



ENHANCING THE STORAGE STABILITY OF BIOTHERAPEUTICS USING NATURAL DEEP EUTECTIC SYSTEMS

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PREFACE

The thesis herein presented reflects the work developed from 2020 to 2024 in the Biocatalysis & Bioenergy (B&B) group under the supervision of Dr. Alexandre Babo de Almeida Paiva from LAQV-REQUIMTE of the Chemistry Department of NOVA School of Science and Technology, and in the Des.Solve group under do co-supervision of Prof. Ana Rita Cruz Duarte from LAQV-REQUIMTE of the Chemistry Department of NOVA School of Science and Technology, within the scope of the doctoral program in Biotechnology of the same university.

This work also includes collaborations with Prof. Mário Diniz from UCIBIO - Applied Molecular Biosciences Unit, Chemistry Department, NOVA School of Science and Technology; Dr. Christoph Held from Laboratory of Thermodynamics, Department of Biochemical and Chemical Engineering, TU Dortmund in Germany; Prof. José Matías Garrido, Chemical Engineering Department, University of Concepción in Chile.

This thesis is organized in different chapters, being chapter 1 a general introduction to different methods of preservation used in the pharmaceutical and food industries, followed by introducing compounds commonly used as preservatives in those processes, as well as different biotherapeutics that are more relevant for the contextualization of this work. Chapter 2 comprises all the materials and methods applied throughout the development of the experimental parts of the work. Chapter 3 focuses on the application of natural deep eutectic systems (NADES) on the stabilisation of ascorbic acid (AA), a toxicity assessment of the said NADES and includes a possible application of the stabilised AA for preservation of fruits. In chapter 4, betaine-based NADES were used to increase the activity and stability of horseradish peroxidase (HRP), with focus on the characterization of the NADES used as well as on the structural characterization of HRP. Chapter 5 concerns a practical application of HRP for the development of NADES-based gels to be used as drug delivery systems. In chapter 6, a new subject will be introduced, as NADES were tested for the preservation of red blood cells. Finally, chapter 7 includes the main conclusions from all the work and the future steps from some of the topics.

The studies herein presented resulted in original scientific publications that are either published or in preparation for submission.

ACKNOWLEDGMENTS

Um doutoramento não se faz sozinho. Após 4 anos de trabalho, conversas e muita ciência tenho de agradecer a todos os que me acompanharam e tornaram o caminho mais fácil.

Primeiro que tudo tenho de agradecer aos meus orientadores, Dr. Alexandre Paiva e Prof. Ana Rita Duarte pelo voto de confiança que fizeram ao aceitar acompanhar este projeto e nunca deixaram de acreditar no meu trabalho, mesmo quando parecia que ia dar tudo para o torto. Obrigada por todas as oportunidades que me deram, tanto a nível científico como pessoal.

Agradeço à Professora Susana Barreiros, que me aceitou no seu laboratório como aluna de mestrado e sempre apoiou o meu percurso estando sempre interessada das novidades dos eutéticos. Deixo também um agradecimento ao Professor Pedro Simões, pelos seus contributos em todas as reuniões, pelo apoio e pelo entusiasmo com que acompanhou este trabalho.

I have to acknowledge the people that were involved in the collaborations established, all the co-authors of the works published during the past 4 years for their scientific contributions to the work herein presented. I would like to address a special thanks to Nicolás Gajardo-Parra for all the work developed over two years of online meetings and endless discussions. Thank you for going out of your way to learn new methods. It was a pleasure to meet you.

A todos os companheiros e visitantes do 4º e 5º pisos, muito obrigado pelo vosso apoio e por todos os momentos de descontração que passamos juntos nas atividades de *team building*, nos almoços de convívio e nas viagens que fizemos juntos. Aos meus colegas de doutoramento, Filipa, Ana, Filipe, Hugo e Joana, foi um gosto partilhar esta viagem convosco. Marta, Rita Gameiro e Rita Craveiro muito obrigada pela vossa amizade e apoio constante. Foi uma sorte ter-vos na minha vida e tenho certeza de que sem vocês os últimos 4 anos não

tenham sido tão divertidos! Por mais almoços no sushi, *Aperol spritz*, passeios em conferências e concertos na vossa companhia.

Obrigada à minha família, em especial aos meus pais, que apesar de saberem que esta vida me afasta fisicamente deles, sempre me apoiaram e incentivaram a não desistir. Bruno, palavras não chegam para te agradecer pela paciência, pelo carinho e por todo o apoio ao longo da loucura que foi o último ano. Contigo o caminho não foi só mais fácil, foi mais bonito. Obrigada à minh'amiga Marta, que fez do longe perto e tem sempre um abraço à minha espera (mesmo que muitas vezes seja virtual).

Por último, tenho de deixar um agradecimento pelo financiamento do Horizon 2020, da União Europeia através do European Research Council (ERC) com o projeto ERC-2016-CoG 725034 e da Fundação para a Ciência e Tecnologia pela bolsa de doutoramento SFRH/BD/148510/2019. Agradeço também à instituição de acolhimento, LAQV – REQUIMTE, apoiada pelo FCT/MCTES (LA/P/0008/2020 DOI 10.54499/LA/P/0008/2020, UIDP/50006/2020 DOI 10.54499/UIDP/50006/2020 e UIDB/50006/2020 DOI 10.54499/UIDB/50006/2020).

“Long live all the mountains we moved, I had the time of my life fighting dragons with you.”

Long Live, Taylor Swift

ABSTRACT

The preservation and equal distribution of biotherapeutics worldwide is hindered by several constraints such as high processing costs, the dependence of the cold chain, the lack of stability of liquid formulations or even the short shelf life that is currently achieved. Hence, this work aimed at developing strategies that increase products' shelf life or even decrease the dependence of the cold chain for preservation. Herein, the stabilisation of different biomolecules using natural deep eutectic systems (NADES) was studied, starting from the simplest, ascorbic acid (AA), moving to more complex structures, such as proteins, and finally cells. The chosen compounds can either have therapeutic application or be used to produce biotherapeutics.

Considering the stabilization of AA, using a NADES composed of betaine and citric acid, it was possible to maintain AA's high antioxidant activity up to 9 days, while the control was only active for 2 days. Moreover, this system showed no toxicity against *Palaemon varians*. The NADES+AA formulations were tested for the preservation of fruits, being more efficient than AA alone.

The activation and thermostability of horseradish peroxidase (HRP) were also studied. It was concluded that upon incubation on betaine-based NADES, HRP was more active, and would acquire a more stable conformation, confirmed by an increase of its unfolding temperature. The enzyme also become thermostable, up to 80 °C when incubated in a NADES composed of betaine, trehalose, and glycerol. The more active and stable HRP was then used to prepare enzymatically crosslinked eutectogels. In comparison to regular hydrogels, these were more stable and had better rheological properties. The eutectogels were used to successfully prepare drug delivery systems of ketoprofen.

The final chapter of this work, focused on the application of NADES as additives for the preservation of red blood cells (RBC). Our study allowed to conclude that, in the conditions tested, NADES were as effective as the standard solution currently used in Europe.

Keywords: Natural deep eutectic systems (NADES), preservation, hydrogels, ascorbic acid (AA), horseradish peroxidase (HRP), red blood cells (RBC)

RESUMO

A preservação e distribuição de fármacos a nível global continua a ser limitada por diversos fatores como o elevado custo das técnicas de processamento, a dependência da cadeia de frio, a falta de estabilidade de formulações líquidas e até mesmo o limite de validade que é possível atingir. Deste modo, o principal objetivo deste trabalho foi desenvolver estratégias que permitam prolongar o prazo de validade destes produtos ou até reduzir a necessidade de recorrer ao uso da cadeia de frio para preservação. Assim, estudou-se a preservação de biomoléculas com solventes eutéticos naturais (NADES), começando por moléculas simples, como o ácido ascórbico (AA) e avançando para estruturas mais complexas como proteínas e também células. Os compostos testados podem ter aplicação terapêutica ou ser usados na produção de biofármacos.

Através da dissolução do AA num NADES composto por betaína e ácido cítrico, foi possível prolongar a sua atividade antioxidante até 9 dias em solução, em comparação com apenas 2 dias no controlo. Também se estudou o perfil toxicológico dos NADES em *Palaemon varians*, permitindo concluir que, nas concentrações testadas os sistemas não são tóxicos. As mesmas formulações de NADES+AA foram testadas para a preservação de frutas, tendo sido mais eficazes do que a solução de AA.

Outro tópico de estudo foi a ativação e estabilidade da enzima peroxidase de rábano (HRP). A sua incubação num NADES à base de betaína permitiu, não só, aumentar a atividade enzimática, mas também conferir uma estrutura mais estável à enzima, aumentando a sua temperatura de desnaturação e, consequentemente a sua estabilidade a temperaturas mais elevadas (80 °C). A HRP mais ativa e estável foi depois usada na preparação de eutectogéis, por *crosslinking* enzimático. Em comparação com hidrogéis tradicionais, os eutectogéis

obtidos eram mais estáveis, apresentando também melhores propriedades mecânicas. Estes géis foram também usados como sistemas de libertação controlada de cetoprofeno.

No capítulo final deste trabalho, testou-se a utilização de soluções à base de NADES para a conservação de eritrócitos. Os resultados obtidos permitiram concluir que, nas condições testadas, os NADES provaram ter a mesma eficácia que a solução atualmente autorizada para uso na Europa.

Palavas chave: Sistemas eutéticos naturais, preservação, hidrogéis, ácido ascórbico, peroxidase de rábano, eritrócitos

LIST OF PUBLICATIONS

Research Articles

Liane Meneses, Nicolás F. Gajardo-Parra, Esteban Cea-Klapp, José Matías Garrido, Christoph Held, Ana Rita Duarte, Alexandre Paiva. 2023. "Improving the Activity of Horseradish Peroxidase in Betaine-based Natural Deep Eutectic Systems". *RSC Sustainability* 1: 886-897. 10.1039/D2SU00127F

Nicolás F. Gajardo-Parra, **Liane Meneses**, Ana Rita C. Duarte, Alexandre Paiva and Christoph Held. 2022. "Assessing the Influence of Betaine-Based Natural Deep Eutectic Systems on Horseradish Peroxidase". *ACS Sustainable Chemistry & Engineering* 10: 12873–1288. 10.1021/acssuschemeng.2c04045

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Ana Rita Jesus, **Liane Meneses**, Ana Rita Cruz Duarte and Alexandre Paiva. 2021. "Natural deep eutectic systems – A new era of cryopreservation", in *Advances in Botanical Research - Eutectic Solvents and Stress in Plants*, Academic Press, UK, pp. 385-409

Communication in Conferences

Liane Meneses, Dimitra Bagaki, Ana Roda, Alexandre Paiva, Ana Rita C. Duarte, "Development of Enzymatically Crosslinked Natural Deep Eutectic Gels: Versatile Gels for Enhanced Drug Delivery", 6th EuChemS Conference on Green and Sustainable Chemistry, Salerno, Italy, 3-6 September, 2023 (Oral Communication)

Liane Meneses, Ana Rita Jesus, Alexandre Paiva, Ana Rita C. Duarte, "Extending the shelf-life of red blood cells using natural deep eutectic systems", 3rd International Meeting on Deep Eutectic Systems, June 19-22, 2023 (Poster Communication)

Liane Meneses, Dimitra Bagaki, Ana Roda, Alexandre Paiva, Ana Rita C. Duarte, "Development Of NADES-based Injectable Hydrogels Through Enzymatic Crosslinking", 2nd Greenering International Conference, Valladolid, Spain, March 21-23, 2023 (Poster Communication)

Liane Meneses, Nicolás F. Gajardo-Parra, Christoph Held, Gabriele Sadowski, Ana Rita C. Duarte, Alexandre Paiva, "Improving the stability and activity of horseradish peroxidase in betaine-based natural deep eutectic systems", 5th International Symposium on Green Chemistry, La Rochelle, France, May 16-20, 2022 (Oral Communication)

Liane Meneses, Ana Rita C. Duarte, Alexandre Paiva, "Stabilization of Horseradish Peroxidase Using Natural Deep Eutectic Systems", 2nd International Meeting on Deep Eutectic Systems, Lisbon, Portugal, June 15-17, 2021 (Oral Communication)

Liane Meneses, Ricardo Correia, Carolina Richheimer, Paula M Alves, Manuel JT Carrondo, Ana Rita C. Duarte, Alexandre Paiva and António Roldão, "Improved storage of influenza HA-VLPs using a trehalose-glycerol natural deep eutectic system", 6th Iberoamerican Meeting on Ionic Liquids, Santiago, Chile, May, 24-26, 2021 (Oral Communication)

Liane Meneses, Ana Rita C. Duarte, Alexandre Paiva, "Improving the Activity of Horseradish Peroxidase in NADES", 1st Greenering International Conference, Almada, Portugal, February 15-16, 2021 (Poster Communication)

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ABBREVIATIONS AND ACRONYMS

3D Three-dimensional

4-AAP 4-aminoantipyrine

A

AA Ascorbic acid

AAD Average absolute deviation

ABTS 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid

AChE Acetylcholinesterase

API Active pharmaceutical ingredient

ATR-FTIR Attenuated total reflection Fourier transform infrared spectroscopy

a_w Water activity

B

Bet Betaine

BHT Butylated hydroxytoluene

BSA Bovine serum albumin

C

CA Citric acid

CAT Catalase

CD	Circular dichroism
cDNB	1-chloro-2,4-dinitrobenzene
ChCl	Choline chloride
CLEA	Cross-linking enzyme aggregate
CLEC	Cross-linking enzyme crystal
COVID-19	Coronavirus disease 2019
CPA	Cryoprotective agent
D	
DES	Deep eutectic systems
DDS	Drug delivery systems
DHA	L-dehydroascorbic acid
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
E	
EC	Enzyme Commission
EC₅₀	Half maximum effective concentration
EDC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
EDTA	Ethylenedinitrilotetraacetic acid
EtGlycol	Ethylene glycol anhydrous
F	
FAO	Food and Agriculture Organization
FBS	Fetal bovine serum
FDA	Food and Drugs Administration

G

G'	Storage modulus
G''	Loss modulus
Glc	D-(+)-glucose
Gly	Glycerol
Glt-Ph	Gelatine-phenol conjugate
GSH	Reduced L-glutathione
GST	Glutathione S-transferase

H

HA	Hyaluronic acid
Hb	Haemoglobin
HaCaT	Human immortalized keratinocyte cell line
HBA	Hydrogen bond acceptor
HBD	Hydrogen bond donor
HES	Hydroxyethyl starch
HRP	Horseradish Peroxidase

I

IBR	Integrated biomarker response
IPST	Instituto Português do Sangue e da Transplantação

K

KBI	Kirkwood-buff integrals
Ket	Ketoprofen
KF	Karl Fisher

L

L929	Mouse fibroblasts of connective tissue cell line
LB	Lactobionic acid
LPO	Lipid peroxidation
LVR	Linear viscoelastic region

M

MEM	Eagle's minimum essential medium
MES	2-morpholinoethanesulfonic acid buffer
MD	Molecular dynamics
MDA	malondialdehyde bis(dimethylacetal)
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

N

Nac	<i>N</i> -acetyl-L-cysteine
NADES	Natural deep eutectic system
NADES-AS	Aqueous solution of natural deep eutectic system
NBT	nitroblue tetrazolium
NHS	<i>N</i> -hydroxysuccinimide
NMR	Nuclear magnetic resonance
NSAID	Non-steroid anti-inflammatory drugs

P

PBS	phosphate buffered saline
PC-SAFT	Perturbed-Chain Statistical Associating Fluid Theory
PEG	Polyethylene glycol
pK_a	Acid dissociation constant

POM	Polarized optical microscope
Pro	DL-proline
PS	penicillin and Streptomycin
PSA	Phenol-4-sulfonic acid sodium salt dihydrate
PVA	Polyvinyl alcohol
PVP	Polyvinylpyrrolidone

Q

QM/MM	Quantum mechanics/molecular mechanics
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R

RBC	Red blood cells
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSA	Radical scavenging activity
RSS	Reactive sulphur species

S

SAG	Saline-adenine-glucose
SAGM	Saline-adenine-glucose-mannitol
SDG	Sustainable Development Goals
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
Sorb	D-sorbitol
Suc	Sucrose

T

TAC	Total antioxidant capacity
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T_{aggregation}	Aggregation temperature
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substance
TBHQ	Tert-butyl hydroquinone
TCA	Thermal energy storage
TES	Trichloroacetic acid
T_{on-set}	Temperature of beginning of protein unfolding
Treh	Trehalose dihydrate
T_{unfolding}	Unfolding temperatures
Tyr	Tyramine hydrochloride

U

UN	United Nations
USA	United States of America
UV	Ultraviolet
UV-Vis	Ultraviolet-visible

V

VLP	Virus like particles
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W

W	Water
WB	Whole blood
WBC	White blood cells
WHO	World Health Organization

X

Xyl	D-(+)-xylose
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INTRODUCTION

The seventeen Sustainable Development Goals (SDG) for 2030, were proposed by the United Nations (UN) back in 2015 (Figure 1.1). Their main objective was to put an end to several problems that are faced worldwide, such as poverty, hunger, lack of proper health conditions, energy production, climate changes and even sustainability of construction, consumption and production (United Nations 2015). Each SDG has its own targets, goals, and indicators, still some of them have common aspects.



Figure 1.1 - Sustainable Development Goals of the United Nations for 2030 (obtained from (United Nations 2015))

It is possible to consider that SDG 2 (Zero Hunger) and 12 (Responsible consumption and production) complement each other since both have targets that involve a more sustainable food supply chain. Specifically, goal 2 focuses on making sure that food is available to all people and that food production is more sustainable. Goal 12 also envisages a more sustainable use of natural resources, as well as to reduce the food loss and waste to halve of the currently registered.

Currently, with the planet's resources reaching its limits, food loss and waste are a major concern. The 2021 report from the UN Environment Programme, showed that in 2019, 931 million tons of food were wasted at a global level, representing 17% of the total processed food. Moreover, the report also shows that the majority of the waste, 61%, comes from households (Forbes, Quested, and O'Connor 2021). The main factors that influence food waste at households include food abundance, in a way that people tend to buy fresh items, instead of having leftovers, along with the human intent to eat things that look and taste good, leaving people to discard food that is still good for consumption (for example, close to the expiry date) (Knorr and Augustin 2022). At the same time as enough food to feed the world is produced, according to the Food and Agriculture Organization (FAO) of the UN, in 2022 about 9% of the world's population was undernourished (FAO et al. 2023). Hence, investing in strategies for the preservation and packaging of edible goods stands as one of the main solutions for the safe delivery of food worldwide (Chan 2022). Nevertheless, in an humanitarian context this is still challenging due to the limited access to technologies, electricity and even lack of affordability (Aste, Del Pero, and Leonforte 2017). A factor that still hinders the safe delivery of food in this context, is the recurrent need of cold chain too guarantee products' safety and quality (Aste, Del Pero, and Leonforte 2017).

On a different note, SDG 3 focuses on the access to good health and well-being to all people. This has been a constant concern, especially for developing countries, where indicators such as maternal and infant mortality are very high, and diseases that are easily treated and prevented in other countries, still represent major concerns. This goal also targets the development of drugs and equal access to essential medicines by developing countries.

The improvement of drugs' production and development can be achieved through the work with enzymes, since they are very efficient biocatalysts, capable of performing numerous chemical transformations. Enzymes are used in several industrial applications such as synthesis of drugs and agrochemicals, production of biopolymers and development of biosensors

(Meghwanshi et al. 2020). There are numerous active pharmaceutical ingredients (API) produced by enzymatic transformations, and it is estimated that by 2033 the market of enzymes for health industries surpasses 1.7 million US\$ (Ghosh 2023).

Besides the improvement of drugs' development, the world-wide distribution of medicines is also a major concern. For instance, vaccines are the medical development that had the highest impact on human health and public well-being. The establishment of vaccination programmes led to the disappearance of several epidemics, such as poliomyelitis, diphtheria, measles or rubella, and to the eradication of smallpox (Schuchat 2011). However, due to constraints concerning vaccine preservation, packaging and transportation they are not equally accessible to everyone (Lopes et al. 2022). This difference became even clearer during the coronavirus disease 2019 (COVID-19) pandemic vaccination process, in which the vaccines required special cold-chain conditions, that were crucial to guarantee effectiveness and safety (Fahrni et al. 2022). Nearly 2 years after the beginning of the vaccination process c.a. 70% of the population of high-income countries was vaccinated, in contrast to only 25% of the population in low-income countries (Duroseau, Kipshidze, and Limaye 2023).

Another problem that was evidenced with the COVID-19 pandemic was the shortage of blood supplies, since blood products have short shelf life, requiring continuous donations (S. R. Xie et al. 2012). Red blood cells (RBC) concentrates are the most used blood product during transfusions. RBC are obtained from the separation of donated blood into its constituents and stored for 42 days at 4°C, after which if they are not used, they are discarded (Islamzada et al. 2022). While developed countries have created systems for blood transportation, in many countries there is limited access to essential blood products (Alghamdi 2023). Nevertheless, the Department of Health and Human Services of the United States of America (USA), determined that in 2017, from the 15.5 million of RBC units collected, 1.7 million were discarded due to their low shelf life (Asghar et al. 2014). Vaccination and blood supply are just two examples of the differences in access to proper healthcare that are found worldwide.

The most common storage and preservation methods rely on drying or refrigeration/freezing, and are common to the food, pharmaceutical and health fields. Both strategies increase the costs of the products, by increasing the costs of transportation and/or energy requirements. Although efforts have been made to find preservation methods without the need for the cold chain there is still a long way to go before this can be achieved.

1.1 Preservation Strategies

Preservation of goods, either foods or biopharmaceutical products, relies on the same principle – changing the properties of the medium to decrease degradation, hence extending shelf life and maintaining the product properties (Aste, Del Pero, and Leonforte 2017). Depending on the type of product, degradation can have different meanings. In the case of food, it could mean microbial proliferation or increased enzymatic activity, that leads to oxidation and browning (Alp and Bulantekin 2021). In the case of biopharmaceuticals, it could be related to unfolding of proteins, disruption of native structure of biomolecules or aggregation, that leads to loss of efficacy or even undesirable effects on the human body (Mastrobattista 2021).

Preservation and stabilization of goods can be performed by a variety of methods. The traditional methods, include drying, refrigeration, freezing, or a combination of these. Nonetheless, these techniques are the basis for most modern preservation methods and are transversal to food, pharmaceutical, and cosmetic industries.

1.1.1 Drying

Drying represents one of the oldest methods of preservation known to man. Going back to the 1800s, drying foods allowed to fulfil the nutritional needs, by improving the consumption of fruits and vegetables. Natural drying systems consisted mostly of using air or sun light to dry food products however this strategy had limitations such as the need of large areas, or the ineffectiveness of sun rays during some time of the year, being mostly used in domestic context. In the early 1900s, artificial drying systems, such as roller and spray dryers were developed, which allowed to deliver 4500 tons of food to soldiers, during World War I (Alp and Bulantekin 2021; Qu et al. 2022).

To this date, drying remains the most used preservation method and drying technologies evolved significantly. The modern strategies include hot air drying or microwave combined with vacuum drying (Qiu et al. 2019). Despite being faster than sun drying, these methods are time consuming, energy intensive and have a high carbon footprint. Therefore, drying methods based on renewable energy sources have also been developed and allow to optimise the use of solar energy, as well as geothermal and biomass. Examples of systems that use renewable energy sources include improved solar drying using thermal energy storage (TES), solar drying

system with phase change material based thermal storage, or solar dryer with pebble-bed TES. These systems store the energy from the sun, during the sunshine hours of drying, that can later be used over the off-sunshine hours, making the process more efficient (Qu et al. 2022).

Food products have a basal microorganism load, which affects their shelf life and safety upon consumption. Microbial growth is dependent on the presence of humidity, hence drying is also crucial for the safety of fresh and raw food (Qiu et al. 2019). In order to ensure the effectiveness of the drying process as preservation technique it is important to guarantee the minimal microbial contamination of the samples before drying. This can be successfully accomplished through the use pre-treatments that allow to decrease the drying time, kill most microorganisms on the surface, and also to maintain properties of the product, such as colour or nutritional values (Alp and Bulantekin 2021). These pre-treatments include dipping in acid, alkaline or salt solutions and dipping in hot/boiling water. Other pre-treatments include spray with gases, ultrasound, high pressure, ultraviolet (UV) irradiation, plasma treatment and even treatment with supercritical carbon dioxide (Alp and Bulantekin 2021).

The percentage of water left after drying depends on the type of food, therefore, instead of determining the water content, it is more important to introduce the concept of water activity (a_w). Water activity is a measure of water mobility and availability to react. It is calculated as the ratio between partial water vapor pressure (of a food, a solvent, etc) and the vapor pressure of pure water (Qiu et al. 2019). Higher a_w values mean that there is more water available to be used in chemical/biochemical reactions, by microorganisms. In the case of food, it is important to reduce a_w below the minimum required for microbial activity, which is 0.6, although most bacteria require 0.87 to be able to grow (Alp and Bulantekin 2021). It is important to note that dried foods are not sterile, and if they are not stored under appropriate conditions some microorganisms can still proliferate. This shows that, although the preservation method is important, proper storage conditions also play a great role in preservation (Waterman et al. 2002).

The same principle is valid for the pharmaceutical industry, in which solid forms are widely used (Dao et al. 2018). Although, most solid forms of drugs are prepared from powders, not including an aqueous portion, water can be present in the final mixture of compounds, due to interaction of the components or from humidity absorption. The control of water activity in these solid formulations is extremely important, since they are not produced under sterile conditions and can have microbial load (Dao et al. 2018). Moreover, water present in these

formulations can also induce several deterioration reactions, such as hydrolysis, that can lead to loss of effect (Waterman et al. 2002).

1.1.2 Refrigeration and Freezing

Refrigeration and freezing are two methods that rely on reducing the temperature of products below room temperature to slow down microbial activity as well as biochemical reactions that lead to deterioration (Sridhar et al. 2021). Refrigerated products are usually kept at temperatures ranging from 4 to 10 °C, being stable for a period of days. Frozen products are exposed to lower temperatures, ranging from -10 to -80 °C, and can be kept for months (Kumar 2019). Both methods allow to have products that are considered minimally processed, since no additives are used during their storage (Daza et al. 1996).

In food preservation, the main advantage of refrigeration is that it has no effect on food sensory properties and nutritional value (Aste, Del Pero, and Leonforte 2017). The drawbacks associated to refrigeration rely mostly on the difficulty to apply the cold conditions immediately after harvest and maintain them until consumption. (Aste, Del Pero, and Leonforte 2017). To achieve maximum extension of the products' shelf life, refrigeration is usually combined with other methods, such as gas absorber membranes, and aseptic or controlled atmosphere packaging (Daza et al. 1996).

Considering freezing for food preservation, its main advantage is that it allows to extend the shelf life of products for longer periods. However, the type of freezing process, whether slow or fast, will impact the sensory properties of the products due to the formation of ice crystals. Slow freezing allows for the formation of big and uneven crystals that can disrupt the microstructure of food, while fast freezing leads to the formation of small and even ice crystals that do not have much impact on the structures, preserving the products' sensory and nutritional properties (Cheng et al. 2017; Zhu, Zhou, and Sun 2019). At an industrial level the most common are the conventional freezing methods that involve slow freezing, such as air blast, direct contact or immersion freezing. Aiming to improve the freezing rate and food quality, new methods have been developed, including high-pressure, ultrasound-assisted, radioactive or dehydration freezing (Cheng et al. 2017; Sridhar et al. 2021).

For both refrigerated and frozen products the maintenance of the cold chain is a critical factor during their processing and transportation. Exposure to higher temperatures even for short

periods of time can have a massive impact on the safety of goods. This becomes even more difficult in humanitarian context but also in some developing regions, that lack conditions of technology, electricity and even affordability (Aste, Del Pero, and Leonforte 2017).

In the pharmaceutical industry, refrigeration is commonly used for biopharmaceutical agents, such as hormones, enzymes or monoclonal antibodies (Mensink et al. 2017). However, these agents show low stability when preserved in liquid formulations and most often will have a short shelf life due to degradation processes such as hydrolysis, aggregation and loss of structure. It has been widely reported that, especially for therapeutic proteins dried formulations are a better option (Z. Wang et al. 2022).

The concern towards the cold-chain is translatable to the pharmaceutical industry, in which the conditions for storage and transport of specific biopharmaceuticals, like proteins and vaccines, are very strict and in the case where they are not maintained, entire batches can end up as waste (Mastrobattista 2021).

1.1.3 Cryopreservation

Cryopreservation is a freezing process in which cells, tissues or even organs, are kept at low temperatures (-80 to -196 °C) for long periods, while maintaining their viability and functionality. At such low temperature metabolic pathways, as well as biochemical and enzymatic processes, are significantly slowed, turning this into the best method for long-term preservation of cells (Bai et al. 2023). Cryopreservation is essential for several biomedical applications, i.e., fertility preservation or cell therapy, but is also important in research laboratories, to keep stocks of cell lines without the high maintenance of live cell cultures (Jesus et al. 2021b).

As for regular freezing, the success of cryopreservation is related to the freezing process and the formation of ice crystals. There are three different methods that are considered cryopreservation, namely static cold storage, slow freezing and vitrification (Dou, Lu, and Rao 2022). Static cold storage consists in keeping the samples at 4 °C, therefore decreasing their metabolic rate. This method is limited to a period of hours, and is mostly used for the preservation of organs during medical procedures. (Dou, Lu, and Rao 2022). Slow freezing (-1 to -5 °C·min⁻¹) ensures that a reduced number of ice crystals is formed inside cells, due to their partially dehydration, hence being commonly used for the cryopreservation of cells and tissues.

Vitrification is a fast-cooling process that uses liquid nitrogen ($> -400\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$), assuring that no ice crystals are formed. This process is mostly used for stem cells or oocytes, that are more sensitive (Dou, Lu, and Rao 2022). Independently of the type of freezing used, cells, tissues, or organs that are cryopreserved should be mixed with cryoprotective agents (CPA) before being cooled to the desired temperature for storage. During freezing, several processes can lead to stress and cell damage, namely the formation of ice crystals and osmotic stress, that can cause dehydration, which in some cases is irreversible. CPA are extremely important during the freezing process, since they allow to minimize the damage caused to cells or tissues as well as maintain their viability and ensuring high post-thawing survival rates (Bai et al. 2023; Jesus et al. 2021b).

1.1.4 Freeze Drying

Freeze drying, or lyophilization, is the most effective technique for the dehydration of products. Through freeze-drying, water is removed from the products through sublimation, meaning that water goes from solid state to gas, under vacuum. This presupposes that the products to lyophilise must be previously frozen. Hence, this technology is not feasible to be applied for all products due to the disadvantages presented when discussing the freezing of edible products. On the other hand, this technology is a good alternative for dried food products since it allows to maintain the original taste, colour, and fragrance without affecting the composition of the products and their nutritional value. This process is widely used in the food industry worldwide as lyophilised products are stable at room temperature, and they are easily reconstituted in water, making them good options not only for specific field operations, such as aerospace or military context, but also for traveling or during hikes, for instance (Liu, Zhang, and Hu 2022). Nevertheless, this method has high costs and high energy demands, compromising its application in some industries (Liu, Zhang, and Hu 2022).

Freeze drying is also widely used in the pharmaceutical industry, especially for novel treatments with therapeutic proteins, such as antibodies, hormones or vaccines (Z. Wang et al. 2022). Additionally, it is commonly used to prepare dry formulations of protein drugs, vaccines and to dry cells or biomaterials, such as polymer scaffolds (Merivaara et al. 2021; Z. Wang et al. 2022). The lyophilization of proteins allows to solve some problems associated to protein stability in solution, such as hydrolysis or aggregation, hence preserving their biological

activity, while simplifying transportation and storage, since they can be kept at room temperature. Still, there are some concerns associated to lyophilisation of therapeutic proteins, such as the preservation of their native structure (Z. Wang et al. 2022; Bolje and Gobec 2021). Moreover, freeze drying commonly requires the addition of excipients, to protect the structure of proteins during the processes, and can also affect the functionality of the final product (Bolje and Gobec 2021). Despite the fact that loss of structure may happen during this process, some proteins are able to fold back into their native structure upon rehydration (Bolje and Gobec 2021).

In the case of cells, freeze drying comes as an alternative to the standard preservation method, cryopreservation. Although freeze dried products are easier to store and transport, freeze drying cells is still a very challenging process, since to preserve whole cells all the cellular components, such as membrane, intracellular structures, and deoxyribonucleic acid (DNA), must be carefully protected. Due to all the stress imposed by the process, optimisation of excipients, freezing, etc, must be carefully performed. Nonetheless, as recently reviewed by Merivaara et al. (2021), several cell types, such as platelets, RBC or mononuclear cells, have been successfully freeze dried with high recovery rates.

1.1.5 Novel Packaging Solutions

The novel packaging solutions for food preservation comprise edible coatings, modified atmosphere packaging and active packaging. In combination with other treatments, these packaging solutions allow to achieve longer shelf life for fresh products (Qiu et al. 2019). Depending on the preservation method, the packaging systems can be impermeable or semipermeable to gases and water vapour and act as barrier to light throughout the expected shelf life, even at room temperature (Kubo et al. 2021).

Edible coatings can be obtained from different materials such as polypeptides, carbohydrates or lipids (Q. Xie, Liu, and Zhang 2022). They are placed directly on the surface of the products and work as a barrier, preventing loss of water and aromas, and protecting against microbial activity and oxidation (Q. Xie, Liu, and Zhang 2022). Overall, edible coatings show several advantages regarding their edibility, low toxicity, low polluting effect and even biodegradability, but their development is still limited to the laboratory scale (Qiu et al. 2019).

Modified atmosphere packaging is an established method to decrease the rate of microbial growth and other deterioration of fresh products. This method reduces gas exchanges in packages, creating a balance between oxygen, carbon dioxide and nitrogen inside the package. It is especially applied in products with high moisture content, like meat and fish, but it has been used for fresh desserts and cheeses. Its efficiency depends on several factors, namely the packaging material, storage conditions and even the products to preserve, hence all these factors should be taken into account when considering using modified atmosphere packaging (Qiu et al. 2019).

Finally, active packaging, is a strategy that promotes interaction between the product, the packaging material, and the atmosphere to extend the shelf life of the product to its maximum. Active packaging can be obtained through different ways, namely through the addition of pads with active agents into the package, through the addition of active agent to the package's polymer (by adsorption, immobilization or direct incorporation), and by using polymers with bioactivity (chitosan, for instance) (Almasi, Jahanbakhsh Oskouie, and Saleh 2021). Active packaging has successfully extended the shelf life of items such as pre-baked bread, fresh vegetables and desserts (Qiu et al. 2019). The method is highly effective however, there are still concerns that the compounds used can migrate to the fresh products, hence the technology is still a research target and has been only authorized in Europe (Qiu et al. 2019).

The use of active packaging has also been adopted by the pharmaceutical industry, to improve storage and safety of their products. The examples of active packaging used on by the pharmaceutical industry include oxygen scavengers and desiccants such as silica gels for moisture control. Recently, the development of intelligent packaging, defined as packages that sense alterations on the storage environment and give that information to the user, became very useful for the storage of sensitive compounds. These systems are one of the strategies that helps to ensure not only effectiveness, but also safety of biopharmaceuticals (Janjarasskul and Suppakul 2018).

1.2 Stabilizer Compounds

As it has been referred, the success of cryopreservation and freeze drying relies on the presence of stabilizers to minimize the effects of ice crystals formation and dehydration. These stabilizers

(or excipients) can be cryoprotective agents (CPA), or lyoprotectants (Z. Wang et al. 2022). CPA are used during cryopreservation processes; they act by supporting the formation of hydrogen bonds with water, hence promoting water vitrification and avoiding the formation of ice crystals that are harmful for structures (Jesus et al. 2021a). Lyoprotectants, are used to provide protection during lyophilization, besides also promoting water vitrification, they also protect by creating preferential interaction with proteins as well as through the water replacement mechanism (Z. Wang et al. 2022). It is important that the chosen molecules have some degree of flexibility, are chemically stable and safe for human consumption (Mensink et al. 2017; Thakral et al. 2021). Hence, stabilizers can be obtained from different families such as sugars and sugar alcohols, amino acids, polymers, surfactants, buffers (Bolje and Gobec 2021; Thakral et al. 2021). Recently, natural deep eutectic systems (NADES) have also been added to the list of stabilizers (Castro et al. 2018; Gertrudes et al. 2017; Jesus et al. 2021a).

The first report of a CPA was in 1949, when Polge and co-workers used glycerol for the cryopreservation of spermatozoa (Polge, Smith, and Parkes 1949). Since this breakthrough, over the last 70 years numerous compounds and biomaterials have been described as CPA. Currently, glycerol and dimethyl sulfoxide (DMSO) are the two most used CPA, due to their high efficacy (Sui et al. 2020). However, both of them present toxicity towards cells, making it crucial that they are removed immediately after thawing (Jesus et al. 2021a). It has been reported that most of these compounds are produced by freeze tolerant organisms, such as the stonefly in Alaska, wood frogs or beetles, as a strategy to survive the exposure to extremely low temperatures (Gertrudes et al. 2017; Jesus et al. 2021b). In order to overcome the disadvantages of the mentioned CPA, alcohols, sugar and sugar alcohols, polymers and amino acids (Jesus et al. 2021a; Sui et al. 2020) have been proposed, in a biomimetic perspective (Jesus et al. 2021a).

On the other hand, the use of stabilizers in the freeze drying process is essential to protect the structures during freezing and drying. There are several compounds that can be used and they can act by promoting vitrification of water (Z. Wang et al. 2022). For instance, stabilizers have different roles on the protection of proteins and interact with each other creating, not only excipient-protein interactions, but also excipient-excipient interactions, hence most formulations used for protein stabilisation are usually a combination of several compounds.

Along this section the main families of stabilisers used in cryopreservation and freeze drying will be introduced.

1.2.1 Sugars and Sugar Alcohols

Sugars such as glucose, sucrose, or trehalose have been described as CPA. Their mechanism of protection towards cells is not fully understood however, one of the theories suggests that sugars replace the water around the cells' membrane, hence stabilizing it. Sucrose and trehalose can also increase the viscosity of aqueous solution, hence allowing vitrification. Sugars can also stabilize proteins in the cells' membrane (Bai et al. 2023). Trehalose and raffinose have been used in combination with other CPA, such as DMSO, improving cell viability (Jesus et al. 2021b).

For protein freezing, sucrose and trehalose, are considered the most efficient stabilizers due to their properties, such as chemical stability, capacity to form hydrogen bonds with water, viscosity in solution, among others (Mensink et al. 2017; Thakral et al. 2021). The main drawback associated to the use of sugars in protein stabilization, is their tendency to crystallize during freezing, which can cause protein aggregation. Overall, disaccharides are better stabilizers for proteins than monosaccharides (glucose, fructose, mannose) or tri-saccharides (such as raffinose) (Thakral et al. 2021).

Considering the sugar alcohols applied as CPA, as it has been referred, glycerol was the first CPA to ever be reported. Glycerol interacts uniformly with the water molecules through hydrogen bonding, hence restricting the formation ice crystals (Bai et al. 2023). In the case of protein stability, sugar alcohols, such as mannitol, sorbitol, or glycerol, are more suitable to protect proteins from heat-induced denaturation. In fact, mannitol is one of the most common excipients in lyophilized formulations and can also act as cryoprotectant (Thakral et al. 2021). In order to overcome the problems with sugar crystallization, bulking agents, such as mannitol, sorbitol or even glycine, are added to the formulations creating a synergistic effect for protein stabilisation in which the sugar remains amorphous, and the other component crystalizes, being responsible for the structure of the lyophilizate (Thakral et al. 2021).

