NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTMENT OF CHEMISTRY

DIOGO BATISTA INÁCIO

BSc in Biochemistry

FUCOPOL-BASED EUTECTOGELS FOR BIOMEDICAL APPLICATIONS

DISSERTATION IN BIOMATERIALS AND NANOMEDICINE

MASTER IN BIOMATERIALS AND NANOMEDICINE

NOVA University Lisbon
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This thesis is a testament not only to my work but also to the generosity and kindness of those who stood by me along the way.

”

“The greatest enemy of knowledge is not ignorance,
it is the illusion of knowledge.”

— Attributed to both Stephen Hawking and Daniel J. Boorstin, en

Abstract

In recent years, sustainable soft materials have attracted considerable notoriety and attention. These materials are typically highly flexible, biocompatible, and tunable, making them suitable for applications in biomedicine, pharmaceuticals, and advanced materials.

This thesis investigates the production of eutectogel membranes composed by the polymer FucoPol and phenolic-based eutectic solvents, aiming to combine the bioactive properties of phenolic compounds and FucoPol with the structural and mechanical properties of this polymer, for potential wound dressing applications.

FucoPol was synthesized by the bacterium *Enterobacter* A47, and the eutectic solvents were prepared by mixing choline chloride with the phenolic compounds 2-Phenylethanol, Pyrogallol, and Gallic Acid. FucoPol and eutectic solvents were combined to produce eutectogel membranes via dry casting method. These membranes demonstrated the controlled release of phenolic compounds at non-toxic concentrations, suggesting their potential for safe skin contact applications. Among the eutectic mixtures, ChCl:2-phenylethanol exhibited the lowest cytotoxicity and optimal diffusion rate. Additionally, this eutectic solvent exhibited significant antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. The membranes also displayed high thermal stability, with no signs of degradation or structural changes up to 150°C .

These findings indicated that the prepared eutectogel membranes were stable, non-toxic, and exhibited promising antimicrobial properties, making them suitable for biomedical and pharmaceutical applications. Future research should focus on further characterizing the mechanical properties and exploring additional biological activities, such as anti-inflammatory and antioxidant effects, associated with phenolic compounds and FucoPol.

Keywords: FucoPol, Eutectogel, Eutectic Solvents, Phenolic Compounds

Resumo

Recentemente, materiais "soft"sustentáveis vêm atraindo uma atenção notável. Estes materiais são tipicamente bastante flexíveis, biocompatíveis e moduláveis, tornando-os apropriados para aplicações em biomedicina, farmacêutica e materiais avançados. Esta tese investiga a produção de membranas de eutectogéis, compostas por FucoPol e solventes eutéticos derivados de fenóis, com o objetivo de juntar as propriedades bioativas dos compostos fenólicos e do FucoPol, com as propriedades estruturais e mecânicas do mesmo, para uma potencial aplicação como cobertura para feridas.

O FucoPol foi sintetizado pela bactéria *Enterobacter* A47 e os solventes eutéticos foram preparados misturando cloreto de colina com os compostos fenólicos: 2-feniletanol, pirogalol e ácido gálico. As membranas foram produzidas ao aplicar o método de evaporação do solvente, à combinação entre a solução de FucoPol e os solventes eutéticos. Estas membranas demonstraram uma libertação controlada de compostos fenólicos a concentrações máximas não letais, demonstrando o seu potencial para serem aplicadas em contacto com a pele seguramente. Entre as misturas eutéticas, a ChCl:Fenilethanol exibiu a citotoxicidade mais baixa e uma difusão ideal em testes antibacterianos. Adicionalmente, este solvente eutético demonstrou uma atividade antibacteriana relevante para todas as bactérias testadas: *Escherichia coli*, *Staphylococcus aureus* e *Pseudomonas aeruginosa*. Em termos de estabilidade térmicas, as membranas de eutectogéis não demonstraram nenhum sinal de degradação, nem alteração da sua estrutura atém uma temperatura de 150 ºC.

Estes resultados indicam que as membranas de eutectogéis preparadas eram estáveis, não tóxicas e com propriedades antimicrobianas promissoras, tornando-as uma escolha apropriada para aplicações biomédicas e farmacêuticas. Trabalhos futuros deverão focar em analisar em maior pormenor as propriedade mecânicas das membranas, bem como explorar atividades biológicas adicionais, como efeitos anti-inflamatórios e antioxidantes, que são propriedade já associadas a compostos fenólicos e ao FucoPol.

Palavras-chave: FucoPol, Eutectogél, Solventes Eutéticos, Compostos Fenólicos

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1 Introduction

The development of sustainable soft materials has gained significant attention in recent years, particularly in the fields of biomedicine, pharmaceuticals, and advanced materials. Eutectogels, which are gel-like materials formed by incorporating deep eutectic solvents (DES) into a polymeric network, have appeared as promising candidates due to their biocompatibility, low toxicity, tunable mechanical properties, and green synthesis approaches. This thesis explores the incorporation of selected phenolic compounds into eutectogels, using a polysaccharide-based gelator, FucoPol, as the structuring agent.

1.1 Soft Materials

Soft materials have been increasingly studied in recent years due to their macroscopic characteristics. These materials present some structural order but lack the large three-dimensional atomic organization of solids, allowing them to flow under specific conditions [2].

Soft materials are highly sought in fields such as soft robotics and electronics [3, 4], due to their mechanical properties similar to biological matter, such as stretchability and capacity to self-heal [5]. Other applications include wound dressing or drug delivery systems, as these materials can incorporate bioactive compounds within or as part of its structure, enabling direct interaction with the body or provide a controlled release of these compounds [6, 7].

Inside the broad category of soft materials, there are sub-types with more specific properties, such as liquid crystals, polymer melts, and colloids.

Liquid crystals represent an intermediate state of matter between isotropic liquid and crystalline solid. As such, they share high fluidity and inability to support shear forces, typical of liquids, while also exhibiting anisotropic electrical, magnetic and optical characteristics of crystals. This dual nature makes liquid crystals unique, possessing both isotropic and anisotropic properties [8].

Polymer melts are liquids consisting of entangled polymers without any solvent. This creates an incompressible viscoelastic liquid, which behaves more like a solution

of disconnected monomers rather than as a collection of entire polymer chains. The properties of the melt are primarily determined by the monomer interactions, while the entanglement of the chains, and therefore the polymer size, affects the fluidity of the melt [9].

Colloids were first studied in the 1840s by Francesco Selmi, who investigated pseudo-solutions of sulfur and silver iodide in water [10]. The definition of colloids has evolved over the years, but they can generally be described as a mixture in which insoluble particles of one compound are dispersed in other substance. This composition implies a dispersed phase and a dispersing medium. Depending on the states of matter of these phases, colloids can be classified into different types as present in Table 1.1. Independent on their classification, colloids exhibit a key property, which is their propensity to aggregate and sediment. This property is influenced by factors such as particle size of the suspended particles and dispersity (the smaller and more uniformly dispersed the particles, the lower their propensity to aggregate and settle) [10].

Table 1.1: Colloid classification adapted from Terence Cosgrove, 2005 [10].

Medium/Phase		Dispersed Phase		
		Gas	Liquid	Solid
Dispersion Medium	Gas	No Records	Liquid aerosol	Solid aerosol
	Liquid	Foam	Liquid emulsion	Sol
	Solid	Solid Foam	Solid emulsion	Solid Sol

1.1.1 Gels

Gels are categorized as solid emulsions with a liquid dispersed phase in a solid dispersing medium. However, in the context of gels, these phases are typically referred to as liquid phase and gelator, which usually is a polymer.

Gels consist of a tri-dimensional crosslinked structure composed by the gelator, which encapsulates a liquid medium. While physical interactions such as entanglement are not classified as crosslinking, they can still contribute to gel formation. The liquid medium within the gel network can be water, organic solvents, or ionic solvents, giving rise to hydrogels, organogels, and ionic gels, respectively. [11]. These different types of gels have similar overall mechanical properties, but their characteristics and functionality are primarily determined by specific compounds used to create these gels.

Hydrogels are biocompatible materials that resemble soft tissue and use hydrophilic gelators that retain aqueous solutions inside their structure. Due to their properties, they are used in tissue engineering, drug delivery, intraocular lenses, and as wound dressing [12, 13]. The amount of liquid phase retained by a hydrogel depends on factors such as chain length, hydrophilicity, and the polymer density in the gel [14].An important

characteristic of hydrogels is swelling, which is the volume change that occurs as they absorb water. Swelling directly influences mechanical properties of gels [15].

On the other hand, organogels, can be synthesized via two different approaches that lead to different gel structures. One method involves using a high molecular weight hydrophobic gelator that forms a network incorporating an organic liquid phase, similar to hydrogel. Alternatively, low molecular weight gelators can be employed in an organic liquid, and will self-assemble into fibers, strands, or larger structures that provide rigidity to the structure [16].This latter method creates a thermally reversible gelation, where the gel transitions between a gel and a sol state, or vice-versa, depending on the temperature relative to its gel transition temperature [16].This reversibility is due to London forces, which are part of the Van der Waals forces and stabilize the self-assembled structures. These forces are much weaker when compared with bonds like ionic and covalent, making gel highly responsive to temperature changes [17].

Lastly, ionic gels are formed by encapsulating an ionic liquid phase in a gelator matrix, which has different properties from the previous ones. These gels exhibit enhance stability and are particularly well suited for electronics such as batteries, or wearable sensors. Due to their mechanical properties and compatibility with electronic systems, ionic gels have been highly studied for soft electronics[18, 19]. When talking about ionic gels, it is implied that the liquid phase is an ionic liquid (IL). However, deep eutectic solvents (DES), who have similar characteristics to ILs, can be used as a liquid phase, creating an eutectogel, which will discussed in detail throughout the next sub-sections

1.2 Deep Eutectic Solvents

1.2.1 Deep Eutectic Solvents Vs Ionic Liquids

Deep eutectic solvents (DES) are not single, pure compounds; rather, they are eutectic mixtures in which the individual components are interconnected through hydrogen bonding. These mixtures can be composed of a huge diversity of compounds as long as at least one acts as a hydrogen bond donor (HBD) and other as a hydrogen bond acceptor (HBA) [20]. In contrast, ILs are a combination of organic cations with organic (a Lewis acid) or inorganic anions, forming a new pure compound through ionic forces (Coulomb Forces) [21].

