Letícia Maria Rui Fialho

Licenciada em Bioquímica

Preparation of biopolymer structures based on FucoPol

Dissertação para obtenção do Grau de Mestre em Biotecnologia

Orientadora: Doutora Maria Filomena Andrade de Freitas
Senior Researcher, FCT-UNL

Co-orientadora: Professora Doutora Maria Alexandra Núncio de Carvalho Ramos Fernandes,
Professora Auxiliar, FCT-UNL

FACULDADE DE CIÊNCIAS E TECNOLOGIA
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Resumo

O FucoPol é um exopolissacárido (EPS) com elevado peso molecular (> 10^6 Da), produzido pela bactéria *Enterobacter* A47, principalmente composto por açúcares neutros (fucose, galactose, glucose e ácido glucorónico) e grupos acilo (acetato, sucinato e piruvato).

O objetivo principal desta tese foi desenvolver estruturas poliméricas baseadas no FucoPol. Em primeiro lugar, o FucoPol foi produzido utilizando o glicerol como única fonte de carbono para o cultivo de *Enterobacter* A47. Um rendimento de 7,74 g L^{-1} foi atingido no final de 96 h de cultura. O rendimento volumétrico global foi de 1,97 g L^{-1} dia^{-1} e o rendimento líquido de FucoPol em glicerol foi de 0,085 g g^{-1}. O polímero resultante foi extraído do caldo e utilizado para testar diferentes procedimentos de gelificação. Com base no carácter aniónico do polímero, foram testados diferentes sais de catiões mono-, di e trivalentes (KCl, KNO_3, NaCl, NaH_2PO_4, $CaCl_2$, $CaSO_4$, $MgCl_2$, $MgSO_4$, $CuSO_4$, $CuCl_2$, $ZnSO_4$, $ZnCl_2$, $FeSO_4$, $FeCl_2$, $FeCl_3$) para avaliar a capacidade de gelificação de FucoPol. A gelificação de FucoPol ocorreu na presença dos sais divalentes testados à exceção dos sais de cálcio, à temperatura ambiente (25 °C) em meio alcalino (NaOH 2M), enquanto que para $FeCl_3$, a gelificação do FucoPol ocorreu na presença de uma solução aquosa de $FeCl_3$, à temperatura ambiente (25 °C), com um pH baixo.

A libertação de cobre e ferro das esferas de FucoPol ($FeCl_3$, $CuSO_4$, $CuSO_4$/$FeCl_3$) foi avaliada em diferentes soluções biológicas (NaCl (0.9%), SGF, SIF, PBS e DMEM). As esferas de FucoPol testadas mantiveram a sua forma nos ensaios realizados em NaCl e desintegraram-se em SGF. As esferas de $CuSO_4$/FucoPol mantiveram a sua forma durante o ensaio em PBS e desintegraram-se em DMEM e SIF. As esferas de $FeCl_3$/FucoPol tornaram-se mais translúcidas ao longo dos ensaios em DMEM, PBS e SIF enquanto as esferas de $CuSO_4$/$FeCl_3$/FucoPol mantiveram a sua forma durante estes ensaios.

A citotoxicidade do FucoPol avaliada em diferentes linhas celulares (fibroblastos, A2780, A549, HCT116 e MCF7) através do ensaio MTS. Os resultados mostraram que o FucoPol não é citotóxico para as linhas celulares testadas. A citotoxicidade das esferas de FucoPol ($FeCl_3$, $CuSO_4$ e $CuSO_4$/$FeCl_3$) foi avaliada através do método de Azul de Tripano para as células K562 e os resultados sugerem que as esferas de $FeCl_3$/FucoPol não são citotóxicas e as esferas preparadas com cobre parecem afetar a viabilidade das células K562.

Palavras-Chave: FucoPol; capacidade gelificante; esferas de FucoPol; ensaios de viabilidade.

Abstract

FucoPol is a high molecular weight exopolysaccharide (EPS) secreted by the bacterium *Enterobacter* A47 that is composed of the neutral sugars fucose, galactose and glucose, and the acidic sugar glucuronic acid. It also has substituent acyl groups (acetyl, succinyl and pyruvyl). The presence of glucuronic acid, together with succinyl and pyruvyl, confer the polymer an anionic character.

The main goal of this thesis was to develop polymeric structures based on FucoPol. Firstly, FucoPol was produced using glycerol as the sole carbon source for the cultivation of *Enterobacter* A47. A production of 7.74 g L^{-1} was reached at the end of 96 h of cultivation. The overall volumetric productivity was 1.97 g L^{-1} day^{-1} and the net yield of FucoPol on glycerol was 0.085 g g^{-1}. The produced polymer was extracted from the broth and used to test different gelling procedures. Based on the anionic character of the polymer, different salts of mono-, di- and trivalent cations were tested (KCl, KNO_3, NaCl, NaH_2PO_4, $CaCl_2$, $CaSO_4$, $MgCl_2$, $MgSO_4$, $CuSO_4$, $CuCl_2$, $ZnSO_4$, $ZnCl_2$, $FeSO_4$, $FeCl_2$, $FeCl_3$) to evaluate the gelling capacity of FucoPol. The gelation of FucoPol occurred in the presence of divalent cations tested excepting calcium salts, at room temperature (25 ºC) in alkaline media (NaOH 2M), while for $FeCl_3$, gels formed only by mixing FucoPol solution with salt aqueous solution at room temperature (25 ºC), with a low pH.

Release of copper and/or iron from FucoPol gel beads ($FeCl_3$, $CuSO_4$,) was evaluated in different biological solutions (NaCl (0.9%), SGF, SIF, PBS and DMEM). FucoPol beads tested maintained their shape in NaCl and disintegrated in SGF. $CuSO_4$/FucoPol beads maintained their shape during the assay in PBS, and disintegrated in DMEM and SIF. $FeCl_3$/FucoPol beads became more faint and translucent in DMEM, SIF and PBS while $CuSO_4$/$FeCl_3$/FucoPol beads maintained their shape in these media.

The cytotoxicity of FucoPol in different human cell lines (fibroblasts, A2780, A549, HCT116, MCF7) was assessed by MTS assay and results shown that FucoPol was not cytotoxic. Cytotoxicity of FucoPol beads was assessed by Trypan Blue method for K562 cells and results suggest that Fe/FucoPol beads were not cytotoxic, and FucoPol beads prepared with copper affect the viability of K562 cells.

Keywords: FucoPol; gelling capacity; FucoPol gel beads; viability assays;

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Abbreviations

A2780	Ovarian carcinoma cell line
A549	Lung adenocarcinoma
bFGF	basic fibroblast growth
CDW	Cell dry weight
COO^-	Carboxylate groups
Cryo-SEM	Cryo scanning electron microscopy
CTR1	Copper transporter 1
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DO	Dissolved oxygen concentration
DOX	Doxorrubicin
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EPS	Exopolysaccharide
FBS	Fetal bovine serum
FTIR	Fourier-transform infrared spectroscopy
HCT116	Colorectal carcinoma cell line
HH	Hereditary Hemochromatosis
HPLC	High performance liquid chromatography
ICP – AES	Inductively coupled plasma – atomic emission spectroscopy
IM	Imatinib
K562	Chronic myelogenous leukemia cell line
LB	Luria Bertani
MCF7	Breast adenocarcinoma cell line
MEM	Minimum essential medium
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
N. A.	data not available
OD	optical density
OD450	optical density at 450 nm
p 53	cytoplasmatic protein
PBS	phosphate-buffered saline
PDI	Polydispersity index
PMS	Phenazinemethosulfate

ROS	Reactive oxygen species
SEC-MALLS	Size Exclusion Chromatography – Multi-Angle Laser Light Scattering
SEM	Scanning electron microscopy
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SLPM	Standard liters per minute
TF	Transferrin
TFA	Trifluoroacetic acid
TFR1	Transferrin receptor 1
VEGF	Vascular endothelial grown factor
WD	Wilson disease

Variables

% (v/v)	Volume/volume percentage
% (w/v)	Weight/volume percentage
°C	Celsius degrees
h; min; s	Hours; minutes; seconds
kDa; Da	Kilodalton; Dalton (10^{-3} kg mol^{-1})
Kg; g; mg; µg	Kilogram; gram (10^{-3} kg); milligram (10^{-6} kg); microgram (10^{-9} kg)
L; mL; µL	Liter; milliliter (10^{-3} L); microliter (10^{-6} L)
m; cm; mm; µm	Meter; centimeter (10^{-2} m); millimeter (10^{-3} m); micrometer (10^{-6} m)
M; mM; µM	Molar (mol L^{-1}); millimolar (10^{-3} M); micromolar (10^{-6} M)
M_n	Number average molecular weight
mol %	Molar percentage
M_w	Average molecular weight
Pa; mPa	Pascal; millipascal (10^{-3} Pa)
ppm	part(s) per million
rpm	Rotations per minute
wt.%	Weight percentage

1. Introduction

1.1. Natural Polysaccharides

Plants, crustacean, algae, as well as a wide range of microorganisms, represent sources of a wide diversity of natural polysaccharides (Torres et al., 2011). The use of polysaccharides from natural origin instead of synthetic polysaccharides decreases the negative environment impact, once that natural polysaccharides are biodegradable, biocompatible and non-toxic (Ferreira et al., 2014; Kumar, Mody, and Jha 2007).

Microorganisms usually have higher growth rates than plants, crustacean and algae, being the microbial production of polysaccharides more amenable to process manipulation, allowing for improved yields, properties and productivity (Alves et al., 2010; Torres et al., 2011). Furthermore, microbial polysaccharides present a greater variety of structures and physicochemical properties, rendering them applicability in areas ranging from their use as biomaterials or as rheology modifiers of aqueous systems, to therapeutic agents (Freitas et al., 2017).

The main characteristics/properties of polymers obtained from different natural sources are presented in Table 1, as well as their possible applications and their producing organisms. The interest for bacterial polysaccharides has increased, in the last decades, due to their high commercial value in different areas, such as pharmaceuticals, biomedicine, food and cosmetics (Alves et al., 2010; Freitas, Alves, & Reis, 2011).

Table 1 – Summary table of the main characteristics (composition, molecular weight), properties and applications of natural polysaccharides produced by different organisms (n.a., data not available).

Polymer	**Alginate**	**Gellan Gum**	**Xanthan Gum**	**Hyaluronan**	**Colanic Acid**	**FucoPol**	**λ- Carrageenan**	**Guar Gum**	**Arabic Gum**	**Bacterial Cellulose**
Organisms	*Pseudomonas aeruginosa* and *Azotobacter vinelandii*	*Sphingomonas paucimobilis*	*Xanthomonas* spp.	*Streptococcus* sp.	*Escherichia* coli, *Shigella* spp., *Salmonella* spp. and *Enterobacter* spp.	*Enterobacter* A47	*Sarcothalia lanceata*	*Cyamopsis tetragonoloba*	*Acacia senegal and Acacia seyal*	*Acetobacter* spp.
Sugar monomers	Glucuronic acid and mannuronic acid	Glucose, rhamnose, glucuronic acid, acetate, glycerate	Glucose, mannose and glucuronic acid	Glucuronic acid, acetylglucosamine	Fucose, glucose, glucuronate and galactose	Fucose, glucose, galactose and glucuronic acid	Galactose	Mannose and galactose	Galactose, arabinose, rhamnose, glucuronic acid	Glucose
Molecular weight (Da)	$(0.3 – 1.3) \times 10^6$	5.0×10^5	$(2.0 – 50) \times 10^6$	2.0×10^6	$2 \times 10^4 – 6 \times 10^5$	$(2.0 – 10.0) \times 10^6$	n.a.	$(1\text{-}2) \times 10^6$	$1 – 2 \times 10^6$	$\approx 10^6$
Properties	Gelling capacity, film forming	Stability over wide range of pH, gelling capacity, thermoreversible gels	High viscosity, stable over wide ranges of temperature, pH and salt concentrations	Biological activity, high hydrophilic and biocompatible	Gelling capacity	Film forming, emulsifying capacity, flocculation capacity, biological activity due to fucose content	thickener, viscosifier, gelling and stabilizers agents	Thickeners and stabilizers agents, gel forming properties	Stabilizer, thickening and emulsifier agent	High crystallinity; Insoluble in the most solvents; High tensile strength; Moldability
Applications	Food hydrocolloid and medicine	Cosmetics, personal products, pharmaceutical/medical applications and foods	Cosmetics, pharmaceutics, personal care products and petroleum industry	Medicine, cosmetics, food	Cosmetics and personal care products	Food, cosmetics, pharmaceuticals and medicine	Food and pharmaceutical applications	Food, pharmaceuticals, paper, textile, explosive, oil well drilling and cosmetics industry	Food, textile, pottery, lithography, cosmetic and pharmaceutical industries	Foods, oil recovery; Biomedical audio speaker diaphragms
Reference	Nwodo et al., 2012	Fialho et al., 2008	Nwodo et al., 2012	Chong et al., 2005	Nwodo et al., 2012	Alves et al., 2010; Freitas et al., 2011	Running et al., 2012	Mudgil et al., 2014	Ali et al., 2009	Nwodo et al., 2012; Freitas, Alves & Reis 2011

1.2. Bacterial Polysaccharides

According to their cellular localization, bacterial polysaccharides can be categorized into intracellular polysaccharides (e.g., glycogen), capsular polysaccharides (e.g., K30 antigen) or exopolysaccharides (EPS) (e.g., xanthan gum, alginate and hyaluronic acid) (Rehm, 2010). Bacterial EPS are high molecular weight biopolymers (10 to 1000 kDa) secreted by the cells that either form a capsule linked to the cell surface or form a slime weakly linked to cell surface (Kumar et al., 2007). Bacterial EPS have been extensively used in high value-applications, such as food, cosmetic, pharmaceutical and medical applications or processes, where they are mostly applied as thickening, stabilizing, binding and structure creation agents, as a result of their non-Newtonian behaviour and high viscosity in aqueous media (Freitas et al., 2011a; Rehm, 2010).

1.2.1. Composition of bacterial EPS

Bacterial EPS can be classified as homopolysaccharides or heteropolysaccharides, according to their chemical composition (Nwodo et al., 2012). Homopolysaccharides are composed of one type of monosaccharide and heteropolysaccharides are usually composed of repeating units with different sizes, varying from disaccharides to octosaccharides (Sutherland, 2001).

In heteropolysaccharides, glucose, galactose and mannose are the most common monomers. Other neutral sugars (e.g., rhamnose and fucose), some uronic acids (e.g., mainly glucuronic and galacturonic acids) and amino-sugars (e.g., glucosamine or galactosamine) are also frequently presents (Freitas et al., 2011a; Nwodo et al., 2012).

EPS have commonly non-sugar components, such as acyl groups (e.g., acetyl, pyruvil, succinyl) (Torres et al., 2011). The presence of some of those substituents, as well as uronic acids and amino-sugars, confers them an anionic character, increases their lipophilicity and affects their capacity to interact with other polysaccharides and cations (Freitas et al., 2011a).

The properties of EPS are determined by their chemical composition, molecular structure, average molecular weight and distribution (Freitas et al., 2011a).

1.2.2. FucoPol

L-fucose, a rare sugar in nature, is a constituent of some polysaccharides, including bacterial EPS, that may give additional biological properties compared to those composed of more common sugar monomers (Roca et al., 2015). Fucose-containing polysaccharides have demonstrated interesting biological activities, namely acceleration of wound healing (Péterszegi et al., 2003) and can be used as skin moisturising agent in cosmetic industry (Cescutti et al., 2005). Fucose and its oligosaccharides have potential medical applications due to their anti-cancer and anti-inflammatory effects (Cescutti et al., 2005).

Microorganisms producing fucose-containing polysaccharides have been reported, such as *Clavibacter michiganensis* (clavan) (Vanhooren and Vandamme, 2000), *Klebsiella pneumoniae* I-1507 (Fucogel) (Vanhooren and Vandamme, 2000) and a marine bacterium of the species *Enterobacter cloacae* was also reported to produce a polymer composed of fucose, galactose, glucose, and glucuronic acid in a molar ratio of 2:1:1:1 (Iyer, Mody, and Jha, 2005). Some members of the Enterobacteriaceae family synthesize colanic acid (Table 1), a fucose-containing EPS (Freitas et al., 2011b).