1.2.2 Amino acids

Amino acids, such as betaine and proline, have been widely used as CPA for cell preservation. Betaine is able to penetrate cells, is highly biocompatible and has low toxicity. This compound protects cells from dehydration and can also preserve the proteins on the cells' membrane by forming a monolayer (Jesus et al. 2021b). It has also been reported that membrane proteins'

are less likely to unfold when solvated in the presence of betaine (Bai et al. 2023). Betaine is often combined with other stabilizers to be used as CPA. Proline has also been used for several years however, it was not used for the cryopreservation of mammalian cells until recently. In combination with trehalose or ethylene glycol it allowed to successfully preserve RBC and oocytes, respectively (Jesus et al. 2021b). Recently, three neutral amino acids (β -alanine, γ -aminobutyric acid and ϵ -aminocaproic acid), have been reported for their CPA properties, acting as ice inhibitors and osmotic stress reducers along with being easy to remove from cells (Sui et al. 2020).

In protein stabilization, amino acids are more common in liquid formulations, however they can also be used in freeze-dried formulations. Usually, amino acids are combined with a sugar, since they have low stabilising effect, which is improved by the protective effect given by sugars. The most used amino acid in lyophilised formulations is arginine, which further helps to improve protein solubility as well as to reduce the viscosity of the solution. Other amino acids such as proline, histidine or lysine have also been reported for the improvement of protein stability. The mechanisms behind protein stabilisation with amino acids are not clear but can include preferential interaction, change in free volume and molecular motility (Thakral et al. 2021).

1.2.3 Polymers

Polymers act as ice growth inhibitors or freezing point depressors, and they can also have an important role on ice recrystallization, during thawing (Bai et al. 2023). Polyvinyl alcohol (PVA), hydroxyethyl starch (HES), polyethylene glycol (PEG) or alginate are some examples that have been described as CPA. Nonetheless, to achieve high cell viability post-thawing, polymers are usually combined with other CPA.

For protein stability, polymers are used in solution, during freezing and/or during freeze drying. The most relevant examples include PEG, polyvinylpyrrolidone (PVP) and dextran. These polymers are more beneficial for protein stability when combined with sugars and can allow the storage of the freeze-dried formulations at room temperature (Thakral et al. 2021).

1.2.4 Surfactants

Surfactants have the ability to stabilise proteins in two different ways. One of the strategies is through binding of the surfactant to interfaces, hence avoiding interactions of the protein with the interfaces that lead to aggregation or denaturation. The other way is by interacting directly with the protein, forming a complex that can prevent the protein from establishing other interactions. Non-ionic surfactants, like polysorbates and poloxamer, are the most used in protein formulations. As for other components, surfactants can be combined with mannitol, to improve the properties of the preservation solution (Thakral et al. 2021).

1.2.5 Buffers

The pH of a protein solution, either it is frozen or lyophilized, must be kept stable during all steps of the processes, and in the final formulation after reconstitution. Hence, the addition of buffer to a protein formulation is also a critical step since pH is one of the main aspects of protein stability. Protein formulations that are kept frozen also require the control of pH during freezing. The most widely used buffers include sodium and potassium phosphate, histidine, citrate, or tris buffers. Buffers should be chosen for each protein, having in mind the protein's acid dissociation constant (pK_a), the crystallisation of its components during freezing and lyophilization and the adequate concentration. The association of buffers and sugars is very common, since sugars are able to reduce salt crystallization, which also reduces the pH shift during concentration. Bulking agents, like mannitol or glycine, can also be combined with buffers to minimize the pH shift (Thakral et al. 2021).

1.2.6 Natural Deep Eutectic Systems

Deep eutectic systems (DES), described for the first time in 2003, are solvents obtained from the mixture of two or more components, that at a specific molar ratio have a melting point lower than the one of the initial components, becoming a stable liquid at room temperature (Abbott et al. 2003). Later, in 2011, Choi and co-workers, introduced NADES as a possible third liquid phase present in cells, that acts as the link between hydrophobic and hydrophilic media, being able to solubilize otherwise insoluble molecules (Y. H. Choi et al. 2011).

NADES are obtained from the mixture of naturally occurring components, mostly primary metabolites, such as sugars, amino acids, organic acids, polyols and water. DES and NADES are produced by the formation of hydrogen bonds between hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA), which causes a decrease in the melting point of the mixture in comparison to the melting point of the original compounds (Paiva et al. 2014). Other intermolecular interactions, such as van der Waals and electrostatic forces can also be involved in the formation of NADES (Santana et al. 2019). A very interesting characteristic of NADES preparation is that it fully complies with the green chemistry metrics, since their production does not generate waste or byproducts, hence having 100% yield of production and atom economy. Consequently, NADES have a theoretical E-factor of 0 (Paiva et al. 2014). NADES also offer other advantages such as biocompatibility, biodegradability, sustainability and low cost of preparation (El Achkar, Fourmentin, and Greige-Gerges 2019).

The high number of compounds' combinations to prepare NADES, allows the design of systems in a tailor-made way, ensuring that they fit the envisaged application in the best way possible. It also means that their physical-chemical properties can be very distinct, depending on the type of components used and even on their molar ratio (Paiva et al. 2014).

Generally, NADES are characterized by their high viscosity which can hinder some applications. One of the solutions to overcome this problem is the addition of water, since even in small amounts, it can cause a significant decrease on the system's viscosity (up to 10-fold) (Castro et al. 2018; Craveiro et al. 2016; Dai et al. 2013). The amount of water in the NADES can also influence other properties of the systems, namely, conductivity, and even biocompatibility. A property of NADES, that creates a range of opportunities for their application, is their high thermal stability, even below 0 °C, along with the evidence that they are able to decrease the glass transition temperature of water (Craveiro et al. 2016). Although NADES are usually described as biocompatible and non-toxic, understanding their toxicity is also of the utmost importance, since it is not always equivalent to the one of the original constituents. With this concern, researchers have created a model for the prediction of NADES' toxicity, in which sugars and polyols show the lowest likelihood for toxicity (Halder and Cordeiro 2019).

Considering the properties that have been discussed, the applications of DES and NADES have also grown exponentially over the past 20 years, and include extraction, biocatalysis, electrochemistry, CO₂ capture and even biomedical applications (Paiva et al. 2014). In the extraction field, NADES have been used as media for the extraction of phenolic compounds

from different matrixes, such as medicinal plants, as safflower (Dai, Verpoorte, and Choi 2014), and agri-food wastes, such as tomato, pears, olives and onions (Fernández et al. 2018). In biocatalysis, NADES have also been applied as solvents, co-solvent, enhancer, or stabilizer of enzymes such as catalases, lipases, laccases, and peroxidases (Pätzold et al. 2019). In the field of biomedical applications some examples are the cryopreservation of cells (Castro et al. 2018; Jesus et al. 2021a), vaccines preservation (Correia et al. 2021), anti-cancer treatment (Pereira et al. 2019) or drug delivery systems (DDS) (Mokhtarpour, Shekaari, and Shayanfar 2020). In which concerns the stabilization of different (bio)molecules, NADES have been used to stabilize several compounds and biological agents, such as proteins, nucleic acids (El Achkar, Fourmentin, and Greige-Gerges 2019) or antioxidants (Silva et al. 2018).

The application of NADES in specific fields, such as biocatalysis and biomedical applications, will be explored in more detail further in this introduction.

1.3 Strategies for the Preservation of Biotherapeutics

1.3.1 Antioxidants

As defined by Halliwell and co-workers, antioxidants are compounds that, even at low concentrations, are able to delay or prevent the oxidation of a substrate (Halliwell 1996). The protection can be obtained by a variety of mechanisms, such as inhibition of generation of reactive oxygen species (ROS), scavenging capacity of ROS, metal chelating capacity, single oxygen quenching activity or hydrogen peroxide decomposition activity (Gulcin 2020). Antioxidants play important roles in food products since they help maintaining the colour, taste, and texture of products during storage. On the other hand, they also protect the human body from the effects of ROS, playing a key role on slowing the development of several chronic diseases (Gulcin 2020). Over the past years, antioxidants have become an indispensable group of additives due to their ability to preserve products but also due to their health benefits.

Free radicals, also known as reactive species, are highly unstable molecules, since they have unpaired electrons that make them more active for chemical reactions with other molecules. They are produced during normal cellular processes, such as mitochondrial respiration, and can be ROS, reactive sulphur species (RSS) or reactive nitrogen species (RNS) (Gulcin 2020). Although they are formed during biochemical processes in the organism, the compounds can be harmful for cellular structures like DNA, lipids or proteins, hence the overproduction of reactive species is usually balanced by the action of antioxidants (Parcheta et al. 2021). The cell's defence mechanisms include enzymatic antioxidants, such as superoxide dismutase or catalase, that repair oxidative damage. In situations where the production of reactive species outbalances the cellular antioxidant mechanisms, the cell enters a state of oxidative stress that can lead to cell death. Non-enzymatic antioxidant systems to regulate ROS production, can be found in fruits and vegetables for example (Parcheta et al. 2021). Natural antioxidants are present in all plant parts, from the roots to the fruits and leaves. The main families of natural antioxidants are the phenolic compounds, vitamins, and carotenes. The phenolic compounds' family includes several classes such as phenolic acids, flavonoids, and tocopherols, for instance and each of these groups has numerous constituents, with different structures (Gulcin 2020). The most well-known antioxidant vitamins are vitamin A, also known as retinol, vitamin C, also known as ascorbic acid, and vitamin E, also known as tocopherols, which are represented in Figure 1.2 (Gulcin 2020).

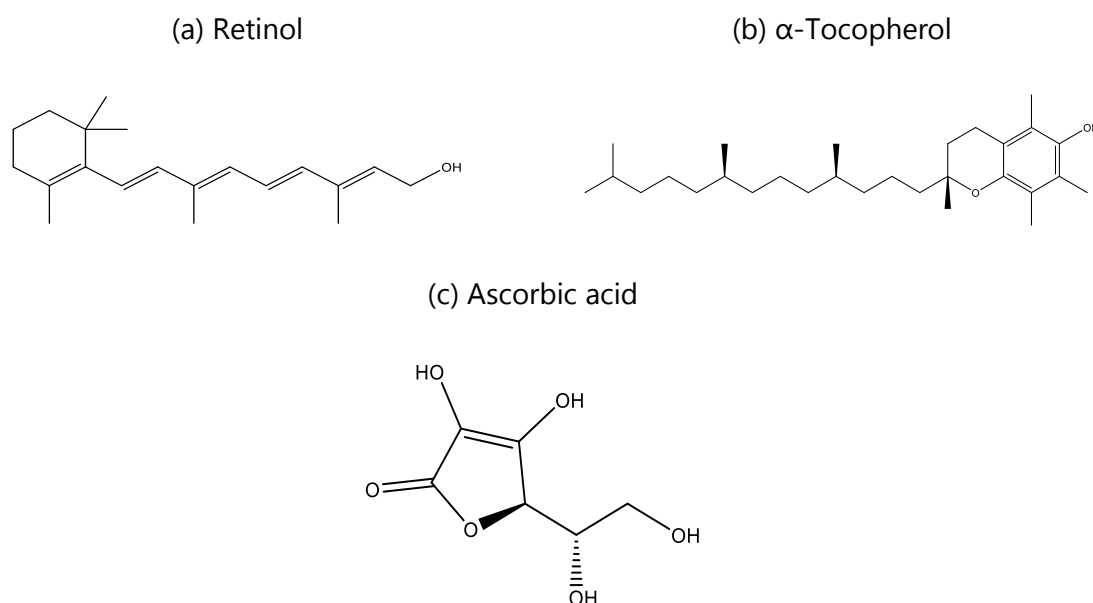


Figure 1.2 – Chemical structures of the most common antioxidant vitamins (a) retinol, (b) α -tocopherol and (c) ascorbic acid.

Vitamin A, or retinol, comes from the carotenoid family. Carotenoids are lipid-soluble colorants produced by plants and microorganisms. In the human diet they are obtained through red and orange fruits, like tomatoes or carrots. They are responsible for several biological effects such as mediation of growth factors, cell regulation and differentiation or stimulation of the immune system. Overall, carotenoids are associated with reducing the risk of cancer and cardiovascular diseases. Lycopene, β -carotene, and astaxanthin are examples of the most important carotenoids on the human diet. Retinol, is a result of the breakdown of β -carotene and is known for its benefits to skin (Gulcin 2020).

Vitamin E, or tocopherols, are phenolic compounds that are lipid soluble. Tocopherols are mostly found in leafy green vegetables and fat-rich foods such as vegetable oils, nuts, and seeds. The most abundant isomer is α -tocopherol, and it also has the highest antioxidant activity of the family. Despite being liposoluble it is commonly found in the plasma and red cells, granting protection to membranes that are lipidic structures (Gulcin 2020).

Antioxidants are also available for human consumption as food supplements, cosmetics, or pharmaceuticals. Depending on their origin, they can be classified as natural, as the ones described above or synthetic. Synthetic antioxidants are widely used in the food, pharmaceutical and cosmetic industries due to their ease of availability, low cost and high activity (Parcheta et al. 2021). The most produced synthetic antioxidants are phenolic

compounds, such as butylated hydroxytoluene (BHT) or tert-butyl hydroquinone (TBHQ) (Gulcin 2020).

1.3.1.1 Ascorbic Acid

Vitamin C, ascorbic acid (AA) or ascorbate, is a powerful antioxidant with low toxicity, that has two active forms: the reduced, L-ascorbic acid, and the oxidised, L-dehydroascorbic acid (DHA). Vitamin C and the strategies for its stabilization will be discussed in more detail, since it is one of the most widely used antioxidants for a wide range of applications. Moreover, there is still a wide interest in the development of preservation strategies for AA, which will be one of the focuses of this work.

In Nature, AA is mostly found in citrus fruits and kiwis, but is also found in some vegetables, such as leafy greens and broccoli. AA is water soluble, and is a very effective antioxidant that plays a role in several physiological processes and biochemical reactions, however it is only obtained through diet, since the human body is not capable of synthesising it (Parcheta et al. 2021; Gulcin 2020; Caritá et al. 2020). AA is responsible for the regulation of collagen synthesis, and its deficiency causes scurvy, a severe disease that is rare nowadays. AA is used in several products in the food, pharmaceutical and cosmetic industries (Caritá et al. 2020).

The main problem of using AA in formulations concerns its instability. AA is highly unstable and degrades under several conditions, such as in aqueous formulation, high pH, and in the presence of oxygen and metal ions (Caritá et al. 2020). When exposed to certain conditions AA is reversibly oxidised into DHA that can later be irreversibly hydrolysed into 2,3-diketogulonic acid (Yin et al. 2022). Several attempts have been made to reduce the extent of AA oxidation during preparation and storage, including limiting the amount of oxygen present, maintaining a low pH, reducing the water content, adding other antioxidants or chelating-agents, and increasing the viscosity of the formulations (Caritá et al. 2020).

AA can be protected through the effect of other compounds present in solution, hence one of the strategies is to add low molecular weight compounds to the mixture that can provide protection to AA. Since the oxidation of AA into DHA is reversible, it is feasible to add compounds that can promote the reverse reaction, such as the synthetic antioxidant TBHQ or glutathione. Ferulic acid can also be used, however it grants protection through a different

mechanism. It is hypothesised that it acts as sacrificial substrate for oxidation in solution, since it has a higher redox potential than AA (Yin et al. 2022). Another strategy for commercial products is to use AA derivatives, that can be either hydrophilic, by introducing a glucose residue or a phosphate group, or lipophilic, as acid 6-palmitate, for instance (Yin et al. 2022). Despite the higher stability of the AA derivatives, they are not as effective as AA and significantly increase the price of the final formulation (Caritá et al. 2020).

Food and pharmaceutical industries also stabilise AA using carriers, that can interact chemically or physically with it and create a layer of protection against the environment conditions. A correct design of the stabilisation agents is important, not only, for the maintenance of AA efficiency and activity, but also for its release in the interest site. For instance, considering its application in food supplements, it is ideal that AA is released in the small intestine, where it is absorbed. Thus, the carrier should be chosen considering the different conditions along the gastrointestinal tract, since the bioavailability of AA is dependent on the release and solubility (Yin et al. 2022).

One of the examples of carriers for the delivery of AA are proteins. They are used to prepare carriers with chemical interaction, which seemed promising for the food industry, due to their safety and nutritional value. However, their application at industrial level is limited since they present a low loading capacity and also poor stability in conditions that mimic the stomach and intestine, causing a fast release of the encapsulated AA (Yin et al. 2022).

Physical barriers prepared by combination two or more compounds have a better performance in the stabilization of AA. These particles are known for allowing a better loading of AA, that is retained in the particle's core either by encapsulation or adsorption. The particles can be obtained from the mixture of proteins and polysaccharides, such as chitosan, gum arabic, sodium alginate, maltodextrin, or pectin. The most common techniques to prepare particles include spray drying, spray chilling and complex coacervation. Due to its low cost, easy scale up and continuous operation, spray drying is the most used at industrial level (Yin et al. 2022). For instance, particles prepared by spray drying using a mix of sodium alginate and gum arabic, have shown a loading of 90% of AA with increased thermal stability (Yin et al. 2022). Chitosan is widely used in the preparation of nanoparticles due to its chelating and crosslinking properties. In combination with AA, chitosan forms hydrogen bonds, which retains the antioxidant on the polysaccharide, while maintaining its high activity. The size and charge of the chitosan particles also provides better absorption of AA, hence increasing the residence

time of AA in the digestive tract. Although chitosan particles have stronger interaction with AA than protein particles, their encapsulation efficiency is also low (Yin et al. 2022; Caritá et al. 2020). Overall, microparticles that act as physical barriers in the protection of AA can retain higher amounts of AA than nanoparticles.

Another class of particles are liposomes, which, have also been widely studied for the encapsulation of AA. Liposomes are amphiphilic molecules that are mostly prepared with phospholipids, but can be modified with surfactants, polymers, or terpenes. Their high interest as delivery systems relies of the fact that can carry both hydrophilic and hydrophobic components, present low toxicity, and high biocompatibility (Caritá et al. 2020). Liposomes with chitosan coating have successfully been used for the encapsulation of AA with 80% encapsulation efficiency, envisaging applications in the food and cosmetic industries (Jiao et al. 2018).

In the pharmaceutical industry, microemulsions and micelles are widely used and have also been studied for the delivery of AA. Emulsions are complex mixtures composed of oil, water, and surfactants, and are classified depending on the size of their droplets. Microemulsions (10-100 nm), have low interfacial tension, high interfacial area and can improve the solubility of hydrophilic and hydrophobic compounds. Microemulsions for the stabilisation of AA have been prepared and the results have shown that high amounts of AA are delivered through the skin and the antioxidant capacity of AA is also increased (Caritá et al. 2020).

1.3.2 Proteins

1.3.2.1 Enzymes

Enzymes are the biggest group of proteins to be known and are essential in several processes in the human body, such as metabolic pathways, gene expression and even immune system reactions (Vandenberghe et al. 2020). Enzymes are considered efficient biocatalysts, since they are able to increase the reaction rates, without changing the equilibrium constant or going through changes in their structure. They are even considered more efficient than conventional chemical catalysts, due to their high specificity and selectivity, which make them suitable to be applied for several ends, such as clinical diagnosis, drugs and food formulation and even environmental remediation (Narváez and Domínguez 2005). Since there are numerous known

enzymes, they have been classified by the Enzyme Commission (EC), according to the reactions they catalyse, following the EC number. Since 2018, there are seven classes of enzymes: oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases and translocases (Vandenbergh et al. 2020).

As all proteins, enzymes are mostly composed of a sequence of amino acids linked through amide bonds, which forms their primary structure. This amino acid chain arranges itself in different structures, with the most common being helical (α -helix), pleated sheets (β -sheet) or turns, which make up the secondary structure of a protein. Finally, the secondary structures will interact creating a three-dimensional (3D) arrangement, that is thermodynamically stable in its environment, called tertiary structure, in which the active site, prosthetic group and other cofactors are available for reaction. Enzymes composed of more than one polypeptide chain, can also acquire a quaternary structure, created by the interaction of their 3D subunits (Narváez and Domínguez 2005). Nevertheless, their characteristics and activity rely on multiple factors such as amino acid sequence, 3D structure, stability and even interaction with other molecules (Vandenbergh et al. 2020).

When in liquid formulation, one of the parameters that highly influences enzymatic structure is water activity (a_w). Water is necessary to ensure enzymatic mobility; nevertheless, in excess it can promote interactions that change the enzyme conformation, leading to loss of structure (Hendrickx et al. 1992). Enzyme conformation depends on an essential hydration shell, which acts as a lubricant that allows conformational mobility and molecular environment adaptation (Klibanov 1989). An environment with controlled a_w can also positively affect the enzyme thermostability, preventing heat inactivation (Setikaite et al. 2009).

Even though the application of enzymes in reactions has made several industrial processes more competitive, their application has been limited by their instability, efficiency and specificity, high production costs and difficulty to separate and recover from the reaction mixture after use. Several efforts were made to solve these concerns, such as study of enzymatic structure, screening of natural enzymes and immobilization of the enzymes. With these improvements, enzymes have been successfully used in the chemical, pharmaceutical, cosmetic, food, textile and paper industries (J. M. Choi, Han, and Kim 2015).

Enzyme immobilization is the attachment or confinement of the enzyme on a solid support, thus improving its catalytic activity. The advantages of using immobilized enzymes include the

increased stability of the enzyme, the possibility to use the same enzyme in several reaction cycles, the easiness to recover and separate the enzyme from the reaction media and finally, the possibility to create multi-enzyme reaction processes (H. H. Nguyen and Kim 2017). The most common methods of enzyme immobilization are: adsorption, covalent bonding, cross-linking and entrapment (Figure 1.3) (H. H. Nguyen and Kim 2017; Brady and Jordaan 2009).

Adsorption is the simplest immobilization method as it is achieved through the establishment of weak bonds, namely Van der Waal's forces, electrostatics, and hydrophobic interactions. This method is considered simple and low cost and does not affect the enzyme, but it also carries drawbacks, namely leaching of the enzyme. This can be caused by changes on temperature or pH since the bonds connecting the enzyme to the support are so weak (H. H. Nguyen and Kim 2017).

From the methods mentioned, the most used for enzyme immobilization is covalent bonding. With this technique, the functional groups of enzyme and support are connected through covalent bonds, which are more stable. In order to not impair enzyme's activity, the non-essential functional groups should be involved in the formation of bonds. Binding the enzyme to the support generally involves two main steps: (1) activation of the support surface and (2) coupling of the enzyme to the activated support. Covalent bonding is one of the preferred methods since it provides low leaching of enzyme, high uniformity on the support, and control of the amount of enzyme immobilized. The main disadvantage of this method is that high degree of enzyme denaturation can occur during the immobilization process. Moreover it also requires high reagent volume to process small amounts of protein, and it can reduce enzyme's activity (H. H. Nguyen and Kim 2017).

Cross-linking represents the strongest binding method used for immobilization of enzymes, in which the enzymes are irreversibly connected among each other by covalent bonds, creating a 3D structure that is not attached to a support matrix. This process requires the use of cross-linking reagent, such as glutaraldehyde, and the most common cross-linking methods are cross-linking enzyme aggregate (CLEA) and cross-linking enzyme crystal (CLEC). The advantages of this methods are the minimal enzyme leaching, due to the strong covalent bonds, and the possibility to change the stabilising agents to change the environment and increase enzyme's stability. The use of glutaraldehyde represents the major drawback of cross-linking enzymes, since it can cause conformational changes that can lead to loss of activity (H. H. Nguyen and Kim 2017).

Lastly, entrapment, is a method in which the enzyme gets trapped within a polymeric network. During biocatalytic processes, the network allows the transference of reaction constituents, while the enzyme is kept in the matrix. Enzymatic entrapment is performed by mixing the enzyme in a polymer solution that is later polymerized. This method allows to achieve minimal enzyme leaching, if the pore size is adequate, improves stability, and allows to adjust the environment for each enzyme. The major disadvantage of the immobilization by entrapment is the mass transfer limitation, imposed by the polymeric network that is formed (H. H. Nguyen and Kim 2017). The polymeric networks for entrapment can be prepared by several methods such as photopolymerization, electropolymerization, silica sol-gel process or micro-encapsulation (H. H. Nguyen and Kim 2017; Brady and Jordaan 2009).

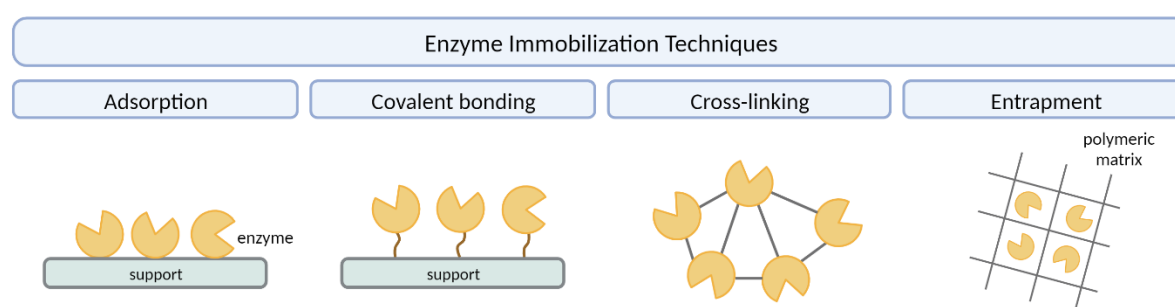


Figure 1.3 – Traditional methods for enzyme immobilization. Adapted from (H. H. Nguyen and Kim 2017). (Created with BioRender.com).

Recently, NADES have been applied to biocatalysis since they offer several advantages, such as improved activity and thermostability of enzymes, better solubility of substrates along with higher selectivity (El Achkar, Fourmentin, and Greige-Gerges 2019). The behaviour of enzymes in NADES is correlated with each systems' intrinsic characteristics such as viscosity, water content, water activity, and pH, which makes essential to carry case-by-case studies, since different combinations will have different characteristics (Pätzold et al. 2019). Recently, Arnodo and co-workers, reviewed the numerous reports of biocatalysis in DES and NADES, organizing them by reaction class in order to create a summary with over 70 examples of more sustainable options for traditional reactions (Arnodo et al. 2023). Besides being applied in reactions as solvent or substrate, NADES have also been used for the stabilization of enzymes, namely, to improve their thermostability, for immobilization and in the preparation of nanoparticles as presented in Table 1.1.

Table 1.1 - Examples of DES and NADES used for enzyme stabilisation or immobilization.

DES/NADES	Enzyme	Results	Ref.
<i>Choline chloride (ChCl)-based NADES</i>			
Trehalose: ChCl	Lysozyme	NADES promoted thermostability and protein refolding, after heating at 90 °C.	Xin et al. (2017)
ChCl: lactic acid	Papain	Immobilization of papain on chitosan treated with DES improved its thermostability. The presence of DES in solution also improved the activity of free papain.	Lin et al. (2020)
ChCl: glycerol	<i>Pseudomonas stutzeri</i> lipase	Immobilized enzyme in CLEA was active in DES-based media.	Guajardo, Ahumada, and Domínguez de María (2021)
ChCl: polyols ChCl: sugars	Lysozyme	Both DES types improved the thermal stability of the enzyme without inducing denaturation. The native tertiary structure of the protein was maintained in all DES.	K. Park et al. (2022)
ChCl: fructose: water	<i>Candida rugosa</i> lipase (CRL)	Nanocellulose treated with organic solvent and NADES was used as support and co-solvent for CRL immobilization. Immobilized CRL showed specific activity higher than free CRL, with increased thermostability, catalytic activity and reusability up to 7 cycles.	Putra et al. (2022)
ChCl: polyols Ethylammonium chloride: polyols	Fatty acid decarboxylase	The enzyme showed improved selectivity towards the decarboxylation of C18 over C16, when used in DES. The immobilization in DES improved the reaction yield over 10 times, in comparison with the free enzyme.	Chanquia et al. (2022)

ChCl: lactic acid	α -Amylase	Lignin was extracted from corncob residues using DES and the DES-lignin mixture was used as carrier for enzyme immobilization. The immobilized enzyme showed increased pH and thermal stability, retaining 80% of its initial activity after 28 days storage and 70% after being reused 12 times.	Zhang et al. (2023)
ChCl: urea	Carbonic anhydrase (CA)	DES and CA were successfully encapsulated by microfluidics and thermally crosslinked for the capture of CO ₂ with high performance.	Zhao et al. (2023)
<i>Betaine (Bet)-based NADES</i>			
Bet: xylitol	Laccase <i>Trametes versicolor</i>	In NADES based medium, enzyme's residual activity at 70 °C is 10-fold higher than without NADES.	Delorme, Andanson, and Verney (2020)
Bet: polyols	Laccase POXA1b variants	Higher residual activity after incubation at 70 and 90 °C in the presence of NADES.	Varriale et al. (2022)
Bet: urea	β -glucosidase	Fe ₃ O ₄ nanoparticles synthesised in NADES were used to immobilize the enzyme with 86% immobilization yield, high thermos, pH and storage stability.	Deng et al. (2022)

1.3.2.1.1 Horseradish Peroxidase

Since it is one of the objects of study of this thesis, HRP will be discussed with more detail.

Horseradish peroxidase (HRP) is part of the oxidoreductase class (E.C. 1.11.1.7). The enzyme is present in the roots of the perennial herb *Amaracia Rusticana*, and is produced on a large scale due to its industrial application in clinical diagnostic kits, immunoassays or cancer therapy (Vojinović et al. 2004; Veitch 2004; Krainer and Glieder 2015).

HRP has been thoroughly studied and has its catalytic centre (Veitch 2004), crystal structure (Gajhede et al. 1997) and amino acid sequence (Welinder 1976) well characterized. The secondary structure of HRP is mostly α -helical with a small β -sheet regions. The heme-group

is located in the centre of HRP, between the distal and proximal domains (Veitch 2004). HRP can carry oxidation reactions of a series of organic and inorganic compounds using hydrogen peroxide as substrate, which makes it a great model protein to be used in stability studies (Veitch 2004; Wu et al. 2014).

HRP is also widely used as cross-linking agent, especially in the production of injectable polymers, such as hydrogels (Saghati et al. 2021). Hydrogels are polymeric materials, reticulated in a three-dimensional form. Water is their dispersion medium, hence they are not complete solids nor liquids. This allows them to have solid behaviour and maintain their shape and volume, while having the thermodynamic properties of a liquid, which facilitates the diffusion of solutes inside the matrix. Along these properties, hydrogels are also characterised by high elasticity and plasticity, decreased friction, biodegradability and biocompatibility (Li, Lu, and Liu 2023). Since they were first developed in 1960, hydrogels have been applied in the food industry, materials and biological engineering and biosensors. The use of natural polymers, such as collagen or hyaluronic acid (HA), for hydrogel preparation, raised attention for their application in tissue engineering (Hoffman 2012; Li, Lu, and Liu 2023). Considering their preparation, hydrogels can be divided into chemical and physical hydrogels. In chemical hydrogels the polymeric chains are connected by covalent bonds, hence making them more stable. Polymers can be crosslinked by a variety of agents, such as radiation, chemical crosslinkers or enzymes (Trombino et al. 2019).

1.3.2.2 Therapeutic Proteins

The development of the recombinant DNA technology back in the 1970's, was a moment of change for the field of biotechnology. Ever since, the research in therapeutic proteins became the main application of this technology, with insulin being the first recombinant protein approved for medical use (Walsh 2003). Nowadays, therapeutic proteins are more accepted and widely used, and the examples include antibodies, vaccines, enzymes, growth factors and even anticoagulants (W. Wang 1999; Z. Wang et al. 2022). These have gathered a lot of attention due to their high affinity and activity, along with low toxicity and evident biological role (Z. Wang et al. 2022). Due to their high molecular weight and instability, therapeutic proteins are commonly formulated as aqueous solutions and delivered to the human body through injection, instead of orally as for other medicines (W. Wang 1999). Nevertheless, liquid formulations come with disadvantages concerning protein stability since they promote

hydrolysis, aggregation, and denaturation, which lead to short shelf life and the requirement of cold storage conditions.

As response to the limitations of liquid formulations, the possibility of freeze dry proteins has been investigated, considering that the solid-state is a stable form for proteins, while making transportation and storage easier and maintaining their biological activity (Z. Wang et al. 2022). Overall, freeze drying represents a valuable method for the preparation of chemically and physically stable proteins. Nevertheless, the functionality of proteins relies mainly on their tertiary and secondary structures and the several steps involved in the freeze drying process, such as dissolution of the proteins, freezing and primary and secondary drying, represent stress factors that can negatively influence the proteins' structure or storage stability.

Hence, it is important develop new stabilizing strategies. On the one hand, there is the option to use liquid formulations, such as in high-concentration protein formulations, simplifying the administration process (Thakral et al. 2021; Mensink et al. 2017). On the other hand, the development of strategies that avoid the use of the cold chain can also be a good path, since a great part of the costs associated to therapeutic proteins come from the high requirements for storage and transportation (Lopes et al. 2022).

Recently, Correia and co-workers tested the possibility to use NADES as preservative of virus like particles (VLP) based vaccines, as well as its efficacy in the protection of VLP when exposed to higher temperatures. The authors successfully demonstrated the potential of a NADES composed of trehalose and glycerol, in a molar ratio of 1:30, to preserve influenza VLP. Moreover, in an accelerated study it was concluded that the NADES under study was able to maintain the integrity and activity of VLP exposed to 50 °C, for 4 hours (Correia et al. 2021). These results show the ability of NADES to be used for the preservation of compounds under non-refrigerated conditions, which could be translated to other therapeutic proteins.

1.3.3 Cells

1.3.3.1 Mammalian cells

The methods for cell preservation can be classified, depending on their temperature range, in hypothermic preservation (1 to 35 °C) and cryopreservation (-80 to -196 °C) (Ma et al. 2021). Preserved mammalian cells are one of the main pillars of cell-based medicine and of clinical

investigation. Proper preservation of the cells is the key to prevent degradation during shipment and storage for long periods. Nowadays, there is a wide variety of cell lines available commercially, that can come from tumours or immortalized cell lines. Cells are also available from non-profit organization, such as biobanks, or primary cells collected during procedures such as biopsies (Dewhurst, Molinari, and Sayer 2021; Ma et al. 2021).

Cryopreservation is considered the gold-standard method for cell preservation for long-term storage and has been explained in more detail in section 1.1.3 of this chapter. Concerning transportation, cryopreserved samples can be transported either using dry ice or liquid nitrogen, which allow to keep the required temperatures. Maintaining the low temperatures is extremely important due the inherent toxicity of DMSO, the most used CPA, towards cells after thawing, that can lead to decrease of viability (Dewhurst, Molinari, and Sayer 2021).

Hypothermic preservation can be used for short-term preservation, up to a maximum of three days. In this case, cells are kept bellow physiological temperature, hence their metabolism and oxygen demand are decreased to a state called "cell pausing" (Ma et al. 2021). Compared to cryopreservation, hypothermic preservation allows for easier storage which correlates with lower cost and does not require the use of CPA. It is mostly used in remote research or treatment centres and for transport of biological samples from the collection site to the laboratory (Ma et al. 2021). Hypothermic preservation is also used for organs preservation, however there is high risk for hypothermia-induced damage. Nevertheless, protectant solutions composed of amino acids, saccharides and salts have been developed, allowing to preserve organs up to three days, by decreasing the production of ROS, slowing the cell cycle, and decreasing cell swelling that can lead do cell death. Still, the efficiency of these solutions is limited and hypothermic preservation of cells and organs is still under extensive research, hence the development of new protective solutions is of the utmost importance (Ma et al. 2021).

Cells can also be preserved for short periods, up to 24 hours, at normothermic conditions, at temperatures close to 37 °C. This is mostly applied in organs intended for transplantation, but cells can also be kept in culture media for transportation. However, due to exhaustion of nutrients and oxygen, it is limited to 24 hours (Dewhurst, Molinari, and Sayer 2021).

The main bottleneck in cell preservation is the compatibility and efficiency of the traditional protectants that are currently used, however some alternatives emerged in the past years, such

as the application of NADES as CPA. This was first reported in 2018, by Castro and co-workers, in which a NADES composed of trehalose and glycerol in a molar ratio of 1:30 was used as a substitute to DMSO in the preservation of mouse fibroblasts of connective tissue cell line (L929). The results showed that the tested NADES was as effective as the gold-standard CPA, with the advantage of being composed of natural and biocompatible molecules (Castro et al. 2018). More recently Jesus and co-workers, published a list with more than 15 combinations of sugars, amino acids and polyols that are able to form stable NADES and tested them as CPA in the cryopreservation of L929 and human immortalized keratinocyte cell line (HaCaT). Their results showed that, besides having low toxicity, the NADES are efficient in the role of CPA. It was also observed that not removing the NADES immediately after thawing, improved the recovery of the cells, hence represent an advantage in comparison to traditional CPA (Jesus et al. 2021a).

1.3.3.2 Red Blood Cells

According to the World Health Organization (WHO), in 2018, over 118 million blood donations were collected worldwide. The collected blood can be used for "whole blood" (WB) transfusion or processed to separate its various components. Blood is composed of plasma, red blood cells (RBC), white blood cells (WBC), and platelets. Combined, these cells provide blood its main functions since, RBC are responsible for the transport of oxygen and carbon dioxide between the lungs and tissues, WBC are part of our immune system and platelets are responsible for homeostasis and coagulation (Glenn and Armstrong 2019).

RBC, or erythrocytes, are the main component of blood, comprising more than 90% of the cells present in the blood. Blood cells are produced in the red bone marrow, and the differentiation of stem cells into RBC takes 5 to 7 days. They reach the blood stream with the well-known biconcave disc shape, without nucleus or any other organelles, and remain in circulation for about 120 days. This adaptation allows RBC to have higher contact surface for gas exchange and to have more room for haemoglobin, that accounts for c.a. 33% of RBC mass (Glenn and Armstrong 2019).

Haemoglobin (Hb) is a metalloprotein with high affinity towards oxygen, that is present on the cytosol. Each Hb molecule contains four atoms of iron than bind reversibly to four molecules of oxygen, forming oxyhaemoglobin, which carries the oxygen from the lungs to the tissues where it is needed. The affinity of haemoglobin towards oxygen can be influenced by

alterations of pH or temperature, which can happen due to some diseases (Glenn and Armstrong 2019).

Blood products are essential in hospitals and treatment centres, as they are needed for several cases, such as during most surgeries, for anaemia treatment, during resuscitation of patients, and even for care of premature infants. Hence, blood donations are needed worldwide due to the high demand of blood products, but also because blood products have limited shelf life (S. R. Xie et al. 2012). To maximise the application of all blood constituents, WB is divided into RBC, WBC, and platelets. After separation, RBC are mixed with an additive solution, and stored at 4 °C for a maximum of 42 days (Islamzada et al. 2022).

The standard method for RBC preservation is refrigeration with additive solution. The first additive solution to ever be used was a simple mixture of citrate, that acts as anticoagulant, and glucose, that feeds the cells' metabolism. The storage solutions currently used are a result of the intensive study of storage lesions of RBC and are composed of several compounds, such as citrate, glucose, phosphate, and adenine. These additive solutions also provide extra volume and more nutrients, hence extending the shelf life of RBC. The first additive solution to be developed was saline-adenine-glucose (SAG), which allowed the preservation of RBC for 35 days with high *in vivo* recovery and low haemolysis. However, it was observed that the extent of haemolysis was highly dependent on the donor and was one of limiting factors of further extending the shelf life of RBC. Hence, mannitol was added to the solution, saline-adenine-glucose-mannitol (SAGM), since it works as a membrane stabilizer and free radical scavenger. Currently, SAGM is used as the standard additive solution in Europe and in the USA and allows to preserve RBC for 42 days (Hess 2006).

Despite these improvements, it has been estimated that 10% of the collected blood units are discarded due to their short shelf life. This not only represents a waste of blood donations and funds, but also numerous health complications caused by blood shortage in some areas (Asghar et al. 2014). Also, it has been reported that the transfusion of older RBC bags can lead to complications that are related with storage lesions which include structural, metabolic and morphologic changes on RBC (Islamzada et al. 2022).

Cryopreservation of RBC is one of the options to achieve longer preservation period and reduce storage lesions. In Europe, RBC cryopreservation is performed by fast freezing method using low concentrations of glycerol as CPA. There are some limitations associated to the use of

glycerol as CPA, such as the need to remove it after thawing to limit the extent of haemolysis. Hence other CPA should be considered for this application. PVA has been tested for the cryopreservation of RBC, however it is still not as effective as glycerol. Unfortunately, due to the high costs of maintaining the blood at -80 °C and the need of specialized people, cryopreservation of RBC is limited to specific situations, such as natural calamities or military settings (Asghar et al. 2014).