Although both materials have similar compositions, their synthesis differs significantly (figure 1.1). Ionic liquids can take up to 48 h to synthesize and require multiple steps with different reagents and volatile organic solvents, generating waste and by-products. This process can be divided into two main steps: the formation of the desired cation and the subsequent anion exchange [21]. Meanwhile, DES synthesis only requires mixing the components under heat and magnetic stirring until a homogeneous liquid forms, a process that can take anywhere from minutes to a few hours depending on the materials used. Among other methods for synthesizing DES, there is the grinding method, which

requires mixing the compounds at room temperature using a mortar and pestle until a liquid is formed, achieving 100% yield and atomic efficiency [22].

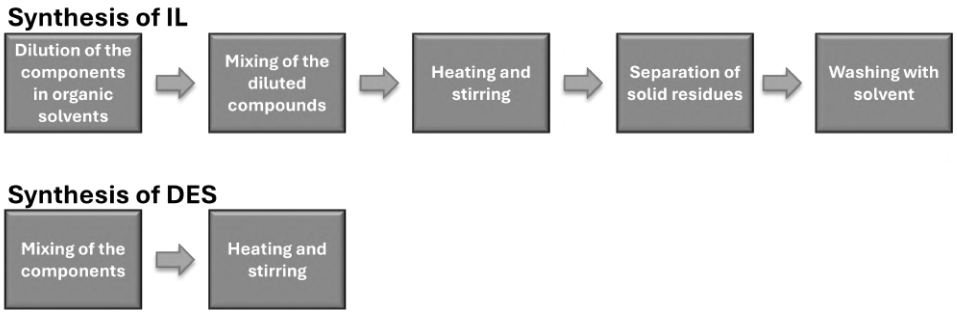


Figure 1.1: Comparison between IL and DES syntheses[23]

The physicochemical properties of ILs and DES can be highly customized based on the selected components, making them suitable for a broad range of application [23].

In the beginning, ILs were considered green, stable and non-flammable. However, it is now known that many of them exhibit different degrees of toxicity, hazardous properties and non- or low biodegradability. In contrast, DES delivered similar performance while being more environmentally friendly, less expensive to produce, and generally more biodegradable, stable and less hazardous [23]. Taking all this into account, this thesis focused on the use of DES as the liquid phase to design eutectogels.

1.2.2 Eutectic Solvents

Deep eutectic solvents (DES) have been studied extensively for a wide range of applications. Although in some cases, the term is erroneously used to describe mixtures that do not meet its defining characteristics. Specifically, the eutectic temperature of a DES should be significantly lower than the theoretical value, and the mixture should remain in a liquid state at its operating temperature. If these criteria are not met or are unknown, the term "eutectic solvent" is a more appropriate designation [24].

1.2.3 DES subtypes

DES systems are composed of a hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA). However, their structure can be further defined using the general formula: $Cat^{+}X^{-}zY$, where *Cat* represents the cation, typically from an ammonium, phosphonium, or sulfonium salt, X- is the anion of the salt (commonly a halide anion), Y is either a Lewis or Bronsted- Lowry Base, and z is the number of Y molecules [25]. This formula facilitates modifications for different types of eutectic mixtures (Tabela 1.2).

Table 1.2: Different types of eutectic mixtures[26]

Type	General Formula	Terms

I	$Cat^+X^-zMCl_x^-$	M:Al, Fe, Zn, Ga, In, Sn
II	$Cat^+X^-zMCl_x \cdot yH_2O$	M:Cr, Fe, Co, Ni, Cu
III	Cat^+X^-zRZ	Z:$CONH_2$, COOH, OH
IV	MCl_x+RZ	M:Al, Zn Z:$CONH_2$, OH
V	RZ_1+RZ_2	$Z_{1,2}$=OH, COOH

1.2.4 NADES

In 2011, Choi et. al[27] reported the existence of eutectic mixtures composed of metabolites that occur in large amounts in cells, as a possible explanation for the solubility of intracellular compounds that are insoluble in both aqueous and lipid phases. Due to the origin of the starting materials for these compounds, they denominated them Natural Deep Eutectic Solvents (NADES). These NADES were mainly composed of sugars, amino acids, choline, and various organic acids including citric acid and lactic acid, among others (Table 1.3). They have been shown to increase significantly the solubility of poorly water-soluble natural compounds, as well as macromolecules such as proteins and polysaccharides [27].This happens due to their spatial arrangement that allows them to form hydrogen bond with solutes, giving them increased solubility of polar and non- polar compounds [28, 29]. They also were shown to enhance the activity of certain enzymes by stabilizing their substrates. This stabilization allows for a higher concentration of substrate to be dissolved in the medium, which increases the product formation, as described by the equation of Michaelis-Menten (equation 1.1). Another way in which NADES improve enzymatic activity was by enhancing the NADH electro-regeneration, a fundamental factor to increase the production on some enzymes [30]. These findings strongly support the first hypothesis by Choi and coworkers [27].

$$S + E \rightleftharpoons SE \rightleftharpoons P, \qquad S\text{= Substract} \quad E\text{= Enzyme} \quad P\text{= Product} \tag{1.1}$$

Table 1.3: List of the first NADES synthesized by Choi et al. [27]

Combination	Molar Ration
ChCl : Citric acid	2:1, 3:1
ChCl : Malic acid	1:1, 2:1, 3:1
ChCl : Maleic acid	1:1, 2:1, 3:1
ChCl : Aconitic acid	1:1
ChCl : Glucose : water	1:1:1
ChCl : Frutose : water	1:1:1
ChCl : Sucrose : water	1:1:1
Pro : Citric acid	1:1, 2:1, 3:1
Glucose : Malic acid	1:1
Frutose : Malic acid	1:1

Sucrose : Malic acid	1:1
Glucose : Citric acid	1:2
Trehalose : Citric acid	1:2
Sucrose : Citric acid	1:1
Glucose : Maleic acid	1:4
Sucrose : Maleic acid	1:1
Frutose : Glcucose	1:1
Frutose : Sucrose	1:1
Glucose : Sucrose	1:1
Sucrose : Glucose : Frutose	1:1:1

1.2.5 THEDES

Due to the green and biodegradable properties of DES, it wasn't long before the idea of using them in therapeutic applications appeared. In 1998, Paul W. Scott reported the use of eutectic solvents to enhance the permeation of ibuprofen for transdermal drug delivery [31], which was the first reported use of DES with active pharmaceutical ingredients (API's) creating what would later be identified as Therapeutic Deep Eutectic Solvents (THEDES).

For an eutectic solvent to be classified as a THEDES, at least one of its constituents must be an API or fully incorporate the API into its structure [32]. Although their structural and functional properties are not yet fully understood, they are being vastly studied using several production techniques and applications. These includes:

- Electrospinning by encapsulating THEDES in gelatin to produce electrospun fibers [33].
- Nanopercepitation, which involves dissolving lignin in DES and then introducing small drops of this solution into water.
- Supercritical CO2 processing, in which DES formation occurs inside a reactor using supercritical CO2, a well-established method to produce fine particles in an environmentally friendly manner [34, 35].

Regarding their applications, THEDES, are being explored for various drug delivery methods, such as oral delivery in particular to enhance the bioavailability RA-XII (an anti-tumor drug) [36] or celecoxib, where the supercritical technique achieved the highest dissolution rates compared to other tested methods [35]. Although several drug delivery routes have been tested, transdermal administration is the safest and most convenient. Many API's have difficulty to penetrate the skin, but THEDES have shown the ability to enhance their solubility and permeability, while not affecting their biomedical properties [12, 37].

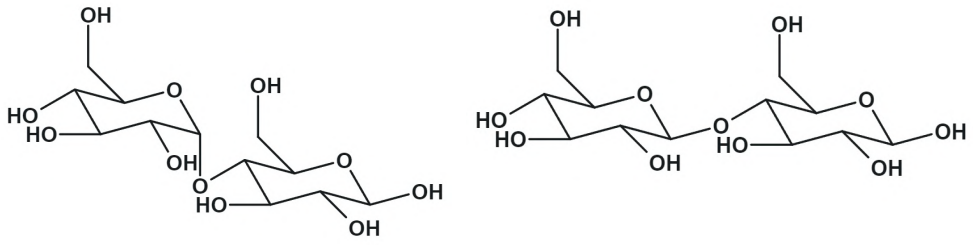
Some notable examples of THEDES applied in previous studies include:

- Arginine: Glycerol incorporated with ibuprofen [12].
- Choline Chloride:(L-(+)-tartaric acid diethyl ester) incorporated with diclofenac diethylamine as a model drug [38].
- Lidocaine: ibuprofen DES incorporated with artemisinin THEDESex2.

1.3 Polysaccharides

As previously mentioned, a gel is composed of a liquid phase and a gelator, which is generally a polymer, such as a polysaccharide.

Polysaccharides are bio-macromolecules composed of long chains of monosaccharides units connected by *alfa* or *beta* glycosidic bonds (Figure 1.2), They can be classified as homo or heteropolymers, depending on their monomer composition. Homopolysaccharides consist of only one type of monomer, whereas heteropolysaccharides are composed of two or more different monomers [39].



α bond β Bond

Figure 1.2: α and β represented in glucose molecules

Polysaccharides are primarily composed of sugars (monosaccharides), but some also contain acyl groups that modify their chemical and functional properties. The presence of functional organic groups, such as sulfate, acetyl, or phosphate groups, can influence solubility, charge, and biological activity. Moreover, such variety in composition affects other properties as thickening, gelling, emulsifying, stabilizing, binding and film-forming ability [40]. Their mechanical properties come from their molecular size, but also from the interactions between their monomers and inherent characteristics. They can be functionally modified to enhance cross-linking, or to improve the incorporation of other compounds such as the previously mentioned DES, or APIs for medicinal applications [41]. Polysaccharides can be obtained from algae, animals, plants and microorganisms, such as bacteria (e.g. xanthan) (Figure 1.3a) [42]. Owing to their natural origin, polysaccharides usually possess high biodegradability and biocompatibility making them prime materials for bio-applications, such as drug delivery, wound dressing, scaffolds and others [43–46].

