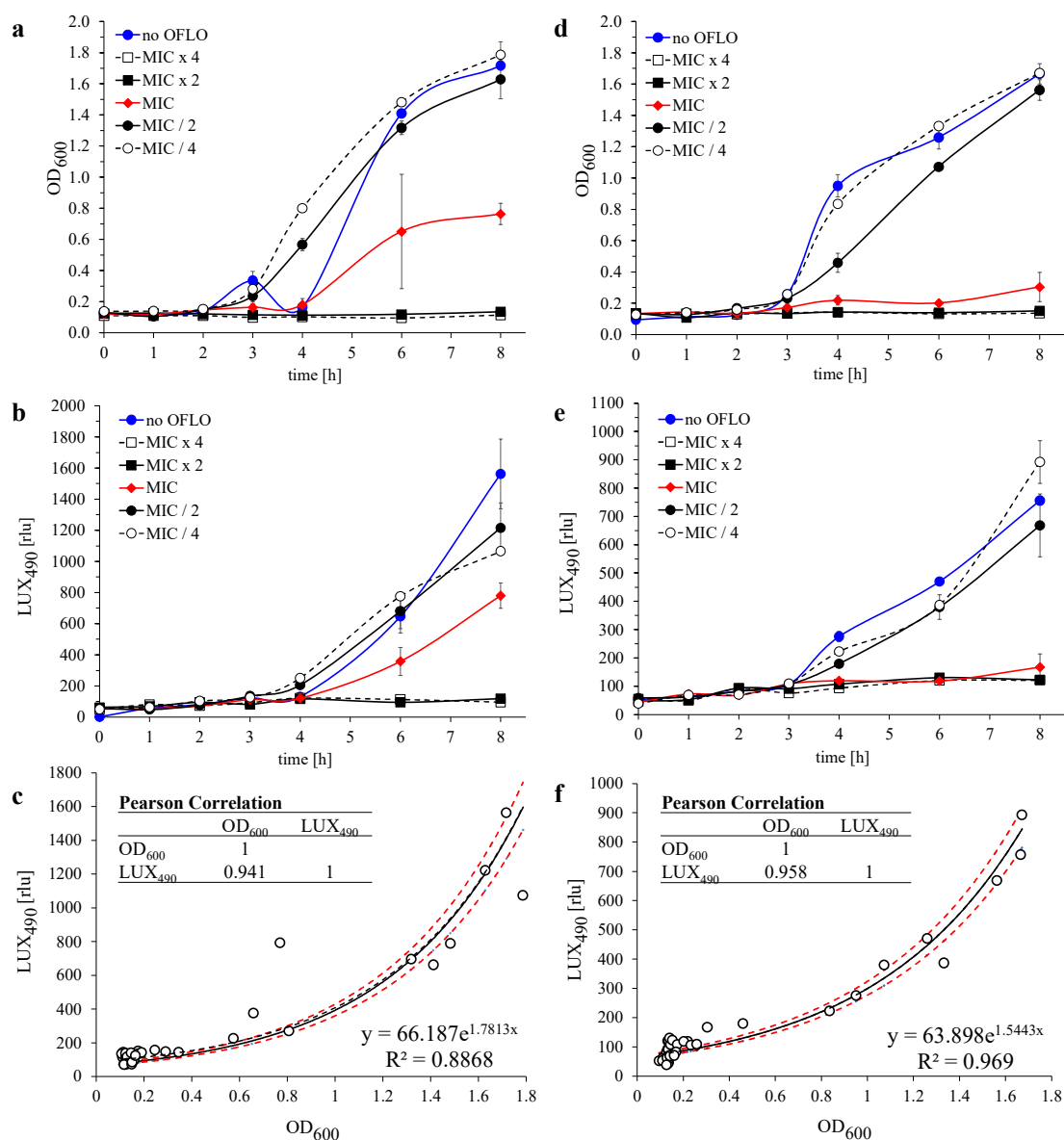


Figure S - 7.4 - *E. coli* Lux growth data under shaking (a,b) and static (d,e) conditions, collected as OD_{600} and LUX_{490} ; successive dilutions from MIC = 30 ng/mL. Plotting of all average OD_{600} vs LUX_{490} with respective exponential regressions and Pearson correlations, for shaking (c) and static (f) conditions.

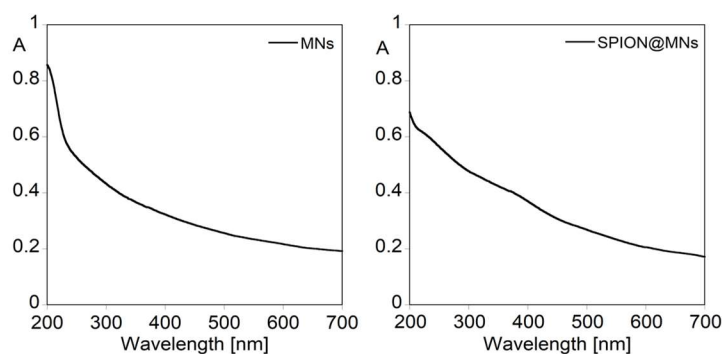


Figure S - 7.7.5 - Absorbance spectra of MNs and SPION@MNs, in water.

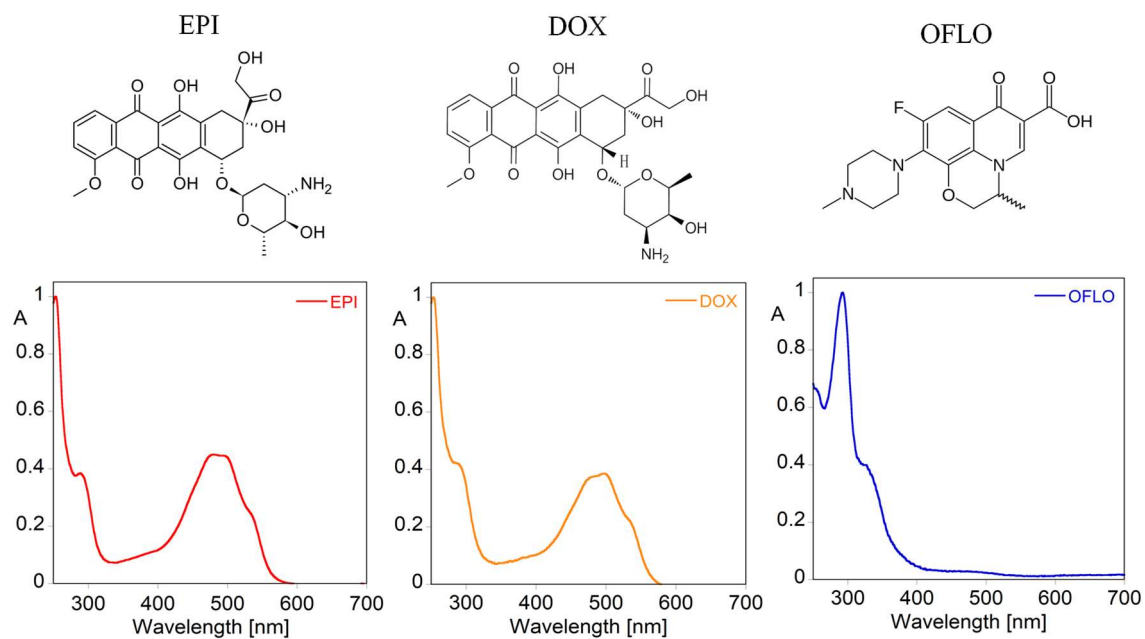


Figure S - 7.6 - Structural representation of all model drugs and respective absorbance spectra: epirubicin (EPI), doxorubicin (DOX) and ofloxacin (OFLO).

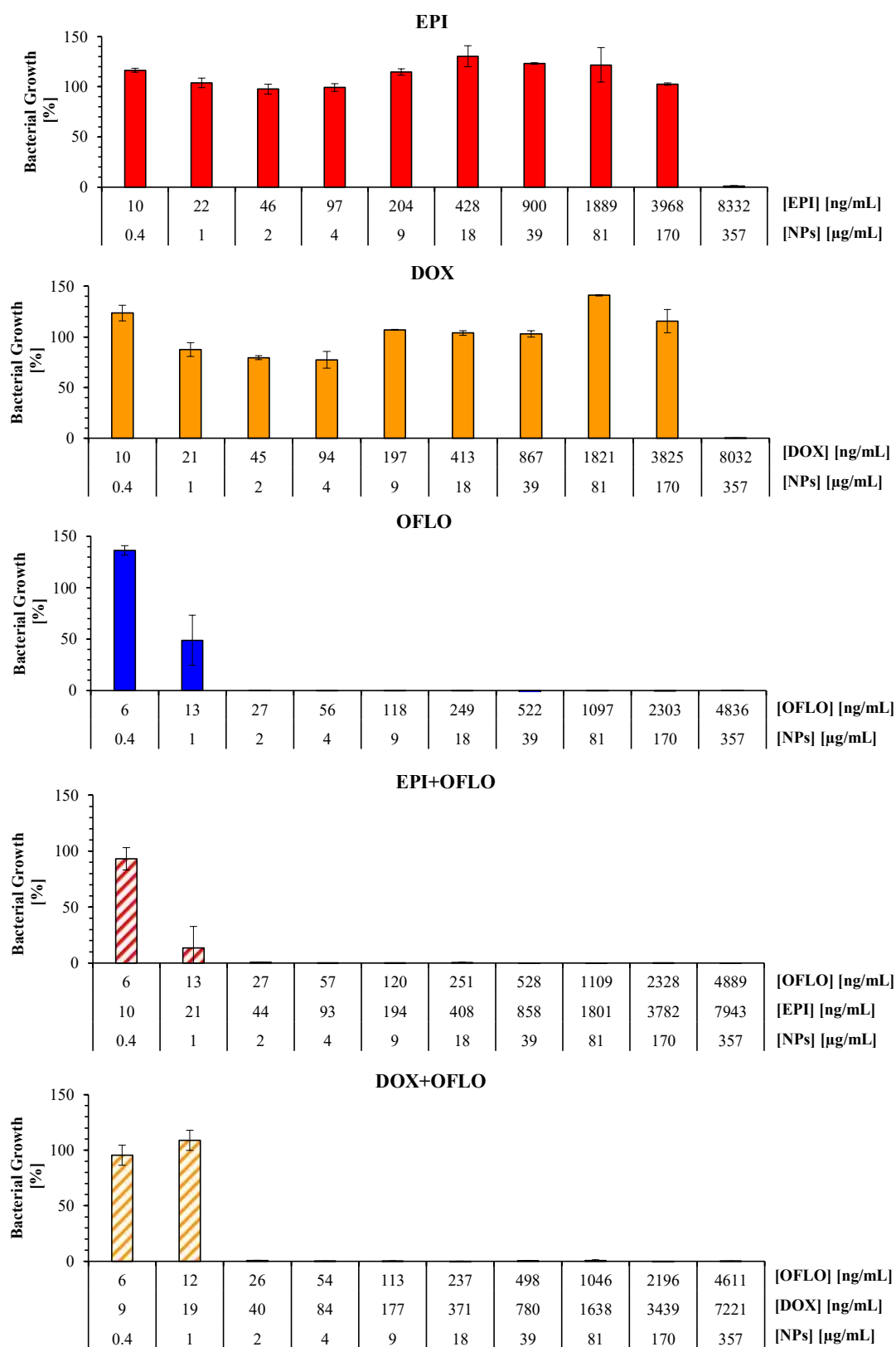
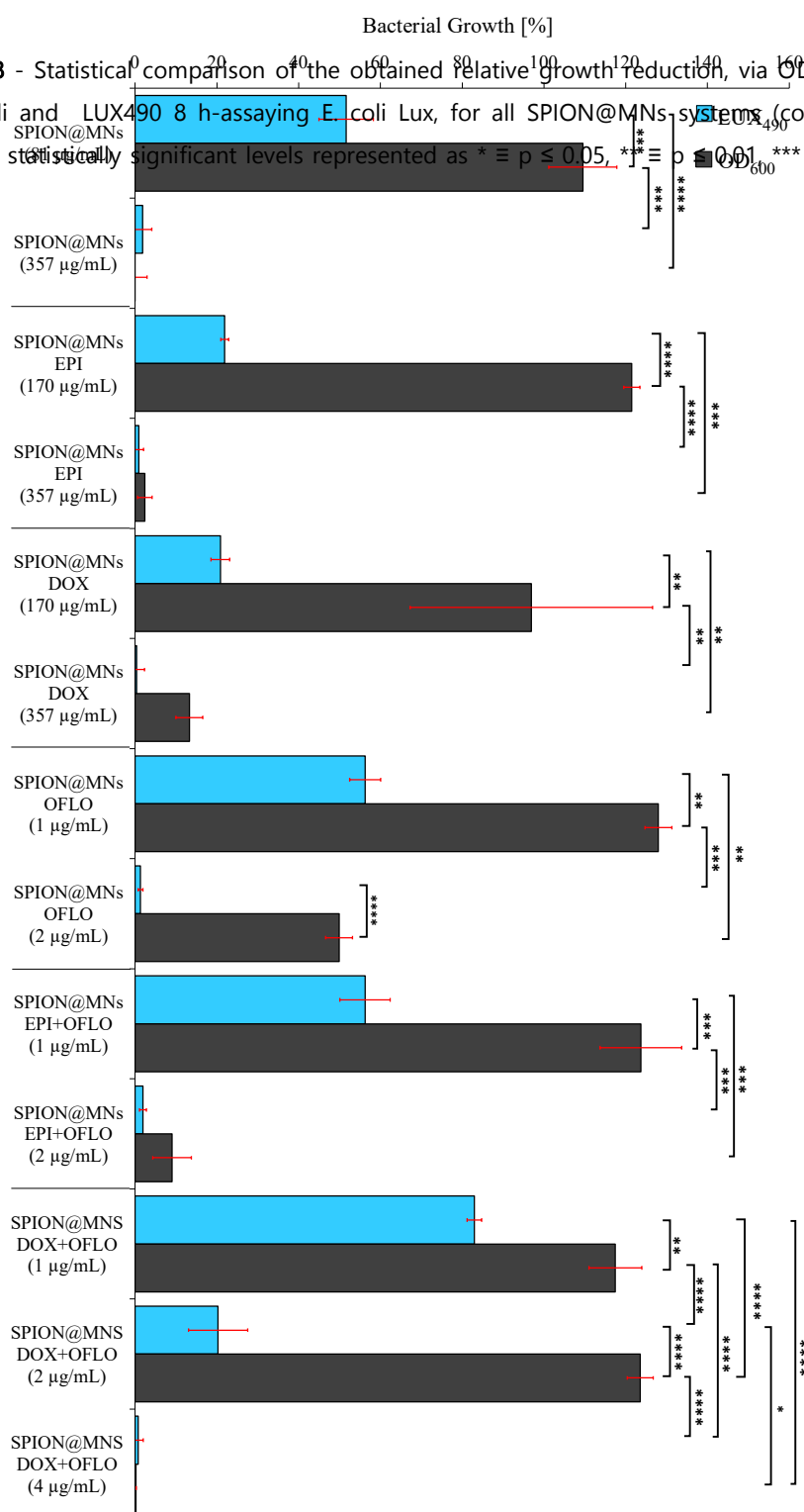


Figure S - 7.7 - Antimicrobial activity of single and combinatory drug controls on the parental *E. coli* strain, measured as the bacterial growth calculated by OD₆₀₀; drug concentrations in ng/mL and respective equivalent nanoparticles concentrations in μg/mL.

Figure S - 7.8 - Statistical comparison of the obtained relative growth reduction, via OD600 24 h-assaying the parental E.coli and LUX490 8 h-assaying E. coli Lux, for all SPION@MNs systems (concentrations related to nanoparticle); statistically significant levels represented as * $\equiv p \leq 0.05$, ** $\equiv p \leq 0.01$, *** $\equiv p \leq 0.001$, **** $\equiv p \leq 0.0001$.



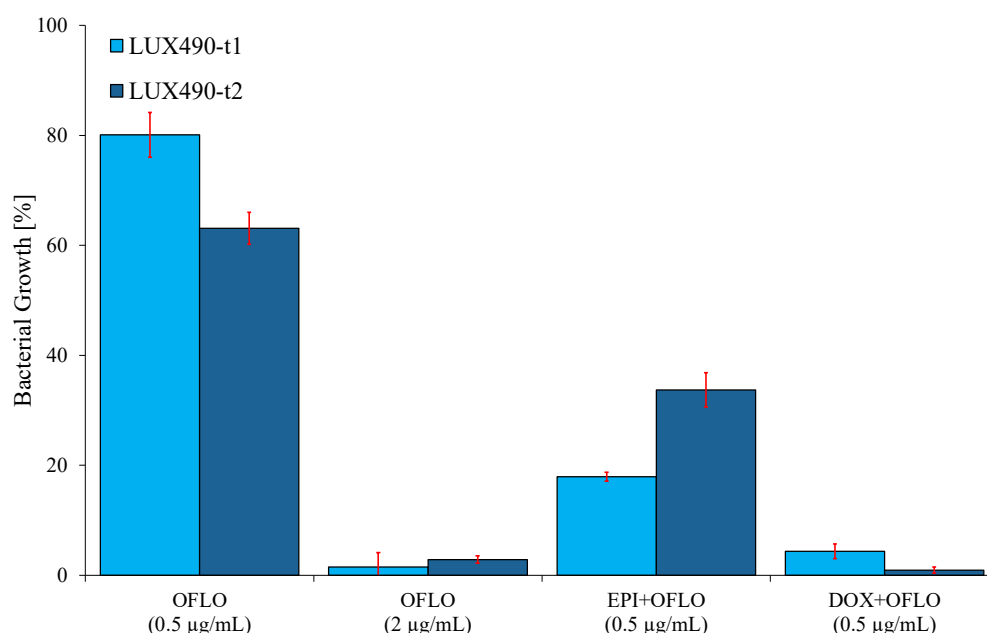
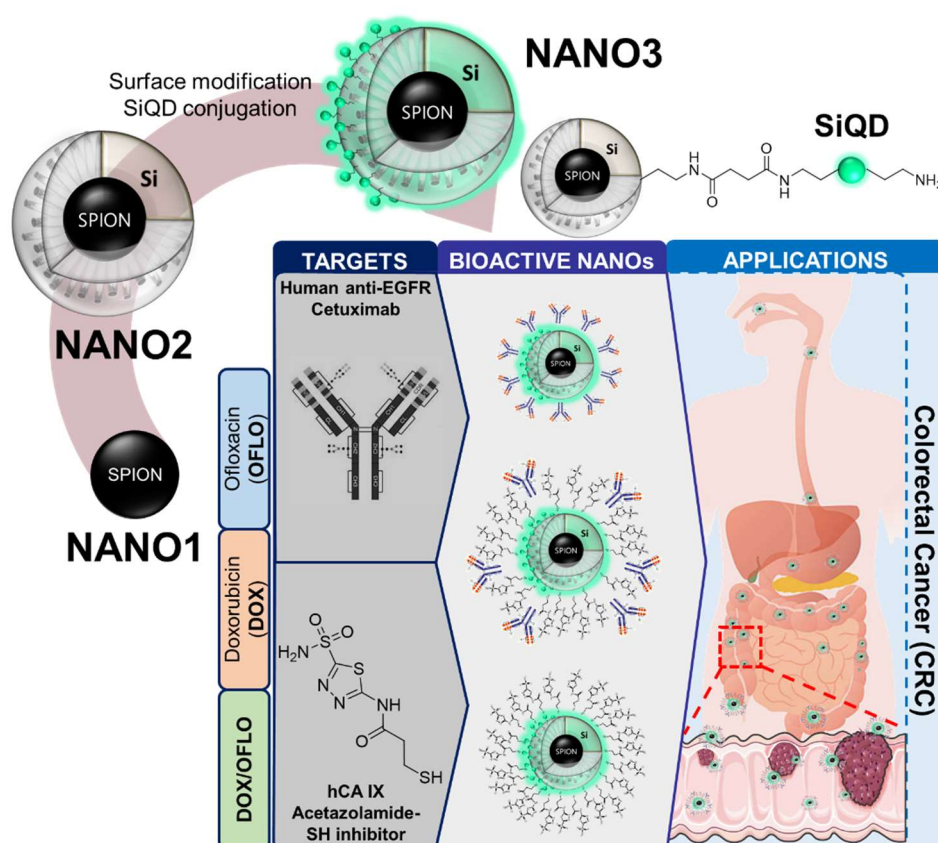


Figure S - 7.6 - Statistical comparison of the MIC₅₀ / MIC₉₀ obtained, via LUX₄₉₀ 8 h-assaying of *E. coli* Lux, for trial 1 (t1) and trial 2 (t2), of all OFLO-containing drug combinations; statistically significant levels represented as * $\equiv p \leq 0.05$, ** $\equiv p \leq 0.01$, *** $\equiv p \leq 0.001$, **** $\equiv p \leq 0.0001$.

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A MAGNETO FLUORESCENT BIOCOMPATIBLE, COMBINATORY AND DUAL TARGETING NANOTHERAPY AGAINST COLORECTAL CANCER



Keywords: Dual-targeting, CRC, anti-tumoral, nanotherapy, oral administration

Adapted from GA Marcelo et al.

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A Magneto Fluorescent Combinatory And Dual-Targeting Nanotherapy Against Colorectal Cancer

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Abstract: The need for non-invasive therapies capable of conserving drug efficiency and stability while having specific targetability against colorectal cancer (CRC), has made nanoparticles preferable vehicles and principal building blocks for the development of complex and multi-action anti-tumoral approaches. Owing to their diverse sizes, morphology and composition, nanoparticles have shown outstanding versatility and availability for complex manipulations and modifications to render them the desirable assets towards a specific problem. For that, here in we report the production of a combinatory anti-tumoral nanotherapy with a dual-targeting capacity towards CRC lines. The above reported magneto-fluorescent NANO3 systems were used as nanocarriers for a combined DOX/OFLO formulations and later modified with an anti-epidermal growth-factor receptor (EGFR) - Cetuximab - and an anti-human carbonic anhydrase IX (hCA IX) - acetazolamide derivative. The synthesis of this novel anti-CA IX acetazolamide derivative (AZM-SH) was also successfully reported for the first time and its inhibitory activity towards hCA IX completely assessed. The cytotoxicity of the obtained materials was thoroughly evaluated, with only DOX-containing nanosystems showing significant and even competitive cytotoxicity when compared to that of free DOX. Interestingly, surface modification with AZM promoted an increased cellular uptake in HCT116 cell line, surpassing even those functionalized with Cetuximab. However, a combined biofunctionalization did not show improved cellular uptake, pointing towards possible competing mechanisms. A proof-of-concept for an oral administration was also successfully attained, with a combined Eudragit® (S100+L100) enteric coating effectively protecting NANO3 systems from the extreme conditions of the stomach and high absorption of small intestine, and ensuring a more intense delivery/release under colonic conditions.

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8.1 Introduction

Colorectal cancer (CRC) is the second deadliest cancer and the third most common malignancy worldwide ¹. Due to its characteristics, it can become highly aggressive and develop resistance to several therapeutic strategies ^{2, 3}. All these aspects contribute to the alarming rising prevalence of CRC worldwide ⁴, making the development of more efficient anticancer therapies, of utmost importance.

Nanoparticle-based materials have been widely developed and successfully employed as anticancer tools or as platforms to improve the efficacy of already known therapies, with metallic/metal-oxide nanoparticles being often used to this end ^{5, 6}. Other nanosystems have also gained particular interest, specifically those containing mesoporous silica nanoparticles/structures whose highly porous matrix renders them with unique cargo delivery capacities, surface manoeuvrability, targetability or even spectroscopical activity ^{7, 8}. Amongst these, precision diagnosis and therapeutic systems are of particular interest, due to their increased performance and variability ⁹⁻¹¹.

Specific characteristics of tumour-like environments, such as hypoxia and acidic extracellular pH, can thus be utilized as targets, for specific delivery of nanoparticle-based systems. Cancer cell environments oftentimes present uncontrolled growth that results in improperly structured and functioning vasculature, low oxygenation and upregulation of homeostasis-associated enzymes, as carbonic anhydrases (CAs, EC 4.2.1.1) ¹².

CAs are ubiquitous metalloenzymes that catalyse the simple physiological hydration of carbon oxide to bicarbonate ions and protons ($\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+$), with most containing in their active centre a zinc ion (Zn^{2+}) essential for catalysis. Besides pH and CO_2 homeostasis regulation, CAs are also responsible for CO_2 and HCO_3^- transport between metabolizing tissues and lungs, electrolytes secretion, some biosynthetic processes (i.e. glucogenesis, lipogenesis and urea genesis), bone resorption and tumorigenicity ¹². Their broad activity spectrum arises from the 15 presently known isozymes, with CAs I, II, III, VII and XIII residing in cytosol; CAs IV, IX, XII, XIV and XV being associated with membranes; CAs VA and VB occurring in mitochondria; and CA VI existing only as a secreted isoform ¹³.

The occurrence of an elevated number of CA isoforms in cellular membranes of numerous organs and the association of their overexpressed activity to some types of tumours, have thus sparked special interest in CAs as potential targets for tumour diagnosis and therapy ^{14, 15}. The human (h)CA IX is an example of a tumour associated CA isoform, with several groups associating it to tumour activity, cell proliferation, cell adhesion and malignant

cell invasion^{16, 17}, being thus also commonly considered as a potential biomarker for tumour diagnosis¹⁸. More specifically, hCA IX's overexpression levels and increased activity have been recently associated to higher rates of hypoxia survival and cell invasion in tumours^{19, 20}.

The inhibition of CAs has been increasingly reported as a potential pathway for cancer therapy, by using CA-directed pharmacological agents as CAs inhibitors, capable of imparting CAs activity and normal cell mechanisms^{21, 22}. Among others, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide, commonly known as Acetazolamide (AZM) and its analogues, have exhibited outstanding and selective inhibitory activities towards CAs, being extensively used clinically as diuretics, anti-glaucoma or anti-convulsant agents²³⁻²⁵. Regarding anti-tumoral therapies, specific CA and hCA IX inhibitors have also been reported as potential hypoxia-activable inhibitors and targeting agents^{14, 26-28}.

The development of novel inhibitors capable of targeting CAs, specifically hCA IX, is thus of high interest specially if they can be conjugated with pro-drugs or with nanometric vectors, rendering them targetability to the overexpressed hCA IX in cancerous environments.

Associated with CA IX action and overexpression in hypoxic and invasive tumors, like CRC^{18, 19}, is also the commonly overexpressed epithelial-growth factor receptor (EGFR) which mediates cancer initiation, progression, and proliferation^{29, 30}. This association makes anti-EGFR agents also good targeting probes, with several works reporting the conjugated use of several anti-EGFR antibodies, like the commercial Cetuximab (CTX), with therapeutic molecules (drugs, pro-drugs, etc.) and nanometric vectors for either diagnosis or therapeutics in CRC³¹⁻³³.

Considering this, herein we report the synthesis of a novel AZM-derivative containing a sulfhydryl anchoring moiety, and its subsequent biofunctionalization on superparamagnetic, fluorescent iron oxide-cored mesoporous silica nanoparticles (NANO3-SH). This system was successfully evaluated as a potential anticancer tool against CRC cell lines. The development of a dual anti-EGFR/anti-hCA IX (CTX+AZM) targeting nanoprobe is also reported and its performance as a precision therapeutic tool against CRC compared with that of each individual NANO3-achored antigen.

Notwithstanding the method of administration of drugs for therapeutic purposes is one critical step in the development of new ground-breaking therapies. The unsuccessful accounting of possible complex interactions with body's solid and liquid interfaces, and all related arising hinderances, dictates the successful rate of said therapies, even for those showing outstanding performances *in silico/in vitro*. Not only that, but with the ever growing

pursuit for non-invasive techniques, orally administered and inhalation-based products have gained particular interest in pharmaceutical industries/market ^{34, 35}.

These points are particularly familiar to CRC which, due to its body location, sees many oral delivery therapies being developed ³⁶⁻³⁸, of which some might be withdrawn. Most withdrawals are not only related to problems of stability when in contact with body fluids constituents, pH and temperature, but also to problems of long circulation instability and peripheral body accumulation in organs/members other than the colon ^{34, 39}. Some of the major barriers to the development of an oral administered therapy against CRC are the passage time, intrinsic high absorption and extreme pH conditions of the whole gastrointestinal tract ³⁹. Its success goes, thus, through the protection of the active compound of interest with an external resistant, but also manoeuvrable, agent. Such agent(s) must be capable of both resisting extreme denaturing conditions and having a stimuli-responsive behaviour (i.e., pH, time, etc.) to correctly dispense the therapeutic compound at the targeted site/time ⁴⁰. For anti-CRC applications, it is crucial that the selected formulation is capable of by-passing stomach extreme conditions and then small intestine absorption, before reaching the colon. For that, in a proof-of-concept, NANO3 -SH systems were protected with an enteric coating formulation of Eudragit® polymers (PMMA-MMA copolymers), that showed prominent capacity to effectively deliver the herein developed

8.2 Materials and Methods

8.2.1 5-amino-1,3,4-thiadiazole-2-sulfonamide (AZM-NH₂) synthesis

Following the procedure patented by Kandula ⁴¹, 1.5 g (6.75 mmol) of 5-acetamino-1,3,4-thiadiazole-2-sulfonamide (**AZM**) were thoroughly dissolved in a mixture of ethanol (10.0 mL) and HCl 37% (3.0 mL, 12M) and refluxed for 4.5h. Refluxing was followed by cooling and solvents evaporation under vacuum. The resulting solid residue was redissolved in MilliQ H₂O (7.5 mL) and neutralized to pH 7.0 with a 5 M aqueous solution of NaOH. Upon neutralization a white precipitate was formed, which was collected by filtration and recrystallized in water, overnight (scheme 1).

The product, 5-amino-1,3,4-thiadiazole-2-sulfonamide (**AZM-NH₂**), was collected in the form of white crystals (yield of 40 % wt.), ESI+QTOF MS: [C₂H₄N₄O₂S₂+H]⁺ 180.9848 *m/z* (error – 3.8 ppm). ¹H NMR spectrum (DMSO-d₆, 400 MHz) δ (ppm): 7.81 (s, 2H, H11-NH₂),

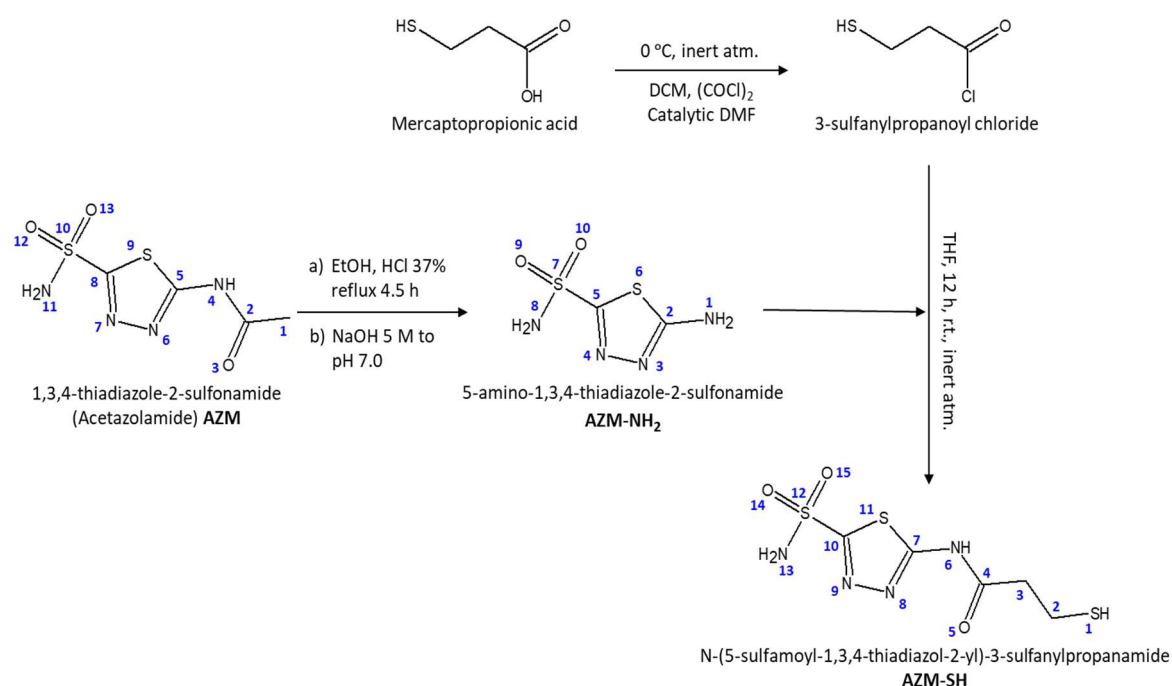
8.05 (s, 2H, H1-NH₂). ¹³C NMR spectrum (DMSO-d₆, 400 MHz) δ (ppm): 158.61 (C2), 172.05 (C5). The product was also characterized by ATR-FTIR.