Some authors have also discussed the possibility to freeze dry platelets and RBC, since it would considerably increase the shelf life of these products. This could represent a major advance, since freeze dried products do not require cold storage which would facilitate transportation of RBC to remote areas. Even though several studies have been performed this strategy for RBC preservation remains in a very preliminary stage (Merivaara et al. 2021).

1.4 Aims of the Thesis

As it has been explained throughout this introduction the preservation of biotherapeutics is still limited by numerous factors such as high costs, the need of the cold-chain, lack of stability of aqueous formulations or even the short shelf life achieved by the strategies in use. In this work we studied the stabilisation of different biomolecules, starting from the simplest, AA, and moving on to more complex structures, such as proteins, and finally cells.

The study with AA (Chapter 3), was performed with the food industry in mind, hence a simple formulation was developed using chemicals already approved for human consumption. In this chapter the antioxidant activity of AA in several NADES formulations was assessed. A set of toxicological assays was also performed against the animal model *Paleoman varians*. Finally, a proof-of-concept application was developed, by testing the ability of the NADES+AA formulations to preserve fresh-cut fruits.

Moving on to the study with proteins, horseradish peroxidase (HRP) was used as a model protein. In Chapter 4, the activity and thermostability of HRP was evaluated in the presence of betaine-based NADES. Moreover, the influence of a_w of the said systems in the enzymatic activity was also assessed. Chapter 5 presents a potential application the highly active and thermostable HRP in the preparation of natural deep eutectogels to be used as DDS for non-steroid anti-inflammatory drugs (NSAID).

Lastly, Chapter 6, focuses on the preservation of RBC using NADES as substitutes for the currently used additive solutions. With this work, our goal was to determine whether it could be possible to extend the shelf life of RBC past the currently 42 days. This is an important topic, since annually millions of blood donations are wasted due to being over the determined shelf life.

MATERIALS AND METHODS

2.1 Materials

The chemicals used in different parts of the work are herein reported:

- *Chapter 3*

Choline chloride (ChCl, $\geq 98\%$, CAS 67-48-1), L-ascorbic acid (AA, 99%, CAS 50-81-7) and citric acid monohydrate (CA, $\geq 99\%$, CAS 5949-29-1), obtained from Sigma-Aldrich (St. Louis, Missouri, USA), trehalose dihydrate (Treh, CAS 6138-23-4) kindly provided from Hayashibara Co., LDA (Okayama, Japan) and betaine anhydrous (Bet, $>97\%$, CAS 107-43-7) obtained from TCI (Tokyo, Japan) were used for the preparation of NADES. ChCl was dried and kept in a closed flasks in the oven at 40 °C, until used.

On the determination of the antioxidant activity the reagents used were: 1,1-diphenyl-2-picrylhydrazyl (DPPH, CAS 1898-66-4, Sigma-Aldrich) and methanol (p.a., CAS 67-56-1, Carlo Erba, Emmendingen, Germany).

For the toxicological assays several reagents were used, namely, reduced L-glutathione (GSH, CAS 70-18-8) and 1-chloro-2,4-dinitrobenzene (cDNB, CAS 97-00-7), xanthine (CAS 69-89-6), nitroblue tetrazolium (NBT, CAS 298-83-9), xanthine-oxidase (XOD, CAS 9002-17-9), hydrogen peroxide 30% (CAS 7722-84-1), sodium dodecyl sulfate (SDS, CAS 151-21-3), thiobarbituric acid (TBA, CAS 504-17-6), acetylcholine iodide (CAS 2260-50-6), 5,5'-dithio-bis-2-nitrobenzoic acid (CAS 69-78-3), myoglobin (CAS 100684-32-0) and potassium phosphate buffer, from

Sigma-Aldrich. Ethylenedinitrilotetraacetic acid (EDTA, CAS 60-00-4) was purchased from Riedel-Haen, Berlin, Germany. 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS, CAS 30931-67-0) was obtained from Alfa Aesar, Germany. Trichloroacetic acid (TCA, CAS no. 76-03-9) was obtained from Panreac, Spain, and malondialdehyde bis(dimethylacetal) (MDA, CAS no. 102-52-3) was purchased from Merck, Darmstadt, Germany.

▪ *Chapters 4 and 5*

For NADES preparation the compounds used were: D-(+)-xylose (Xyl, $\geq 99\%$, CAS 58-86-6, Sigma-Aldrich), glycerol (Gly, $\geq 99.5\%$ CAS 56-81-5, Sigma-Aldrich), D-sorbitol (Sorb, $\geq 98\%$, CAS 50-70-4, Sigma-Aldrich), DL-proline (Pro, 99% , CAS 609-36-9, Sigma-Aldrich), trehalose dihydrate (CAS 6138-23-4, Hayashibara Co., LDA), betaine anhydrous ($> 97\%$, CAS 107-43-7, TCI) and sucrose (Suc, CAS 57-50-1, Cmd Chemicals, Funchal, Portugal).

For enzymatic reactions, phenol-4-sulfonic acid sodium salt dihydrate (PSA, 98% , CAS 10580-19-5, Sigma-Aldrich), 4-aminoantipyrine (4-AAP, $\geq 99\%$, CAS 83-07-8, Sigma-Aldrich) and hydrogen peroxide 30 % solution (H_2O_2 , CAS 7722-84-1, Sigma-Aldrich) were used. Lyophilized powder of peroxidase from horseradish (HRP, type I, $89.63 \text{ U} \cdot \text{mg}^{-1}$ solid, CAS 9003-99-0, Sigma-Aldrich) was used without further purification.

For the preparation of hydrogels, the following compounds were used: gelatine from bovine skin (~ 225 Bloom, type B, CAS 9000-70-8, Sigma-Aldrich), 2-morpholinoethanesulfonic acid buffer (MES, CAS 145224-94-8, Sigma-Aldrich), sodium phosphate monobasic ($\geq 99\%$, CAS 7558-80-7, Sigma-Aldrich), *N*-hydroxysuccinimide (NHS, $\geq 98\%$, CAS 6066-82-6, Thermo Scientific, Kandel, Germany) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, $\geq 98\%$, CAS 25952-53-8, Thermo Scientific) and tyramine hydrochloride (Tyr, CAS 60-19-5, TCI). Ketoprofen (Ket, $\geq 98\%$, CAS 22071-15-4, Sigma-Aldrich), was used for the drug delivery test.

▪ *Chapter 6*

For NADES preparation the following compounds were used: betaine anhydrous ($> 97\%$, CAS 107-43-7, TCI), glycerol ($\geq 99.5\%$ CAS 56-81-5, Sigma-Aldrich), D-sorbitol ($\geq 98\%$, CAS 50-70-4, Sigma-Aldrich), DL-proline (99% , CAS 609-36-9, Sigma-Aldrich), trehalose dihydrate (CAS

6138-23-4, Hayashibara Co., LDA), sucrose (CAS 57-50-1, Cmd Chemicals), D-(+)-glucose (Glc, $\geq 99.5\%$, CAS 50-99-7, Sigma-Aldrich), ethylene glycol anhydrous (EtGlycol, 99.8%, CAS 107-21-1, Sigma-Aldrich), *N*-acetyl-L-cysteine (Nac, for biochemistry, CAS 616-91-1, Sigma-Aldrich), lactobionic acid (LB, 97%, CAS 96-82-2, Sigma-Aldrich).

General reagents used: phosphate buffered saline (PBS) tablets (Sigma-Aldrich) prepared as indicated by the supplier (one tablet/200 mL, yielding 10 mM phosphate buffer, pH 7.2-7.6), sodium chloride (NaCl, $\geq 99.5\%$, CAS 7647-14-5 Sigma-Aldrich), D-(+)-glucose monohydrate (puriss., 5996-10-1, Sigma-Aldrich), and adenine ($\geq 99\%$, CAS 73-24-5, Sigma-Aldrich).

For cell culture, Eagle's minimum essential medium (MEM, Corning), fetal bovine serum (FBS, Corning), antibiotic-antimycotic solution of penicillin and streptomycin (PS, Corning), CellTiter96® AQueous One Solution Cell Proliferation Assay (Promega) and carbon dioxide (CO₂, 99.95%, CAS, 124-38-9, Air Liquide).

2.2 Methods

2.2.1 Preparation and Characterization of Natural Deep Eutectic Systems

2.2.1.1 Preparation of Natural Deep Eutectic Systems

The natural deep eutectic systems (NADES) prepared and used throughout this work are listed in Table 2.1. The systems were all prepared using the heating and stirring method (Meneses et al. 2019). Using this method, the individual components of the NADES were weighed into flasks, after which they were closed and heated at 40 – 60 °C, with constant stirring, until a transparent and viscous fluid was obtained. All the NADES prepared were kept in closed flasks until used.

Table 2.1 – Code name, molar ratio and components of the NADES prepared throughout this work. The chapter in which they are used is also indicated.

Code	Molar ratio	Components	Chapter	Ref.
ChCl:AA	2:1	Choline chloride, ascorbic acid	3	Silva et al., (2018)
ChCl:CA	1:1	Choline chloride, citric acid	3	Dai et al., (2013)
CA:Treh	2:1:3	Citric acid, trehalose, water	3	Dai et al., (2013)
Bet:CA	1:1:3	Betaine, citric acid, water	3	Dai et al., (2013)
BXylW	2:1:6	Betaine, xylose, water	4	Jesus et al. (2021)
BTrehGlyW	2:1:3:5	Betaine, trehalose, glycerol, water	4	Jesus et al. (2021)
BSorbW	1:1:3	Betaine, sorbitol, water	4,6	This work
BSucProW	5:2:2:21	Betaine, sucrose, proline, water	4	Jesus et al. (2021)
BGly	1:2	Betaine, glycerol	4,5,6	Rodrigues et al. (2021)
BTrehW	4:1:10	Betaine, trehalose, water	6	Jesus et al. (2021)
BEtGlycol	1:3	Betaine, ethylene glycol	6	Rodrigues et al. (2021)
BGlySorbW	1:2:1:3	Betaine, glycerol, sorbitol, water	6	Jesus, Duarte, and Paiva (2022)
TrehGlcSucW	1:1:1:11	Trehalose, glucose, sucrose, water	6	Jesus et al. (2021)
BProW	1:2:10	Betaine, proline, water	6	Sarmiento et al. (2023)
ProGlySorbW	1:1:1:3	Proline, glycerol, sorbitol, water	6	Jesus et al. (2021)
TGly	1:30	Trehalose, glycerol	6	Castro et al. (2018)
GlyGlc	4:1	Glycerol, glucose	6	Jesus et al. (2021)
BNacW	1:1:3	Betaine, acetyl cysteine, water	6	Sarmiento et al. (2023)
GlyLB	4:1	Glycerol, lactobionic acid	6	This work

The molecular mass ($\text{g}\cdot\text{mol}^{-1}$) of the prepared NADES was calculated according to Equation 2.1, in which X and M represent, respectively, the molar fraction and molecular mass of each component.

$$M_{NADES} = X_A \times M_A + X_B \times M_B + X_C \times M_C \quad \text{Equation 2.1}$$

2.2.1.2 Determination of Water Content

The water content the NADES prepared was determined by Karl-Fisher (KF) titration, performed in an 831 KF Coulometer (Metrohm, Herissau, Switzerland) with generator electrode without diaphragm, using Hydranal Coulomat AG (Honeywell, Charlotte, North Carolina, USA) as reagent. For each sample, the water content was determined in triplicate.

2.2.1.3 Determination of Water Activity

The water activity (a_w) of the prepared NADES was determined using a AwTherm - Water Activity meter (Rotronic, Bassersdorf, Switzerland). The measurements were performed in equilibrium mode, at 37 and 60 °C. All measurements were performed in triplicate.

2.2.1.4 Determination of Viscosity

The viscosity of the NADES was determined using a MCR 102 rheometer (Anton Paar, Graz, Austria), fitted with a 50 mm parallel plate geometry (PP50, Anton Paar), using a 1 mm gap. Before the measurement, samples were pre-equilibrated at 50 °C for 10 minutes, after which, a temperature scan from 50 to 10 °C was performed ($5\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$), under a constant shear rate of 10 s^{-1} . When determining the viscosity of samples with water contents over 50 wt.%, the gap was changed to 0.5 mm with all the other conditions kept the same. All measurements were performed in triplicate.

2.2.1.5 Determination of Density

The density of the systems were determined using an Anton Paar SVM 3001 viscometer (Graz, Austria) in a temperature range from 20 to 80 °C ($\pm 0.03\text{ }^{\circ}\text{C}$), with 10 °C steps. The measurements were performed in triplicate for each sample. The pressure of the equipment was 100 kPa, and the uncertainty of density measurements was $0.0002\text{ g}\cdot\text{cm}^{-3}$. For chapter 4, the dynamic viscosity of the NADES was also determined using this equipment.

2.2.2 Stabilisation of Ascorbic Acid

2.2.2.1 Preparation of solutions of NADES

To determine the stabilization of AA in aqueous solutions of NADES (NADES-AS), AA was dissolved in the different NADES by heating at 40 °C and gently stirring the mixture, for a few minutes. The mixtures were allowed to cool down, and water was added to obtain final concentrations of 0.5 mg·mL⁻¹ of AA and 125 mg·mL⁻¹ of NADES. The antioxidant capacity of the samples was analysed at time points of 0, 2 and 5 days. In the cases where high antioxidant activity was detected additional time points were considered (7 and 9 days). All solutions (in duplicate) were stored at room temperature and exposed to light. As control, the same time points were analysed on a solution of 0.5 mg·mL⁻¹ of AA.

2.2.2.2 Determination of antioxidant activity

The antioxidant activity of all the samples was determined by following the DPPH assay as described by Pedras et al. (2017). The assay allows for the determination of the percentage of radical scavenging activity (%RSA) of each sample, calculated through Equation 2.2. Briefly, 150 µL of sample were added to 4 mL of DPPH reagent solution in methanol and left in the dark for 40 min, after which the absorbance at 517 nm was measured in a ultraviolet-visible (UV–vis) spectrophotometer (Genesys 50, Thermo Scientific, Waltham, MA, USA). Water was used as blank sample. The test solutions were diluted from 0.5 to 0.05 mg·mL⁻¹ of AA and the half maximum effective concentration (EC₅₀) of AA was determined. All samples were analysed in triplicate.

$$\%RSA = \frac{A_{DPPH} - A_{sample}}{A_{DPPH}} \quad \text{Equation 2.2}$$

2.2.2.3 Toxicological Assessment

2.2.2.3.1 Exposure of *Palaemon varians* to NADES

Palaemon varians were obtained from a local producer (Alcochete, Portugal) and transported alive in a cooler box to the laboratory facilities. The healthy animals were acclimatized at laboratory conditions for at least 24 h in aquaria (10 L) with continuous aeration and water recirculation (>6 mg O₂·L⁻¹, with a carbon filter, at room temperature). Then the animals were

transferred to new tanks (15 L) and acclimatized in saline artificial water (salinity ≈ 33 ‰, CORAL PRO SALT, Red Sea, Israel) at a pH between 8.0 and 8.2, subject to a photoperiod of 12/12.

Before performing any assays, 10 animals were sacrificed to assess their initial status (T0). For the toxicity assessment, the animals ($n=70$; 460.9 ± 250.3 mg) were randomly distributed by seven groups, as shown in Table 2.2. The concentrations of NADES tested were 1, 5 and 10 %w/v. Each solution was placed in the aquaria in a specific ratio based on animals' weight. During the assay, the animals were fed every 24 h with store-bought flakes (Tetra brand, Blacksburg, VA, USA). The pH and temperature of the water were checked daily, as well as cumulative mortality. After seven days, *P. varians* were euthanized through quick freezing at -45 °C and weighted. Their digestive system and muscle were removed, weighted, and stored at -45 °C until further processing.

Table 2.2 - Number of animals (*P. varians*) and weight for group (mean $\pm$ SD, mg) in the toxicity assay.

NADES	Concentration (mg _{NADES} ·g _{animal} ⁻¹)	Number of animals	Animal weight (mg)
Control	-	10	612.7 $\pm$ 306.9
Bet:CA	0.29	10	316.7 $\pm$ 200.4
	1.43	10	353.6 $\pm$ 246.1
	2.77	10	487.2 $\pm$ 237.8
ChCl:AA	0.29	10	476.8 $\pm$ 221.2
	1.42	10	442.8 $\pm$ 228.9
	2.80	10	529.5 $\pm$ 233.8

2.2.2.3.2 Sample Treatment

Using a tissue homogenizer (Parkside, Landau, Germany), the digestive system and muscular tissue were homogenized respectively, with 1 mL and 2 mL of cold PBS (pH 7.4). The tissue homogenates were kept in the freezer (-45 °C) overnight, and centrifugated at 4 °C ($15000\times g$, 10 min). The supernatant was collected and stored at -45 °C. In order to normalize the results of the biochemical assays, the total mass of cytosolic proteins was determined through the Bradford method, using bovine serum albumin (BSA) as standard, in concentrations ranging from 0 to 4 mg·mL⁻¹ (Bradford 1976).

2.2.2.3.3 Glutathione S-transferase Activity

The protocol to determine the activity of glutathione-S-transferase (GST) (EC 2.5.1.18) was adapted from Habig, Pabst, and Jakoby (1974). The assay is based on the synthesis of a conjugate between GSH and cDNB. Briefly, 180 μL of substrate solution (GSH 200 mM and cDNB 100 mM, in PBS) were added to 20 μL of sample in a 96-well microplate (Greiner Bio-one, Austria). The total enzyme activity was determined by recording the absorbance at 340 nm, every minute for a total of 6 minutes, in a microplate reader (Synergy HTX, BioTek, Winooski, VT, USA). The change in absorbance per minute was calculated and the reaction rate at 340 nm was determined using a molar extinction coefficient (ϵ) of $0.0053 \mu\text{M}^{-1}\text{cm}^{-1}$. The results are expressed as $\text{nmol}\cdot\text{min}^{-1}\text{mg}^{-1}$ cytosolic protein.

2.2.2.3.4 Superoxide Dismutase Activity

The determination of the activity of superoxidase dismutase (SOD) (EC 1.15.1.1) was adapted from Sun, Oberley, and Li (1988). In this method, superoxide radicals ($\bullet\text{O}_2^-$) are generated by the reaction of xanthine with XOD, leading to the reduction of NBT to formazan. SOD competes with NBT for the dismutation of $\bullet\text{O}_2^-$, inhibiting its reduction. The degree of inhibition is equivalent to the activity of SOD. In a 96-well microplate, 200 μL of PBS (50 mM, pH 8.0) were mixed with 10 μL of xanthine 3 mM, 10 μL of NBT 0.075 mM and 10 μL of EDTA 3mM and 10 μL of standard or sample. To start the reaction, 10 μL XOD were added, and the absorbance at 560 nm was recorded every 2 minutes for 26 minutes, using a microplate reader. The SOD activity was determined as the percentage of inhibition of NBT-diformazan and was expressed as $\text{U}\cdot\text{mg}^{-1}$ cytosolic protein.

2.2.2.3.5 Acetylcholinesterase Activity

Acetylcholinesterase (AChE, EC 3.1.1.7) activity was measured using the method described by Ellman et al. (1961). Briefly, 250 μL of mix solution (PBS 50 mM, acetylcholine iodide 75 mM and 5,5'-dithio-bis-2-nitrobenzoic acid 1 mM) were added to each well, along with 50 μL of sample or buffer (blank). The absorbance at 415 nm was read every minute during a total of 10 min in a microplate reader. The AChE activity was expressed in $\text{nmol}\cdot\text{min}^{-1}\text{mg}^{-1}$ cytosolic protein.

2.2.2.3.6 Catalase Activity

The catalase (CAT, EC 1.11.1.6) activity was determined with a method adapted from Beers and Sizer (1952). The consumption of H_2O_2 by catalase was measured by the decrease of absorbance at 240 nm. Briefly, a substrate solution of H_2O_2 (0.036% w/w) in potassium phosphate buffer (50mM; pH 7.0) was prepared. Then, 193 μL of substrate solution and 7 μL of sample were mixed in a 96-well microplate (UV-Star, Greiner-bio-one, Germany) and the absorbance at 240 nm was recorded for 3 min, with 13 seconds' intervals. CAT's activity was determined considering the initial velocity of the reaction and the $\epsilon(\text{H}_2\text{O}_2)$ $0.04 \mu\text{M}^{-1}\text{cm}^{-1}$. The results were expressed in $\text{nmol}\cdot\text{min}^{-1}\text{mg}^{-1}$ cytosolic protein.

2.2.2.3.7 Total antioxidant capacity

Total antioxidant capacity (TAC) was determined according to the method described by Kambayashi et al. (2009). Briefly, in a 96-well microplate, 10 μL of the sample, 10 μL of myoglobin 90 μM , 150 μL of ABTS 600 mM were mixed. To start the reaction, 40 μL H_2O_2 500 μM were added. The microplate was incubated for 5 min, after which the absorbance at 415 nm was recorded in a microplate reader. The quantification of TAC was performed using a calibration curve prepared with Trolox, in concentration between 0 and 0.33 mM. The results are expressed in $\text{nmol}\cdot\text{mg}^{-1}$ cytosolic protein.

2.2.2.3.8 Lipid peroxidation

The lipid peroxidation (LPO) assay was adapted from the thiobarbituric acid reactive substance (TBARS) protocol (Uchiyama and Mihara 1978). Briefly, in a microtube, 5 μL of sample were mixed with 45 μL of PBS (pH 7-7.4). Then, 12.5 μL of SDS 8.1%, 93.5 μL of TCA 20% and 93.5 μL of TBA 1% were added. To this mixture, 50.5 μL of MQ-grade ultrapure water were added and then microtubes were mixed in a vortex (30 seconds). With lids punctured, the microtubes were incubated for 10 minutes in boiling water, after which they were immediately transferred to ice to cool. Then, 62.5 μL of MQ-grade ultrapure water were added to each microtube. The microtubes were then centrifuged ($5000\times g$, 1 min). The samples were transferred to 96-well microplates and the absorbance at 530 nm was recorded. All samples were analysed in

duplicate. As standard, a calibration curve (0 - 0.3 μM TBARS) was prepared with MDA. The results are expressed in $\text{pmol}\cdot\text{mg}^{-1}$ cytosolic protein.

2.2.2.4 Statistical analysis

To compare the different treatments, an integrated biomarker response (IBR) index was calculated, and the respective radar plots were constructed. IBR was calculated following the protocol described by Beliaeff and Burgeot (2002) using Excel. Briefly, an overall average (m) and standard deviation (s) of all data for each biomarker was computed and then standardised for each situation to obtain $y=(x-m/s)$. Then $Z=-Y$ or $Z=Y$ were be calculated to determine if the biological effect was inhibitory or activator, respectively. Score (S) value was calculated as $S = Z+|\text{Min}|$, where Min is the minimum value for all calculated Y , in each biomarker, and $S \geq 0$. The scores (S) were then plotted as stars and the IBR calculated trough Equation 2.3 and Equation 2.4.

$$IBR = \sum \sin \beta (S_i \cdot \cos \beta + S_{i+1} \cdot \cos \beta) \quad \text{Equation 2.3}$$

$$\beta = \tan^{-1}((S_{i+1} \cdot \sin \alpha) / ((S_i - S_{i-1}) \cdot \cos \alpha)) \quad \text{Equation 2.4}$$

In these equations, S_i and S_{i+1} are two successive clockwise points (radius coordinates) of a given star plot, $\alpha = 2\pi/n$ with n representing the number of biomarkers used. The IBR was calculated using the same parameter ordering for all tissues. Statistical analyses were performed using one-way ANOVA or Kruskal-Wallis tests followed by Dunnett's multiple comparison test, depending on if the parametric assumptions were fulfilled or not.

2.2.2.5 Validation of NADES-AS as coating material

To investigate the potential of NADES-AS to be used as coating material for the preservation of fresh-cut fruits and vegetables, a proof-of-concept test was performed with russet apple slices. Apples were washed and dried and slices were cut and immediately dipped in the test solutions for 1 min. The tested solutions were $125 \text{ mg}\cdot\text{mL}^{-1}$ of NADES and $125 \text{ mg}\cdot\text{mL}^{-1}$ of NADES with $0.5 \text{ mg}\cdot\text{mL}^{-1}$ of AA. Water and AA ($1 \text{ mg}\cdot\text{mL}^{-1}$) were used as negative and positive controls, respectively. After dipping, the excess solution was removed in paper towels and the

slices were placed in petri dishes, at room temperature. The changes in apple's appearance were observed and pictures were taken at 0, 1, 2 and 24 h.

2.2.3 Stabilisation of Horseradish Peroxidase in NADES

2.2.3.1 Enzymatic Activity Assay

HRP in NADES mixtures were prepared by suspending HRP in pure NADES. After that, PBS (100 mM, pH 7) was added, resulting in the dissolution of all the components in PBS, hence obtaining a NADES-AS, containing 1 mg·mL⁻¹ HRP, and 20 wt.% NADES. The enzymatic activity of HRP in the presence of the NADES was determined using a colorimetric method adapted from Wu et al. (2014). Briefly, in a cuvette, 950 µL of PSA, 950 µL of 4-AAP, and 50 µL of H₂O₂, were added to 1 mL of PBS, yielding the final concentrations of 10 mM, 2.4 mM, and 2 mM, respectively. After homogenization, this solution was used as a blank reference. Then, 50 µL of NADES-AS was added, and the increase in absorbance at 490 nm was followed for 1 minute in a UV-Vis spectrophotometer (Genesys 50, Thermo Scientific). The molar extinction coefficient (ϵ) of 5560 M⁻¹·cm⁻¹ was used as determined elsewhere (Vojinović et al. 2004). The assays were performed in triplicate at 25 °C. The concentration of HRP in NADES-AS was determined using the Lowry method for protein quantification (Lowry et al. 1951), using bovine serum albumin (BSA) as standard, in concentrations ranging from 20 to 100 µg·mL⁻¹ at 25 °C.

2.2.3.2 Evaluation of HRP thermostability

Enzyme activity of HRP was tested upon incubation in NADES for 6 h and 24 h at 37 °C. To assess HRP activity under thermal stress (that will be further addressed as "thermostability"), two temperatures were tested, 60 and 80 °C. For the assays at 60 °C, the enzyme was incubated for 4 h and 24 h. For the assays at 80 °C, the tested incubation times were 2 h and 4 h. For all temperatures tested, after each incubation time, 3 mL of PBS were added to each mixture and these solutions were used to assess the activity of HRP, using the enzymatic activity assay described before. PBS (100 mM, pH 7) was used as control medium for all the conditions tested. All incubations were performed in triplicate. Control experiments, using NADES solutions without additional incubation time (considered as 0 h), were performed for all the systems, as well as for PBS.

2.2.3.3 Impact of water activity on HRP activity

Water is usually used to reduce the viscosity of NADES, however, it can play a key role in protein hydration shell dynamics, modifying the enzyme activity. To evaluate the impact of the water activity of BGly on HRP activity, the enzyme was suspended in the BGly formulations with different water contents (5 to 20 wt.%) and incubated at 37 °C, for 6 h and for 24 h. As described before, after each incubation time, PBS (100 mM, pH 7) was added to the incubated mixtures and the enzyme's activity was assessed using the enzymatic activity assay described before.

2.2.3.4 Thermal stability with nanoDSF

In this work, unfolding temperature ($T_{unfolding}$) was measured using the nanoDSF apparatus Prometheus NT.48 (NanoTemper, Munich, Germany). The method is based on the difference in measured fluorescence between tryptophan and tyrosine, present in abundance before and after the denaturation process, respectively. For this, the fluorescence ratio F350/F330 is used as previously described in the literature (Jaworek et al. 2021). Furthermore, the equipment has a back-reflection technology that detects the aggregation of the sample, by the attenuation of the light that passes through the cell, which is collected from its reflection on the surface of the sample. The equipment was charged with 10 μ L of the HRP (0.5 μ M) in NADES (20 wt.%) solution in buffer (PBS, 100 mM, pH 7). For data collection and data processing, the software PR.ThermControl, version 2.1.2, was used.

2.2.3.5 Circular Dichroism

Circular dichroism (CD) spectra were obtained between 190 and 250 nm in a Chirascan qCD spectrometer (Applied Photophysics, Leatherhead, UK) equipped with a Quantum Northwest TC125 temperature controller. After incubation in the conditions described before, HRP and NADES were dissolved in PBS (100 mM, pH 7) to the final concentrations of 5 μ M and 5 wt.%, respectively. Spectra were obtained at 25 °C, using a 0.1 mm path length. The secondary structure contents were calculated using CONTIN-LL (Provencher & Glockner Method), with reference data set SP175 (Lees et al. 2006), in the DICHROWEB web server (<http://dichroweb.cryst.bbk.ac.uk>).

2.2.4 Preparation of enzymatically crosslinked hydrogels

2.2.4.1 Synthesis of gelatine-phenol conjugates

Synthesis of gelatine-phenol (Glt-Ph) conjugates was adapted from Sakai et al. (2009) and Chuang et al. (2015). Gelatine from bovine skin was dissolved at 2 %w/v in MES buffer (50 mM, pH 6) at 60 °C. After complete dissolution of gelatine, the solution was let to cool to room temperature. The following compounds were added to the solution to initiate the reaction: 57.6 mM of Tyr, 38.3 mM of EDC, and 19.2 mM NHS. The pH was corrected to 6 using NaOH (10 M). The reaction proceeded overnight (16-18h) at 25 °C, under constant stirring. To stop the reaction, 50 mM sodium phosphate was added to the media and stirred for 1 hour.

The resultant polymer solution was dialyzed against deionized water, using SnakeSkin Dialysis tubing membranes (MWCO: 10,000, Thermo Scientific) for a minimum of 48 h, until no absorbance peak was detected at 275 nm in a UV-Vis spectrophotometer (Genesys 50, Thermo Scientific). The dialysed polymer was lyophilized for 48 hours and stored in closed flasks until use.

2.2.4.2 Quantification of gelatine modification extent

The conjugation reaction was confirmed by attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) performed in a Perkin Elmer Spectrum Two spectrometer. The samples – gelatine, Tyr or Glt-Ph – were placed directly in the ATR-FTIR cell, and spectra were recorded from 4000 to 400 cm^{-1} resolution with 16 scans, at room temperature.

To quantify the extent of the conjugation, 0.1 %w/v Glt-Ph was dissolved in water and the absorbance at 275 nm was determined using a UV-Vis spectrophotometer (Genesys 50, ThermoFischer Scientific). The percentage of conjugated tyramine in gelatine was calculated through a calibration curve prepared with Tyr (0.000625 to 0.2 %w/v) (Sakai et al. 2009). The total mass of tyramine in Glt-Ph was calculated using Equation 2.5. The yield of modification was calculated using Equation 2.6.

$$Total\ Tyr\ (mg) = Tyr\ \left(\frac{W}{v}\%\right) \cdot product\ mass\ (g) \cdot 10000 \quad \text{Equation 2.5}$$

$$Yield\ (\%) = \frac{Total\ Tyr\ (mg)}{Initial\ Tyr\ (mg)} 100\% \quad \text{Equation 2.6}$$

2.2.4.3 Preparation of Hydrogels

To prepare standard hydrogels, a 6.25 w/v.% Glt-Ph polymer solution in PBS was prepared, by heating at high temperature and low stirring. After complete dissolution of the polymer, the hydrogel was produced by gently mixing Glt-Ph and HRP solution, and later adding the H₂O₂ solution to start the reaction. Two concentrations of HRP solution were tested and the concentration of each component in the hydrogel was 5 %w/v Glt-Ph, using 5 or 10 units.ml⁻¹ HRP and 3mM H₂O₂ (Sakai et al. 2009).

2.2.4.4 Preparation of Eutectogels

For the preparation of eutectogels, the NADES BGly (1:2) was used. Due to high viscosity of the mixture, water was added to the NADES, at a ratio of 90 wt.% BGly and 10 wt.% water (90BGly). Using 90BGly, a 6.25 %w/v polymer solution was prepared, by heating at high temperatures and low stirring and let to rest overnight, before using to prepare gels. The gels were prepared in the same rational as before, freshly prepared solution of HRP was added to Glt-Ph in NADES solution and gently mixed, and H₂O₂ was added for the crosslinking reaction to start. The final concentration of the components in the eutectogel were, 5 %w/v Glt-Ph, 5 or 10 U·ml⁻¹ HRP and 3 mM H₂O₂.

2.2.4.5 Characterization of Eutectogels

2.2.4.5.1 Determination of gelation time

Determination of the gelation time was conducted by preparing the gels on a stirring plate at 37 °C. The Glt-Ph solution and HRP were poured in 6-well plates and stirred with magnetic stirrer at 60 rpm. After homogenization, H₂O₂ solution was added to start the crosslinking. The gelation time was considered as the time elapsed between the addition of H₂O₂ and the impairment of movement of the magnetic stirrer. The experiments were performed in triplicate (Sakai, 2007, 2009).

2.2.4.5.2 Stability Tests

Hydrogels and eutectogels were subjected to two stability assays, with and without immersion in PBS. For the tests with immersion, samples of both gels were placed in 1 mL of PBS in closed flasks, and kept in the oven at 37 °C, until complete dissolution in the buffer. For the non-immersed samples, prepared gels were stored in 6-well microplates and placed in the oven at 37 °C. The samples were inspected periodically, to determine any changes, and kept until the gels lost form or until 1 year was completed.

2.2.4.5.3 Determination of rheologic properties

The viscosity of the injection solution (Glt-Ph and HRP in 90BGly), in function of shear rate was determined in a MCR102 rheometer (Anton Paar) fitted with a 25 mm, 2° cone-plate geometry (CP25-2, Anton Paar), using shear rates ranging from 0.01 to 1000 s⁻¹, at a constant temperature of 25 °C. All measurements were performed in triplicate.

The rheological properties of the prepared hydrogels and eutectogels were determined using a MCR102 rheometer (Anton Paar) fitted with an 8 mm parallel plate geometry with sandblasted surface (PP08/S, Anton Paar) in oscillatory method. The measurements were performed in the linear viscoelastic region (LVR) of the materials, determined by amplitude sweep at a frequency of 1 Hz, 37 °C and within a shear strain (γ) ranging from 10⁻⁶ to 10 (LVR from 10⁻⁶ to 0.5) (Nicholls, 2017). Frequency sweep analysis was performed under controlled deformation (using a shear strain of 0.2, in the LVR). The gels were equilibrated at 37 °C for 10 min and the storage (G') and loss (G'') modulus were determined in a frequency range from 10 to 0.1 rad·s⁻¹. The properties of the eutectogels were analysed after preparation and at 1 month, 3 months and 1 year. All measurements were performed in triplicate.

2.2.4.6 Preparation of drug delivery systems

2.2.4.6.1 Dissolution of ketoprofen in NADES

The dissolution of ketoprofen in NADES was determined by adding 10 mg of Ket to 1 g of NADES, and mixing it in a closed vial, for several hours until a transparent liquid was obtained. The dissolution was confirmed in a polarized optical microscope (POM) using the Olympus BX-53-F2 (Olympus Iberia, Barcelona, Spain) microscope. A droplet of the mixture was placed on

a microscope glass slide and then observed. The images were obtained with an equipped camera (Olympus SC50) using the software Olympus stream start 2.3.

2.2.4.6.2 Incorporation of ketoprofen in eutectogels

To prepare eutectogels with Ket, 10 mg·g⁻¹ of drug were added to the polymer solution in 90BGly, after complete dissolution of Glt-Ph. The gels were then prepared as described before, using only 10 U·ml⁻¹ of HRP. All samples were prepared in triplicate, stored at 37 °C over-night and used for release studies in the following day.

2.2.4.6.3 Drug release and solubility studies

For the release studies, two types of samples were used – eutectogel prepared with Ket (10 mg·g⁻¹) and BGly with dissolved Ket (10 mg·g⁻¹). Each sample, was placed in a 1.5 mL glass vial, closed with a cellulose membrane (0.45 µm, Sartorius, Germany). The vials were then placed in 15 mL tubes, containing 2 mL of PBS as release media, with the membrane facing down. The tubes were kept in a thermostat water bath (Grant, LSB 12 Aqua Pro, United Kingdom) at 37 °C, stirring at 60 rpm. At pre-determined time points, 100 µL of the released media were collected and replaced by the same volume of fresh PBS.

The amount of ketoprofen released from the samples was determined using a calibration curve of ketoprofen in PBS with concentrations ranging from 1 to 10 mg·L⁻¹. The absorbance of the samples at 262 nm was determined in a microplate reader (VICTOR Nivo TM, PerkinElmer, USA). The solubility of Ket in PBS was determined as the maximum amount of ketoprofen released to the PBS, once equilibrium was reached.

The release profile of ketoprofen in PBS was obtained by calculating M_t/M_{inf} , where M_t represents the mass of ketoprofen released at time t and M_{inf} represents the total mass of ketoprofen released at equilibrium.

2.2.5 Preservation of Red Blood Cells

2.2.5.1 Red blood cells concentrate

Red blood cells (RBC) concentrates were provided by Instituto Português do Sangue e da Transplantação (IPST) at the end of shelf-life, under the protocol "Preservation of Blood Cells using Natural Deep Eutectic Systems". The provided RBC were preserved in additive solution composed of sodium chloride (8.77 g.L⁻¹), adenine (0.169 g.L⁻¹), glucose monohydrate (9.0 g.L⁻¹) and mannitol (5.25 g.L⁻¹), hence called SAGM.

Prior to the experiments, RBC were washed in PBS. First, RBC were centrifuged (1000xg, 10 min), to remove the plasma with old SAG-M and any contaminants from haemolysis during storage. Then, 10 mL of PBS were added, RBC were resuspended and centrifuged (1000xg, 10 min). This step was repeated 2 more times. The obtained washed RBC were then used on experiments. To use as standard, 30 mL of unwashed RBC were kept during the entire experiment.

2.2.5.2 Haemolysis assay

A screening assay of the haemolytic capacity of several NADES was performed using the haemolysis assay adapted from Nave et al. (2016). In the screening assays, two concentrations of NADES were tested, namely 8 and 4 % (w/v). For the assay, 200 µL of washed RBC were mixed with 200 µL of NADES solution and incubated in a thermostat water bath (Grant, LSB 12 Aqua Pro, United Kingdom) at 37 °C for 1 hour. After incubation the samples were centrifuged (800xg, 10 min) and the absorbance of the supernatant was read at 550 nm with reference filter at 620 nm, in a microplate reader (VICTOR Nivo TM, PerkinElmer). The percentage of haemolysis of each sample was calculated with comparison to a positive control, water yielding 100 % haemolysis, and negative control, PBS yielding 0 % haemolysis (Equation 2.7).

$$Haemolysis (\%) = \frac{AbsS - AbsN}{AbsP - AbsN} \times 100 \quad \text{Equation 2.7}$$

2.2.5.3 Determination of osmolality of NADES-solutions

Osmolality of NADES solutions were determined using a semi-micro osmometer (K-7400S, Knauer, Berlin, Germany) calibrated with 0, 400 and 850 mOsm.kg⁻¹ solutions. For each measurement, 100 µL of NADES solution was used, and all measurements were performed in triplicate.

2.2.5.4 Cytotoxicity Assays

The cytotoxicity of NADES solutions was evaluated against model cell line of mouse fibroblasts of connective tissue (L929, DSMZ – German Collection of Microorganisms and Cell Culture GmbH), grown MEM, with 1.5 g.L⁻¹ sodium bicarbonate, non-essential amino acids, L-glutamine and sodium pyruvate, supplemented with 10% FBS and 1% PS. The cells were cultured in a humidified incubator at 37 °C, with 5% CO₂.

When confluent, the cells were placed in 96-well plates at a density of 1.0-1.5x10⁴ cells.well⁻¹. After 24 h in the plate, media was removed and each NADES solution was added, and the cells were incubated for 24h, at 37 °C, with 5% CO₂. Control cells were incubated only with complete media. After 24 h, media was removed and cell viability was evaluated using the CellTiter 96® AQueous One Solution Cell Proliferation Assay, which is based on tetrazolium active component ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, MTS). After incubation for 2.5 hours, the amount of formazan product was measured in a microplate reader (VICTOR Nivo™, PerkinElmer, USA) at 490 nm, as absorbance is directly proportional to the number of viable cells in culture. Cell viability was expressed as percentage of cells exposed to NADES vs control cells.