The bacterium *Enterobacter* A47 (DSM 23139) was previously reported to produce a high molecular fucose-containing biopolymer, which was named FucoPol (Freitas et al., 2011a). FucoPol is an heteropolysaccharide composed of fucose (32-36 mol %), galactose (22-26 mol %), glucose (28 – 37 mol %), glucuronic acid (9-10 mol %) and acyl groups, particularly, succinyl (2 – 3 wt.%), pyruvyl (13-14 wt.%) and acetyl (3-5 wt.%) (Torres et al., 2015). This polymer has high molecular weight (5×10^6 Da) and a low polydispersity index (1.3) (Torres et al., 2015).

Several properties of FucoPol have been studied, such as flocculating activity (Freitas et al., 2011), film forming capacity (Ferreira et al., 2014, 2016), emulsion forming and stabilizing capacity (Freitas et al., 2011, 2014). FucoPol forms viscous solutions with shear thinning behavior (Cruz et al., 2011; Torres et al., 2015); has high flocculating activity, which envisages its potential for colloid and cell aggregation in several applications such as water treatment, food and mining industries (Freitas et al., 2011a).

1.3. Polysaccharide Gels

Hydrogels are three-dimensional, hydrophilic, polymeric networks, with chemical or physical cross-links, capable of imbibing large amounts of water or biological fluids (Coviello et al., 2007).

Some hydrophilic biopolymers, such as polysaccharides, have gelling ability, having as examples alginate, curdlan, colanic acid and gellan gum (Freitas et al., 2011a; Nwodo et al., 2012).

The physicochemical properties of biopolymers (e.g., molecular dimensions, surface activity, polarity and thermal stability) and environmental conditions (e.g., temperature, ionic strength, pressure/shear, pH and water activity) influence the different type of gels structures (Nazir et al.,2017). The capacity of some EPS to establish polymeric matrices allows their *in vitro* manipulation to shape them as structured materials (e.g., nanoparticles, scaffolds or hydrogels) that can be applied to specific biomedical applications including drug delivery, imaging, tissue engineering and wound dressing (Freitas, et al., 2011a).

Hydrogels can be classified according to:

- Type of biopolymer network: Gel structures can be composed of **simple networks** (consisting of a single component as happens with polysaccharides or proteins; **binary/mixed networks** (composed of two or more components); **composite/filled network** (composed of different particles, for example using fat globules along the biopolymer network) (Nazir et al., 2017).

- Type of gelation mechanism: gels have three main mechanisms of gelation (Burey et al., 2008), namely **ionotropic gelation** that occurs through the linkage of polymer chains with ions, frequently cation-mediated gelation of negatively charged polysaccharides; **cold-set gelation** that occurs when an aqueous polymer solution is prepared at high temperatures or at a boiling point and then cooled down to room temperature; **heat-set gelation** occurs by unfolding/expansion of native structures (e.g., polymer chains, native proteins) and their subsequent rearrangement into a network. This technique is the least common used in forming gel particles and it is usually required in food applications (Burey et al., 2008).
- Type of cross-linking: gels can be classified according to their cross-linking mechanism into **physical cross-linking,** that include entangled chains, hydrogen bonding, hydrophobic interaction and crystallite formation, and **chemical cross-linking** where gels are formed by covalent cross-linking of polymers (Maitra and Shukla, 2014).

1.3.1. Gel particles

Recently, research has been focused on the creation of novel structures (micro/nano spheres, polymer beads and capsules) with edible safe materials, wherein bioactive compounds (e.g., antioxidants, vitamins, probiotics or prebiotics) are encapsulated and applied in food, pharmaceutical, medical or cosmetic products (Burey et al., 2008; Freitas et al., 2011a).

The gel network gives the particles both the rigidity of a soft solid while providing a permeability to small molecules and ions (Perez-Moral et al., 2013).There are several techniques that can be used to prepare particles from polysaccharides.

The matrix material is selected using different factors, such as particles size, charge, permeability of the surface, and the degree of biocompatibility and toxicity (Alves et al., 2016).

The preparation of gel particles should use nontoxic solvents, enable large production and be commercially sustainable (Alves et al., 2016).

The methods used to prepare particles based on bacterial polymers are presented in Table 2, as for example ionotropic gelation, emulsification and spray drying.

Emulsification is one of the methods commonly used to prepare particles which comprises blending of immiscible liquid using mechanical stirring (Alves et al., 2016). This technique is generally applied for the encapsulation of bioactive agents in aqueous solutions (Alves et al., 2016).

Spray Drying consists in atomizing a polymeric solution into a stream of hot air, which induces a rapid solvent evaporation from the droplets, leading to the formation of microspheres or microcapsules (Alves et al., 2016). Spray drying has been used with excellent results for microencapsulation of flavors, probiotics and drug delivery (Burey et al., 2008).

Extrusion or Ionotropic gelation is a technique commonly used to produce gel particles. Hydrogel beads are formed by dropping, through a needle, a polymeric solution into an aqueous solution containing polyvalent cations (e.g. Ca^{2+}, Cu^{2+}, Al^{3+}) (Alves et al., 2016).

Coacervation is a technique where, biopolymers with two opposite charges interact with each other, through electrostatic attraction, forming coacervants. A core of ingredients or bioactive compounds are encapsulated by precipitation of biopolymer-coacervate around it (Alves et al., 2016). Polymer coacervation is a well-established technique which is often used for reversible gelation and encapsulation of biological materials, molecules or cells (Burey et al., 2008).

Precipitation procedure is based on dissolving a biopolymer and the active agent/ingredient in a good solvent. Then, the solvent quality is decreased by providing conditions to spontaneous formation of biopolymer nanoparticles or microparticles. This process is widely used in pharmaceutical industry because there is no need of specific equipment (Alves et al., 2016).

Table 2- Several techniques available to prepare gel particles from bacterial polymers, advantages and disadvantages.

Method	Advantages	Disadvantages	Polymers used with these method	Reference
Ionotropic gelation **Extrusion**	Prevent the use of organic solvents and extreme conditions of pH and temperature.	Capsules generally have high porosity. Technique mainly used at lab scale.	Gellan gum, Xanthan gum	Nedovic et al.,2011; Alves et al.,2016
Coacervation	Heat sensitive ingredients may be encapsulated since technique is performed at room temperature.	Used non-approved solvents. Particles agglomeration and variability from batch to batch is common.	Xanthan chitosan	Oliveira & Mano, 2011; Alves et al., 2016
Spray-drying	Simple continuous method, economic, fast and easy to scale up.	Difficult to control particle size, moderate yield of small batches. Can degrade highly temperature sensitive compounds.	Chitosan, bacterial cellulose, hyaluronan	Alves et al., 2016; Lourenço et al., 2017
Emulsification	Simple method to produce hydrogel particles	Difficult to control shape and size distribution	Xanthan gum, gellan gum	Oliveira & Mano 2011; Alves et al., 2016
Precipitation	Simple method. No need special equipment. Easy to scale up	Difficult to control shape and size distribution.	Polyhydroxyalkanoates (PHAs)	Alves et al., 2016

1.4. Relevance of iron and copper in human body

1.4.1. Iron

Iron is a component of several metalloproteins and plays a crucial role in vital biochemical activities, such as oxygen sensing and transport, electron transfer and catalysis, macromolecule biosynthesis, necessary for cell growth and proliferation (Heath et al., 2013; Papanikolaou & Pantopoulos, 2005).

Iron participates in the transfer of electrons via oxidation-reduction (REDOX) reactions that results in the fluctuation of iron between its ferric (+3) and ferrous (+2) states (Mackenzie et al., 2008). This predisposition to undergo REDOX reactions is largely responsible for the biological significance of

iron and responsible for its toxicity (Mackenzie et al., 2008). **Fenton reaction** occurs when ferrous iron donates an electron in a reaction with hydrogen peroxide to produce the hydroxyl radical, a reactive oxygen species (ROS). This reaction can promote lipid peroxidation and lead to oxidative damage of DNA and other macromolecules (Torti & Torti, 2013).

Each day, approximately 25 mg of iron are needed for erythropoiesis and other vital functions but only 1 to 2 mg of iron comes from intestinal iron sources (Mackenzie et al., 2008).

Changes in intracellular iron availability can have significant effects on cell cycle regulation, cellular metabolism, and cell division (Heath et al., 2013). Deviations from normal iron levels have been indicated in the pathogenesis of aging, neurodegenerative disease, cancer, and infection (Mackenzie et al., 2008).

The primary method of total iron body regulation is via modulation of iron absorption through duodenum, because humans do not have an efficient mechanism to excrete excess iron (Heath et al., 2013).

Other mechanisms for iron regulation, including release of iron from cellular storage depots and recycling of iron from protein sources, are critically important to provide for organismal iron requirements (Mackenzie et al., 2008).

Significant interest has emerged in the possibility that iron depletion strategies have an antiproliferative effect on tumor cells due to the enhanced dependence on iron metabolism in neoplastic cells, once that iron plays a crucial role in cell growth and proliferation (Heath et al., 2013).

1.4.2. Copper

Copper is required for the function of over 30 proteins, including superoxide dismutase, ceruloplasmin, lysyl oxidase, cytochrome c oxidase, tyrosinase and dopamine-β-hydroxylase (Collins & Knutson, 2010).

Copper ingested daily in the diet is estimated to be between 0.6 and 1.6 mg (Denoyer et al., 2015). The mechanism by which dietary copper is absorbed is distinct from that of iron, although complementary mechanisms seem plausible given the atomic properties of the two highly reactive and structurally similar transition metals (Collins & Knutson, 2010).

While the redox activity of copper is essential for enzymatic reactions, that property can be potentially toxic (Denoyer et al., 2015), because copper participates in Fenton reaction, which can promote lipid peroxidation or oxidative damage of DNA (Gammella et al., 2016).

Copper excess or deficiency of which cause impaired cellular functions and eventually cell death, similar of what happens with iron (Papanikolaou & Pantopoulos, 2005).

Systemic copper deficiency generates cellular iron deficiency, which in humans results in diminished work capacity, reduced intellectual capacity, decrease growth, alterations in bone mineralization, and decline immune response (Papanikolaou & Pantopoulos, 2005).

1.5. Relevance of copper and iron in diseases

1.5.1. Iron

The mechanisms for iron-induced neoplastic transformation are poorly characterized but it is clear that iron overload disrupts the redox balance of the cell and generates chronic oxidative stress, which modulates signaling networks related to malignant transformation (Papanikolaou & Pantopoulos, 2005).

High iron concentrations are found in the brains of patients with conditions such as Parkinson's and Alzheimer's disease, or more rare conditions such as Huntington's disease. In these disorders, iron-induced oxidative stress, combined with defective antioxidant capacities, promotes neuronal death and neurodegeneration (Papanikolaou & Pantopoulos, 2005)**.**

Secondary iron overload develops as a result of repeated blood transfusions, which are frequently applied for the treatment of various anemias (Papanikolaou & Pantopoulos, 2005)**.**

An insufficient iron supply, unmet demand for iron or substantial loss of iron will lead to a lack of hemoglobin, resulting in iron deficiency anemia (Crielaard et al., 2017)**.**

Both the beneficial and deleterious effects of iron have a role in cancer. For example, iron may accelerate tumor initiation by enhancing the formation of free radicals, as well as function as a nutrient that fosters tumor cell proliferation (Torti & Torti, 2013).

Iron is a target for cancer therapy. Iron chelators, transferrin receptor 1 (TFR1) antibodies and cytotoxic ligands conjugated to transferrin (TF) represent some ways in which iron is being exploited therapeutically (Torti & Torti, 2013).

It is believed that iron-induced chronic oxidative stress eventually leads to mutations in critical gatekeeping or DNA repair genes. This view is supported by the increased lipid peroxidation and frequency of mutations in the tumor suppressor p53 gene, identified in nontumorous liver samples from Hereditary Hemochromatosis (HH) and Wilson disease (WD) patients (Papanikolaou & Pantopoulos, 2005).

HH is an autosomal recessive disorder in which inappropriate high absorption of dietary iron eventually leads to iron accumulation in tissue parenchymal cells and severe organ damage (Papanikolaou & Pantopoulos, 2005)**.**

WD is an autosomal recessive copper overload disorder that manifests primarily in the liver and brain (Denoyer et al., 2015).

The potential of iron as a carcinogenic agent appears to be mainly related to its ability to promote oxidative stress (Papanikolaou & Pantopoulos, 2005).

1.5.2. Copper

Copper is a redox active metal that can enhance the production of ROS, which subsequently can damage most biomolecules (Denoyer et al., 2015). Oxidative stress and chronic inflammation are intrinsically linked to malignant transformation of cells (Denoyer et al.,2015). Therefore, it has been proposed that elevated copper in tissues or serum may be a risk factor for carcinogenesis (Denoyer et al., 2015). Copper homeostasis is normally maintained between the uptake (predominantly by copper transporter 1(CTR1)), the intracellular system responsible for its distribution to target proteins (through glutathione and the metallochaperones), and the efflux transporters, Cu-activated ATPases (Clifford et al., 2016). Internalization of CTR1 in the presence of excess extracellular copper is reversible and CTR1 returns to the plasma membrane when extracellular copper levels are reduced (Clifford et al., 2016). This regulatory endocytosis serves to protect the cell against excessive Cu accumulation by lowering the plasma membrane content of CTR1 and hence Cu entry (Clifford et al., 2016).

High serum copper concentrations are associated with a variety of cancers including lymphoma, reticulum cell sarcoma, bronchogenic and laryngeal squamous cell carcinomas, cervical, breast, stomach and lung cancers (Denoyer et al., 2015).

Exposure of wild type mice to 20 mM copper ($CuSO_4$) in drinking water for up to 2 years did not increase the incidence of cancer, suggesting that copper is not carcinogenic (Denoyer et al., 2015). Nevertheless, no clear association between copper level and cancer incidence has been found to corroborate this hypothesis (Denoyer et al., 2015).

1.6. Relevance of iron and copper in cancer

1.6.1. Iron

Several studies have indeed shown a correlation between the incidence and mortality of cancer and systemic iron levels, as measured by transferrin saturation or serum ferritin, this correlation is generally weak, has shown conflicting outcomes and is often confounded by the presence of other patholo-

gy (Crielaard et al., 2017). However, there is clear evidence to indicate that deregulated iron homeostasis on a local (microenvironment and/or cellular) level is associated with tumor progression (Crielaard et al., 2017). In context of tumor progression, one can envision that proliferating cancer cells, which require substantial amount of iron to support their grown will attempt to increase their iron uptake (Crielaard et al., 2017). Proliferating cancer cells can also increase iron availability by modulating iron storage capacity by producing ferritin and by limiting their iron export by downregulation of ferroportin (Crielaard et al., 2017).

A connection between iron and cancer growth has been demonstrated in several forms of cancers with breast cancer, prostate cancer and glioblastoma, as establish in examples, it seems reasonable that many, but not all cancer types demonstrate a similar iron dependency (Crielaard et al., 2017).

1.6.2. Copper

The involvement of copper in cancer has been studied for decades and there have been numerous reports on copper levels being aberrant in cancerous tissues of tumor-bearing mice and in cancer patients (Denoyer et al., 2015). Copper had a little impact on the proliferation of both human fibroblasts and arterial smooth muscle cells (Denoyer et al., 2015).

Angiogenesis involves the migration, proliferation, and differentiation of endothelial cells to form new blood vessels. This process is controlled by angiogenic simulating factors (e.g., angiogenin, vascular endothelial grown factor (VEGF), basic fibroblast growth (bFGF)) as well as inhibitors (e.g., angiostatin and endostatin) (Denoyer et al., 2015). Copper can directly bind the angiogenic growth factor angiogenin and enhance its affinity for endothelial cells and also regulate the secretion of angiogenic molecules and copper can be required for the expression of certain angiogenic factors (Denoyer et al., 2015).