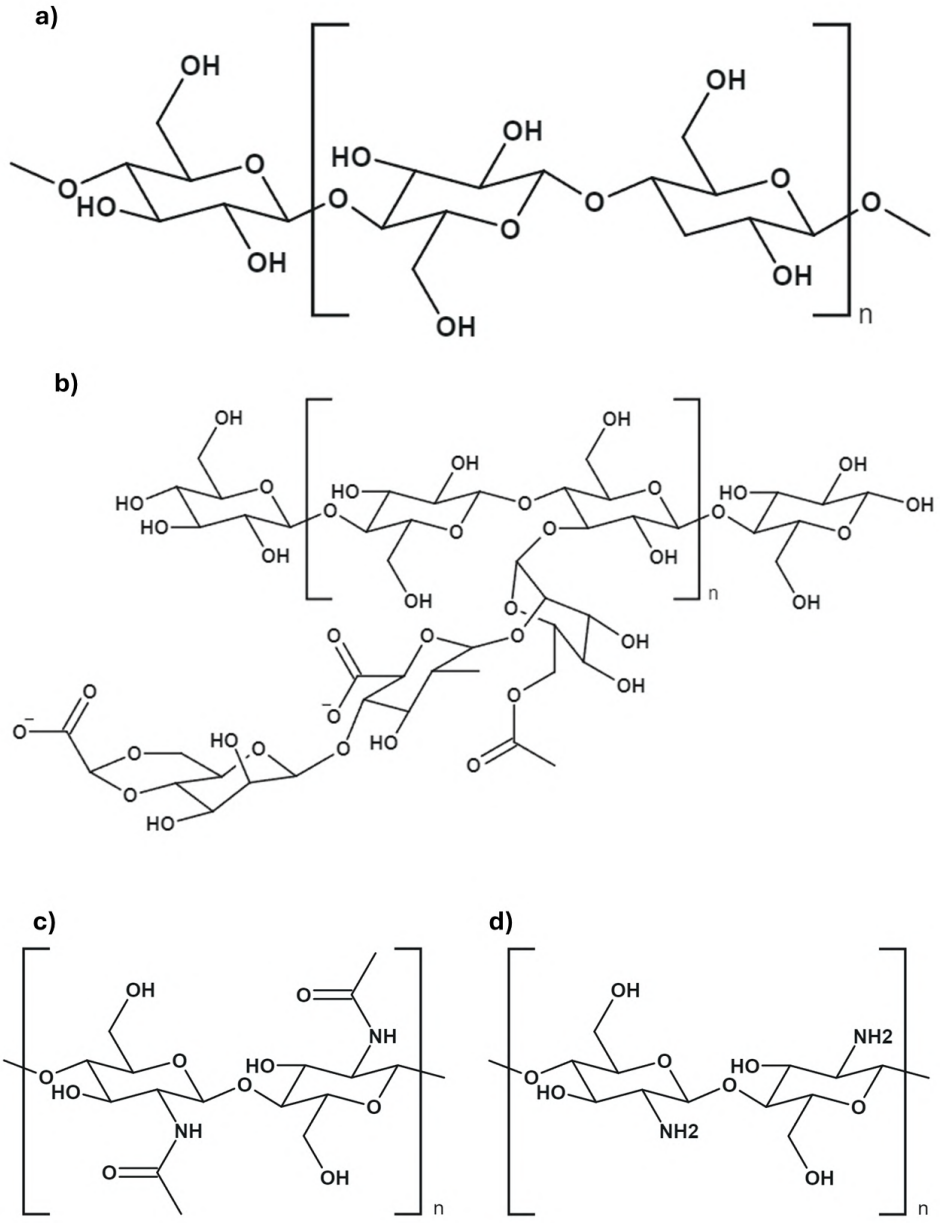


Figure 1.3: Polysaccharides examples:

(a) Cellulose, a natural homopolysaccharide composed of glucose that can be synthesized by plants, algae, and microbes with structural function (b) Xanthan, a natural anionic heteropolysaccharide secreted by the microorganism *Xanthomonas campestris* by aerobic fermentation and consisting of glucose, mannose and glucuronic acid (c) Chitin, a natural homopolyssacharide composed of N-acetylglucosamine produced by fungi and animals with a structural function (d) chitosan, a synthetic homopolysaccharide synthesized from chitin by deacetylation and can possess cationic charge at pH levels under 7

1.3.1 FucoPol

FucoPol is an anionic, natural heteropolysaccharide secreted by bacteria and with a high molecular weight (1.7 - 5.8 $\times$ 10^6 Da average). What makes this polymer noteworthy

is its fucose-rich composition (32-36 mol%). It is also composed by glucose (28-34 mol%), galactose (25-26 mol%), glucuronic acid (9-10 mol%), pyruvate (3.7-14 wt.%), acetate (3.5-6.8 wt.%), and succinate(0.6-3 wt.%) [47].

Fucose-rich polysaccharides have been widely applied, for their bioactive properties, in different fields such as immunomodulatory drugs, where high molecular weight polymers were able to prevent liver fibrosis [48], as an antitumor drug capable of inhibit Lewis lung carcinoma end even reduce it [49], and have been shown to possess antioxidant activity [50].

FucoPol, is an exopolysaccharide (EPS) produced by the *Enterobacter* A47 using glycerol as the sole carbon source. This polymer has demonstrated great versatility in different applications due to its unique physicochemical and bifunctional properties, including emulsifying ability [47], photoprotective effect [51], adhesive capabilities [52], and cryoprotective potential [53].

Due to its functional versatility, FucoPol has been modified and engineered for various applications. FucoPol has been crosslinked with citric acid, resulting in biodegradable films that showed great potential in the food industry [54]. Has been electrospun combined with other polymers, such as polyethylene oxide and pullulan, to fabricate nanofiber systems, making viable candidates for cosmetic, pharmaceutical and biomedical applications [55]. FucoPol was also cationically cross-linked using copper(II) and iron(III) to produce hydrogels that possessed varying mechanical properties and low citotoxicity, which showed potential as drug delivery system [56].

1.4 Eutectogel

All the abovementioned aspects about gels, DES, and polysaccharides, particularly FucoPol, encouraged the exploration of the bioactivity, low-cytotoxicity, mechanical properties, and environmental sustainability of these compounds by combining them into eutectogels. These gels have low volatility, that increases stability and shelf-life, high customization [57], which provides an ability to adapt to the desired applications, and a green and simplistic preparation when using mostly bio-based compounds. However, these advantages are largely dependent on the initial component used in the eutectic solvent. A potential drawback is their sensitivity to external conditions, especially their tendency to absorb water due to extensive hydrogen bonding networks [58]. This property may affect performance and consistency, but can also be used to alter their functional properties as needed. An example of this is a super-strong adhesive produced by YC. Wan et al., which can be effortlessly be detached by soaking in water, without damaging the system [59]. To improve the resilience and stability of eutectogels, different strategies had been explored by crosslinking the polymer (gelator) using methods such as photo-polymerization [60] or physical crosslinking [5].

The choice of gelator depends on the desired application and required material properties, for example, YC. Wan et al used a synthetic homopolymer of acrylic acid, poly(acrylic

acid) as gelator [59]. This polymer has many carboxyl functional groups with promotes enhanced water absorption, pH sensitivity and responsiveness to ionic strength [61]. Other example is the usage of a cellulose-based framework reinforced with polyacrylamide in conjunction with DES, to create an eutectogel for a zinc-ion hybrid super capacitors, offering great mechanical properties and a high ion transfer capacity [62]. Eutectogels incorporating NADES and xanthan gum as gelator, have been reported, yielding materials with tunable mechanical, thermal, and rheological properties by adjusting water content [63].

Among the components of eutectogels, DES offer the greatest versatility, as they can be readily modified to suit specific applications. One of the most commonly used hydrogen bond acceptors (HBA) in biobased DES formulations is choline chloride (ChCl). This natural molecule is commonly grouped with the B-complex vitamins. Structurally, it consists of a quaternary ammonium salt with an alcohol group (Figure 1.4) allowing it to interact with an assortment of hydrogen bond donors, (HBDs) such as alcohols, sugars, hydroxy acids, and urea [64, 65]. The ability of ChCl-based DESs to preserve the bioactivity of target compounds makes them particularly attractive to be applied in biomedical, cosmetic and food industries [66].

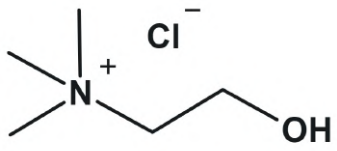


Figure 1.4: Choline Chloride molecule

While HBAs are relatively standardized, HBDs are chosen based on their desired characteristics. Some examples of HBDs reported in the literature are:

- Urea, as the first reported HBD in a DES, in combination with ChCl [67]. This molecule is a widely available simple organic compound. It is non-flammable, nontoxic, biodegradable, cost-effective, and produces DES in conjugation with ChCl, with high ionic conductivity [68].
- Glycerol, which is a green solvent completely soluble in water, with a short-chain alcohol backbone, making a suitable HBD. However, it has limited solubility in some organic solvents, and a tendency for its backbone to react and create unwanted side reactions [69], which can be mitigated by stabilizing these compounds as HBD in an eutectic solvent [70].

Phenolic compounds have gained interest as HBDs due to their unique properties. DES based on phenolic compounds have been reported as ideal solvents for extraction and purification of organic molecules and hard-to-isolate compounds [71, 72]. Moreover, many

phenolic compounds show antibacterial, anti-inflammatory, and antioxidant activity [73, 74], making them valuable candidate for natural deep eutectic solvents (NADES) and therapeutical eutectic solvents (THEDES) [75, 76].

1.5 Thesis motivation

This study specifically focuses on three phenolic compounds, namely pyrogallol, gallic acid, and 2-phenylethanol, due to their distinct bioactivities and potential biomedical relevance. The rationale for selecting these molecules stems from their varied reactivity, toxicity profiles, and functional applications, allowing for a comparative evaluation of how DES-based eutectogels can modulate their properties. By combining the eutectic solvents with the structural integrity of FucoPol, and the bioactivity of phenolic compounds, this work aims to develop novel eutectogels with enhanced properties.