2.2.5.5 Preservation of RBC in NADES

Fresh NADES solutions and SAGM (as indicated in the blood bags) were prepared and sterilized. Washed RBC were mixed with NADES or SAGM in a ratio of 1:1 and the tubes were kept at 4 °C for a maximum of 42 days. The RBC were analysed weekly to assess morphology, determine the extent of haemolysis, quantify glucose, lactate, and ROS. For morphology, 20 µL of homogenised RBC suspension were collected. Then the tubes were centrifuged (1000xg, 10 min) and 1 mL of supernatant was collected and replaced with fresh solution. Unwashed RBC and washed RBC resuspended in fresh SAGM were used as controls for the experiment.

2.2.5.5.1 Morphological characterization

Collected RBC were diluted at a ratio of 1:1000 in 0.9 wt.% NaCl solution. Then, 100 μ L of each sample were placed in 96-well plate and left to stabilize, at room temperature, for 30 minutes, after which they were observed in an inverted microscope (Axio Vert A1, Zeiss, Oberkochen, Germany).

2.2.5.5.2 Haemolysis quantification

The extent of haemolysis during storage was directly quantified on the supernatant obtained from RBC suspensions in NADES. The protocol was followed as described in section 2.2.5.2, with the difference that incubation was only performed for the positive (water) and negative (PBS) controls.

PRESERVATION OF ASCORBIC ACID USING NADES

3.1 Introduction

Ascorbic acid (AA), also known as vitamin C, carotenoids and other plant pigments, are examples of antioxidants included in the human diet (Halliwell 1996). AA is able to control the oxidative process that leads to food rancidity, due to its effectiveness in scavenging free radicals (Ravetti et al. 2019). Citric acid (CA), which is also a natural antioxidant, is a weak organic acid, used mostly as preservative and flavouring agent in food and beverages. It can also be found in cosmetics and detergents due to its cleaning and chelating properties, or in combination with active pharmaceutical substances for pain relief or as blood anticoagulant agent (Ciriminna et al. 2017).

One of the main drawbacks associated to the use of natural antioxidants, whether in food, pharmaceutical or cosmetic applications, is their high sensitivity to light exposure, especially when solubilised. Hence, there is a need to find ways to increase the lifespan of natural antioxidants when incorporated into such formulations (Yin et al. 2022).

NADES are generally described as biocompatible and biodegradable, however, assessing the biocompatibility and environmental impact of NADES is also a major concern and must be performed on a case-by-case basis. Despite this, there has been a growing number of papers focusing on studying of toxicity of NADES to the environment and living organisms, rather than cell lines and bacteria (Ferreira, Oliveira, et al. 2022).

Considering the importance of finding solutions to extend the shelf life of formulations containing powerful antioxidants, the incorporation of AA in a eutectic mixture was assessed in this chapter. The incorporation was evaluated with 1) AA as a component of NADES and 2) by adding AA to the NADES to determine whether its antioxidant properties could be preserved under these conditions. Furthermore, the toxicity of the systems was assessed using shrimp (*Palaemon varians*) as biological model and the applicability of NADES-based solutions to preserve fresh-cut fruits was evaluated on apple slices.

3.2 Results

3.2.1 Characterisation of NADES

The NADES were characterized in terms of their water content and viscosity. It is important to state that, even though ChCl was dried before use, it is a highly hygroscopic compound, and the water content of the systems comprising ChCl was mostly related to its presence. The systems CA:Treh and Bet:CA had been previously prepared by the evaporation method (Dai et al. 2013), however in this work they were prepared by the heating and stirring method. To do that it was necessary to determine the minimum amount of water needed, since these systems could not be successfully prepared without water. Through Karl-Fisher titration the water content of the prepared NADES was determined, ranging from 9 to 14 % (Table 3.1). Addressing the concerns about higher water contents present in hydrophilic NADES, Jesus et al. (2021), recently confirmed the formation of hydrogen bonds and the importance of water in NADES, through nuclear magnetic resonance (NMR) studies. In the systems analysed, there was a clear interaction between the OH groups of the NADES components and water even in systems with higher percentages of water (>20 %). This indicates that up to those water contents the NADES structure is maintained, and water may work as hydrogen bond facilitator (Dai et al. 2015).

Table 3.1 – Water content (%) of the prepared NADES.

System	Water Content (%)
ChCl:AA	8.9 ± 0.3
ChCl:CA	11.7 ± 0.5
CA:Treh	13.8 ± 0.6
Bet:CA	14.2 ± 0.6

Moreover, the viscosity of the NADES was determined in a temperature range from 10 to 50 °C and the respective viscosity curves are represented in Figure 3.1, where it is possible to see that the systems are highly viscous. The viscosity of the NADES CA:Treh, (first described in (Ferreira, Meneses, et al. 2022)) is specially high (c.a. 150 Pa·s⁻¹) being 10 times higher than the rest of the systems, at 20 °C. Figure 3.1 also shows that viscosity decreases exponentially for higher temperatures, being much similar between the NADES, for the range of temperatures from 40 to 50 °C. This behaviour was expected and is very similar to the one found in other

NADES (Jesus et al. 2021a; Correia et al. 2021), for which a significant decrease in NADES' viscosity was observed for higher temperatures or with addition of small percentages of water.

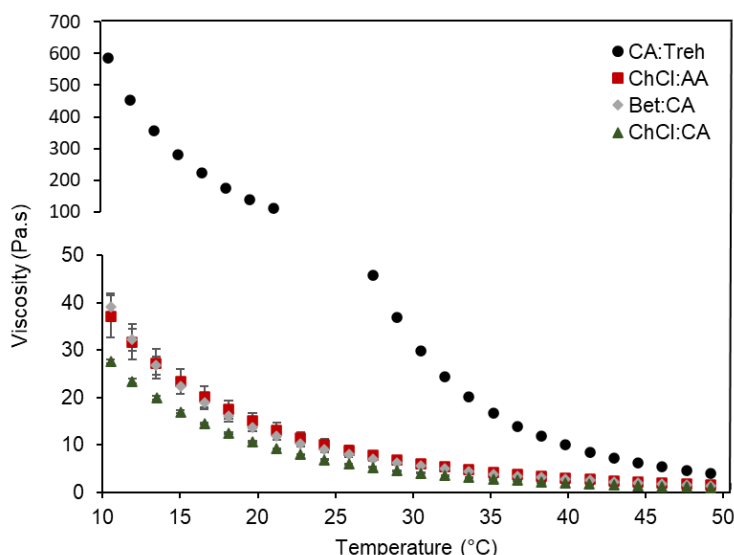


Figure 3.1 - Viscosity (Pa.s) as a function of temperature (10 to 50 °C) of the NADES ChCl:AA, ChCl:CA, CA:Treh and Bet:CA.

3.2.2 Antioxidant activity of AA in aqueous solutions of NADES

In order to determine if the chosen NADES had the capacity to stabilize the antioxidant activity of AA, the compound was dissolved, at 40 °C, in each of the NADES, and these mixtures, after cooling to room temperature, were solubilized in water to form an aqueous solution of NADES (NADES-AS). In pre-determined time points, the DPPH assay was performed to a maximum of 9 days.

The results obtained show that the control solution, pure AA (Figure 3.2) shows a drastic decrease of antioxidant capacity after only two days of storage, with an increase of the EC_{50} of almost 4-fold. In the system in which AA is one of the compounds, ChCl:AA, (Figure 3.2) there is a less pronounced decrease of antioxidant activity after 5 days, which corresponds to an increase of 2-fold on the EC_{50} value. It has been reported that when in formulation, ChCl:AA (2:1), AA is able to maintain its EC_{50} over at least six months (Silva et al. 2018). Nevertheless, in the said study the analysis was performed to the pure system, while herein it was analysed in aqueous solution with a concentration of AA of $0.5 \text{ mg}\cdot\text{mL}^{-1}$, which is more relevant in terms

of application development for food or cosmetic industries. Other authors have also reported the high antioxidant activity of this NADES (Ling et al. 2020).

In which concerns the antioxidant capacity of AA after incorporation in the systems ChCl:CA, CA:Treh and Bet:CA, the results show the same trend – the antioxidant activity of AA is maintained for at least 5 days upon solubilisation in water (Figure 3.2). It is possible to observe that the system with betaine was the best one in the preservation of the antioxidant activity of AA in solution, showing a 2-fold increase of the EC_{50} by the 9th day.

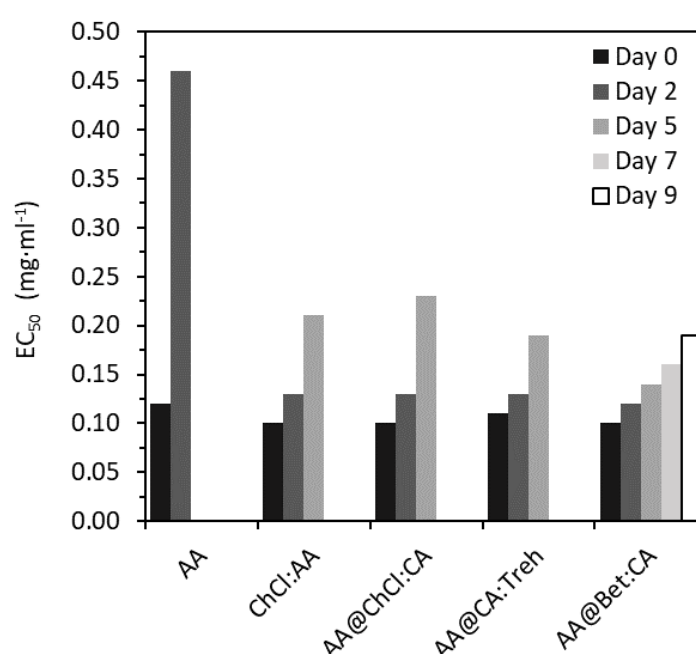


Figure 3.2 - Variation of the EC_{50} of AA ($mg \cdot mL^{-1}$) at 0, 2, 5, 7 and 9 days of testing at the different conditions of storage.

To assure that the antioxidant activity was not influenced by the presence of CA, which is also a natural antioxidant, the DPPH assay of the NADES-AS without AA was performed in the same range of concentrations (Figure 3.3). It was concluded that the %RSA of the NADES *per se* was 12, 4 and 3% for the higher concentrations of ChCl:CA, CA:Treh and Bet:CA, namely, hence not causing an overestimation on the quantification of AA's antioxidant activity. Moreover, an additional stability assay was conducted for CA:Treh. In this case, the samples were prepared without the dissolution of the AA in the NADES prior to the addition of water, i.e., AA and NADES were dissolved in water as two independent entities. In those experiments the

behaviour of AA was similar to the one presented with AA alone, with a severe decrease of the antioxidant activity over a short time period (5 days) (Figure 3.4).

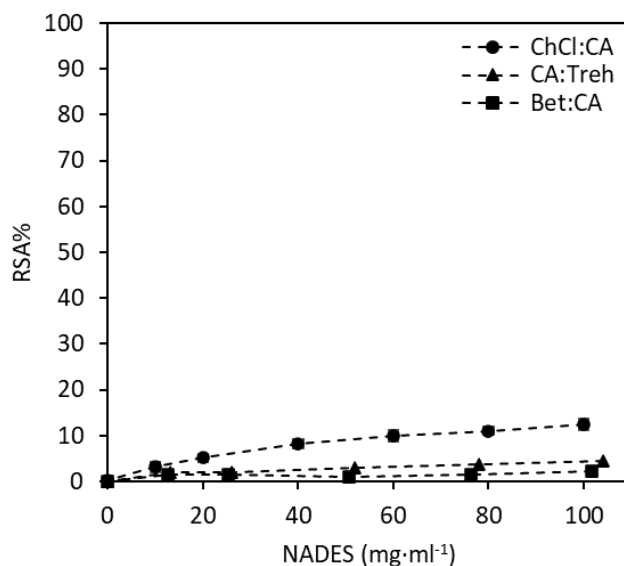


Figure 3.3 - Antioxidant activity of the NADES ChCl:CA, CA:Treh and Bet:CA in the same range of concentrations that were used to dissolve AA (10 to 10 mg·ml⁻¹).

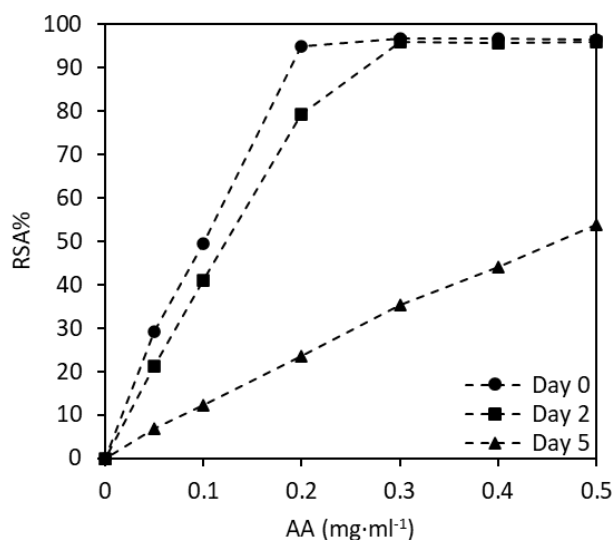


Figure 3.4 - %RSA of 0.5 mg·ml⁻¹ AA and 120 mg·ml⁻¹ CA:Treh dissolved in water (without AA being dissolved in NADES before the addition of water).

In previous studies, it has been described that interactions between the constituents of a DES are maintained if the system contains up to 50% of water content and for contents above that the interactions are lost (Dai et al. 2015). In this case, the storage solutions had a water content of c.a. 90%, however the antioxidant activity of AA remains high. The same type of behaviour

has been observed with NADES applied in cryopreservation, in which the effect of the NADES dissolved in growth medium is not the same as the individual components (Jesus et al. 2021a). It has also been recently described that antioxidants stabilized in the system ChCl:AA presented a cluster structure which were observed by transmission electron microscopy (Ling et al. 2020). This could be one of the explanations for the different behaviour observed in samples in which AA and NADES were simultaneously dissolved in water.

3.2.3 Toxicity Assays

In products intended for human consumption, it is essential to study the capability of the newly developed systems to cause oxidative stress in cells and living organisms. In this work we evaluated the toxicity of NADES in *P. varians*, by exposing the animals to water with three concentrations of NADES, namely 1, 5 and 10 %w/v. These concentrations mimic a realistic concentration of the systems considering the application in development, namely for the food industry. The assay was only performed with ChCl:AA, which comprised AA in its formulation, and Bet:CA, the system that provided AA the best protection against oxidation. The toxicity of CA:Treh (2:1), had been previously studied and reported. This system's toxicity was studied using *Danio rerio* (zebrafish) as animal model, and the results showed that CA:Treh has low toxicity both when present in water and when injected in the animals, results that were promising for its application in pharmaceutical, cosmetic and food industries (Ferreira, Meneses, et al. 2022). Moreover, the toxicity of several betaine-based NADES was also assessed in zebrafish, and the results have shown that the systems did not present toxicity on the range of concentrations studied (Ferreira et al. 2023).

The evaluation of several stress indicative biomarkers allowed to calculate the integrated biomarker response (IBR) index of the conditions evaluated. In comparison to the control animals, the IBR of the muscle tissue and digestive system remained constant for most of the conditions tested. One of the exceptions can be seen on the muscle and digestive systems of animals exposed to the higher concentration of ChCl:AA, $2.80 \text{ mg}_{\text{NADES}} \cdot \text{g}_{\text{animal}}^{-1}$ (Table 3.2). In these conditions, the highest IBR was obtained for digestive systems, which can be explained by the high activity of superoxide dismutase (SOD) and acetylcholinesterase (AChE) along with the higher total antioxidant capacity (TAC) (Figure 3.5(A)). Regarding the muscle tissue of the animals exposed to the higher concentration of ChCl:AA, the IBR had the lowest value obtained

(Table 3.2). The decrease can be justified by the decrease of AChE and CAT activities, as well as the decrease of lipid peroxidation (LPO), despite the increase of the activity of SOD (Figure 3.5(B)). The other exception concerns the muscle tissue of the animals exposed to 1.42 $\text{mg} \cdot \text{g}_{\text{animal}}^{-1}$ of ChCl:AA, whose IBR was also lower than the one of control samples, due to a decrease of the activities of AChE and CAT and decrease of LPO.

Table 3.2 - IBR index values calculated for the digestive system and muscle of the animals exposed to the different concentrations of NADES (1, 5 and 10 %) and control.

Sample	Concentration ($\text{mg}_{\text{NADES}} \cdot \text{g}_{\text{animal}}^{-1}$)	Digestive System	Muscle
Control	-	27.41	29.21
Bet:CA	0.29	25.92	28.06
	1.43	25.41	29.62
	2.77	26.13	28.64
ChCl:AA	0.29	27.15	28.12
	1.42	26.49	22.92
	2.80	31.36	21.34

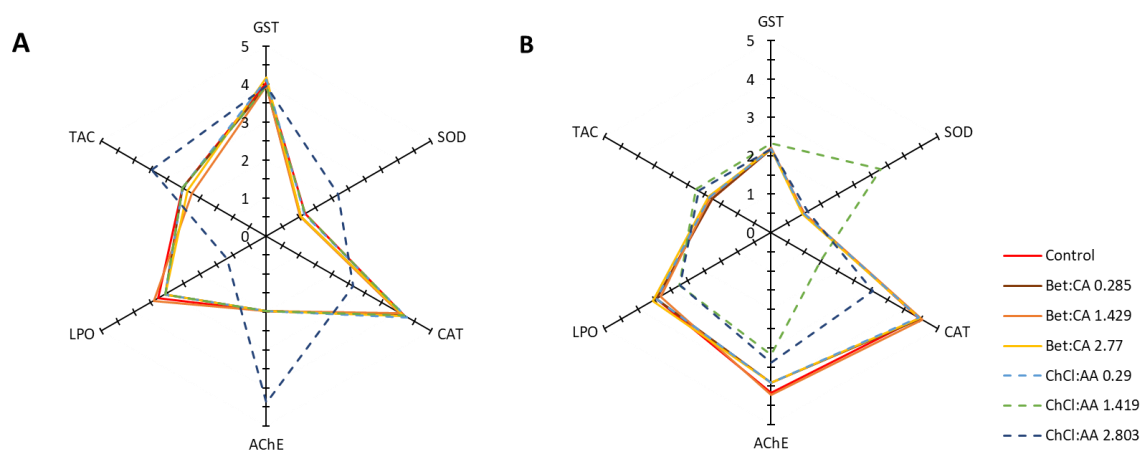


Figure 3.5 - Radar chart for the IBR values of each NADES concentration ($\text{mg} \cdot \text{g}^{-1}$ animal) for (A) the digestive system and (B) muscle. Full lines represent Bet:CA and dashed lines represent ChCl:AA.

Regarding oxidative stress, there were no significant alterations observed upon the exposition to Bet:CA. Considering the exposition to ChCl:AA, out of the eight biomarkers studied, only two (SOD and CAT) were significantly affected ($p < 0.05$; Appendix A, Figure A.1.1 and Figure A.1.2). Catalase activity was significantly different between the digestive systems of animals exposed to 1.42 $\text{mg}_{\text{NADES}} \cdot \text{g}_{\text{animal}}^{-1}$ and to 2.80 $\text{mg}_{\text{NADES}} \cdot \text{g}_{\text{animal}}^{-1}$ of ChCl:AA ($p < 0.05$). With respect

to SOD, a significant increase of activity was observed ($p < 0.01$) when comparing the control muscle to the one exposed to $1.42 \text{ mg}_{\text{NADES}} \cdot \text{g}_{\text{animal}}^{-1}$ of ChCl:AA. A significant increase of activity was also observed, although with lower level of significance ($p < 0.05$) between the muscles of the animals exposed to ChCl:AA in the concentrations $0.29 \text{ mg}_{\text{NADES}} \cdot \text{g}_{\text{animal}}^{-1}$ and $1.42 \text{ mg}_{\text{NADES}} \cdot \text{g}_{\text{animal}}^{-1}$.

SOD is partially responsible for the conversion of ROS into hydrogen peroxide and water (Pandey and Rizvi 2010). Hence, an increase on production of ROS, causes an increase of the enzyme's activity. On the other hand, catalase is in part responsible for the conversion of hydrogen peroxide into molecular oxygen and water (Pandey and Rizvi 2010). The exposure of the different organs to ChCl:AA, lead to an increase of the activity of both catalase and SOD. Hence, the increase of catalase activity can be associated with the need to remove the H_2O_2 that was that was produced by SOD, under oxidative stress (Song, Choi, and Park 2020). This correlation was observed for these two enzymes, in samples that were exposed to the higher concentrations of ChCl:AA, hence they can be associated with the toxicity of the concentrations tested.

The high variances observed are associated with external elements, such as gender and genetic predisposition (Gagnon and Hodson 2012) however no significant changes were observed when comparing the animals in the beginning (T0) and animals used for as control on the assay.

Overall, the results suggest that Bet:CA presents no significant toxicity against this animal model, at the concentrations tested (up to $2.77 \text{ mg}_{\text{NADES}} \cdot \text{g}_{\text{animal}}^{-1}$). On the other hand, considering ChCl:AA, only the lower concentration tested ($0.29 \text{ mg}_{\text{NADES}} \cdot \text{g}_{\text{animal}}^{-1}$) presented no toxicity.

3.2.4 NADES-AS solution for the preservation of fresh cut fruits

Considering the capacity of NADES-AS to act as stabilizers of AA and their low toxicity towards the animal models, their applicability as preservation solution for fresh-cut fruits and vegetables was tested.

The results obtained for sliced russet apple that were dipped in solutions of NADES (with and without AA) and AA are presented in Figure 3.6. It is possible to observe that for the studied period of 24 h, the formulation of NADES with dissolved AA, outperformed the AA alone, one of the alternatives currently used in the food industry. It is also interesting to see that the low concentration of AA added to NADES is enough to reduce the browning of the slice, in comparison to NADES alone.

Despite being preliminary results, the advantages of these systems over the conventional solution of AA were clearly observed. It is further expected that performing the assays in ideal storage conditions (such as controlled atmosphere) would only improve the results.

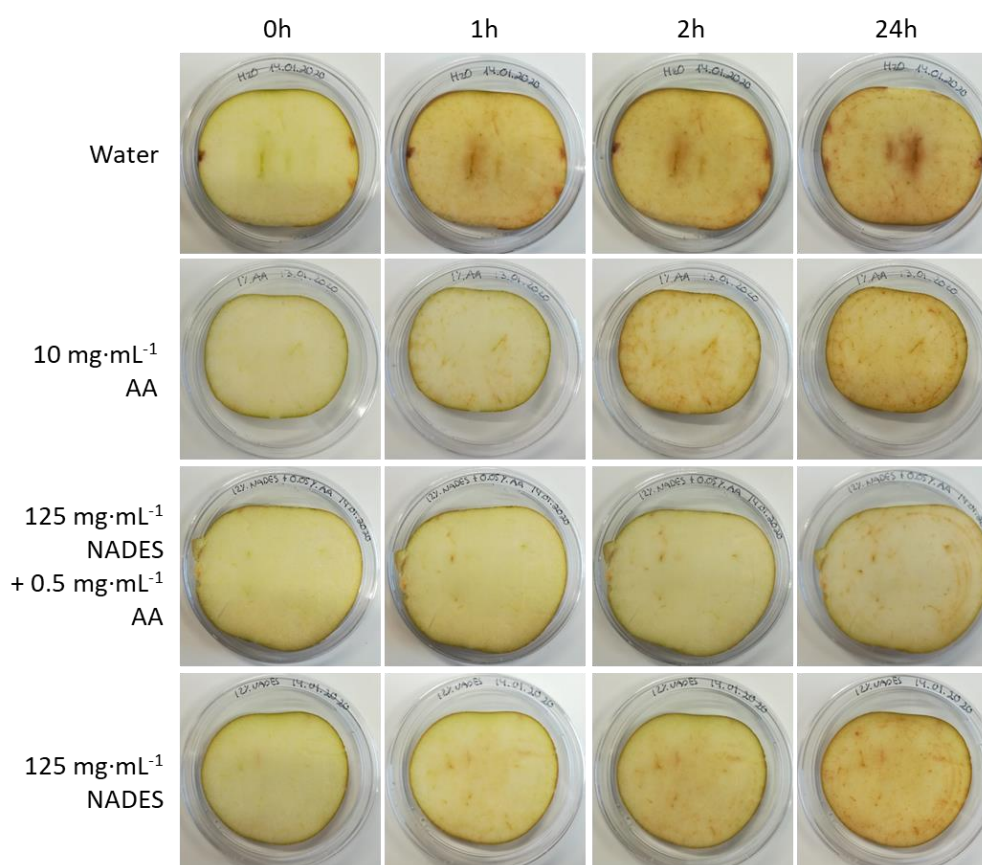


Figure 3.6 - Pictures of russet apples over 24 h of testing in water, 10 mg·mL⁻¹ AA, 125 mg·mL⁻¹ NADES + 0.5 mg·mL⁻¹ AA or 125 mg·mL⁻¹ NADES.

3.3 Conclusion

In this study several NADES were prepared to stabilize AA. These systems were characterized in terms of their water content and viscosity. It was observed that although the system's water content was within a small range (9 to 14%), the viscosity of CA:Treh was 5-times higher than that of the other NADES, specially below 40 °C. The capacity of the different NADES to preserve the antioxidant properties of AA in aqueous solutions was also assessed. The results showed that dissolving AA in Bet:CA, is more beneficial than having AA incorporated in the formulation ChCl:AA. Of the systems tested, Bet:CA had the longest effect on retaining the antioxidant properties of AA, with improved stability up to 9 days. Regarding the toxicological assays towards *P. varians*, it was observed that the presence of Bet:CA in the water did not present significant toxicity (up to $2.77 \text{ mg}_{\text{NADES}} \cdot \text{g}_{\text{animal}}^{-1}$), while the system ChCl:AA showed toxicity at the higher concentrations tested (1.42 and $2.80 \text{ mg}_{\text{NADES}} \cdot \text{g}_{\text{animal}}^{-1}$), due to alterations observed in two biomarkers of oxidative stress (SOD and CAT activity), when compared with the respective control. The preliminary assay of the application of NADES-AS as preservation solution for fruits and vegetables has shown promising results. The apple slices preserved with NADES+AA solutions showed the less browning over a 24 h period. It is known that the food industry, constantly seeking strategies that can extend the shelf-life of fresh products. Hence, the possibility of applying these NADES in food industry is also presented. These findings could also be transferred to the cosmetic or pharmaceutical industries, in which AA is widely used or in the cosmetic field, where the incorporation of AA in formulations can still be quite challenging.

The following chapter was adapted from the publications:

Nicolás F. Gajardo-Parra, Liane Meneses, Ana Rita C. Duarte, Alexandre Paiva and Christoph Held, Assessing the Influence of Betaine-Based Natural Deep Eutectic Systems on Horseradish Peroxidase, ACS Sustainable Chemistry & Engineering (2022), 10, pp. 12873–1288, DOI: 10.1021/acssuschemeng.2c04045

Liane Meneses, Nicolás F. Gajardo-Parra, Esteban Cea-Klapp, José Matías Garrido, Christoph Held, Ana Rita Duarte, Alexandre Paiva, Improving the Activity of Horseradish Peroxidase in Betaine-based Natural Deep Eutectic Systems, RSC Sustainability (2023), 1, 886-897, DOI: 10.1039/D2SU00127F

STABILISATION OF HRP USING BETAINE-BASED NADES

4.1 Introduction

The application of enzymes at an industrial scale, implies that numerous reaction conditions must be taken into consideration due to their lack of stability regarding temperature, pH, or solvent. Hence, the stabilization of enzymes becomes crucial for their application in industrial processes such as treatment of wastewaters, tissue engineering, biosensors, or drug delivery systems (B. W. Park et al. 2012; Chapman, Ismail, and Dinu 2018). Immobilization of enzymes, has become a routine process and made several industrial processes feasible and greener (Basso and Serban 2019).

Although HRP is a considerably thermostable enzyme, its structural stability and biocatalytic activity are essential for the inclusion in industrial processes (Chapman, Ismail, and Dinu 2018). Various approaches have been described to increase enzyme activity without decreasing stability, such as protein engineering, high-pressure operation, protein immobilization, and the addition of co-solvents (Iyer and Ananthanarayan 2008). Another important factor for enzyme stability is a_w . The knowledge of a_w of the NADES allows to design media for enzyme storage and stabilization without compromising their enzymatic activity due to inadequate moisture content. So far, the stabilization of HRP in NADES has been only studied using choline-based systems. (Wu et al. 2014; Papadopoulou et al. 2016).

Bioprocesses significantly benefit from predictive methods that substantially reduce the number of required trial and error experiments (Kontogeorgis et al. 2021). Perturbed-Chain Statistical Associating Fluid Theory (PC-SAFT) has been used to predict physicochemical properties of different compounds and mixtures (Gross and Sadowski 2001; 2002). Equations of state and thermodynamic methods have been used in the literature to demonstrate the effect of different cosolvents on enzymatic properties, including kinetics (Gajardo-Parra et al. 2022; Knierbein et al. 2019) and thermal stability (Tadeo et al. 2009). Diverse molecular dynamics methods, including classical molecular dynamics (MD) (Vrbka et al. 2006; Filizola and Loew 2000) and quantum mechanics/molecular mechanics (QM/MM) (Derat et al. 2007) have been used to describe HRP and different mutations (Heering, Smith, and Smulevich 2002).

In this work, we proposed the use of betaine-based NADES to improve the enzymatic activity and thermostability of HRP. Five different NADES were characterized by measuring density, viscosity, and a_w . These properties were obtained experimentally, and, out of the scope of this thesis, modelled using PC-SAFT. The influence of these NADES in the activity and structure of HRP was also assessed, using nanoDSF to obtain the denaturation temperature to evaluate the effect of NADES on the thermal stability of HRP, and circular dichroism (CD) to quantify the conformational changes in the secondary structure of HRP. Moreover, the thermostability of HRP after prolonged exposure to higher temperatures (60 and 80 °C) was evaluated. Betaine was used in these systems as a substitute for choline chloride.

4.2 Results and Discussion

4.2.1 NADES characterisation

4.2.1.1 Viscosity

The presence of betaine as HBA turns the obtained NADES into highly viscous liquids; it is known from the literature that this is caused by the strong molecular interactions that can affect molecular mobility (Gajardo-Parra et al. 2020). As shown in Figure 4.1(A), BTrehGlyW has the highest viscosity, possibly due to the low flexibility for molecular mobility that hydrogen bonds interactions and structure provide among the NADES constituents (betaine, trehalose, glycerol) and water. BSucProW and BSorbW have similar viscosities values. It would be expected that BSucProW, due to the higher complexity of its structure offered by the additional component, would have a higher viscosity than BSorbW. However, due to the higher water content of BSucProW (19.3 wt.%) the viscosities of the two NADES almost overlap, as it can be noticed in Figure 4.1(A). Due to the low water content, BGly is one the systems with higher viscosity, while BXylW has the lowest viscosity of the NADES. This can be caused by a combination of factors, namely, its simple chemical structure, low density, and higher water content. As expected, the viscosity decreases drastically with the temperature for all NADES, as shown in Figure 4.1.

Side by side with temperature, water addition is known to decrease the viscosity of these systems (Castro et al. 2018; Craveiro et al. 2016). We have studied its effect on the system BGly and as shown in Figure 4.1(B), adding 20 wt.% water, at 20 °C, reduces the viscosity from ~2600 mPa·s down to ~60 mPa·s. Although water addition is an essential tool for reducing the viscosity in industrial applications, it is crucial to make sure the non-disruption of the molecular interactions between the HBA - HBD of the NADES, that for choline chloride based NADES starts at around 40% molar of water (Kaur, Gupta, and K. Kashyap 2020). Nevertheless, the exact influence of water concentrations on the behaviour of betaine-based DES is not yet completely known.

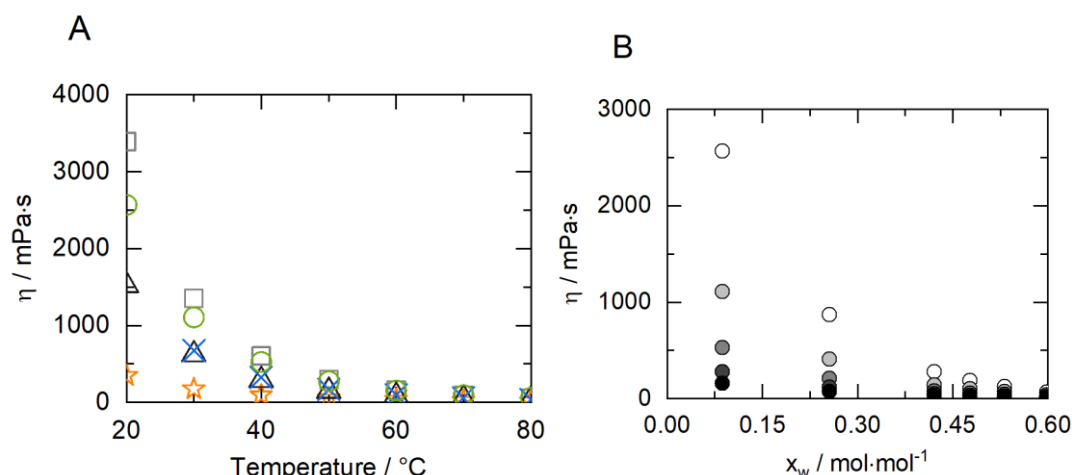


Figure 4.1- Experimental viscosity (mPa·s) as function of the temperature for (A) BXylW (stars), BTrehGlyW (square), BSorbW (triangle), BSucProW (cross), and BGly (circles) and (B) BGly as function of water mole fraction at different temperatures: 20 °C (white), 30 °C (light gray), 40 °C (gray), 50 °C (dark gray), 60 °C (black).

4.2.1.2 Density

The density of the NADES was determined experimentally in a temperature range from 20 °C to 80 °C and PC-SAFT was used to model the data. The density of the systems ranged from 1.210 g·cm⁻³, for BXylW and BGlyW, to 1.280 g·cm⁻³, for BTrehGlyW at 40 °C. These values are similar to other betaine-based systems with polyols reported by Rodrigues et al. (Rodrigues, Cardeira, et al. 2021). Moreover, Kucan et al. studied the density of BGly in a different molar ratio (1:3), which also fell within the range obtained in this study, 1.20 g·cm⁻³, at 15 °C and 1.23 g·cm⁻³, at 55 °C (Kučan et al. 2018). Altamash et al. (2018), has reported the density of NADES combining betaine and other compounds, such as acids, and the values range between 1.2 and 1.3 g·cm⁻³.

As expected, the density decreased linearly with increasing temperature for all systems, as shown in Figure 4.2(A). BGly and BXylW have a similar density, which is lower than the other systems under study, although having a significantly different amount of water. On the one hand, the differences in density can be attributed to electrostatic forces and hydrogen bonds between HBA, HBD, and water, which decreases the free volume in the mixture and increases the density. In other words, the more OH groups within the NADES the higher the density (Maugeri and Domínguez De María 2014; Haifeng et al. 2008). On the other hand, the spatial orientation influences density of NADES due to the steric effect of aromatic groups or large

sugars, as in the case of systems comprising trehalose. As shown in Figure 4.2(B), water addition causes a decrease in density.

The liquid densities for the systems used in this work were modelled with PC-SAFT using the individual-component approach. Although modelling density is straight forward for equations of state, modelling density of NADES is challenging. The reason is that the HBA and HBD are solids (Cea-Klapp et al. 2020; Zubeir et al. 2016) except glycerol, and parameters of HBA and HBD could thus not be fitted to density of the pure HBA and HBD in the original references for the parameters of HBA and HBD (Table A.2.1), respectively. Thus, it was necessary to use binary interaction parameters to correlate the density of the systems under study. The modelling results were within an overall average absolute deviation (AAD) of 0.43%. This is an excellent result, and it shows that fitting binary parameters to experimental density is a valid option. Furthermore, these parameters were used to predict other properties (see next section).

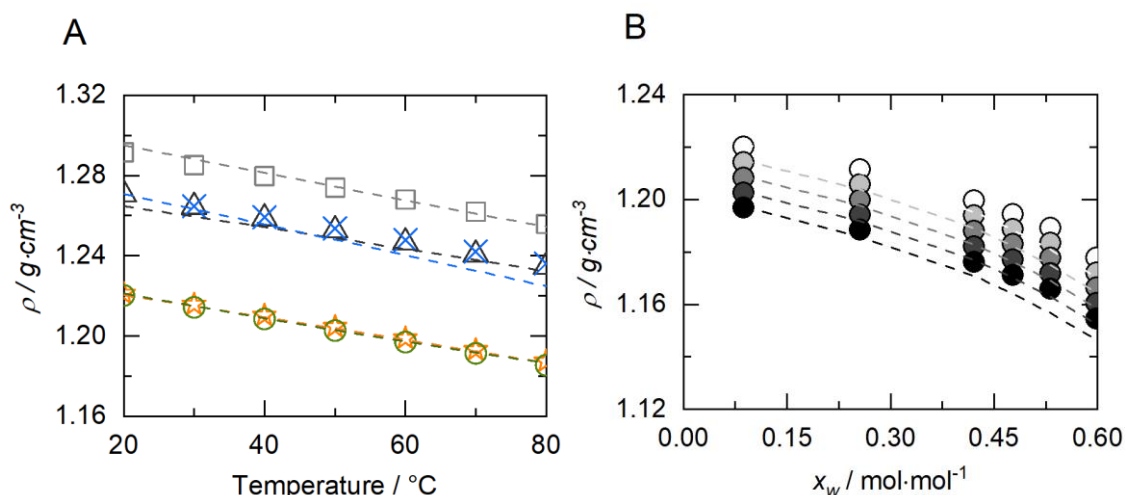


Figure 4.2 - Density ($\text{g}\cdot\text{cm}^{-3}$) in function of the temperature of (A) BXylW (stars), BTrehGlyW (squares), BSorbW (triangles), BSucProW (cross), and BGly (circles) and (B) BGly as function of mass fractions of BGly: 100% (white), 95% (light gray), 90% (gray), 85% (dark gray), 80% (black). Dashed lines represent PC-SAFT calculations with parameters reported in Table A.2.1 and Table A.2.2.

4.2.1.3 Water activity

Since these systems were chosen based on their potential use in biocatalytic applications, determining water activity is quite relevant. The water activity of the systems under study was simultaneously predicted using PC-SAFT and determined experimentally and the results demonstrate that the predicted values are in accordance with the results obtained, validating

the model used. First, the experimental data is discussed. The experimental results for water activity at defined water contents of the NADES used in this work are shown in Table 4.1. Except for BGly, all the NADES needed the addition of water (ranging between 40 and 70 mol%) to be prepared (Table 4.1). These water mole fractions correspond to water mass fractions between 14 and 22 wt.% water, respectively. From the results presented in Table 4.1, it is possible to observe that the a_w values of the systems at 37 °C (except for BGly) are close to 0.4, despite the mixtures were prepared with very different amounts of water content. Even though some NADES present high water mass fractions, NMR studies of the NADES herein used and reported elsewhere, prove that in these conditions water is part of the hydrogen bond network that is involved the formation of the supramolecular structure of the NADES (Jesus et al. 2021a).

Table 4.1 - Water content (wt.%) and a_w at 37 °C and 60 °C at 100 kPa of the systems under study.

System	Water content (wt.%)	a_w at 37 °C	a_w at 60 °C
BXylW	21.9 ± 0.2	0.444 ± 0.004	0.469 ± 0.005
BTrehGlyW	14.6 ± 0.3	0.394 ± 0.002	0.413 ± 0.005
BSorbW	14.3 ± 0.5	0.433 ± 0.009	0.417 ± 0.008
BSucProW	19.3 ± 1.2	0.443 ± 0.001	0.461 ± 0.007
BGly	1.7 ± 0.1	0.071 ± 0.004	0.082 ± 0.003

Figure 4.3(A) shows the qualitative agreement for a_w obtained by the predictions with PC-SAFT without using any binary interaction parameters between HBA and HBD. The NADES BGly contains only residual water (< 2 wt.%), and, as so, a_w is lower than for the other studied NADES. As expected, a_w increases upon addition of water to BGly (Figure 4.3(B)). At the maximum water mole fraction studied ($x_w=0.6$, corresponding to ≈ 20 wt.% water), the a_w was found to be 0.43, which falls within the a_w values determined for the other systems (Table 4.1).