An obvious role for copper in metastasis is through regulating angiogenesis, which is fundamental process required for metastatic potential. However, there is growing evidence that copper also directly influences the ability of cancerous cells to invade and metastasize (Denoyer et al., 2015).

1.7. Motivation

As mentioned above, some hydrophilic biopolymers, such as polysaccharides, have gelling ability, having as examples alginate, curdlan, colanic acid and gellan gum (Freitas et al., 2011; Nwodo et al., 2012). The physicochemical properties of biopolymers (e.g., molecular dimensions, surface activity, polarity and thermal stability) and environmental conditions (e.g., temperature, ionic strength, pressure/shear, pH and water activity) influence the different type of gels structures (Nazir et al., 2017). So,

the objective of this thesis was to produce FucoPol, a fucose-containing exopolysaccharide secreted by *Enterobacter* A47, and testing its the gelling capacity using mono-, di- and trivalent cations through ionotropic gelation. Then, the stability of FucoPol beads in different biological media was evaluated. Additionally, the cytotoxicity of FucoPol and FucoPol beads was assessed.

2. Materials and Methods

2.1. FucoPol Production

2.1.1. Inoculum preparation

Enterobacter A47 (DSM 23139) was cryopreserved at -80 ºC in 20 % (v/v) glycerol. Reactivation from the stock culture was performed by plating in Chromagar (CHROMagar™ Orientation) and incubation during 24 h at 30 ºC, to obtain isolated colonies.

The pre-inoculum was prepared by inoculating a single colony, grown on the Chromagar plate, in 50 mL Luria Bertani (LB) medium (peptone, 10 g L^{-1}; yeast extract, 5 g L^{-1}; sodium chloride, 10 g L^{-1}; pH 7.0). The pre-inoculum was incubated in an orbital shaker, at 30 ºC and 200 rpm, during 24 h.

After that, the inoculum for the bioreactor assay was obtained by transferring 20 mL of the LB grown culture into 200 mL Medium E*, supplemented with glycerol (200 g L^{-1}; Scharlau, 86-88% w/w) and incubated (30 ºC, 200 rpm), during 72 h. Medium E* had the following composition: K_2HPO_4, 5.8 g L^{-1}; KH_2PO_4, 3.7 g L^{-1}; $(NH_4)_2HPO_4$, 3.3 g L^{-1} (pH 7.0); and was supplemented with commercial glycerol (Scharlau, 86-88%), 1 mL of mineral solution and 10 mL of a 100 mM $MgSO_4$ solution. The mineral solution had the following composition (per liter of 1 N HCl): $FeSO_4 \cdot 7H_2O$, 2.78 g; $MnCl_2 \cdot 4H_2O$, 1.98 g; $CoSO_4 \cdot 7H_2O$, 2.81 g; $CaCl_2 \cdot 2H_2O$, 1.67 g; $CuCl_2 \cdot 2H_2O$, 0.17 g; $ZnSO_4 \cdot 7H_2O$, 0.29 g).

All media were sterilized by autoclaving (Uniclave 77, Portugal) at 121 ºC during 20 min.

All the steps involved in the handling of the bacterial strain and media were performed under aseptic conditions, in a laminar flow chamber (Heraeus SB 48, Germany).

2.1.2. Bioreactor assay

FucoPol production was performed in a 2 L bioreactor (BioStat B-plus, Sartorius), using 1.7 L of Medium E*, supplemented with 40 g L^{-1} glycerol. The bioreactor was inoculated with 200 mL of inoculum and was operated with controlled pH (7.0 ±0.02) and temperature (30 ± 0.1 ºC).

The bioreactor was operated in batch mode in the first day of cultivation. After that, it was maintained in fed-batch mode, by supplying a feeding solution (Medium E*, with a glycerol concentration of 200 g L^{-1}) at a constant rate of 5 mL h^{-1}. The aeration rate was maintained constant at 0.42 SLPM (standard liter per minute), and the dissolved oxygen (DO) concentration was maintained at 10 % of the

air saturation during the fed-batch assay by automatic adjustment of the stirrer speed (300-800 rpm) by two six-blade impellers.

Culture broth samples were periodically recovered from the bioreactor for measurement of the apparent viscosity and quantification of cell dry weight, polymer and nutrients. The samples were centrifuged (8000 g, 10 min; 20 ºC) for cell separation. The cell-free supernatant was stored at -20 ºC for determination of glycerol and ammonium concentration, as well as quantification of FucoPol.

2.1.3. Analytical techniques

2.1.3.1. Cell growth

Cell growth was monitored by measuring the optical density at 450 nm (OD450) (VWR V-1200 spectrophotometer) of the samples collected during the cultivation run. The cell dry weight (CDW) was estimated as described by Antunes et al. (2017) , considering that one unit of OD450 is equivalent to a CDW of 0.26 g L^{-1}.

2.1.3.2. Apparent viscosity

The viscosity of culture broth samples was measured at different shear rates in the range of 0.01 s^{-1} to 1.67 s^{-1}, using a digital viscometer (FungiLab Alpha Series, Spain).

2.1.3.3. Determination of glycerol concentration

The concentration of carbon source (glycerol) in the cell-free supernatant samples was determined by high-performance liquid chromatography (HPLC) with a VARIAN Metacarb 87H column. The analysis was performed at 50 ºC with sulphuric acid (0.01 N H_2SO_4) as eluent, at a flow rate of 0.6 mL min^{-1}.

Glycerol (Scharlau, 86-88% w/w) was used as standard at concentrations between 0.051 g L^{-1} and 1.638 g L^{-1}.

2.1.4. Determination of ammonium concentration

Ammonia concentration in the cell-free supernatant were determined by colorimetry using a flow segmented analyser (Skalar 5100, Skalar Analytical, The Netherlands).

Standard solutions of ammonium chloride (NH_4Cl) were also prepared (4 – 20 ppm). This analysis was performed in duplicate.

2.1.5. Polymer quantification

The cell-free supernatant samples were dialyzed with a 12,000 MWCO membrane against deionized water at constant stirring and temperature (25 ºC) during 48 h. The water was changed often and the conductivity was measured during the dialysis process, until it reached a value below 10 µS cm^{-1}.

Sodium azide (50 mg L^{-1}) was added to the dialysis water to prevent microbial degradation of the polysaccharide that could occur during the dialysis process.

The dialyzed samples were lyophilized and weighed to determine the polymer concentration during the cultivation run.

2.2. FucoPol extraction and characterization

2.2.1. FucoPol extraction and purification

The cell-free supernatant obtained by centrifugation of the culture broth was microfiltrated (Sartocon® Hydrosart® Cassette; 0.2 µm) to eliminate low molecular weight impurities (proteins, ions, etc.) present in the culture broth.

Ultrafiltration performed in a Sartocon® Slice 200 Hydrosart® Cassette (100 µm) was done to concentrate the polymer solution, which was lyophilized.

2.2.2. FucoPol characterization

2.2.2.1. Sugar composition

In order to analyze EPS composition in sugar monomers and acyl groups, EPS dried samples (≈ 5 mg) were hydrolyzed with trifluoracetic acid (TFA) (0.1 mL TFA 99%) at 120 ºC through 2 hours.

The identification and quantification of sugar monomers presents in the hydrolysate was performed by liquid chromatography (HPLC) using a CarboPac PA10 column (Dionex) equipped with an amperometric detector as described by Freitas et al. (2014). The analysis was performed at 30 ºC with sodium hydroxide (NaOH 4 mM) as eluent, at a flow rate of 0.9 mL.min^{-1} (Torres et al., 2014). D-(+) Fucose (98%, Sigma), D-(+) glucose anhydrous (99%, Scharlau), D-(+)-Xylose (98%, Merck), D-(+)-Galactose (99%, Fluka), D-(+)-mannose (99% Fluka), L-rhamnose monohydrate (99%,

Fluka), D-glucoronic acid (98%, Alfa Aesan) and D-(+) galacturonic acid monohydrate (97%, Fluka) were used as standards (1- 50 ppm).

2.2.2.2. Molecular mass distribution

Average and number molecular weights (M_w and M_n respectively), as well as the polydispersity index (M_n/M_w), were determined by size exclusion chromatography coupled with multi-angle light scattering (SEC-MALLS), as described by Torres et al. (2012). FucoPol solutions (2 g L^{-1}) were dissolved in 0.1 M Tris-HCl, NaCl (0.2 M), pH 8.1 buffer, which was also the Sec mobile phase. The SEC columns (PL aquagel-OH mixed 8 µm, 300 x 7.5 mm) were equilibrated during 24 hours before running the analysis at a flow rate of 1 mL. min^{-1} at room temperature. To follow the molecular mass distribution and the purity of polysaccharide, signals from MALLS were recorded in parallel and treated with Astra (V 4.73.04). A dn/dc of 0.190 mL g^{-1} was assumed to calculate the average molecular weight of FucoPol.

The analysis was performed by Dr. Christian Grandfils at the Interfacultary Research Centre of Biomaterials (CEIB), University of Liège, Belgium.

2.3. FucoPol gelation

2.3.1. Development of the gelation procedure

The ability of FucoPol to form gels in the presence of cations was tested based on procedures described in the literature for other polysaccharides (Ching, Bansal, and Bhandari, 2015; Running et al., 2012). Several mono-, di- and trivalent cations were selected for testing, namely, Na^+, K^+, Ca^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, and Fe^{3+} for testing. The salts used in the different tests were: $CuCl_2 \cdot 2H_2O$ (EMSURE ®), $CuSO_4 \cdot 5H_2O$ (PRS, Panreac), $ZnCl_2$ (Scharlau extrapure), $FeCl_3 \cdot 6H_2O$ (Sigma Aldrich), $FeCl_2 \cdot 4\ H_2O$ (Sigma Aldrich), $FeSO_4 \cdot 7H_2O$ (Sigma Aldrich), NaCl (Sigma Aldrich), KCl (Panreac), KNO_3 (Sigma Aldrich), $CaSO_4 \cdot 2H_2O$ (ACROS organics), $CaCl_2 \cdot 2H_2O$ (AppliChem, Panreac), $ZnSO_4 \cdot 7H_2O$ (Panreac), $MgSO_4 \cdot 7H_2O$ (Labchem), $MgCl_2 \cdot 6H_2O$ (Labchem), NaOH (eka), NaH_2PO_4 (Panreac).

FucoPol (1 g) was dissolved in 200 mL of deionized water under stirring (400 rpm, Heidolph MR 3001 K plate) for 2 h. This FucoPol solution was used for the different gelation tests.

Five procedures were tested, as follows:

Method 1 (Figure 2.1 - a) – An aqueous FucoPol solution with a concentration of 5 g L^{-1} (25 mL) was mixed with NaOH 2 M (5 mL). Then, the salt (50 mg) was added.

Method 2 (Figure 2.1 - b) – The aqueous salt solution (25 mL; 2 mg mL) was mixed with NaOH 2 M (5 mL). FucoPol (125 mg) was added to give a final polymer concentration of 5 g L^{-1}.

Method 3 (Figure 2.1 - c) – The salt in solid form (50 mg) was added to NaOH 2 M (1 mL) and the resulting mixture was mixed with the 5 g L^{-1} FucoPol solution (4 mL).

Method 4 (Figure 2.1 - d) – The salt in solid form (50 mg) was added to the 5 g L^{-1} FucoPol solution (4 mL) and mixed until complete solubilization of the salt. Then, NaOH 2 M (1 mL) was added.

Method 5 (Figure 2.1 - e) – The salt (50 mg) was first dissolved in deionized water (1 mL) and the resulting solution was mixed with the 5 g L^{-1} FucoPol solution (4 mL). After, NaOH 2 M (1 mL) was added.

All mixtures were homogenized by hand shaking and left standing for 30 minutes.

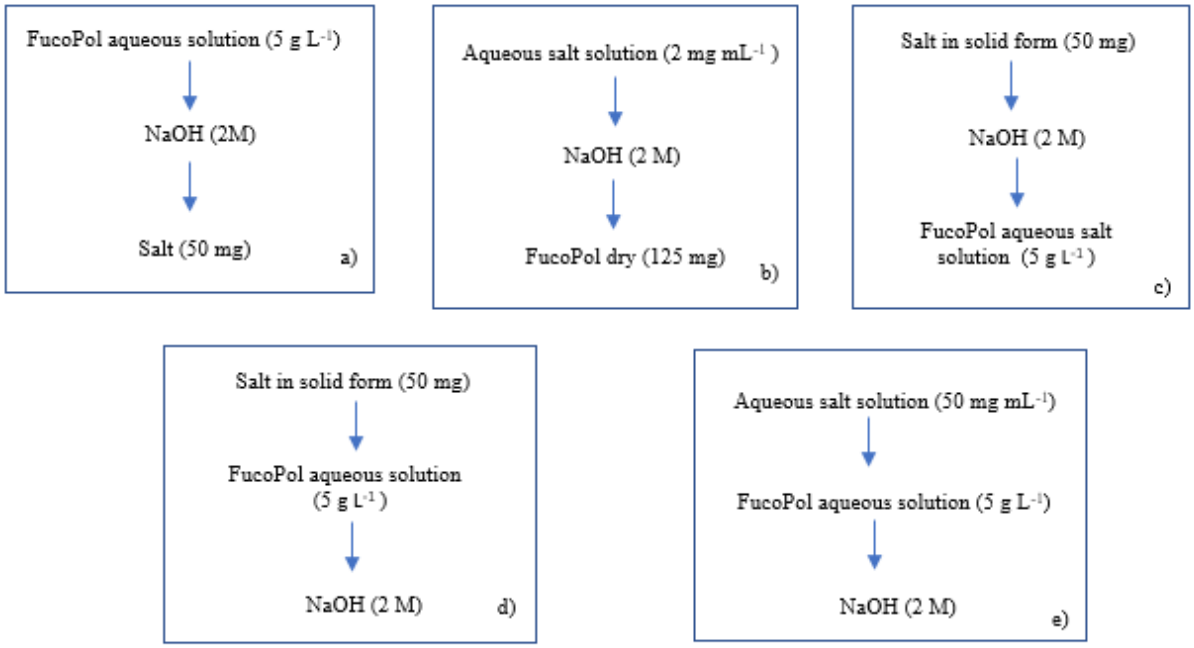


Figure 2.1 - Scheme of the addition order of components of methods tested for FucoPol gelation; a) Method 1; b) Method 2; c) Method 3; d) Method 4; e) Method 5.

2.3.1.1. Effect of NaOH concentration

The effect of NaOH concentration was evaluated with method 4 for the cations that gelled in the previous tests for this method, using different concentrations of NaOH (2 M or 4 M).

2.3.1.2. Effect of cation/anion in gel formation

Different salts ($CuSO_4$, $CuCl_2$, $CaCl_2$, $CaSO_4$, $MgSO_4$, $MgCl_2$, KCl, KNO_3, $ZnCl_2$, $ZnCl_2$ NaCl, NaH_2PO_4) of the same cation were tested to evaluate the effect of anion in FucoPol gelation through method 4, that was described in section 2.3.1.1.

2.3.2. Preparation of gel particles (beads)

FucoPol beads were prepared with different methods and using di- ($CuCl_2$, $ZnCl_2$, $CaCl_2$ and $FeSO_4$) and trivalent cations ($FeCl_3$).

FucoPol beads were prepared with a FucoPol solution of 10 g L^{-1} (1 % w/v), a concentration similar to that reported for alginate gelation (Burey et al., 2008).

2.3.2.1. Preparation of FucoPol beads with divalent cations

The FucoPol solution (10 g L^{-1}) was prepared by mixing with deionized water and stirring for 1 hour, at room temperature.

Then, 2 mL of the FucoPol solution (10 g L^{-1}) were added to 1 mL of aqueous divalent cation (50 mg mL^{-1}). This mixture was placed in a syringe (B|BRAUN®) and extruded through a needle (FINE-JECT ® 23 G – 0.6 x 25 mm) into a NaOH solution (30 mL; 0.2 – 2 M) under constant stirring (300 rpm; Heidolph MR 3001 K plate), at room temperature (25 ºC). The beads were kept under stirring for 20 min.). Afterwards, the beads were washed with deionized water to remove excess NaOH. The pH and conductivity were measured over the washes until pH was between 6.5 – 7.5 and the conductivity was below 10 µS cm^{-1}.