8.2.2 3-sulfanylpropanoyl chloride synthesis

The chlorination of 3-mercaptopropionic acid was accomplished by a typical Vilsmeier mechanism, as detailed by Mohapatra et al ⁴². A solution of 3-mercaptopropionic acid (0.87 mL, 10.0 mmol), in DCM (5 mL), was firstly cooled to 0 °C, under N₂ atmosphere. To this mix, catalytic amounts anhydrous DMF were added, followed by a dropwise addition of oxalyl chloride (2.0 mL, 20 mmol). The resulting mixture was then slowly stirred for 4.5 hours at room temperature. Solvents and excess oxalyl chloride were removed by evaporation under vacuum. The product, 3-sulfanylpropanoyl chloride, was obtained in the form of a dark-brown liquid and characterized by ¹H-NMR and ¹³C-NMR.

8.2.3 N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)-3-sulfanylpropanamide (AZM-SH) synthesis

In an adapted protocol from those described by de Simone et al ²⁶ and Bolla and Nangia ⁴³, 90 mg (0.5 mmol) of AZM-NH₂ were dissolved in THF (5 mL) and mixed with 50 µL (1 mmol) of 3-sulfanylpropanoyl chloride, at room temperature and N₂ atmosphere. After 12h of continuous stirring, the solvent was evaporated under vacuum and the obtained green precipitate left to dry, for 48h under vacuum. The dry pellet was then redissolved in MilliQ H₂O, tuning from its original green coloration to a brownish-white colour, and filtered under vacuum with 5 consecutives washes in H₂O, DCM and diethyl ether.



Scheme 8.1 Synthetic workflow on the preparation of N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)-3-sulfanylpropanamide (AZM-SH) salt. Numbering system of atom (blue) used for the assignment of ^1H and ^{13}C NMR chemical shifts.

The resulting product, N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)-3-sulfanylpropanamide (**AZM-SH**, FW = 268.34), was dried at 37 °C and collected in the form of brownish-white powder (70.4 % wt. yield, mp 203.6 $\pm$ 1.0 °C), ESI+QTOF MS: $[\text{C}_5\text{H}_8\text{N}_4\text{O}_3\text{S}_3 + \text{H}]^+$ 268.9831 m/z (error – 2.5 ppm) and $[\text{C}_5\text{H}_8\text{N}_4\text{O}_3\text{S}_3 - \text{H}]^+$ 266.9853 m/z (error – 2.2 ppm). Elemental analysis of $\text{C}_5\text{H}_8\text{N}_4\text{O}_3\text{S}_3$ as, found (calculated): C 21.64% (22.38%), H 2.28% (3.00%); N 17.41% (20.88%), S 29.66% (35.85%).

^1H NMR spectrum (CD_3CN , 400 MHz) δ (ppm): 1.30 (s, 1H, H1 -SH) 2.70-2.97 (dt, 2H, H2 -CH₂), 3.23-3.37 (dt, 2H, H3 -CH₂), 6.43 (d, 2H, H13 -NH₂, J = 33.8 Hz), 10.62 (br s, H, H6 -NH-). ^{13}C NMR spectrum (DMSO-d_6 , 400 MHz) δ (ppm): 24.04 (C1), 34.56 (C2), 161.29 (C3), 164.94 (C4), 170.78 (C5). The product was also characterized by ATR-FTIR. A summary of all synthetic steps is depicted in Scheme 1.

8.2.4 Modelling studies and molecular docking simulations

Docking simulations were accomplished in AutoDock4, using the hCA IX crystal structure and active site, from the 3IAI protein complex, available at Protein Data Bank ⁴⁴. Docking parameters were defined according to AutoDock4_{zn} forcefield, adapted to the docking of small molecules to zinc metalloproteins ⁴⁵. Ligand (AZM-SH) and receptor (hCA IX)

were prepared separately. Ligand coordinates were processed with Openbabel⁴⁶ software to add hydrogens at pH 7.0. AutoDockTools v.1.5.6⁴⁷ script *prepare_ligand4.py* with default settings was used to add Gasteiger-Marsili charges, merge non-polar hydrogens, and assign atom types. Number of torsions was set to 7 to include its rotatable amide bond. The receptor structure was prepared using AutoDockTools script *prepare_receptor4.py* with default settings, except the removal of water molecules, to assign Gasteiger-Marsili charges, merge non-polar hydrogens, add polar hydrogens and assign atom types. The charge of the zinc ion was left to its default value (0.0) when using Autodock4_{Zn} forcefield (Supple. Mat.). The TZ pseudo-atom responsible for the attractive interaction in the tetrahedral coordination arrangement of zinc was added to the receptor molecule and to the standard forcefield table (Supple. Mat.). Docking search parameters followed a Genetic algorithm, with 50 runs and a population size of 300. 3D representations of AZM, hCA IX and docked complexes were performed with UCSF Chimera⁴⁸. Modulations with the normal AutoDock4 forcefield, without the presence of the TZ pseudo-atom were also run as comparison.

Best fitting models were chosen for predictions with the lowest inhibition constant (*K_i*), the lowest binding energy and RMSD (< 2.0 Å), and the highest binding efficiency.

8.2.5 CA *in vitro* inhibition assay

Experimental *in vitro* AZM-SH inhibition activity was assessed using the CA Inhibitor Screening Kit (Colometric), by BioVision Inc., following the manufacturer's instructions. The kit uses the esterase activity of an active CA on an ester substrate, producing a chromogenic product easily quantified by absorbance, at 405 nm. In the presence of a CA specific inhibitor, enzymatic activity decreases, accompanied by a reduction in the product's absorbance. AZM-SH inhibitor 2 mM stock solution was prepared in 0.01 M PBS pH 7.4 and dilutions up to 2 pM were done in the same buffer. The assay was prepared in a 96-well plate for AZM-SH concentrations of 2 mM, 1 mM, 0.5 mM, 0.2 mM, 20 μM, 2 μM, 20 nM, 2 nM, 0.2 nM, 2pM and the absorbance collected in a microplate reader at 405 nm, over the course of 60 min for 30 time points. Inhibition constant (*K_i*) was obtained as *in vitro* IC₅₀ and compared to that of the standard AZM.

8.2.6 Synthesis of NANO3-SH

NANO3 were obtained as thoroughly described in the Supple. Mat. Briefly, magnetic cores (NANO1) were obtained via the aqueous co-precipitation of Fe²⁺ and Fe³⁺ ions, in the

presence of oleic acid as morphological agent and as surface stabilizer. Silanization of NANO1 surface with a mesoporous silica layer was achieved after ligand exchange with CTAB (morphological agent and pore modulator) followed by TEOS precipitation, to yield NANO2. NANO2 surface was further aminated by precipitating APTES, rendering NANO2-NH₂, and modified with succinic anhydride via ring opening amidation reaction to form succinamic acid (succinic acid-amine) surface groups, yielding NANO2-COOH. NANO3 were obtained by EDC/NHS cross-linkage of NANO2-COOH with the NH₂ groups of the synthesized SiQDs, in a SiQD excess to avoid inter-NANO3 cross-linkage. The same strategy was used to thiolate NANO3 surface NH₂ groups with thioglycolic acid (TGA), producing NANO3-SH. NANO3-SH activation of its surface thiol moieties was attained using Ellman's reagent (DTNB), whose colorimetric change from transparent to yellow (412 nm) allows for a simultaneous estimation of the present -SH groups and degree of activation. All materials, at each step, were thoroughly characterized by SEM/TEM, DLS, TGA, N₂ sorption (physisorption), FTIR, XRD and absorption/fluorescence spectroscopy. Magnetic characterization was also done and is also detailed in Supple. Mat.

8.2.7 NANO3 biofunctionalization with CTX-SH and AZM-SH

Human colorectal carcinoma cells (HCT 116), human colorectal adenocarcinoma cells (HT-29) and normal human colon fibroblasts (CCD112-CoN) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). HCT116 and HT-29 cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) containing 10% heat-inactivated fetal bovine serum and 1% antibiotic-antimycotic solution (Gibco) at 37°C in a humidified 10% CO₂ atmosphere. CCD112-CoN cells were maintained in Minimum Essential Medium with Earle's salts (MEM, Gibco) containing 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, Non-Essential Amino Acids (Gibco) and 1% antibiotic-antimycotic solution.

For each four 5 mg NANO3-SH sample, 400 µL of a cetuximab (CTX) 1 mg/mL stock solution in 0.01 M PBS pH 7.4, were mixed with 8 µL of a 1.93 mg/mL solution of 2-iminothiolane hydrochloride in 0.01 M PBS (pH 8.5), with 0.1 mM EDTA, for 1h at 4 °C (in the dark). Each batch of the resulting thiolated cetuximab (CTX-SH) was 4 times centrifuged in a 10K MWCO Vivacon® membrane at 13.500 rpm and 10 °C, for 5 min. The CTX-SH was solvent exchanged into a final volume of 100 µL of 0.01 M HEPES, with 0.1 mM EDTA ([CTX-SH]_{final} = 4.0 µg/µL).

For the biofunctionalization of the already activated NANO3-SH, 5 mg batches were used for each type of modification (i.e., single or dual modification). The 4 µg/µL (27.4 mM) CTX-SH solutions, in 0.01 M HEPES buffer, and a 1 mg/mL (3.73 mM) solution of AZM-SH, in 0.1 M TrisHCl with 0.1 mM EDTA, were used as stock solutions. Briefly, 5 mg of NANO3 were resuspended in 0.1 M Tris HCl (pH 8.05) to a total volume of 1.7 mL and mixed, for 3h at 4 °C (in the dark), with: (1) 25 µL of CTX-SH stock solution; (2) 53 µL of AZM-SH stock solution; or (3) 25 µL and 53 µL of CTX-SH and AZM-SH stock solution, respectively. All samples were recovered by centrifugation, washed twice with 0.01 M PBS (V = 1 mL, pH 7.4), dried under vacuum and sterilized by UV for 30 min (253.7 nm, 200 V, 50 Hz).

8.2.8 Drug loading and release assays

For drug loaded nanoparticles, as-synthesised NANO2-COOH had first their pores emptied via NH_4NO_3 solution route, as detailed in Supp. Mat.. Ofloxacin (OFLO) and doxorubicin (DOX) were used as antimicrobial and anti-tumoral model drugs and were loaded either alone or conjugated in a 1:1 wt. ratio. A 2 mg/mL doxorubicin stock solution was prepared in milliQ H_2O and a 2 mg/mL ofloxacin stock solution in 0.01 M PBS (pH 7.0). Loadings were performed in batches of 20 mg of template-free NANO2-COOH. Briefly, 20 mg of NANO2 were resuspended in 3.2 mL of the corresponding drug stock solution and diluted in the same volume of the complementary solvent (Table S-1). Co-loading of OFLO and DOX was performed similarly with 20 mg of NANO2-COOH being resuspended in 3.2 mL of OFLO and 3.2 mL of DOX stock solutions.

Each sample was collected by centrifugation (10,000 rpm for 10 min) and washed 4 times with 1 mL of PBS 0.01M (pH 7.0). All supernatants were isolated and used to estimate, by mass balance, the loading capacity of NANO2-COOH for each drug, *via* UV-vis spectroscopy at 330 (OFLO) and 480 (DOX) nm. The loaded nanoparticles were then submitted to the same surface modifications described above, yielding NANO3@D, NANO3@O and NANO3@DO and their DOX and OFLO content assessed by the same procedure. Each product was further modified with CTX-SH and AZM-SH, accordingly.

pH-dependent release studies were conducted in PBS 0.01M, with pH of 7.4 and pH 5.0, at 37 °C. Briefly, 1 mg of each loaded NANO3 was resuspended in 2 mL of buffer solution and shaken for up to 72h. After centrifugation (12,000 rpm for 5 min), the released drug content in the supernatant was measured for the 5, 15, 30, 45, 60, 90 min and 2, 3, 4, 24, 72 h time points, by fluorescence: OFLO at pH 7.4 (λ_{exc} = 300 nm, λ_{exc} = 460 nm), OFLO at pH 5.0 (λ_{exc} = 300 nm, λ_{exc} = 490 nm) and DOX (λ_{exc} = 480 nm, λ_{exc} = 560 nm).

8.2.9 Enteric Coatings & Simulated Digestive Track Release

The enteric coating of the final formulations was achieved using Eudragit® polymers, Eudragit® L100 (E-L100) and Eudragit® S100 (E-S100), as model enteric coatings. Two types of coatings were prepared, one with only E-S100 and another with E-S100 followed by E-L100. 0.2% w/v stock solutions of each polymer were prepared in ethanol. Briefly, 1 mg of each nanoparticle formulation was resuspended in 1 mL of H₂O miliQ and mixed with 250 µL of E-S100 stock solution overnight, at room temperature (in the dark). With all ethanol evaporated, all NANO3-S100 formulations were recovered by centrifugation (10 000 rpm, 15 min) and kept away from humidity in a desiccator. For further modification with E-L100, the selected products were treated with the same approach, but this time using E-L100 stock solution, yielding NANO3-SL100 formulations. All surface modifications were followed by DLS/Zeta characterization.

For a simulated gastric release, all formulations were suspended in the appropriate medium under continuous stirring at 37 °C, for the respective passage time of the simulated gastric section. All samples were collected (11 000 rpm, 3 min) after the appointed time and the release amount of drugs spectroscopically quantified. Firstly, the prepared formulations (1 mg) were resuspended in 2 mL of 0.025 M HCl (pH 1.75) for 2h to simulate stomach conditions, followed by solvent exchange into another 2mL of 0.01 M PBS (pH 6.5) for 5h to simulate small intestine conditions and, lastly, by another solvent exchange into 2 mL of 0.01 M PBS (pH 7.0) for 15h to simulate colonic conditions. Note that in this last part the samples were quantified after 5h and 15 h of incubation to assess the release of the drugs at ascending colon conditions and later at the end of the colon/rectum, respectively.

8.2.10 CA IX and EGFR expression in CRC cell lines

HCT-116 and HT-29 cells in exponential growth were collected. 106 cells of each cell line were pelleted, washed twice with PBS and lysed using M-PER Extraction Reagent (Thermo Scientific). The total protein content was determined via a BCA protein assay (Thermo Scientific). Proteins from extracts from both cell lines were separated by size using SDS-PAGE and transferred into a PVDF membrane for Western Blotting. Epidermal Growth Factor Receptor (EGFR) and Carbonic Anhydrase IX (CA IX) levels were quantified after immunoblotting with anti-EGFR (Sc-03, Santa Cruz Biotechnology) and anti-CAIX (ref. 10107-R003, Sino Biological) respectively.

8.2.11 Cytotoxicity and Cellular Uptake

The cytotoxicity of biofunctionalized NANO3-SH systems (i.e., NANO3-CTX, NANO3-AZM and NANO3-CTX+AZM) and their loaded counterparts (with DOX and OLFO) was tested in HT-29 and HCT-116 human CRC cell lines using the PrestoBlue assay. Cells were seeded into a 96-well plate at a cell density of 3.0×10^3 cell/well and incubated for 24h before nanoparticles were added at a concentration from 1 to 300 $\mu\text{g/mL}$. After 72 h, 20 μL of prestoBlue reagent were added to each well and the plates incubated for 4h at 37 °C. The effect on cell viability was measured using a multilabel plate reader (Victor 3 PerkinElmer). Its fluorescence was quantified at $\lambda = 615$ nm (after excitation at $\lambda = 531$ nm), and its absorbance was monitored at $\lambda = 570$ nm. Cell cytotoxicity was evaluated in terms of cell growth inhibition in treated cultures and expressed as a percentage of the control conditions. Each experiment was repeated three times, and each concentration was tested in triplicates.

For cellular accumulation determination, HT-29 and HCT-116 cells were plated in 35 mm-cell culture dishes and were grown overnight. Cells on exponential growth were plated 24-well plates, at a density of 20,000 cells per dish. Cells were grown overnight and treated with 5, 10 and 25 $\mu\text{g/mL}$ of each nanoparticle for 3 hours (untreated cells were used as negative controls). After treatment, cells were lysed with PBS buffer with 1% of SDS and fluorescence was measured in a Cary Eclipse fluorometer (samples were excited at 480 nm, and emission light was collected at 554 nm). The relative fluorescence of the samples was compared to the fluorescence of the nanoparticles in medium before the treatment.

Cellular uptake experiments were also performed on HT-29 and HCT-116 cell lines. Cells on exponential growth were plated in MatTek® 35mm dishes, at a density of 40,000 cells per dish. Cells were grown overnight and treated with 25 $\mu\text{g/mL}$ of NANO3@D-CTX, NANO3@D-AZM and NANO3@D-CTX+AZM for 3 hours (untreated cells were used as negative controls). After treatment, cells were counter-stained with Hoechst and CellMask and observed in a Confocal Microscope Olympus FV1000.

8.3 Results and Discussion

8.3.1 Synthesis of AZM-SH and anti-CAIX action

The potential of acetazolamide and acetazolamide derivatives to serve as selective vehicles for the delivery of cancer specific drugs and their inhibition activity over cancer related CAs has been hinted in several works, with special attention to the hCA IX isoform ¹⁵.

^{49, 50}. With this in consideration, herein we propose the synthesis of a thiol moiety containing AZM-derivative, as a possible hCA (CA IX included) inhibitor and targeting agent in the delivery of anti-cancer drugs. The interest in the inclusion of a terminal thiol to the original structure of AZM arises from the potential formation of disulphide bonds with anti-cancer drugs of interest, or with nanovectors with intrinsic anti-tumoral activity or with drug delivery capacity, for a targeted anti-cancer therapy.

To synthesise the new compound, the amide in AZM was firstly hydrolysed to produce an available terminal NH₂ group, other than that of the sulfonamide moiety. By activation of the carboxylic acid moiety of the 3-mercaptopropionic acid, *via* Vilsmeier mechanism, and reaction with compound AZM-NH₂, the new amide derivative (AZM-SH) was obtained. Intermediate AZM-NH₂ and final product AZM-SH were characterised accordingly and compared to initial AZM.

8.3.1.1 Chemistry

The ¹H and ¹³C NMR spectra of AZM-NH₂ (Figure S-8.2) confirm the total hydrolysis of the of the amide bond in AZM. This step is characterised by the disappearance of the 2.25 ppm -CH₃ signal in the AZM ¹H spectrum (Figure S-8.1) and a right shift of the 13 ppm signal to 8.05 ppm, corresponding to the transformation of the secondary amide moiety into the terminal primary amine in AZM-NH₂. Moreover, the loss of the acetamide partition of AZM was verified with the vanishing of its correspondent 22.83 ppm and 161.57 ppm signals in the ¹³C NMR spectrum of AZM-NH₂.

The successful binding of 3-sulfanylpropanoyl chloride to the amine (1) of AZM-NH₂ was confirmed by ¹H and ¹³C NMR spectra of AZM-SH, in DMSO-d₆ and CD₃CN (Figure S-8.3). Due to water presence in the product, both deuterated solvents were used to attain full insight on all peaks. The reconstitution of the >NH signal at 13.13 ppm (DMSO-d₆) and the surgency of a doublet at 8.38 ppm (DMSO-d₆), corresponding to the sulfonamide -NH₂, in the ¹H NMR spectrum of the product (Figure S-8.3.b), are the first hints at the successful synthesis of AZM-SH (Figure 8.1.a). These signals are in accordance with the chemical shifts of the same groups in AZM's NMR spectrum (Figure S-8.1) and with those described for other thiadiazole-derived ligands ^{51, 52}.

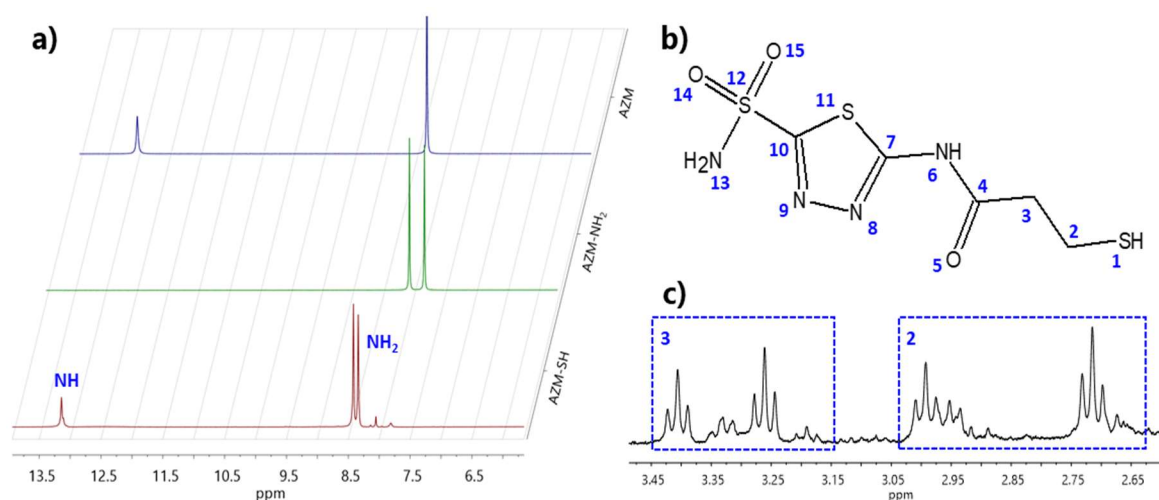


Figure 8.1 - (a) Stacked ^1H NMR spectra of AZM, AZM- NH_2 and AZM-SH (DMSO-d_6 , 400 MHz), for δ between 6-14 ppm. (b) AZM-SH structure and atom number assignment. (c) Close-up of the ^1H NMR spectrum of AZM-SH (CD_3CN , 400 MHz) and chemical shift assignment of sulfanylpropanamide C(2) and C(3) protons, seen as two crossed doublet-triplet (dt) signals.

Although not perceptible in the DMSO-d_6 ^1H NMR of AZM-SH, in CD_3Cl the signals relating the sulfanylpropanamide ($\text{NHCO-CH}_2\text{-CH}_2\text{-SH}$) C(2) and C(3) protons are visible between 3.45-3.15 ppm and 3.05-2.65 ppm as a set of two doublet-triplet like signals⁵³, thus supporting the successful synthesis of AZM-SH, by the inclusion of 3-sulfanylpropanoyl via amide bond formation. The obtained chemical shifts and crossings of the signals of C(2) and C(3) protons have been pointed before, by Schuetze et al⁵⁴, to arise from a certain degree of dimerization of the 3-sulfanylpropanoyl segment of the molecule via disulphide bond (Figure 8.1.c) The dimerization of the molecule is also hinted by the splitting of the $-\text{NH}_2$ signal at 8.38 ppm, $J = 24.4$ Hz (Figure 8.1.a), comprising the asymmetry created by intermolecular hydrogen bonding between the $>\text{NH}/-\text{NH}_2$ and $-\text{C}=\text{O}$ moieties of adjacent molecules (Figure 8.2), similarly to those formed in some reported AZM dimers^{55, 56}.

Complete conversion of AZM- NH_2 into AZM-SH was also confirmed by the latter's ^{13}C NMR spectrum, in DMSO-d_6 (Figure S-8.3.c).

ATR-FTIR spectra of both intermediate AZM- NH_2 and final product AZM-SH, also translate the above-mentioned modifications of the original AZM (Figure S-8.4). Assigned vibration nodes (Table S-8.1) are in accordance with those described in literature for acetazolamide and its derivatives, specifically those concerning analogous groups, as $-\text{SO}_2\text{NH}_2$, amide ($-\text{C}=\text{O}$, $>\text{N-H}$ and C-N-C) and thiadiazolic ring^{55, 57, 58}. The appearance of preferable asymmetrical vibrational modes, in both AZM and AZM-SH, and the presence of particularly broader signals at low frequencies, demonstrates their similar appetency to form

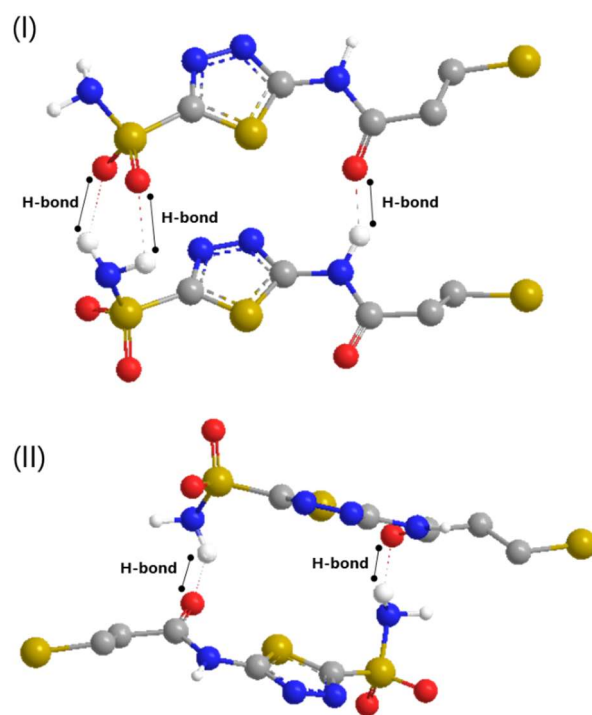


Figure 8.2 - Suggested dimeric conformations of AZM-SH attained via hydrogen bonding.

intermolecular hydrogen bonds and dimeric conformations^{55, 59}. Special attention is brought to the vibrations concerning the >NH (amide) and -SO₂NH₂ groups in the spectrum of AZM-SH, whose significant broadening supports the already hinted formation of dimers, via hydrogen bonding.

The successful syntheses of the intermediate AZM-NH₂ and the final product AZM-SH were also confirmed with electron spray ionization (ESI) mass spectrometry (MS) analysis (Figure S-8.5). With a nominal mass and a monoisotopic mass of 268 Da and 267.97585 Da (calculated), respectively, AZM-SH - [M] - was detected in two ionic forms: [M+H]⁺ (268.9831 *m/z*) and [M]⁺ (266.9853 *m/z*). Whereas the first ion corresponds to the addition of a hydrogen atom to an amine moiety (Figure S-5.b), the second ion represents the lability of the molecule and is the result of an internal S...O close contact interaction (Figure S-8.5.c). This type of intramolecular interaction has been already reported and confirmed in literature for AZM or similar AZM derivatives⁶⁰, being achievable with the loss of the hydrogen atom of terminal sulfhydryl of AZM-SH.

8.3.1.2 Docking modelling and *in vitro* inhibition studies

The effect of AZM-SH as potential inhibitor of human CAs, specifically the hCA IX isoform, was initially predicted by modelling the docking mechanism of AZM-SH into hCA IX active site. The AZM-less chain A of the 3IAI model, available at PDB, was used for the

determination of the best docking result. The ligand-receptor interaction depicted in the experimentally obtained 3IAI PDB model was taken as a comparative standard interactive conformation between AZM and hCA IX.

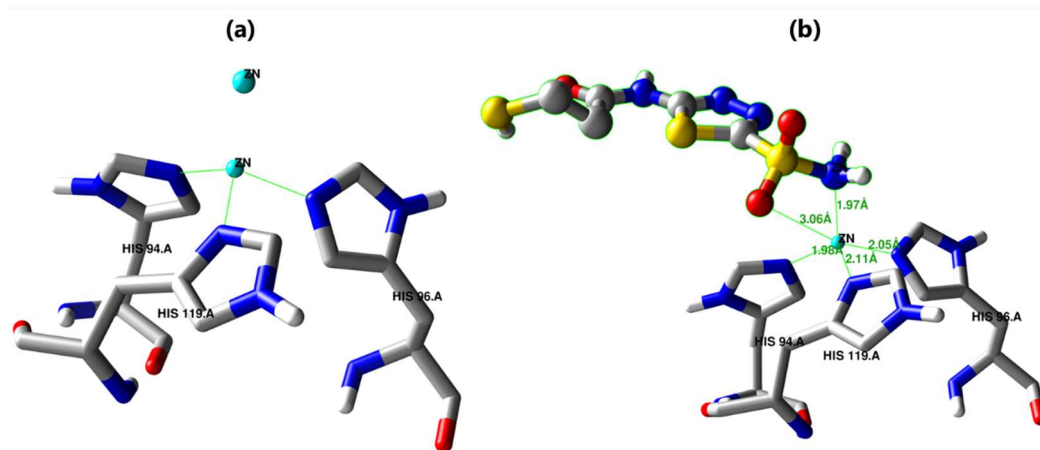


Figure 8.3 - (a) Tetrahedral zinc geometry, with TZ pseudo-atom located at the unoccupied corner of the ideal coordination tetrahedral geometry. (b) AZM-SH zinc coordination via NA (nitrogen acceptor) atom type interaction with the attractive component (TZ pseudo-atom) of the zinc ion.

The AutoDock4_{Zn} forcefield was preferred over traditional AutoDock4 forcefield, as its performance in properly reproducing zinc-coordination geometries has been proven to outcome the other's predictions⁴⁵. The chosen forcefield parameters consider the most common biological zinc coordination geometry, the tetrahedral one (Zn_{3,1} and Zn_{4,0}), where AutoDock atom types NA (nitrogen HB acceptor), N (nitrogen non-HB acceptor), OA (oxygen HB acceptor), and SA (sulphur HB acceptor) and their interaction and positioning regarding the ZN type atom play an important role in the proper docking of ligand molecules (Figure 8.3.a). Moreover, the inclusion of a TZ pseudo-atom mediating the attractive component of the zinc ion, allows a new directional tetrahedral potential for the NA atom type and more accurate docking results, with lower binding energies and RMSD values and higher binding efficiencies (Table 8.1).

Table 8.1 - Docking performances for similar conformational modulations, using AutoDock4 and AutoDock4_{Zn} forcefields.

	Binding Efficiency	Binding Energy (kcal/mol)	RMSD (Å)	K_i
AutoDock4	66 %	-10.20	1.13	61.92 nM
AutoDock4 _{Zn}	93 %	-13.35	0.0	65.36 pM

As expected, the highest binding efficiency (93 %) and the lowest binding energy (-13.89 kcal/mol), RMSD (0.0) and inhibition constant (K_i : 65.36 pM) were obtained for a conformation where the sulfonamide moiety formed a direct interaction (1.97 Å) with the Zn^{2+} ion in a tetrahedral orientation, *via* coordination by the N(13)-atom of the primary amine. Like the reported AZM - hCA IX interactive complex, the Zn^{2+} ion is also coordinated (3.06 Å) by one of the adjacent O-atoms of the sulfonamide moiety (Figure 8.3.b). Likewise, the non-coordinating O-atom and the H(13)-atom of the amine, present in the sulfonamide moiety, were predicted to form hydrogen bonds with the GLU106 (2.72 Å) and THR199 (1.98 Å) residues of hCA IX, respectively (Figure S-8.6.a) similar to other reported results^{26, 61, 62}.

Hydrogen bonding was also calculated to occur between the hydrophilic =N(8)-, >N(6)H, =O(4), -S(1)H groups of the ligand, adjacent water molecules and the THR200, PRO201, GLN67 GLN92 receptor residues, forming a continuous H-bond network facing the outer surface of hCA IX active site (Figure S-8.6.b-c). Accordingly, the alignment of the aliphatic C(3) and C(4) of the sulfanylpropanamide segment of AZM-SH with internal hydrophobic moieties of the LEU198, LEU141, VAL121, VAL143 residues (Figure S-6.d) contribute to the overall stability of the obtained conformation.

A similar conformation was obtained using the standard AutoDock4 forcefield, and without the inclusion of the TZ pseudo-atom, but only for a lower binding efficiency and higher binding energies, RMSD value and K_i (Table 8.1).

The preferable AutoDock4_{Zn} predicted ligand-receptor interactions and spatial arrangement (Figure S-8.6) indicate that the chemical modification of the original AZM, to include the sulfanylpropanamide segment, has little to no hampering impact on the inhibition role of the final AZM-SH ligand, making it a good potential inhibitor for the CRC-associated hCA IX isoform.