It can be further seen from Figure 4.3(B) that a_w and the water mole fraction are different, and the difference is most pronounced at equimolar NADES:water ratio. Further, activity is lower than the mole fraction; that is, activity coefficients of water (γ_w) must be lower than one ($a_w = x_w \gamma_w$). The γ_w values modelled with PC-SAFT are lower than one for the systems under study; that is, water interactions in the NADES mixtures are more substantial than in pure water. This is caused by the strong hydrogen bonding of the NADES constituents with water. As Baz et al. (2019) noticed, the individual-component approach provides flexibility to the model without losing quality in the predictive results, achieving an AAD of 7.76%. However, by increasing the amount of water in the mixture, the HBA-HBD interactions weaken rapidly (Fisher et al. 2020).

Hence, incorporating a binary interaction parameter increases the accuracy of PC-SAFT modelled a_w in the BGly + water dilutions, as shown in Figure 4.3(B). It is important to note that for the system BGly one binary parameter between betaine and glycerol was fitted to independent experimental data (density data, cf. section 4.2.1.2); the availability of this single parameter allows predicting shape of the a_w curve within an AAD of 16.2%, a satisfying agreement from the experimental data with water uptake up to $x_w = 0.6$ (Figure 4.3(B)).

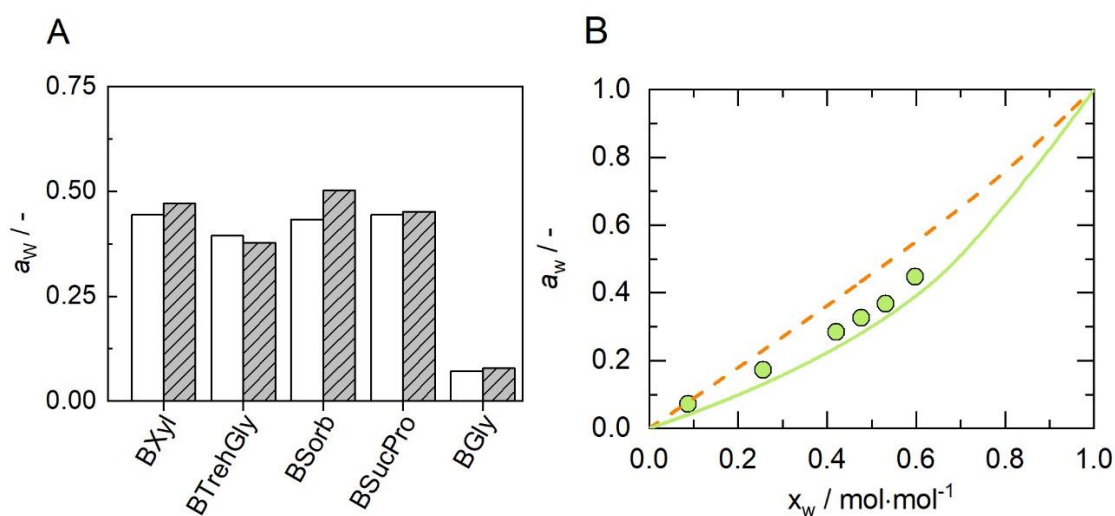


Figure 4.3 - a_w values at 37 °C and 100 kPa. (A) NADES with compositions in Table 4.1. Experimental (white bars), PC-SAFT predictions $k_{ij} = 0$ (stripped bars). (B) Water influence on BGly. Experimental data (circles), PC-SAFT predictions with $k_{ij} = 0$ between betaine and glycerol (dashed line), PC-SAFT predictions using k_{ij} between betaine and glycerol fitted to density (full line). PC-SAFT parameters are reported in Table A.2.1 and Table A.2.2.

4.2.2 Influence of NADES on enzymatic activity of HRP

The enzymatic activity of HRP at 37 °C was studied in NADES-AS, using PBS (100 mM, pH 7) as control. The activity was assessed by a colorimetric method to determine the production of a dye, by the oxidation of PSA in the presence of 4-AAP (Vojinović et al. 2004). Figure 4.4 illustrates that in all the NADES-AS, there was an increase of the enzymatic activity and reaction rate, compared to the control buffer. The addition of BTrehGlyW, BSucProW, and BGly lead to an increase in enzymatic activity of approximately 60 %. The NADES that caused the highest impact on enzymatic activity was BSorbW, in which the enzymatic activity increased two-fold compared to the control buffer.

In literature, only the effect of choline-based DES had been studied on HRP; however, discordant results were found. While one study shows the improvement of HRP activity in the presence of DES (Wu et al. 2014), more recent results indicate that HRP's activity decreased, especially for higher DES concentrations (Papadopoulou et al. 2016). Moreover, as recently reviewed, most DES used for protein stabilization and activation are based on choline derivatives (Yadav and Venkatesu 2022). These two findings were the driven force for the development of this work. On the one hand, it was important to study the impact of NADES on HRP activity and stability. On the other hand, replacing choline chloride in such applications has become urgent due to its hygroscopic behaviour, as well as the limitations to its application imposed by several industries. Betaine-based NADES have been used for some preservation ends, such as for protein stabilization (Yadav and Venkatesu 2022) or cryopreservation (Jesus et al. 2021a), hence this was our starting point for choosing this family of NADES.

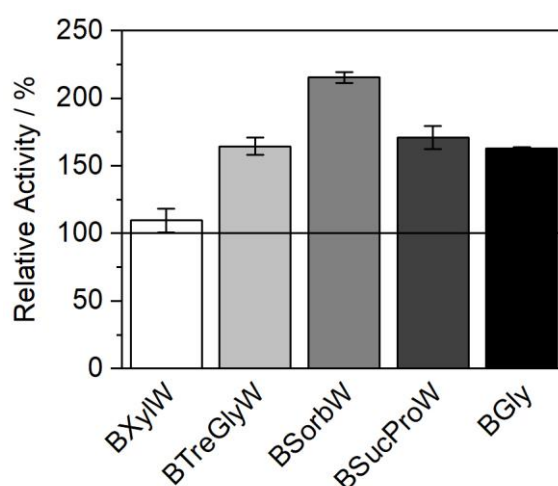


Figure 4.4 - Relative enzymatic activity of HRP in five different NADES-AS using PBS (100 mM, pH 7) as control at 37 °C and 100 kPa.

4.2.3 HRP activity after incubation at 37 °C

The impact of the presence of NADES on enzymatic activity was also evaluated upon incubation in pure NADES and PBS, which was used as control. Two incubation times were tested for the temperature of 37 °C, namely 6 h and 24 h. The activity at these conditions was determined using the same activity protocol as described before and are reported as “residual activity”, which was normalized to the value obtained at the start of the measurements (t=0). The results

obtained are illustrated in Figure 4.5. Overall, all the systems tested, including PBS, kept HRP active throughout the 24 h of incubation. From Figure 4.5 it is also possible to observe that PBS and BGly caused a decrease of about 20% compared to initial activity, while BSorbW, BSucProW, and BXylW maintained it close to the initial 100%, and BTrehGlyW increased it by 20% of the HRP's activity, after 6 h of incubation, which was maintained constant until 24 h. The reasons leading to the maintenance/increase in HRP's activity will be further discussed in combination with other results.

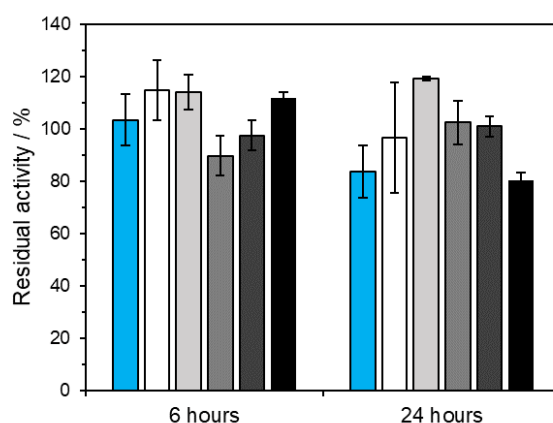


Figure 4.5 - Residual enzymatic activity of HRP after incubation for 6 and 24h at 37 °C (n=3) in PBS (light blue), BXylW (white), BTrehGlyW (light gray), BSorbW (gray), BSucProW (dark gray) and BGly (black).

4.2.4 Thermostability of HRP in the presence of NADES

Further studies were performed to understand if the chosen NADES could also impact the enzyme's thermostability over longer periods. Therefore, to assess the thermostability under thermal stress, HRP was incubated in pure NADES at two different temperatures, 60 and 80 °C.

The results obtained from the incubation at 60 °C (for 4 and 24 h) can be observed in Figure 4.6(A). When exposed to this temperature, the differences in enzymatic activity were more noticeable than at 37 °C. It was possible to observe that two of the tested NADES maintained high enzymatic activity, namely, BTrehGlyW and BSucProW. Particularly, in the presence of BSorbW there was a decrease of activity of c.a. 25%, compared to the initial one, after 24 h of incubation on those systems. When incubated in the systems BGly and BXylW, the enzymatic activity was reduced to 42% and 27%, respectively. After incubation for 24 h at 60 °C in control

media, enzymatic activity was reduced to residual values. Although HRP's activity was reduced in the presence of some NADES, it was always higher than in the presence of PBS alone.

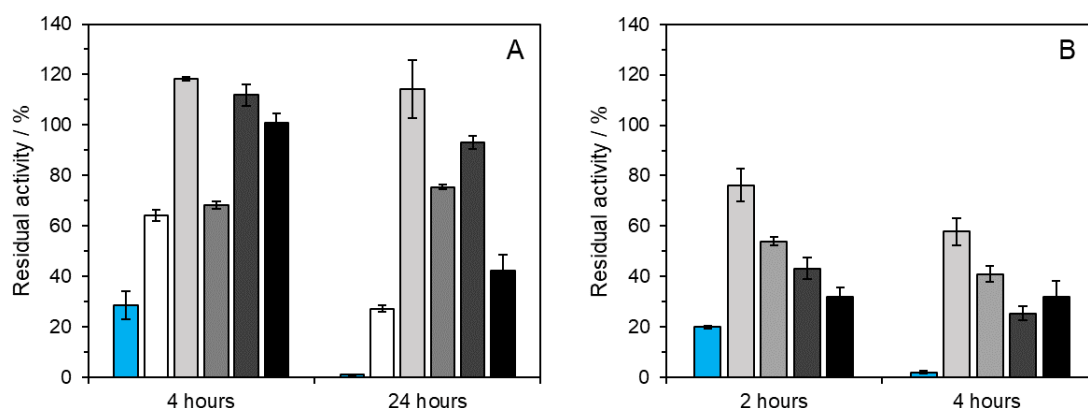


Figure 4.6 - Enzymatic activity of HRP after incubation for (A) 4 and 24h at 60 °C (n=3) and (B) 2 and 4h at 80 °C (n=3), in in PBS (light blue), BXylW (white), BTrehGlyW (light gray), BSorbW (gray), BSucProW (dark gray) and BGly (black).

Furthermore, the thermostability of HRP at 80 °C was also evaluated. In this case, the incubation time points studied were 2 and 4 h. As shown in Figure 4.6(B), the enzymatic activity decreased with the incubation in all the media tested; however, the impact of incubation temperature on enzyme activity was not the same for all NADES. Since the system BXylW had the lowest performance in the assays at 60 °C, it was discarded for the tests at 80 °C. The system with the best thermal stability on HRP was BTrehGlyW, in which the enzyme kept c.a. 60% of the initial activity after 4h of incubation at 80 °C. When incubated in BSorbW for 4h, the enzyme kept 41% of the initial activity. BSucProW and BGly showed low thermal stability, decreasing the activity to 30% of the initial value. The complete loss of enzyme activity was observed only in the PBS systems, in 24 h for systems incubated at 60 °C and in 4 h for systems incubated at 80 °C.

Betaine-based systems had already proven their thermal protective behaviour at high temperatures (80 and 90 °C) in other enzymes, such as laccase (Khodaverdian et al. 2018). Nonetheless, it was observed that among betaine-based DES the results have wide variability and are dependent on their composition. Some researchers have studied the effect of sugars on the activity of HRP and observed that in systems with equal viscosity, sugars such as trehalose and glucose, and with sorbitol had less impact on the enzymatic activity of HRP (Neri et al. 2010; 2014).

4.2.5 Temperatures of unfolding of HRP

The denaturation temperature was measured to determine the impact of NADES on the protein unfolding process. As thermal stabilization mediated by the co-solvents directly influences unfolding temperature (Wessner, Nowaczyk, and Brandenbusch 2020), it was expected that the NADES used in this work also increase unfolding temperatures ($T_{unfolding}$); this could indeed be observed, as shown in Figure 4.7(A). Table 4.2 shows the variation of $T_{unfolding}$ and aggregation temperature ($T_{aggregation}$) at ambient pressure, under a heating rate of $0.7\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$, in the control buffer as well as in the different NADES-AS.

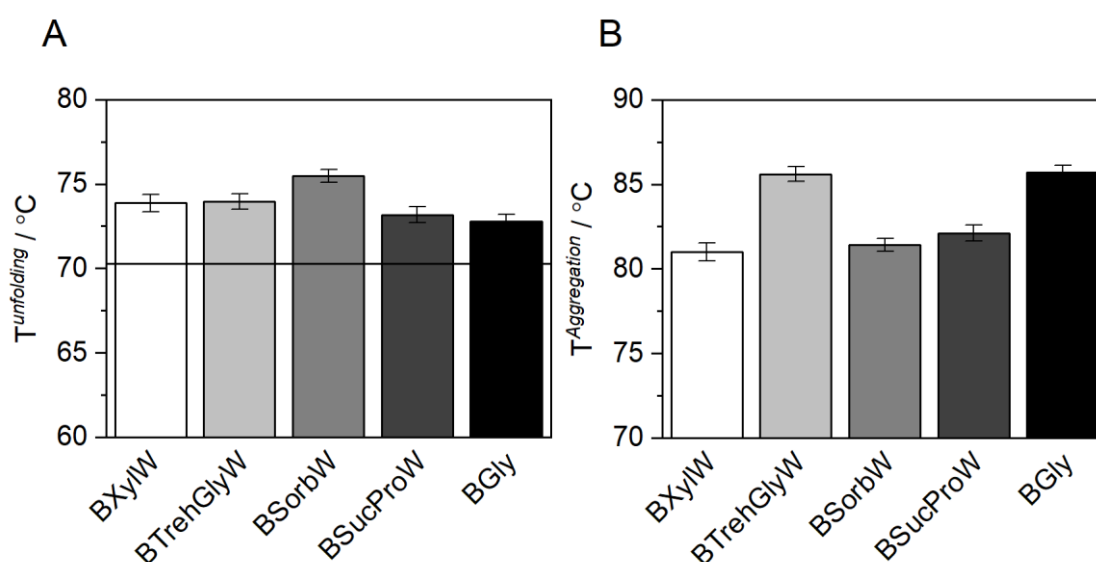


Figure 4.7 - Thermal stability of HRP in presence of NADES-AS used in this work. (A) Experimental unfolding temperature and (B) aggregation temperature. Experiments performed at 100 kPa and pH 7. The horizontal continuous black line represents the unfolding temperature in neat buffer.

Table 4.2 – Unfolding (T_{on-set}) and aggregation temperatures of HRP in the presence of NADES compared to control buffer (PBS).

System	T_{on-set} ($^{\circ}\text{C}$)	$\Delta T_{unfolding}$ ($^{\circ}\text{C}$)	$T_{Aggregation}$ ($^{\circ}\text{C}$)
PBS	57.1	-	-
BXylW	54.5	3.59	81.0
BTrehGlyW	55.9	3.67	85.6
BSorbW	39.8	5.18	81.4
BSucProW	40.6	2.89	82.1
BGly	52.1	2.50	85.7

HRP follows the denaturation model proposed by Lumir and Eyring (Lumry and Eyring 2002), in which an intermediate state can be observed before unfolding. This intermediate state is determined by the melting of the tertiary structure of the protein near the distal heme group, without significant changes in the secondary structure (Chattopadhyay and Mazumdar 2000). As shown in Table 4.2, in the presence of BSorbW and BSucProW the addition of NADES-AS decreases considerably, the beginning of the protein unfolding (T^{on-set}) in comparison to the experiment in a neat buffer. This result could indicate that NADES-AS promotes the HRP intermediate state coupled with changes in the secondary structure, an effect previously observed in other enzymes (Papadopoulou et al. 2016; Yadav et al. 2020). This is accompanied by a slow unfolding, where the enzyme exhibits a boost in activity, ending later than the control in neat buffer. Hydrogen bond-like interactions of the exposed distal heme pocket, at temperatures below $T^{unfolding}$, might promote an increase in the enzymatic activity. Nevertheless, NADES addition causes the appearance of enzyme aggregates at high temperatures, as shown in Figure 4.7(B), which have a known effect against activity due to blocking active sites (Smeller, Fidy, and Heremans 2004; B. Wang et al. 2017). $T^{unfolding}$ values are in the range of what is reported in the literature, namely in the range between 70 °C and 85 °C (Haifeng et al. 2008; Chattopadhyay and Mazumdar 2000; Pina et al. 2001; Holzbaur, English, and Ismail 1996). In addition, protein aggregation is observed at temperatures between 80 and 85 °C in the presence of NADES as shown in Figure 4.7(B) and Table 4.2, which was not observed for the control in PBS buffer.

4.2.6 Structural studies of HRP in the presence of NADES

The secondary structure of HRP in different solutions was assessed by CD, and measurements in PBS (100 mM, pH 7) were used as a control. Figure 4.8(A) compares the HRP's CD spectra in PBS vs. the five NADES-AS herein studied, obtained from 190 nm to 240 nm. All the CD spectra obtained have similar shapes, with slight intensity differences at 205 nm, which can be attributed to changes in α -helix contents (Gui et al. 2006). It is also possible to observe that there are no signs of protein denaturation, which is usually characterized by a broad negative band below 200 nm (Doderio, Quirolo, and Sequeira 2011).

To obtain more information about the HRP structure, the relative content of each major secondary structure (α -helix, β -sheet and turns) and the random coil of HRP were determined

and can be observed in Figure 4.8(B). The CONTIN-LL method was used (via the DICHROWEB web server) (Lees et al. 2006). The native HRP structure was the following: 31% α -helix, 9% β -sheet, 16% turns and 44% random coil. It can also be seen in Figure 4.8(B) that the presence of NADES did not significantly change the random coil ($\sim 42\%$) and turns ($\sim 15\%$) content of HRP compared to PBS control, according to the information obtained from the spectra analysis. However, it was possible to detect some alterations in the α -helix and β -sheet contents of HRP depending on the NADES in solution. In the presence of BXylW, the contents of α -helix and β -sheet become nearly identical (20% α -helix and 23% β -sheet), while in the presence of BSorbW α -helix was increased at the cost of decreased β -sheet content (35% α -helix and 5% β -sheet).

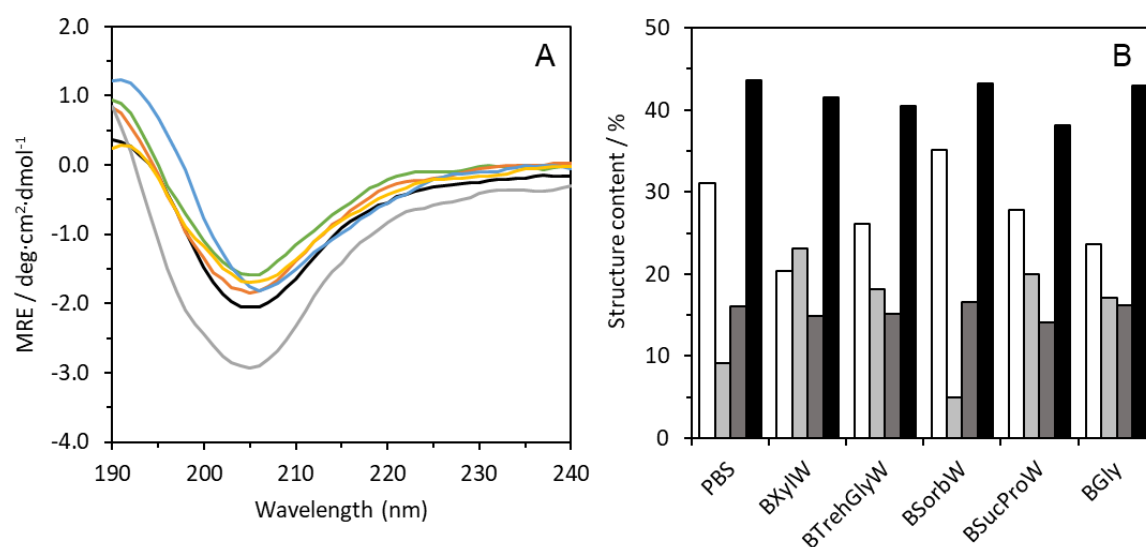


Figure 4.8 – Circular dichroism results. (A) Mean residue ellipticity (MRE) of HRP (5 μ M) dissolved in PBS (black line), BXylW (green line), BTrehGlyW (orange line), BSorbW (gray line), BSucProW (blue line) and BGly (yellow line) solutions; and (B) relative content of secondary structures of HRP in control and NADES: α -helix (white), β -sheet (light gray), turns (dark gray) and random coils (black).

HRP is described as a protein with high content of α -helix secondary structure and small β -sheet regions (Veitch 2004). Hence, in the presence of BSorbW, the structural changes favoured the increase of α -helix content by 13%, which can be related to the rise in activity (Figure 4.4). There is evidence that HRP's activity can be facilitated by higher α -helix and lower β -sheet contents (Wu et al. 2014). As previously demonstrated, a reduction of the relative activity of HRP to 12%, was associated to a decrease of α -helix structure of c.a. 18% (Gui et al. 2006). More studies show the same relation between loss of activity and drop in the α -helix content of HRP, upon different treatments (Zhong et al. 2005; Guo et al. 2022; Han, Cheng, and Sun

2019). These conformational changes are added to positive molecular interactions generated by co-solvents within the protein's active site, as demonstrated previously in the literature (Jaworek et al. 2021; Toledo et al. 2019).

The structure of HRP was also evaluated upon incubation at higher temperatures, using the final time point of each temperature, 24 h at 37 and 60 °C and 4 h at 80 °C. Figure 4.9 shows the secondary structure contents of HRP after incubation in these conditions. In general, in all the systems tested, including the control in PBS, as happened for the control experiments without incubation, there were no significant changes in the contents of random coils (~ 43%) and turns (~ 16%), with the major structural changes being observed between the α -helix and β -sheet contents.

Incubation in PBS at higher temperatures resulted in a complete loss of enzymatic activity. It was expected that this loss would represent a total loss of the secondary structure of the enzyme; however, the results (Figure 4.9(A)) show that there was a decrease of the α -helix content at 60 and 80 °C, concomitant with an increase of the β -sheet, which correlates to the loss in activity. The same behaviour was observed for BXylW (Figure 4.9(B)), which was the NADES with a less thermal protective effect on HRP.

In contrast, BTrehGlyW was the system in which HRP presented the highest thermostability. After the incubation in this system (Figure 4.9 (C)), the secondary structure content shows that the α -helix content was stable at all the incubation temperatures tested, which correlates with higher activity. As mentioned before, in BSorbW, the initial secondary structures content was different from the native enzyme and promoted a faster reaction rate. Incubation in this NADES highly promoted the presence of α -helix (Figure 4.9(D)); however, this conformation did not provide increased protection at a higher temperature. After incubation in BSucProW (Figure 4.9(E)), the presence of β -sheet was favoured without causing a major decrease in the α -helix content; however, this was also reflected in the activity of the enzyme, which decreased. Finally, the results were very surprising when the enzyme was incubated in BGly. Figure 4.9(F) shows that upon incubation at the three temperatures tested, there was an increase in α -helix with the correspondent decrease in the β -sheet content. However, in this system this did not correlate with an increase of activity, and this might be due to the fact that besides structure, the hydration level of the environment i.e. the hydrogen bond network between protein-water and NADES-water influences enzyme activity not monotonically (Sanchez-Fernandez et al.

2022; Belviso et al. 2021). In this case the system BGly presented a very low water content and water activity, which may be related to the lower activity in this system.

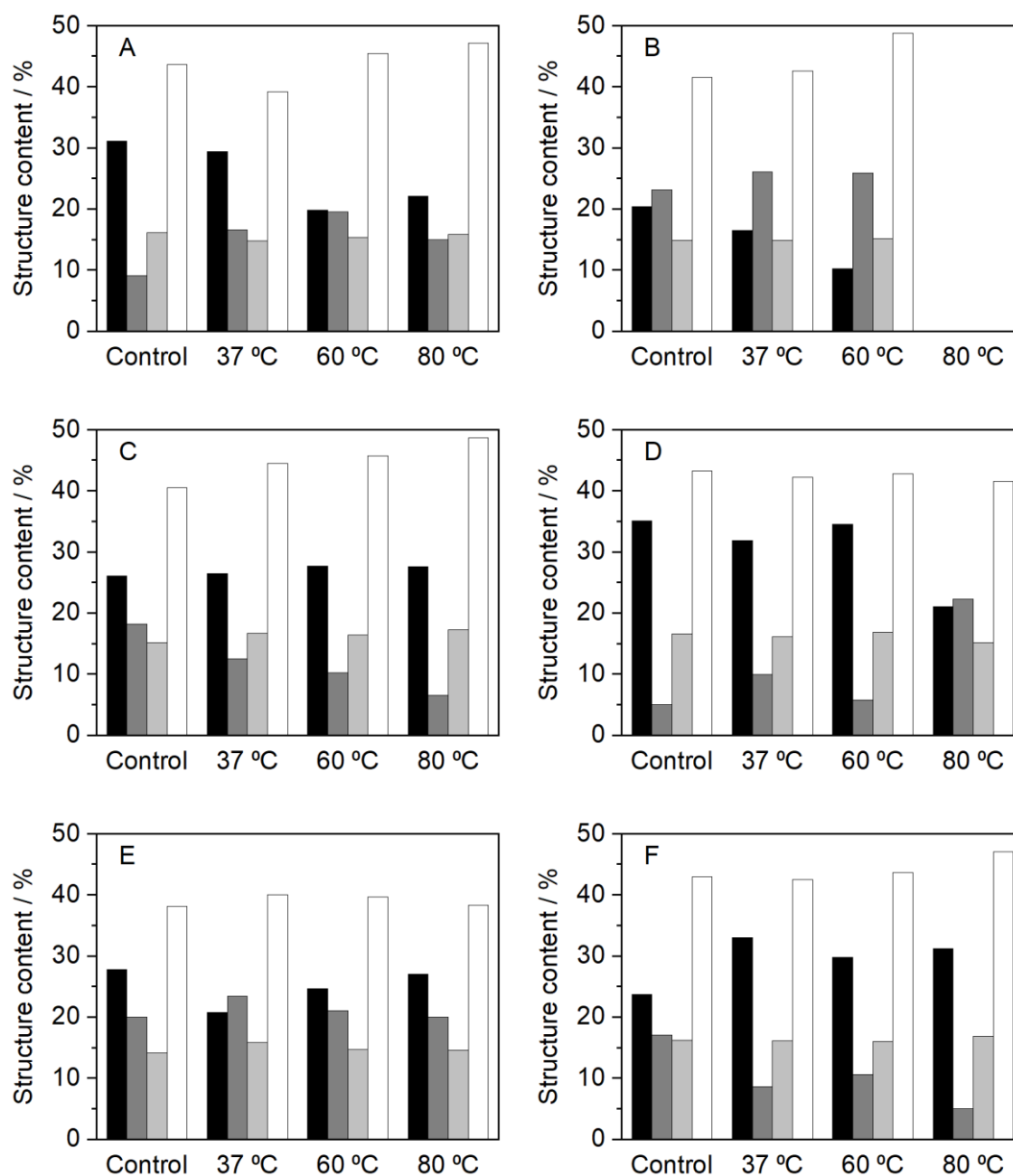


Figure 4.9 - Relative content of the secondary structures of HRP, determined by CD, after 24 h of incubation, at 37 and 60 °C, and 4 h at 80 °C, in the different media tested, namely, (A) PBS, (B) BXylW, (C) BTrehGlyW, (D) BSorbW, (E) BSucProW and (F) BGly. α-helix (black), β-sheet (dark gray), turns (light gray) and random coil (white). The control in each system represents the secondary structure content of HRP in the respective media, at initial time, without any incubation.

4.2.7 Influence of a_w in HRP's activity

The impact of water activity (a_w) on the enzymatic activity of HRP was also studied since this is one of the factors that highly influences enzymatic activity (Neri et al. 2010). For this purpose, the system BGly was used since it had the lowest water content (1.7 wt.%, from residual water in the initial components). Table 4.3 shows the exact water content (wt.%) and a_w of the BGly dilutions, prepared by adding water to the original NADES, finally yielding the 4 different mixtures with different water activity. We observed that each 5 wt.% increase in the water content caused an increase of c.a. 0.1 in the water activity. Increasing the water content of BGly to 21.1 wt.% allowed to achieve an a_w of 0.448, which is close to the a_w of the other systems used in this study (Table 4.1), making the NADES comparable at nearly isoactivity conditions of water.

Table 4.3 - BGly formulations prepared with different amounts of water, with respective water content (wt.%), water activity (a_w).

Formulation	Water added (wt.%)	Water content (wt.%)	a_w , 37 °C
BGly	0	1.7 ± 0.0	0.071 ± 0.004
BGly95	5	5.8 ± 0.1	0.172 ± 0.001
BGly90	10	11.5 ± 0.1	0.283 ± 0.003
BGly85	15	16.9 ± 0.3	0.367 ± 0.001
BGly80	20	21.1 ± 0.2	0.448 ± 0.000

The influence of a_w on enzymatic activity was evaluated after incubation in each of the BGly formulations for 6 and 24 h, at 37 °C. The same procedure was used without any incubation time to determine the influence of the systems on the enzymatic activity shown in Figure 4.10(A). The relative activity presented for BGly is different than the one shown in Figure 4.4, since a different enzyme batch was used. Based on our experience with enzymatic reactions in NADES, we expected that increasing a_w would increase the enzymatic activity; however, the results obtained were quite different. The influence of NADES in reaction rate is presented in Figure 4.10(A), and it is noticeable that without incubation, i.e., time for the enzyme to adapt to the environment, the activity increases for BGly with 5 wt.% of water, being lower for the remaining 3 water contents. However, Figure 4.10(B) shows the results of activity after incubation in BGly, and it is visible that HRP's activity was promoted at 10, 15, and 20 wt.% water, and higher water contents indeed yielded higher enzymatic activities, as expected. Nevertheless, the incubation with BGly95 caused a decrease in enzymatic activity down to 60%

from the control value. Hence, it is possible to observe that while the presence of NADES has impact on the reaction rate, the incubation time allows the enzyme to achieve a conformation that is more favourable for its catalytic activity.

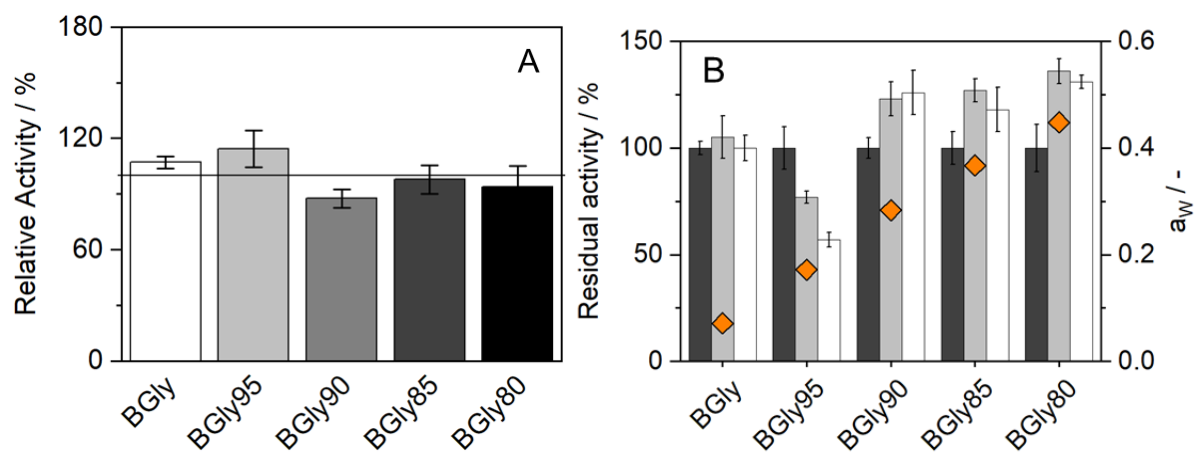


Figure 4.10 – Relative enzymatic activity of HRP in BGly formulation (A) at $t=0$ h ($n=3$); and (B) after incubation in BGly formulations at time 0 h (black), 6 h (grey) and 24 h (white). Diamonds represent water activity of the samples ($n=3$).

The possible explanations to the hydrogen bond network disruption/reorganization, induced by the presence of NADES, or by the different water contents of the NADES BGly, were studied by molecular dynamics (MD) simulations that were performed out of the scope of this thesis. The complete results are presented in Appendix A.2.

4.3 Conclusions

In this work, the influence of five betaine-based NADES on the activity and conformation of HRP was studied. First, density, viscosity, and a_w were measured from 20 °C to 80 °C at a pressure of 100 kPa. Even though the water mole fractions of the studied systems varied strongly, a_w values of all the systems were measured to be around 0.4. For the system BGly, the influence of water content on a_w was measured. Density and a_w were modelled with PC-SAFT. PC-SAFT achieved an overall average absolute deviation of 0.432% and 7.76% for densities and a_w , respectively. The important conclusion is that binary parameters that were fitted to density were able to predict a_w values successfully. The ability to use this predictive power of PC-SAFT to characterize a_w values of NADES will allow the generation of tailor-made solvents for different enzymes in the future, thereby optimizing the design of biocatalytic processes.

As demonstrated in this case study, the presence of NADES in solution, promoted an increase in the thermal and structural stability of HRP. The approach of using NADES in enzyme solutions contributes to a broader insight into biocatalytic reactions in crowded environments and ultimately aims at optimizing the enzymatic environment towards improved stability and efficiency. Overall, an increase of unfolding temperature was observed, and the aggregation appeared at higher temperatures. A transition state before denaturation is promoted by the presence of NADES systems, which could increase enzymatic activity due to the exposure of the heme pocket. On the other hand, the changes in the composition of the secondary structures, α -helix, and β -sheet, show how the protein is restructured in the presence of NADES, by the hydrogen bond network. These conformational changes, more specifically the increase in α -helix content, increased enzymatic activity with the system BSorbW showing a 2-fold increase in HRP's activity. This improvement reflects the suitability of NADES to be used as efficient co-solvents in biocatalytic reactions as a preserving agent against denaturation and for significant enhanced activity.

The thermostability of HRP in both buffer and in NADES at moderate up to high temperatures was also evaluated. The results show that it was possible to improve the activity of HRP in the presence of NADES, especially BSorbW and BTrehGlyW. Moreover, HRP's activity was not impaired after 24h incubation in BTrehGlyW at 60 °C, contrary to the results obtained for PBS and for all other NADES under study. The system BTrehGlyW has also shown the best performance in protecting the enzyme at incubation at 80 °C. The structural studies conducted by CD analysis have shown that NADES that improve enzyme activity the most facilitate higher

contents of the α -helix structure in HRP. Besides thermostability, the effect of water activity on enzymatic activity was studied using the system BGly. The overall results have shown that increasing the water activity leads to an increase in enzymatic activity of HRP, but this advantage drops at a certain critical amount of water in BGly, namely at about 5 wt.% water.

It is worth mentioning that the MD simulations, performed out of the scope of this thesis, accompanied the experimental results. These simulations, allowed to conclude that NADES reduced the flexibility of HRP, leading to a greater stability of the active site. This was promoted by the water exclusion effect that is caused due to the interactions between NADES and the surface of HRP. A correlation between enzyme activity and Gibbs energy of solvation was found, especially when the calculation breaks down into hydrophobic residues. Thus, predicting solvation energies allowed to estimate the beneficial effect of NADES on HRP enzymatic activity. Furthermore, protein-solvent interactions were analysed using minimum distance functions and Kirkwood-buff integrals (KBI), demonstrating that the interaction between the NADES and the protein surface generated an increase in water on the protein surface, which helps to stabilize the active site.

PREPARATION OF NATURAL DEEP EUTECTOGELS FOR DRUG DELIVERY

5.1 Introduction

Enzymatically crosslinked hydrogels offer a wide range of advantages, such as high efficiency and selectivity, fast gelation rate, the use of mild reaction conditions and the fact that no toxic cross-linking agents are used (Li, Lu, and Liu 2023; Trombino et al. 2019). Moreover, they show great mechanical properties and can be prepared *in situ*, in the form of injectable hydrogels. Injectable hydrogels are particularly interesting for tissue engineering since they are able to fill any free space, can be formulated with cells, and do not require surgical implantation or removal, if they are biodegradable (L. S. Wang et al. 2010). The major concern associated with the preparation of injectable hydrogels is the control of the gelation time, which is closely related to the precursor/crosslinker concentration, and should be thoroughly optimised, not only to ensure adequate mechanical properties, but also to guarantee proper growth rate and differentiation of cells inside the polymeric matrix (L. S. Wang et al. 2010; F. Lee, Bae, and Kurisawa 2015).

HRP is one of the most used enzymes to crosslink phenol-groups due to the ease to tune the final hydrogel's characteristics by adjusting the concentrations of HRP and hydrogen peroxide (H_2O_2). HRP has been used in combination with numerous polymers, such as HA, gelatine, chitosan or alginate, with the main condition that these polymers must be modified with tyramine or hydroxyphenylpropionic acid to have phenol groups available (F. Lee, Bae, and Kurisawa 2015; Trombino et al. 2019). In the presence of phenolic hydroxyl groups and H_2O_2 ,

HRP promotes the crosslinking through C-C and C-O coupling of the aromatic ring, which ends up forming the hydrogel (Roberts et al. 2016).

Over the past few years, several authors combined NADES and polymers to prepare hydrogels. For instance, Bianchi et al. reported the preparation of a gelatine-based hydrogel combined with choline chloride: glycerol, for the prolonged release of drug nanocrystals through mucosal delivery (Bianchi et al. 2022). Focusing on the delivery of drugs, the preparation of alginate based hydrogels has also been reported, using ibuprofen as model drug (Pedro et al. 2022). Another example of the incorporation of NADES in gels, includes self-polymerization of 2-hydroxyethylmethacrylate with choline chloride: fructose for the delivery of indomethacin (Mukesh et al. 2016) and with choline chloride: ascorbic acid for the delivery of sunitinib malate (Mokhtarpour, Shekaari, and Shayanfar 2020).

The number of publications concerning the use of NADES for the preparation of drug delivery systems (DDS) using non-steroidal anti-inflammatory drugs (NSAID) is scarce and most focus on the application of NADES as enhancers of solubility in aqueous media (Roda, Paiva, and Duarte 2021). Nevertheless, the use of ibuprofen in the DES formulation, allowed to improve its solubility in buffer, while achieving a controlled release profile (Aroso et al. 2015). Recently, the preparation of alginate hydrogels with NADES was also assessed for the delivery of ibuprofen, which was successful (Pedro et al. 2022).

Following the work developed for the improvement of HRP activity and thermostability in NADES (Chapter 4), this chapter presents a practical application of the combination of NADES and HRP. The main goal of this work was to prepare enzymatically crosslinked gels using NADES as dispersion medium, in replacement of water, that will be called eutectogels. Gelatine was used as model polymer, since it is easily available, and this work was developed as a proof-of-concept. The gels were characterized in terms of their gelation time, stability, and rheological properties. Their feasibility to be used as DDS for a NSAID, ketoprofen, was also evaluated. To the extent of our knowledge this is the first report of the preparation of eutectogels through enzymatic crosslinking.