Pyrogallol is a phenolic molecule that can be found in nature, mostly as a metabolite or derivative of other phenolic compounds, such as tannic acid or gallic acid, originated from hardwood trees or other natural sources [77, 78]. Although pyrogallol has shown good bioactive properties, including even anti-cancer mechanism, it is also known for its strong oxidative nature, producing oxygen radicals that contribute to its significant toxicity [78, 79]. A previous study reported the use of pyrogallol and choline chloride DES to produce an eutectogel [80], but not with intention to improve its biological activity or using polysaccharides as gelator.

Gallic Acid is another natural phenolic compound found in plants, such as tea leaves and fruits like berries [81, 82]. It has shown great biological properties, including anti-inflammatory, antioxidant , antibacterial and anticarcinogenic activity, while also possessing relatively low toxicity [83, 84]. Although gallic acid is commonly used to synthesize deep eutectic solvents, to our knowledge, it has never been used to produce eutectogels with polysaccharides.

2-Phenylethanol is also a natural phenolic compound that can be produced by yeast fermentation, more specifically, by *Saccharomyces cerevisiae* [85]. This alcohol also occur naturally and exhibits antimicrobial activity[86], however, it is mostly used in the food industry and as a fragrance raw material [87, 88]. DES have been reported to provide a method of extraction and separation of 2-phenylethanol [89, 90], however, the idea of implementing it into the eutectic system as HBD was a novelty the beginning of this thesis. To the best of our knowledge, the first reported implementation of this compound in an eutectic solvent was recently documented by H. Qezelje et al. (2024) using ChCl as the HBA, which in combination other methods demonstrated promising analytical capabilities [91].

Therefore, the main objective of this thesis was developing a novel gel-like biomaterial, using FucoPol polymer and the aforementioned phenolic compounds, in order to apply them in biomedical applications, as these compounds present promising characteristics in these fields.

2

Materials and Experimental Procedure

2.1 FucoPol Production

2.1.1 Media

LB medium per liter (distilled water): NaCl, 10g; Yeast extract (Sigma-Aldrich), 5 g; tryptone (Millipore), 10 g

Medium E per liter (distilled water): K_2HPO_4 (Sigma-Aldrich), 5.4 g; KH_2PO_4 (Sigma-Aldrich), 3.7 g; $(NH_4)2HPO_4$ (Sigma-Aldrich), 3.3 g. 10 mL of $MgSO_4$ (Sigma-Aldrich) 100 mM, 1ml of mineral solution.

The Mineral solution is constituted by (per liter of HCl 1N): $FeSO_4.7H_2O$ (Sigma-Aldrich), 2.78 g; $MnCl_2.4H_2O$ (Sigma-Aldrich), 1.98 g; $CoSO_4.7H_2O$ (Sigma-Aldrich), 2.81 g; $CaCl_2.2H_2O$ (Sigma-Aldrich), 1.67 g $CuCl_2.2H_2O$ 0.17 g; $ZnSO_4.7H_2O$, 0.29 g.

Glycerol (86-88%, Fisher Scientific) was the carbon source added to medium E, after sterilization, for a final concentration of 40 g/L.

After dissolution, media were sterilized per 20 min, at 121 °C. After cooling, LB Agar, down at approximately 50 °C, it was poured into sterile Petri dishes.

Bioreactor feeding solution: 500 mL of glycerol (86-88%, Fisher Scientific) and sterilize it through autoclavation at 120 °C, for 20 minutes. 50 mL of sterilized medium E, 5 mL of Mg SO_4 solution 100 mM, and 0.5 mL of mineral solution were added to glycerol in a flux chamber, to guarantee it remained sterile.

2.1.2 Bacterial Strain and Inoculum preparation

Enterobacter A47, stored at -80 °C in 20% glycerol (99%, Sigma-Aldrich) was reactivated by streaking onto LB Agar plate. The plate was inoculated at 30 °C for 24 h, to allow colony formation.

Enterobacter A47 inocula were prepared by adding 1 colony to 50 mL of LB medium, in a 250 mL baffled shake-flask, which was then incubated during 16 h, at 30 °C, in an orbital

shaker (New Brunswick Scientific) at 200 rpm. After, bioreactor assay was inoculated with 400 mL of inocula (2 x 200 mL LB medium), incubated for 16 h at 30 °C, and 200 rpm.

2.1.3 Bioreactor operation

Enterobacter A47 cultivation assays were performed in a 10 L bioreactor (BioStat B-Plus, Sartorius, Germany), with a working volume of 8 L. The experiment started with an initial batch phase and was followed by a fed-batch phase (respectively growth and production phase). The feeding solution was supplemented at rate of 4.5 $mL.h^{-1}$. The bioreactor had temperature controlled 30°C (± 0.1), and the pH was set at 7, by automatically adding sodium hydroxide (NaOH) 5 N and hydrochloric acid (HCl) 2 N solutions. The formation of foam was automatically suppressed by adding Antifoam A (Sigma). To maximize bacterial growth and EPS production, the experiment was aerated at 0.125 vvm (Volume of air per reactor's Volume per minute) throughout the entire cultivation process. To maintain the dissolved oxygen concentration (DO) at 10%, the stirrer with two six-blade impellers, varied its stirring speed between 300-800 rpm.

Samples were taken at 0, 24, 48, and 72 hours after the inoculation to measure the absorbance at 600 nm to ascertain cell growth.

After 72 h, the reactor was ended and the cultivation broth was stored at 4 ºC the bacterial broth in dark glass bottles sealed with parafilm.

2.1.4 FucoPol Extraction and Purification

Cultivation broth was diluted at a 1:4 dilution due to its high viscosity. The diluted bacterial broth was centrifuged at 13000×g for 30 minutes at 4 °C (fixed-angle rotor 12356 at 8000 min-1 Sigma 6-16S). After centrifugation finished, the supernatant was stored in a glass Schott flask and put in an oven at 70°C for 1h to denature proteins and other heat-sensitive macromolecules, allowing their subsequent removal. The treated solution was then subjected to a second round of centrifugation under the same conditions, but for 20 minutes. Following the second centrifugation, the supernatant was transferred into a glass Shott flask sealed, stored in a cold room at 4°C. To prevent bacterial growth, sodium azide was added.

To purify the twice centrifuged solution, it was used a 100 kDa MWCO membrane with 0.1 m^2 surface area(Hydrosart, Sartorius, Germany), inserted in a crossflow module (Sartocon Slide Holder, Sartorius, Germany). The process was executed at room temperature. The Purification procedures followed a diafiltration step and an ultrafiltration step. The first step was executed by operating the aforementioned system in the diafiltration mode, where the retentate solution was kept at a constant volume by adding deionized water continuously. When the conductivity of the retentate solution reached a value below 150 µS/cm, no more water was added until solution had reduced to a volume of around 10% the initial one, by operating the module in an ultrafiltration mode. The concentrated solution was then freeze dried at -96 °C and 0.030 bar, for 48 h. The resulting solid FucoPol

was collected in a sample flask and stored inside a sealed box containing silica gel to maintain its dehydrated state.

Following the purification process, the filtration system was cleaned and decontaminated to remove any residual polymer. First, distilled water was pumped through the system to flush out any remains of the polymer solution. This was followed by the circulation of a 1 M NaOH solution, for 30 minutes at 40 °C, through the system, with both retrieval and permeate outlets recirculating back into the solution. After that, distilled water was pumped again to remove any residual alkaline solution. To evaluate if the membrane was appropriately clean, a sample from the retrieval outlet was tested in terms of conductivity and pH. In case the values were outside the range for distilled water, additional rinsing was performed until acceptable levels were achieved.

2.1.5 FucoPol Characterization

2.1.5.1 Sugar Composition

To infer the FucoPol composition, a small sample were dissolved in deionized water (approximately 5 mL with 5 g of polymer) and hydrolyzed with 100 μL of trifluoroacetic acid (TFA, 99 %) at 120 °C, for 2h. The resulting solution was then filtered and used to identify and quantify the monosaccharides present by HPLC. The used column was a CarboPac PA10 (Thermo Dionex), equipped with a pulsed amperometric detector (Dionex ICS3000, ThermoFisher Scientific Inc.). The analysis was performed at 30 °C with sodium hydroxide (NaOH 4 mM) as eluent, at a flow rate of 0.9 mL.min-1. D-(+)-galactose (99%, Fluka), D-(+)-glucose anidra (99%, Scharlau), D-(+)-fucose (98%, Sigma), D-(+)-xylose (99%, Merck), L-rhamnose monohydrate (99%, Fluka), D-(+)-mannose (99%, Fluka), D-glucuronic acid (98%, Alfa Aesar) and D-(+)-galacturonic acid monohydrate (97%, Fluka) were used as standards (1-50 ppm).

This process was executed in the analytical laboratory from the Laboratório Associado para a Química Verde (LAQV) REQUIMTE.

2.2 Preparation of Eutectic solvents

A standard heating procedure was used to prepare the eutectic solvents, as this was the most commonly method reported in literature [75, 92]. Briefly, the process involves mixing the compounds under magnetic stirring while heating the vial to the specified temperatures in Table 2.1. Once a homogeneous and transparent liquid was formed, the vials were either used immediately or stored in a 40 °C oven to preserve their characteristics. Considering that phenylethanol-based eutectic mixtures had not been reported in the literature, the same heating technique was applied while adjusting the proportions of choline chloride and 2-phenylethanol until a homogeneous and transparent liquid was obtained.

Table 2.1: Eutectic solvent production process; solvents proportion and mixing temperatures

Nomenclature	HBA	HBD	Proportion	Mixing Temperature (°C)
ChCl:Py	ChCl	Pyrogallol	1:1	60
ChCl:GA	ChCl	Gallic Acid	1:1	100°
ChCl:SA	ChCl	Salicylic Acid	1:1	100
ChCl:VA	ChCl	Vanillyl Alcohol	1:1 2:3	100
ChCl:CoA	ChCl	Coumaric Acid	2:1	100
ChCl:CfA	ChCl	Caffeic Acid	2:1	100
ChCl:PE	ChCl	Phenylethanol	1:1 1:2 2:1 1:3 1:4	40

2.3 Preparation of FucoPol-Based Membranes

The eutectogel membranes were prepared using the dry casting method, which involves casting a polymer solution containing FucoPol, a solvent, a polymer, and the desired eutectic mixture to form the membrane. A mixture of water and methanol (9:1) was used as the solvent. The same method was applied to produce pure FucoPol membranes without adding a eutectic solvent to the casting solution.