Experimental inhibition activity of the synthesised AZM-SH against CAs was assessed using an esterase mediated assay and calculated after appropriate Boltzmann regression (Figure 8.4). The obtained AZM-SH IC_{50} of 52.4 ± 0.5 nM ($n=3$) is well within the same order of magnitude as that reported by the kit's manufacturer for the standard AZM (IC_{50} 16.3 ± 2.0 nM), and as other IC_{50} reported in the literature for other AZM derivatives^{61, 63}. The assay

confirmed the final product's efficient inhibition activity towards CAs, namely hCA IX, thus

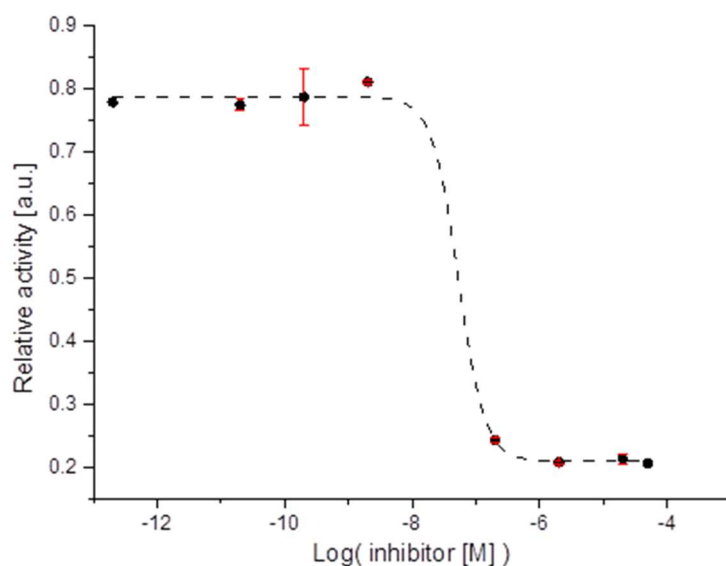


Figure 8.4 - CAs relative activity in the presence of AZM-SH and respective Boltzmann regression for the determination of its IC_{50} .

supporting its use as potential targeting agent in future anti-cancer therapies.

8.3.2 NANO3-SH characterization

As demonstrated in a previous work of our group (Chapter 6), the synthesis of NANO3-SH resulted in well-defined spherical, single core-shell particles with an average diameter of 60 nm and a shell porous matrix with pores of *ca.* 2.5-3.0 nm. A step-by-step synthetic approach ensured the successful incorporation of a superparamagnetic magnetite core (NANO1, of *ca.* 12 nm) within a mesoporous silica shell of high surface area and porous volume (NANO2, of 900 m²/g and 0.9 cm³/g, respectively), typical of this class of materials⁶⁴. These were confirmed by Brunauer Emmett and Teller (BET) and Barrett-Joyner-Halenda (BJH) methods, as well as by SEM and TEM analysis.

Subsequent NANO2 surface modifications followed a similar stepwise approach, from the grafting of APTES moieties, to its amidation by succinic anhydride (rendering them terminal -COOH groups and total surface charge inversion), and the later cross-linkage of SiQDs to the particles surface via EDC/NHS mechanism. All surface modifications were thoroughly confirmed by UV-vis and FTIR spectroscopy, thermogravimetric analysis (TGA) and physisorption analysis. Result of the continuous capping of its surface, NANO3 decrease in surface area and porous volume (580.33 m²/g and 0.443 cm³/g, respectively) confirms its

efficient surface functionalization. Despite the decrease in available surface area, its mesoporosity is still competitive with most available nanocarriers for therapeutical use ⁶⁴⁻⁶⁶, rendering NANO3 with a high availability for drug loading and delivery.

NANO3 simultaneous magnetic and fluorescent properties arise from the successful, and not-reported-so-far combination of biocompatible and biodegradable SPIONs and SiQDs in one single system ⁶⁷. While the former is incorporated within the NANO3 core, the latter is anchored to its surface, contributing with a pH-dependent gate-like role in drug delivery (see Chapter 6). Despite all surface modifications, XRPD analysis and VSM magnetization loops of NANO3 confirm the conservation of the core's single reverse spinel phase and superparamagnetic (i.e., the capacity to instantly orient and magnetize itself to an external field). This confirms, thus, NANO3's potential for magnetic hyperthermia, with a specific loss power of $295 \pm 47 \text{ W/g}_{\text{Fe}}$. On the other hand, the slightly blue shifted, but yet conserved SiQD fluorescence after grafting to NANO surface, demonstrates that there is no apparent interaction from the magnetism of the core, with SiQDs well-distanced from it to avoid energy transfer phenomena. Consequently, NANO3 fluorescence can be used independently as sensor to media pH (see Chapter 6) and as cellular uptake probe, with a bluish-green maximum emission centered at 515 nm.

The assessment of NANO surface charges by DLS and zeta potential throughout the synthetic steps, was a in important tool to, not only quickly confirm surface modifications, but also evaluate NANO3's polydispersity and stability in the selected medium. This factor has a high impact in the performance of the nanovectors, by having an active role in drug delivery and cell uptake processes ⁶⁸. As depicted in Figure 8.5, the thiolation of NANO3 surface via EDC/NHS cross-linkage of SiQD available amines and TGAc carboxylic groups, rendered the final NANO3-SH with increased stability in physiological medium, translated by an increase in zeta potential (ζ) from +12.4 mV to + 21.5 mV and a clear reduction of its hydrodynamic diameter and polydispersity. The more robust net positive charge after thiolation makes NANO3-SH particularly interesting for cellular therapeutic applications, namely CRC therapy, as positively charged particles have been recurrently reported to have preferential binding activity to cell membranes and increased uptake ⁶⁸. Moreover, enhanced cellular uptake has been reported for several thiol containing nanoparticles, that covalently bind/interact with cell surface thiol groups, increasing by a 100-fold membrane transfection efficiency and endocytosis ⁶⁹.

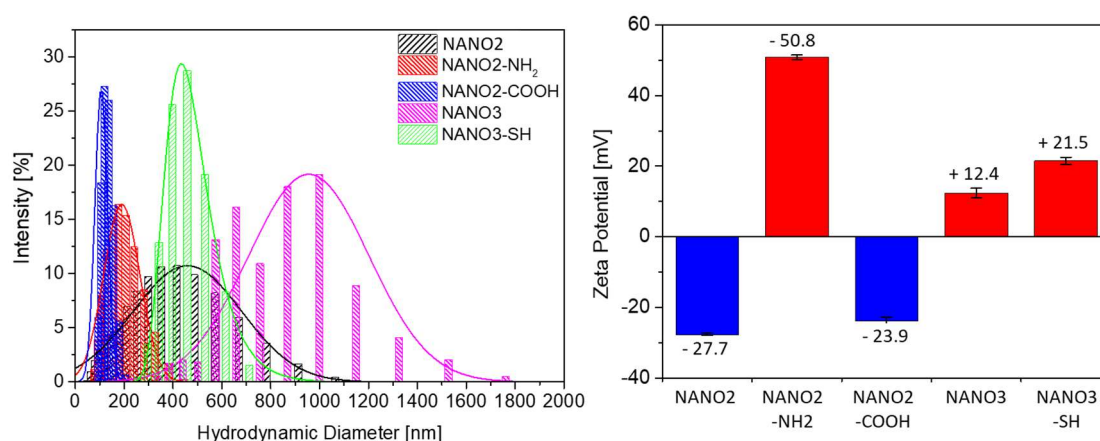


Figure 8.6 - (left) DLS hydrodynamic diameter of NANO1 to NANO3-SH systems, and (right) respective surface ζ .

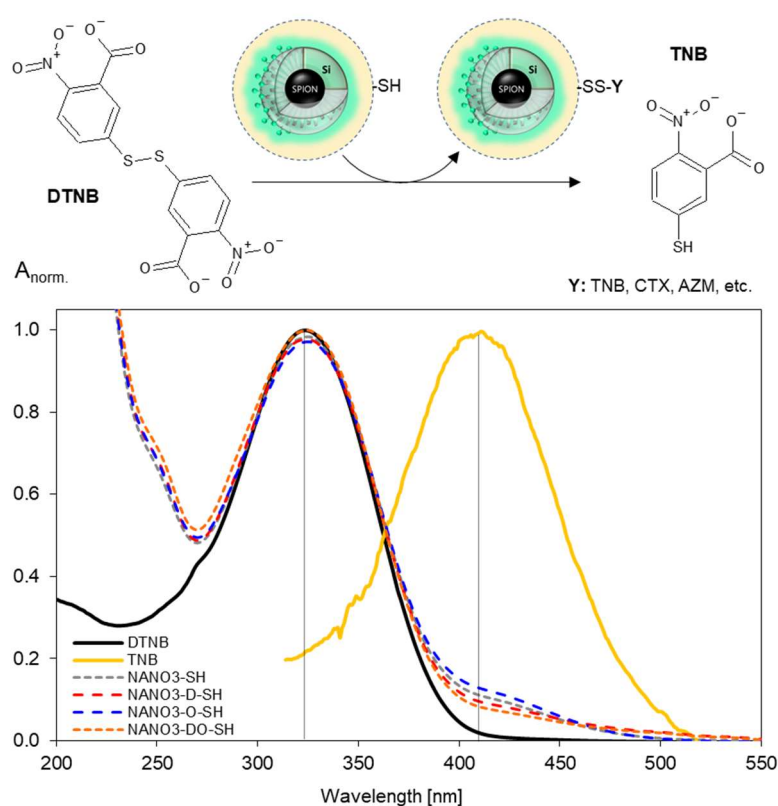


Figure 8.5 - - Molecular interaction of Ellman's reagent with thiol moieties and corresponding spectroscopic response (i.e., absorbance band red shift to 412 nm) upon the formation of TNB free species; NANO3-SH systems spectroscopic signature upon thiol determination and activation.

The introduction of thiol terminal moieties to NANO3-SH systems sought also to

create binding points for further biofunctionalization with anti-CAIX and anti-EGFR species. Consequently, it was important to not only assess the thiolation ratio, but also necessary to activate these thiol moieties and catalyse further linkages. Ellman's reagent (DTNB) has been repeatedly reported as a good visual and spectroscopical agent for the estimation of disulphide bonds and existing thiol moieties, owing it to the surgency of a quantifiable absorbance band at 412 nm (translated by a yellow coloured solution)⁷⁰⁻⁷² (Figure 8.6). Thiolation was applied to bare NANO3-SH (0.33 $\mu\text{g}_{\text{SH}}/\text{mg}_{\text{NANO}}$) and to its loaded counterparts, NANO3@D-SH (0.28 $\mu\text{g}_{\text{SH}}/\text{mg}_{\text{NANO}}$), NANO3@DO-SH (0.25 $\mu\text{g}_{\text{SH}}/\text{mg}_{\text{NANO}}$) and NANO3@O-SH (0.38 $\mu\text{g}_{\text{SH}}/\text{mg}_{\text{NANO}}$), rendering them with 0.25, 0.25, 0.19 and 0.29 nmol possible S-binding, active sites for further modification with thiol containing biomolecules/small molecules like the herein synthesized CTX-SH and AZM-SH.

A last assessment on the NANO3-SH physical stability was done by exposing the nanosystem to simulated in-body conditions, this is, at 37 °C under continuous stirring in 0.01 M PBS at physiological pH, for one month. From the TEM micrographs (Figure S - 8.7) a total decomposition of the structured material is seen with all magnetic cores segregated from the present silica, and the later spread through the whole field with no structured order.

8.3.3 Loading and Release assays

The empty porous matrix of NANO3 was used to load both model anticancer and antibiotic, in single and dual formulations. The loading of both drugs was done in an intermediate step of NANO3-SH synthesis, via electrostatic interactions with non-templated NANO2-COOH, before pores capping with SiQDs and thiolated species. Accessibility to pores is high at this stage, where surface moieties are yet malleable and do not produce a high steric impediment on drugs migration. Moreover, the correct selection of loading media's pH (pH 7.4) was important to maximize attractive interactions between the negatively charged -COOH surface moieties, pores silanol groups, and both DOX ($\text{pK}_{\text{a-NH}_2} = 8.2$, $\text{pK}_{\text{a-phenol}} = 9.5$) and OFLO ($\text{pK}_{\text{a-COOH}} = 6.1$, $\text{pK}_{\text{a(piperiziny ring)}} = 8.2$)^{73, 74} which are partly positively charged at physiological pH (Figure S-8.8.a).

DOX and OFLO were successfully loaded inside NANO2-COOH, and its drug content was evaluated by UV-Vis at each surface modification step, till the final product NANO3-SH, before its biofunctionalization (Figure S-8.8.b). The obtained loaded nanosystems and its corresponding drug content are: NANO3@D-SH with 287.5 $\mu\text{g}_{\text{DOX}}/\text{mg}_{\text{NANO}}$, NANO3@DO-SH with 274.0 $\mu\text{g}_{\text{DOX}}/\text{mg}_{\text{NANO}}$ and 140 $\mu\text{g}_{\text{OFLO}}/\text{mg}_{\text{NANO}}$, and NANO3@O-SH with 182.8 $\mu\text{g}_{\text{OFLO}}/\text{mg}_{\text{NANO}}$. The high encapsulation capacities are in accordance with those reported in

previous studies for similar mesoporous system and selected drugs, with OFLO successfully replicating the results obtained in other works of our group⁷⁵⁻⁷⁸. Regarding NANO3@DO-SH formulation, the relative reduced DOX solubility at the selected medium pH contributes to the preferential loading of DOX, in detriment of OFLO encapsulation.

The pH-dependent release capacity of each loaded system was performed with their non-biofunctionalized versions (i.e, NANO@*drugs*-SH, drugs = D, O, DO), to simulate the prominent stressful conditions in colorectal cancer microenvironments where redox imbalance and high levels of GSH promote disulphide bonds breakage^{79, 80}. CTX-SH and AZM-SH disulphide bridges with NANO3-SH systems are mostly broken at such conditions, with NANO3@*drug*-SH systems being representative of the release interface during its therapeutic action, either inside or outside CRC cells. The simulation of cancer microenvironment and its role in drug delivery from NANO3@*drug*-SH nanosystems was achieved by studying drug release in both physiological (pH 7.4) and acidic (pH 5.0) conditions (Figure 8.8.a and 8.8.b) for 72 h. When compared with the results obtained in previous studies (Chapter 6) for non-thiolated NANO3 systems, at the same conditions, one concludes that although the final functionalization with thiol moieties does not change the release tendencies for different pHs, it changes the total released quantities of drugs. This effect is particularly significant for total released OFLO from both NANO3@O-SH and NANO3@DO-SH, which see, respectively, a 10-fold and 3-fold reduction when compared to their non-thiolated counterparts (see Chapter 6). Notwithstanding, the presence of DOX in a combinatory formulation promotes, again, a higher release of OFLO (although lower than that of the aforementioned NANO3 systems). The slow release of OFLO from NANO3 systems is however an interesting result, as a more time-paced delivery of the antibiotic could promote an initial therapeutic burst of anti-cancer activity by DOX, followed by a later,

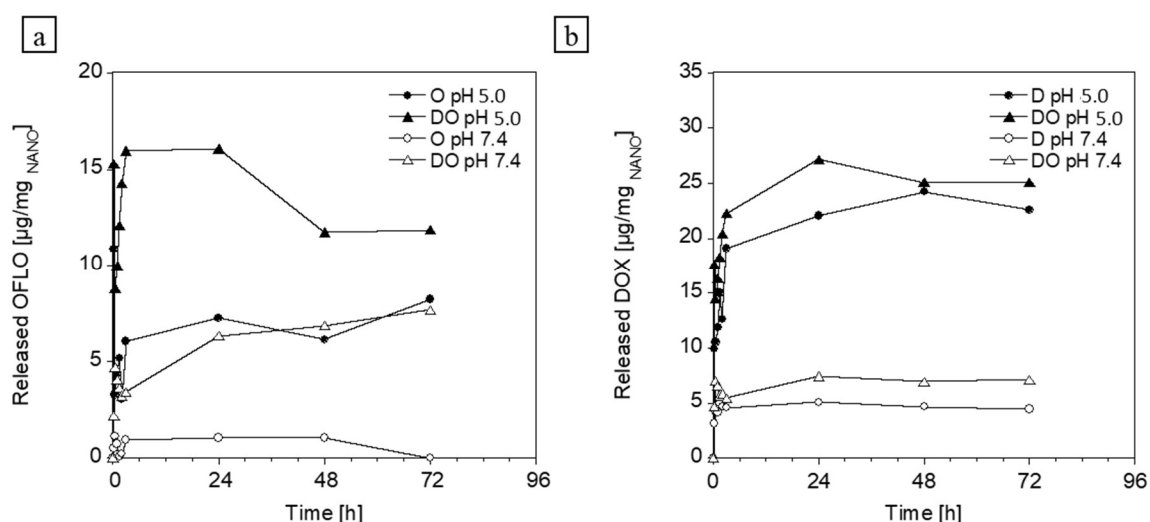


Figure 8.7 - (a) Olfoxacin (OFLO) and **(b)** Doxorubicin (DOX) pH-stimulated release profiles from NANO@O-SH, NANO@DO-SH and NANO@D-SH, at pH 7.4 and 5.0.

long-term, and continuous antimicrobial action of OFLO.

Conversely, DOX release either remains almost invariable or is enhanced. While for NANO3@D-SH released DOX at pH = 7.4 is the same as that of NANO3@D, at acidic conditions it sees a 2-fold increase. Regarding NANO3@DO-SH, no changes were seen when compared to its non-thiolated counterpart. Overall, the release of drugs, DOX in particular, is favored in acidic microenvironments and in DOX:OFLO dual formulations.

8.3.4 *Proof-of concept*. Enteric Coatings & Gastric Simulated Release

For that, in a proof-of-concept, NANO3@*drugs*-SH systems underwent an extra modification: a layered protective enteric coating with Eudragit® polymers (PMMA-MMA copolymers). These enteric polymers are well-known protective excipients for active pharmaceutical compounds, capable of regulating its release at different conditions of interest, be them time-based, pH-based or temperature-based^{40, 81, 82}. Of all available Eudragit® polymers, E-S100 and E-L100 polymers were selected based on their renown reputation for colon targeted drug delivery^{83, 84} and their dissolution pH-thresholds, with the former preferably dissolving at pH > 6.0 and the later at pH > 7.0. Their dissolution pH coincides with those of small intestine and colon sections (i.e., ascending, transverse and descending), where a proper dissolution of their polymeric matrix promotes the release of encapsulated NANO3 systems and the effective/local delivery of their targeting and therapeutic agents to CRC cells.

Two distinct encapsulation approaches were performed: (i) a single encapsulation with E-S100 (i.e., NANO3@*drugs*-S100), and (ii) a dual 1:1 encapsulation with E-S100 and E-L100. (i.e., NANO3@*drugs*-SL100); to assess the effective delivery to the colon and understand the protective role of the enteric matrices in NANO3 systems delivery. The successful coating of NANO3-SH and NANO3@*drug*-SH particles was followed zeta potential after the addition of each enteric coating as a total inversion to negative surface charges was expected⁸². The total inversion of their zeta potential (in deionized water, pH ~6.5), from a net-positive thiolated system to an net-negative system with average $\zeta = -24.0 \pm 1.7$ mV, confirmed indeed the successful coating by E-S100. A secondary coating with E-L100 was again confirmed by zeta potential, with obtained NANO3-SH materials showing consistent negative zeta potentials.

A simulated digestive track delivery and colonic-targeted release was achieved by exposing coated NANO3 systems to different pH and stirring times, in accordance with the conditions and passage times typical of each section. As perceived in Figure 8.8, and in line

with previously reported works on E-S100 and E-L100^{84, 85}, the overall release of drug-loaded nanoparticles in the simulated digestive fluids is higher at colonic pH than at small intestine and stomach pH. This demonstrates the capacity of the enteric coatings to protect the encapsulated NANO3@*drugs*-SH systems from external conditions, conditionate its release for the appropriate section of the digestive track and increase its bioavailability. Regarding single E-S100 and dual E-SL100 formulations, the results show that at a pH of 1.75 and 6.5 the dual enteric formulation shows an overall better capacity to control/retard the release by dispensing less NANO3@*drugs*-SH to the medium than E-S100 formulations at the same pHs. The early relaxation and dissolution of the E-S100 expedites NANO3@*drugs*-SH exposure to surrounding media and the release of the loaded-drugs at an early stage. This tendency is delayed in the dual-enteric formulations, for which at only pH 7.4 (i.e. in the colon) the external E-L100 coating is dissolved and a higher dispensing of nanoparticles to The surrounding media is permitted. The obtained results are in accordance with other reports that use the same model drugs^{86, 87} supporting the application of these enteric coatings in the oral administration of a sustained delivery to colon.

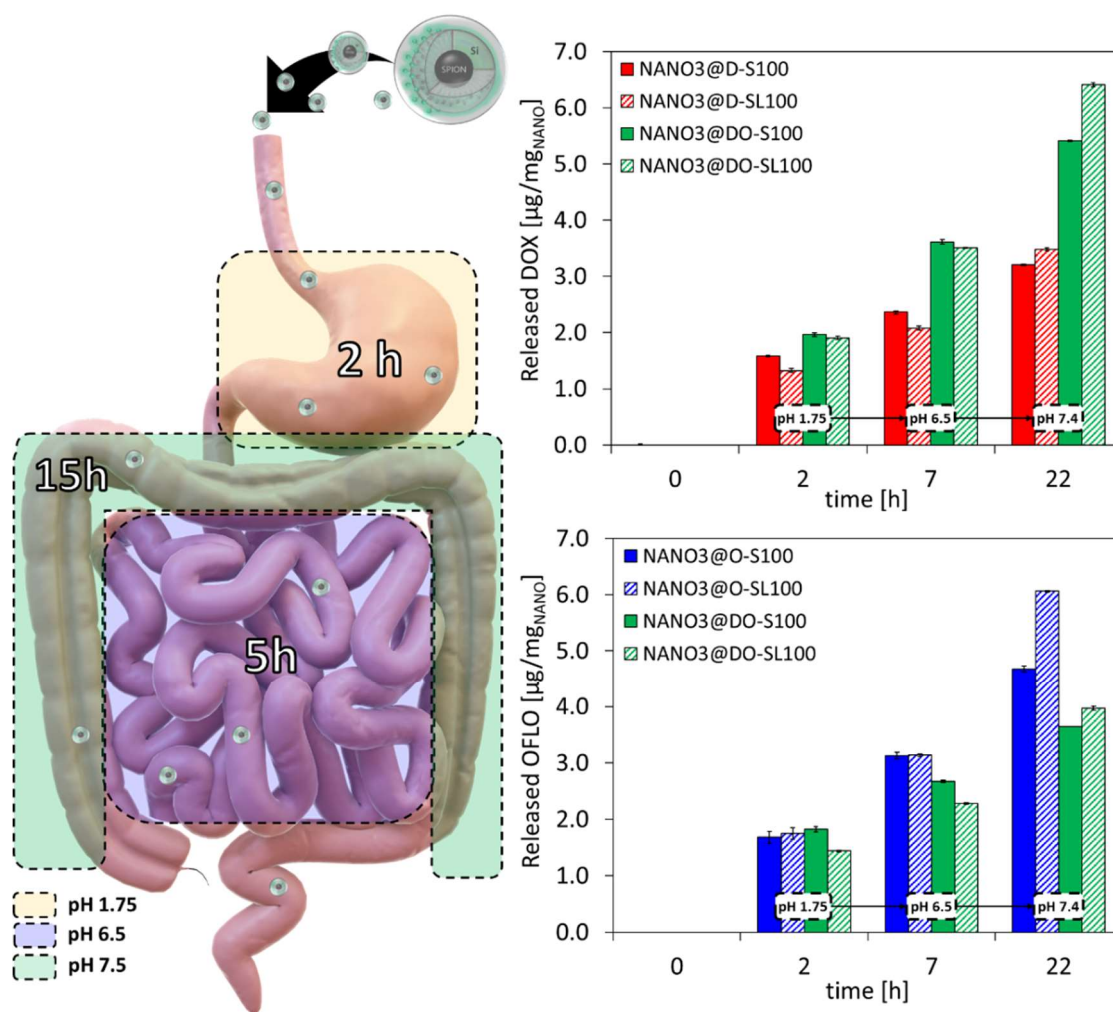


Figure 8.8 - Simulated digestive track release from enteric-coated NANO3@*drugs* systems (n=3), with medium pH and exposure time fitting the biological conditions of the selected sections of the digestive track (yellow – stomach, blue – small intestine, green – colon)

8.3.5 Biofunctionalization

The biofunctionalization of the already activated NANO3-SH systems with the previously modified CTX-SH and the synthesized AZM-SH was achieved by promoting the formation of disulfide bonds between nanoparticles and biological species. Biofunctionalized nanoparticles were obtained in 3 distinct final formulations: (1) single functionalization with thiolated CTX-SH (NANO3-CTX and NANO3@*drugs*-CTX), (2) single functionalization with AZM-SH (NANO3-AZM and NANO3@*drugs*-AZM); and (3) dual functionalization with CTX-SH and AZM-SH (NANO3-CTX and NANO3@*drugs*-CTX). A summary of all final formulations is depicted in Table 8.2.

Table 8.2 - Summary of all final NANO3 formulations, with drug and targeting species content.

Sample	Drug loading			Biofunctionalization			
	NANO3 (mg)	DOX (mg/g _{NANO})	OFLOX (mg/g _{NANO})	CTX (mg/g _{NANO})	AZM-SH (mg/g _{NANO})	CTX (#/NANO)	AZM-SH (#/NANO)
NANO3-CTX	5.0	-	-	13.65	-	5.54	-
NANO3-AZM	5.0	-	-	-	0.27	-	58.91
NANO3-CTX+AZM	5.0	-	-	5.62	0.33	2.28	72.39
NANO3@D-CTX	5.0	278.5	-	14.12	-	5.73	-
NANO3@D-AZM	5.0	278.5	-	-	0.32	-	69.85
NANO3@D-CTX+AZM	5.0	278.5	-	7.05	0.22	2.86	47.64
NANO3@DO-CTX	5.0	274.0	140.0	8.38	-	3.40	-
NANO3@DO-AZM	5.0	274.0	140.0	-	0.48	-	106.87
NANO3@DO-CTX+AZM	5.0	274.0	140.0	11.18	0.61	4.54	133.80
NANO3@O-CTX	5.0	-	182.8	9.76	-	3.96	-
NANO3@O-AZM	5.0	-	182.8	-	0.42	-	93.41
NANO3@O-CTX+AZM	5.0	-	182.8	6.13	0.35	2.49	77.97

All samples were resuspended in 1 mL PBS 0.01 M (pH 7.4) and then dried under vacuum.

#/NANO is equivalent to number of molecules per nanoparticle

Surface bound CTX content in NANO3 systems was assessed by forced reduction of cross-linking disulfide bonds and gel electrophoresis quantification (free CTX was used as standard) (Figure S-8.9). Surface bound AZM content was confirmed and determined by UV-vis spectroscopic analysis, at 412 nm, of the differential content of generated free TNB upon biofunctionalization of the activated (TNB containing) NANO3-SH/NANO3@*drugs*-SH, with AZM or CTX+AZM.

From Table 8.2, a significant increase in the co-functionalization of CTX and AZM occurs in the dual-loaded NANO3 systems when compared to non-loaded NANO3 system. This might result from a higher availability of surface thiol groups, that derives from a increased surface charge provided by the dual loading of OFLO and DOX (35 ± 0 mV for the dual loaded nanosystem and 21.5 ± 1.0 mV for the non-loaded NANO3-SH). A similar trend is seen for NANO3@D-SH which has a $\zeta = 20 \pm 0$ mV, comparable to that of the empty NANO3-SH. This will result in less cross-linking phenomena between surface thiols and subsequently, in a higher biofunctionalization rate for AZM and CTX. When regarding OFLO-containing NANO3 systems, there is also a variation on the surface charge (26.6 ± 0.9 mV), however it is not as significant as that provided by the dual loading. This results in a more efficient biofunctionalization of the CTX and AZM molecules than that presented by the non-loaded system, but not as effective as that provided by the dual loaded system.

The occurring variations in surface charge are a response to the presence of one or two drugs and are influenced by the loading mechanisms of the drugs to the particles. These are modulated by their water solubility and electrostatic interactions between drugs and particles. The presence of ofloxacin in the systems always results in a higher surface charge, possibly due to the presence of a fluor atom in the molecule, which increases stereochemical impediment in the porous matrix, but is also modulated by ofloxacin solubility in water (1.4 mg/mL), which is higher than that presented by doxorubicin (0.3 mg/mL)^{73, 88}. Doxorubicin presents thus a lower water solubility which favours a DOX loading into an more internal section of the mesoporous matrix, in detriment of a more external OFLO encapsulation.

8.3.6 Cytotoxicity and antitumor activity

The cytotoxicity of the biofunctionalized NANO3-SH systems was assessed against HCT-116 and HT-29 cell lines, as representative CRC models (Table 8.3). These cells were selected on their hCA IX and EGFR overexpression, which was confirmed by western blot and where we can see high intensity bands for anti EGFR and anti CA IX in HCT-116 cell line and HT-29 cell line, respectively. These results indicate a EGFR overexpression in HCT-116 cells,

whereas for HT-29 cell line there is overexpression of hCA IX receptor. The house keeping α -tubulin protein, was used as control to normalize and ensure the presence of a similar cell concentration between the two lines (Figure 8.9).

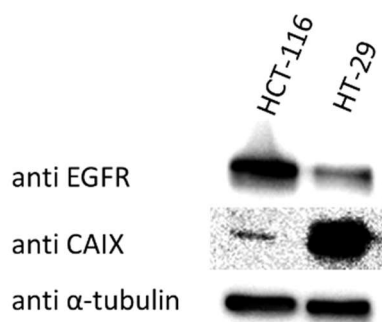


Figure 8.9 - Western blot for the overexpression levels of EGFR and CA IX in HCT-166 and HT-29 CRC cell lines.

As per Table 8.3, the absence of toxicity of the free OFLO supports the non-toxic role of the antibiotic in its nanometric formulations, which is confirmed by the absence of toxicity from NANO3@O systems, regardless of the chosen CRC cell line. An equal lack of cytotoxicity was also observed for the empty NANO3 systems which demonstrates the systems' biocompatibility and possible usage in a human directed therapy. This is aligned with the cytotoxic profiles reported in literature for similar composite nanomaterials, whose constituents have shown non to virtually-null cytotoxicity^{8, 89, 90}.

The toxicity/antitumor activity of the prepared materials derives only from DOX presence in their formulation, having a more accentuated action against HCT-116 line than HT-29 line even in its free form. The obtained IC₅₀ for the NANO3 systems account with the total released DOX after 72h and are in line with those obtained for the free drug. This demonstrates that the introduction of a protective nanometric material, capable of shielding DOX from harsh external conditions and in-body pharmacokinetics (i.e., absorption, distribution, metabolism, and excretion)⁹¹⁻⁹³, does not hinder the efficacy of the encapsulated drug and drug delivery.

Table 8.3 - IC₅₀ values of biofunctionalized NANO3 and NANO3@*drugs* against HCT-116 and HT29 CRC cell lines, for 72h. Concentrations given as equivalent DOX concentrations (in μM).

Compound	72h	
	IC ₅₀ (μM) HCT-116	IC ₅₀ (μM) HT-29
NANO3-CTX	>100	>100
NANO3-AZM	>100	>100
NANO3-CTX+AZM	>100	>100
NANO3@D-CTX	0.25 $\pm$ 0.02	0.88 $\pm$ 0.01
NANO3@D-AZM	0.23 $\pm$ 0.01	1.06 $\pm$ 0.24
NANO3@D-CTX+AZM	0.25 $\pm$ 0.01	1.13 $\pm$ 0.05
NANO3@DO-CTX	0.28 $\pm$ 0.02	1.32 $\pm$ 0.20
NANO3@DO-AZM	0.31 $\pm$ 0.02	1.26 $\pm$ 0.09
NANO3@DO-CTX+AZM	0.28 $\pm$ 0.03	1.39 $\pm$ 0.42
NANO3@O-CTX	>100	>100
NANO3@O-AZM	>100	>100
NANO3@O-CTX+AZM	>100	>100
DOX	0.19 $\pm$ 0.03	0.86 $\pm$ 0.17
OFLO	>200	>200

The effect of different biofunctionalizations is, however, not perceived after a 72h exposure of the selected cell lines to the NANO3 systems. At this exposure time we might be looking at the cumulative/maximum effect of these nanoparticles, with no opportunity to understand and visualize the impact that the biofunctionalizations might have on cellular uptake and short-term toxicity.