2.3.2.1.1. Preparation of $FeCl_3$/FucoPol beads

The FucoPol solution (10 g L^{-1}) was prepared as described above.

Then, 1 mL of FucoPol solution (10 g L^{-1}) was placed in a syringe (B|BRAUN®) and extruded through a needle (FINE-JECT ® 23 G – 0.6 x 25 mm) into an aqueous $FeCl_3$ solution (1,33 x 10^{-5} mM of Fe^{3+}) under constant stirring (300 rpm; Heidolph MR 3001 K plate), at room temperature (25 ºC).

The $FeCl_3$/FucoPol beads formed were kept under stirring (300 rpm) in the ferric chloride solution for 30 minutes and then the beads were washed with deionized water until removing the unreacted crosslinked agent (Fe^{3+}). The pH and conductivity were measured to ensure that the unreacted crosslinker has been removed where pH was between 6.5-7.5 and conductivity was below 10 µS cm^{-1}. After that, FucoPol beads were used for stability tests in different media and/or lyophilized at -109 ºC.

2.3.2.1.2. Preparation of $CuSO_4$/FucoPol beads

The FucoPol solution (10 g L^{-1}) was prepared as described above.

Then, 50 mg of $CuSO_4$ were dissolved in 1 mL of distilled water and were added 2 mL of FucoPol (10 g L^{-1}). This mixture was placed in a syringe (B|BRAUN®) and extrude through a needle (FINEJECT ® 23 G – 0.6 x 25 mm) into a NaOH solution (30 mL; 2 M) at stirring (300 rpm; Heidolph MR 3001 K plate). The $CuSO_4$/FucoPol beads were removed from NaOH solution 2 min. after being formed.

These beads were washed with deionized water until removing the NaOH and $CuSO_4$ in excess. The pH and conductivity were measured until pH were between 7.5-7.8 and conductivity was below 10 µS cm^{-1}. The beads were lyophilized with no success. The fresh FucoPol beads were used for stability tests in different media.

2.3.2.1.3. Preparation of $CuSO_4$/$FeCl_3$/FucoPol beads

$CuSO_4$/FucoPol beads, prepared as described in previous section, were transferred to an aqueous $FeCl_3$ solution (1,33 x 10^{-5} mM of Fe^{3+}) and kept stirred during 15 min (300 rpm; Heidolph MR 3001 K plate). After that, the beads were ready to be used for the stability tests in different media and/or to be lyophilized. The beads were not washed because they were very fragile, and the washes could destroy them.

2.3.3. Characterization of the beads

2.3.3.1. Cryo scanning electron microscopy (Cryo-SEM) of FucoPol beads

Cryo-SEM of $FeCl_3$/FucoPol beads and $CuSO_4$/$FeCl_3$/FucoPol beads were performed with Tabletop Microscope TM3030 (Hitachi in high technologies, America). Samples were observed at low temperature (-4 ºC) and a magnification in the range of 30 - 1200 x.

2.3.3.2. Metal content

For quantification of the metal content, 150 mg of $CuSO_4$/FucoPol, 100 mg of $FeCl_3$/FucoPol and 30 mg of $CuSO_4$/$FeCl_3$/FucoPol fresh beads were placed in a hydrolysis tube. Deionized water (5 mL) and 500 µL of TFA (Sigma Aldrich) were added to the mixture. The hydrolysis was performed at 120 ºC for 4 hours.

The samples were filtered (0.45 µm nylon; WHATMAN) and analyzed by ICP-AES (Horiba Jobin-Yvon, France, Ultima, model equipped with a 40,68 MHz RF generator, Czerny-Turner monochromator with 1,00 m (sequential), autosampler AS500 and CMA (Concomitant Metals Analyzer) in Analytical Services by Carla Rodrigues.

2.4. Metal release in different biological solutions

The metal release from the FucoPol beads was evaluated in different media:

- Simulated Gastric Fluid (SGF) without pepsin: prepared by dissolving 20 g of sodium chloride (NaCl – Sigma Aldrich) in 70 mL of hydrochloric acid (HCl -Sigma Aldrich) and completed with sufficient water to make 1000 mL. Then pH was adjusted to 1.2 ±0.1, either with 0.2 N NaOH or 0.2 N HCl (Lourenço et al., 2017).

- Simulated Intestinal Fluid (SIF) without pancreatin: prepared by dissolving 6.8 g of monobasic potassium phosphate (KH_2PO_4 – Sigma Aldrich) in 250 mL of deionized water, and adding 77 mL of 0.2 N sodium hydroxide (NaOH). Then, the solution was diluted with deionized water to a final volume of 1000 mL and the pH was adjusted to 6.8 ± 0.1 with either 0.2 N NaOH or 0.2 N HCl (Lourenço et al., 2017).

- Phosphate-buffered saline (PBS) was prepared by dissolving 80 g of NaCl, 2.0 g of KCl, 14 g of NaH_2PO_4 and 2.9 g of KH_2PO_4 in 800 mL of deionized water and pH was adjusted to 6.8 ± 0.1 with either 0.2 N NaOH or 0.2 N HCl. The final solution was diluted in distilled water (1:10).

- sodium chloride solution (NaCl) (0.9 %): prepared by dissolving 0.45 g of NaCl (Sigma Aldrich) in 50 mL of deionized water.

- Dulbecco's Modified Eagle Medium (DMEM) was purchased from Life Technologies and was supplemented with 10 % of FBS (fetal bovine serum) and 1 % antibiotic/antimycotic solution (Invitrogen Corp.).

To evaluate the metal release of FucoPol beads, a certain number of fresh beads were placed in different media (NaCl (0.9%), SGF, SIF, PBS and DMEM). For the metal release from the FucoPol beads in DMEM, different amounts of FucoPol beads (150 mg of $CuSO_4$/FucoPol, 100 mg of $FeCl_3$/FucoPol and 30 mg of $CuSO_4$/$FeCl_3$/FucoPol) were placed in 30 mL of DMEM. Samples were collected over time to measure the pH and conductivity, as well as to quantify by ICP analysis the metal released into solution.

2.5. Cytotoxicity of FucoPol and FucoPol beads in different cell lines

Different cell viability assays were used to evaluate the cytotoxicity of FucoPol and FucoPol beads. MTS assay is an indirect method used in cell viability determination, where MTS is bioreduced into formazan, whose quantity is proportional to number of viable cells in culture (Strober, 2001). Trypan Blue Method is a direct method for cell viability determination, where viable and unviable cells are distinguished through integrity of cellular membrane (Corporation 2006).

2.5.1. Cell Culture

Five adherent cell lines (colorectal carcinoma cell line (HCT116), an ovarian carcinoma cell line (A2780), a breast adenocarcinoma cell line (MCF7), lung adenocarcinoma (A549), normal human fibroblasts) and one suspension cell line (chronic myelogenous leukemia cell line - K562) were purchased from ATCC® (www.atcc.org).

HCT116 and A549 cell lines were grown in DMEM (Life Technologies, USA) supplemented with 10 % (v/v) FBS and 1 % (v/v) antibiotic/antimycotic solution (Invitrogen) and maintained at 37 ºC in a humidified atmosphere of 5 % (v/v) CO_2 (Sutradhar et al., 2016). MCF7 cell line and normal fibroblasts were grown in similar conditions, supplemented with 1% MEM non-essential amino acids (Invitrogen Corp.) (Sutradhar et al., 2016).

K562 cell line was cultured in DMEM supplemented with 10% of FBS at 37 ºC with 5% CO_2 and 99% relative humidity (Vinhas et al., 2017).

2.5.2. Cell viability – MTS assay

HCT116, A2780, A549, normal fibroblasts and MCF7 cells were seeded in 96 well plate at a density of 0.75 x 10^5 cells per well. Media was removed 24 hours after seeding and replaced with fresh media (DMEM) containing FucoPol (0-2000 µg mL^{-1}) or Doxorubicin (DOX, 0.4 µM).
After 48 hours of incubation in the presence or absence of FucoPol or DOX, cell viability was evaluated with CellTiter 96 ® AQueous Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, EUA) as have been described in Sutradhar et al., (2016). Briefly, cells harvested as mentioned in previous section were centrifuged at 500 *g* for 5 min, discarding the supernatant and resuspending in fresh medium. Cell density assessment was required in order to obtain 0.75 x 10^5 cells mL^{-1} in each well of 96-well plates (VWR, Radnor, Pennsylvania, USA). Applying trypan blue exclusion method, cell count was performed using a hemocytometer (Hirschmann, Eberstadt, Germany) loaded with a solution of cell suspension 1:10 and trypan blue solution 1:5 (Sigma, St. Louis, USA) diluted in fresh growth medium. Cells were observed using an Olympus CXX41inverted microscope, Tokyo, Japan. Trypan blue is a dye

selective to cells with loss of membrane permeability thus is used to exclude unviable cells from count. Final assessment of cell density (in cells mL^{-1}) is given by equation (eq. 1):

$$\frac{total\ cell\ count\ from\ 4\ quadrants}{4} \times 10 \text{ (Dilution factor)} \times 10^4\ (chamber\ volume) \quad \text{(eq.1)}$$

After a 24 h cell seeding at 37 ºC in a 5% CO_2 (v/v) and 99% (v/v) humidity atmosphere, a range of concentrations of each compound diluted in fresh medium, were added to the cells with a subsequently incubation period of 48h.

The CellTiter 96 ® AQueous Assay is composed of solutions of [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, MTS (inner salt) and phenazinemethosulfate (PMS), an electron coupling reagent (Corporation 2006). MTS is bioreduced by cells into formazan product. The conversion of MTS into an aqueous soluble compound (formazan) is accomplished by dehydrogenase enzymes found in metabolic active cells (Sutradhar et al. 2016). So, the quantity of formazan produced (measured by the amount of 490 nm absorbance) is directly proportional to number of living cells in culture (Corporation 2006). After an incubation period of approximately 45 minutes under 37 ºC with 5% CO_2 and 99% relative humidity, absorbance measurement at 490 nm of each well was determined with Tecan Infinite F200 Microplate Reader (Tecan, Männedorf, Switzerland).

Obtained data was normalized relative to control sampler to obtain cell viability for each FucoPol concentration using equation (eq.2).

$$\text{Cell Viability (\%)} = \frac{Sample\ absorbance\ (490\ nm)}{Control\ absorvance\ (49o\ nm)} \times 100 \qquad \text{(eq.2)}$$

2.5.3. Cellular viability – Trypan Blue Exclusion Method

Trypan blue is a dye selective to cells with loss of membrane permeability, giving a blue color to non-viable cells (Strober, 2001). K562 cells were seeded at a density of 2.5 x 10^4 cells per well (24-well plates) containing FucoPol beads (150 mg) or Imatinib (IM, 0.1 µM; positive control) or Dimethyl sulfoxide (DMSO) (control for Imatinib). After 3, 24 and 48 hours of incubation in the presence or absence of the FucoPol beads, IM and DMSO, cell viability was evaluated using the Trypan Blue Exclusion Method. This method relies on counting the number of viable cells (in a hemocytometer (Hirschmann, Eberstadt, Germany)) using an Olympus CXX41 inverted microscope after the incubation of each cell suspension (exposed or not to the presence of FucoPol beads, IM or DMSO) with Trypan Blue

(1:2).Viable cells maintain their cellular membrane integrity excluding the dye and dead cells lose their cellular integrity becoming blue (Vinhas et al., 2017).

The percentage of viable cells is given by the following equation (eq.3):

$$\text{Viability (\%)} = \frac{Number\ of\ viable\ cells}{Number\ of\ total\ cells} x\ 100 \qquad \text{(eq.3)}$$

2.5.4. Preparation of samples for Inductively coupled plasma – atomic emission spectroscopy (ICP-AES) analysis

Cells incubated with $CuSO_4$/FucoPol beads for 24 hours were centrifuged at 500 g for 5 minutes. After centrifugation, 1 mL of *aqua régia* was added to each pellet and 550 µL of *aqua régia* was added to each supernatant. The samples were digested for 48 hours and then were sent to be analyzed by ICP-AES as a service.

3. Results and discussion

3.1. FucoPol production and characterization

The bioreactor was operated in batch mode during the first day, and after that fed-batch mode was initiated by feeding the culture with a mineral solution supplement with glycerol (200 g L^{-1}) at a constant rate of 5 mL h^{-1}. Figure 3.1 presents the CDW, FucoPol production, glycerol and ammonia concentrations over cultivation time.

In the first day of cultivation, *Enterobacter* A47 grew at a specific grown rate of 0.28 h^{-1}, a value in accordance with the range reported in literature (0.26-0.32 h^{-1}) for cultivation of *Enterobacter* A47 in glycerol (Antunes et al., 2017). During the exponential growth phase of *Enterobacter* A47, the CDW reached a maximum of 7.61 g L^{-1} at 28.5 h of cultivation, a value within the range reported in literature (5.80–7.68 g L^{-1}) (Antunes et al., 2017).

The fed-batch phase was initiated at 8 h of cultivation, when the glycerol concentration in the medium was 41.85 g L^{-1}. After 28.5 h of cultivation, cell growth was suppressed by imposing nitrogen limiting conditions (<0.1 g NH_4^+ L^{-1}). The feeding solution containing 0.9 g NH_4^+ L^{-1} was used to feed the bioreactor at a constant flow, but the ammonium concentration in the cultivation broth stayed at a residual value (below the detection limit).

FucoPol production was initiated in the batch phase and was continued during the fed-batch phase, reaching the maximum of 7.74 g L^{-1} at the end of the cultivation run, which corresponds to the values reported in literature of FucoPol production using glycerol as carbon source (7.23 – 7.97 g L^{-1}) (Antunes et al., 2017; Torres et al., 2014).

The glycerol concentration remained practically constant from 28.5 h till 55.5 h of cultivation run, which means that the glycerol that was entering in the culture was totally consumed by *Enterobacter* A47. After 70.5 h of assay, glycerol concentration was maintained below 2.0 g L^{-1}, evidencing that the culture had increased the glycerol consumption rate. No cellular growth was observed in this phase, meaning that the glycerol that has been consumed during this period was used mostly for FucoPol production.

The overall volumetric productivity of FucoPol was 1.97 g L^{-1} day^{-1}, reaching the productivities values for FucoPol with glycerol as carbon source reported in literature (1.89-2.04 g L^{-1} day^{-1}) (Torres et al., 2014). The net yield of FucoPol on glycerol (0.085 g L^{-1}) was slightly below of the values reported in literature (0.10-0.17 g L^{-1}) (Torres et al., 2014).

The apparent viscosity of the broth increased from 1.1 mPa.s^{-1} to 10.87 mPa.s^{-1} (measured at 0.83 s^{-1}) from the beginning (0h) till 49.5 h of the cultivation run. At 73.5 h of cultivation run, the apparent viscosity of the broth was 1679.58 mPa s^{-1} (measured at 0.01 s^{-1}). During the 94.5 h of the assay, the viscosity of culture broth increased two orders of magnitude, concordant with the values presented in previous studies (Torres et al., 2011a; Torres et al., 2014).

The viscosity of microbial culture broths may be influenced by physical as well as biological parameters including the cultivation medium used, biomass concentration, the size of both cells and cell aggregates formed, morphological parameters and the products being secreted into solution (Alves et al., 2010). So, the viscosity increase may be related to the accumulation of FucoPol in the broth, as well as changes of the biopolymer´s composition and molecular weight (Alves et al., 2010; Torres et al., 2011a). Furthermore, the establishment of novel or stronger interactions among polymer molecules or between polymer chains and other components of the broth can contribute for viscosity increase (Torres et al., 2011).