2.3.1 Preparation of the casting solution

For all the produced membranes, the FucoPol solution was prepared at a concentration of 1.5% (W/W) by dissolving FucoPol in the solvent mixture under magnetic stirring at room temperature, for 30 minutes, until a homogeneous solution was obtained. Depending on the membrane type:

- Pure FucoPol membranes were cast directly from the FucoPol solution.
- Eutectogel membranes were prepared by incorporating the eutectic solvent into the FucoPol solution under magnetic stirring before casting.

The eutectic solvents incorporation was done by measuring the its desired quantity into a beaker, and then adding the FucoPol solution under magnetic stirring until an homogeneous solution is formed.

As the incorporation of eutectic solvents into FucoPol membranes was novel, an initial assessment was performed to determine the maximum feasible percentage of eutectic solvents. Membranes were prepared with the content of the eutectic solvent ranging from 20% to 50% in increments of 10%. For the subsequent tests, formulations containing 20%, 35%, and 50% eutectic solvents were selected to optimize polymer consumption, while still obtaining data on the effect of increasing quantities of the eutectic mixture on the final product.

2.3.2 Casting and Drying Process

Once the FucoPol solution or eutectogel formulation was prepared, it was poured into 10 cm Teflon mold, which was chosen for its ease of membrane extraction. The mold was then placed in an oven at 45 °C for 48 h or until fully dried was achieved. The drying time increased with higher eutectic solvent content, because these formulations were more viscous and hampered water evaporation.

Once the membranes were formed and dried, they were carefully removed from the mold and stored in Petri dishes at room temperature in a dry environment until further testing.

2.4 Characterization of the eutectogels

2.4.1 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR-ATR technique was employed using a FTIR spectroscope (Perkin Elmer FT-IR spectrometer) equiped with ATR sampling equipment. The resolution was 0.5 cm^{-1} and the number of scans was eight. The samples were placed in the absorbance chamber and corrected by applying the ATR-correction function of Perkin Elmer Spectrum (Waltham, MA, USA) software in the region of 4000–400 cm^{-1}.

2.4.2 Thermal Properties

Thermogravimetric analysis (TGA) was performed using a thermogravimetric analyzer (PerkinElmer). The samples were heated from 25 to 600 °C at a heating rate of 10 °C/min under a nitrogen atmosphere. The peak degradation temperature corresponds to the temperature where the maximum decrease in mass occurs.

Regarding the Differential scanning calorimetry (DSC), it was performed using a PerkinElmer 8500 DSC equipment. The samples analyzed were small, unweighted strips of membranes, which were put through a first heating from 25° C to 150° C at 10° C per minute, followed by a 3 minutes isotherm. A cooling process from 150° C to -70° C at 10° C per minute followed by a 10-minute isotherm, and finally a heating process prom -70° C to 150° C at 10° C per minute. The eutectic solvents, however, were first heating from 25° C to 100° C at 10° C per min, followed by a 3 min isotherm. A cooling process from 100° C to -70° C at 10° C per minute followed by a 10-minute isotherm, and finally a heating process from -70° C to 100° C at 10° C per minute.

Both the TGA and DSC analysis were executed in POLYMAT-University of the Basque Country, San Sebastián, Spain.

2.4.3 Ionic Conductivity

Electrochemical impedance spectroscopy was executed to calculate the ionic conductivity (σ) of the eutectogel membranes. A layered cell composed of stainless steel/eutectogel/stainless

steel cell was put together to measure the eutectogel samples. The distance between the electrodes (l) was kept fixed at approximately 0.543 mm using a silicone spacer ring with an inner area (A) of 0.502 cm^2. Measurements were taken from 85 to 25 °C at 10 °C intervals where a 10 mV amplitude perturbation was applied, at the frequency range of 1 MHz to 10 mHz at open circuit potential conditions in a potentiostat galvanostat 302N from Autolab coupled to a Microcell HC temperature controller.

The experimental results were fitted using a nonlinear regression algorithm which was implemented in a Scribner software, the Zview®. To calculate the ionic conductivity, the ohmic resistance (Rb) of the eutectogels at 0.1Hz was inserted in the following equation:

$$\sigma = \frac{1}{R_b} \frac{l}{A} \tag{2.1}$$

Electrochemical impedance spectroscopy was executed in POLYMAT-University of the Basque Country, San Sebastián, Spain.

2.4.4 Contact Angle

Contact angles were measured using a Drop Shape Analyzer (DSA 25 B, Kruss GmbH, Germany) and the sessile drop method. The membrane samples were fixed to a microscope slide, and a droplet of water from a 0.8 mm needle was dropped on top of the membrane to measure the angle formed between the droplet and the membranes surface. The data were collected in a dynamic procedure instead of a fixed one, which means that several contact angle values were measured for the same droplet in a short time range and then an average was calculated. The time frame for these values should be small (less than 1 second), to avoid possible absorption or evaporation of the liquid, which in this case was distilled water.

2.4.5 Antibacterial activity tests

A disc diffusion test was used to qualitatively assess the antibacterial activity of the membranes. In this method, absorbent paper discs were soaked with the liquid compounds under analysis, while the membranes were cut to a similar size to facilitate the comparison of inhibition zones against different bacterial strains.

The prepared discs and membrane were then placed on an agar plate that had been previously and uniformly inoculated with one of three bacteria, namely *Escherichia coli* (*E.coli*), *Staphylococcus aureus* (*S. Aureus*), and *Pseudomonas aeruginosas* (*P. aeruginosas*). Then plates were incubated at 37 °C, for 24 h. During this period, the compounds present in the discs or membranes diffuse through the agar, generating a radial gradient, where depending on the antibacterial activity of the tested material, the bacteria will not grow in that circular zone.

2.4.6 Citotoxicity tests

Cytotoxicity tests using the VERO cell line were performed to determine the toxicity of the proposed membranes and their eutectic solvents. This was achieved by evaluating the percentage of surviving cells compared to the negative control, indicating cellular viability. The VERO cell line is a renal epithelial cell line from the green African monkey, and the test is executed during a 5-day planning.

On day 1, the membranes and eutectic solvents were dissolved in Dulbecco's Modified Eagle Medium (DMEM) low-glucose medium, with the highest concentration to be tested, as the remaining samples were serially diluted from this one.

On day 2, cells were seeded into two 96-well plates. A 15 ml cell suspension was prepared with 60000 cells/ml, and 100 L was added to 72 wells. Each plate corresponded to 5 different concentrations (prepared from 1:2 serial dilutions). Control wells on each plate were constituted by positive control (10% DMSO) and negative control (low-glucose medium), and three replicates for each test condition, which were then incubated at 37 °C for 24 h.

On day 3, the medium in each well was replaced with either the different test materials, 10% DMSO for the positive control, or low-glucose medium for the negative and medium control. The plates were then incubated for 48 h at 37 °C.

On day 5, the wells were washed with a PBS solution and filled with 0.02 mg/mL resazurin solution in 50% culture medium and 50% PBS solution. The plates were incubated for 2 h, and the absorbance was measured at 570 and 600 nm.

This procedure was repeated three times. In the first test, 35% eutectogel membranes and higher concentrations of eutectic solvents were used, while in the other two tests, 20% eutectogel membranes and lower eutectic solvent concentrations were used.

2.4.7 Franz Diffusion Cell Testing

This procedure consisted of two identical small glass vials, opened on top and with a sideways sampling port, filled with a receptor solution, which in this case was a PBS buffer solution to slightly mimic the interstitial environment, where the biomaterials would diffuse. Samples of these two solutions were taken at intervals of 30 min during the first two hours, and at 60 min intervals for 6 h. This time division was selected to cover the possible behavior of the eutectogels, as diffusion of the eutectic solvents could occur rapidly or for an extended period of time.

To mimic the skin as a physical barrier, filter paper was placed over the upper opening of the vial, with the desired membrane positioned on top. This setup prevents direct contact between the membrane and the receptor solution. Additionally, to replicate the physiological environment, the tests were performed at 37 °C, which is similar to the human body temperature.

Eutectogel membranes with 35% phenylethanol and a diameter of 2.5 mm and were tested. The samples were analyzed by high-pressure liquid chromatography (HPLC) to

quantify the diffusion of the eutectic solvent and fucopol.

The PBS solution was produced by dissolving 4g of NaCl, 0.1g of KCl, 0.72g Na_2HPO_4, and 0.1225g of KH_2PO_4 in 500 mL of distilled water

2.4.7.1 Phenylethanol Quantification

The phenylethanol quantification was executed by high performance liquid chromatography (HPLC) Thermo Scientific Vanquish Core equipped with a column ACE 3 4.6 mm × 150 mm, connected with a PDA detector, set at 215 nm. The testing samples were diluted in deionized water and filtered through 0.2 µm centrifuge filters. The seperation occured at 25°C and the volume injected was 20 µL. A gradient method comprising water/acetonitrile was applied as follows: 0–15 min 90/10 (flow 0.5 mL/min), 15–30 min 70/30 (flow 0.5 mL/min); 30–33 min 90/10 (flow 0.5 mL/min). Phenylethanol (99 %, Sigma-Aldrich) was used as the standard at concentrations between 0.005 and 0.3 g/L.

3

Results and Discussion

The FucoPol was successfully produced by the *Enterobacter* A47 using glycerol as carbon source. After freeze drying the purified EPS solution, it was obtained around 2 grams of purified FucoPol (Fig 3.1) per liter of purified solution. This FucoPol had a similar composition to literature, namely it was composed of fucose, glucose, galactose, glucuronic acid, pyruvate, acetate, and succinate, as stated in section 1.3.1.