8.3.7 Cellular uptake and targetability

To better understand the possible influence of the different biofunctionalizations (CTX, AZM and CTX+AZM) in the NANO3 system, internalization assays were conducted for both cell lines. The exposure period for these assays was of 3h to try to distinguish the short time effects of each biofunctionalization in cellular uptake, for at 72h a maximum cumulative uptake was detected. After the exposure time, cells were stained and visualized by laser scanning confocal microscopy (LSCM). As depicted in Figure 8.10, three distinct fluorescent signals were collected, refereeing to cellular membrane staining (green), nuclei position (blue) and DOX distribution (red). DOX was mostly localized in the cytoplasmic environment of both cell lines, confirming the successful internalization of all NANO3@D systems. Moreover, a qualitative analysis of HT-29 micrographs points towards a more intense internalization of NANO3@D systems, than at HCT116. This difference in red fluorescent signals between cell

lines might point to the existence of either different uptake mechanisms, or different kinetic uptake responses for the assay's timing⁹⁴⁻⁹⁷.

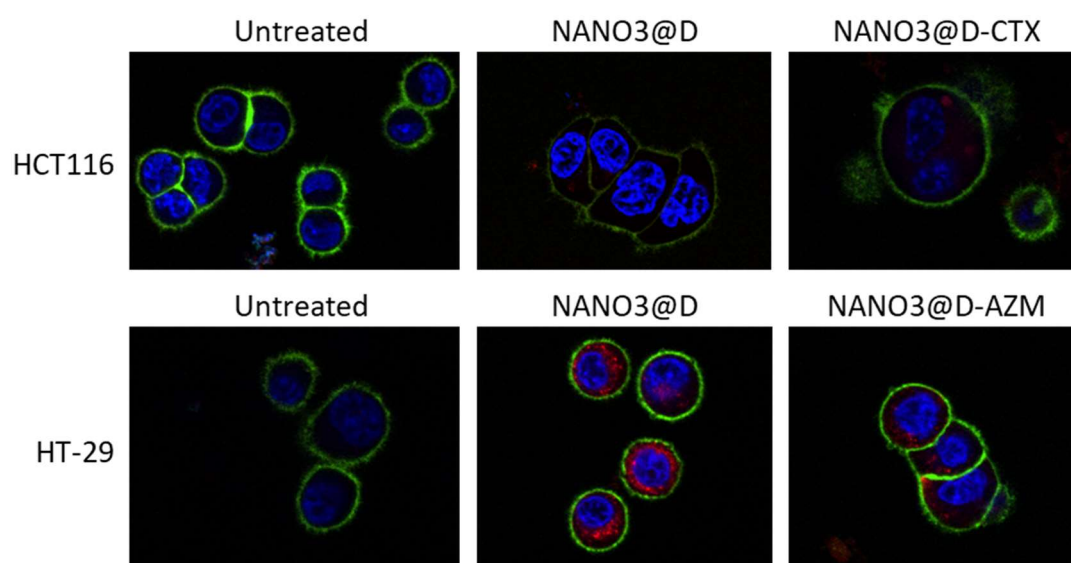


Figure 8.10 - LSCM images of HCT-116 and HT-29 cell lines treated for 3h with NANO3@D, NANO3@D-CTX and NANO3@D-AZM, according to their specific overexpressed targets (untreated cells were used as negative controls).

For a quantitative analysis of the uptake profiles, cellular accumulation assays were performed. These assays were performed with an exposure period of 3 hours, similarly to those presented in the aforementioned uptake assays with LSCM.

Results depicted in Table 8.4 confirm the overall higher uptake of DOX-containing nanoparticles in the HT-29 cell line, regardless of the type of biofunctionalization. Its independency on particle concentration and type of biofunctionalization might be an indirect consequence of the selected concentrations, which exert enough pressure to force HT-29 cells to use non-targeted specific uptake pathways. In regard to HCT-116 cell line (a low expressing CA IX line) a preferable uptake was observed for NANO3@D-AZM ($10.26\% \pm 0.49\%$), with an increase of 2-fold when compared to the non-functionalized NANO3@D ($5.93\% \pm 0.32\%$), at $5\mu\text{M}$. An increased uptake of the CTX-containing formulation was also observed as expected, owing to its overexpression of EGFR. The somehow contradictory lower uptake of NANO3@D-CTX+AZM for the same cell line, regardless of the tested concentrations, could point to slower uptake kinetics caused by the presence of two targeting probes that might be competing with each other. Notwithstanding, by crossing this result with the system's cytotoxicity we confirm that this does not hamper the overall anti-tumor activity, after 72h of exposure. Finally, despite the above results, extended uptake

studies at lower concentration ranges and kinetic studies are advised to completely evaluate the tumor targetability mechanism of the herein prepared NANO3 systems.

Table 8.4 - Internalization rates of different NANO3@D systems, at different equivalent DOX concentrations (5, 10 and 25 μ M) in HCT-116 and HT-29 CRC cell lines. Internalization rates given as the relative fluorescence of the samples against the fluorescence of the nanoparticles in medium before treatment (excitation at 480 nm, emission collected at 550 nm).

% Internalization			
NP concentration	25 μ M	10 μ M	5 μ M
HCT-116			
NANO3@D	6.97% $\pm$ 0.02%	6.16% $\pm$ 0.95%	5.93% $\pm$ 0.32%
NANO3@D-CTX	9.78% $\pm$ 2.01%	5.81% $\pm$ 0.62%	9.27% $\pm$ 0.51%
NANO3@D-AZM	9.67% $\pm$ 2.17%	8.18% $\pm$ 1.69%	10.26% $\pm$ 1.96%
NANO3@D-CTX+AZM	7.61% $\pm$ 0.51%	7.38% $\pm$ 0.51%	6.17% $\pm$ 0.38%
HT-29			
NANO3@D	11.13% $\pm$ 0.43%	9.92% $\pm$ 0.68%	9.72% $\pm$ 0.37%
NANO3@D-CTX	9.22% $\pm$ 1.64%	7.68% $\pm$ 0.88%	8.84% $\pm$ 0.59%
NANO3@D-AZM	8.29% $\pm$ 1.63%	9.29% $\pm$ 0.87%	10.05% $\pm$ 0.77%
NANO3@D-CTX+AZM	9.40% $\pm$ 1.23%	9.63% $\pm$ 0.75%	10.07% $\pm$ 0.84%

8.4 Conclusions

In light of the current need of new, non-invasive and targeting therapies against CRC, here in we successfully detail the synthesis a new acetazolamide derivative (AZM-SH), with anti-hCA IX inhibitory activity, and of a dual-cargo, dual-targeting magneto fluorescent nanosystem (NANO3) as possible vehicle for a new oral administered anti-CRC therapies. Beyond their already confirmed antimicrobial activity through the inclusion of an OFLO-containing formulation and hyperthermia properties (reported in Chapter 6), the NANO3 systems showed consistent and competitive anti-tumoral activity against both tested CRC cell lines. The inclusion of CTX and AZM derivatives as targeting agents at the surface of NANO3 showed beneficial for their uptake in HCT-116 cell line, particularly those functionalized with AZM-SH. Future studies are needed to gather more knowledge on the nanosystems' uptake kinetics and concentration influence in HT-29 cell line. Serum starvation trials are also to be run to assess NANO3 targetability in the absence of competing growth factors and proteins. An oral delivery approach was confirmed through a proof-of-concept modification of the

drug-loaded nanoparticles. with the inclusion of a combinatory enteric coating of E-S100 and E-L100 successfully delaying the release in simulated gastric and small-intestine conditions and favoring it in colonic conditions. Coating of biofunctionalized NANO3 with enteric polymers is also a protocol to be optimized to ensure AZM and CTX stability and function, with future release assays including a final cytotoxic assay to check their therapeutic efficacy.

8.5 Acknowledgments

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8.7 Supplementary Materials

8.7.1 Materials and Methods

8.7.1.1 Materials

Iron(III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 97.0-102.0%), iron(II) chloride hydrate ($\text{FeCl}_2 \cdot \text{H}_2\text{O}$, 99%), ammonium nitrate (NH_4NO_3 , 99.999%), N-hydroxysuccinimide (NHS, 98+%), ethylenediaminetetraacetic acid tetrasodium salt hydrate (EDTA, 98%) and tetraethyl orthosilicate (TEOS, $\geq 99.999\%$ metals basis) were bought at AlfaAesar. *n*-cetyltrimethylammonium bromide (CTAB), (3-aminopropyl) triethoxysilane (APTES, $\geq 98\%$), thioglycolic acid ($\geq 99.9\%$), succinic anhydride ($\geq 99\%$ GC), (+)-sodium L-ascorbate (crystalline, $\geq 98\%$), N-3-dimethylaminopropyl-*n*-ethylcarbodiimide hydrochloride (EDC, $\geq 97\%$), acetone ($\geq 99.5\%$ GC), 4-morpholineethanesulfonic acid (MES, $\geq 99\%$), thioglycolic acid (TGAc, 98%), 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's Reagent, DTNB, $\geq 98\%$), 2-2-iminothiolane hydrochloride ($\geq 98\%$ (TLC)), 5-acetamino-1,3,4-thiadiazole-2-sulfonamide (Acetazolamide, AZM, $\geq 99\%$), 3-mercaptopropionic acid (99%), anhydrous N,N-dimethylformamide (DMF, 99.8%), anhydrous tetrahydrofuran (THF, 99.9%), TRIS (hydroxymethyl)aminomethane hydrochloride (TRIS HCl), doxorubicin hydrochloride (DOX, 98.0-102.0% (HPLC)), DL-Dithiothreitol (DTT), β -mercaptoethanol (molecular biology grade) and phosphate-buffered saline (PBS, tablets) were purchased from Sigma Aldrich. Ethanol absolute (EtOH, extra pure), hydrochloric acid (HCl, 37%) and sodium hydroxide (NaOH) were bought at Scharlab. SL. Oleic acid (OA, 65.0 - 88.0 %) was acquired from Honeywell Fluka. Ofloxacin (OFLO, 98%) was bought from ThermoFisher. Deuterated reagents as dimethyl sulfoxide-D6 (DMSO-d6), acetone-D6 and acetonitrile-D3 (CD_3CN) were purchased from Acros Organics. Anti-EGFR Cetuximab (CTX 5.2mg/mL, 100%) was acquired from Selleckchem. Eudragit-L100® and Eudragit-S100® were supplied by Evonik Operations GmbH. All reagents were used as acquired, without any further purification, and all solutions, unless otherwise indicated, were prepared with deionized Millipore milliQ water.

8.7.1.2 Instrumentation

ATR-infrared spectra were collected in a Tensor 27 (Bruker, Massachusetts, United States), and ^1H / ^{13}C nuclear magnetic resonance spectroscopy (NMR) performed in a Bruker FT-NMR Avance 400 (Ettlingen, Germany) spectrometer, at 300K, from the Departamento de Química, Universidad Politécnica de Valencia. Mass spectroscopy analysis was performed by

ESI+QTOF MS in a SCIEX 6600 plus TripleTOF. of the SCSIE - UVEG of the University of Valencia. Melting point was determined in a Gallenkamp Melting Point apparatus (England) from the Bioscope Group LAQV-NOVAFCT. Elemental analysis was performed by CHNS method in a Elemental analyzer Thermo Finnigan-CE Instruments Flash EA 1112 CHNS series. from the Analysis Laboratory at LAQV-NOVAFCT. 96-well plate sampling analysis was performed in an EnSpire™ multilabel reader (Perki Elmer. Singapore Pte. Ltd.) from Departamento de Química. Universidad Politécnica de Valencia.

Temperature controlled incubations and release studies were performed in a HC24 PCMT Thermo-shaker (Grant Instruments. Cambridge. UK). Magnetization studies were performed in a Vibrating Sample Magnetometer (VSM) Lakeshore 7304 (Lake Shore Cryotronics. Ohio. USA). with a maximum applied magnetic field of 1.45 T. HRFSEM (and EDS analysis) and SEM images were taken with a GeminiSEM 500 Fiel Emission Scanning Electron Microscope (ZEISS Oxford Instruments) and a FEI Helios NanoLab 450S DualBeam – FIB with UHREM FEG-SEM. TEM images and EDS analysis were acquired in a 200 kV JEM 2100F JEOL–GATAN Oxford Nanomegas. with X-Ray Detector (EDX) DIGISTAR/ADT 3D/ASTAR. from Universitat Politècnica de València (UPV). DLS experiments were performed in a Malvern Nano ZS Zetasizer (633 nm laser diode). UV–Vis absorption spectra were obtained in a Jasco V-650 spectrophotometer and fluorescence spectroscopy measurements taken in a HORIBA Scientific FLUOROMAX-4 spectrofluorometer. from the Bioscope Group LAQV- FCT NOVA. and a JASCO FP-8300 spectrophotometer. from UPV. Powder X-ray diffraction (PXRD) measurements were taken on a D8 Advance diffractometer (Bruker. Massachusetts. United States). Thermogravimetric analyses were carried out on a TGA/SDTA 851e balance (Mettler Toledo. Columbus. Ohio. USA) in an oxidizing atmosphere (air. 80 mL min⁻¹) Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was performed in an ICP Ultima model (Horiba Jobin-Yvon. France). N₂ adsorption-desorption isotherms were recorded in a Tristar II Plus automated analyzer (Micromeritics. Norcross. GA. USA). The samples were degassed at 90 or 120 °C under vacuum overnight. ATR-infrared spectra were collected in a Tensor 27 (Bruker. Massachusetts. United States). and ¹H / ¹³C nuclear magnetic resonance spectroscopy (NMR) performed in a Bruker FT-NMR Avance 400 (Ettlingen. Germany) spectrometer.

Hyperthermia studies were conducted under an AC magnetic field. obtained with a water cooled two-spire Helmholtz coil. operated by an Ambrell EASYHeat 0224 power generator. Samples were placed in a thermostatic chamber with temperature controlled by the flow of heated air between the sample container and the cork-insulator that isolates the

system from the coils. The whole ensemble is isolated from external air convection by an acrylic chamber.

8.7.1.3 AutoDock4ZN forcefield parameters

As detailed by Santos-Martins et al. ¹, pre-processing the receptor molecule to add TZ pseudo-atoms was achieved by the python script `zinc_pseudo.py`, available at the AutoDock website, with the following syntax:

```
zinc_pseudo.py -r Receptor.pdbqt -o Receptor_tz.pdbqt
```

After generating the Grid Parameter File (`.gpf`), it was update with the keyword `parameter_file AD4Zn.dat` to specify `AD4Zn.dat` as the standard forcefield table. The new TZ atom type is specified in the `AD4Zn.dat` forcefield table, as having zero energetic contribution in Van der Waals interactions, H-bonds and desolvation. The keyword `nbp_r_eps` (see in AutoDock user manual for complete description) was used to define the specific pairwise potential between atom types involved in Zn interactions. The same keyword is used in the grid parameter file (`.gpf`) with the following syntax:

```
nbp_r_eps req e n m A1 A2
```

where, *n* and *m* are the L J potential coefficients (*n* = 12 & *m* = 6 in AutoDock4_{Zn}), and A1 and A2 are the type of involved atom. The final forcefield is implemented with the following entries:

```
nbp_r_eps 0.25 3.8581 12 6 NA TZ
nbp_r_eps 2.1 0.6391 12 6 OA Zn
nbp_r_eps 2.25 1.2617 12 6 SA Zn
nbp_r_eps 1.0 0.0 12 6 HD Zn
nbp_r_eps 2.0 0.001 12 6 NA Zn
nbp_r_eps 2.0 0.0493 12 6 N Zn
```

The ϵ value for the Zn-N interaction is the standard value computed from the forcefield table. The keyword `nbp_r_eps` updates the pairwise interaction radius to 2.0Å.

8.7.1.4 Superparamagnetic iron oxide nanoparticles synthesis – NANO1

The synthesis of well dispersed superparamagnetic iron oxide (Fe₃O₄) nanoparticles (NANO1) was achieved by a modification of the co-precipitation approach detailed by Zhang et al ². Briefly, 2.4 g of FeCl₃.6H₂O and 1.0 g of FeCl₂.H₂O were stirred until total dissolution in 10 mL of miliQ H₂O, at 80 °C, under continuous Ar bubbling. 5 mL of NH₄OH (25 wt.%) were

then added. and the resulting black solution stirred for an additional 1h. at 80 °C under continuous Argon (Ar) bubbling. After. 425 µL of oleic acid were injected into the mixture. which was bubbled for an additional 5 min and then sealed in Ar atm. to stir for 1.5h at 80 °C. The OA- coated NANO1 were magnetically decanted with the aid of a neodymium magnet. washed 10 times with miliQ H₂O to remove OA excess and transferred to 20 mL of chloroform. NANO1 concentration was estimated through the total content of Fe. determined by ICP-AES; the size of NANO1. determined by TEM; and its crystalline lattice structure ³.yielding *ca.* 6.8×10^{15} particles/mL (2.4×10^{17} particles/g).

8.7.1.5 Mesoporous silica shell surface growth and surface modifications – NANO2

In a typical approach. 150 mg of CTAB were dissolved in 10 mL of miliQ H₂O. mixed with 0.74 mL of NANO1 and sonicated for 1h. in an ultrasound bath at 35 KHz and 50 °C. The resulting emulsion was then heated to 70 °C and stirred for 30 min to evaporate the residual chloroform. Then. 30 mL of miliQ H₂O. 10 mL of ethylene glycol and 0.7 mL of NH₄OH (25 wt.%). were added. in this order. to the mixture which was stirred for at 70 °C. After 30 min of homogenization. 750 µL of TEOS were introduced dropwise to the reaction which was stirred for 3h at 70 °C. The as-prepared sample - NANO2 - was cooled to room temperature. centrifuged. washed in MeOH 3 times and left to dry at 70 °C.

For the modification of NANO2 surfaces. 50 mg of NANO2 were resuspended in 12 mL of miliQ H₂O and mixed with 1.2 mL of a THF solution containing 350 µL of APTES. for 24h at room temperature. The resulting NANO2-NH₂ were collected by centrifugation and washed with H₂O and acetone. before being resuspended in 5 mL of acetone and mixed with 3 mL of a 1.5 M succinic anhydride solution in acetone. The mix was stirred for another 24 h. at room temperature. and the samples collected by centrifugation and washed in MeOH. CTAB removal from the pores of the resulting NANO2-COOH samples was achieved by resuspension in 20 mL of 3 mg/mL methanolic solution of NH₄NO₃. for 1h at 60 °C (3 times).

8.7.1.6 Template Removal

Pores template removal from NANO2-COOH. was achieved after resuspension in a 30 mg/mL NH₄NO₃ methanolic solution (50 mL) and stirring for 1h at 60 °C. This process was repeated twice over. and the product obtained in the form of a light brown magnetic powder. respectively. after washing in methanol and drainage at air.

8.7.1.7 Silicon quantum dots (SiQDs) synthesis

In an adaption of the synthesis detailed by Marcelo et al ⁴, green emissive silicon quantum dots (SiQDs) were prepared by dissolving 50 mg of sodium ascorbate in 10 mL milliQ H₂O and mixing with 2 mL of APTES. The reaction was initially stirred for 40 min at 50 °C and aged for 24h at room temperature.

8.7.1.8 Magneto-fluorescent nanoconjugates - NANO3

Magneto-fluorescent nanoconjugates (NANO3) were obtained by conjugation of SiQDs' amine terminal moieties and NANO2- COOH, via a two-step EDC/NHS crosslinking reaction in a 1:3:3 COOH:EDC:NHS molecular ratio. Briefly, 20 mg of NANO2-COOH were resuspended in 4 mL MES Buffer 0.1 M (pH 6.0), containing 19.2 mg EDC and 14.4 mg NHS, and stirred for 30 min at room temperature. To the resulting mixture, 4 mL of SiQD were added and stirred for 24h at room temperature, in dark. The SiQDs were brought to pH 8.0 before conjugation with the modified NANO2-COOH, to ensure optimal reaction conditions. The resulting nanoparticles – NANO3 – were collected (10.000 rpm for 10 min) and washed with milliQ H₂O and MeOH.

8.7.1.9 NANO3 thiolation and activation

NANO3 thiolation was accomplished via EDC/NHS cross-linkage with thioglycolic acid (TGAc). For that, 2.5 mL of a 304 µg/mL TGAc aqueous solution were mixed with 1.25 mL of 0.1 M MES buffer (pH 6.8) containing 12.75 mg EDC and 9.75 mg NHS, for 30 min. Then, 25 mg of NANO3 were resuspended in 1.25 mL of 0.1 M MES buffer (pH 6.8) and mixed with the prepared TGAc solution, overnight at room temperature (in the dark). Each 25 mg batch of the yielded NANO3-SH was washed twice in 0.1 M TrisHCl (pH 8.05) and dried at air.

For the activation of NANO3 surface thiol groups, for further modifications with biological active species, each 25 mg batch of NANO3-SH were resuspended in 10.63 mL of 0.1 M TrisHCl (pH 8.05), with 0.1 mM EDTA, and mixed with 4.16 mL of a 0.18 mg/mL DTNB in 0.1 M TrisHCl (pH 8.05), for 3h at room temperature (in the dark). The resulting activated NANO3-SH were recovered by centrifugation and washed twice in the same buffer.

8.7.1.10 CTX bound content determination

For the determination of the content of bound CTX to the surface of NANO3-SH systems, a forced reduction of all disulfide bonds was performed over 5 mg/mL suspensions of each nanosystem in 0.01M PBS 7.4. For that, 5 µL of the suspension were mixed with 15 µL

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of a x4 reductive buffer containing 40 mM of DTT and 10% w/w of β -mercaptoethanol (see detailed Bioscope Research Team protocol below) and incubated for 45 min at 95 °C. Non functionalized NANO3-SH (5 mg/ml) were used as negative controls and free CTX (1 mg/mL) as positive controls and standards for quantification. The reduced samples were loaded, in duplicates (10 μ L), onto a 12 % electrophoresis acrylamide gel and run Mini-PROTEAN® Tetra Cell apparatus at 200 mV, 400 mA. The gel was revealed overnight in Coomassie R-250, then washed with water and digitalized in a Genomics Solutions' Propic IV. Image treatment and band intensity/area analysis allowed for the determination of CTX content.

8.7.2 Tables

Table S - 8.1 - Assignment of all collected FT-IR peaks, for AZM, AZM-NH₂ and AZM-SH.

AZM	AZM-NH ₂	AZM-SH	
FT-IR wavenumber (cm ⁻¹)			Assignment
-	3676	3676	ν_1
-	-	3549	ν_2
-	-	3491	ν_{as} N-H
-	3425 <i>str</i>	-	R-NH ₂ <i>stretch</i>
-	-	3375 <i>brd sh</i>	ν_{as} RSO ₂ N-H/-NH ₂
-	3317 <i>str</i>	-	R-NH ₂ <i>stretch</i>
3294 <i>str</i>	-	3248 <i>str</i>	ν_{as} RSO ₂ N-H/-NH ₂
3175 <i>med</i>	3170 <i>wk</i>	3170 <i>brd</i>	ν_s RSO ₂ N-H/-NH ₂
3090	-	3086 <i>brd</i>	ν N-H (RCONH)
-	2974	2974	ν_6
2889	2889	2889	ν_7
2773	-	2723	ν ring-NH ₂
2685	2638	-	ν_9
-	-	2515 <i>wk</i>	ν SH
2361	2341	2345	ν_{10}
1678 <i>str</i>	-	1678 <i>str</i>	C=O <i>stretch</i> ν CO-NH-R
-	1601 <i>str</i>	-	ν ring-NH ₂
1543	-	1528	ν CO-NH-R ν_{as} ring C=N
-	1497	-	ν azole ring
1431	-	1427	ν_s ring C=N ν n-propyl/ethyl
-	1447	-	ν R-SO ₂ -NH ₂
1362	-	1362	ν_{as} SO ₂
-	1335	-	ν ring-NH ₂
1311	-	1304	ν_{as} C-N-C
1284	-	1265	ν C-N ring
1246	1246	-	ν_{12}
1173	1173	1162	ν_s SO ₂
1119	1138	-	ν_{13}
1084	-	1084 <i>med</i>	ν n-propyl/ethyl
1045	1057	1057	ν_{14}
1011	-	1011	ν_{15}
972	-	972 <i>wk</i>	ν n-propyl/ethyl ν N-N
906	-	923	ν S-N ν n-propyl/ethyl
-	-	864	ν n-propyl
837	-	-	ν ethyl
818	-	-	ν ethyl
-	-	802 <i>wk</i>	ν SH
-	-	771 <i>med</i>	ν_1 n-propyl
702	-	702	ν_{as} C-S ring
671	-	-	ν_{20}
-	648	652	ν_{21}
617	-	-	ν_{22}
579	579	582	δ R-SO ₂ -NH ₂
555	-	-	ν_{23}
505	-	505	ν_{24}
455	482	-	ν_{25}
409	420	-	ν_{26}

wk, weak; *med*, medium; *str*, strong; *sh*, shoulder
s, symmetric; *as*, asymmetric

8.7.3 Figures

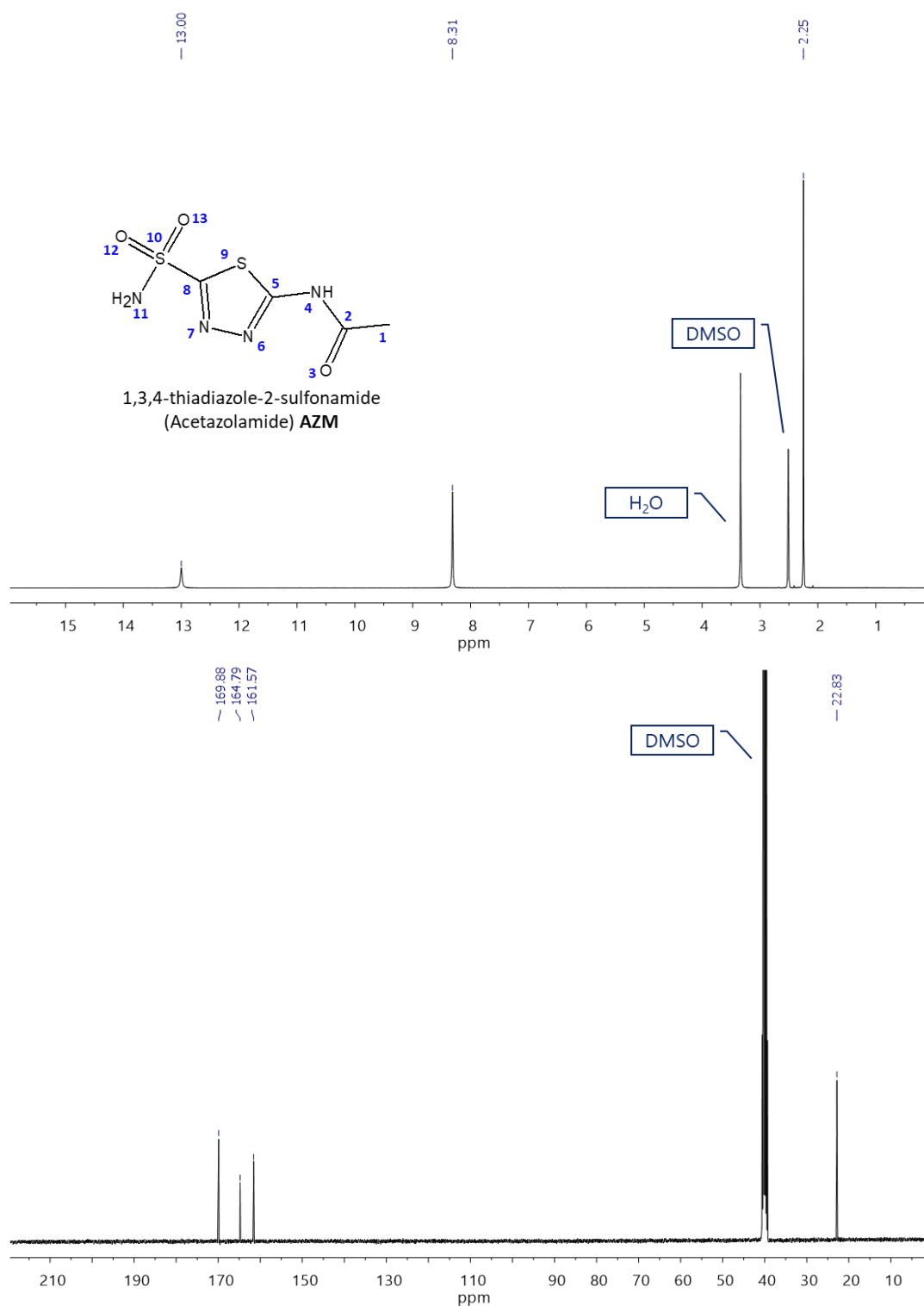


Figure S - 8.1 - (top) ^1H NMR spectrum of AZM: (DMSO- d_6 , 400 MHz) δ (ppm): 2.25 (s, 3H, H1 - CH_3), 8.31 (s, 2H, H11 - NH_2) and 13.00 (s, 1H, H4 - NH); spectral contaminants: 3.34 (s, 2H, H_2O) and 2.51 (t, 6H, DMSO). **(bottom)** ^{13}C NMR of spectrum AZM: (DMSO- d_6 , 400 MHz) δ (ppm): 22.83 (C1), 161.57 (C2), 164.79 (C5) and 169.88 (C8); spectral contaminants: 39.37-40.62 (m, DMSO).

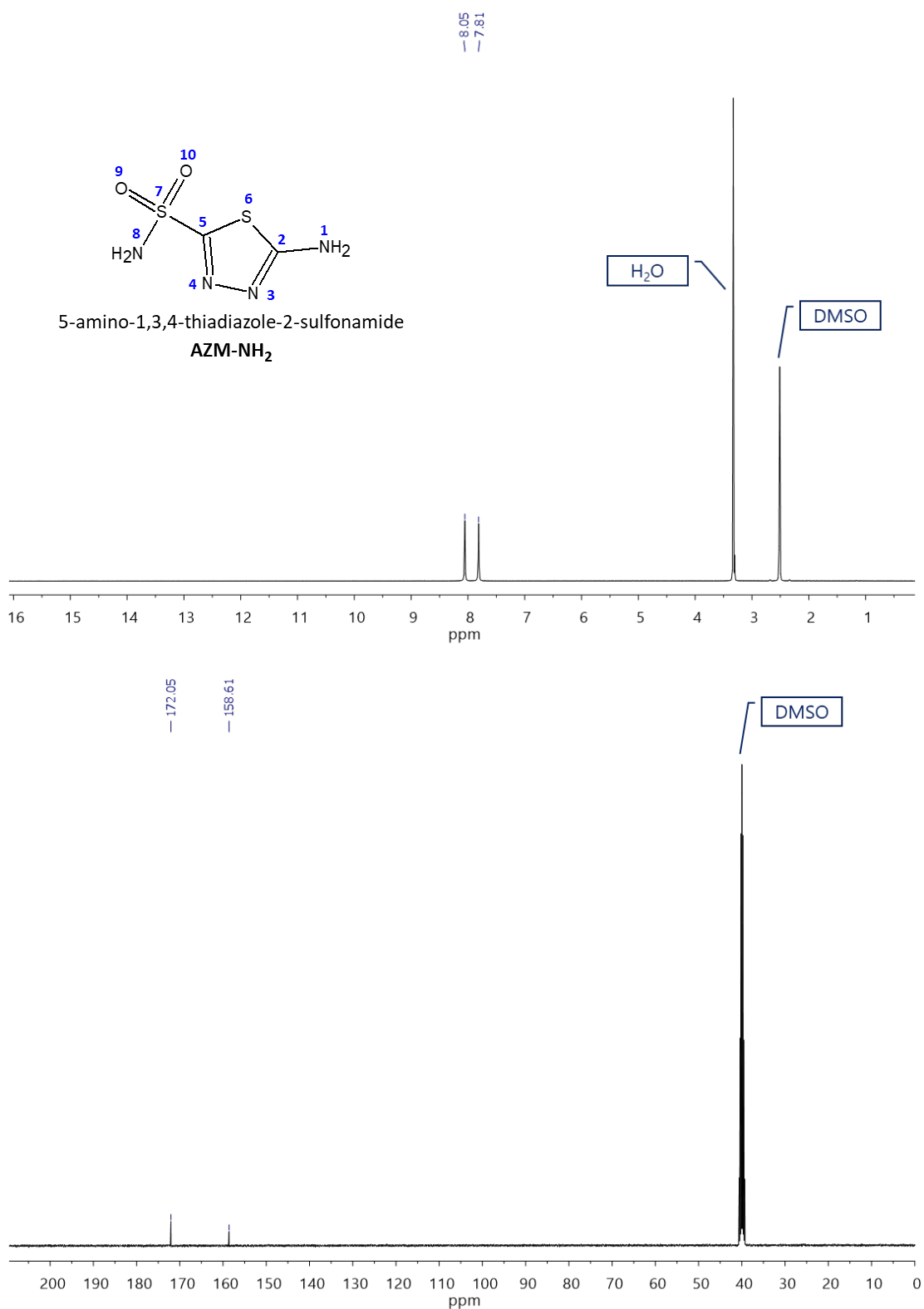


Figure S - 8.2 - (top) ¹H NMR spectrum of AZM-NH₂: (DMSO-d₆, 400 MHz) δ (ppm): 7.81 (s, 2H, H8 -NH₂), 8.05 (s, 2H, H1 -NH₂); spectral contaminants: 3.33 (s, 2H, H₂O) and 2.51 (t, 6H, DMSO). **(bottom)** ¹³C NMR of spectrum AZM-NH₂: (DMSO-d₆, 400 MHz) δ (ppm): 158.61 (C2), 172.05 (C5); spectral contaminants: 39.35-40.61 (m, DMSO).