5.2 Results and Discussion

5.2.1 Synthesis of gelatine-phenol conjugates

The gelatine-phenol (Glt-Ph) conjugates were obtained using bovine skin gelatine and tyramine hydrochloride. The synthesis was performed through the carbodiimide-mediated condensation of the amino groups of tyramine and the carboxyl groups of gelatine, which allows for the binding of a phenol groups to a carboxyl group, as shown in Figure 5.1 (Chuang et al. 2015). The cross-linking of protein chains occurs through tyrosine residues; however they can be inaccessible or be present in small amounts, hence the incorporation of more phenol groups is essential for hydrogel production (Sakai et al. 2009).

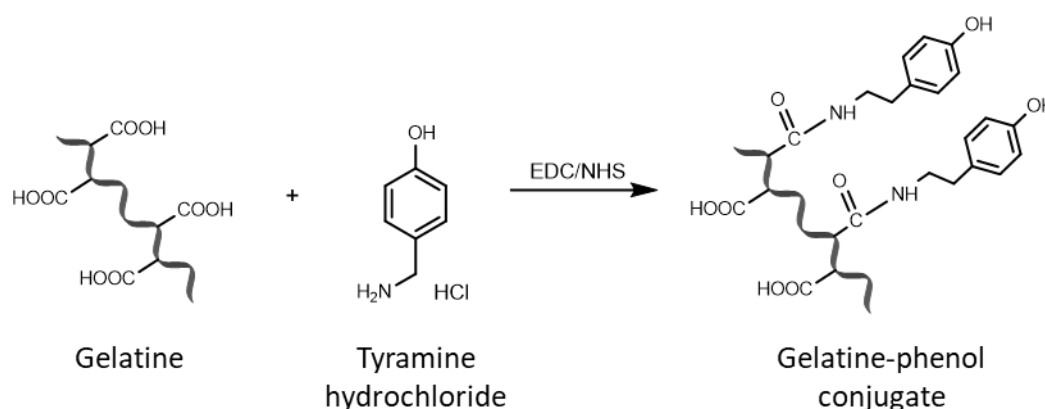


Figure 5.1 – Schematic representation of the conjugation reaction used for the synthesis of gelatine-phenol conjugates.

The synthesis of Glt-Ph conjugates was performed as described by Chuang et al., using fixed concentrations of gelatine, Tyr, EDC and NHS (Chuang et al. 2015). The reaction was carried out over-night at 25 °C, as indicated in Table 5.1.

The success of the conjugation reaction was determined through UV-Vis and through ATR-FTIR analysis. Performing a UV-Vis scan, using a 0.1 w/v.% Glt-Ph solution, allowed to confirm that the conjugation reaction had taken place, since a peak at 275 nm was only visible in the presence of tyramine, confirming that it had been incorporated by gelatine during the reaction. To further confirm the conjugation, ATR-FTIR analysis was performed. The spectra obtained are visible in Figure 5.2. The spectra obtained for gelatine and tyramine were in accordance with others found in literature (N. H. Nguyen et al. 2022; Tran et al. 2013). Briefly, in gelatine's spectrum, it is possible to see the stretching of amine bonds, at 3300 cm⁻¹ and bending at 1590

cm⁻¹. The carbonyl groups of gelatine are visible at wavenumbers 1640 cm⁻¹ (Tran et al. 2013). As for tyramine hydrochloride, it was also possible to identify the bands corresponding to amine I vibration, at ~3070 cm⁻¹ and from 800 to 600 cm⁻¹, along with band at 1500 cm⁻¹ corresponding to the C=C of the phenol group (N. H. Nguyen et al. 2022). In the spectrum of the conjugated polymer (Glt-Ph), it is possible to identify bands that correlate to ones found both in gelatine and in tyramine, as well as the disappearance of the bands from 800 to 600 cm⁻¹, associated to the amine group of tyramine, indicating the formation of the bond between tyramine and gelatine. There was also an increase in intensity of the bands around 1300 and 1100 cm⁻¹, associated to the stretching of C-N bonds, which are formed during the condensation reaction (N. H. Nguyen et al. 2022).

With the confirmation that the reaction was successful, the yield of tyramine incorporation was quantified, through UV-Vis, using a calibration curve prepared with tyramine hydrochloride. Although the reaction conditions were kept the same, the yield of Tyr conjugation was not stable, varying from 5.60% to 8.47% (Table 5.1).

Table 5.1 – Reaction conditions used for synthesis of Glt-Ph conjugates and respective Tyr conjugation yield. The reaction was carried out using 2 %w/v gelatine, 1 %w/v Tyr, 38.3 mM EDC and 19.2 mM NHS.

Experiment	Glt-Ph1	Glt-Ph2	Glt-Ph3
pH	6.0	6.0	6.0
Temperature (°C)	25	25	25
Tyr conjugation yield	8.47%	7.10%	5.60%

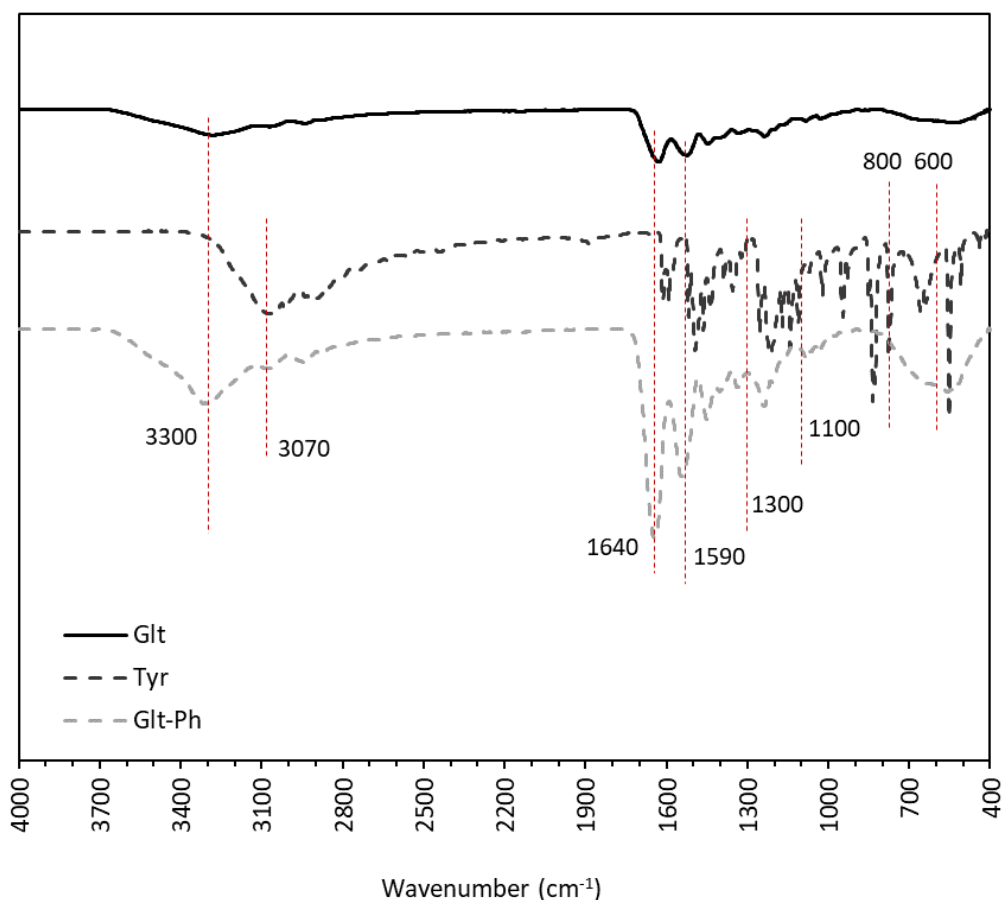


Figure 5.2 – ATR-FTIR spectra of gelatine (Glt, full black line), tyramine hydrochloride (Tyr, dashed black line) and the conjugate Glt-Ph (dashed grey line). The spectra were obtained between 4000 and 400 cm^{-1} , in transmittance mode.

5.2.2 Preparation of Gels and Gelation Time

To access the viability of obtaining gels with the produced Glt-Ph conjugates, hydrogels were prepared using 5 %w/v Glt-Ph in PBS, using two concentrations of HRP (5 and 10 $\text{U}\cdot\text{mL}^{-1}$). Under all conditions tested, hydrogels were formed and the relation between HRP concentration and gelation time was assessed. Higher HRP concentrations yielded lower gelation times, for instance 15 and 0.15 $\text{U}\cdot\text{mL}^{-1}$, lead to 2 and 13 minutes of gelation time, respectively.

For the preparation of eutectogels, betaine: glycerol (BGly) in a molar ratio of 1:2 was used. This NADES has been thoroughly characterized in Chapter 4, section 4.2.1. After several tests, it was concluded that Glt-Ph was not soluble in pure NADES therefore, a mixture of 90 wt.% BGly and 10 wt.% water (BGly90) was used for the preparation of the eutectogels.

Eutectogels were then successfully prepared using the same conditions described for hydrogels, 5 %w/v Glt-Ph dissolved in BGly90, and two concentrations of HRP were used, namely 5 and 10 U·mL⁻¹. As it was observed during the preparation of hydrogels, the gelation time was closely related to the HRP concentration. During the preparation of eutectogels, the same trend was observed, with the gelation times being 52 and 30 seconds for 5 and 10 U·mL⁻¹ of HRP, respectively (Figure 5.3).

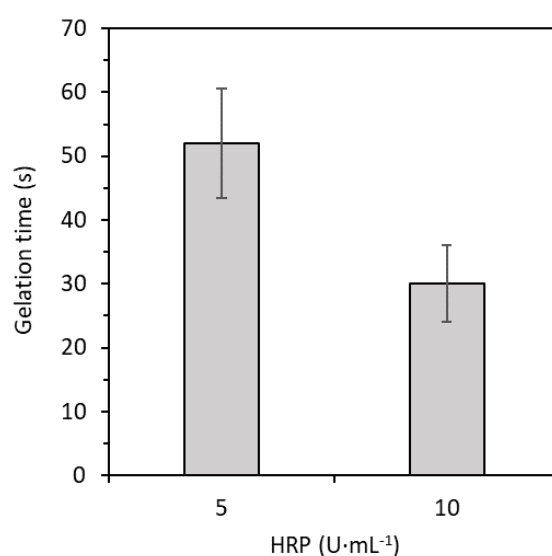


Figure 5.3 – Gelation time of eutectogels (mean±SD) prepared with 5 %w/v Glt-Ph in a BGly90 matrix, using different concentration of HRP.

The relation between gelation time and HRP concentration had already been described by Sakai et al. (2009). The authors observed the same behaviour, increasing concentrations of HRP lead to lower gelation times. The gelation times obtained by these authors are not comparable to the ones obtained in this work, since the concentration of H₂O₂ used was much higher (50 mM against 3 mM) (Sakai et al. 2009). In a more recent work, other authors obtained similar results, in a time range closer to the one obtained in our work. Mubarok et al. determined that with concentrations of HRP of 1, 5 and 15 U·mL⁻¹, the gelation time was 57, 35 and 15 seconds, respectively (Mubarok, Qu, and Sakai 2021). Nevertheless, the most important aspects when considering *in vivo* applications of hydrogels, are that hydrogels should be successfully obtained in less than 1 minute, along with the fact that they can be prepared using lower amounts of hydrogen peroxide, since it is a strong oxidant which can have toxic effects on cells and live tissues (Sakai et al. 2009; Mubarok, Qu, and Sakai 2021).

5.2.3 Characterization of the produced gels

5.2.3.1 Stability at 37 °C

To determine the stability of the prepared gels, samples were placed in an oven at 37 °C, either immersed or not in PBS, and checked periodically to determine any changes to its surface or integrity. The gels kept immersed in PBS, either PBS-based hydrogels and eutectogels, showed low stability, being completely disintegrated in the liquid after 24 hours or 5 days, respectively. For what concerns the gels which were not immersed the results were much different. PBS-based hydrogels would lose their structure and become liquid after a maximum of 24 hours. However, the eutectogels were more stable, and maintained the gel-like appearance up to 1 year after preparation.

These results indicate that the structure created in the eutectogels is more robust than the one obtained in the conventional hydrogels. This can be linked to the presence of BGly, since the dense hydrogen bond network of NADES improves, not only, the link between its components, but also the protein-NADES bonds, creating a more stable 3D structure (Pedro et al. 2022).

5.2.3.2 Rheological Properties

Since the eutectogels were prepared envisaging their application as drug delivery systems, possibly in an injectable form, their rheological properties were also determined.

Viscosity is one of the key aspects on the design of an injectable material and indicates the resistance to deformation of a material in response to stress (Chen et al. 2017). Hence, viscosity *vs.* shear rate was determined for a mixture of BGly, Glt-Ph and HRP (the possible mixture used for injection), at a fixed temperature of 37 °C. The results obtained are presented in Figure 5.4, showing that the mixture presents a shear thinning behaviour, characterized by a decrease in viscosity for increasing shear rates (Chen et al. 2017), which is a good indicator of injectability. Moreover, the viscosity of the mixture is within the range described for injectable hydrogels (Chen et al. 2017).

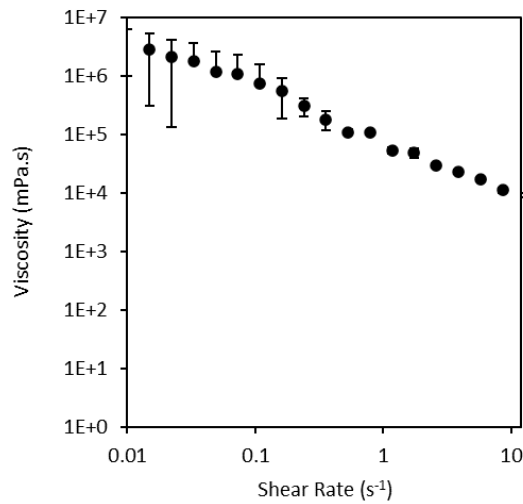


Figure 5.4 – Viscosity vs. shear rate obtained for the mixture of 5 %w/v Glt-Ph in 90BGly.

Besides the viscosity of the injectable material, when designing a hydrogel it is also important to assess storage (G') and loss (G'') moduli, which provide information about the elastic and viscous behaviour of the material, correlating to its ability to absorb or dissipate energy (Chen et al. 2017).

The frequency sweep study for the PBS-based hydrogels (Figure 5.5) shows a typical hydrogel profile, with G' consistently above G'' , which is representative of elastic behaviour and also of crosslinked hydrogels (Alizadeh Sardroud et al. 2017; Stojkov et al. 2021). It can also be observed that the different concentrations of HRP (5 and 10 $U \cdot ml^{-1}$), have no impact on the rheological properties of the hydrogels, since the same resistance to deformation was obtained in both samples.

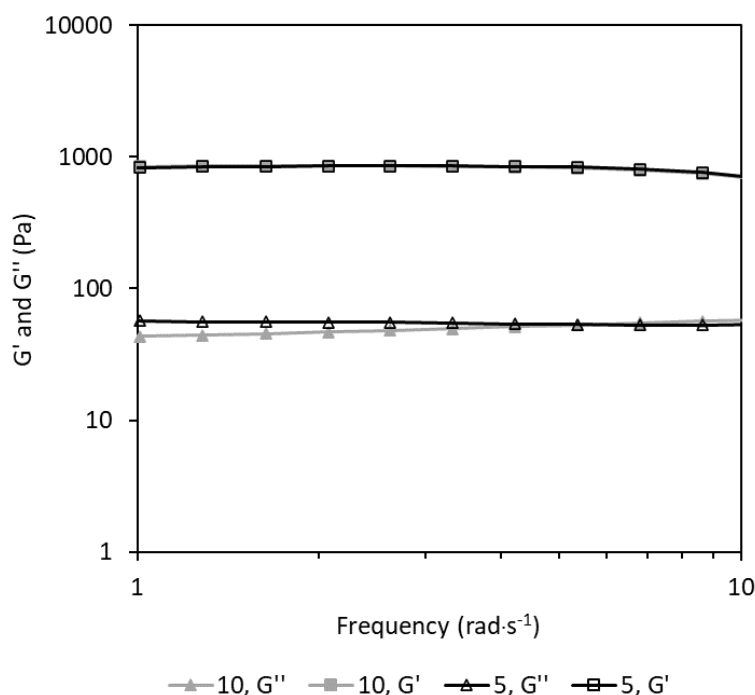


Figure 5.5 – Frequency sweep of PBS-based hydrogels prepared with different HRP concentrations: 5 U·mL⁻¹ (black open symbols) and 10 U·mL⁻¹ (grey filled symbols). Storage (G') and loss (G'') moduli as a function of frequency (rad·s⁻¹).

To determine if the storage time had any influence on the properties, the rheological properties of the eutectogels were determined after preparation and periodically, after 1 month, 3 months and again after 1 year, since the gels were still intact at this point. As can be seen in Figure 5.6, after preparation (blue symbols), the eutectogels produced with both concentrations of HRP, show G' consistently above G'' , indicating that they behave as elastic materials, and there is no cross of G' and G'' , as expected for a crosslinked gel (Stojkov et al. 2021).

Moreover, in all conditions tested, G' shows independence of frequency up to 5 rad·s⁻¹, showing a slight decrease for higher frequencies. It is also possible to observe that, especially for the longer time point (1 year), the absolute value of both moduli becomes higher, indicating higher resistance to deformability. This increase could mean that there is an increase of the gels' stiffness over time, which could be related to the evaporation of water during storage or even to a higher degree of crosslinking, caused by residual HRP activity over time. In comparison to PBS-based hydrogels, eutectogels have lower G' and G'' , indicating that these gels are softer, which can also represent more flexibility (Alizadeh Sardroud et al. 2017). This could be explained by the presence of glycerol in the formulation, since it is a well-known plasticizer for biopolymers, particularly hydrophilic polymers such as gelatine. Its presence can have impact

on the polymers' properties, for instance increasing their flexibility and elasticity and decreasing its Young's modulus (Suderman, Isa, and Sarbon 2018).

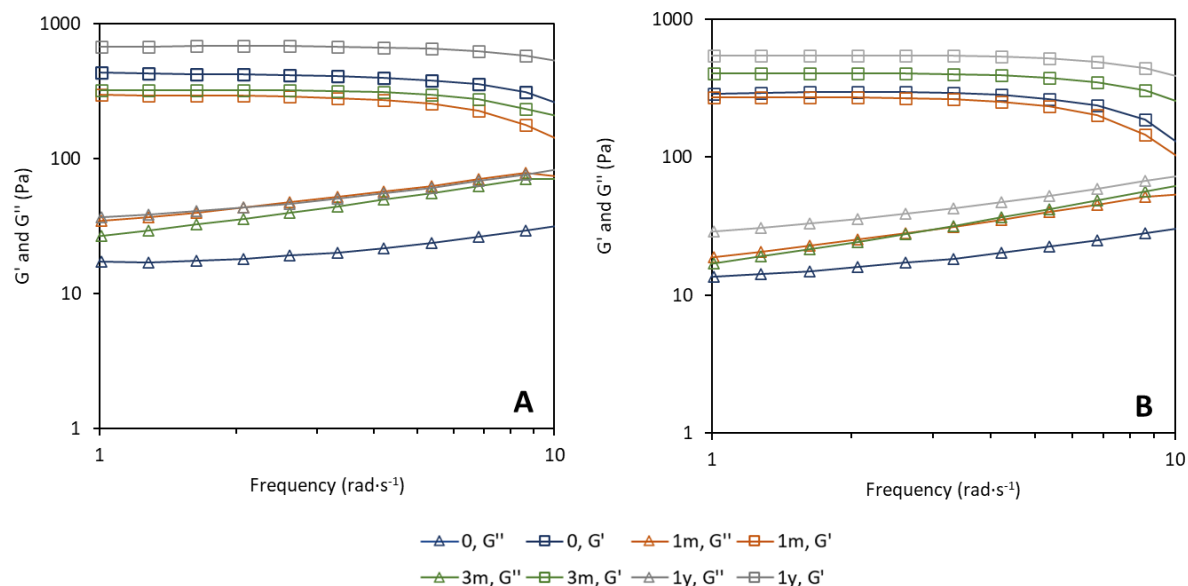


Figure 5.6 - Frequency sweep of eutectogels prepared with (A) 10 U·mL⁻¹ HRP and (B) 5 U·mL⁻¹ HRP. Storage (G') and loss (G'') moduli are represented as a function of frequency (rad·s⁻¹). Blue represents the samples after preparation ($t = 0$), orange represents the samples after 1 month, green after 3 months and grey after 1 year of storage at 37 °C.

5.2.4 Incorporation and Release Profile of Ketoprofen from Eutectogels

Injectable hydrogels offer a wide range of advantages, including their application as drug release matrixes. Herein, we evaluated the incorporation of a NSAID, ketoprofen (Ket), and its subsequential release from the eutectogels, in order to access the feasibility of their application for biomedical ends.

Polarized optical microscopy (POM), was used to confirm the complete dissolution of Ket in NADES, by using a cross polarizer that allows to identify if there were crystals present in the sample, which would mean that the mixtures were not homogeneous (Santos, Leitão, and Duarte 2019). The images collected can be seen in Figure 5.7. The uniform black images represent amorphous and homogenous liquids, confirming the total dissolution of up to 10 mg_{Ket}·g_{carrier}⁻¹ in BGly (Figure 5.7(A)), as well as in the gel precursor mixture, Glt-Ph in 90BGly (Figure 5.7(B)).

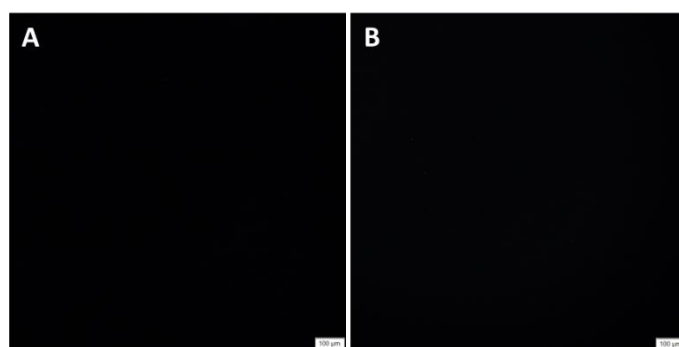


Figure 5.7 – Polarized optical microscopy images of A) ketoprofen dissolved in pure BGly and B) ketoprofen dissolved in gel precursor solution (Glt-Ph in 90BGly).

Hence, eutectogels were successfully produced with the incorporation of Ket, at the final composition of 5 wt.% Glt-Ph in 90BGly, 10 U·mL⁻¹ of HRP, and 3 mM H₂O₂, with 10 mg·g⁻¹ of Ket. The release of Ket was evaluated from the two samples: the eutectogel and BGly alone.

The release of Ket was followed through UV-vis until a plateau was achieved. For both samples, the concentration of Ket determined upon stabilization was assumed as the solubility of ketoprofen in PBS, in the studied conditions and is reported in Table 5.2. The solubilities determined are close to the expected since Ket solubility in PBS has been described to be 500 µg·mL⁻¹ (Cayman 2022).

Table 5.2 – Solubility of Ket in PBS (µg·mL⁻¹) when released from eutectogels and BGly.

Carrier	Ket solubility in PBS (µg·mL ⁻¹)
Ket	500 (Cayman 2022)
Eutectogel	657.5 ± 89.5
BGly	447.6 ± 26.2

The release profile of Ket from BGly and from the eutectogel is shown in Figure 5.8. For BGly, 3 phases of release can be identified. First, there was a fast release over the first 8h, during which 60% of the total Ket are released to the medium. The second phase is comprised between 8h and 2 days, with the release reaching 80%. The third, and last release phase, happened from 2 days until 10 days, with a release of the remaining 20% over 8 days, at a much slower rate. In the case of eutectogel, only 2 phases were observed. The fast release phase also happens over the first 8h however, with only 50% of the total Ket being released. From there until day 10, the release of Ket reached 70% of the total, hence not reaching the limit of Ket dissolved.

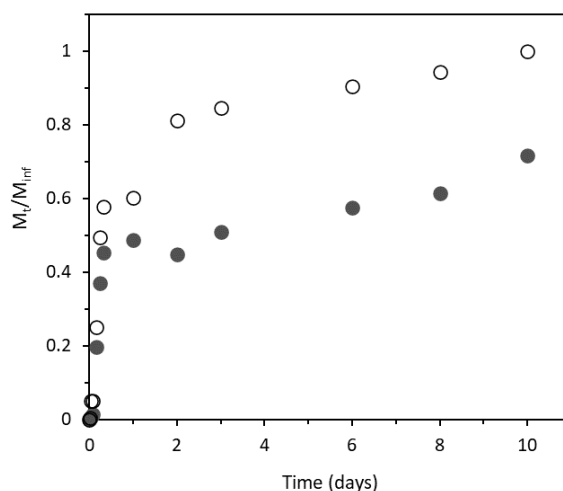


Figure 5.8 – Release profile of ketoprofen from eutectogels (●) and from BGly (○).

The observed profile corresponds to a type II release profile (Bajpai et al. 2008), characterized by a fast release in an initial short time period that tends to stabilize over a longer period. In real conditions, i.e. administration to the blood stream, for instance, these results could be different, since the concentration of drug in plasma is affected by a variety of factors such as stress, physical activity or sleeping. Moreover, physiological conditions, such as presence of enzymes, may interfere with the process and rate of degradation of the eutectogel, which will consequently affect the drug release rate and profile. Nevertheless, as a proof-of-concept work these results seem very promising for the design of injectable drug delivery systems. To optimize the design of the DDS two main conditions should be taken into account, the amount of Ket loaded, and the percentage of gelatine used, since it has been reported that it can influence the release profile of the drug.

The Ket release profile herein obtained was comparable with the one reported by other authors in carriers containing gelatine (Kéri et al. 2020; Raafat and Abd-Allah 2018). When Ket was loaded in hybrid aerogels of silica and gelatine, a sustained release profile was obtained. However, the authors observed that the profile was dependent of the amount of gelatine in the aerogel, with the higher percentages yielding a more controlled profile, without an initial stage of fast release observed for lower gelatine contents (Kéri et al. 2020). On a different study, Ket was loaded into polyvinyl alcohol and gelatine scaffolds reinforced with bioglass to evaluate its potential as drug carrier. It was concluded that the scaffolds could successfully create a controlled delivery of Ket after an initial fast release (Raafat and Abd-Allah 2018).

5.3 Conclusions

Throughout this chapter the preparation of eutectogels using enzymatic crosslinking was discussed. The main goal of this work was to develop a formulation for an injectable gel, to be crosslinked *in vivo*, that could also be used for drug delivery and presented enhanced properties over conventional hydrogels. Using Glt-Ph with an average of 6% tyramine two types of gels were prepared, hydrogels, using PBS as solvent, and eutectogels, in which the aqueous solvent was substituted by BGly.

Once the conjugation of gelatine and phenol (Glt-Ph) was optimized, the conditions for the preparation of PBS-based hydrogels were determined, leading to the optimal conditions of 5 wt.% polymer, 5/10 U·ml⁻¹ HRP and 3 mM H₂O₂, that were later used in the preparation of the eutectogels.

From the results obtained it was concluded that the concentration of HRP had high influence on the gelation time of the gel, reducing it from 52 to 30 seconds, when using 5 and 10 U·ml⁻¹ of HRP, respectively. Moreover, the eutectogels were more stable than regular hydrogels, both in solvent and in the dry form, in which they kept for one year at 37 °C.

Concerning the rheological properties, the injectability of the mixture of 90BGly and Glt-Ph was assessed, and it was concluded that despite its high viscosity it presented shear-thinning behaviour, which is a good indicator of injectability. The prepared eutectogels showed a typical rheological behaviour for gels, with a storage modulus higher than the loss modulus, at frequencies up to 10 rad·s⁻¹. It was also observed that storage at 37 °C had impact in the absolute values of storage and loss moduli, which increased for all the samples tested, independently of the enzyme concentration.

Finally, a proof-of-concept test of the application of the eutectogels as drug delivery systems was conducted. For this, ketoprofen was used as model drug since it was soluble both in the pure BGly and in the gel precursor mix. The release study showed that after an initial period (8h) during which the concentration of drug increased rapidly in the medium, a stable release could be maintained up to 10 days.

Overall, the goals proposed for this work were achieved, since it was possible to obtain eutectogels that had better properties than the PBS-based hydrogels. The evidence of injectability of the precursor mixture and short gelation times are promising for their

application as *in vivo* injectable gels with the possibility to use them as drug release systems. The future steps of this work include biocompatibility assays of the gels, which are crucial for the develop of this system for biomedical applications.

PRESERVATION OF RED BLOOD CELLS IN NADES

6.1 Introduction

Healthy RBC, or erythrocytes, in discocyte shape, measure c.a. 8 μm in diameter and 2 μm in thickness (Figure 6.1(a)), comprising $\sim 90 \mu\text{m}^3$ of cytosol with an osmolarity of $300 \text{ mOsm}\cdot\text{Kg}^{-1}$ (Son et al. 2021). Haemoglobin (Hb) molecules, essential for oxygen transportation are present in the cytosol, representing 1/3 of the total RBC mass (Glenn and Armstrong 2019).

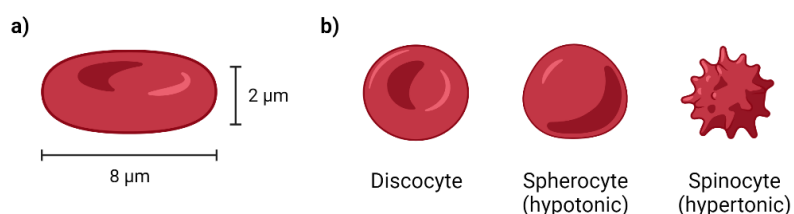


Figure 6.1 – Schematic representation of a) healthy RBC dimension and b) shapes RBC can acquire in media with different tonicity (Created with BioRender.com).

After donation, RBC concentrates are stored in hypothermic storage ($2-6^{\circ}\text{C}$) up to 6 to 7 weeks, depending on the additive solution used (Islamzada et al. 2022). The cryopreservation of RBC is limited to special cases, such as preservation of rare RBC or in military settings (Asghar et al. 2014). According to Instituto Português do Sangue da Transplantação (IPST, IP), in Portugal RBC are stored in saline-adenine-glucose-mannitol (SAGM), at $1-4^{\circ}\text{C}$, for a maximum of 42 days, after which they are disposed (Ministério da Saúde 2007).

During the storage period, cells are prone to suffer storage lesions that cause structural, metabolic and morphologic alterations that will lead to loss of deformability, oxidation and

loss of membrane functionality. The main reasons for the development of storage lesions are hypothermia and semi-closed environment in which RBC are stored. The storage at low temperatures forces RBC metabolism to slow down, and the semi-closed environment only allows gas exchange, hence leading to depletion of nutrients provided by the additive solution (i.e. glucose) and accumulation of metabolic waste (mostly lactic acid, the byproduct of glycolysis), causing acidification of the medium (Yoshida, Prudent, and D'Alessandro 2019). The damage becomes more intense after 2 weeks of storage (Bennett-Guerrero et al. 2007). High extents of storage lesions can have negative impact in the blood's recipients, being associated with post-transfusion morbidity and mortality (Islamzada et al. 2022; Zehnder et al. 2008).

Among several aspects, osmolarity of the additive solutions has a high influence on the shape and volume of RBC, which are directly linked with the Hb concentration in the cytosol (Friebel 2010). In hypertonic medium, water moves from RBC to the medium, causing shrinkage of the cells, turning them into spinocytes (Figure 6.1(b)). On the other hand, exposure of RBC to hypotonic medium, moves water inside of RBC, leading to an increase of cell's volume, changing them into spherocytes (Figure 6.1(b)) (Friebel 2010). Depending on the extent of the damage, the shrinkage process can be reversible, however in advanced stages, the cells lose function due to loss of membrane flexibility and are eventually removed from circulation as microvesicles (Xia et al. 2017). The swelling of RBC is most likely irreversible, with membranes losing their integrity leading to bursting of the cells, a process called haemolysis, that releases the Hb content of the RBC into the cytosol (Friebel 2010). Haemolysis is considered a good indicator of the quality of RBC storage system and can also indicate contamination of the sample. The maximum amount of haemolysis allowed at the end of the storage period has been defined by the European council at 0.8% of total mass of erythrocytes, while the Food and Drugs Administration (FDA) set the limit at 1% (Arif et al. 2017; Ministério da Saúde 2007).

Considering the formulation and biocompatibility of most NADES formulations and their application in preservation processes (Jesus et al. 2021a; 2021b), in this chapter the application of NADES as additive solutions for RBC storage was assessed, aiming to extend the storage limit past the current 42 days, using an environmental friendly alternative.

6.2 Results and Discussion

6.2.1 Haemolysis screening assay

For an initial screening of haemolytic activity, a selection of 14 NADES containing sugars, polyols and amino acids was made as indicated in Table 2.1 (Chapter 2, Materials and Methods). These compositions were selected since sugars are essential for the maintenance of RBC metabolism (Hess 2006). Furthermore, mannitol and sorbitol are also commonly present in additive solutions and provide osmotic support, reducing haemolysis and stabilizing membranes (Arif et al. 2017). For an initial study the NADES-based solutions were prepared with 8% and 4% of NADES, since it has been described that most of the systems show toxicity towards healthy cell lines above 10% (Jesus et al. 2021a).

In this screening the main goal was to understand if NADES would promote haemolysis of RBC. Hence, the haemolysis assay was performed, in which RBC were incubated at 37 °C, for 1h in the presence of NADES solutions and controls. This assay is a relative assay, using PBS as negative control, in which the haemolysis is considered 0%, and using water as positive control, in which haemolysis considered 100%.

As it has been stated, both the limits for haemolysis after long term storage are around 0.8% and 1% in Europe and USA, respectively (Arif et al. 2017). The results obtained in this assay have shown that ten out of the fourteen NADES tested, showed promising results to be used for RBC preservation, since they induced less than 1% of haemolysis to RBC (Figure 6.2, primary axis). From these ten, the highest extent of haemolysis, 0.7%, was determined for the solution with 4% TrehGlcSucW. However, for most of them, there was no defined pattern, since in some cases, the solutions with 8% would cause haemolysis, while the solutions with 4% NADES did not. It is also not possible to create an association of the higher haemolysis extent with components of the used NADES.

The remaining four systems had a very distinct behaviour, namely TGly, GlyGlc, BNaCW and GlyLB. The results obtained for TGly and GlyLB are represented on the secondary axis of Figure 6.2, since these NADES caused high percentages of haemolysis (from 50 to 70%). It is known that glycerol has the ability to permeate cell membranes, which creates an osmotic imbalance, dragging water inside, causing their swelling and, ultimately, bursting (Jay and Rowlands 1975). Hence, this degree of haemolysis can be associated to the higher content of glycerol of these solutions. The capacity of glycerol to change RBC's shape has been previously described (Dou

et al. 2019). However, since in some cases the toxicity of components is different when they are in NADES formulation these systems were also tested in this assay.

The result of the addition of BNaCW and GlyLB to RBC, caused a fast coagulation of RBC, hence not having a quantifiable extent of haemolysis. The coagulant activity of BNaCW and GlyLB may be caused by the present of acidic components, namely *N*-acetyl cysteine and lactobionic acid.

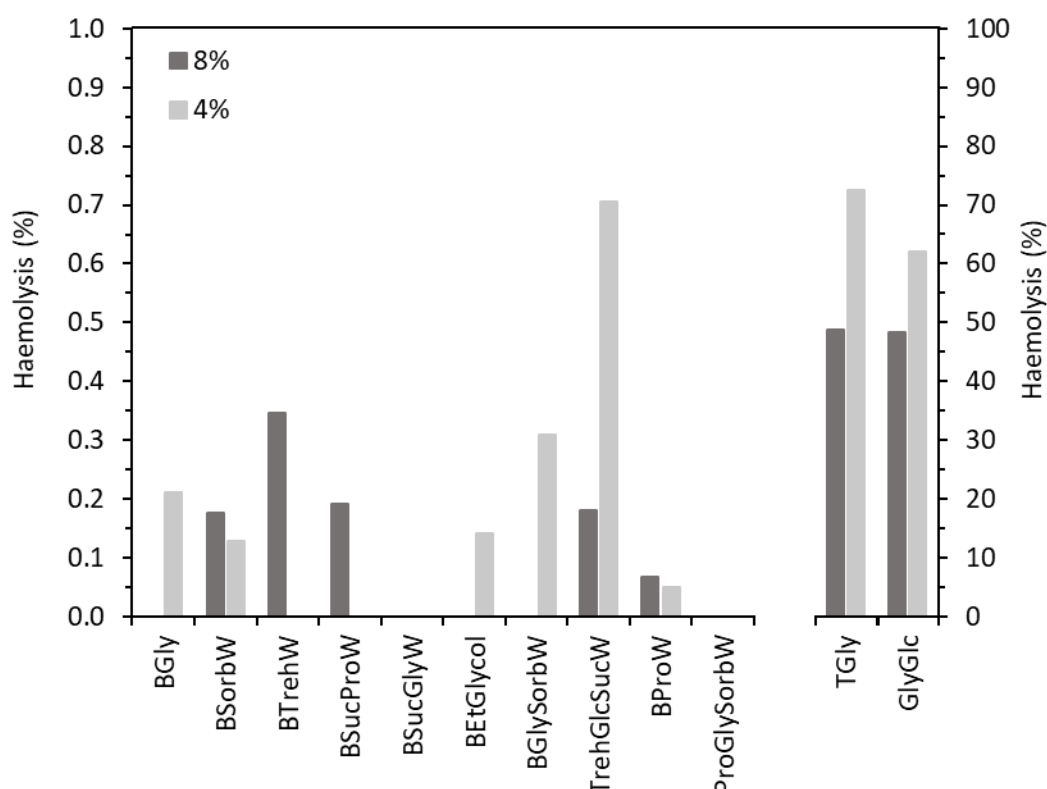


Figure 6.2 – Percentage of haemolysis caused by 8% (dark grey) and 4% (light grey) NADES solutions, relative to PBS (0%) and water (100%). TGLy and GlyGlc are represented on the secondary axis.

6.2.2 Optimization of NADES-solutions

One of the most important characteristics of additive solutions for blood preservation is osmolality since RBC are very sensitive to changes in the osmolality of the media in which they are placed. When subjected to media with the wrong osmolality, RBC can suffer irreversible damage. Plasma's osmolality is reported to be c.a. 300 mOsm·kg⁻¹, the same as cytosol (Son et al. 2021). The additive solution currently used in Europe is composed of sodium chloride,

adenine, glucose and mannitol (SAGM), and is slightly hypertonic with osmolality around 360 mOsm·kg⁻¹ (Figure 6.3).

Hence, we proceeded to determine the osmolality of the NADES solutions that were previously used for the haemolysis screening assay. Figure 6.3 shows the results obtained for all the NADES tested, in both concentrations, in comparison to SAGM, represented by the black line. As it can be seen the used solutions presented an osmolality ranging from 100 to 1000 mOsm·kg⁻¹. In some cases, the osmolality of the solutions was very close to the optimal, namely 4% BGly and 8% BTrehW and BSucProcW. As for the TrehGlcSucW, which had the lowest osmolality (227 and 115 for 8 and 4%, respectively), a correlation with the higher extent of haemolysis can be done. This shows that the medium was hypotonic to RBC, causing water to move towards the cells, hence causing their lysis. On the other hand, solutions with high osmolality, such as BEtGlycol, are hypertonic to RBC and their impact is not visible in the haemolysis assay since they cause dehydration of RBC. Nevertheless, it helps to prove that the absence of haemolysis is not the best indicator for the performance of NADES as preservatives for RBC. Besides these two exceptions, no other correlation between osmolality and haemolysis was found, which indicates that there are multiple factors influencing the outcome.