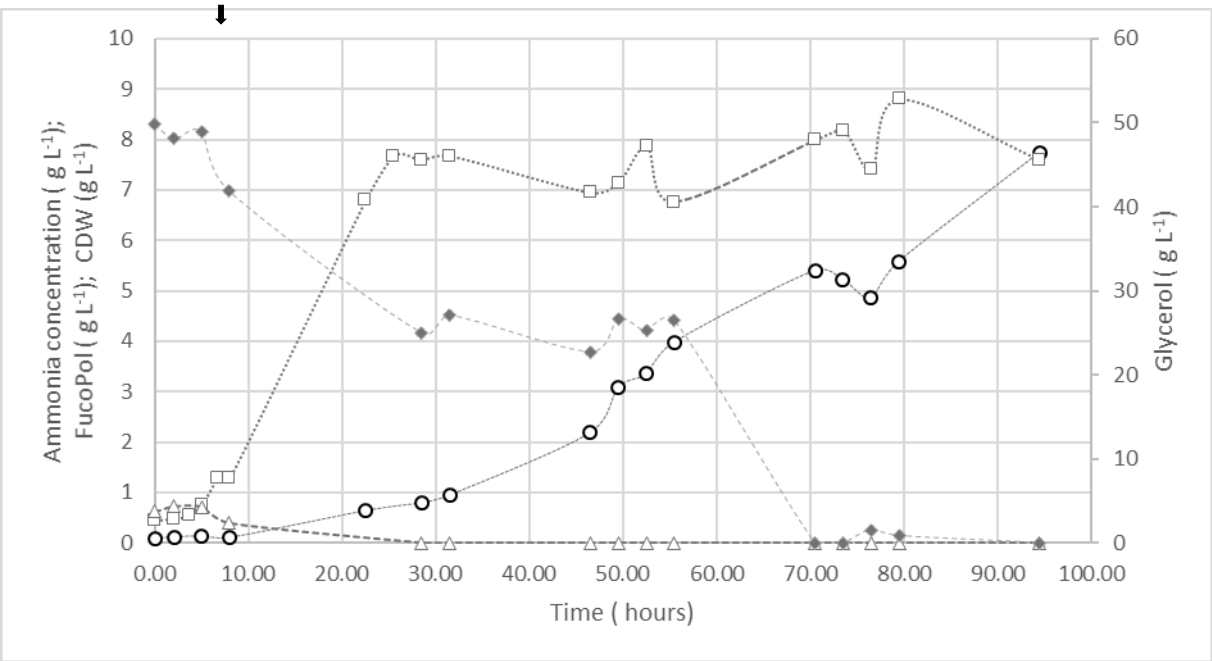


Figure 3.1 –Cultivation profile of *Enterobacter* A47 using glycerol as carbon source (ammonia (Δ), CDW (□), glycerol (♦) and FucoPol (○)). The fed-batch phase was initiated at 8 hours of cultivation (↑).

The chemical composition analysis of the EPS shown that it had the typical FucoPol composition, namely, it was composed of fucose (32-36 mol %), galactose (22-26 mol %), glucose (28 – 37 mol %), glucuronic acid (9-10 mol %) and acyl groups, particularly, succinyl (2 – 3 wt.%), pyruvyl (13-14 wt.%) and acetyl (3-5 wt.%) (Torres et al., 2015). The total inorganic content was 7.92 wt.%, similar to inorganic content of FucoPol after dialysis produced in Alves et al. (2010).

The average molecular weight (M_w) of FucoPol was 4.22 x 10^6 Da, a value in accordance with the range reported in literature for FucoPol (4.19×10^6 - 5.8×10^6 Da) (Torres et al., 2014).

The polydispersity index (PDI) was 1.71, which means that the FucoPol synthetized was homogeneous, similarly to the PDI values reported by Torres et al. (2014) (1.14-1.98). The PDI reflects the degree of heterogeneity of polymer's chain lengths (Torres et al., 2014).

3.2. Preparation of FucoPol gels

3.2.1. Development of the gelation procedure

Several methods to obtain FucoPol gels were tested with different salts and the overall results are presented in Table 3.

Table 3 - Results obtained with the different tested methods (gel formation, (+); no alteration, (-); precipitation, pp; not tested, (■).

Salt	Method 1	Method 2	Method 3	Method 4	Method 5
NaCl	(-)	■	■	(-)	■
NaH_2PO_4	(-)	■	■	(-)	■
KCl	(-)	■	■	(-)	■
KNO_3	■	■	■	(-)	■
$CaCl_2$	pp	(-)	pp	pp	■
$CaSO_4$	■	■	■	pp	■
$MgCl_2$	■	■	■	(+)	■
$MgSO_4$	pp	pp	■	(+)	■
$CuCl_2$	■	■	■	(+)	(+)
$CuSO_4$	pp	pp	pp	(+)	(+)
$ZnCl_2$	■	■	■	(+)	(+)
$ZnSO_4$	■	■	■	(-)	(+)
$FeCl_2$	■	■	■	(+)	(+)
$FeSO_4$	■	■	■	(+)	(+)
$FeCl_3$	■	■	■	(+)	(+)

For the monovalent cations (Na^+, K^+) tested, there were no visible changes observed (i.e., no gel or precipitate were formed) for the procedures of Methods 1 and 4. These results suggested that FucoPol was not able to form gels in the presence of either Na^+ or K^+, under the tested conditions. Hence, these cations were not further tested with the other methods.

Gelation with monovalent cations did not occur for this polymer probably due to insufficient screening of the electrostatic repulsion between carboxylate groups (COO^-) on FucoPol chains, contrary of what happens with gellan gum (Singh & Kim, 2005).

For the divalent cations Ca^{2+}, Mg^{2+} and Cu^{2+}, no gel formation occurred for Methods 1, 2 and 3, and precipitates were formed. For $CuSO_4$, there was a change of the solution's color from blue to purple and the formation of a blue precipitate, while for $CaCl_2$ (Method 3) and $MgSO_4$ (Method 2) white precipitates were formed, with no alteration of the solutions' color.

Method 4 yielded the most interesting results since gels were obtained with several of the tested cations (Figure 2.1). It consisted in adding the reagents at the same concentration but in a different order than that of Methods 1, 2 and 3. The results obtained show that the order of reagents' addition is crucial to the formation of FucoPol gels. With the exceptions of Ca^{2+} salts that still resulted in the formation of white precipitates similarly to the ones obtained with Methods 1-3, and $ZnSO_4$ that did not gel, all other salts resulted in the formation of FucoPol gels.

After a few hours over the gels' formation, the darkening of FucoPol gels formed in the presence of Cu^{2+} was observed, which was probably due to an incomplete dissolution of the salt in the solution. The unreacted copper present in the mixture may have reacted with NaOH and caused the darkening of the gels. This problem was solved by first dissolving the salt in deionized water prior to its mixing with FucoPol solution, as described in Method 5 and can be seen in Figure 3.2. The gels prepared with copper salts maintained their stability and color during a day, after which they were broken.

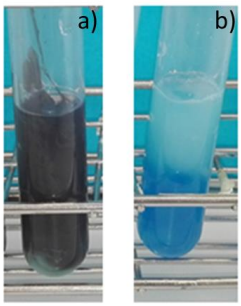


Figure 3.2 – FucoPol gels prepared with $CuCl_2$ (20 mg mL^{-1}) and NaOH (2 M) using method 4 (a) and method 5 (b) that evidencing that excess of copper is reacting with NaOH, which not happens when is prepared an aqueous salt solution.

These results show that the ability of FucoPol to form gels with divalent salts is selective, since of the salts tested, FucoPol only forms gels with copper, zinc and magnesium salts.

Other polymers that have similar properties as reported in Table 1, such as gellan gum form gel in the presence of divalent cations, such as calcium, due to the chemical bond between divalent cations and carboxylate groups of glucuronic acid residues that constitute gellan chains, in addition to the screening effect (Singh & Kim, 2005).

The gels prepared with zinc or magnesium salts were quite unstable and after an hour, a precipitate was formed.

Iron salts ($FeSO_4$, $FeCl_2$, $FeCl_3$) were tested for FucoPol gelation with method 5. The iron divalent salts formed gels with FucoPol, as can be observed in Figure 3.3. Interestingly, FucoPol gelled in the presence of the trivalent iron salt without NaOH, similarly of what happens with λ-carrageenan, where iron works as a gel crosslinker (Running et al., 2012); or what happens to alginate, where iron is able to interact with three carboxyl groups of this biopolymer at same time, forming a three-dimensional bonding structure that results in a more compact gel network (Ching et al., 2015). This hypothesis can be confirmed by analyzing these gels by Fourier-transform infrared spectroscopy (FTIR).

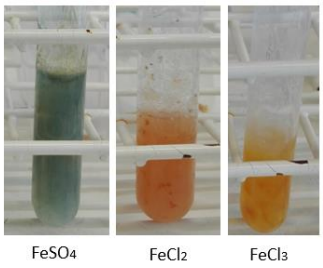


Figure 3.3 – FucoPol gels prepared with different iron salts (20 mg mL^{-1}) using method 5.

FucoPol has glucuronic acid as one of its minor sugar component (9-10 % mol) and acyl groups (succinyl (2 – 3 wt.%) and pyruvyl (13-14 wt.%)) which confers a polyelectrolyte character. This monomer and acyl groups may be responsible for FucoPol gelation, since that process occurs by interaction between the glucuronic acid residues of polymer and cations (Burey et al., 2008).

In general, method 5 allowed the total dissolution of salt in FucoPol solution, which lead to a more homogeneous gel for $CuSO_4$ and $FeCl_3$.

3.2.1.1. Effect of NaOH concentration

As previously mentioned, FucoPol gelled in the presence of copper, magnesium and zinc salts with NaOH (2 M). To evaluate the effect of NaOH concentration on gel formation, two concentrations of NaOH (2 M and 4 M) were used in method 4. Gels were formed with both concentrations of NaOH using different salts, but the stronger gels were obtained with NaOH 2 M. Figure 3.4 shows that FucoPol gels obtained with different salts of which $CuSO_4$ and $FeCl_3$ FucoPol gels seem to be the strongest ones.

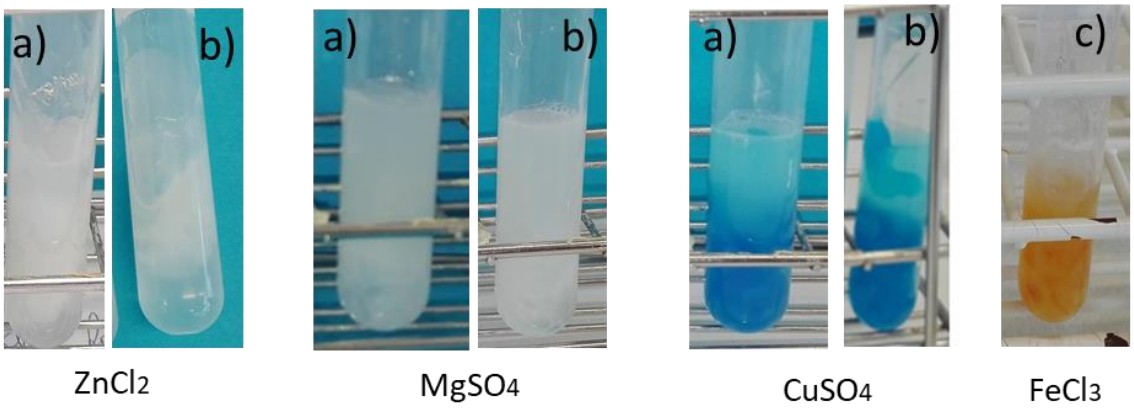


Figure 3.4 – FucoPol gels prepared with different salts (mentioned below each gel tube) and NaOH (a) 2 M; b) 4 M; c) 0 M) using method 4.

3.2.1.2. Effect of cation/anion concentration

The effect of cation concentration was evaluated with method 4 for the salts presented in Table 3 using 2, 10 and 20 mg mL^{-1} of salt.

The different monovalent salts (potassium and sodium) had similar effects independently from the anion used.

Relatively to the divalent salts tested in FucoPol gelation, zinc salt ($ZnCl_2$), more specifically, the anion Cl^- apparently prevented the formation of a strong gel, once that FucoPol form gel and a few minutes later gel entered in syneresis (process in which a gel contracts on standing and exudes liquid). FucoPol gelation has not occurred in the presence of calcium salts, independently of the anion used. For the other divalent salts (copper, magnesium, iron), the tested concentrations contributed to decrease of electrostatic repulsion between FucoPol chains and promote gelation at room temperature (25 ºC), that showed to be stronger and form gel in more quantity as the salt concentration increased, similarly to what happens for gellan gum (Singh & Kim, 2005). Gelation of gellan gum in the case of divalent cations, occurs via a chemical bonding between divalent cations ($CaCl_2$ and $MgCl_2$) and two COO^- groups belonging to glucuronic acid molecules in the gellan chains, in addition to the screening effect.

Summarizing, the tests performed to evaluate the FucoPol gelation showed that no gels were formed in the presence of the monovalent cations potassium and sodium (at least in the tested concentrations); FucoPol was able to form gels with divalent cations such as copper and iron salts, although weaker gels were formed with zinc and magnesium salts ($MgCl_2$, $ZnSO_4$); trivalent salt tested formed the strongest and more stable FucoPol gels. Hence, these salts ($CaCl_2$, $CuCl_2$, $CuSO_4$, $FeCl_3$, $FeSO_4$ and $ZnCl_2$) were selected to test the ability of FucoPol to form beads.

3.3. FucoPol gel beads

3.3.1. Preparation of FucoPol gel beads

FucoPol gel beads were prepared with a similar methodology used for FucoPol gels. A mixture of aqueous salt solution and FucoPol solution (10 g L^{-1}) was prepared and placed into a syringe and extruded through a needle into a NaOH 2 M solution under constant stirring (300 rpm). Different salts ($CaCl_2$, $CuCl_2$, $FeCl_3$, $FeSO_4$ and $ZnCl_2$) with different concentrations (2, 10, 20 mg mL^{-1}) were tested to evaluate the ability of FucoPol to form gel beads.

FucoPol gel beads formed with $CaCl_2$ and $ZnCl_2$ but disintegrated within a few minutes, for all the concentrations tested. This result was similar to the test performed to obtain gels with such salts, where FucoPol and these salts apparently established a weak linkage which ended up dissipating after a few minutes.

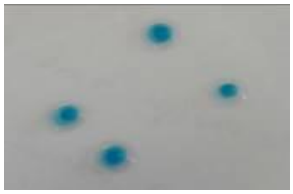
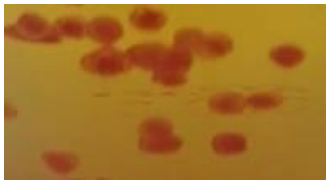
FucoPol formed beads with the salts $CuCl_2$, $FeCl_3$ and $FeSO_4$, which were stronger for higher salt concentrations (20 mg mL^{-1}).

Different needles were used to evaluate the ones that would yield the beads with the best diameter and shape (sphericity). The needles used in the tests were from FINE-JECT with 15 G x 2" (1.8 x 50 mm), 20 G x 1 $^{1/2}$ (0.9 x 40 mm), 21 G x 1" (0.8 x 25 mm) and 23 G (0.6 x 25 mm). The needle that showed better results for this test was the blue needle (23 G), showing beads with good sphericity and diameter, so this needle was used in the next assays.

Different NaOH concentrations (0 -2 M) were tested for these salts to evaluate their influence in the density and strength of FucoPol gel beads. FucoPol formed beads with copper sulfate and iron chloride with different characteristics presented in Table 4, which summarizes the main characteristics of FucoPol beads, including shape, color, size, concentration in cation, mass and density.

The beads formed with $CuCl_2$ (20 mg mL^{-1}) lasted longer when NaOH (2 M) was used, showing less sphericity and density when NaOH concentration was decreased.