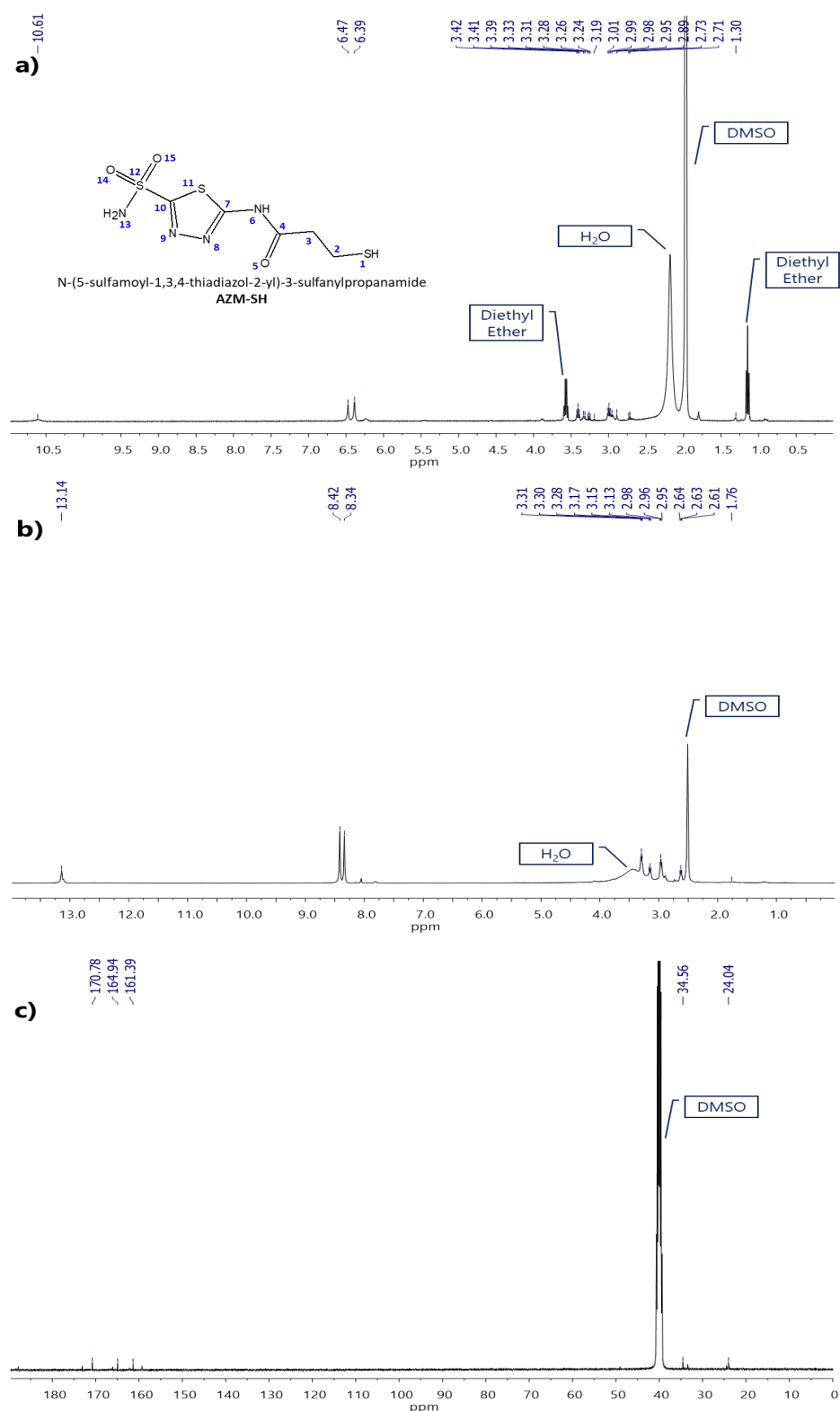


Figure S - 8.3 - (a) ^1H NMR spectrum of AZM-SH (CD_3CN , 400 MHz), spectral contaminants: 3.55 (q, 2H, $-\text{CH}_2$, diethyl ether) 2.22 (s, 2H, H_2O), 1.97 (s, 3H, CH_3CN), 1.15 (t, 3H, $-\text{CH}_3$, diethyl ether). **(b)** ^1H NMR spectrum of AZM-SH ($\text{DMSO}-d_6$, 400 MHz), spectral contaminants: 3.33 (s, 2H, H_2O) and 2.51 (t, 6H, DMSO). **(c)** ^{13}C NMR of spectrum AZM-SH ($\text{DMSO}-d_6$, 400 MHz), spectral contaminants: 39.35-40.61 (m, DMSO).

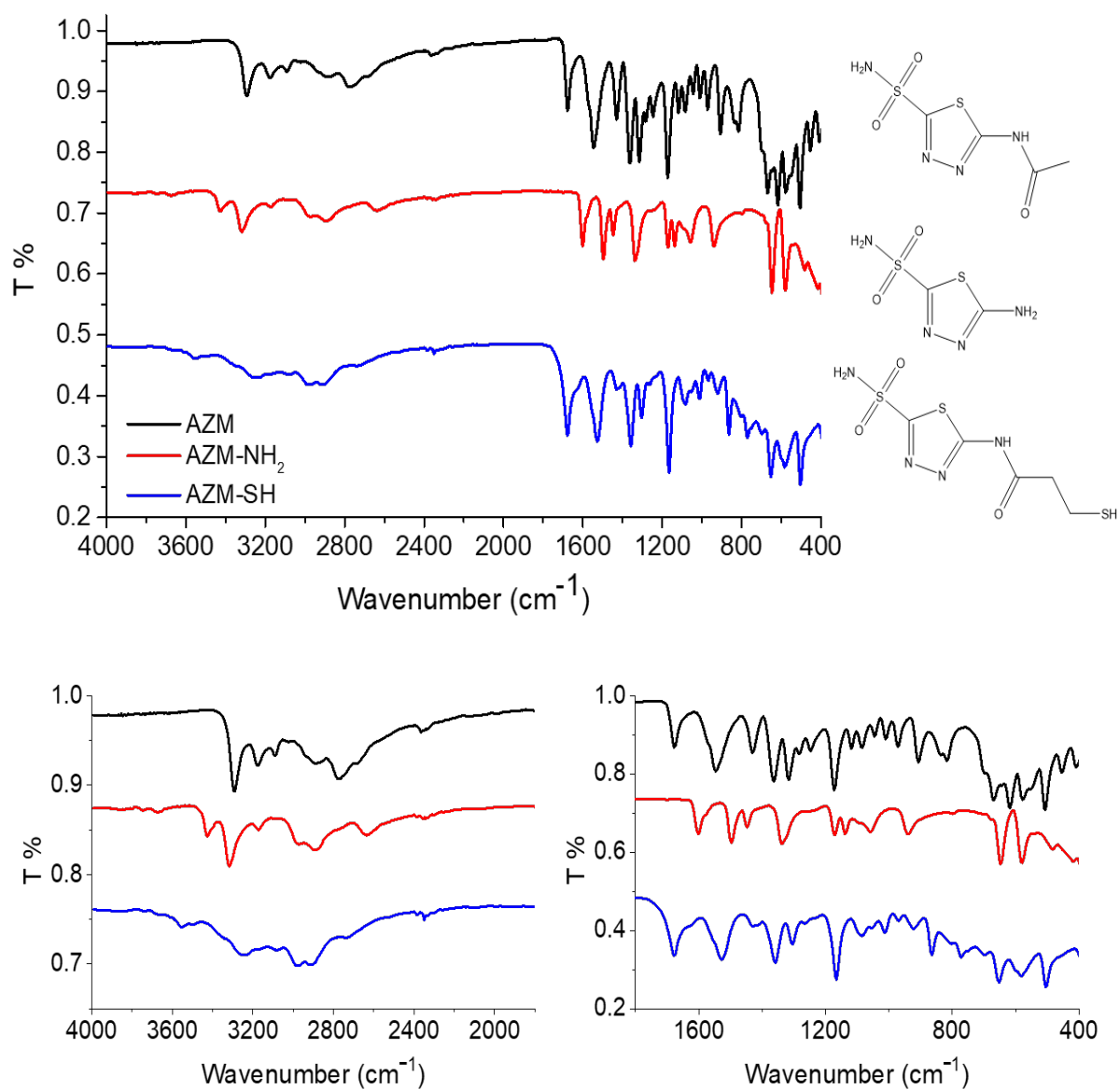


Figure S - 8.4 - (top) Complete ATR-FTIR spectra for AZM, AZ-NH₂ and AZM-SH. **(bottom)** Close-up to the same spectra for the selected wavenumbers ranges.

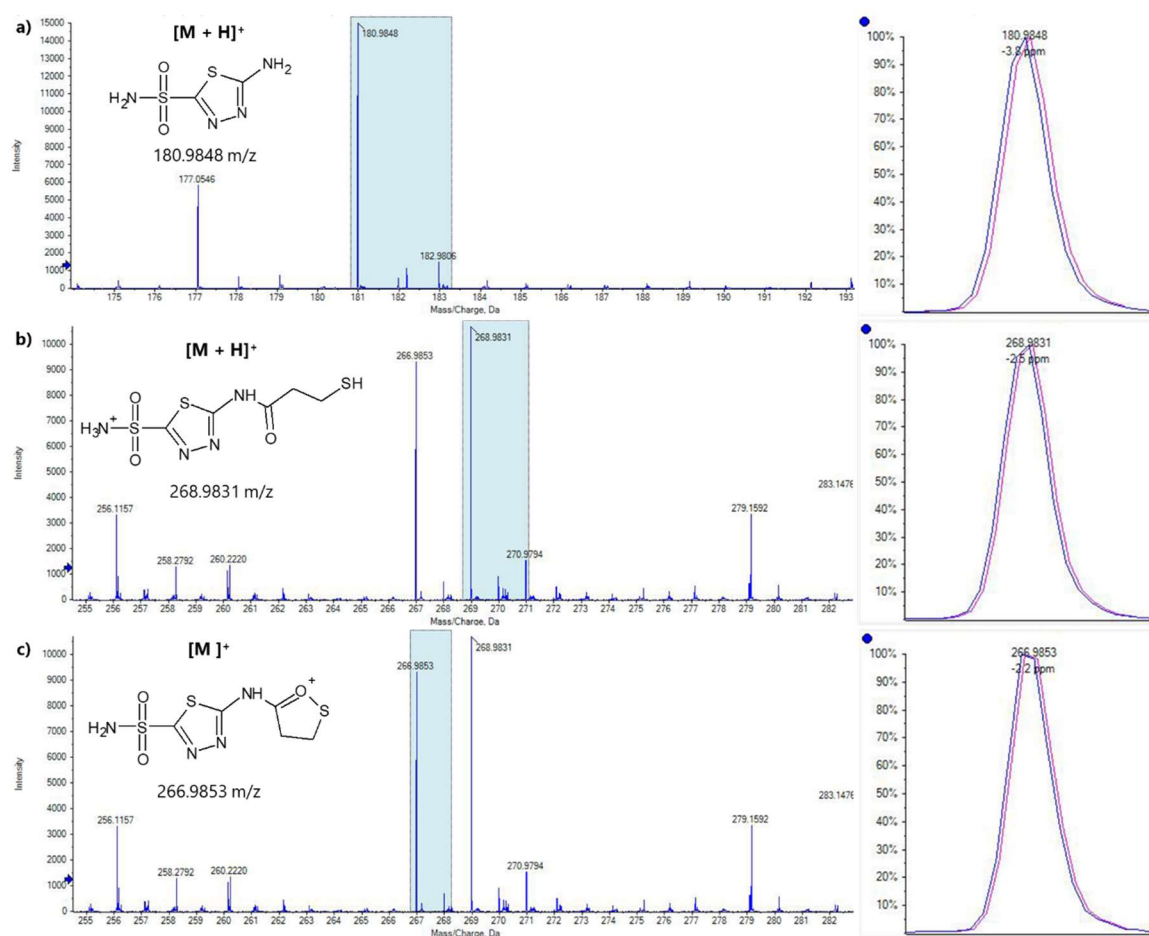


Figure S - 8.5 - ESI+QTOF MS spectra of (a) AZM-NH₂. (b) AZM-SH [M+H]⁺ ion and (c) AZM-SH [M]⁺ ion.

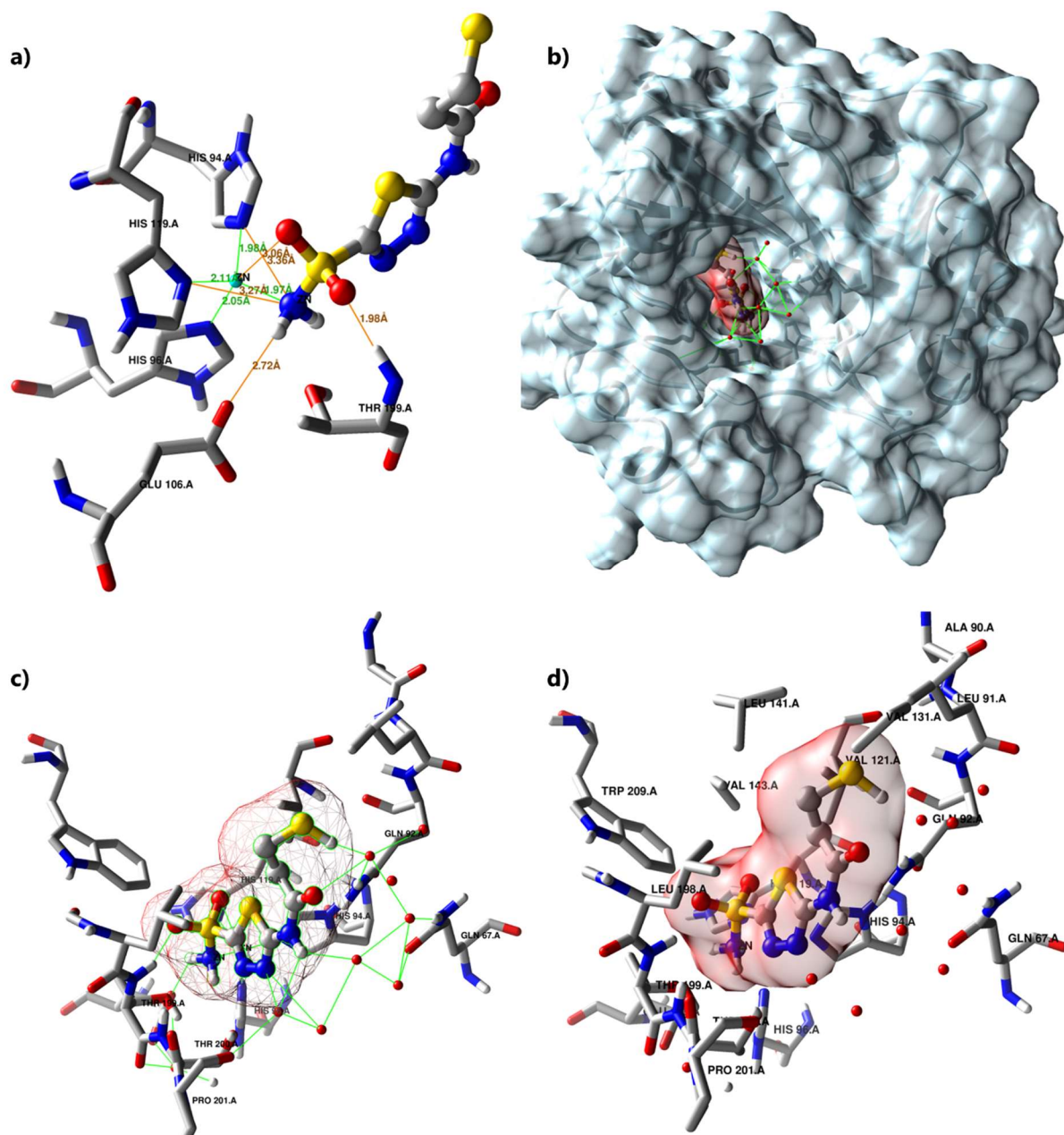


Figure S - 8.6 - (a) Predicted zinc coordination interactions and hydrogen bonds for the AZM-SH sulfonamide moiety, with Zn²⁺ ion and GLU106 and THR199 CA IX residues, respectively. **(b)** Spatial surface representation of AZM-SH binding to CA IX active site. **(c)** Hydrogen bonds network between AZM-SH thiadiazole N(8)-atom, sulfanylpropanamide N(6), O(5) and S(1)H moieties, and water molecules. **(d)** Hydrophobic alignment of the aliphatic AZM-SH sulfanylpropanamide carbons and the hydrophobic residues of CA IX.

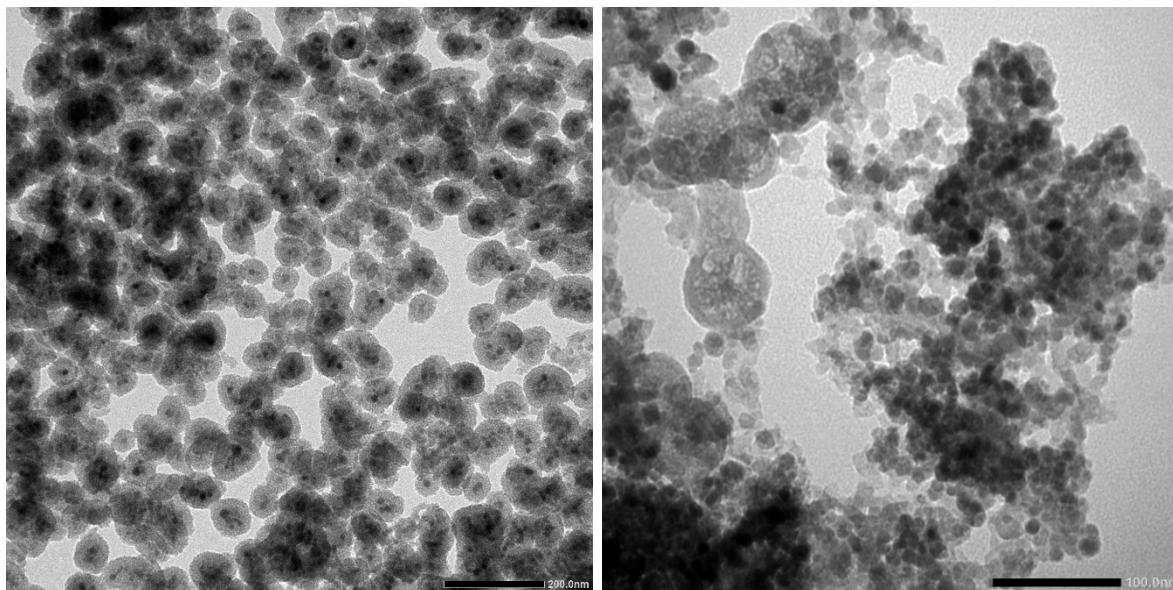


Figure S - 8.7 - TEM micrographs at 0 (*left*) and 1-month (*right*) NANO3-SH systems from degradation studies in physiological simulated conditions; scale bars at 200 μm and 100 μm , respectively.

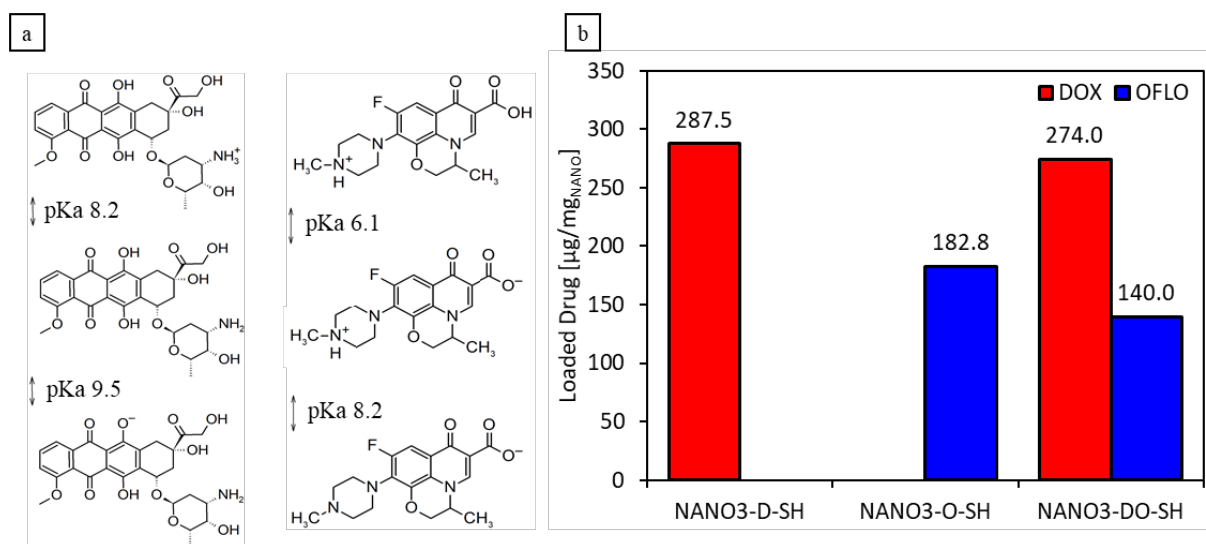


Figure S - 8.8 - (a) DOX and OFLO pKa in aqueous solutions; (b) NANO3-SH systems loading capacities of DOX and OFLO ($n=3$), in single and dual combinatory formulations

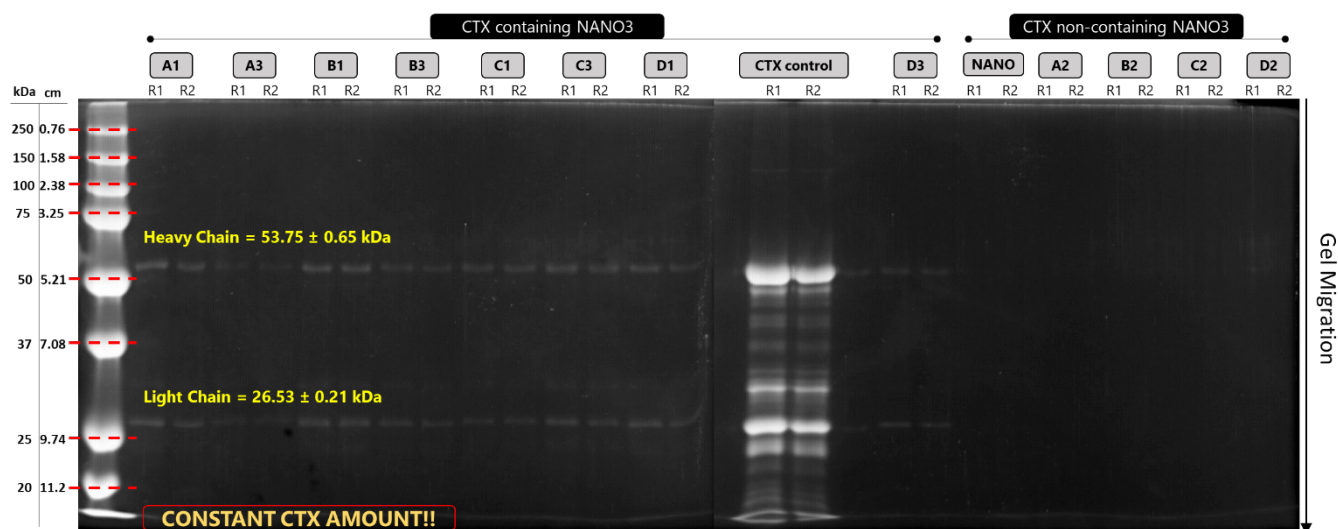


Figure S - 8.9 - Gel electrophoresis of reduced biofunctionalized NANO3-SH systems loaded with DOX and OFLO (n=2), in single and dual combinatory formulations. Samples: A1= NANO3-CTX, A2 = NANO3-AZM, A3= NANO3-CTX+AZM, B1= NANO3@D-CTX, B2 = NANO3@D-AZM, B3= NANO3@D-CTX+AZM, C1= NANO3@DO-CTX, C2 = NANO3@DO-AZM, C3= NANO3@DO-CTX+AZM, D1= NANO3@O-CTX, D2 = NANO3@O-AZM, D3= NANO3@O-CTX+AZM.

8.7.4 Bioscope Research Team Protocol

8.7.4.1 Stock Solutions

The preparation of each stock solution (solution I. solution II. solution III. running buffer. 10% APS. sample (Laemmli SDS-PAGE) 1D buffer solution and staining solution Coomassie R-250) is thoroughly described in Table S-8.3. except for 10% SDS solution that was diluted from an initial 20% SDS stock solution.

With all stock solutions prepared. the formulation of both stacking and running gel is detailed in Table S-8.2 Note that the compositions correspond to the preparation of two 4% acrylamide stacking gels and two 12% of acrylamide running gels.

Table S - 8.2 - Volumetric composition of a 4 mL monomer solution for stacking gel and a 10 mL monomer solution for running gel.

<i>Stock Solution</i>	<i>Stacking Gel</i>	<i>Running Gel</i>	
% Acrylamide	4	12	
<i>Solution I</i>	-	2.5	
<i>Solution II</i>	1	-	
<i>Solution III</i> (acrylamide/bisacrylamide) (37.5:1)	0.52	4	mL
<i>SDS 10%</i>	0.04	0.1	
<i>H2O Milli-Q</i>	2.48	3.4	
APS 10 %	30	70	
TMED	2	5	μL

8.7.4.2 Gel Cassette Preparation

With all Mini-PROTEAN® Tetra Cell components neatly washed (with deionised water) and assembled. the solution was smoothly introduced in the cassette apparatus and immediately overlaid with a 50% (v/v) butanol / 5% SDS solution. to prevent mixing with air. Polymerisation of the running gel lasted up to 30 minutes.

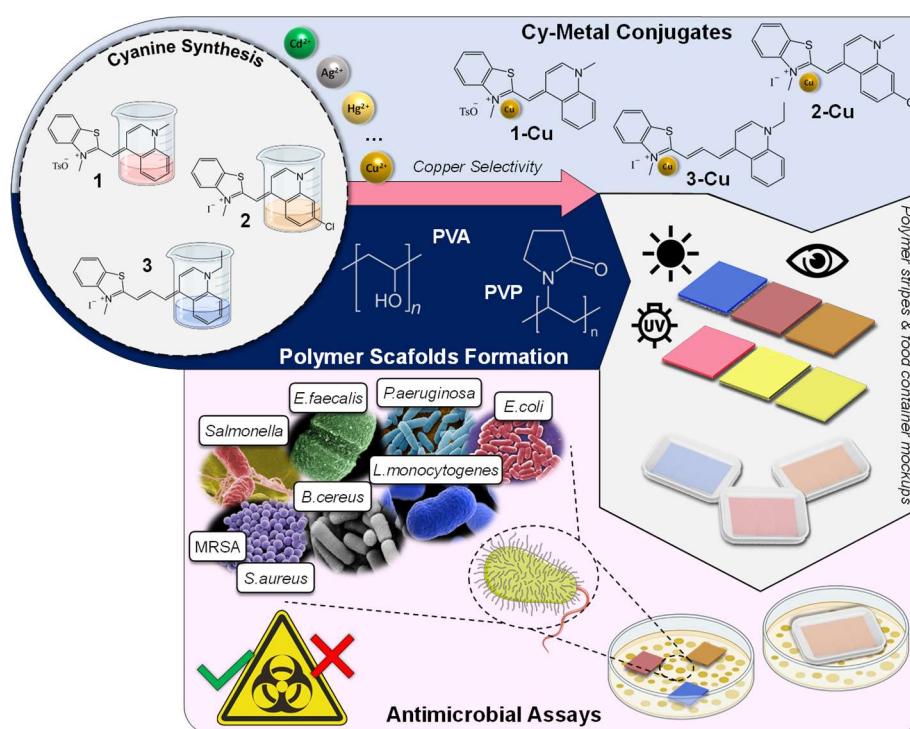
After removing both butanol / SDS solution and post- stacking gel monomer solution was introduced over the running gel interface and left to polymerize. A comb. with desired wells' dimensions. was also introduced onto the cassette apparatus at the same time (in this case. a 10-welled comb of 10 mm each was chosen). After stacking gel polymerisation. the comb was gently removed. and all wells rinsed with deionised water.

Table S - 8.3 - Stocks solution preparation table.

Solution	Reagents	Quantity	Comments
Solution I (running gel)	Tris Base	27.23 g	1.5M Tris-HCl. pH = 8.8
	HCl conc.	until pH 8.8	
	H ₂ O	until 150 ml	
Solution II (stacking gel)	Tris Base	6.06 g	0.5M Tris-HCl. pH 6.8
	HCl conc.	until pH 6.8	
	H ₂ O	until 100 ml	
10% SDS	SDS	10 g	-
	H ₂ O	until 100 ml	
10% APS	Ammonium persulphate	0.1 g	-
	H ₂ O	until 1 ml	
Running buffer (Tris-glycine)	Tris base	30.3 g	pH = 8.3 Dilute 1: 10 before use
	Glycine	144.1 g	
	SDS	10 g	
	H ₂ O	until 1000 ml	
Sample buffer (SDS-PAGE)	Solution II	1.25 ml	Store at -20°C Add 1:1 to protein mixture
	10% SDS	2 ml	
	β -mercaptoethanol	0.5 ml	
	Glycerol	2.5 ml	
	Bromophenol blue (0.5% w/v)	0.2 ml	
	H ₂ O	3.55 ml	
Staining solution Coomassie R-250	Coomassie Blue R-250	0.25 g	0.1% Coomassie Blue R-250 5% Glacial acetic acid 30% Methanol
	Glacial acetic acid	12.5 ml	
	Methanol	75 ml	
	H ₂ O	until 250 mL	
Distaining solution I	Glacial acetic acid	50 ml	5% Glacial acetic acid 30% Methanol
	Methanol	300 ml	
	H ₂ O	until 1000 ml	
Distaining solution II	Glacial acetic acid	35 ml	5% Glacial acetic acid 5% Methanol
	Methanol	25 ml	
	H ₂ O	until 500 ml	

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FUNCTIONAL CYANINE-BASED PVA:PVP POLYMERS AS ANTIMICROBIAL TOOLS TOWARDS FOOD AND HEALTH-CARE BACTERIAL INFECTIONS



Keywords: Polymers • cyanine • antibacterial surfaces •
biomedicine • food science

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Functional Cyanine-based PVA:PVP polymers as antimicrobial tools towards food and health-care bacterial infections

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Abstract: The rising of multidrug-resistant bacteria and their associated proliferation as harmful microorganisms boosted the creation of new antibacterial surfaces and biomaterials with applications ranging from health to food packing. Herein, low-cost antibacterial PVA:PVP copolymers containing cyanine derivatives (1, 2 and 3) and their respective Cu^{2+} complexes were successfully obtained and tested against Gram-negative and Gram-positive bacteria. The possible application in food packing was addressed by covering the surface of typical paper mockups with the doped polymers. All dye-doped polymers presented a broad-spectrum antibacterial effect against Gram-positive bacteria, especially for *B. cereus*, *S. aureus* and MRSA strains, with PVA:PVP@3 and PVA:PVP@3-Cu being the most effective. Moreover, polymers containing cyanine derivatives presented interesting inhibition effects against *P. aeruginosa*, where the production of its characteristic blue/green virulent pigment was not observed. Of the coated paper mockups, PVA:PVP:paper@2 and PVA:PVP:paper@2-Cu were most effective against *B. cereus* and *S. aureus*, while PVA:PVP:paper@3 and PVA:PVP:paper@3-Cu were most effective against the MRSA strain. In these formulations, direct contact inhibition mechanisms appear to be more significant than diffusional mechanisms, due to cyanine release hinderance, making them very interesting and versatile platforms for medical and food applications.