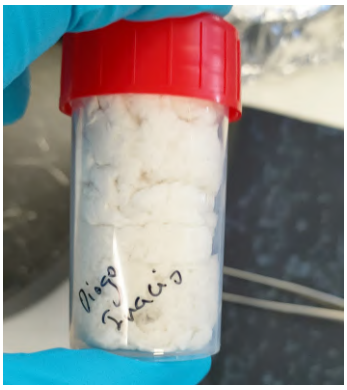


Figure 3.1: Pure FucoPol in a sample flask

3.1 Eutectogel Formation

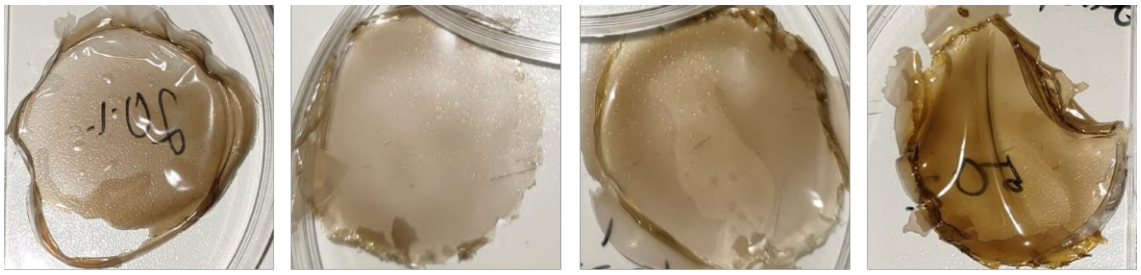
The selection of suitable eutectic solvents plays a crucial role in determining the final properties of a eutectogel. The incorporation of deep eutectic solvents (DES) into polysaccharide-based gels, such as FucoPol, offers a promising strategy to enhance their functionality. However, reaching a structurally stable membrane could be challenging, as the compatibility between DES and the polysaccharide matrix can significantly influence final material properties.

This section explores the initial assessments of different eutectic solvents in FucoPol-based eutectogels, highlighting the challenges encountered and the strategies implemented to optimize membrane formation. Figure 3.2 and 3.3 shows the possibility of incorporating

up to 50% of the ChCl:Py (1:1) and ChCl:PE (1:4) eutectic solvents, into FucoPol, suggesting a good compatibility between these DES and polysaccharides.

20% ChCl:PE | 30% ChCl:PE | 40% ChCl:PE | 50% ChCl:PE

Figure 3.2: ChCl:PE-based FucoPol eutectogel membranes



20% ChCl:Pyro | 30% ChCl:Pyro | 40% ChCl:Pyro | 50% ChCl:Pyro

Figure 3.3: ChCl:Pyro-based FucoPol eutectogel membranes

20% ChCl:VA (2:3) | Using sonication with tween | Using Sonication without tween

20% ChCl:VA (1:1) | Using sonication with tween | Using Sonication without tween

Figure 3.4: ChCl:VA-based FucoPol eutectogel membranes

ChCl:VA membranes presented a significant degree of heterogeneity and a rugged surface. The significant heterogeneity observed in the ChCl:VA membranes may indicate a lack of a uniform dispersion, which may be attributed to differences in viscosity, polarity, or phase separation. The rugged surface morphology further suggested that ChCl:VA may not be effectively integrated into the structure, leading to inconsistencies in the gel matrix. These results led to the search for a different preparation technique aimed at obtaining homogeneous membranes, which in this case was attempted using emulsions generated using a sonication probe.

The natural emulsifying properties of FucoPol and Tween 80 were used to create the emulsions. Although this method yielded better results, the membranes obtained using both methods were still inadequate (Figure 3.4). Additionally, a 1:1 ratio of ChCl and vanillyl alcohol was tested to improve membrane quality; however, similar results led to the decision to replace this eutectic solvent.

To find a replacement for the ChCl:VA eutectic solvent, another aromatic compounds were tested, namely coumaric (ChCl:CoumaricAcid 2:1), caffeic (ChCl:CaffeicAcid 2:1) and gallic acid (ChCl:GallicAcid 1:1). However, coumaric and caffeic acids exhibited significant solubilizing complications in the FucoPol aqueous solution (10% methanol in distilled water), contrary to gallic acid, which produced a clear and adequate membrane. The successful formation of a clear and well-structured membrane using gallic acid suggests that its chemical properties, particularly its hydroxyl functional groups, facilitate strong interactions with the polysaccharide matrix. This result supports the idea that the compatibility of eutectic solvents with FucoPol is largely dictated by their hydrogen bonding capabilities and solubility behavior in aqueous system. Pyrogallol, phenylethanol and gallic acid were further analyzed, and membranes were design using their DES and FucoPol.

3.2 Chemical Structure

Fourier-transform infrared (FTIR) spectroscopy was used to analyze the interaction between FucoPol and the incorporated eutectic solvents to understand their interactions within the membranes. As the ChCl is present in all the eutectic solvents studied, its peaks would appear in all spectra, with the additional peaks corresponding to the hydrogen bond donors (HBDs). Notably, deviations were observed in the C-N stretching peak (around 1325 cm^{-1}), and the C-O stretching peak (around 1040 cm^{-1}), probably resulting from the molecular interactions between ChCl and the respective HBDs, as presented in Figure 3.5. However, an absorption peak at approximately 3200 cm^{-1} in the ChCl scan did not appear for the other eutectic solvents. When comparing the experimental spectrum with the reported theoretical spectrum (figure 3.5), the same peak was missing from the theoretical data. This discrepancy could be explained by the hygroscopic nature of ChCl, which could have absorbed atmospheric moisture prior to the FTIR analysis, causing a significant peak of OH at 3200 cm^{-1}. To correct this issue, future FTIR analysis should

be conducted while minimizing the air exposure of ChCl and drying it beforehand in a ventilated oven at moderate temperatures (around 55 to 65 °C [93]). If those results still showed the same peak, the scan would need to be performed in a controlled atmospheric environment in addition to the previous procedure.

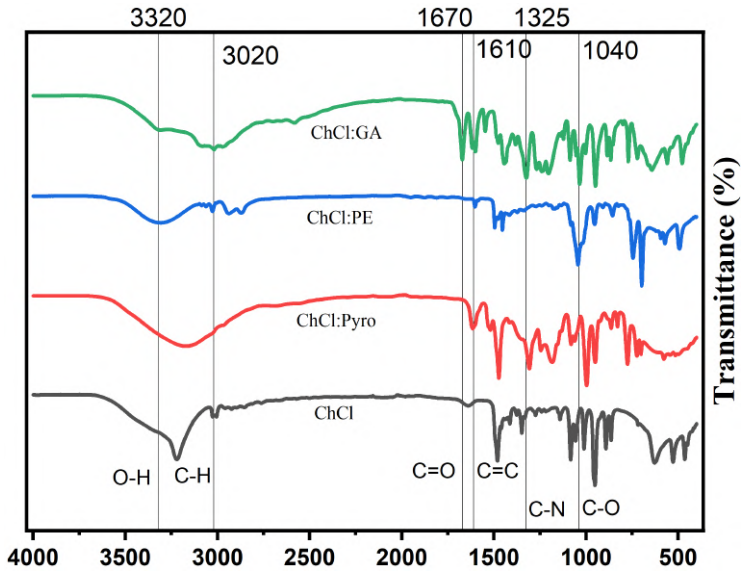


Figure 3.5: ATR-FTIR spectra from eutectic solvents, ChCl:Gallic acid (green line) ChCl:Phenylethanol (Blue line) ChCl:Pyrogallol (red line) and Choline Chloride (ChCl, black line)

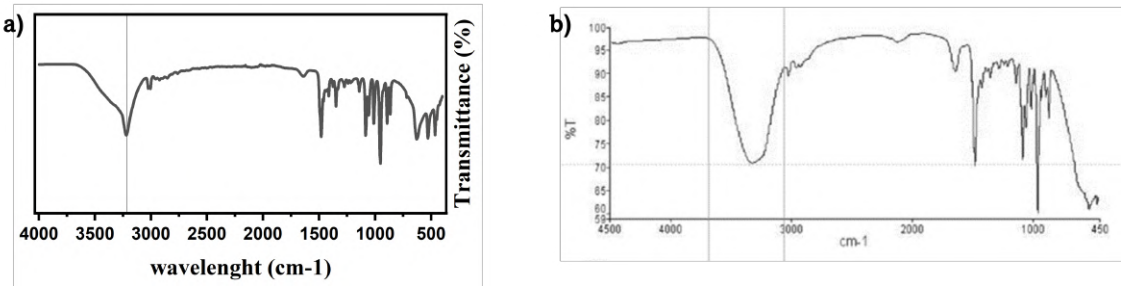


Figure 3.6: FTIR spectra of Choline Chloride a) practical result; b) Theoretical spectra[94]

The spectra of membranes containing different contents of eutectic solvent (Figure 3.7) showed an increase in peak intensity with a rise in eutectic solvent content. This trend can be attributed to the increased concentration of the eutectic solvent within the membrane, leading to greater light adsorption and, consequently, more pronounced peaks. Additionally, a significant increase in the OH- stretching band (3300-3270 cm^{-1}) was detected in 50% eutectic solvent membranes. This increase could stem from the higher water retention in the membrane, as the high viscosity increases the difficulty in evaporating the water content present in the eutectogel. Alternatively, this may be due to the increased concentration of phenolic compounds, as they have a considerable density of hydroxyl (OH-) functional groups.