Table 4 – Main characteristics of FucoPol gel beads prepared with $FeCl_3$ or/and $CuSO_4$

	$FeCl_3$/FucoPol beads	**$CuSO_4$ FucoPol beads**	**$CuSO_4$/$FeCl_3$ FucoPol beads**
Shape	Spheres	Spheres	Flattened
Color	Yellow	Blue	Dark orange
Size (cm)	0.12	0.15	0.10
Concentration of Fe and/or Cu (mg L^{-1})	2.25 (Fe)	8.73 (Fe)	6.73 (Fe) 0.19 (Cu)
Mass per bead (mg)	12.5	50	4.3
Percentage of water (%) [1]	98.88	99.63	97.38
Density[2]	Higher than water	Higher than water	Higher than water
Image			

[1] The percentage of water was obtained through weight of beads before and after being placed in drying oven for 24 h.

[2] Density of beads was observed placing beads in water.

The beads formed with $FeSO_4$ were more stable using NaOH (2 M), but only least with a good aspect (sphericity and color) for 8 hours, after that period an orange cover that became darker with time, which probably was result from iron oxidation, as can be observed in Figure 3.5.

Figure 3.5 – $FeSO_4$/FucoPol beads prepared with NaOH (2 M) minutes after preparation (left) and 8 hours after preparation (right)

In the case of $FeCl_3$, the test was done differently. In this case, a syringe with FucoPol solution (10 g L^{-1}) was extrude through a 0.6 x 25 mm syringe into a $FeCl_3$ aqueous solution under stirring (300 rpm). Different concentrations of this salt (0.22, 0.41, 0.83, 1.67, 3.33, 6.90, 13.83, and 27.70 mg mL^{-1}) were tested to evaluate the effect of salt concentration in the strength of FucoPol gel beads (Figure 3.6). There was an evident effect of salt concentration on the strength of the gel beads: as the concentration of salt decreased from 3.33 to 0.22 mg mL^{-1}, more fragile beads were formed, beads became more flattened and the density of beads decreased, once that beads do not float in water. The concentration of salt selected to prepare the beads was 3.33 mg mL^{-1} because FucoPol beads present spherical form, small diameter and least stable for at least 15 days.

$FeCl_3$/FucoPol beads were formed without NaOH, so in this case the salt has probably worked as a crosslinker, similarly of what happens to gels of other polymers such as λ-carrageenan and alginate (Running et al., 2012; Ching et al., 2015). The gel formed with λ-carrageenan is thermally stable and trivalent cations seem to be more suitable than monovalent ions for balancing the three negative sulfate charges, per disaccharide repeat unit, and promote cooperative inter-chain interactions. In case of alginate, interactions between their carboxyl groups and trivalent cations establish a three-dimensional bonding structure that results in a more compact gel network (Ching et al., 2015).

Figure 3.6 – FucoPol beads prepared with different concentrations of $FeCl_3$ ((a) 13.83 mg mL^{-1}, (b) 6.90 mg mL^{-1}, (c) 3.33 mg mL^{-1}, (d) 0.41 mg mL^{-1}).

Generally, as the NaOH concentration was decreasing in the preparation of FucoPol gel beads, the more unstable were the beads, as can be seen in Figure 3.7., which can indicate that NaOH concentration influences the formation of gel beads.

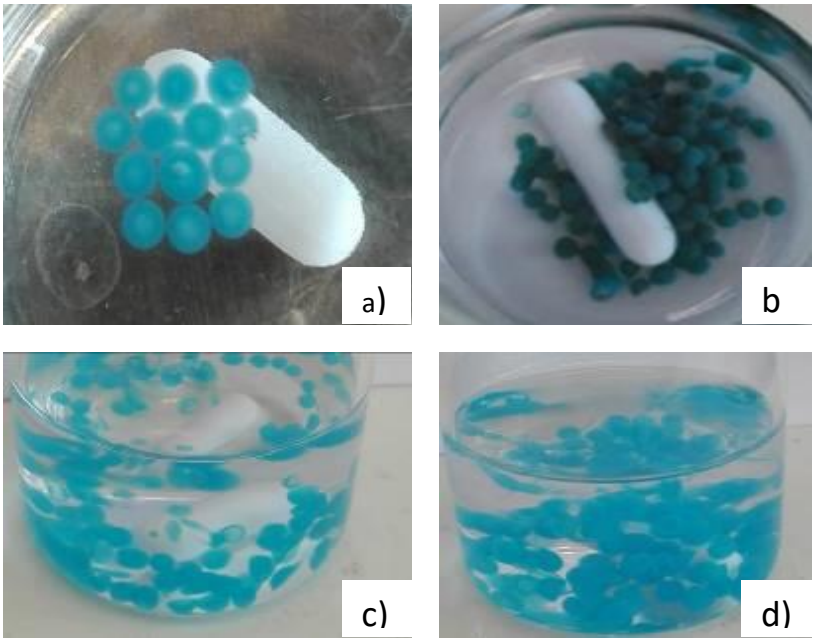


Figure 3.7 – $CuSO_4$/FucoPol beads prepared with different NaOH concentrations (a) 2 M; b) 1 M; c) 0.5 M; d) 0.2 M.

The results from the tests performed with different salts and NaOH concentrations for the formation of FucoPol gel beads showed the need to adequate the method for each salt tested to obtain gel beads with good stability, color and shape.

Three different types of FucoPol beads were prepared using copper salt ($CuSO_4$), iron salt ($FeCl_3$) and the mixture of these salts.

The pH and conductivity were measured to evaluate the removing unreacted reagents for different gel beads, as can be seen in Figure 3.8 and Figure 3.9.

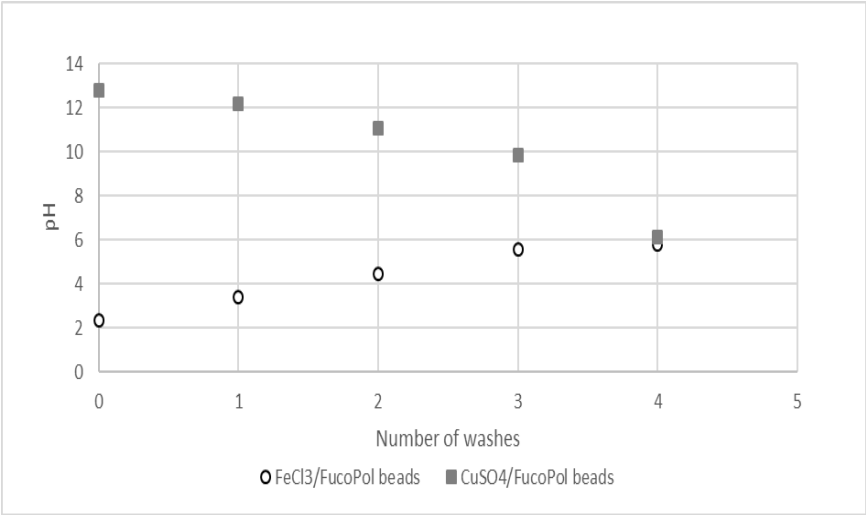


Figure 3.8 – Number of FucoPol beads ($FeCl_3$/FucoPol beads (○) and $CuSO_4$/FucoPol beads (■)) washes as function of pH

From the results presented in Figure 3.8, it is clear that the pH where the gel beads are formed is different derived from the different solutions used, once that $FeCl_3$ solution has an acidic pH (≈ 2.4) and NaOH solution where copper gel beads are formed has a basic pH (≈ 12.8).

After preparation of Fe/FucoPol beads, the initial solution ($FeCl_3$) was removed and replaced for deionized water (pH ≈ 5.5-6.0). Beads were periodically (10 min.) washed with deionized water, where pH and conductivity where measured. According to Figure 3.8, pH increases with 4 periodical washes, once that free ions from $FeCl_3$ solution that did not interacted with FucoPol establishing gel were being removed with increasing number washes.

The preparation solution (NaOH, 2 M) of $CuSO_4$/FucoPol beads was removed and replaced for deionized water (pH ≈ 13.0). Beads were periodically (10 min.) washed with deionized water, where pH and conductivity where measured. As can be observed in Figure 3.8, pH decreased with increasing number of washes, which demonstrate that ions from NaOH solution that did not interacted with Fuco-Pol establishing gel were being removed with increasing number of washes. In the end of washes, the beads have similar pH.

The conductivity measured in function of number of washes (Figure 3.9) shows that the measured parameter decreased over washes.

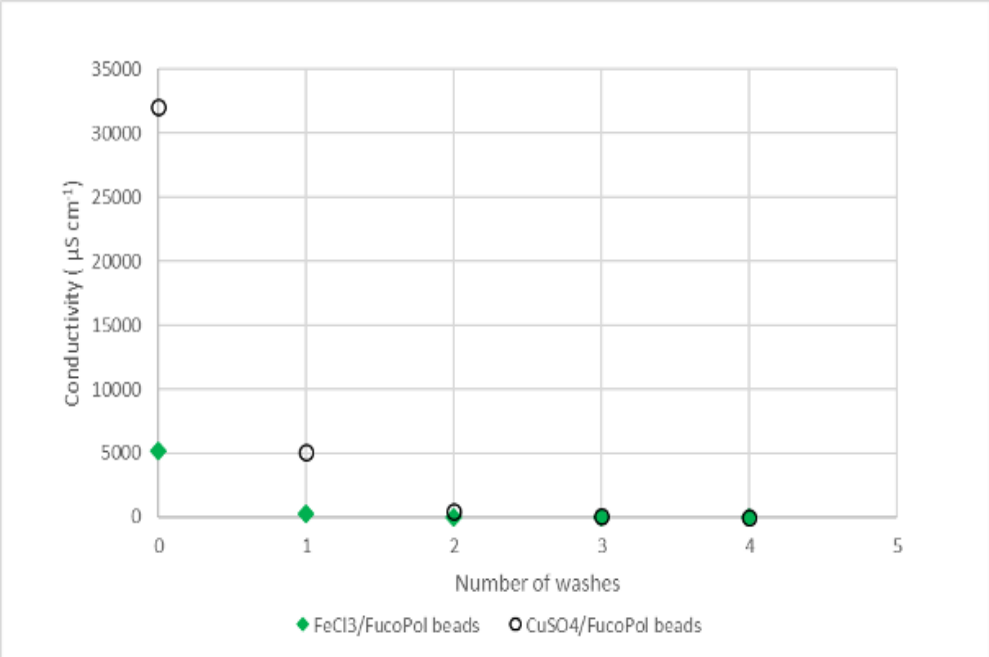


Figure 3.9 – Number of FucoPol beads ($FeCl_3$/FucoPol beads (○) and $CuSO_4$/FucoPol beads (♦)) washes as function of conductivity (µS cm^{-1}).

Conductivity of FucoPol beads preparation solution was measured initially and $FeCl_3$ aqueous solution showed a higher conductivity, probably due to their higher content of ions. As the FucoPol beads preparation solution has been removed and replaced for deionized water, there was a decline of conductivity in the first wash. After that, beads were being washed, were the unlinked cations/anions were being released for the washing solution.

In the end of washes, the conductivity of FucoPol beads was around 10 µS cm^{-1}.

3.3.2. Gel beads characterization

The FucoPol beads formed with iron (alone or with copper sulfate) were the most stable structures and were stable for at least 15 days (then beads start to disintegrate) with the same appearance in aqueous media, at room temperature (25 ºC). FucoPol beads formed with copper sulfate only lasted for 24 hours.

Cryo SEM photographs were used to analyze surface morphology and internal structure of the FucoPol gel beads. From photograph taken at $FeCl_3$/FucoPol beads (magnification of 40 x, Figure 3.10), it is possible to observe spherical beads with a smooth surface, but presenting some fractures. Photographs taken with other magnifications (250 x, 500 x and 1000 x) show that the beads presented different porosities.

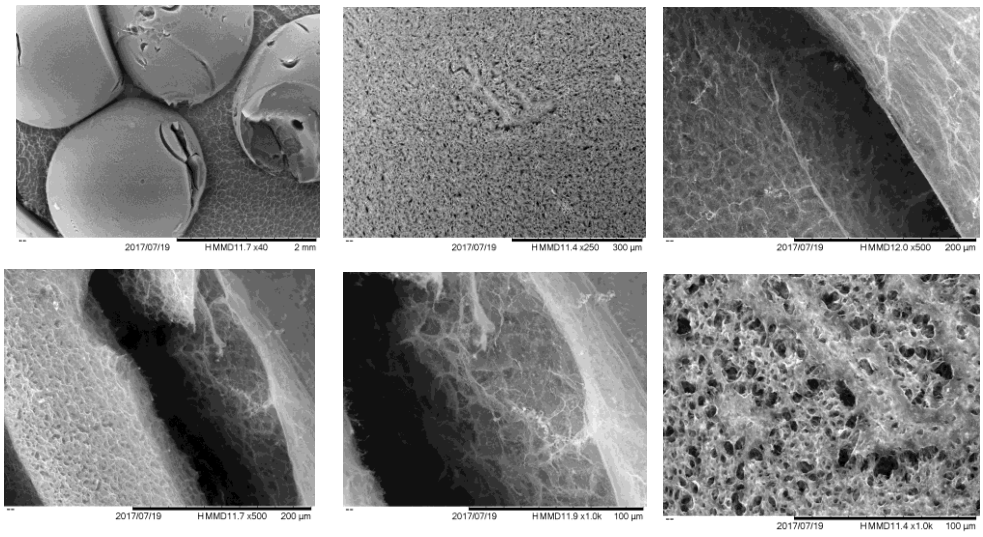


Figure 3.10 – Cryo SEM images of $FeCl_3$/FucoPol beads taken at – 4ºC using different magnifications (40, 250, 500 and 1000 x)

Cryo SEM photograph from $CuSO_4$/$FeCl_3$/FucoPol beads (magnification of 40 x, Figure 3.11) show that these beads have a different surface morphology from $FeCl_3$/FucoPol beads, showing a rough surface and present a flatten form. From photographs with other magnifications (30 x – 1000 x,

Figure 3.11), $CuSO_4$/$FeCl_3$/FucoPol beads present different structures, probably due to the different connections established between polymer and copper or between polymer and iron.

Other polymers such as gellan formed beads with ionotropic gelation method (Alves et al., 2016), similar of what happen for FucoPol. Sodium Alginate/ Sodium Carboxymethyl cellulose (CMC) blend hydrogel beads cross linked with Fe (III) were prepared by ionotropic gelation method (Swamy & Yun, 2015) are presented in Figure 3.12. In this case, beads have metformin encapsulated (a) and released (b). FucoPol beads from Figure 3.10 have a smooth surface than beads formed with alginate/CMC loaded with metformin (Figure 3.12, a). Relatively to FucoPol beads formed with two salts, they present a different morphology from beads presented in Figure 3.12, derived from the different interactions established between FucoPol, copper and iron.