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9.1 Introduction

The increasing demand for the creation of antibacterial surfaces that destroy or inhibit the adherence of microorganisms has found itself boosted by the most recent Worldwide COVID-19 pandemic, that raised populations' awareness over good safety practices, when trying to avoid the spread of harmful microorganisms ^[1]. A similar phenomenon has been seen with the increased need for "ready-to-eat" food that has made consumers and manufacturers more aware of the potential hazards associated with packaging materials. As a matter of fact, this is not a unique and new occurrence, as significant interest in products' hazardousness and safety has been given during the development of antimicrobial biomaterials with application in various sectors, ranging from health to food and personal hygiene ^[2]. This general preoccupation has also been under the spotlight with the increasing news and public awareness on the rise of multidrug resistant bacteria. Consequence of an excessive and careless use of antibiotics multidrug resistance is, not only responsible for the spread of serious bacterial infections, some though eradicated, but also for the increasing expenses in healthcare as well as pharmacological research with the active search of alternative antimicrobial formulations ^[3].

With most efforts being directed to new post-infection clinical treatments, prevention through the decrease of bacteria proliferation presents itself as a sustainable alternative to overcome this problem. This is particularly achievable by the development of novel antibacterial materials ^[4], or by rendering existing materials with antibacterial activity, for a later incorporated in common surfaces or biomedical tools. In literature a vast number of papers can be found related to the design and production of antimicrobial materials ^[5]. Moreover, it is important to note that most antibacterial devices used for food packing, medicine or for the textile industry are made of polymeric materials ^[6].

Polymer-based materials are already present in many medical devices, such as catheters, prosthetic materials and surgical masks aiming for the prevention of bacterial growth ^[7]. Food science and technology areas also apply these polymeric materials for intelligent packaging, by creating sensors capable of interacting with food and providing information on potential deterioration or contamination ^[8]. Among all polymeric materials,

(polyvinyl alcohol) PVA and (Polyvinylpyrrolidone) PVP have been shown to have good stability, low toxicity, hemocompatibility and biodegradability, while being approved by the US Food and Drug Administration and the European Food Safety Authority as safe polymers for biological applications ^[9]. They are widely used for medical purposes (e.g. contact lenses) or as food additives/stabilizers ^[6, 10]. Despite their remarkable properties, when used alone, PVP leads to materials with poor mechanical strength, an issue that could be easily overcome by the addition of other polymers, such as PVA, that render them malleability, often critical for their successful application ^[11]. Furthermore, studies revealed synergistic effects in PVA and PVP comprising hydrogels/films, like those of high water absorbency and antimicrobial activity, that impart them valuable additional functionalities ^[6, 12]. The modification of polymeric matrices to prevent or reduce the growth and adhesion of harmful microorganisms is thus a highly desired goal, with the co-utilization of biodegradable and biocompatible polymeric films, or polymeric-based materials, with antimicrobial actives as promising and innovative solutions for both packaging and healthcare industries ^[4].

The attribution of antimicrobial properties to a surface is usually achieved by the incorporation of either metals, metal oxides, or organic antibacterial compounds, like silver ^[13], iron-oxide ^[14] and methylene blue ^[15], respectively.

Interestingly, a class of cyanine derivatives containing aromatic heterocyclic moieties, as thiazolium, have been described as attractive organic antibacterial compounds, due to their known antimicrobial, anti-inflammatory, analgesic, anti-tubercular and anticancer activities ^[16]. Moreover, this same class of cyanine dyes has also shown to have a preferable absorption in the visible region and a positive net charge arising from their thiazolium moieties. This positive charge allows for electrostatic interactions with bacteria cell membranes, which are known to be mostly negatively charged ^[17]. Wang and co-workers ^[18] reported the light-assisted killing ability of three cyanine derivatives containing thiazolium units, against *Staphylococcus aureus* and *Escherichia coli*. Reactive oxygen species (ROS) was one of the mechanisms proved to kill bacteria upon exposure to white light. To the best of our knowledge, while studies concerning the use of this type of cyanines as biocides are yet scarce, reports on their combination with polymers and metals are practically non-existent.

Copper is an essential metal element for human health and is considered a low-toxic metal for human organisms, supported by its current use in some intrauterine devices ^[19]. Copper ions exhibit high antibacterial activity against a wide range of bacteria, including *Salmonella enterica*, *Campylobacter jejuni*, *E. coli*, *Listeria monocytogenes*, and *S. aureus* ^[20].

Seeking to create breakthrough and low-cost antibacterial polymers for possible use in biomedical and food packing applications, cyanine and Cu²⁺@cyanine-doped solid devices, based on PVA and PVP, were synthesized and characterized. The antibacterial properties of these polymeric devices were tested against gram negative and positive bacteria. Additionally, cyanine and Cu²⁺@cyanine-doped PVA:PVP@paper mockups were prepared and their antibacterial properties evaluated in order to check their applicability in real cases of food packing.

9.2 Results and Discussion

9.2.1 Synthesis

The monomethine cyanine dye **1**, also known as Thiazole Orange ^[21] was prepared in a 53% yield according to the literature ^[22]. The chlorinated analogue dye **2** was obtained in 71% using an improved synthetic procedure reported by Kurutos and co-workers ^[23]. Originally, the synthesis of the same fluorophore **2** was reported in 2017 via method proposed by Vasilev et. al. (dye named TO-7Cl) ^[24], followed by an updated protocol by Kurutos et. al in 2020 (dye named as Cl-TO-1) ^[23]. The newer improved methods both afford highly pure target product (no formation of the blue coloured quinolinium by-product resulting from quinoline self-condensation). Nevertheless, Abdelhameed's method ^[25] also leads to higher yield of the monomethine chromophores (currently 71% of dye **2**). The unsymmetrical trimethine cyanine dye **3** was prepared in 64% yield according to the method shown by Zhytniakivska et. al. ^[26]. The structural identity of the target molecules was confirmed by NMR spectroscopy (**Figures S-9.1 to S-9.9**). The chemical shifts of the 3 cyanine dyes were also in agreement to those reported for the same compounds known from the literature.

9.2.2 Photophysical characterization and sensorial ability to metal ions of dyes 1 to 3

The photophysical characterization of dyes **1** to **3** was performed in acetonitrile at 295 K. and the main photophysical are represented in **Figure 9.1**.

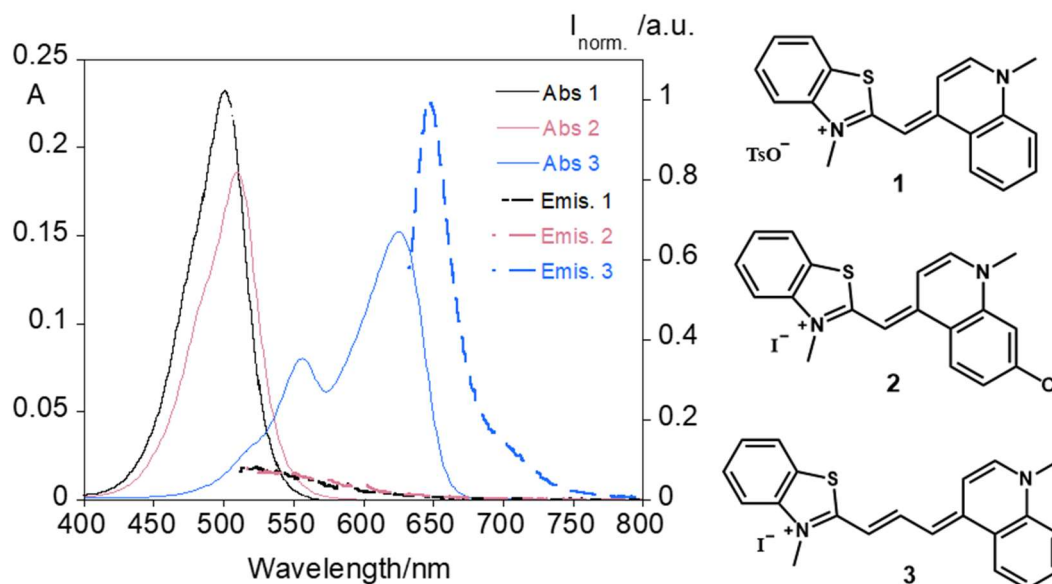


Figure 9.1 - Absorption and emission spectra of dyes **1** to **3** in acetonitrile at 295K. Chemical structures of cyanine derivative **1** to **3**. $[1] = [2] = [3] = 3 \times 10^{-6}$ M.

The cationic unsymmetrical monomethine cyanines **1** and **2** showed an absorption band centred at 501 nm ($\epsilon = 6.3 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$) and 510 nm ($\epsilon = 1.0 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$), respectively. No emission signal was observed for both compounds. On the other hand, the unsymmetrical trimethine cyanine **3** presents an emission signal with a maximum band centred at 647 nm. Concerning the absorption spectra, dye **3** has a maximum band at 624 nm ($\epsilon = 4.6 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$)^[26]. The length increasing of the polymethine chain not only shifts the absorption and emission bands to the red, but also gives some stability to the molecule in the excited state, leading to a stronger fluorescence signal.

The sensorial ability towards Zn^{2+} , Co^{2+} , Cd^{2+} , Ni^{2+} , Cu^{2+} , Hg^{2+} and Ag^+ metal ions were also evaluated in acetonitrile. No spectral changes were observed in the presence Zn^{2+} , Co^{2+} , Cd^{2+} , Ni^{2+} , Hg^{2+} and Ag^+ metal ions (**Figure S-9.10**). All dyes **1** to **3** showed an unprecedented selectivity to Cu^{2+} metal ions (**Figure 9.2**). In the ground state, increasing amounts of Cu^{2+} lead to a decrease in the absorption at 501 nm, 510 nm and 624 nm, for dyes **1**, **2** and **3**, respectively. Moreover, in the case of dye **3** the formation of a new absorption band at 450

nm is observed, as well as a colorimetric behaviour with a change of colour from blue to colourless. Concerning the excited state, a fluorescence quenching at 647 nm (dye **3**) by Chelation Enhancement Quenching Effect (CHEQ) mechanism was visualized with Cu^{2+} ions due to its paramagnetic structure [27].

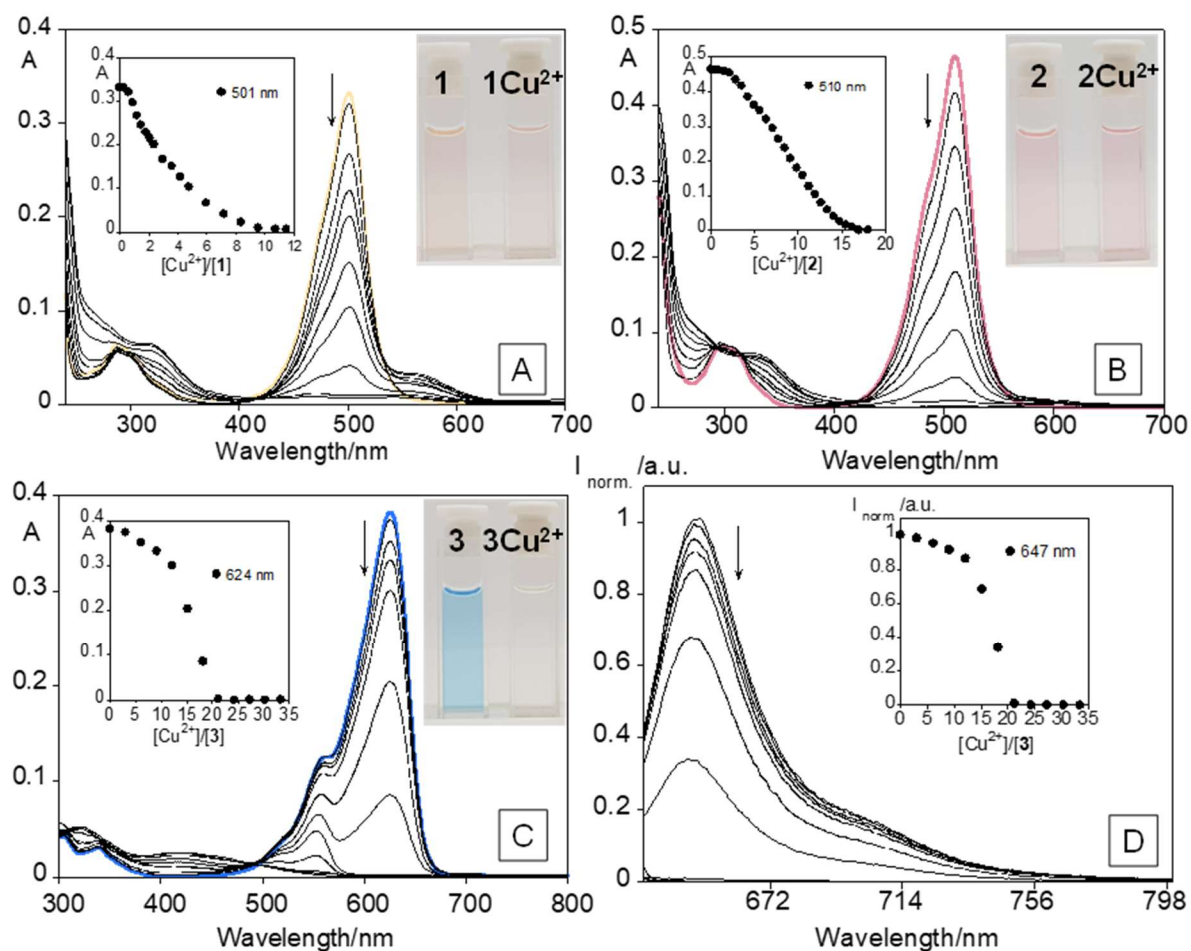


Figure 9.2 - Spectrophotometric (A, B, C) and spectrofluorimetric (D) titrations of dyes **1** (A), **2** (B) and **3** (C, D) with the addition of Cu^{2+} in acetonitrile. The inset represents the absorbance (A, B, C) at 501 nm (A), 510 nm (B) and 624 nm (C); and the emission intensity (D) at 647 nm, as function of $[\text{Cu}^{2+}]/[\text{1}]$ (A), $[\text{Cu}^{2+}]/[\text{2}]$ (B) and $[\text{Cu}^{2+}]/[\text{3}]$ (C, D). $[\text{1}] = [\text{2}] = [\text{3}] = 3 \times 10^{-6}$ M. $\lambda_{\text{exc1}} = 501$ nm, $\lambda_{\text{exc2}} = 510$ nm, $\lambda_{\text{exc3}} = 624$ nm. $T = 295$ K).

Based on the results obtained in solution, the selectivity of cyanine derivatives to Cu^{2+} metal ions, the antibacterial properties of cyanine derivatives **1** to **3** and their copper complexes were evaluated.

9.2.3 PVA:PVP and PVA:PVP:paper mockups supports for dyes 1 to 3 and Cu²⁺ complexes

The immobilization of dyes **1** to **3**, and their copper complexes, in manoeuvrable solid supports is extremely important when looking for possible direct applications. This is an especially important approach for antimicrobial applications where the incorporation of antimicrobial agents into daily objects renders the final products with antimicrobial properties ^[28]. Paper mockups are commonly used in/as food containers and their direct contact with the latter make them interesting solid platforms to introduce antimicrobial agents and help preserve food. The use of polymer-paper composites (e.g., PVA) have found a wide usage as packaging materials, owing it to the visible improvement of the composite barrier properties, as well as thermal stability ^[29]. With that, biocompatible PVA:PVP copolymeric matrices and PVA:PVP embedded paper mockups (PVA:PVP:paper) were prepared and used as scaffolds for dyes **1** to **3** incorporation.

The solid-state photophysical properties of the final products were assessed to understand the possible incorporation of both dyes and dye-Cu complexes within the polymeric matrices. The incorporation of dye **1** in PVA:PVP copolymer resulted in an homogeneous orange polymeric film, that retained both the dye's original coloration and the flexibility of PVA. Film stability and slight rigidity were rendered by the PVP counterpart of the copolymeric matrix. Interestingly, the fluorescence profile of PVA:PVP@**1** is completely different from that of the free, in-solution dye (i.e. no emission), having now a distinct yellowish emission (under 365 nm lamp), centred at its maximum at 550 nm (**Figure 9.3**). This emission, as previously described in literature, is favoured by the predominant rigidity and planarity of polymethine cyanines and their tendency to aggregate, via π - π stacking, into H-aggregates or J-aggregates, that restrict intramolecular motions, reduce nonradiative decays and enhance emission ^[30]. Inside the PVA:PVP scaffolds, the strong blueshift in the fluorescence spectrum indicates a predominance in H-aggregates, and thus the appearance of emission bands in the visible region ^[31]. The unsuccessful production of a usable PVA:PVP@**1**-Cu film owns it to the product's high brittleness and low homogeneity, with **1**-Cu complexes visibly aggregating into macroscopic agglomerates (data not shown).

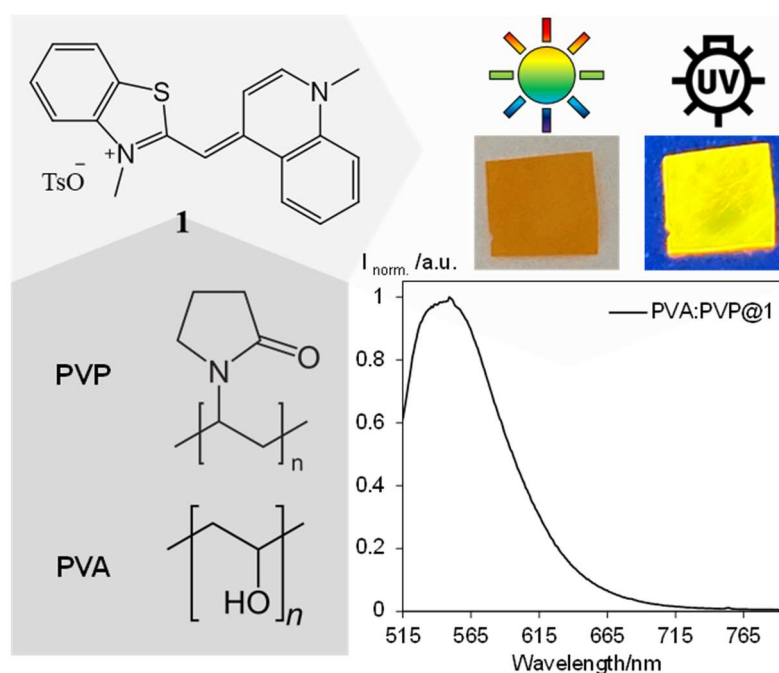


Figure 9.3 - Schematic representation of PVA:PVP@1 constituents, synthetic steps and final product, under white and UV-light, with respective emission spectra after excitation at $\lambda = 501$ nm.

PVA:PVP@2, PVA:PVP@2-Cu, PVA:PVP@3 and PVA:PVP@3-Cu were successfully obtained by the same approach, with all showing good homogeneity, cyanine-related coloration and film robustness (**Figure 9.4**). Similarly to PVA:PVP@1, PVP:PVP@2 and PVA:PVP@2-Cu films show intense fluorescent profiles, that arise from the possible stacking of the dye and dye-copper complex molecules within the polymer matrix. While PVA:PVP@2 has a yellowish emission centred at 550 nm, after complexation with Cu^{2+} its emission maximum left shifts towards 535 nm, resulting in a distinct green fluorescence. Naked-eye color changes are also perceived between the mentioned films, shifting from a strong orange (PVA:PVP@2) to a brownish-transparent coloration (PVA:PVP@2-Cu).

PVA:PVP@3 and PVA:PVP@3-Cu films showed similar colorimetric and fluorometric spectral profiles to those of the free dye and the free complex, with Cu^{2+} complexation inducing a decrease in emission intensity and a slight blueshift of its maxima (from 660 to 650 nm). A loss of color is also perceived when complexing with Cu^{2+} ions, although not as intense as that seen for the free in-solution species.

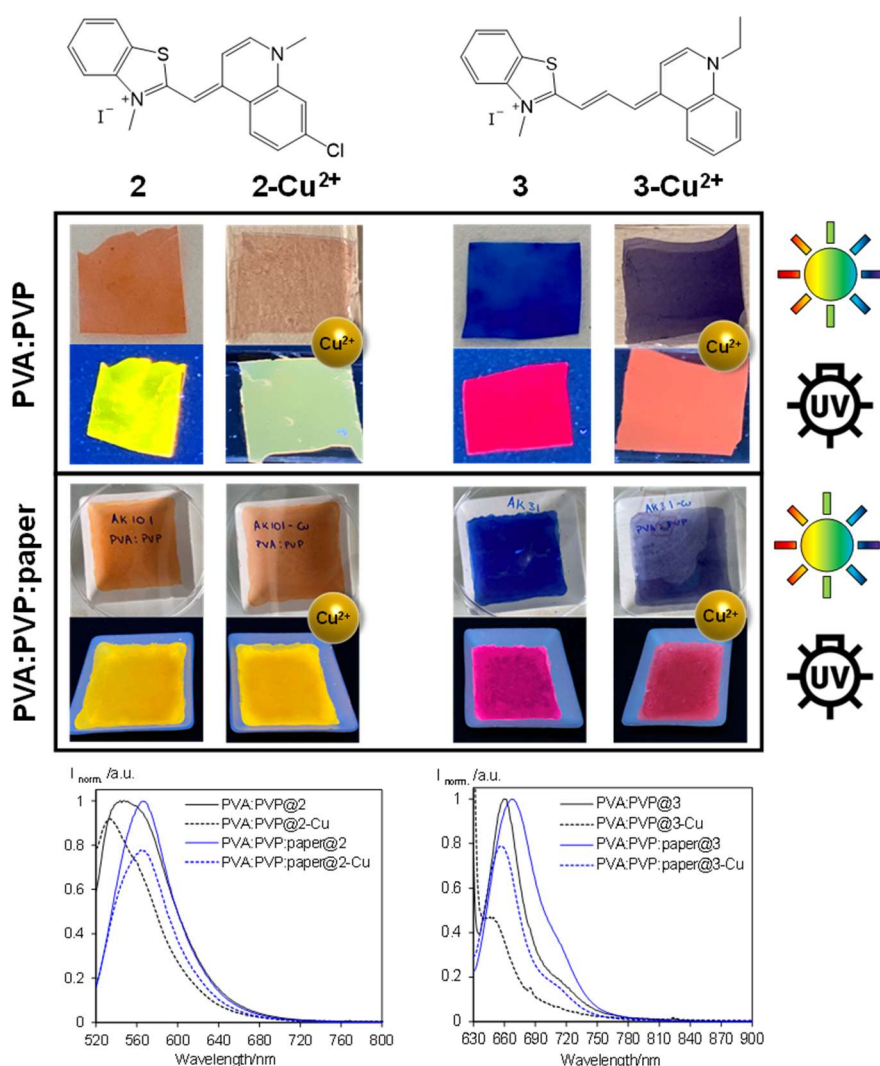


Figure 9.4 - (top) PVA:PVP@2, PVA:PVP@3, PVA:PVP:paper@2, PVA:PVP:paper@3, and their Cu²⁺-complexed counterparts, under white and UV-light. (bottom) Emission spectra of each 2- and 3- containing material after excitation at $\lambda = 510$ nm and $\lambda = 624$ nm, respectively.

This similar behaviour, between PVA:PVP@3, PVA:PVP@3-Cu films and free molecule, suggest that there are less constraints on the dye's molecules inside the polymeric matrix. Such is supported by the structure of dye 3, whose longer trimethine chain and out plane N-ethylation create a higher steric hindrance and reduce PVA and PVP capacity to entrap and induce dye stacking. This is also supported by the smaller blueshift between free-dye emission and that of the polymer, suggesting a smaller formation of H-aggregates inside the matrix.

PVA:PVP:paper mockups of dye 2 and 3, and their Cu²⁺ complexes, were successfully obtained as homogeneous colorimetric scaffolds (Figure 9.4). The obtained PVA:PVP:paper@2 and PVA:PVP:paper@2-Cu have the same colors as their PVA:PVP

counterparts. but differ in their emission spectra with both mockups having emission maxima at 570 nm (yellow) despite the presence/absence of Cu^{2+} ions. Conversely, PVA:PVP:paper@3 and PVA:PVP:paper@3-Cu not only showed the same colors as their respective polymeric films. but also followed the same tendency of the fluorescence profiles of PVA:PVP@3 and PVA:PVP@3-Cu films.

From all immobilizations it can be concluded that the dyes and their copper complexes were successfully entrapped inside both PVA:PVP or PVA:PVP:paper matrices. and are still available for further antimicrobial testing of the obtained scaffolds.

9.2.4 Antibacterial Assays

9.2.4.1 Broth microdilution assays

The antibacterial activity of cyanine derivatives **1**, **2** and **3** was evaluated through broth microdilution assays against some of the common bacterial strains that can be food contaminants and/or are foodborne: *Escherichia coli* (*E. coli* 1. ATCC® 8739™), *Escherichia coli* (*E. coli* 2. ATCC® 25922™), *Staphylococcus aureus* (ATCC® 6538™), methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC® 33591™), *Enterococcus faecalis* (ATCC® 29212™), *Pseudomonas aeruginosa* (ATCC® 9027™), *Listeria monocytogenes* (ATCC® 15313™), *Bacillus cereus* (ATCC® 11778™) and *Salmonella enterica* subsp. *enterica* serovar *Choleraesuis* (ATCC® 10708™). For these assays. the antibacterial activity of the free dyes was evaluated. as well as their respective cyanine-copper complex counterparts (1:3 stoichiometry), due to the high selectivity of these dyes for copper ions. as previously characterized. All bacterial strains were incubated overnight with a gradient concentration of each dye ranging from 1 to 0.05 μM .

Figure 9.5 summarizes the obtained results. indicated as the minimum inhibitory concentration (MIC) for each formulation. MIC values are considered as the lowest concentrations that reduce bacterial growth in 50% (MIC_{50}) or 90% (MIC_{90}) when in comparison to the bacterial control. These values are an indicator of the antibacterial capacity of each dye and complex and allow for a comparative analysis between the antibacterial activity of the formulations.

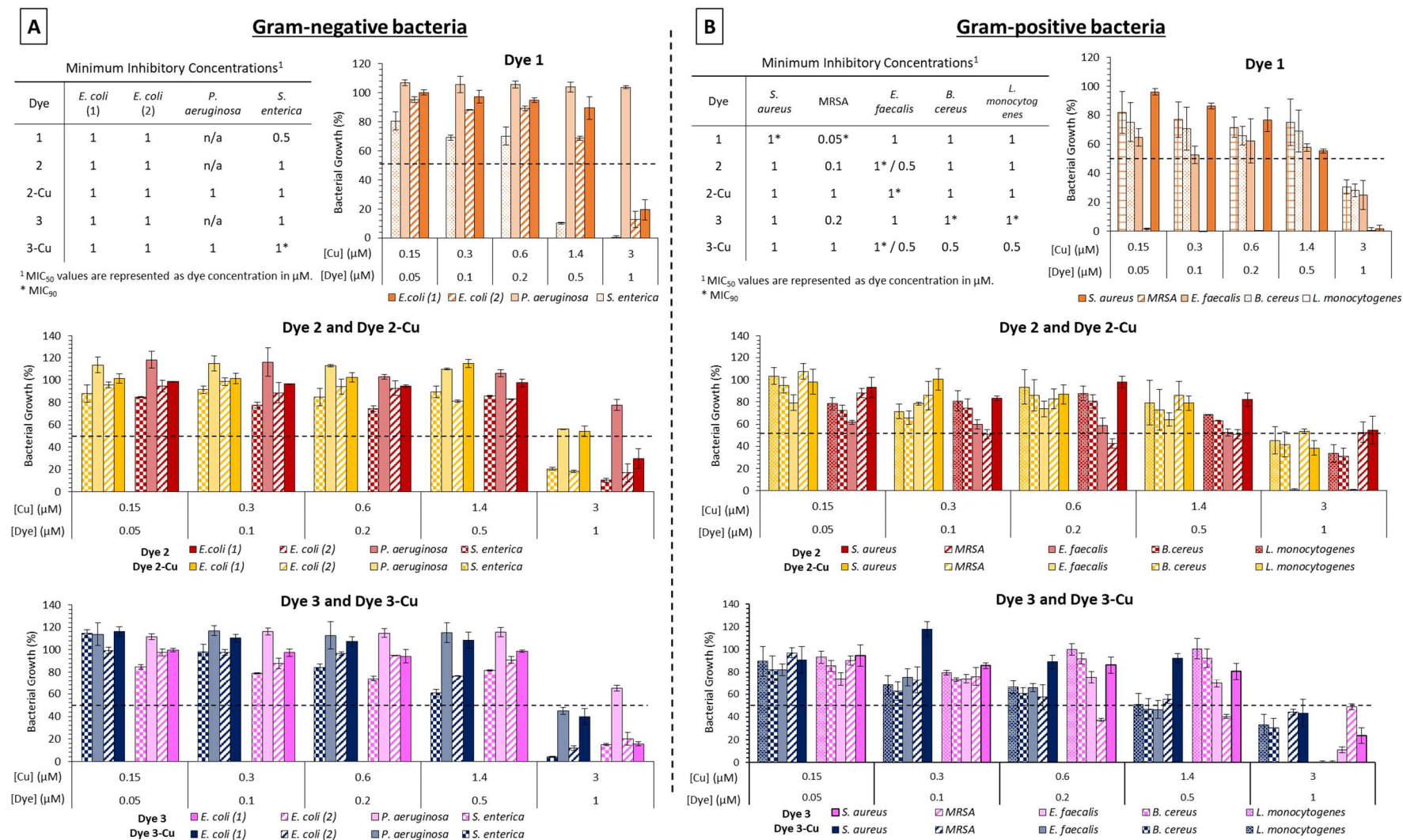


Figure 9.5 - Obtained MICs for all cyanine derivatives (1 to 3 and respective copper complexes- 1:3 stoichiometry) and respective bacterial growth profiles. against Gram-negative (A) and Gram-positive (B) bacteria. Concentrations represented in μM and n/a = no activity.

All cyanine derivatives have antibacterial activity against the majority of the tested bacterial strains, including highly resistant ones such as MRSA or *P. aeruginosa*. Although the activity against *P. aeruginosa* was only detected in the presence of copper complexes (**2-Cu** and **3-Cu**), this is of particular interest as *P. aeruginosa* is known to have a sophisticated copper defense cascade, that targets and exports sole copper ions from its cytosol, thus evading its usual antimicrobial activity^[32]. Remarkably, the complexation of copper with the cyanine derivatives demonstrates, similar to other reported copper complexes, the important role of complexation in the development of new synergistic antimicrobial strategies, by promoting the protection of copper ions for an effective incorporation inside bacteria and increase the amount of reactive oxygen species^[32-33]. Derivatives **2-Cu** and **3-Cu** showed antibacterial activity against all tested bacterial strains, suggesting a non-selective inhibitory behaviour for bacterial growth. Besides *P. aeruginosa*, the presence of copper decreases the MIC₅₀ of derivative **3** in *E. faecalis*, *L. monocytogenes* and *B. cereus*. The MIC₅₀ obtained for these strains with the free derivative was of 1 µM, while with the copper complex it was two-fold decreased to 0.5 µM. Regarding the MRSA strain, the most effective formulation was **Dye 1**, inhibiting bacterial growth at all tested concentrations (MIC₉₀ = 0.05 µM). **Dye 2** (MIC₅₀ = 0.1 µM) and **Dye 3** (MIC₅₀ = 0.2 µM) were also effective against MRSA but with a lower activity. Interestingly, in solution Cu²⁺ ions showed no stand-alone antibacterial activity against any of the tested bacteria.