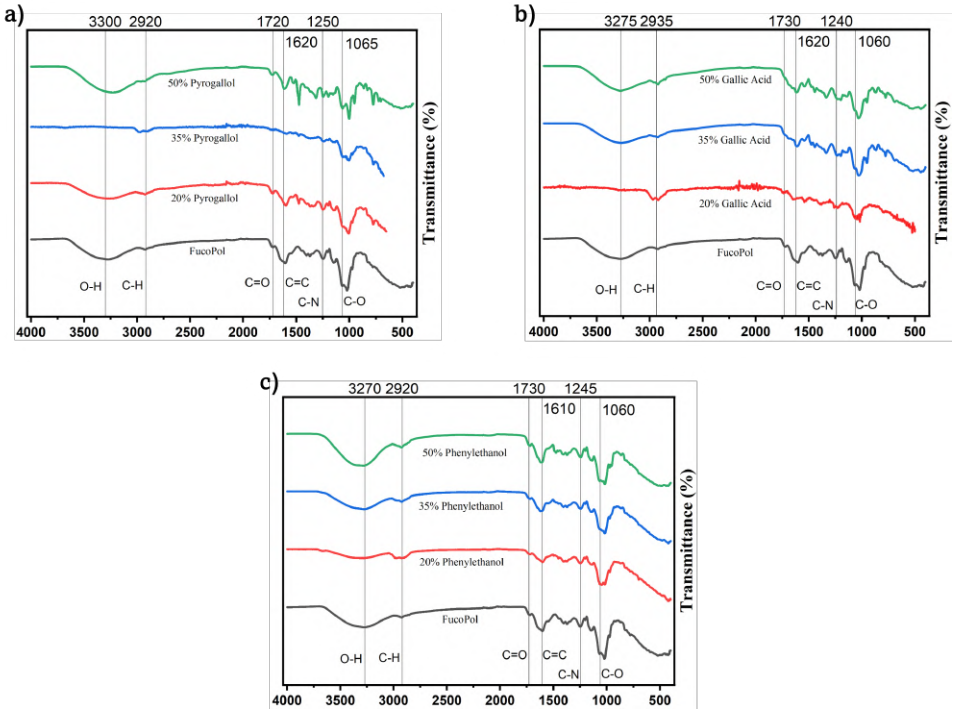


Figure 3.7: FTIR spectra of Eutectogels with increasing percentage of eutectic solvent: a) ChCl Pyrogallol; b) ChCl:Gallic acid; c) ChCl:Phenylethanol. Black line – FucoPol; Red line – 20% concentration; Blue line – 35%; Green line – 50%.

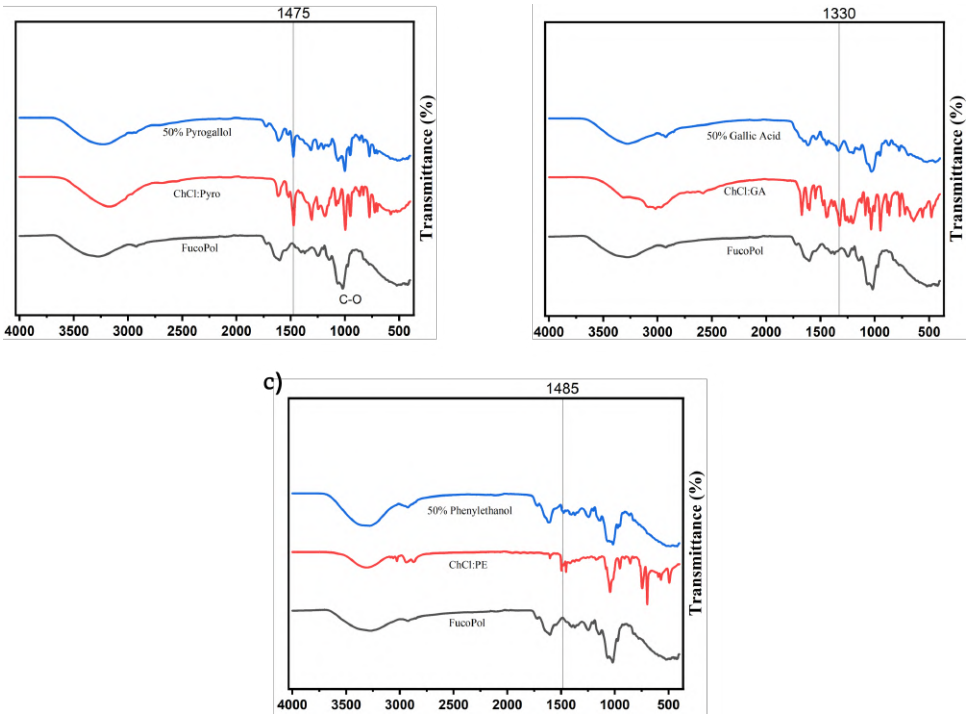


Figure 3.8: FTIR spectra of the 50% Eutectogels, coupled with the respective eutectic solvent and FucoPol spectra; a) ChCl Pyrogallol; b) ChCl:Gallic acid; c) ChCl:Phenylethanol. Black line – FucoPol; Red line – Eutectic Solvent; Blue line – 50% membrane.

Comparing the spectra of the membranes against FucoPol and the corresponding eutectic solvent spectra (figure 3.8), confirms integration of eutectic solvent's peaks within the FucoPol matrix, as evidenced by the preservation of the C-N stretching peak at approximately 1330 cm^{-1}. However, no clear peak deviation or new peaks, indicative of strong molecular interactions, were observed. This lack of spectral deviation suggests that FucoPol may be arranged through physical entrapment, rather than forming strong chemical bonds with them; consequently, the eutectic solvents probably remain dispersed in the FucoPol matrix without significant structural modifications to the polysaccharide's network.

3.3 Contact Angle

To evaluate the impact of mixing eutectic solvents on the water affinity of FucoPol, contact angle analysis was performed using the sessile drop technique. Results of this analysis (Figure 3.9) do not indicate a clear evolution of contact angle with the increase in the eutectic solvent in the eutectogel. However, all the membranes with eutectic solvents presented higher contact angles than pure FucoPol, indicating a slight decrease in the water affinity from the membrane´s surface. This would be expected, as these compounds are more hydrophobic than FucoPol; however, the non-patterned evolution of the contact angle with increasing DES concentration is an unexpected result.

Another characteristic that varies between membranes and could explain the difference in contact angles, could be the roughness of the membrane's surface; which would be analyzed by SEM imaging in the next sections.

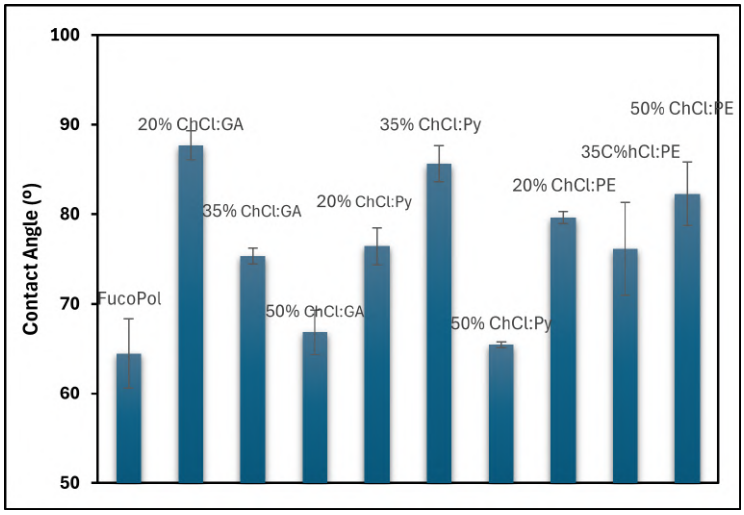


Figure 3.9: Contact Angle Analysis though the Sessile drop method

3.4 Thermal Properties

Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) were used to evaluate thermal stability, water content, and residue weight. A clearer understanding of membrane stability was obtained by analyzing the thermal degradation profiles and residual weights.

This pattern and the values obtained are similar to reported TGA analysis of FucoPol [55, 56, 95], which indicates that the casting technique used during this thesis did not significantly alter the thermal properties of FucoPol. When compared to other bacterial polysaccharides, these results present comparable profiles and degradation temperatures owing to the similar structures of these macromolecules [96–98].

TGA results, summarized in Table 3.1, indicate that the evaporation temperature did not follow a clear trend with increasing eutectic solvent content. However, overall, the eutectogel membranes exhibited higher evaporation temperatures than the pure FucoPol membranes. This can be explained by the stronger binding affinity of eutectic solvents to water molecules, which probably caused cause an increase in the energy required for water evaporation. The variation in evaporation temperatures within the same compound could be due to the thickness differences in the membrane, as larger and denser membranes hinder water loss.

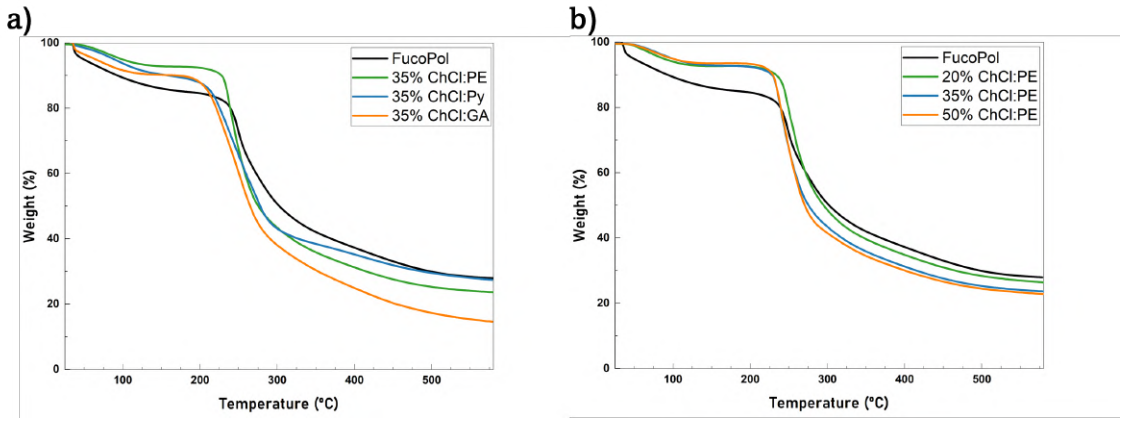


Figure 3.10: TGA analysis of the eutectogel membranes at different concentrations of eutectic solvent: a) Pure FucoPol and 35% eutectogel membranes b) Pure FucoPol and ChCl:PE membranes