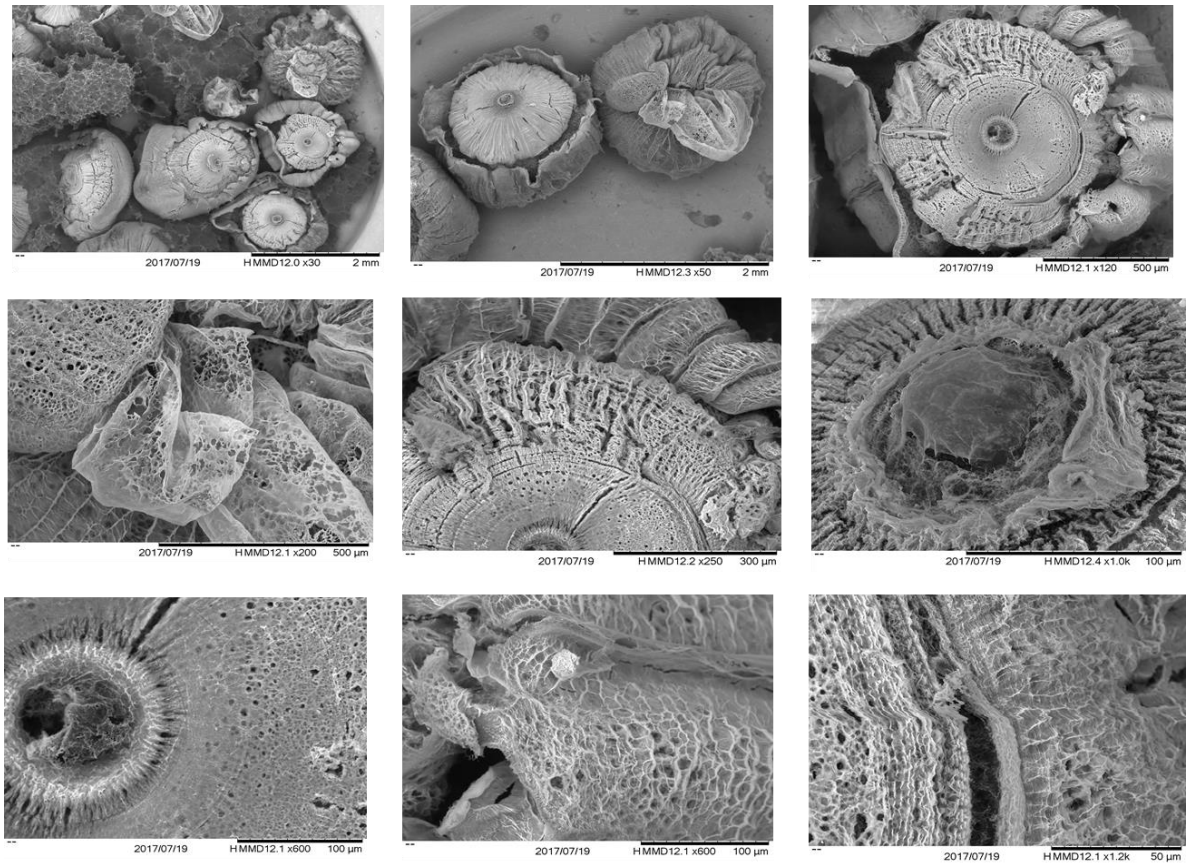


Figure 3.11 – Cryo SEM images of $FeCl_3$/$CuSO_4$/FucoPol beads at different magnifications (30, 50,120, 200, 250, 600, 1000 and 1200 x)

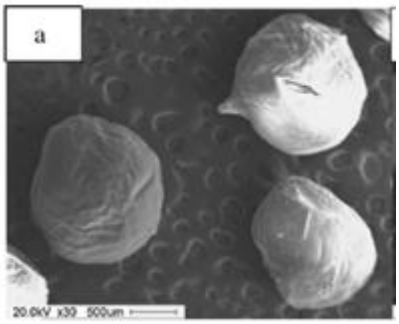


Figure 3.12 - SEM photograph of (a) Metformin hydrochloride-loaded hydrogel beads from Swamy & Yun (2015).

3.3.3. Metal release

The metal release from the FucoPol beads was evaluated in different media for 48 hours. An amount of FucoPol beads was placed in 30 mL of SGF, SIF, water, NaCl (0.9 %) or DMEM and samples of 2 mL were taken to be analyzed by ICP-AES, to determine the concentration of copper and/or iron released from FucoPol beads over time. The assay was performed in triplicate.

FucoPol beads were tested in DMEM, once that cytotoxicity of beads would be tested in this media with human cells. SGF, SIF and NaCl (0.9%) were used in metal release test to simulate the behavior of beads in the human body, in case of oral administration of the beads.

Figure 3.13, Figure 3.14 and Figure 3.15 represent the release profiles of the different FucoPol beads in DMEM.

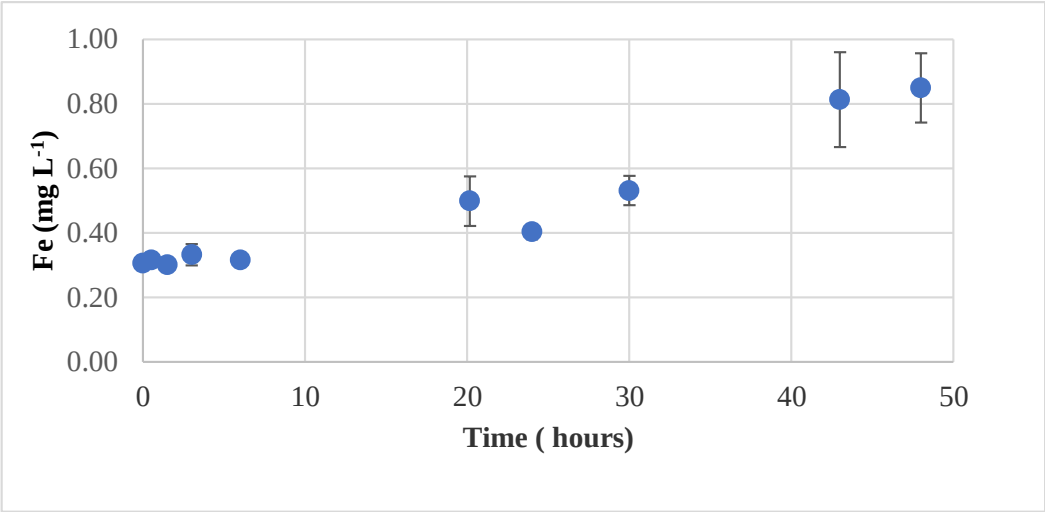


Figure 3.13 – Concentration of iron (mg L^{-1}) measured over time in different samples of DMEM, where Fe/FucoPol beads where released.

The release profile of Fe/FucoPol beads (Figure 3.13) shows that initial concentration of iron in the media was 0.31 mg L^{-1}, a value that increased for 0.50 mg L^{-1} at 20.2 h of assay. Till the end of assay, iron concentration increased gradually to reach a maximum value of 0.85 mg mL^{-1}. During the test, the beads released on average, 0.54 mg L^{-1} of iron, and maintained their shape.

$CuSO_4$/FucoPol beads released, on average, 4.22 mg L^{-1} of copper, thirty minutes after the assay´s beginning (Figure 3.14). During sampling, the beads were noticeably smaller and when the sample was withdrawn at 6 h of assay, the beads were totally disintegrated in DMEM, as there was visible formation of macroscopic precipitate was observed. Till the end of the assay, on average, were released 1.93 mg L^{-1} of copper, probably due to microscopic agglomerates of polymer and copper that were not totally dissolved at 6 h, where beads were not observed. The $CuSO_4$/FucoPol beads released, on average 8.7 mg L^{-1} of copper during the assay, as can be seen in Figure 3.14.

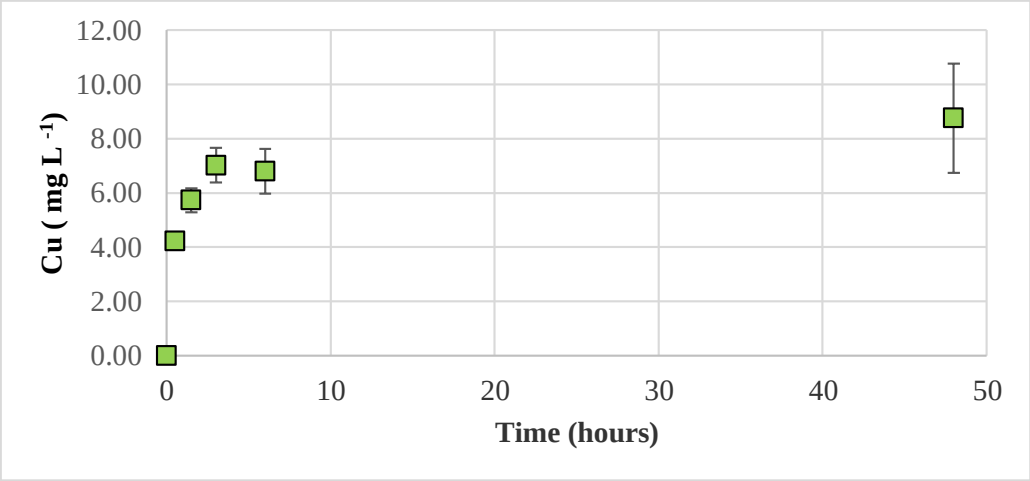


Figure 3.14 – Copper concentration (mg L^{-1}) measured over time in different samples of DMEM, where $CuSO_4$/FucoPol beads where released.

The release profile of $CuSO_4$/$FeCl_3$/FucoPol beads (Figure 3.15) shows how the concentrations of copper and iron oscillated during the assay. There was no iron released from the beads during the assay. Copper concentration increased with the course of the assay, reaching 2.36 mg L^{-1} of copper released on average for 48 hours. The quantification of iron in the beads gave variable results heterogeneous (0.186 – 0.196 mg L^{-1}), which means that the beads can present different contents of iron between them. So, the hydrolyzed beads may not be representative of the real value from the beads placed in DMEM. $CuSO_4$/$FeCl_3$/FucoPol gel beads maintained their shape during the assay.

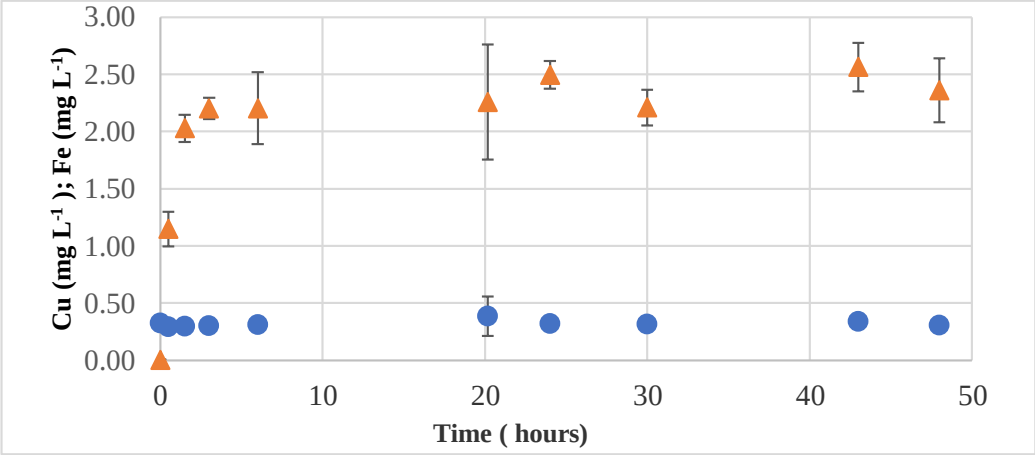


Figure 3.15 - Iron (▲) and copper (■) concentrations (mg L^{-1}) measured over time in different samples of DMEM, where $CuSO_4$/$FeCl_3$/FucoPol beads where released.

Release of copper and iron from FucoPol beads were evaluated in different biological solutions (NaCl (0.9%), SIF, SGF and PBS) over time (Figure 3.16, Figure 3.17, Figure 3.18 and Figure 3.19). FucoPol beads were more stable in NaCl and PBS, maintaining their integrity during the assay. The different types of FucoPol beads were unstable in SGF and were disintegrated in few minutes probably due to the low pH of solution that cause breaking of bounds existing between FucoPol and cations.

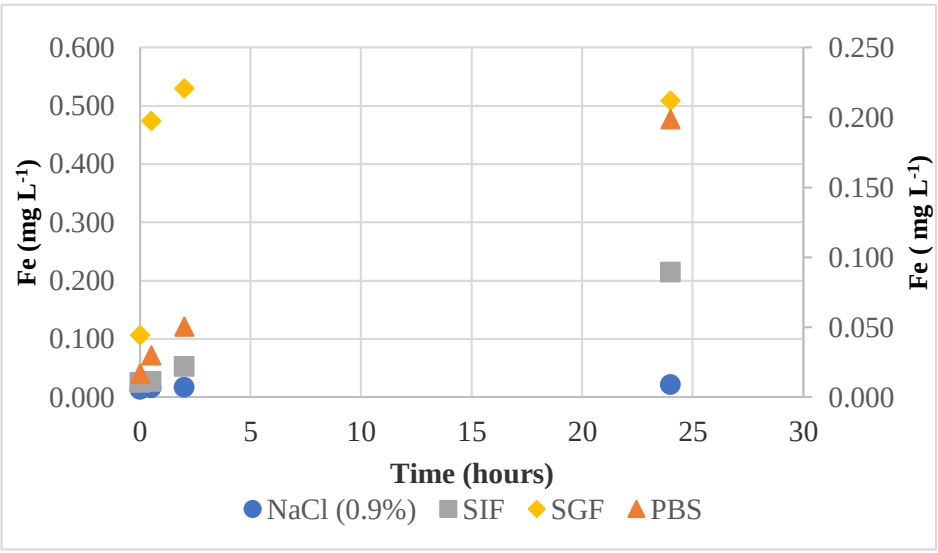


Figure 3.16 – Iron concentration (mg L^{-1}) measured over time in different samples of NaCl, PBS, SIF and SGF where $FeCl_3$/FucoPol beads were released.

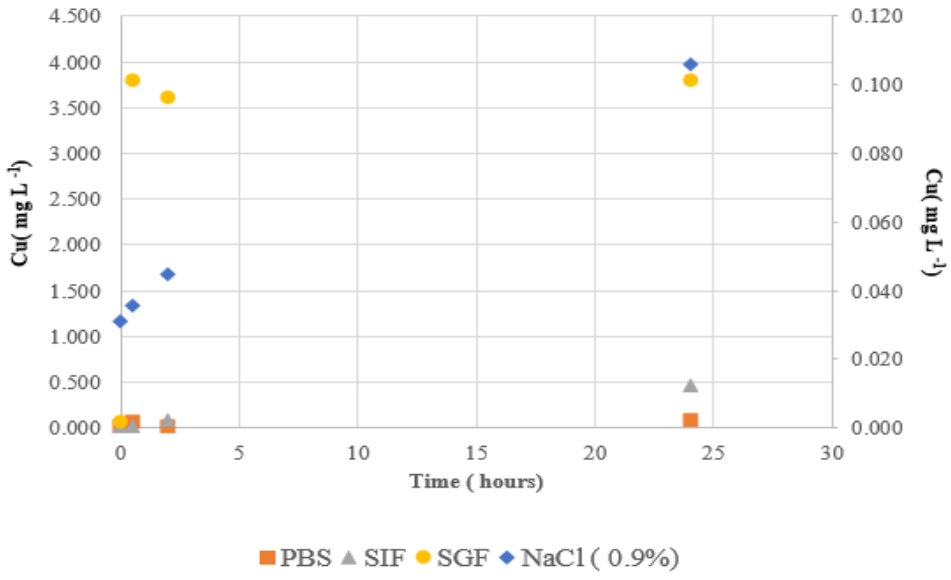


Figure 3.17 - Copper concentration (mg L^{-1}) measured over time in different samples of NaCl, PBS, SIF and SGF where $CuSO_4$/FucoPol beads were released.

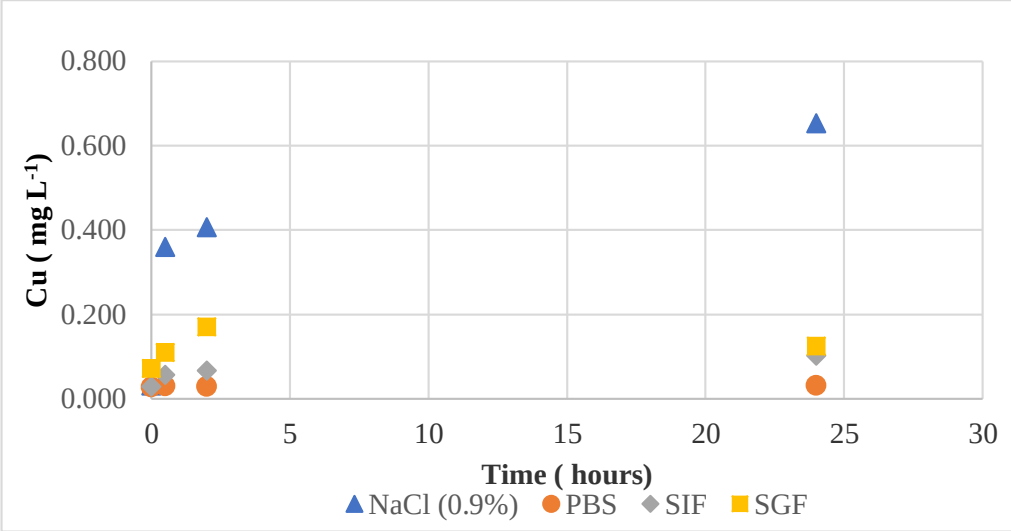


Figure 3.18 - Copper concentration (mg L^{-1}) measured over time in different samples of NaCl, PBS, SIF and SGF where $CuSO_4$/$FeCl_3$/FucoPol beads were released.