Results obtained reveal the potential that the derivatives studied can have as a broad-spectrum antibacterial formulation. To further evaluate and optimize the inhibitory properties of these derivatives, antibacterial assays were performed with the previously characterized dye-doped PVA:PVP polymers, which were further incorporated as a coating for paper mockups as described in the following sections

9.2.4.2 Dye-doped PVA:PVP polymers

All previously described dye-doped PVA:PVP polymers were subjected to antibacterial assays in all previously referred bacterial strains. For these assays, 0.5 x 0.5 cm squares were cut of all polymeric formulations and placed onto inoculated MHA plates and left to incubate overnight, to ensure bacterial growth. Non-doped PVA:PVP polymers were also used as

controls. Polymers doped with cyanine-copper complexes (1:3 stoichiometry) were also evaluated.

Herein, a significant antibacterial activity was considered when no bacterial growth occurred in the agar-polymer interface, or by occurrence of an inhibition halo beyond the physical dimensions of the polymer. No antibacterial activity was detected for the control non-doped polymers, indicating that all activity is derived from the cyanine dyes incorporated onto the polymeric matrix. **Table 9.1** summarizes the results obtained from these assays, indicating the inhibition zones of each polymer for each bacterial strain. The presence of coloration in the agar matrix was also reported.

Table 9.1 - Results of antibacterial assays of PVA:PVP polymers against several bacterial strains. Obtained inhibition halos represented in cm².

	PVA:PVP		PVA:PVP@1	PVA:PVP@2	PVA:PVP@3	PVA:PVP@2-Cu	PVA:PVP@3-Cu
<i>E. coli</i> (1)	Growth		No growth No halo	No growth Halo: 0.81 cm ² Pink agar	No growth No halo Blue agar	No growth Halo: 1.0 cm ² Pink agar	No growth Halo: 0.64 cm ²
<i>E. coli</i> (2)	Growth		No growth No halo Yellow agar	No growth Halo: 1.0 cm ² Pink agar	No growth No halo Blue agar	No growth Halo: 0.7 cm ² Pink agar	No growth No halo
<i>S. aureus</i>	Growth		No growth Halo: 0.64 cm ² Yellow agar	No growth Halo: 1.95 cm ² Pink agar	No growth Halo: 1.21 cm ² Blue agar	No growth Halo: 1.32 cm ² Pink agar	No growth Halo: 1.3 cm ²
MRSA	Growth		No growth Halo: 0.7 cm ² Yellow agar	No growth Halo: 1.2 cm ² Pink agar	No growth Halo: 1.32 cm ² Blue agar	No growth Halo: 1.1 cm ² Pink agar	No growth Halo: 1.8 cm ²
<i>E. faecalis</i>	Growth		No growth No halo Yellow agar	No growth Halo: 0.64 cm ² Pink agar	No growth Halo: 1.21 cm ² Blue agar	No growth Halo: 1.04 cm ² Pink agar	No growth Halo: 0.72 cm ²
<i>P. aeruginosa</i>	Growth		Growth	No growth No halo Pink agar	No growth No halo Blue agar	No growth Halo: 0.49 cm ² Pink agar	No growth Halo: 0.9 cm ² Light blue agar
<i>B. cereus</i>	Growth		No growth No halo Yellow agar	No growth Halo: 1.44 cm ² Pink agar	No growth Halo: 0.72 cm ²	No growth Halo: 1.56 cm ² Pink agar	No growth Halo: 0.72 cm ²
<i>S. enterica</i>	Growth		Growth Yellow agar	Growth Pink agar	No growth No halo Blue agar	Growth	No growth No halo
<i>L. monocytogenes</i>	Growth		No growth No halo Yellow agar	No growth Halo: 1.4 cm ² Pink agar	No growth Halo: 0.9 cm ² Blue agar	No growth Halo: 1.8 cm ² Pink agar	No growth Halo: 1.0 cm ²

All dye-doped polymers present antibacterial activity in a variety of bacterial strains (**Figure S11**), with results suggesting a more substantial antibacterial effect against Gram-positive bacteria (i.e., *E. faecalis*, *L. monocytogenes*, *B. cereus*, *S. aureus* and MRSA strains). Inhibition zones for the two latter were the largest verified amongst all, with areas up to 1.95 cm², in polymer PVA:PVP@2. Interestingly, in the liquid formulations, dye 2 did not produce any inhibitory activity against MRSA, which suggests that the polymeric formulations have an improved antibacterial performance that of those presented by the dyes in solution. Polymers PVA:PVP@3 and PVA:PVP@3-Cu were the only formulations that produced antibacterial activity against almost all bacterial strains (**Figure S-9.11**), particularly against *S. enterica*, mirroring most of the results obtained for the liquid formulations. With only the exception of *P. aeruginosa*. Moreover, polymers PVA:PVP@2 and PVA:PVP@2-Cu produced antibacterial activity against all strains with exception of *S. enterica*.

When comparing the antibacterial response of polymers containing the cyanine derivative with those with the copper-cyanine complex, in general, similar responses were obtained. However, PVA:PVP@2-Cu and PVA:PVP@3-Cu were the only formulations in which it was possible to detect an inhibition halo in *P. aeruginosa*, mirroring the results obtained for the liquid formulations, where copper complex also increased the antimicrobial activity.

Polymers containing cyanine derivatives without the copper complex presented interesting effects in *P. aeruginosa*, performing inhibition without the formation of the blue/green pigment, a virulence factor characteristic of this bacterial strain ^[34]. On the contrary, when in the presence of copper complexes, the formation of this pigment occurs despite the inhibition of bacterial growth at the direct agar-polymer interface. Thus, results suggest that these polymers have potential as a clinical agent to overcome the pathogenicity of this bacterial strain in both nosocomial and communitarian environments.

Polymer PVA:PVP@1 was the least effective formulation, since it was not able to inhibit bacterial growth of *P. aeruginosa* and *S. enterica*. Furthermore, this polymer only inhibited bacterial growth in direct contact with the agar-polymer interface, with no large inhibition zones beyond the polymer in the majority of the tested strains. Similar to that verified for all remaining polymers, PVA:PVP@1 had the best results against both *S. aureus* and MRSA strains.

Considering all obtained results, it appears that these polymers can present a broad-spectrum antibacterial effect, particularly against Gram-positive strains like *B. cereus*, *S. aureus* and MRSA strains (Figure 9.6 and Figure S-9.11).

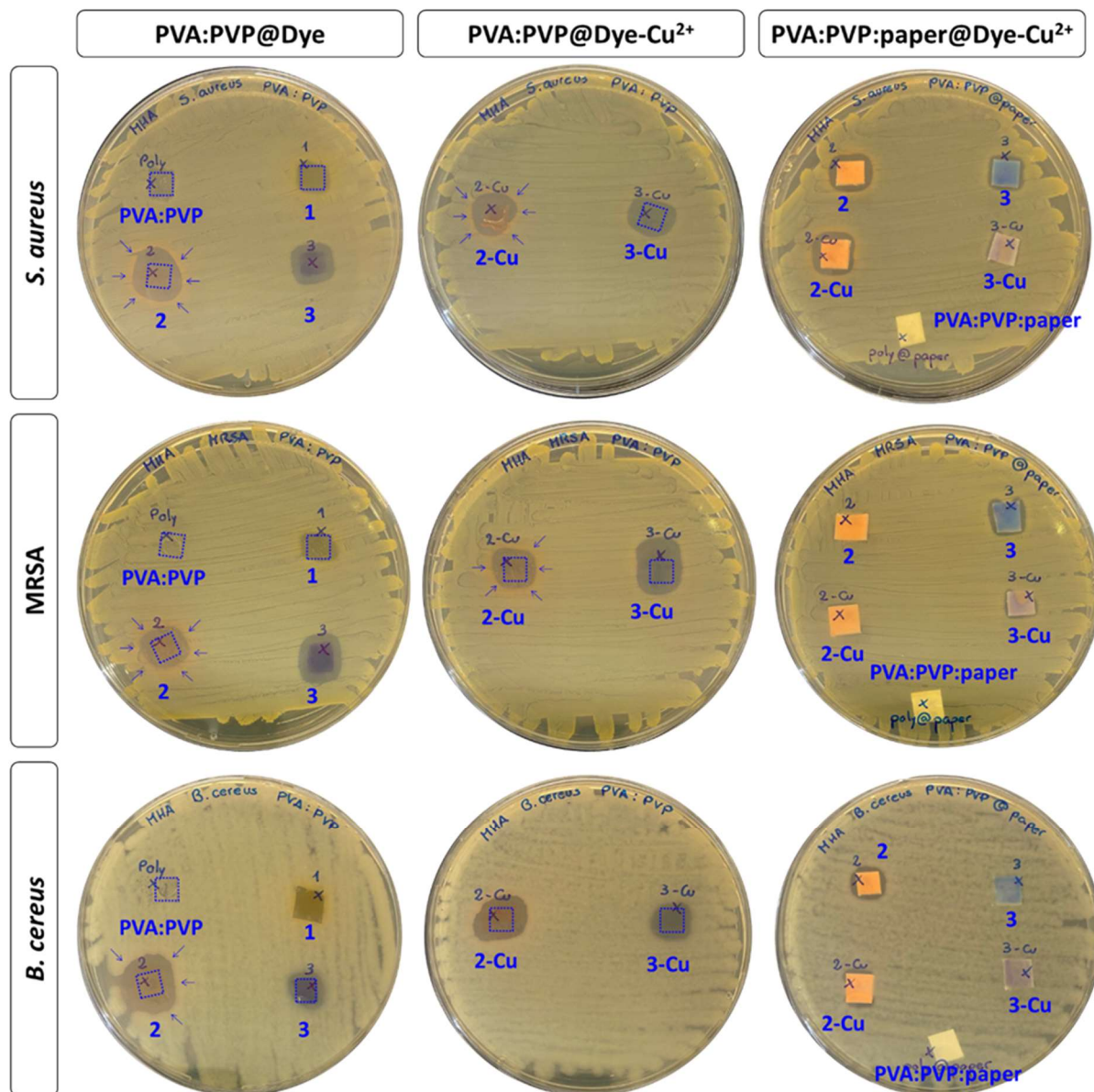


Figure 9.6 - Antibacterial assays of PVA:PVP and PVA:PVP:paper systems against *S. aureus*, MRSA and *B. cereus*. Blue dotted squares delimitate 0.5 x 0.5 cm polymer pieces and blue arrows indicate red colored inhibition zones edges, due to migration of dye 2 from polymeric matrices.

Two types of mechanisms might cause the bacterial growth inhibition presented by these polymers: (i) diffusional mechanisms of the cyanine derivatives from the polymeric matrix, and (ii) surface-dependent inhibition of bacterial adhesion and subsequent growth [35].

The presence of diffusional mechanisms can be confirmed by the occurrence of inhibition zones beyond the polymer area, and subsequent coloration of bacterial borders.

This effect is particularly noticeable in PVA:PVP@2, PVA:PVP@3 and PVA:PVP@2-Cu polymers, where coloration was verified for the majority of bacterial strains. This can be further visualized in **Figure 9.6**, as a slight red coloration was present in the borders of the inhibition areas in polymers PVA:PVP@2 and PVA:PVP@2-Cu. These diffusional mechanisms play an important role in the antibacterial activity of the polymers since it allows for the occurrence of direct non-covalent electrostatic interactions between the positively charged cyanine derivatives and the negatively charged bacterial surface ^[36]. Studies indicate that Gram-positive bacteria present an increased negative surface charge, when in comparison to Gram-negative bacteria, due to the presence of teichoic acids linked to the peptidoglycan present in the bacterial membrane ^[37]. This slightly different surface charge might result in increased electrostatic interactions between the dyes and Gram-positive bacteria, and subsequently, an increased antibacterial effect translated onto a larger inhibition zone. Furthermore, these cyanine derivatives, containing aromatic moieties, are highly hydrophobic molecules, enabling easier interactions with the also hydrophobic environment of bacteria, particularly *Staphylococcus aureus* strains ^[38].

The second effect in place relates to the fact that adhesion is the crucial first step during bacterial infections ^[39]. The tridimensional configuration of the dyes onto the polymeric matrix, might hinder bacterial adhesion to the surface of the polymer, not allowing for its growth in a solid surface ^[40], preventing the pathogenic activity of these microorganisms. These effects can be particularly noticeable in polymers that do not produce inhibition zones beyond the area of the polymer, nor coloration in the bacteria or agar plate. All this suggests the presence of a second mechanism of inhibition, that does not rely only on diffusional mechanisms, but in a direct contact inhibition.

The antibacterial activity of these polymers might be further optimized through the application of cyanine dyes as photosensitizer molecules, activated by light irradiation ^[41], however further studies need to be performed to evaluate this potential.

9.2.4.3 Dye-doped paper mockups

To further evaluate the antibacterial activity of these cyanine derivatives, dye doped PVA:PVP polymers were used as coats for paper mockups. The obtained dye-doped paper mockups were then cut onto 0.5 x 0.5 cm squares and placed onto inoculated agar plates.

with the dye-containing side in contact with the agar. These plates were inoculated with all previously referred bacterial strains and left to incubate overnight at 35 ± 2 °C to ensure bacterial growth. Non-doped paper mockups were also used as controls, with no antibacterial activity detected for these. Once again, all activity is derived from the cyanine derivatives, and not of the solid matrix they are incorporated onto. No paper mockups were produced with cyanine dye 1 since this dye presented the least promising results in the PVA:PVP polymers.

Table 9.2 summarizes the results obtained for the paper mockups. Paper mockups appear to present less antibacterial effects than their PVA:PVP polymer counterparts. The best results obtained were against *B. cereus*, *S. aureus* and MRSA bacterial strains (**Figure 9.6**). PVA:PVP:paper@2 and PVA:PVP:paper@2-Cu were most effective against *B. cereus* and *S. aureus*, while PVA:PVP:paper@3 and PVA:PVP:paper@3-Cu were most effective against MRSA strains.

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Macromolecular Bioscience, 2022, 2200244

Table 9.2 - Results of antibacterial assays of PVA:PVP:paper mockups against several bacterial strains. Obtained inhibition halos represented in cm².

	PVA:PVP:paper	PVA:PVP:paper@2	PVA:PVP:paper@3	PVA:PVP:paper@2-Cu	PVA:PVP:paper@3-Cu
<i>E. coli</i> (1)	Growth	No growth No halo	No growth No halo	No growth No halo	No growth No halo
<i>E. coli</i> (2)	Growth	No growth No halo Orange agar	No growth No halo Blue agar	No growth No halo Orange agar	No growth No halo
<i>S. aureus</i>	Growth	No growth Halo: 1.2 cm ² Orange agar	No growth Halo: 0.72 cm ²	No growth Halo: 1.32 cm ² Orange agar	No growth No halo
MRSA	Growth	Growth	No growth Halo: 0.9 cm ²	Growth	No growth Halo: 0.56 cm ²
<i>E. faecalis</i>	Growth	Growth	No growth No halo	No growth Halo: 1.04 cm ²	Growth
<i>P. aeruginosa</i>	Growth	No growth No halo	No growth No halo	No growth No halo	No growth No halo
<i>B. cereus</i>	Growth	No growth Halo: 0.9 cm ²	Growth	No growth Halo: 1.0 cm ²	Growth
<i>S. enterica</i>	Growth	Growth	Growth	Growth	No growth No halo
<i>L. monocytogenes</i>	Growth	No growth Halo: 0.56 cm ² Orange agar	No growth No halo	Growth	Growth

The antibacterial effects that were derived from these paper mockups are reduced when in comparison with the PVA:PVP polymers. Diffusional mechanisms appear to be less significant than those presented by the PVA:PVP polymers due to the different solid support matrixes, hindering the release of the cyanines to the medium. This can be further confirmed by the absence of coloration in either the agar plate or the bacterial borders of growth. However, mockups continue to present antibacterial activity against *S. aureus*, including resistant strains such as MRSA. In these formulations, direct contact inhibition mechanisms appear to be more significant than diffusional mechanisms. These are similar to those described previously regarding surface-dependent inhibition of bacterial adhesion and growth. The adhesion mechanisms for Gram-positive bacteria, particularly *S. aureus* strains, in hydrophobic environments involves multiple weak interactions with macromolecules [42]. These weak interactions, allow for the inhibition of bacterial adhesion at the paper mockup surface-bacteria interface and subsequent growth inhibition. However, associated to the more complex nature of the solid matrix and no apparent release of the dyes to the medium, the occurrence of larger inhibition zones, beyond the areas of the matrixes does not occur as it did in the polymeric solid matrixes.

These results indicate that the dyes can present antibacterial activity when incorporated in different solid matrixes, being thus a very versatile platform for application in various fields of science.

9.3 Conclusion

Cyanine derivatives 1, 2 and 3 were evaluated as antimicrobial agents in solution and in solid-supported devices. In solution, all dyes showed an unprecedented selectivity to Cu^{2+} metal. All cyanine derivatives showed antibacterial activity against the majority of the tested bacterial strains. The copper complexes have increased antibacterial activity in strains such as *L. monocytogenes* and *B. cereus*. For free dyes, an overall MIC_{50} of 1 μM was determined, with only the dye 3-copper complex reducing it by two-fold to 0.5 μM . Based on these results, dyes 1 to 3 and their copper complexes were immobilized into PVA:PVP polymers in order to optimize the antibacterial effects of the described cyanine dyes. Polymers doped with cyanines 2 and 3 were particularly effective against Gram-positive bacteria, with inhibition zones of up to 1.32 cm^2 and 1.8 cm^2 for *S. aureus* and *L. monocytogenes*.

respectively. Broth microdilution assays were also performed for cyanines 1 to 3 with antibacterial effects being detected for all dyes. These antibacterial effects are thought to be mainly mediated by reversible electrostatic interactions between the positively charged cyanine dyes and the negatively charged bacterial surface. Overall, these dyes and dye-doped polymers are very promising antibacterial agents against a wide variety of bacterial strains, presenting effects even against resistant bacterial strains such as MRSA and *P. aeruginosa*. Further studies need to be performed to fully optimize and understand these effects, for possible future applications. These materials could be potentially utilized as sterile surfaces for high contact points or medical environments, providing an increased layer of security against antibiotic-resistant bacterial strains.

9.4 Experimental Section

Materials: Copper (II) trifluoromethanesulfonate ($\text{Cu}(\text{TfO})_2$, > 98%). Cadmium (II) trifluoromethanesulfonate ($\text{Cd}(\text{TfO})_2$, > 98%). Mercury (II) trifluoromethanesulfonate ($\text{Hg}(\text{TfO})_2$, > 98%). Nickel (II) trifluoromethanesulfonate ($\text{Ni}(\text{TfO})_2$, > 98%). Cobalt (II) trifluoromethanesulfonate ($\text{Co}(\text{TfO})_2$, > 98%). Zinc (II) trifluoromethanesulfonate ($\text{Zn}(\text{TfO})_2$, > 98%) and Silver (I) trifluoromethanesulfonate ($\text{Ag}(\text{TfO})$, > 98%) were acquired from Solchemar. Polyvinyl alcohol (PVA, 27 kDa) and polyvinylpyrrolidone (PVP, 29 kDa) were bought from Sigma-Aldrich. Absolute anhydrous ethanol was acquired from Carlo Erba Reagents. Paper mockups were used as bought from Auchan®.

All solvents and reagents were of analytical reagent grade (Panreac and Carlo Erba) and were used as received.

Synthesis of cyanine derivatives 1 to 3: All chemicals and solvents used in the synthesis were acquired from Sigma-Aldrich, Alfa-Aesar, and Acros Organics and used as such. Deuterated DMSO- d_6 was purchased from Eurisotop. The fluorophores 1-3 used in the current investigation were obtained according to methods already reported in the literature [22-23, 26]. The target cyanines were purified by recrystallization from ethanol, and NMR spectroscopy was used to confirm the compounds' identities. ^1H -NMR and ^{13}C -NMR spectra were recorded in DMSO- d_6 at 25 °C using 5 mm tubes at 500.13 MHz and 125.77 MHz, respectively on a Bruker Ultrashield Plus 500 spectrometer. All chemical shifts are given in parts per million (ppm), with a precision of 0.01 ppm. The coupling constants J are measured in Hz and are accurate to 0.1 Hz. A copy of the NMR spectra can be found in the related supplementary material file (Figures S-9.1 to S-9.9).

Photophysical characterization and titrations: The UV-Vis absorption spectra were recorded with a JASCO V-650 spectrophotometer and the fluorescence emission spectra by an HORIBA Scientific FLUOROMAX-4 spectrofluorimeter. The linearity of the fluorescence emission vs. the concentration was checked in the concentration used (10^{-4} – 10^{-6} M). A correction for the absorbed light was performed when necessary. Spectra of solid samples were collected with a Horiba-Jobin-Yvon Fluoromax-4® spectrofluorimeter using an optic fibre connected to the equipment. by exciting the solid compounds at appropriated λ (nm).

The spectroscopic characterizations and titrations were performed using stock solutions of the studied compounds (ca. 10^{-3} M). prepared by dissolving the appropriate amount of dye **1** to **3** in acetonitrile in a 4 mL volumetric flask. The studied solutions were prepared by appropriate dilution of the stock solutions up to 10^{-5} – 10^{-6} M. Titrations of dyes **1** to **3** were carried out by the addition of microliter amounts of standard solutions of Cu^{2+} , Cd^{2+} , Hg^{2+} , Ni^{2+} , Co^{2+} , Zn^{2+} , Ag^{2+} metal ions in acetonitrile. All measurements were performed at 25 °C.

Synthesis of dye-doped PVA:PVP polymeric films: PVA: PVP polymer films were obtained by dissolving 200 mg of PVA and 50 mg of PVP in 1.5 mL miliQ water. at 80 °C. under continuous stirring. 2 mL of EtOH containing 0.5 mg of the cyanine dyes **1** to **3**. were added onto the hot mixture and briefly sonicated for 5 min. The resulting mixture was poured onto Teflon plates and the films were obtained by slow evaporation at room temperature (~48 hours). Non-doped PVA: PVP films were also prepared as controls.

Each film was cut into approximately 0.5 x 0.5 cm pieces to perform the assays with bacteria. Polymers of each cyanine dye were also made with the addition of 3 equivalents of $\text{Cu}(\text{TfO})_2$. following the same protocol described above.

Synthesis of dye-doped PVA:PVP@paper mockups: Dye-doped paper mockups were obtained using the same protocol and ratios as described above. The polymer-dye mixtures were poured onto paper mockups and left to dry at air (~24 hours). Non-doped PVA: PVP@paper mockups were also prepared as controls. Dye- Cu^{2+} doped mockups were also prepared by the addition of 3 equivalents of $\text{Cu}(\text{TfO})_2$ to each dye stock solution and by following the same protocol described above. Each paper support was cut into approximately 0.5 x 0.5 cm pieces for the antibacterial assays.

Antibacterial Assays: The antibacterial activity of the obtained compounds and supports was evaluated against *Escherichia coli* (*E. coli* 1. ATCC® 8739™). *Escherichia coli* (*E. coli* 2. ATCC® 25922™). *Staphylococcus aureus* (ATCC® 6538™). methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC® 33591™). *Enterococcus faecalis* (ATCC® 29212™). *Pseudomonas aeruginosa* (ATCC ® 9027™). *Listeria monocytogenes* (ATCC® 15313™).

Bacillus cereus (ATCC® 11778) and *Salmonella enterica* subsp. *enterica* serovar *Choleraesuis* (ATCC® 10708™). Bacteria stocks were kept frozen at – 70 °C in broth containing glycerol (15% v/v). Initial bacterial suspensions of all strains were inoculated in TSA and incubated overnight at 35 ± 2 °C. Isolated colonies were transferred to saline medium (NaCl. 0.85% m/v). the turbidity of the suspension was adjusted to 0.5 on the McFarland scale (approx. 1.5×10^8 CFU/mL) (DEN-1B McFarland Densitometer. Grant-bio).

The antibacterial activity of free cyanines **1** to **3** and of their Cu²⁺ complexes was assessed by the broth microdilution method in 96-well microplates with cyanine concentrations ranging from 1 to 0.05 µM. Each well was inoculated with the above prepared bacterial suspensions (10^6 CFU/mL in each well). The microplates were incubated. in the dark. at 35 ± 2 °C for 24 h. The minimum inhibitory concentration (MIC) was assessed by optical density (OD. 600 nm) quantification (UV–Vis CLARIOstar spectrophotometer. BMG Labtech). Cu²⁺ was also tested alone and used as negative control.

To evaluate the bactericidal effect of the prepared doped polymer films and doped polymer@paper mockups. 0.5 x 0.5 cm pieces of each were placed onto Mueller-Hinton Agar (MHA) plates inoculated with each bacterial strain. The plates were then incubated at 35 ± 2 °C for 24 hours. Non-doped PVA:PVP films and PVA:PVP@paper mockups were also evaluated as controls. Bacterial growth was visually evaluated in MHA plates and assessed as inhibition zones. in squared centimetres (cm²). Asepsis was achieved in a VBH Laminar Flow Hood (Steril). Incubations were done in a Mermmet Incubator B50 (Vikat Ekinox).

9.5 Acknowledgements

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9.7 Supporting Information

9.7.1 Figures

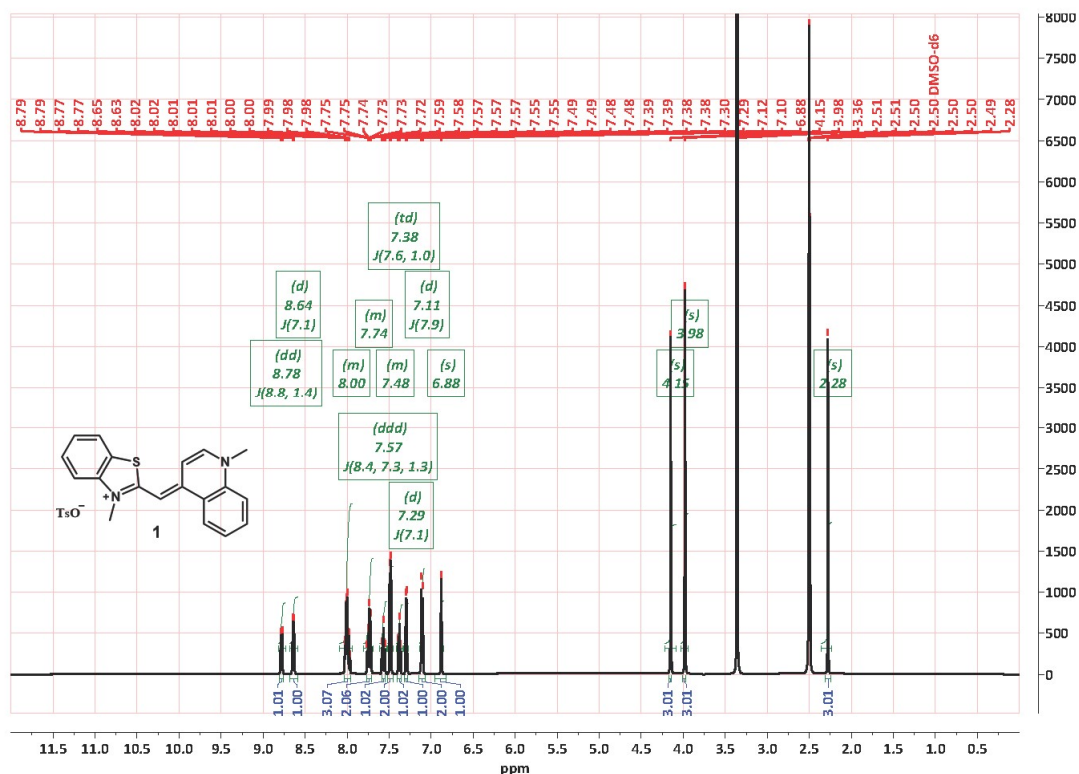


Figure S -9.1 - ^1H -NMR spectrum of cyanine dye 1 recorded in DMSO-d_6 (whole spectrum).

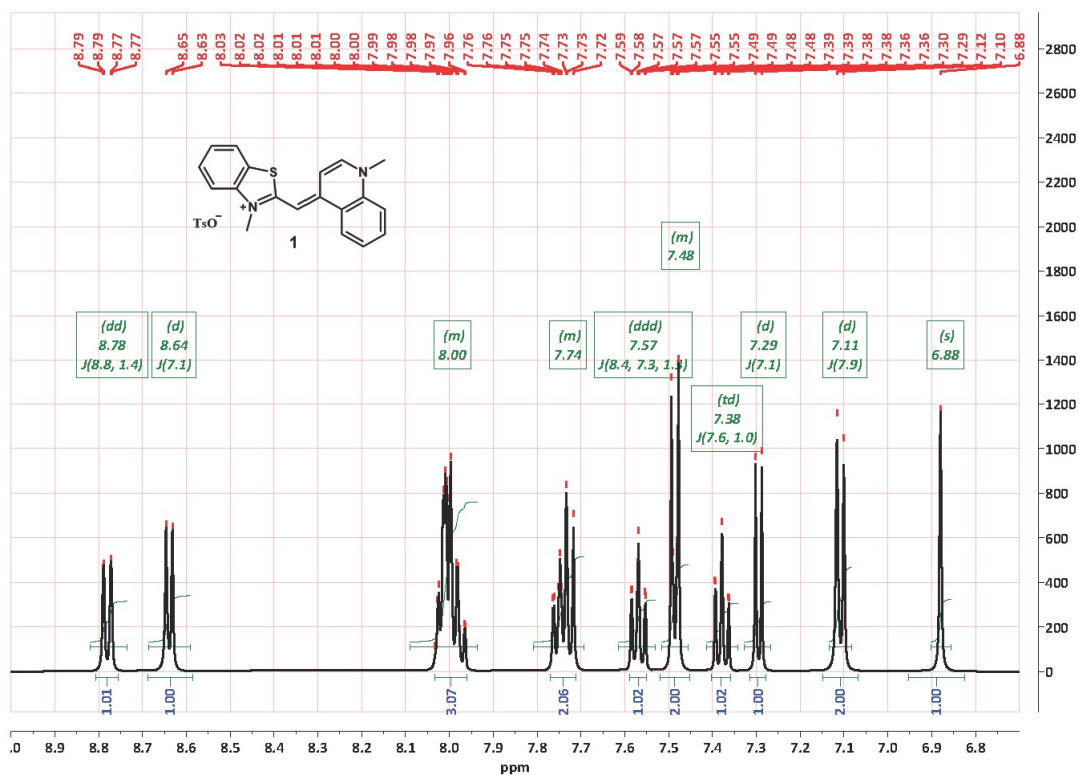


Figure S - 9.2 - ¹H-NMR spectrum of cyanine dye 1 recorded in DMSO-d₆ (aromatic expansion).

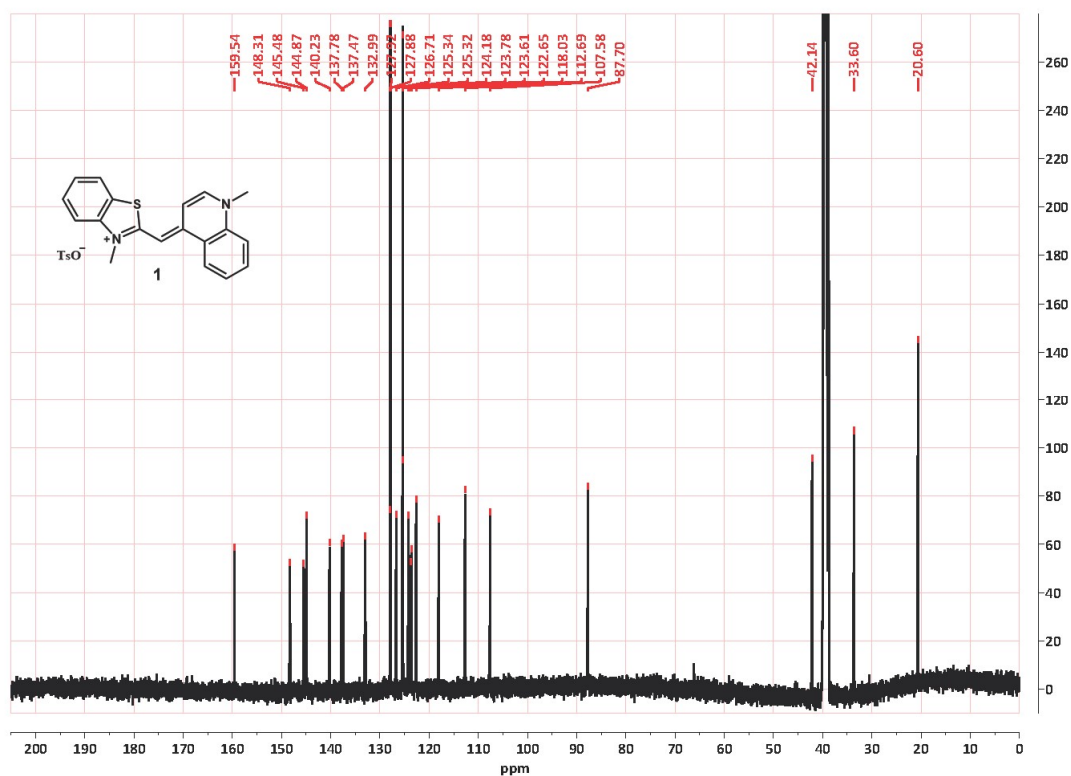


Figure S - 9.3 - ¹³C-NMR spectrum of cyanine dye 1 recorded in DMSO-d₆ (whole spectrum).