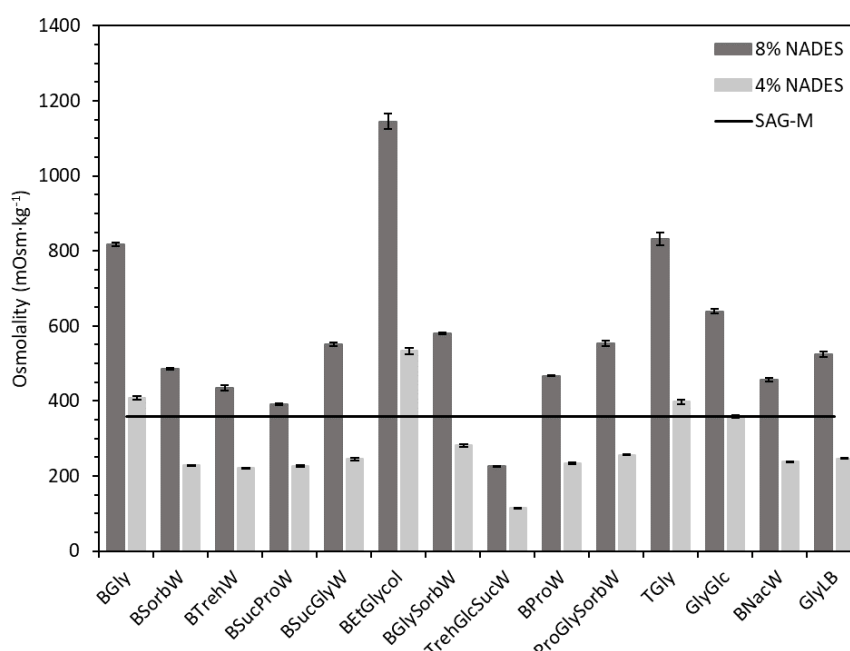


Figure 6.3 – Osmolality (mOsm·kg⁻¹) of 8% (dark grey) and 4% (light grey) NADES solutions. The black line represents the standard additive solution, SAGM.

These findings led to an optimization of the percentage of NADES in solution, to achieve optimal osmolality of the additive solution and minimize the damage caused to RBC. For this process, TGly, GlyGlc BNaCW and GlyLB were not considered. Moreover, the system TrehGlcSucW was also not considered, since the percentage required to achieve optimal osmolality was estimated to be 13%. Table 6.1 shows the percentage of each NADES needed to achieve osmolality within the optimal range (considered from 350 to 390 mOsm·kg⁻¹), which ranged from 2.7% to 7.7%. Considering these as the optimal percentages of NADES to be used in an additive solution, the remaining assays were performed using these concentrations.

Table 6.1 – Correction of NADES percentage in solution to obtain the required osmolality for RBC preservation.

Solution	%NADES	Osmolality (mOsm·kg⁻¹)
SAGM	-	358
BGly	3.8	370 ± 2
BSorbW	6.5	360 ± 5
BTrehW	7.5	370 ± 2
BSucProW	7.7	361 ± 4
BSucGlyW	6.0	366 ± 5
BEtGlycol	2.7	350 ± 3
BGlySorbW	5.6	388 ± 1
BProW	6.4	364 ± 1
ProGlySorbW	5.4	361 ± 1

6.2.3 Biocompatibility of NADES

According to previous studies that reported the biocompatibility of the NADES used, it was expected they would not present high toxicity towards healthy cell lines. Nevertheless, since the concentrations of NADES had to be optimized to meet the requirements of osmolality and covered a wide range of values, their cytotoxicity was evaluated in L929 cell line, as previously described for some of the NADES herein used (Jesus et al. 2021a). Considering the application as RBC preservation media, the cytotoxicity of SAGM was also assessed.

The results of the cell viability assay, pictured in Figure 6.4, show that the highest impact on viability was caused by SAGM or solutions with more than 5% NADES. Comparing the

biocompatibility of NADES to SAGM's, the only system with highest toxicity was BSucProW with less than 60% viability.

The low viability obtained in the presence of SAGM suggested that this may not be the best assay to conclude about biocompatibility of RBC preservation solutions, since SAGM is the option approved for clinical used. Nevertheless, it allowed to conclude that the NADES in study did not present increasing toxicity (in comparison to SAGM) and could be further used in the long-term assays of RBC preservation.

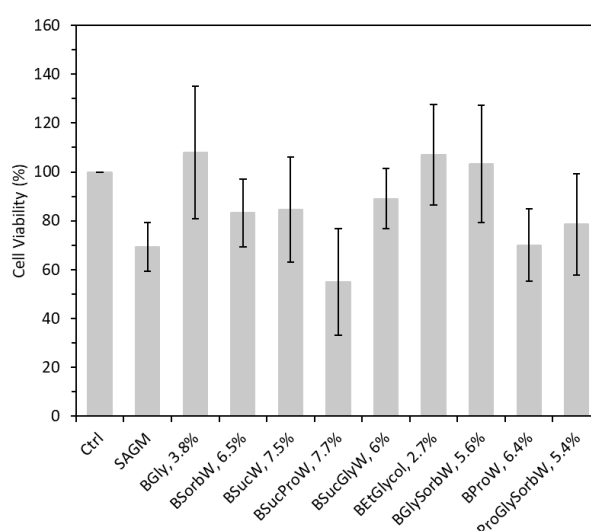


Figure 6.4 – Cell viability of L929 in the presence of NADES solutions in the optimized concentrations. The results are expressed as average and SD (n=3).

6.2.4 RBC preservation in NADES

Considering the findings of the osmolality of NADES solutions combined with their biocompatibility towards healthy cell lines, the NADES listed in Table 6.1 were used for a long-term study of RBC preservation, in which NADES solutions were used as main additive solution. During the duration of the assay, samples of RBC were collected to verify their morphology. Moreover, samples of the additive solution were also collected to quantify their extent of haemolysis during storage. The results obtained for these parameters will be discussed in detail.

6.2.4.1 Characterisation of RBC

The RBC concentrates used in this work were already at the end of shelf life (over 42 days of storage) hence as soon as obtained, the samples were characterized to establish the conditions of the starting material.

Overall, the concentrates provided had low extent of haemolysis in accordance with legislation, below 0.8%. Moreover, by visual analysis, the morphology of the RBC was not extensively affected, with most of the cells in the form of discocytes, the typical biconcave disc shape (Figure 6.5). As expected, some spinocytes and spherocytes were also detected in the observed sample. Despite being stored under the proper conditions, the deterioration of RBC still occurs, being accentuated from between 2 and 3 weeks of storage (William et al. 2023).

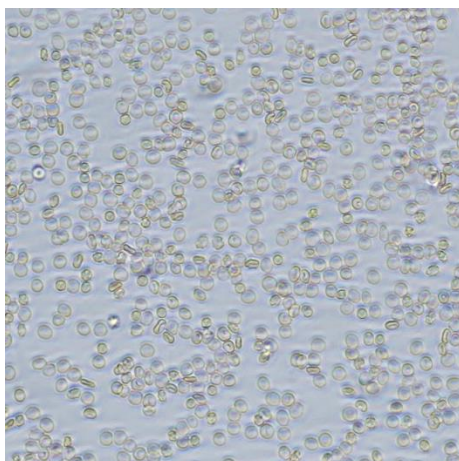


Figure 6.5 – RBC obtained from IPST at receiving day (day 0), at a dilution of 1:1000 in NaCl 0.9% (amplification x20).

6.2.4.2 Haemolysis during storage

During the storage period, haemolysis was quantified from the supernatant obtained from RBC after centrifugation, using the same method described before. Since most studies reported in literature use fresh RBC samples, it was difficult to establish a correlation between the extent of haemolysis in this work and the ones already described. Due to this constraint, two internal standards were used. One sample of the original RBC, as obtained from IPST, was kept in a separate flask and was also analysed in each time point. The other control was a sample stored in SAGM, in which the RBC were processed in the same way as for the NADES samples, to remove any debris that accumulated during storage, and fresh SAGM was added for the assay.

The samples were kept for a total of 42 days. It is important to mention that the nine NADES solutions were tested, however BGly and BEtGly caused high extent of haemolysis during the first week of analysis, hence they were removed from the assay. Figure 6.6 shows the results obtained for the remaining NADES as well as controls (RBC and SAGM). Briefly, it was observed that the haemolysis in the RBC sample was faster, with a total of 15% detected after 11 days of assay. The sample with fresh SAGM, was our positive control, and it is possible to see that the extent of haemolysis increased gradually, reaching a maximum of 7% at day 42. Considering this value, we could conclude that some of the NADES solutions were equally effective in the preservation of RBC, namely BSucProW, BSucGlyW, BGlySorbW and ProGLySorbW, in which haemolysis after 42 days was also around 7%. The worst storage performance was obtained for BSucW, in which the extent of haemolysis increase exponentially on the last week of assay.

It is important to mention that these assays were performed with RBC at end-of-life. Legislation limits the extent of haemolysis to 1%, and these results are far over that limit, however they are influenced by several factors, with the main being the fact the RBC were past their defined shelf life, which acelerates the metabolic decay of RBC. Moreover, in the preparation of the assay and during its duration the RBC were exposed to air and subjected to several steps of centrifugation and resuspension, all contributing to an acelerated degradation of RBC.

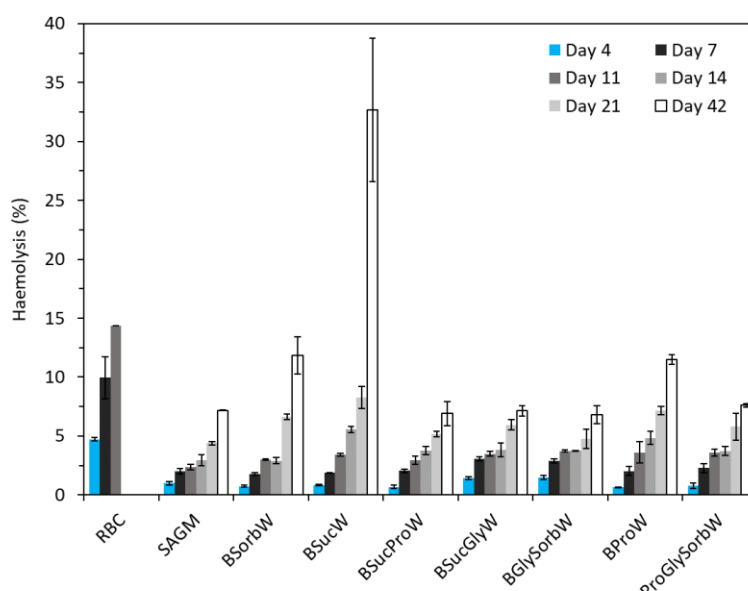


Figure 6.6 – Extent of haemolysis during storage of RBC for 4 (blue), 7 (black), 11 (dark grey), 14 (grey), 21 (light grey) and 42 (white) days in the presence of SAGM and NADES solutions in the percentages described in Table 6.1. RBC represents the original sample obtained from IPST without medium change (n=2).

6.2.4.3 RBC morphology during storage

To observe the morphology of RBC at each time point, the samples obtained from the storage flasks were diluted at 1:1000 in NaCl 0.9%, which is considered isotonic for the cells. Even in high dilution it was difficult to obtain clear images. The most representative images are shown in Figure 6.7 (the full set of results is shown in Appendix A, Figure A.3.1), and it was possible to detect differences in the number and shape of the RBC between the different storage conditions.

The original RBC samples presented visible differences from day 0 to 42. Although there was no visible reduction of the cell number, most cells have the appearance of spinocytes, a sign of dehydration, associated with possible irreversible damage and lack of function. For the samples preserved in fresh SAGM, there was no evident decrease of cell number, and most cells presented show regular discocyte shape, even after 42 days of storage.

The most pronounced difference was observed for BSucW, a system in which after 42 days, the number of cells was reduced, corroborating the high extent of haemolysis described before. Moreover, the cell's shape was also altered, showing mostly spinocytes and spherocytes.

From the four NADES that yielded the best results, only two are shown in Figure 6.7, namely BGlySorbW and ProGlySorbW. In these systems, the performance was similar of the one of fresh SAGM, and the images show that, after 42 days of storage, a high number of cells can still be observed. Moreover, most cells from both samples showed a majority of the discocyte shape.

Changes on the RBC's shapes happen naturally during storage however, they should be limited. Other authors have recorded the change of healthy RBC concentrates, with majority of discocytes and stomatocytes, to a sample composed of mostly spherocytes over a period of 71 days (Manon Bardyn 2019). The echinocytosis process has several intermediate stages that can be reversed after transfusion, however when the cells get to the end stage – spherocytes – it is no longer reversible and can cause serious problems to the recipient of blood transfusion. Due to extensive microvesiculation, spherocytes are rigid and non-deformable, being capable of obstructing capillaries during circulation, they are more sensitive to lysis and are able to activate the immune system for their removal. The consequences of infusion of high number of spherocytes include organ failure and decrease of defences, especially in critically ill people (Xia et al. 2017)

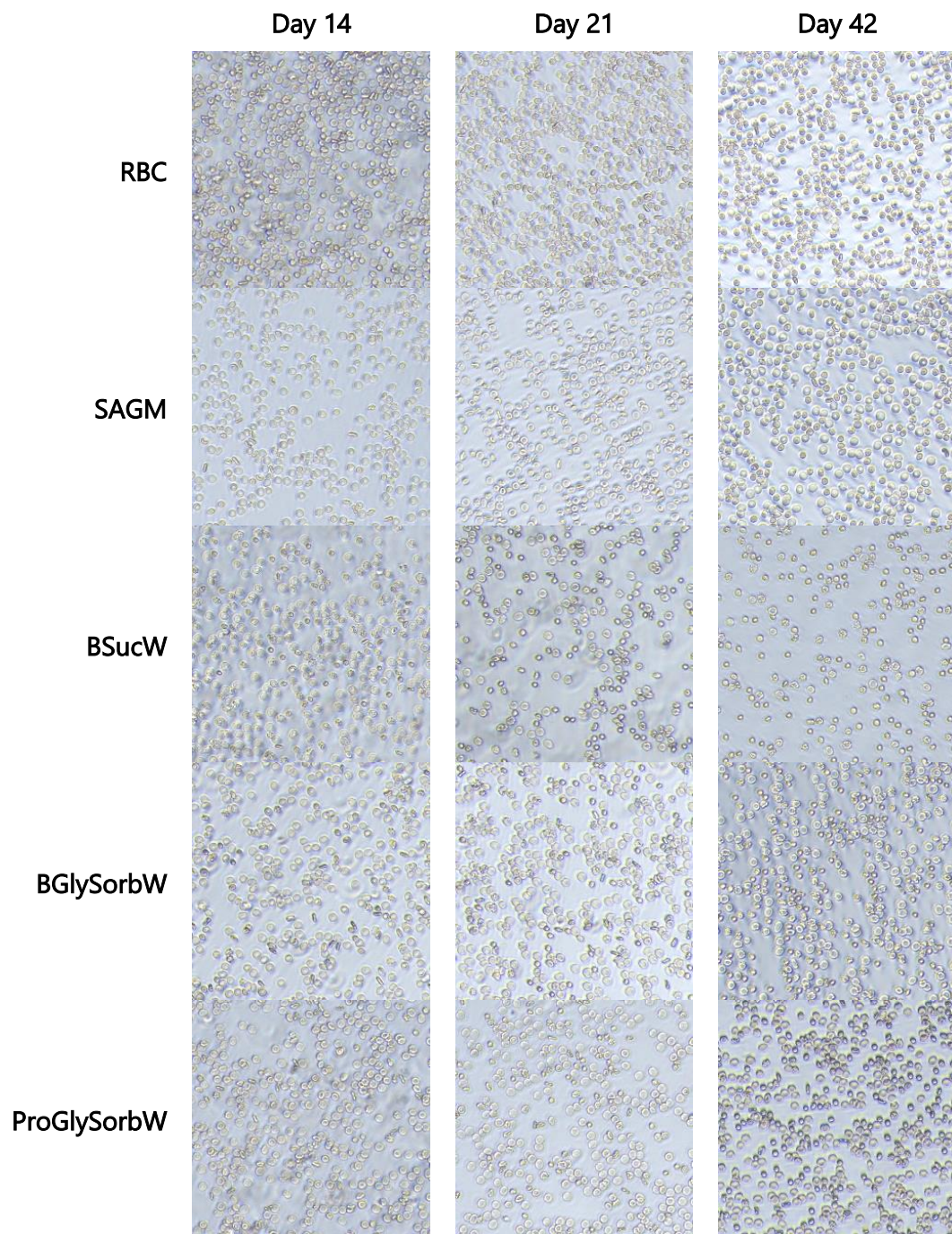


Figure 6.7 – RBC collected at 14, 21 and 42 days of storage in different additive solutions in a dilution of 1:1000 in NaCl 0.9% (amplification x20).

6.3 Conclusions

The work herein presented shows a proof-of-concept study of the application of NADES for the preparation of additive solutions used for RBC preservation. A total of 14 NADES were screened for their haemolytic capacity, leading to the conclusion that almost all the systems showed low or residual haemolytic capacity. The exceptions were determined for systems with higher amounts of glycerol, that caused high percentages of haemolysis, and NADES with acidic profile, which caused immediate coagulation of the RBC. It was also concluded that the osmolality of the NADES solutions tested for the haemolysis capacity was not ideal, hence the NADES concentration in solution was adjusted to be slightly hypertonic in comparison to RBC, as happens for SAGM.

Considering the osmolality of these solutions and the lack of toxicity towards healthy cell lines, the same NADES-based formulations were used on a long-term assay of RBC preservation. The cells were kept for a total of 42 days and the results show that the systems BSucProW, BSucGlyW, BGlySorbW and ProGlySorbW present a performance comparable to the one of SAGM, the additive solution currently used in Europe. The morphological characterization of the RBC showed that, in the systems with the best results, not only the number of cells was maintained, but also that most of the cells present were in the discocytes form, considered as healthy.

Hence, it is believed that the results obtained allow to conclude that these systems show potential to be applied in RBC preservation, by having a similar performance as the option that is currently used and promoting the discocyte form of RBC. The consolidation of these results would require testing the performance of NADES-based solutions in the preservation of fresh RBC, to determine if they are able to keep the extent of haemolysis below the regulatory limits, and eventually extend RBC's shelf life past the current 42 days. It would also allow to determine other stress indicators, such as glucose, lactates and even ROS.

Testing on fresh RBC would be essential to confirm NADES' real potential to be used as blood preservatives and establish the difference between using NADES or SAGM. Nevertheless, the approval of a new additive solution for blood preservation would require intensive clinical research to ensure patient safety, since the risks associated to the transfusion of RBC with high extent of storage lesions are extremely high.

CONCLUSIONS AND FUTURE PERSPECTIVES

The work developed for this thesis was focused on the application of NADES as media for the preservation and stabilization of different types of molecules and biological compounds. Despite the evolution of preservation and storage methods, of both food and biopharmaceuticals, the amount of goods wasted is still very high. This waste is related to breaks of the cold chain, to packaging malfunctions or due to the short shelf life that are possible to obtain. The focus of this work was laid not only on simple molecules, such as antioxidants, but also in more complex structures that could mimic biopharmaceuticals, such as proteins and cells. Moreover, the findings regarding stability were used to develop proof-of-concept applications.

Starting from simple molecules, the development of a strategy for the stabilisation of AA was proposed. From the NADES tested, it was concluded that the best protective action was obtained when AA was dissolved in Bet:CA, being even better that including AA in the NADES formulation (ChCl:AA). Bet:CA allowed to keep AA's antioxidant activity in aqueous media up to 9 days. Moreover, the toxicological profile of the ChCl:AA and Bet:CA was also assessed. These assays lead to the conclusion that Bet:CA showed no significant toxicity towards shrimps (*P. varians*), while ChCl:AA was only not toxic at the lowest concentration tested. Finally, considering the improved stability of AA and the evidence of no toxicity, the feasibility of using the mixture NADES+AA in the preservation of fresh cut fruits was assessed. In a preliminary assay, it was observed that apple slices preserved with NADES+AA showed less signs of oxidation, than the ones preserved in AA. These results seem promising, especially for the

stability of the active form of AA. The gathered knowledge could be useful, not only to the food industry, but also in the pharmaceutical or cosmetic industries. Considering the application of a NADES formulation in the food industry, it would be important to test its performance using the proper storage conditions (i.e. modified atmosphere) and compare with commercially available strategies.

Then moving on to more complex structures, the stability of HRP in NADES was evaluated. Besides the findings of enzymatic stability and activity, an extensive characterization of several betaine-based NADES was performed. The characterisation includes NADES' water content and activity, viscosity, and density. Moreover, the experimental results were successfully used to model density and water activity, using PC-SAFT calculations. Concerning HRP's activity, it was seen that the presence of NADES in the reaction media was enough to improve the enzymatic activity. Furthermore, incubation in NADES at high temperatures (60 and 80 °C), provided increased thermal and structural stability to the HRP, that were promoted by several conformational changes. One of the changes was the increase of the unfolding temperature, along with the presence of aggregation at higher temperatures, suggesting that NADES promoted an intermediate folding state, prior to denaturation. Moreover, CD studies shown that the presence of NADES promoted higher contents of α -helix secondary structure, associated to increased enzymatic activity. The system BTrehGlyW was especially effective to provide thermostability to HRP. An additional study of the influence of a_w on enzymatic activity was also conducted, allowing to conclude that increasing the NADES water activity increased the enzymatic activity. Using molecular dynamics simulations, it was possible to determine that the NADES promoted a greater stability of the enzyme's active site, by the water exclusion effect caused by the interactions NADES-HRP.

The stabilisation of HRP in NADES led to the development of an application using the highly active and stable HRP on the preparation of enzymatically crosslinked natural deep eutectic gels – eutectogels. The main goal was to develop an injectable formulation that could be crosslinked *in vivo*, to act as a drug delivery system. Using BGly as solvent and a gelatine-phenol conjugate, the eutectogels were obtained with gelation times below 60 seconds. The eutectogels also showed high stability, being kept up to 1 year, without noticeable visual changes. Concerning the rheological properties, despite its high viscosity, the formulation showed shear thinning behaviour, a good indicator of injectability. In frequency sweeps, the eutectogels presented the typical profile of crosslinked gels, with storage modulus consistently higher than loss modulus, which was also determined for the 1-year-old samples. To evaluate

the use of the eutectogels as drug delivery systems, ketoprofen was dissolved in the NADES, that was then used to prepare the gels. The release study allowed to conclude that during the first 24 h ketoprofen was released at a high rate that become constant up to 10 days. In conclusion, stable eutectogels were prepared, with the required properties to be injected and crosslinked *in vivo* and with potential to be used as drug delivery system. The development of the eutectogels for a specific application would require biocompatibility assays of the gels would be needed, optimization of the design of the DDS, and tests of the co-injection of the eutectogels precursors.

Finally, NADES were tested for the preservation of the most complex compounds under study - red blood cells. Several NADES-based solutions were applied as additive solutions for the preservation of RBC. After the optimization of the NADES concentrations to achieve the correct osmolality, the systems were tested for the long-term preservation of RBC. With this assay it was possible to conclude that four of the tested systems showed a similar performance to the one of SAGM, in the same conditions. These are very preliminary results that need to be further validated. For instance, conducting an assay with fresh RBC would be necessary to determine if NADES are able to keep the extent of haemolysis bellow the regulated limit. Nevertheless, the conclusions open a path for the development of new additive formulations for RBC.

Overall, the main goals proposed for this work have been achieved. New applications of NADES for the storage and preservation of materials have been proposed for simple antioxidant compounds, proteins, and RBC. This work led to the development of two proof-of-concept applications of the stabilised compounds, namely the preservation of fresh-cut fruits with NADES+AA solutions, as well as the application of HRP in the production of eutectogels to be used as drug delivery systems. These findings support and encourage the application of NADES in preservation and stabilization processes. We believe that the possibility to extrapolate these findings to more sensitive biopharmaceuticals, can open several paths of research in a near future, leading to the disruption of several constraints currently faced by the pharmaceutical industry.

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APPENDIX A

A.1 Preservation of Ascorbic Acid using NADES

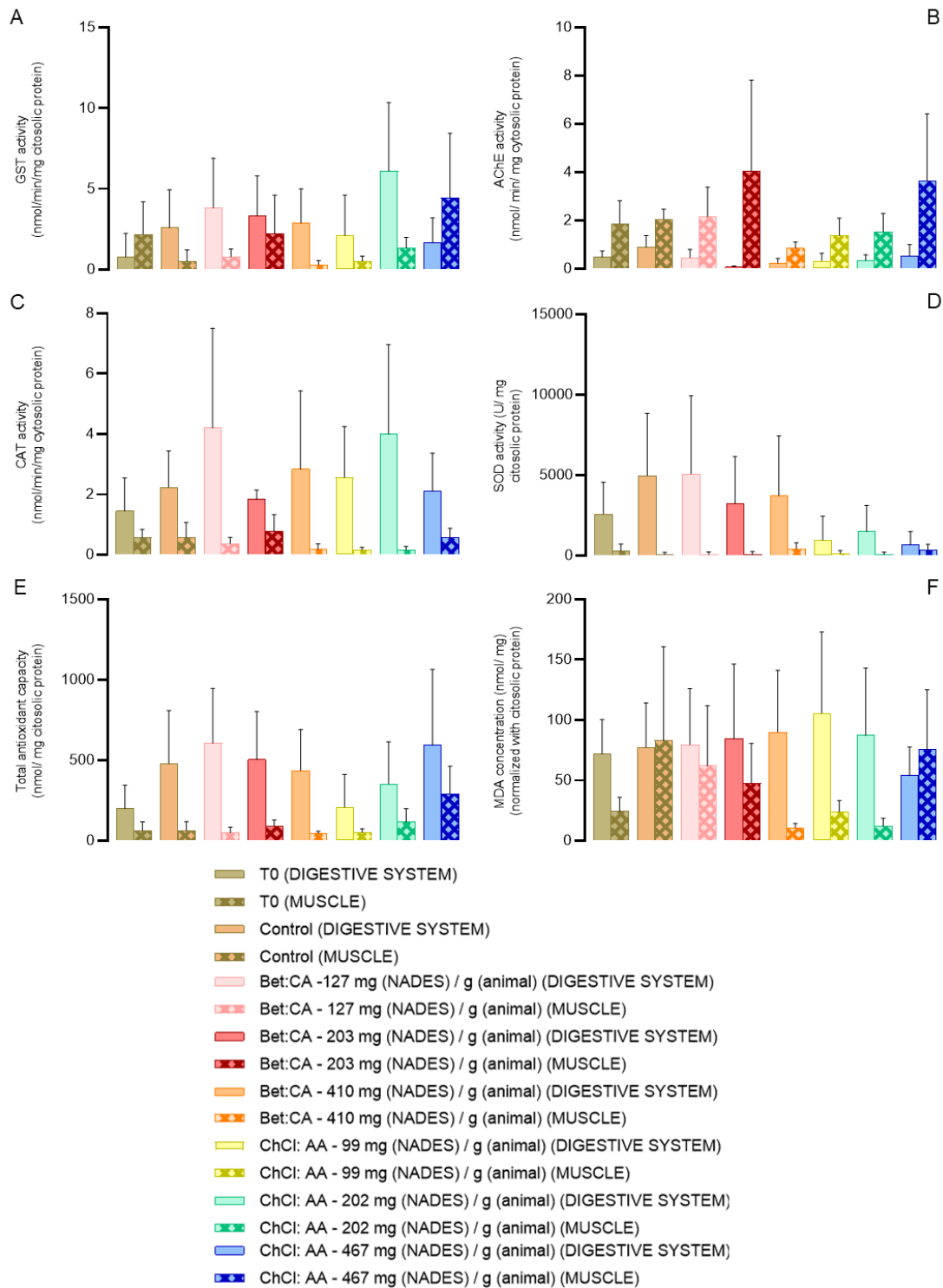


Figure A.1.1 - (A) Glutathione-S Transferase, (B) Acetylcholinesterase, (C) Catalase and (D) Superoxide dismutase activity, (E) Total antioxidant capacity, (F) MDA concentration in two different systems, digestive system and muscle, after *P. varians* exposed to different concentrations of Bet:CA and ChCl:AA. Data expressed as mean $\pm$ SD (* $p < 0.05$).

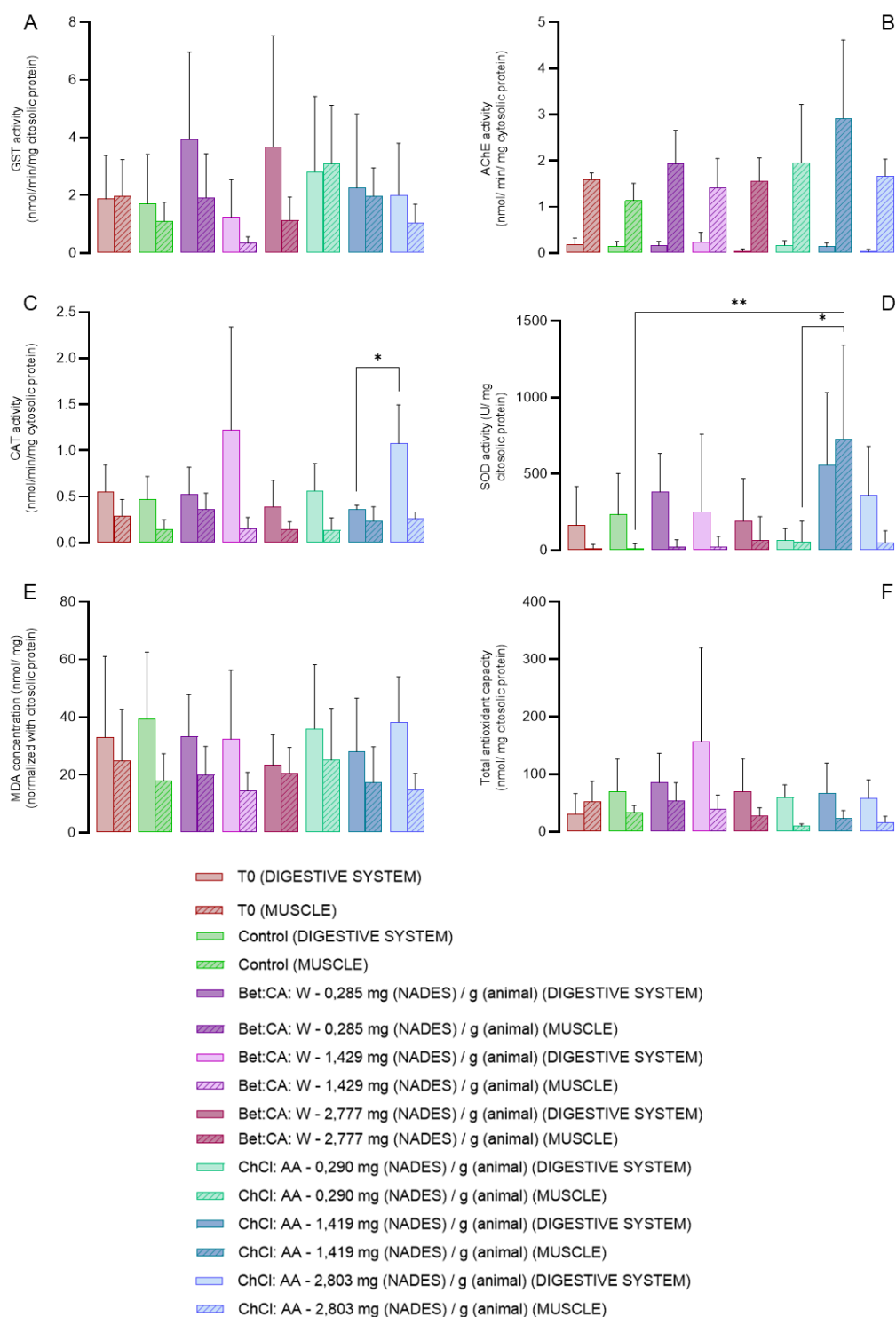


Figure A.1.2 - (A) Glutathione-S Transferase, (B) Acetylcholinesterase, (C) Catalase and (D) Superoxide dismutase activity, (E) Total antioxidant capacity, (F) MDA concentration in two different systems, digestive system and muscle, after *P. varians* exposed to different concentrations of Bet:CA and ChCl:AA. Data expressed as mean $\pm$ SD (* $p < 0.05$, ** $p < 0.01$).

A.2 Stabilisation of HRP using Betaine-based NADES

A.2.1 PC-SAFT parameters

Table A.2.1 - PC-SAFT parameters used to calculate density and water content of NADES.

Compound	M_w (g/mol)	m_i^{seg}	σ_i (Å)	u_i/k_B (K)	N_{sites}	ϵ^{AiBi}/k_B (K)	κ^{AiBi}	Ref.
Water	18.0153	1.205	*	353.945	1:1	2425.70	0.045099	Cameretti and Sadowski (2008)
Betaine	117.148	8.466	2.547	266.59	1:1	2541.62	0.03840	Held and Sadowski (2016)
Glycerol	92.094	2.007	3.815	430.82	1:1	4633.47	0.00189	Held and Sadowski (2016)
Xylose	150.13	6.012	2.888	283.22	4:4	5000.00	0.10000	Held et al. (2013)
Trehalose	378.33	13.692	2.856	319.85	8:8	5000.00	0.10000	Held et al. (2013)
Sucrose	342.30	14.886	2.827	297.39	8:8	5000.00	0.10000	Held et al. (2013)
Sorbitol	182.17	7.230	2.960	226.94	6:6	5000.00	0.10000	Held et al. (2013)
Proline	115.13	6.7117	2.6562	33.35	1:1	2146.37	0.02350	B. S. Lee and Kim (2010)

*For water, a temperature-dependent segment diameter $\sigma_i = 2.7927 + 10.11 \cdot \exp(-0.01775 \cdot T) - 1.417 \cdot \exp(0.01146 \cdot T)$ was used.

Table A.2.2 - PC-SAFT binary interaction parameters k_{ij} used to calculate density and water content of NADES.

Binary System	k_{ij}	Ref.
water-betaine	-0.0922	Held and Sadowski (2016)
water-glycerol	-0.005	Held and Sadowski (2016)
water-xylose	$-0.0342 + 0.000258(T/K - 298.15 \text{ K})$	Held et al. (2013)
water-sorbitol	$-0.0591 + 0.000093(T/K - 298.15 \text{ K})$	Held et al. (2013)
water-trehalose	$-0.0274 + 0.000219(T/K - 298.15 \text{ K})$	Held et al. (2013)
water-sucrose	$-0.0371 + 0.0002560(T/K - 298.15 \text{ K})$	Held et al. (2013)
water-proline	-0.1235	B. S. Lee and Kim (2010)
betaine-glycerol	$0.219 - 0.0016(T/K - 298.15 \text{ K})$	This Work*
betaine-xylose	$0.110 - 0.0038(T/K - 298.15 \text{ K})$	This Work*
betaine-sorbitol	$0.200 - 0.001(T/K - 298.15 \text{ K})$	This Work*
betaine-trehalose	$0.27 - 0.001(T/K - 298.15 \text{ K})$	This Work*
trehalose-glycerol	$0.3 - 0.001(T/K - 298.15 \text{ K})$	This Work*
proline-betaine	$-0.18 - 0.01(T/K - 298.15 \text{ K})$	This Work*

* Fitted to experimental density data between 20°C and 80°C

A.2.2 Molecular Dynamics simulations

A.2.2.1 Protein Flexibility upon NADES addition

To obtain molecular insights into NADES-induced enzyme stabilization, molecular dynamics (MD) simulations were performed, primarily focused on the interactions between solvents and the surface of HRP at nanoseconds timescale. An extensive summary of relevant properties, calculated from the MD simulations, is shown in Table A.2.3. The effect of co-solvent on the dynamics of HRP was monitored by calculating the root mean square fluctuation (RMSF) of the C α atoms of all 306 residues.

Table A.2.3 - Simulation results obtained from MD for the different NADES used in this work.

	BXylW	BTrehGlyW	BSorbW	BSucProW	BGly	PBS
ΔG_s (kcal/mol)	-32.92 $\pm$ 3.95	-28.12 $\pm$ 4.69	-24.89 $\pm$ 2.44	-24.84 $\pm$ 4.33	-28.01 $\pm$ 4.71	-31.28 $\pm$ 1.4
SASA (nm²)	173.45 $\pm$ 2.02	166.47 $\pm$ 4.02	160.79 $\pm$ 5.74	153 $\pm$ 2.87	156.95 $\pm$ 3.71	159.03 $\pm$ 2.72
R_g (nm)	2.1 $\pm$ 0.01	2.04 $\pm$ 0.02	2.02 $\pm$ 0.03	1.98 $\pm$ 0.02	2.00 $\pm$ 0.02	2.01 $\pm$ 0.03
RMSD (nm)	0.38 $\pm$ 0.07	0.31 $\pm$ 0.08	0.21 $\pm$ 0.15	0.18 $\pm$ 0.04	0.17 $\pm$ 0.04	0.21 $\pm$ 0.02
D_{ARG38-HIS42} (nm)	0.66 $\pm$ 0.11	0.58 $\pm$ 0.03	0.58 $\pm$ 0.02	0.60 $\pm$ 0.12	0.65 $\pm$ 0.16	0.63 $\pm$ 0.12
X_{hydrophobic}/X_{hydrophilic}	1.077 $\pm$ 0.02	1.091 $\pm$ 0.01	1.106 $\pm$ 0.02	1.078 $\pm$ 0.03	1.083 $\pm$ 0.04	1.065 $\pm$ 0.02
$\Delta G_{s,hydrophobic}$ (kcal/mol)	31.93 $\pm$ 2.29	33.54 $\pm$ 2.48	35.96 $\pm$ 2.3	33.97 $\pm$ 2.15	35.08 $\pm$ 0.29	31.85 $\pm$ 1.8
H-Bonds	653.41 $\pm$ 6.34	637.45 $\pm$ 17.12	592.67 $\pm$ 10.61	575.83 $\pm$ 18.11	574.85 $\pm$ 20.83	627.84 $\pm$ 9.01

* ΔG_s : estimated solvation free energy, SASA: solvent-accessible surface area, R_g: radius of gyration, RMSD: root-mean-square deviation

The RMSF profile is overall similar for the NADES used in this work, except for three highly flexible regions: C-terminus and the N-terminus, where the RMSF values are noticeably high, reflecting the flexible nature of HRP in these regions. Secondly, the helix $\alpha 5$ region (residues 77–90) shows a remarkable decrease in the RMSF values as compared to native folds, as shown in Figure A.2.1. Helix $\alpha 5$ is close to the active site (residues 38–43), and its stabilization could contribute to maintaining a closed HRP conformation. A special flexibility suppression of Helix $\alpha 5$ is observed upon the addition of catalytic boosters NADES such as BSorbW and BSucProW

(Figure A.2.1 (C,F)). Unlike the other NADES, BXylW (Figure A.2.1(E)) shows a clear increment in the region of the residues 1–100 compared with the PBS system.