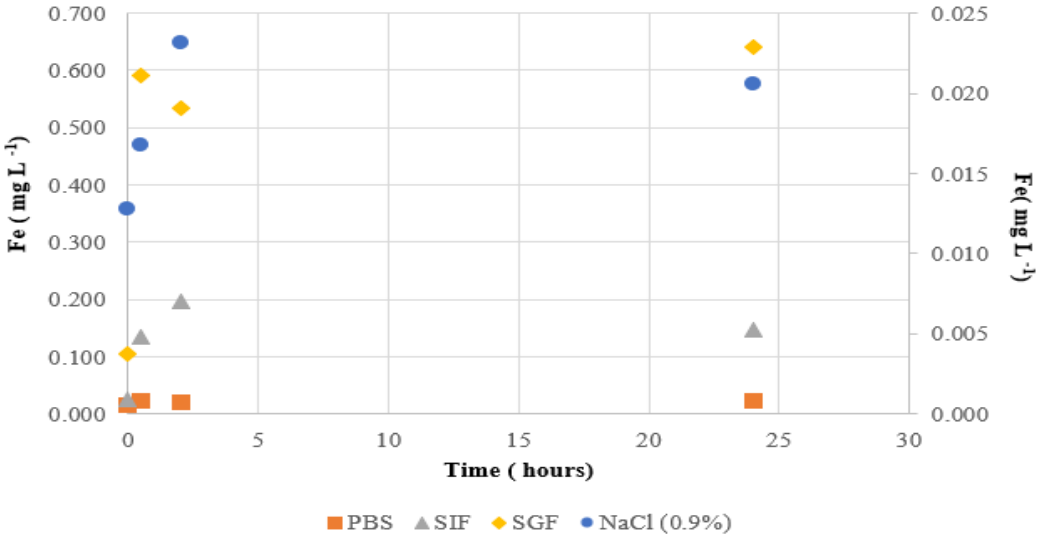


Figure 3.19 - Iron concentration (mg L^{-1}) measured over time in different samples of NaCl, PBS, SIF and SGF where $CuSO_4$/$FeCl_3$/FucoPol beads were released.

$CuSO_4$/FucoPol beads released on average 0.075 mg L^{-1} of copper in NaCl (0.9%) within 24 hours. In PBS medium, 0.078 mg L^{-1} of copper were released. The simulated fluids registered the highest copper release, reaching 0.435 mg L^{-1} for SIF and 3.739 mg L^{-1} for SGF. These beads maintained their shape during the assay in NaCl and PBS, and disintegrated in DMEM, SIF and SGF after 6h, 24h and a few minutes, respectively.

$FeCl_3$/FucoPol beads released on average 0.009 mg L^{-1} of copper in NaCl (0.9%) for 24 hours. In PBS media, 0.182 mg L^{-1} of iron were released. The simulated fluids registered the highest iron release, reaching 0.190 mg L^{-1} for SIF and 0.403 mg L^{-1} for SGF. These beads maintained their shape during the assay in NaCl, while for other media tested (DMEM, SIF, PBS), beads became more faint and translucent. Beads disintegrated in SGF, after a few minutes.

$CuSO_4$/$FeCl_3$/FucoPol beads released on average 0.622 mg L^{-1} of copper and 0.008 mg L^{-1} of iron in NaCl (0.9%) for 24 hours. In PBS media, 0.004 mg L^{-1} of copper and 0.057 mg L^{-1} of iron were released. The simulated fluids registered the highest copper and iron release, reaching 0.073 mg L^{-1} of copper and 0.122 mg L^{-1} of iron in SIF, whereas SGF registered 0.019 mg L^{-1} of copper and 0.534 mg L^{-1} of iron. These beads maintained their shape during the assay in NaCl, DMEM, PBS and SIF (appeared to be more translucent) and disintegrated in SGF.

According to the literature, normal levels of body iron range from 50 to 60 mg/kg in males, 35–40 mg/kg in females, and very low in children and young women (Huang, 2003), so the concentration of iron released by the beads was not toxic under normal conditions.

The normal adult liver contains from 18 to 45 mg Cu/g dry weight, while the blood contains about 6 mg of Cu (Gaetke, 2003).

3.3.4. Cytotoxicity tests

The cytotoxicity of FucoPol was evaluated in different human cell lines (fibroblasts, A 2780, A549, MCF-7, HCT116), in order to use this polymer as drug delivery vehicle similarly of what actually happens with alginate (Tønnesen & Karlsen, 2002). Figure 3.20 present the treated results from MTS assay, where formazan produced is directly proportional to number of living cells in culture.

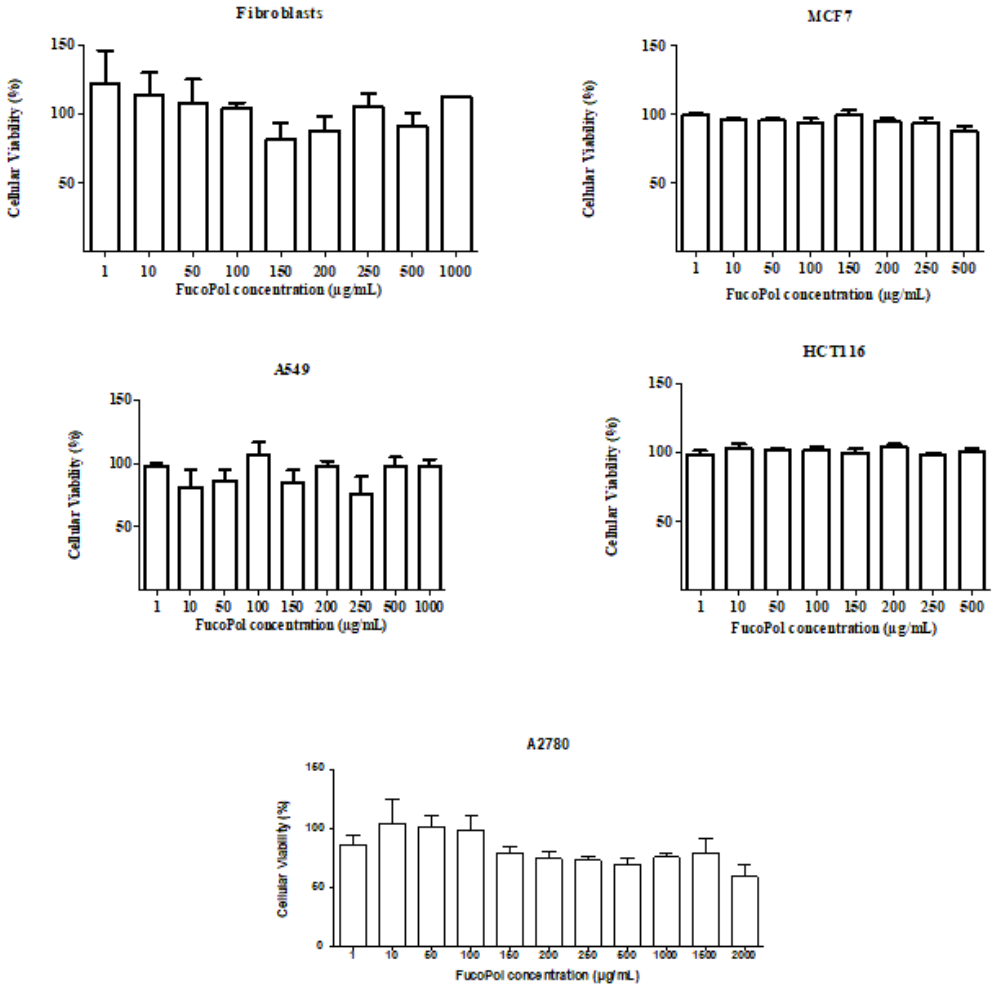


Figure 3.20 – Cytotoxicity of FucoPol in fibroblasts, HCT116, A549, MCF7 and A2780 cells were treated with increasing concentrations of polymer for 48 h and cell viability was determined by MTS assay. The results showed are expressed as mean ± SEM (standard error mean) of three independent assays.

From the results shown in Figure 3.20, FucoPol presents no cytotoxicity for the different cell lines tested. The A2780 cell line was the most sensitive line, nevertheless, the reduction of cell viability was lower than 50%s for a concentration up to 2000 µg mL^{-1} of FucoPol.

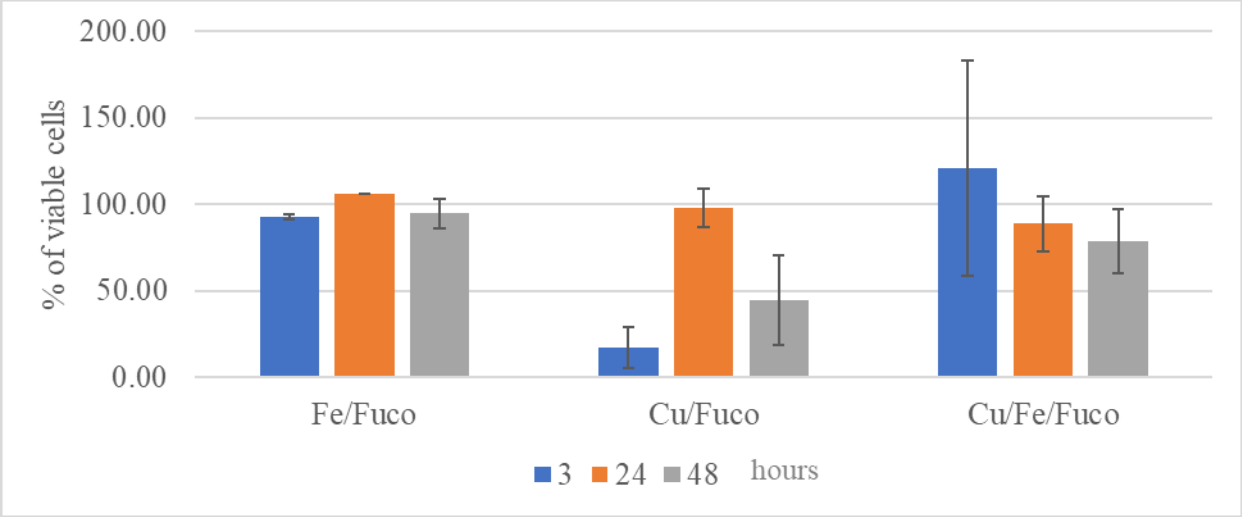


Figure 3.21 - Percentage of viable K562 cells in the presence of $FeCl_3$/FucoPol beads (Fe/Fuco), $CuSO_4$/FucoPol beads (Cu/Fuco) and $CuSO_4$/$FeCl_3$/FucoPol beads (Cu/Fe/Fuco) at 3, 24 and 48 hours of incubation using the Trypan Blue Exclusion Method.

The Figure 3.21 evidence that the Fe/FucoPol beads are not cytotoxic for K562 cells during the 48 hours of incubation. These results are in agreement with the release profile of iron from beads in Figure 3.13, where iron concentration released on average was 0.54 mg L^{-1} that demonstrate not be cytotoxic for the cellular line tested.

Concerning the Cu/Fe/FucoPol beads there is a slightly decrease in the number of viable K562 cells (30 %) over time that might be correlated with the release of copper (2.236 mg L^{-1}) from the beads (Figure 3.20 and Figure 3.21). The results obtained from the incubation of K562 cells with Cu/FucoPol beads show that from 24 hours till 48 hours reveal that the decrease of viable K562 cells (54%) is related with the release of copper (8.7 mg L^{-1}) from beads (Figure 3.14 and Figure 3.21). At 3 hours of incubation of K562 cells with Cu/FucoPol probably a non-homogeneous sample was taken from the culture because the results not concordant with % of viable cells at 24 and 48 hours of assay.

In order to better understand amount of copper that entered in cells, another assay was performed but this time, cells were centrifuged at 500 *g* during 5 min, and pellet and supernatant were separated and analyzed by ICP-AES, for determination of intra- and extracellular copper concentration. The control sample were the K562 cells, that did not present intra- or extracellular copper. In the samples

containing 150 mg of $CuSO_4$/FucoPol beads, showed 7.52 ± 3.53 % of intracellular copper and 92.48 ± 3.53 % of extracellular copper from total amount of copper of 0.161 ± 0.05 mg.

Data from literature suggest that serum copper levels of normal patients have 1.14 mg L^{-1} and patients with acute lymphocytic leukemia present 3.28 mg L^{-1} (Gupte & Mumper, 2009).

Summarizing, the FucoPol beads prepared with iron does not seem to affect the viability of K562 cells. The FucoPol beads prepared with copper and iron seem to slight affect the viability of K562 cells, probably due to copper release. It is possible that FucoPol beads prepared with copper affect the viability of K562 cells.

The potential application of FucoPol beads was their use as drug delivery vehicle. However more studies are needed to achieve this application, but these studies allowed to understand the release profile of different type of beads and the cytotoxic effects in K562 cells.

4. Conclusions and Future work

In this thesis, FucoPol, a fucose-containing polysaccharide was produced by *Enterobacter* A47 using glycerol as sole carbon source. The main objective was to prepare polymeric structures, namely gels, based on FucoPol that could be useful for drug delivery.

FucoPol was tested in fibroblasts, A2780, HCT116, A549 and MC7 and showed not to be cytotoxic.

The next step was to develop a method to obtain FucoPol gels. Here, several methods were tested, and effect of NaOH and cation/anion concentration were tested in FucoPol gel formation. Method 5 was selected to prepare FucoPol gels with different salts ($CaCl_2$, $CuCl_2$, $CuSO_4$, $FeCl_3$, $FeSO_4$ and $ZnCl_2$) that were also tested for preparation of FucoPol beads.

Then, FucoPol gel beads were developed with the same principle used for FucoPol gels. This time, effect of NaOH concentration and diameter of needle were tested. The NaOH concentration that advantaged the beads formation was 2 M, and diameter of needle that shown better results, namely sphericity of beads has as dimensions 0.6 x 25 mm (23 G). Different concentrations of $FeCl_3$ were tested in the preparation of FucoPol gel beads, yielding better results (sphericity, color, size) with 3.33 mg mL^{-1} of $FeCl_3$. Optimal concentration of salt used to prepare $CuSO_4$/FucoPol beads was 20 mg mL^{-1}. So, $FeCl_3$/FucoPol, $CuSO_4$ FucoPol and $CuSO_4$/$FeCl_3$/FucoPol gel beads were obtain with different procedures and their cytotoxicity was tested. Cytotoxicity of FucoPol beads was tested for K562 cells. Fe/FucoPol beads weren´t cytotoxic to this cell line. Cu/FucoPol beads and Cu/Fe/FucoPol beads showed some cytotoxicity for K562 cells.

FucoPol gel beads ($FeCl_3$ and $CuSO_4$/$FeCl_3$) were observed with Cryo-SEM that was useful to observe that FucoPol beads have different surfaces.

FTIR analysis of FucoPol gels and FucoPol beads will be important to understand how FucoPol structure interact with cations.

In future, preparation methods of FucoPol gels and beads can be optimized. FucoPol gel beads can be prepared and loaded with a drug evaluating the encapsulation efficiency and cytotoxicity for human cell lines having as purpose a biomedical application. Gel beads can be also useful for food applications (encapsulation of aromas, essential oils, and vitamins), in cosmetic applications as base substance in cosmetic vehicles or in agriculture, used in the encapsulation of phytochemicals.

The study of gel properties (rheology and mechanical tests) have an important contribute to evaluate the gel strength. Another study that is important is related with the evaluation of stability of FucoPol gels and beads at different temperatures over time.

To finish, the gelling ability of FucoPol opened the possibility to design different structures based on FucoPol suitable for several applications.

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