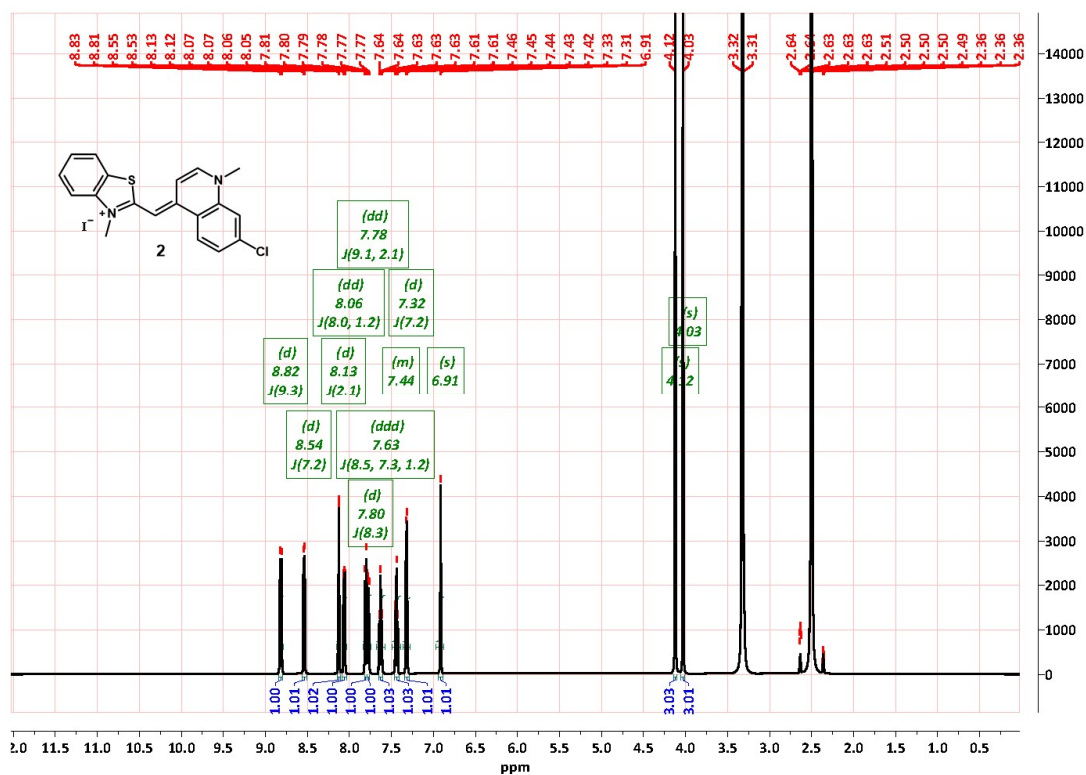


Figure S - 9.4 - ^1H -NMR spectrum of cyanine dye 2 recorded in DMSO-d_6 (whole spectrum).

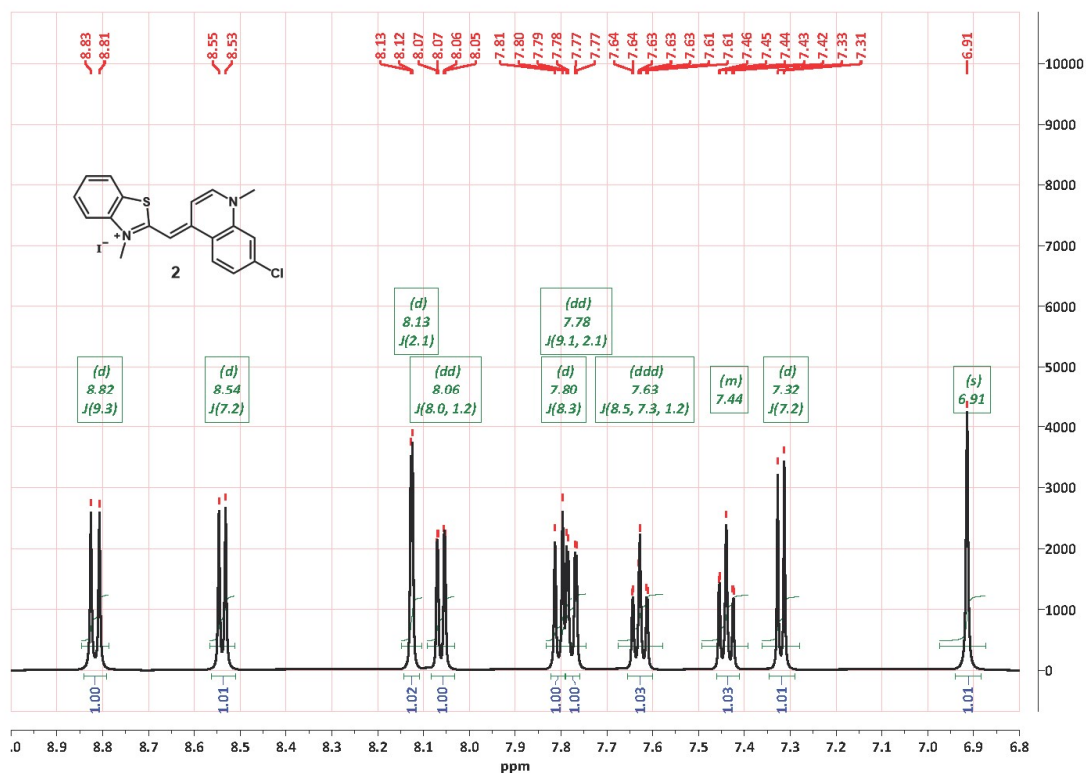


Figure S - 9.5 - ^1H -NMR spectrum of cyanine dye 2 recorded in DMSO-d_6 (aromatic expansion).

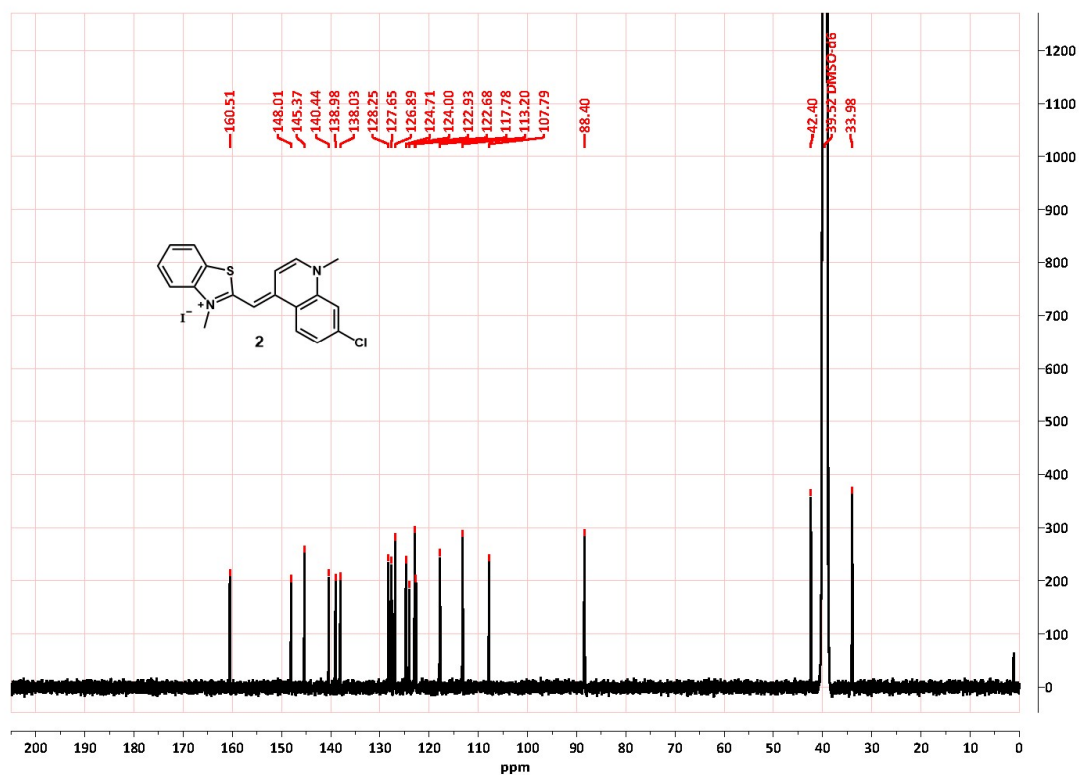


Figure S - 9.6 - ^{13}C -NMR spectrum of cyanine dye 2 recorded in DMSO- d_6 (whole spectrum).

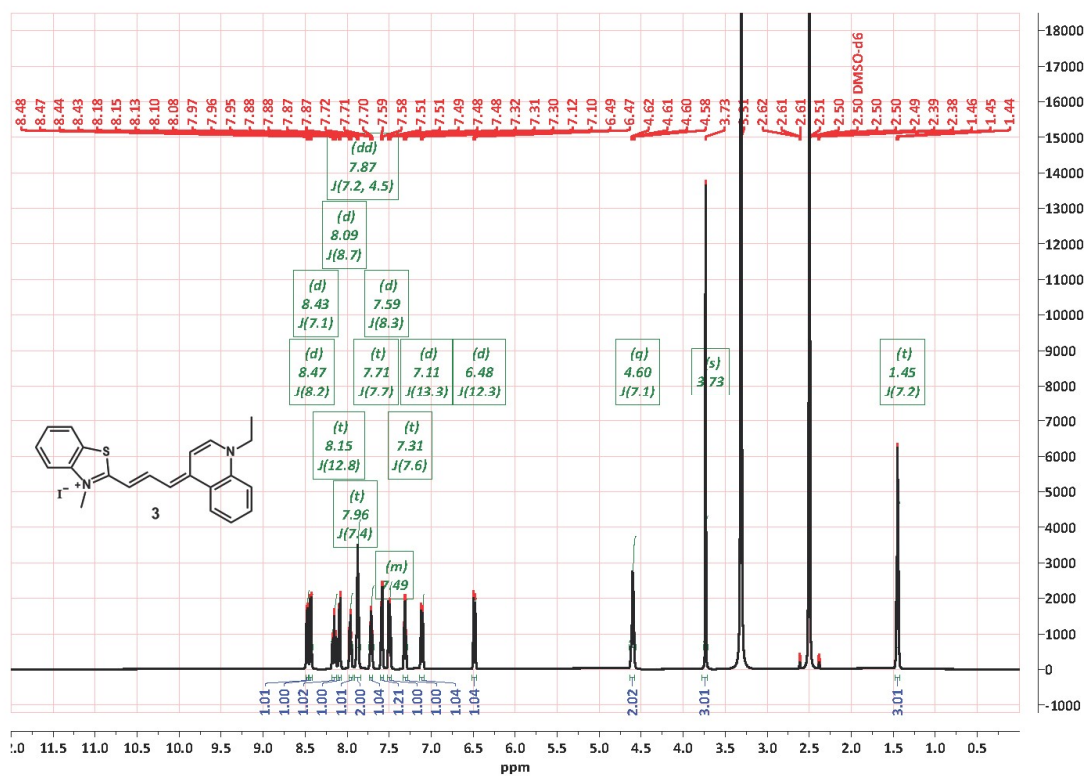


Figure S - 9.7 - ^1H -NMR spectrum of cyanine dye 3 recorded in DMSO- d_6 (whole spectrum).

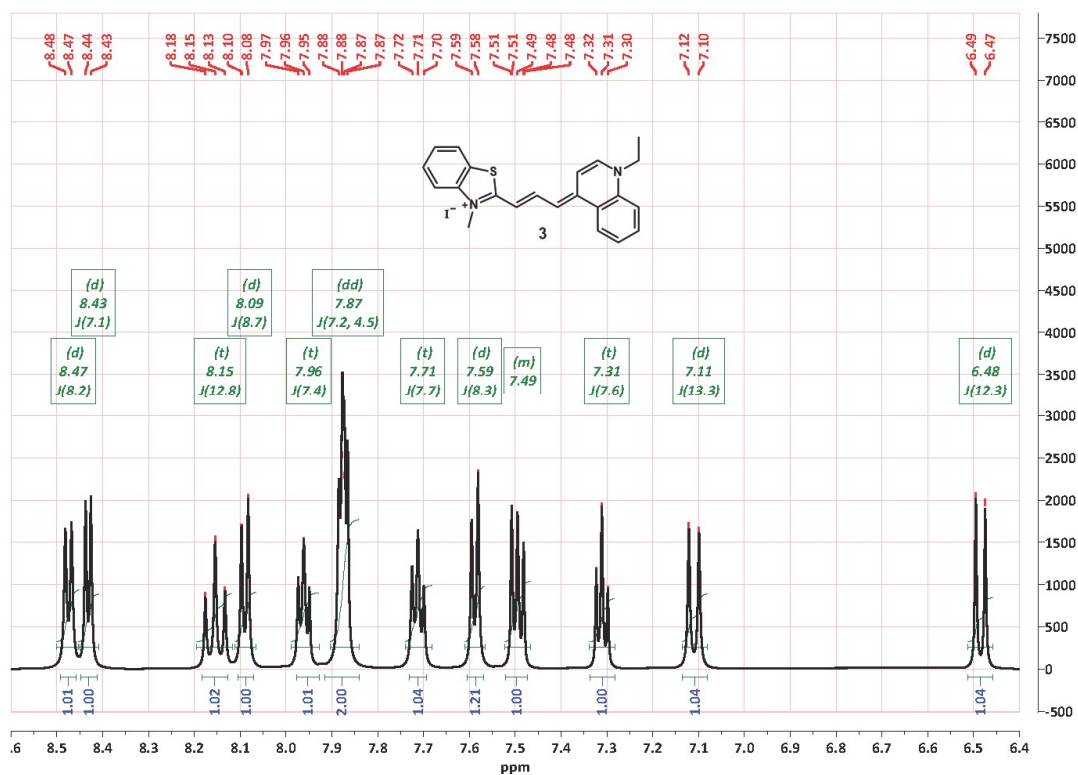


Figure S - 9.8 - ^1H -NMR spectrum of cyanine dye 3 recorded in DMSO-d_6 (aromatic expansion).

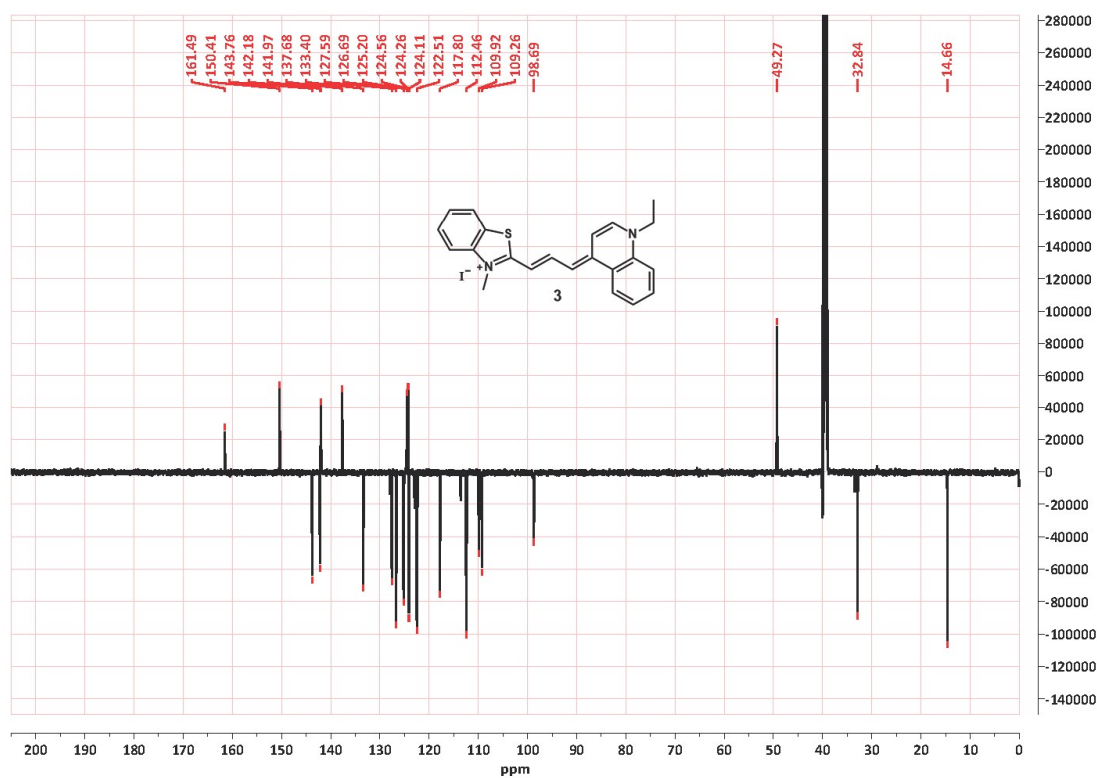


Figure S - 9.6 - Attached Proton Test-NMR spectrum of cyanine dye 3 recorded in DMSO-d_6 (whole spectrum).

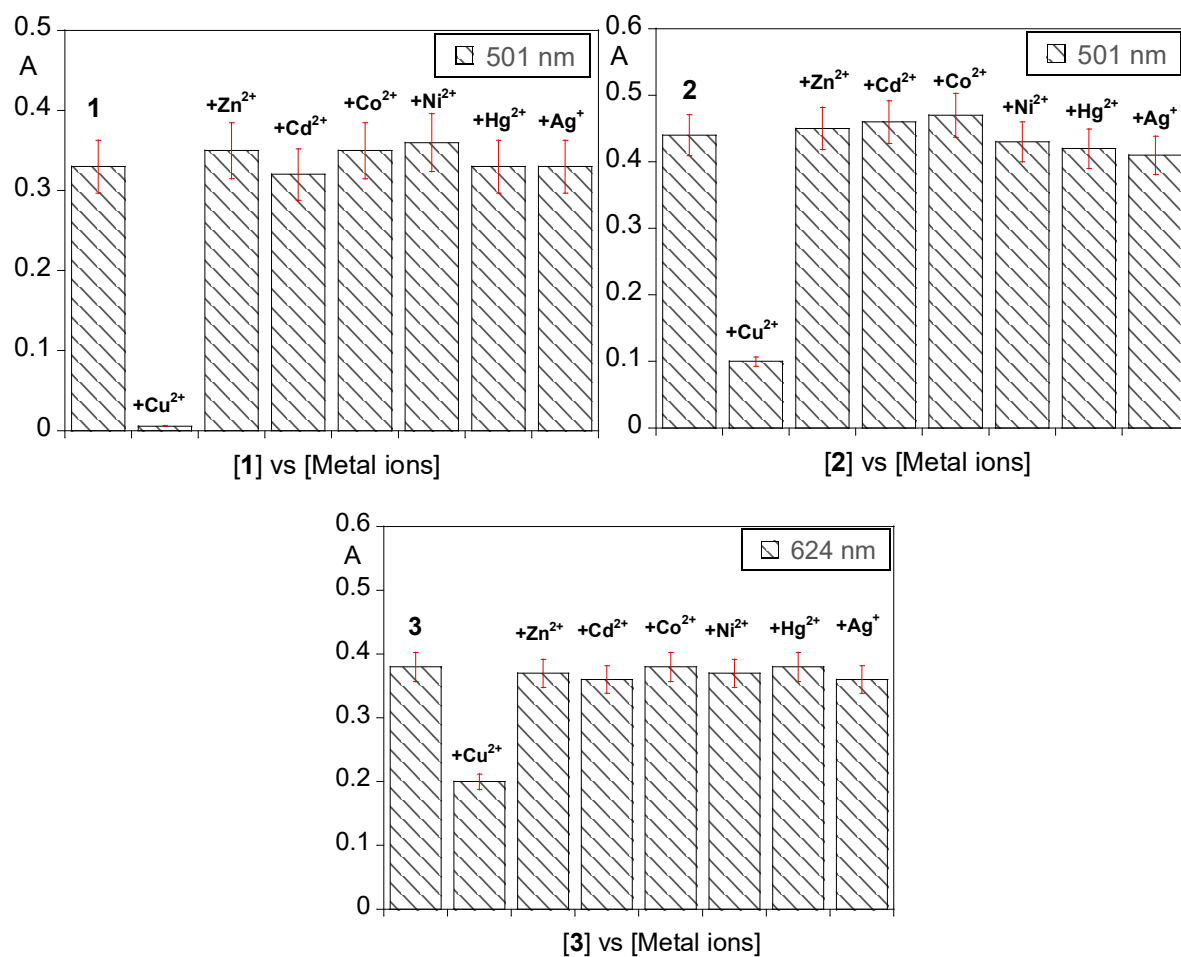


Figure S - 9.10 - Absorption at 501 nm for dyes **1** and **2**, and 624 nm for **3**, after addition of 10 equivalents of metal ions in acetonitrile, respectively. $[1] = [2] = [3] = 3 \times 10^{-6} \text{ M}$.

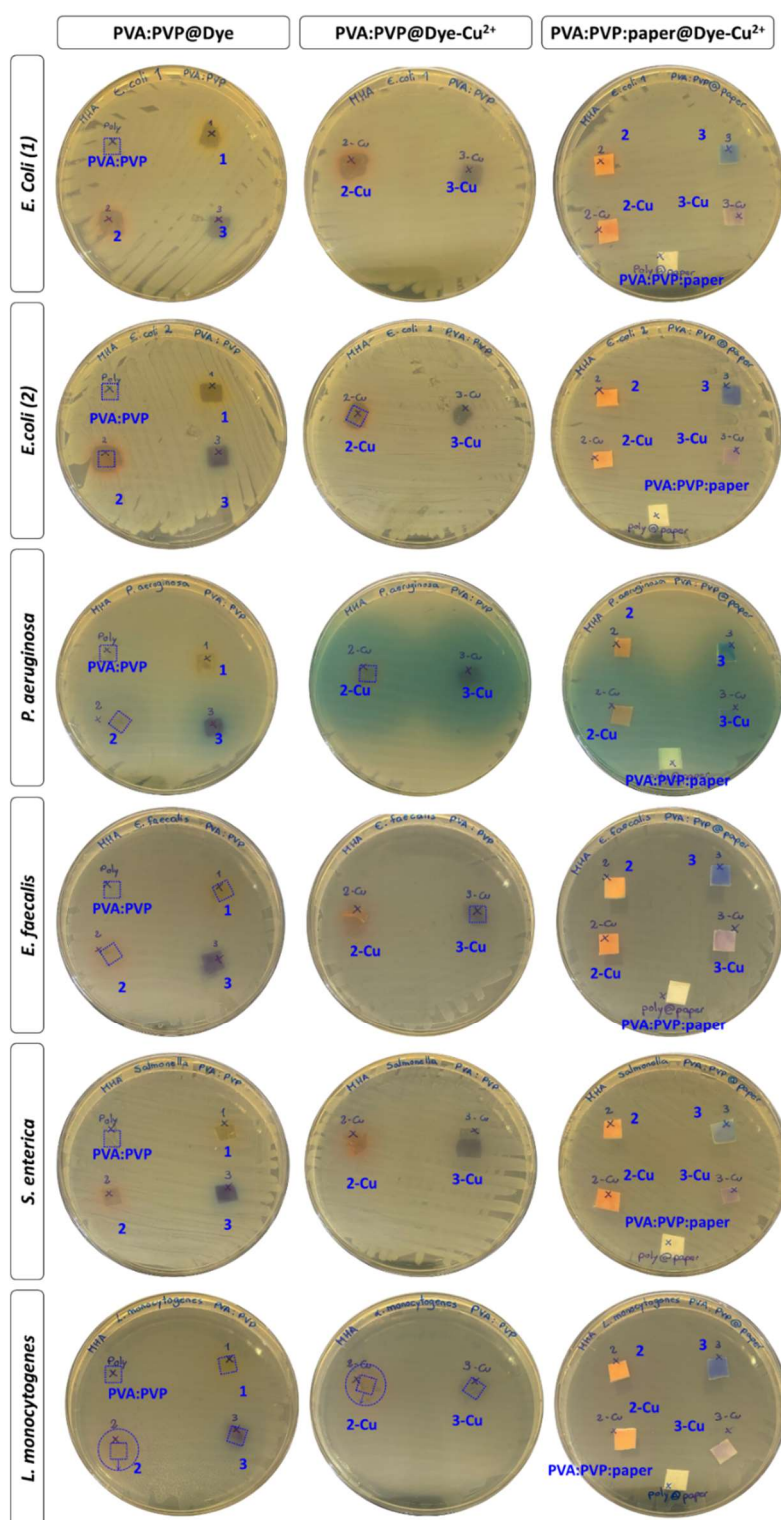


Figure S - 9.11 - Antibacterial assays of PVA:PVP and PVA:PVP:paper systems against *Escherichia coli* (*E. coli* 1), *Escherichia coli* (*E. coli* 2), *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Listeria monocytogenes* and *Salmonella enterica*.

GENERAL CONCLUSIONS AND PERSPECTIVES

In light of the current need for a sustainable oral therapy against colorectal cancer (CRC) and associated-opportunistic bacterial infections, the current thesis was successfully conducted for the production of a magneto-fluorescent nanoparticle-based therapy, using sustainable, green, biodegradable and biocompatible building blocks. In its parts, this thesis reported:

1. The successful synthesis of luminescent silicon/silica-based nanocarriers with optimal cellular uptake sizes of *ca.* 70-80 nm, for the effective pH-dependent delivery of DOX as model drug against CRC. The *in vitro* biological effects of the DOX-loaded nanoparticles were evaluated on human cancer cell lines of CRC, with the produced materials inducing noticeably cellular uptake and cytotoxicity with effects comparable to that of free drug. The bare SiQD mesoporous nanocarrier showed low toxicity and promising load and release capacity of DOX in acidic cancer environment due to their surface disulfide and amine moieties, while keeping its fluorescence in cellular environment.

2. The proteomic analysis of the nano-bio interaction is an important tool in the estimation of any nanomaterial's cytotoxicity, highlighting the levels of expression of certain genes/proteins in the targeted cells' proteome. From the above mentioned nanocarriers we confirmed that surface functionalized nanoparticles, particularly those containing thiol/disulfide and amine moieties, can be used in future targeted therapies to induce significant alterations in key proteins. These alterations not only disrupt the normal function and control of the cells, but also amplify the effect of potential therapeutic cargo (e.g., DOX) by simultaneously delivering it into the cells while weakening its defense mechanisms.

3. The preparation of core-shell mesoporous silica nanoparticles as optimal carriers for the delivery of amoxicillin and ofloxacin as model antibiotics against gram-

positive and gram-negative harmful bacterial strains. The study highlighted the capacity of the mesoporous nanoparticles to mask the presence of the selected antibiotics to the targeted bacteria, and by-pass resistance mechanisms (specially β -lactam resistance ones) and locally dispense them at bacteria membranes. This resulted in impactful 10- and 20-fold reductions of MIC₅₀ and MBC. Moreover, the tested materials showed the outstanding capacity of mesoporous silica matrices to hold high amounts of OFLO within its pores and of conserving its powerful antimicrobial properties until release, particularly against CRC-related bacteria.

4. The synthesis of nanoparticles to address opportunistic bacterial infections during cancer treatments Core-shell NANO3 were successfully synthesized by combining biocompatible and biodegradable materials, comprising of a superparamagnetic Fe₃O₄ core, a highly mesoporous silica shell and the above-referenced fluorescent silicon QDs as pore gates. The system showed a high predisposition to simultaneously incorporate and deliver OFLO and DOX as model anticancer drug and antibiotic, with a preferable release in acidic media like that of invasive tumors and bacterial infections. Hyperthermia-mediated release and heat generation under an external AMF were also confirmed for all NANO3 systems, by achieving febrile temperatures capable of stimulating the immune system and enhanced drug release. OFLO-containing NANO3 showed effective antimicrobial activity against *E. coli* and *E. faecalis* strains, with AMF application increasing the overall antibacterial activity and extending inhibitory activity to otherwise non-susceptible strains like *S. aureus*, MRSA and *P. aeruginosa*. Thus, confirming its potential application in a combined anti-CRC/antimicrobial nanotherapy.

5. The successful establishing of a luminescence-based assay specific for the assessment of nanoparticles antimicrobial activity that contributes to the standardization of methods in potential industrial applications of nanoparticles. The developed method gives researchers a fast (8 hours assay vs original 24h) and sensible methodological toolbox, in which luminescence allows for a direct measurement of bacterial growth phase and distinction between MIC₅₀ and MIC₉₀ concentrations. This approach has the particularity of having reduced overall variability and uncertainty by removing nanoparticle suspensions' and colorimetric compounds' spectral interferences. Such is the case of MNs and SPION@MNs colorimetric formulations (i.e., with colorimetric DOX and EPI) where otherwise determined MIC₅₀ were correctly assessed as rather MIC₉₀, moving the real assessment of MIC₅₀ to lower concentrations. This is possible since luminescence is directly correlated with the metabolic

response of the bacteria to the provided stimulus, and not to the physical behavior of the suspension, providing more accurate results.

6. The synthesis a new acetazolamide derivative (AZM-SH), with anti-hCA IX inhibitory activity, and of a dual-cargo, dual-targeting magneto fluorescent nanosystem (NANO3) as potential oral administered anti-CRC therapy. Beyond their above-confirmed antimicrobial activity and hyperthermia properties, NANO3 systems showed consistent and competitive anti-tumoral activity against HT-29 and HCT-116 CRC cell lines. The functionalization of CTX and AZM derivatives as targeting agents at the surface of NANO3 promoted an increased uptake in HCT-116 cell line, particularly those with the in-house synthesized AZM-SH. Its oral delivery approach was also confirmed by the proof-of-concept protection of NANO3 systems with a combinatory enteric coating of Eudragit® S100 and L100, capable of delaying the release in simulated gastric and small-intestine conditions and favoring it in colonic conditions. Thus, we demonstrate the applicability of the here in prepared system as a potential, stimuli responsive anti-CRC nanocarrier, with potential for an effective oral delivery.

7. The use of cyanine derivatives as potential alternative antimicrobial agents in both solution and solid-supported PVA:PVP devices. The dyes showed an unprecedented selectivity to Cu^{2+} metal, which showed increased antibacterial activity against *L. monocytogenes* and *B. cereus* with overall MIC_{50} of 1 - 0.5 μM . Composite dye-doped PVA:PVP membranes were particularly effective against Gram-positive bacteria, like *L. monocytogenes* and *S. aureus*, the later linked to CRC incidence. Moreover, these dyes and dye-doped polymers were also effective against resistant bacterial strains such as MRSA and *P. aeruginosa*, supporting their potential future use as sterile surfaces for high contact points or medical environments/materials, providing an increased layer of security against antibiotic-resistant bacterial strains.

For all above said, it should be concluded that the present thesis was successful in the preparation and optimization of a potential combined anti-CRC and antimicrobial nanotherapy, with a confirmed proof-of-concept for an oral administration.

In a future perspective, and to make possible the delivery of the herein reported materials to CRC patients several, further studies are envisaged. Proteomic studies on cells

exposed to NANO3 systems to assess possible impacts on cell membranes and triggered membrane mechanisms are ongoing. Thorough uptake kinetics studies are also planned. While toxicity studies are currently being executed on healthy colon model cell lines (CCD112Con). *in vivo* studies are the next step to assess the validity of the herein detailed nanotherapy. Complete digestive track delivery studies are also programed to be performed in an improved digestion method (INFOGEST 2.0). to support the adequacy of the therapy to an oral administration.



2022

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BIODEGRADABLE MAGNETO-LUMINESCENT MESOPOROUS NANOPARTICLES
AS NEW NANOBIOMEDICAL TOOLS IN CANCER TREATMENT