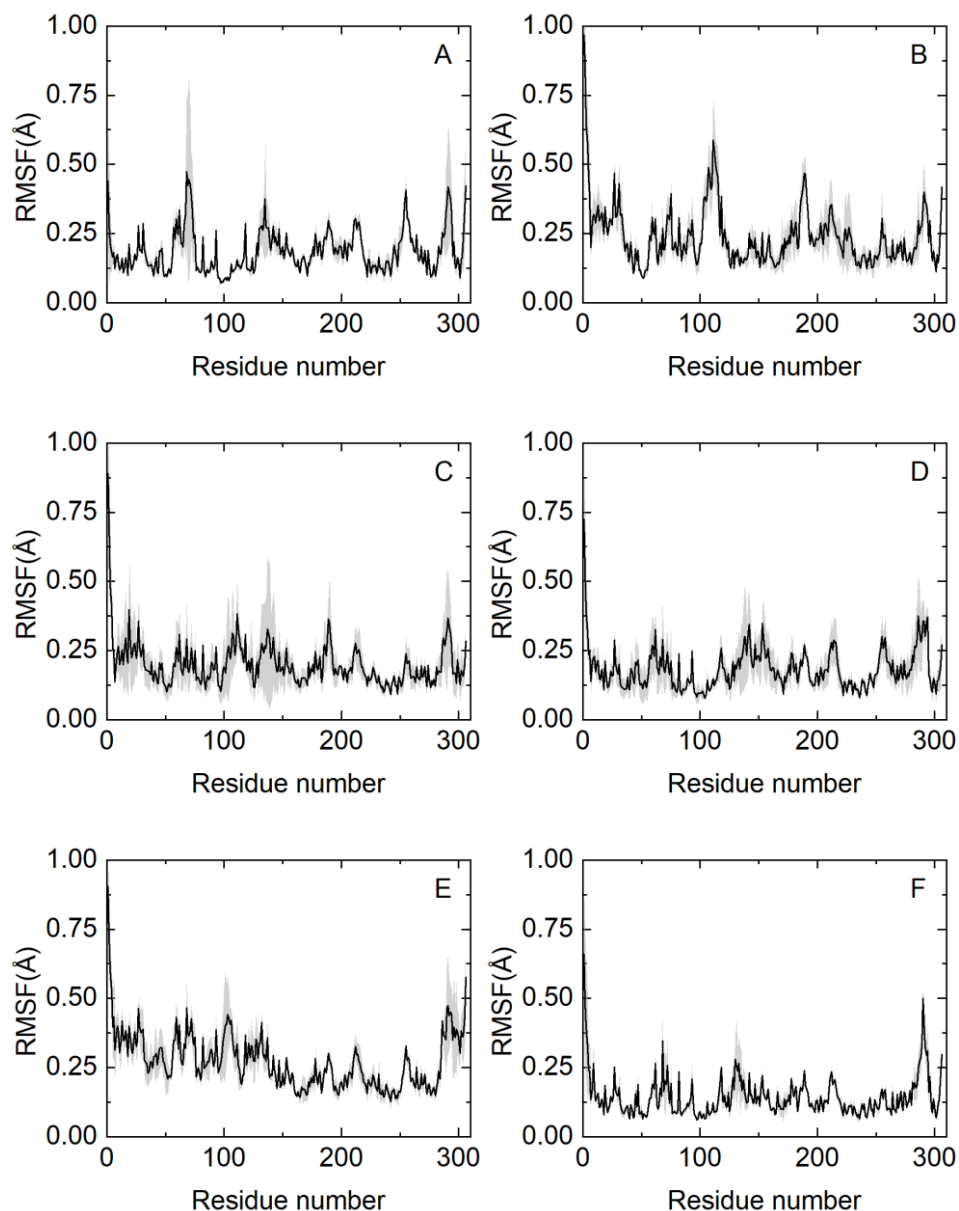


Figure A.2.1 - Protein alpha carbons RMSF of HRP in NADES systems from molecular dynamics. (A) PBS, (B) BTrehGlyW, (C) BSorbW, (D) BGly, (E) BXylW and (F) BSucProW. Average values from the last 50 ns of simulations, and shaded area is standard error computed from 3 independent runs.

HRP remains folded in all the NADES used in this work, as depicted by the root mean square deviation ($\text{RMSD}_{\text{C}\alpha}$), which indicates $\text{C}\alpha$ atoms coordinated with the equilibrated crystal structure as a reference, shown in Figure A.2.2(A). A small $\text{RMSD}_{\text{C}\alpha}$ (as for BSorbW) value implies that HRP stability of the folded state is promoted, reducing the conformational stability (Qiao,

Shi, and Shao 2021). In an analogous fashion, the distance between the centres of mass of the active pocket residues, ARG-38 and HIS-42, is reduced in systems with NADES that are more catalytically active (i.e. BSorbW). Additionally, Figure A.2.2 (B) shows the radius of gyration (R_g), which hints to the protein compactness in NADES environment. The number of hydrogen bonds between HRP and water was computed for all systems as shown in Table A.2.3. The number of HRP-water hydrogen bonds is reduced in all NADES systems, which might promote the experimentally observed increase in the enzymatic activity compared to PBS. On exception is BXylW, which promotes a higher density of water around HRP surface due to a stronger protein volume-exclusion effect. Strong hydrogen bond donors such as sorbitol, sucrose, or glycerol compete with betaine and water for HRP surface association sites, displacing HRP hydrogen bonding sites. The direct interaction of NADES donor groups with the surface of HRP excludes water from the second shell solvation, thereby decreasing HRP-water hydrogen bonds. This type of stabilization by the exclusion of water, is characteristic of the solvation of uncharged polar residues on the protein surface (Qiao, Shi, and Shao 2021).

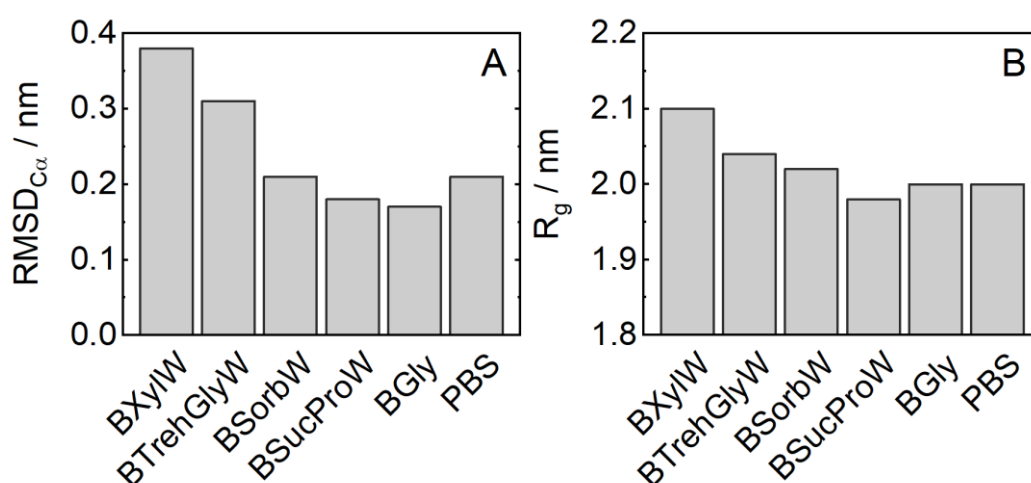


Figure A.2.2 - (A) The RMSD and (B) the R_g for NADES used in this work.

A.2.2.2 Surface area and Gibbs energy of solvation of HRP

To explore the HRP-solvent (water or NADES or water+NADES) dynamics, the solvent-accessible surface area (SASA) was studied. It is known that improved protein folded state stability of some enzymes appears to be related to reduced surface area, increased hydrophobicity and protein core-packed density (Arnold 1993). Figure A.2.3(A) shows the total SASA area and Figure A.2.3(B) shows Gibbs solvation energy calculated with the Eisenberg &

McLachlan method of the HRP in the NADES used in this work (Eisenberg and McLachlan 1986). A reduced SASA could be caused by a decrease in the volume of the substrate pocket, thus lowering protein flexibility and promoting catalytic activity. It is generally accepted that protein folded state stability then improves because a small surface area reduces the unfavourable surface energy and increases the attractive interior packing interactions. A low SASA (i.e. BSucProW) value suggest NADES components accumulations over HRP surface, promoting thermostability and an increase in the enzymatic activity, meanwhile SASA value is the highest in BXylW, the system with lower activity and thermostability as observed experimentally, suggesting an accumulation of water on the surface of HRP. This is in agreement with the results on hydrogen bonding in the previous section. Ultimately, the Gibbs solvation energy calculated from SASA strongly correlates to the enzymatic activity discussed in this paper and previously reported work on the discussed NADES systems (De Oliveira and Martínez 2019). That is, low Gibbs energy of solvation values (i.e BXylW and PBS) correlates with the lowest enzymatic activity obtained experimentally.

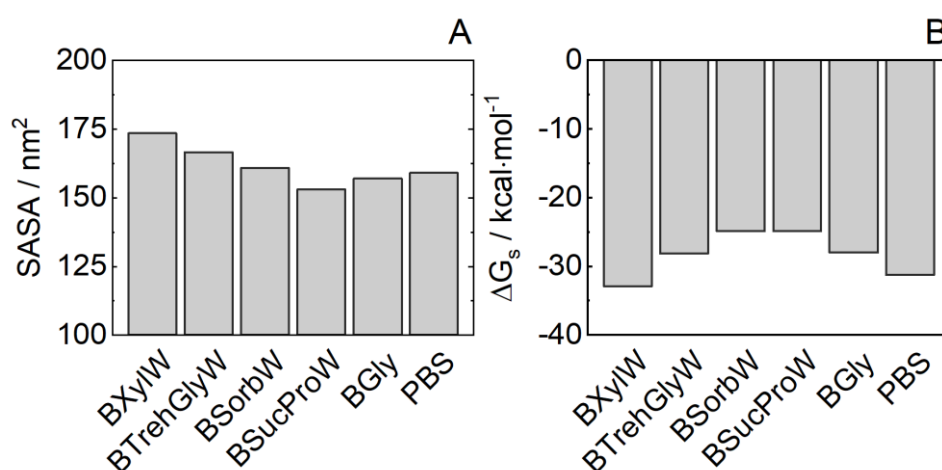


Figure A.2.3 - Protein (A) SASA and (B) Gibbs energy of solvation of HRP in NADES systems from molecular dynamics. Average values from the last 50 ns of simulations, standard errors computed from 3 independent runs are shown in Table A.2.3.

Although the accumulation of water molecules on the protein surface does not necessarily mean a decrease in enzymatic activity, it can cause the reorientation of the hydrophobic and hydrophilic side chains. Thus, the kind of interaction of NADES with specific residues on the protein surface are responsible to promote or decrease the non-monotonic dynamics of water molecules in the protein active site. In the case of sorbitol, hydrophobic interactions around basic HRP residues (e.g., arginine) are encouraged; this could improve folded state protein stability due to the highly hydrophobic reaction pocket of wild-type HRP (Tatoli et al. 2009).

Furthermore, an important correlation between HRP activity and Gibbs energy of solvation of hydrophobic residues can be observed for NADES systems as shown in Figure A.2.4. On the contrary, xylitol interacts with the protein around non-polar aromatic residues, increasing water density in HRP surface and increasing solvent-accessible surface area.

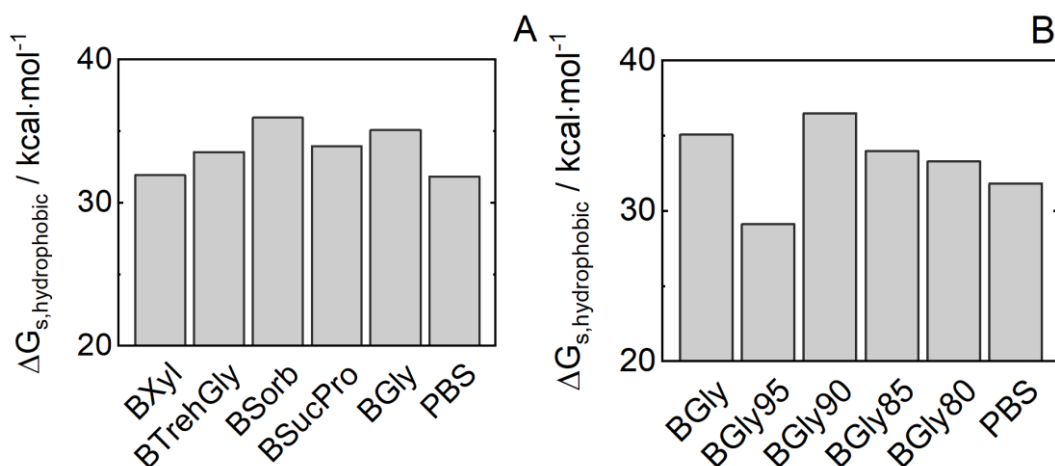
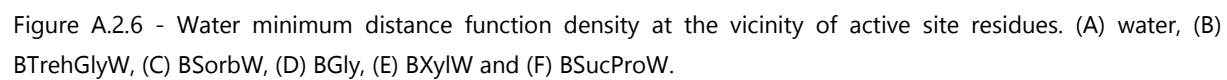
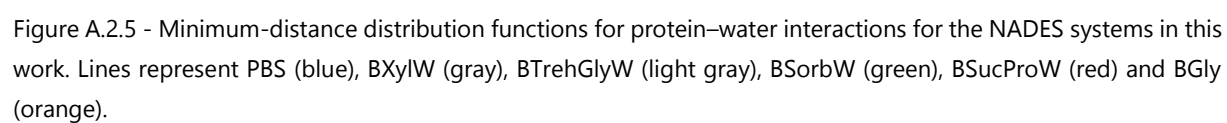


Figure A.2.4 - The estimated solvation free energy (ΔG_s) of the hydrophobics groups for (A) NADES used in this work and (B) BGly dilutions.

A.2.2.3 Protein solvation - MDDF and KBI

In this work, the Minimum-Distance Distribution functions (MDDF) were used as a practical way to obtain insights into the interaction between a solute with complex geometry (e.g., protein surface) and a solvent (water or NADES components). Figure A.2.5 shows the MDDF for HRP-water, reaffirming what was previously stated, showing a water accumulation in the first solvation shell due to the volume-exclusion effect, except for BSucProW where the water is repelled from the surface into larger distances. Figure A.2.6 displays the variation in the local density of water around the active pocket of HRP in NADES. An accumulation of water can be observed in the protein solvation shell (at $\sim 1.8\text{--}2.5$ Å), especially in the NADES that allowed high enzymatic activity (i.e., BSorbW and BSucProW). This increase in water density occurs at hydrogen-bonding distances and at the second water layer (at $\sim 2.5\text{--}3.5$ Å), most notably around residues ARG38, and HIS42. Interestingly, no significant changes were observed for BXylW compared to PBS control, although BXylW displaces the most water to the surface (Figure A.2.6). The MDDFs for the all the systems are shown in Figure A.2.7 and Figure A.2.8.



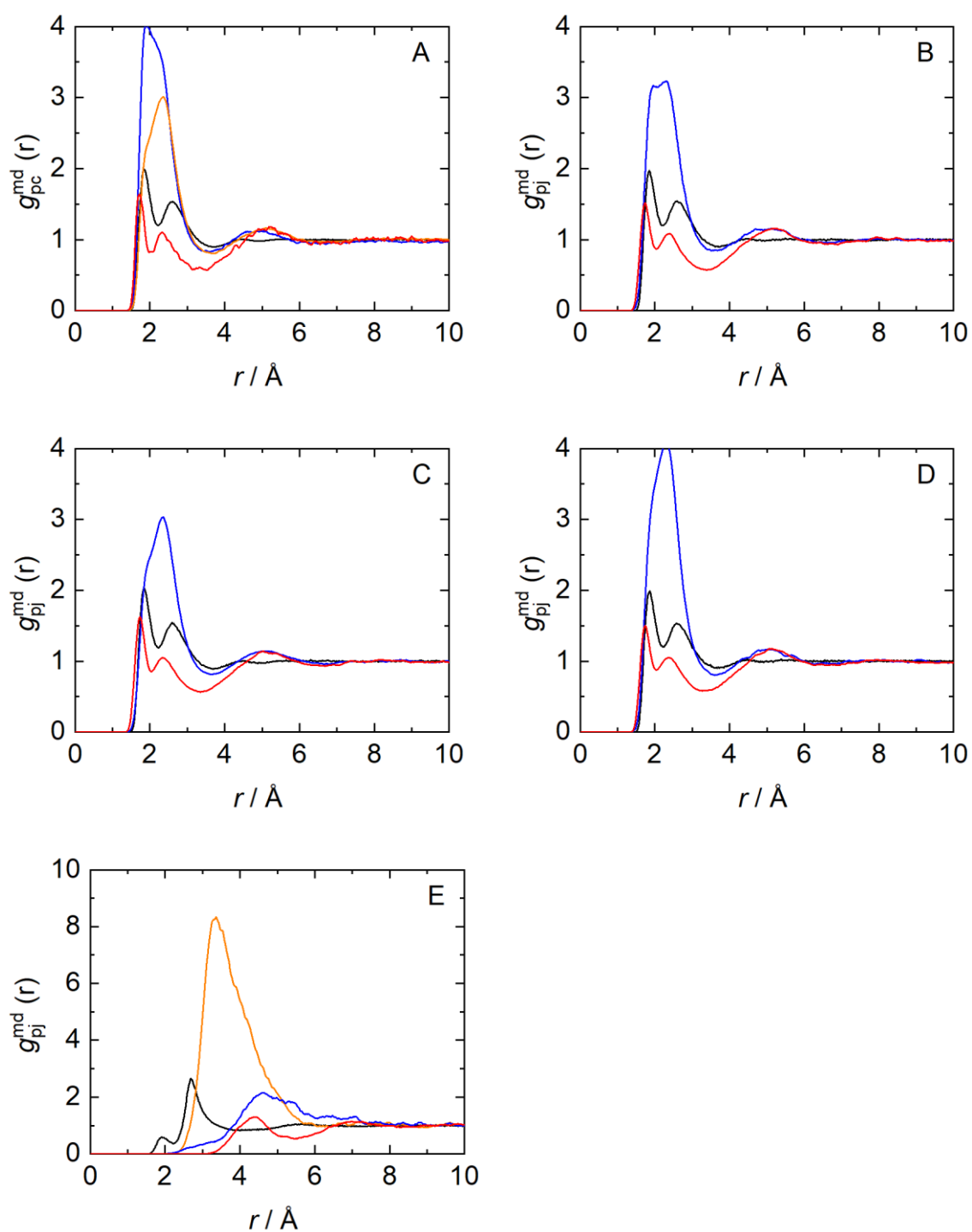


Figure A.2.7 - Minimum-Distance Distribution Functions ($g_{pj}^{md}(r)$) between protein and solvent for: (A) BetTrehGlyW, (B) BSorbW, (C) BGly, (D) BXylW and (E) BSucProW. Solvents: water (black), betaine (red), second cosolvent (blue), third cosolvent (orange).

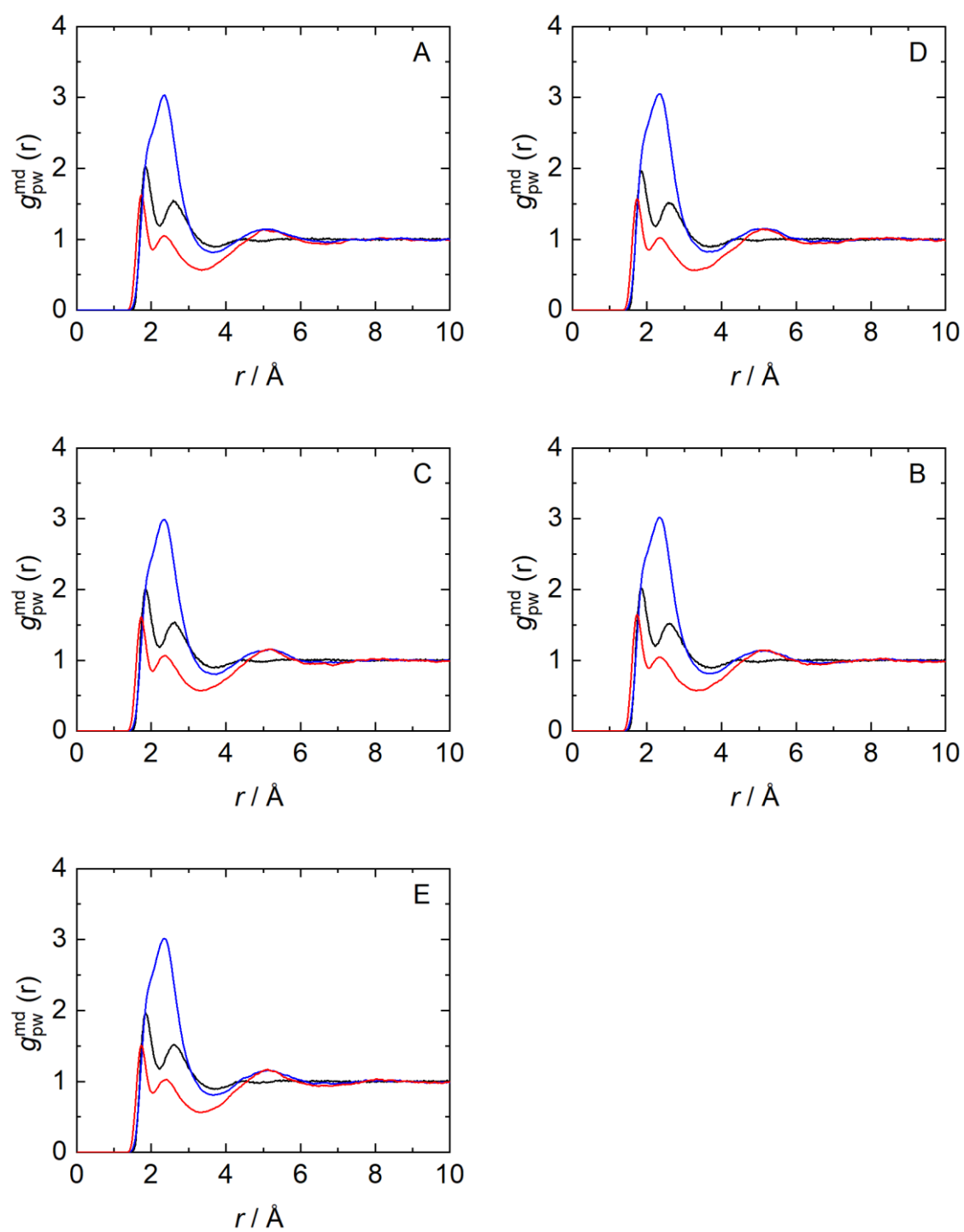


Figure A.2.8 - Minimum-Distance Distribution Functions ($g_{pj}^{md}(r)$) between protein and solvent for: (A) BetGly, (B) BGly95, (C) BGly90, (D) BGly85 and (E) BGly80. Solvents: water (black), betaine (red), glycerol (blue).

To reflect the volume-exclusion effect associated with protein-solvent interactions at short distances, the Kirkwood Buff Integral (KBI) profiles were computed according to Equation A.2.1,

$$G_{pj}(R) = \frac{1}{\rho_j} \int_0^R [n_{pj}(r) - n_{pj}^*(r)] S(r) dr \quad \text{Equation A.2.1}$$

where $S(r)$ is the surface area element at distance r , n_{pj} is the atoms-average number density of solvent atoms which are minimum-distance atoms to the solute at r , n_{pj}^* as the number of minimum distances that would be observed at r if there were no solute–solvent interactions, and ρ_j is the molar density of the solvent j in the bulk. The KBI profiles determine whether there is excess or exclusion of each NADES constituents around the protein. At short distances, $r < 1.5 \text{ \AA}$, the KBIs drop to very negative values, which is caused by the excluded volume of protein and the solvent molecules; this could be compensated at long distances by favourable HRP-solvent interactions. The accumulation of water and betaine in the solvation shells offsets only a part of the exclusion volume. For most of the NADES' constituents, the KBI increases for $r > 1.5 \text{ \AA}$, but converges into negative values at long distances. A lower value of KBI indicates that the NADES constituents accumulate more effectively on the protein second solvation shell, promoting water molecules into the first solvation shell. On the contrary, DL-proline accumulates in the first solvation shell, solvating the protein surface, as depicted by a prominent peak of proline at short distances in Figure A.2.7(E), at $r \sim 3.0 \text{ \AA}$.

Once the G_{pc} have been computed, they were used to calculate the preferential hydration parameters according to Equation A.2.2, usually used to correlate protein stability under the effect of a solvent.

$$\Gamma_{pc}(R) \approx \rho_c [G_{pc}(R) - G_{pw}(R)] \quad \text{Equation A.2.2}$$

Where G_{pc} and G_{pw} are the converged values of the KBI at an R distance, for NADES constituents and water respectively, and ρ_c is the density in the bulk of the NADES component. At about $\sim 8 \text{ \AA}$, all KB integrals are essentially converged, and therefore $R=8$ was used in this work. The G_{pc} and Γ_{pc} values for each NADES component found in this work are listed in Table A.2.4 and and Table A.2.5. Γ_{pc} are negative for all concentrations except for DL-proline, which exhibits positive value and thus preferentially solvating the protein. Thus, the rest of NADES constituents preferentially solvate HRP, which is consistent with previous reports that water

accumulation in the protein surface favours folded-state protein stabilization. This shows two different stabilization mechanisms for HRP. On one side, BSorbW, by promoting a greater density of water around the protein, generates an initial boost in the activity that cannot maintain for a long time. On the contrary, other NADES (i.e. BSucProW & BTrehGlyW) cause an accumulation of NADES constituent around the surface of HRP with a lower initial activity boost but greater residual activity over long times and high temperatures.

Table A.2.4 - Kirkwood Buff Integral between protein-cosolvent (G_{pc}) for the different NADES used in this work.

$KBI_{pi} =$	<i>W</i>	<i>B</i>	<i>Gly</i>	<i>Xyl</i>	<i>Treh</i>	<i>Sorb</i>	<i>Suc</i>	<i>Pro</i>
Water	-26.02 ± 0.06		-	-	-	-	-	-
BXylW	-23.82 ± 0.07	-49.6 ± 0.7	-	-22.7 ± 0.7	-	-	-	-
BTrehGlyW	-24.05 ± 0.04	-48.9 ± 0.5	-28.2 ± 0.4	-	-34.7 ± 0.62	-	-	-
BSorbW	-24.12 ± 0.05	-47.9 ± 0.6	-	-	-	-30.0 ± 0.3	-	-
BSucProW	-26.32 ± 0.07	-48.2 ± 0.5	-	-	-	-	-26.7 ± 0.7	42.5 ± 0.7
100 wt% BGly	-24.06 ± 0.04	-47.8 ± 0.2	-27.5 ± 0.7	-	-	-	-	-
95 wt% BGly	-24.10 ± 0.14	-48.3 ± 1.0	-28.0 ± 0.3	-	-	-	-	-
90 wt% BGly	-24.14 ± 0.01	-47.5 ± 0.7	-28.1 ± 0.4	-	-	-	-	-
85 wt% BGly	-24.34 ± 0.16	-49.0 ± 0.8	-27.2 ± 0.7	-	-	-	-	-
80 wt% BGly	-24.41 ± 0.09	-48.8 ± 0.9	-28.1 ± 0.3	-	-	-	-	-

*W: water, B: betaine, Gly: glycerol, Xyl: D-(+)-xylose, Treh: trehalose, Sorb: D-sorbitol, Suc: sucrose, Pro: DL proline.

Table A.2.5 - Preferential interaction parameters between protein-cosolvent (Γ_{pc}) for the different NADES used in this work.

$\Gamma_{pi} \setminus i =$	<i>B</i>	<i>Gly</i>	<i>Xyl</i>	<i>Treh</i>	<i>Sorb</i>	<i>Suc</i>	<i>Pro</i>
BXylW	-23.4 ± 0.4	-	0.5 ± 0.3	-	-	-	-
BTrehGlyW	-10.4 ± 0.3	-2.6 ± 0.4	-	-2.5 ± 0.3	-	-	-
BSorbW	-14.3 ± 0.4	-	-	-	-3.5 ± 0.2	-	-
BSucProW	-12.4 ± 0.4	-	-	-	-	-0.1 ± 0.2	14.1 ± 0.2
100 wt% BGly	-17.4 ± 0.4	-4.7 ± 1.0	-	-	-	-	-
95 wt% BGly	-17.1 ± 1.0	-5.2 ± 0.5	-	-	-	-	-
90 wt% BGly	-15.4 ± 0.5	-5.0 ± 0.5	-	-	-	-	-
85 wt% BGly	-15.4 ± 0.6	-3.5 ± 1.0	-	-	-	-	-
80 wt% BGly	-14.2 ± 0.4	-4.1 ± 0.3	-	-	-	-	-

*W: water, B: betaine, Gly: glycerol, Xyl: D-(+)-xylose, Treh: trehalose, Sorb: D-sorbitol, Suc: sucrose, Pro: DL-proline

A.2.2.4 Gibbs energy of solvation of HRP in NADES+water

It was shown that adding 5 wt.% water to BGly (i.e., BGly95) causes a dramatic loss in enzymatic activity of HRP. The conditions of water content in BGly were inherited also to MD simulations. As shown in Figure A.2.9(A), 5 wt.% water addition to BGly increases $RMSD_{Ca}$, thus promotes an increment in the HRP flexibility, and this correlates well with the drop that was experimentally found in enzymatic activity for BGly95 compared to BGly. Interestingly, an increase in the amount of protein-water hydrogen bonding (cf. Table A.2.3) and protein flexibility correlates with a decrease in enzymatic activity after incubation. This could be explained as a rearrangement of the hydrogen bonds network due to the inclusion of water in the BGly system. After this disruption (>10 wt.% water), probably caused by the competition between NADES and HRP for the hydrogen bonds of the water, the BGly+water system seems to be stabilized in a re-organized structure, recovering the ability to prevent protein flexibility (decreasing values for $RMSD_{Ca}$ and R_g). The water organization between the solvation shell and the bulk might directly affect the stabilization of the transition state (Lees et al. 2006).

The same trend was observed for Gibbs energy of solvation of HRP in Figure A.2.10(A), whereupon water addition, the reorganization of the hydrogen bond network causes an increase in SASA (Figure A.2.11) and, consequently, a decrease in Gibbs energy of solvation. In a further step, we distinguished between hydrophilic and hydrophobic Gibbs energy of solvation based on the nature of the residues involved in the calculation. Particularly interesting is the prominent reduction in hydrophobic solvation energy shown in Figure A.2.10(B), which

may be responsible for the inability of BGly95 to maintain enzyme stability, which is compensated for at higher water contents with a decrease in hydrophilic solvation energy.

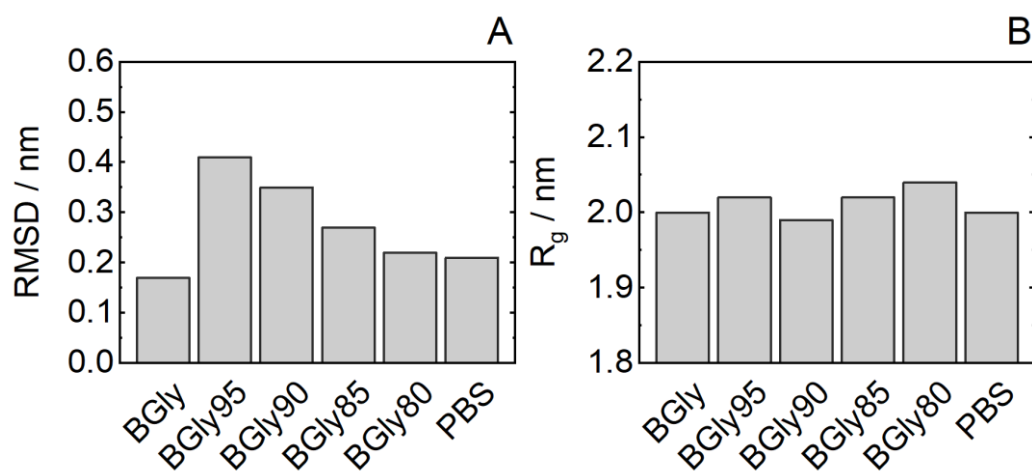


Figure A.2.9 - (A) The RMSD and (B) the R_g for BGly dilutions used in this work.

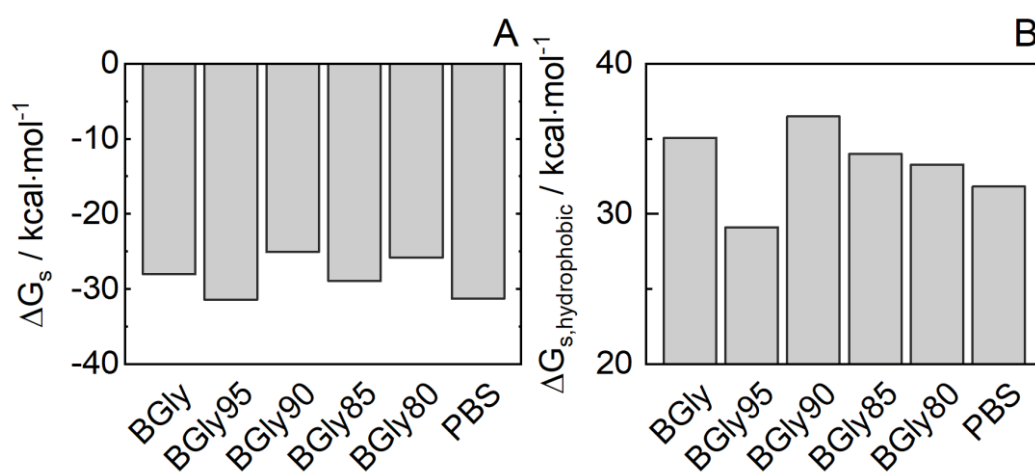


Figure A.2.10 - (A) Estimated Gibbs energy of solvation of total HRP (ΔG_s) and (B) of only the hydrophobic HRP groups for BGly dilutions used in this work.

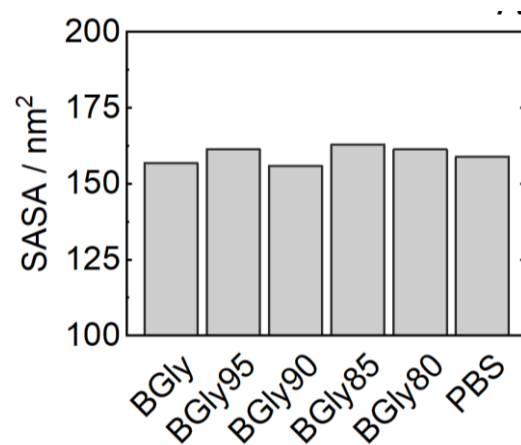
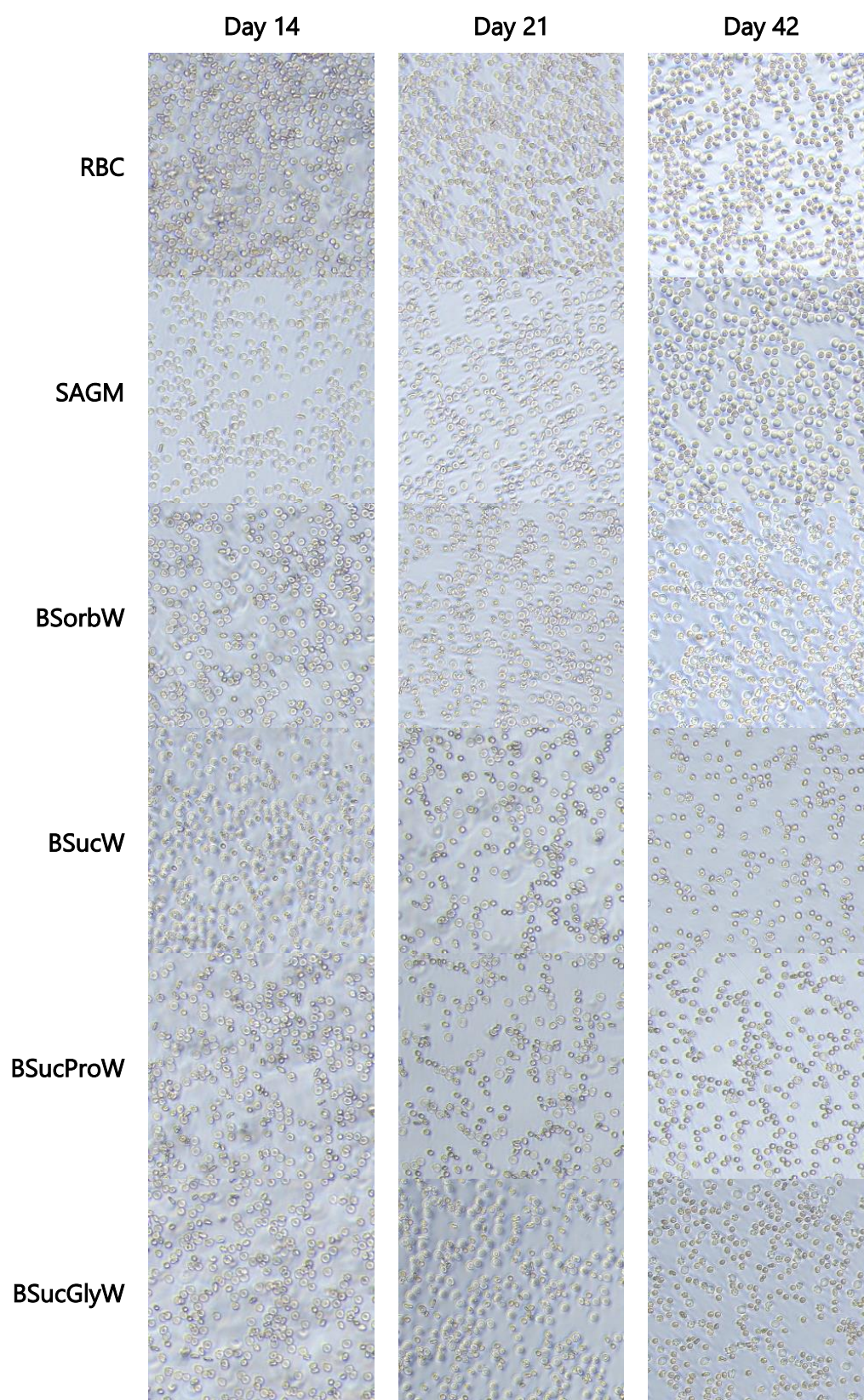


Figure A.2.11 - The solvent accessible surface areas (SASA)

A.3 Preservation of Red Blood Cells in NADES



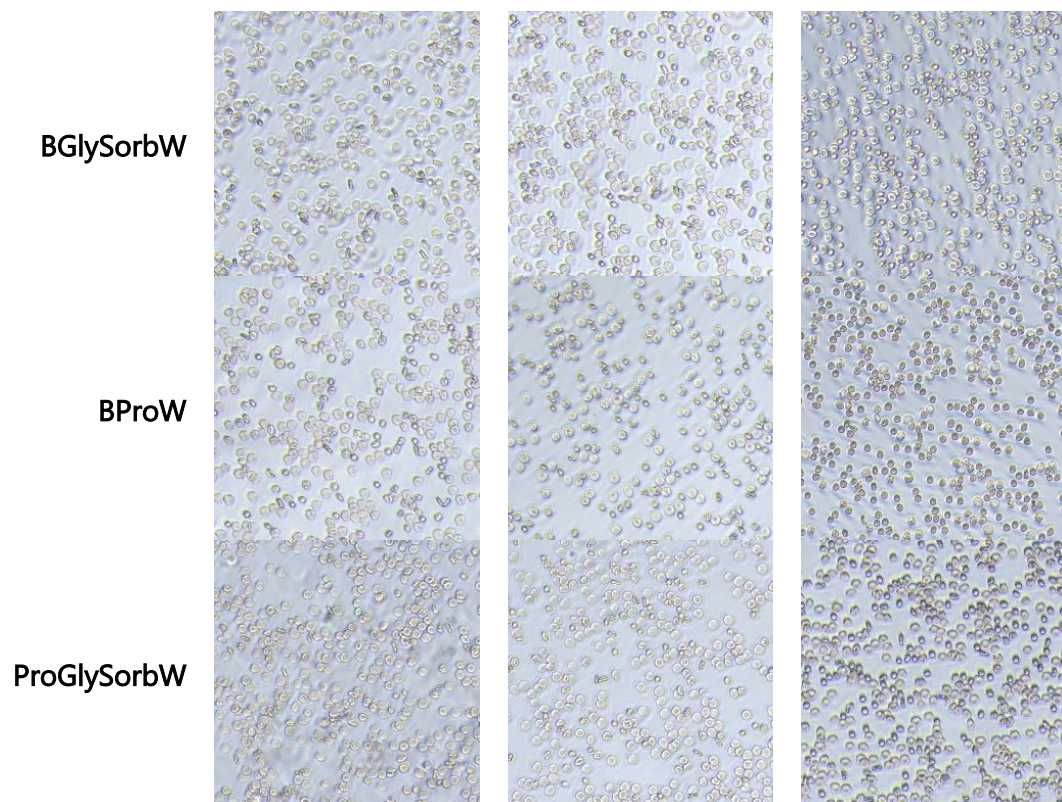


Figure A.3.1- RBC collected at 14, 21 and 42 days of storage in different additive solutions in a dilution of 1:1000 in NaCl 0.9% (amplification x20).



2024

LIANE PEREIRA DE MENESES

ENHANCING THE STORAGE STABILITY OF BIOTHERAPEUTICS
USING NATURAL DEEP EUTECTIC SYSTEMS