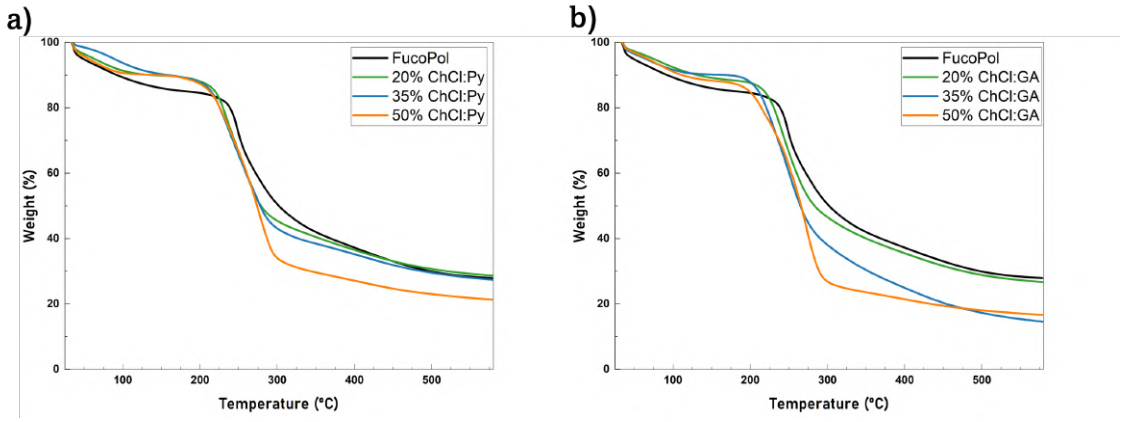


Figure 3.11: TGA analysis of the eutectogel membranes at different concentrations of eutectic solvent: a) Pure FucoPol and ChCl:Py membranes b) Pure FucoPol and ChCl:GA membranes

Table 3.1: TGA data

	Evaporation Temperature	Water Weight	T5%	Peak Degradation Temperature	Residue Weight
FucoPol	62.3°C	15%	240°C	250°C	28%
20% ChCl:Pyro	65.7°C	11%	222°C	236°C	29%
35% ChCl:Pyro	85.4°C	10%	216°C	239°C	27%
50% ChCl:Pyro	61.6°C	10%	213°C	275°C	21%
20% ChCl:GA	75.3°C	12%	224°C	242°C	27%
35% ChCl:GA	66.6°C	10%	210°C	249°C	15%
50% ChCl:GA	74.1°C	12%	207°C	272°C	17%
20% ChCl:PE	69.8°C	7%	240°C	257°C	26%
35% ChCl:PE	82.4°C	7%	232°C	247°C	23%
50% ChCl:PE	74.8°C	6%	232°C	250°C	23%

The water weight percentage, which remained relatively constant in varying concentrations of the same eutectogel membranes, was significantly higher in the pure FucoPol membranes. This suggests that the presence of eutectic solvents reduces the tendency of the membranes to bond water molecules, possibly by promoting a tighter polymeric matrix, thereby lowering their capacity to retain water. An eutectic solvent content of 20% seemed to be the percentile of eutectic solvent necessary to minimize the water content, as no substantial changes in water weight were observed beyond this concentration. ChCl:PE presented the significantly lower value of water weight from all the eutectogels. This could have been caused by its samples being sent to analysis at a different time, with different weather and transport conditions. However, as this eutectic solvent had the

highest proportion of phenolic compound to choline chloride (4:1 respectively), this could suggest that are the phenolic compounds that decrease the water retention capabilities of the membrane either by modifying the FucoPol matrix, or altering its water affinity. This will be confirmed during contact angle analysis in later sections.

The fact that the water content did not increase across the different eutectic solvent percentages contradicts the hypothesis in Section 3.2. This means that the increase in the OH- band intensity in the FTIR spectra for the 50% membranes is most likely due to the increase in phenolic OH- groups rather than the additional water content. To confirm this, FTIR and TGA should be performed using the same membrane sample under controlled humidity conditions.

The TGA data, particularly for the ChCl:Py membranes, revealed that the onset of the degradation of the eutectogel membranes (T5%) was lower than that of FucoPol membranes. This initial degradation likely corresponds to the free eutectic solvent, which increased at higher percentages within the membranes. However, when examining the derivative weight variation (from the more detailed TGA results found in Appendix A Figure A.1 to A.10), both primary and secondary degradation peaks (corresponding to the FucoPol and eutectic solvent degradation, respectively) shifted to higher temperatures with increasing eutectic solvent content. This indicates a mutual stabilization effect between FucoPol and eutectic solvent. In the ChCl:GA membranes this correlation is not as clear, however, the increase in the degradation peak temperatures follows a similar stabilization mechanism. In the ChCl:PE membranes this process does not occur, however, the derivatives possess a singular peak and follow a smoother curve than pure FucoPol. This might indicate a higher stabilization and interaction between this eutectic solvent and FucoPol.

The last data recoverable from the TGA is the residue weight, which corresponds to the fraction of FucoPol's carbon backbone that remains unreacted during the heating process, should theoretically decrease with increasing percentages of eutectic solvent content, as choline chloride-based DESs fully degrade. However, in the case of the membranes with a high percentage of the eutectic solvents containing gallic acid and 2-phenylethanol, an unexpected shift from this trend was observed. Coupled with deviation from the expected evolution in the peak degradation temperatures, this might indicate some degree of heterogeneity in the membranes, with eutectic solvent being unevenly distributed throughout the membrane. This heterogeneity, not seen by the naked eye, may have caused the fragments used for the TGA testing to have different quantities of eutectic solvent, which led to these results deviating from the expected ones.

DSC analysis confirmed that, within -50 to 150 °C, no significant structural changes nor glass transition can be detected. Which is confirmed by the absence of peaks or valleys in these DSC spectrum (Fig 3.12), which indicates that the material retained its structure stability under these conditions.

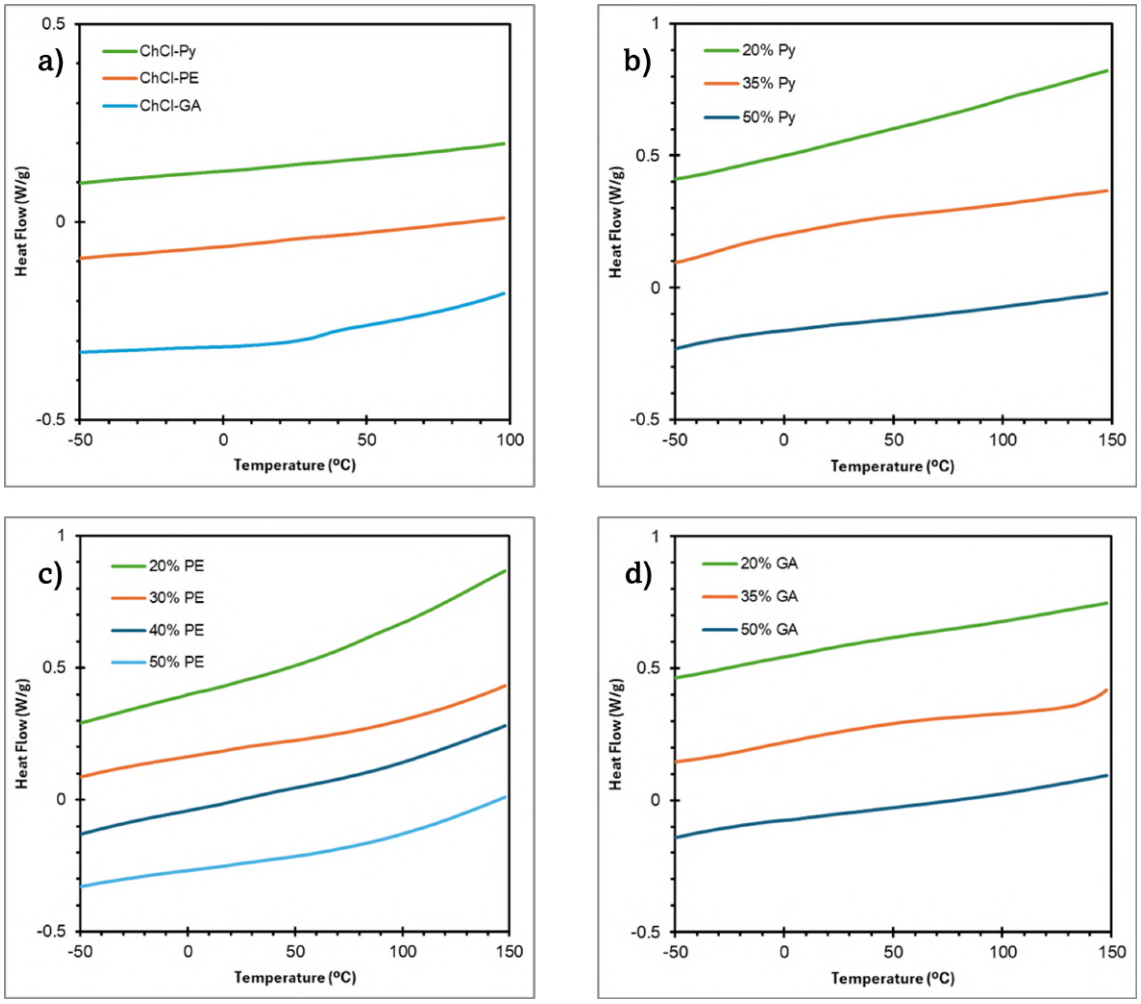


Figure 3.12: DSC analysis of eutectogel membranes with different percentages of eutectic solvent: b) ChCl:Pyro, c) ChCl:PE, d) ChCl:GA; and those same eutectic solvents: a) Green line: ChCl:Pyro, Orange line: ChCl:PE, Blue line: ChCl:GA

3.5 Conductivity

Conductivity analysis provides valuable insights into the ionic behavior and potential applications of membranes. However, several factors, including membrane integrity and the structural consistency, can significantly affect the accuracy of these measurements. In this study, challenges such as bubble formation and damage during transportation (measurements performed at the POLYMAT-University of the Basque Country, San Sebastián, Spain) did not allow reliable data to be obtained.

Nonetheless, a single intact membrane, ChCl:PE, that is 35% phenylethanol membrane, allowed for preliminary assessment, offering a starting point for further investigations into the influence of eutectic solvents on membrane conductivity (Figure 3.13). These conductivity values are similar to those of other dry polysaccharides, such as dry chitosan (approximately 10^{-9} and 10^{-10} S.cm^{-1} [99]) and gum arabica (approximately 1.5×10^{-6} S.cm^{-1} [100]). However, in the absence of conductivity data for pure FucoPol membranes,

it is not possible to determine the extent to which the eutectic solvent influences the membrane conductivity. Further experiments, particularly those including pure FucoPol samples and additional eutectic solvent concentrations, would be necessary to draw meaningful conclusions.

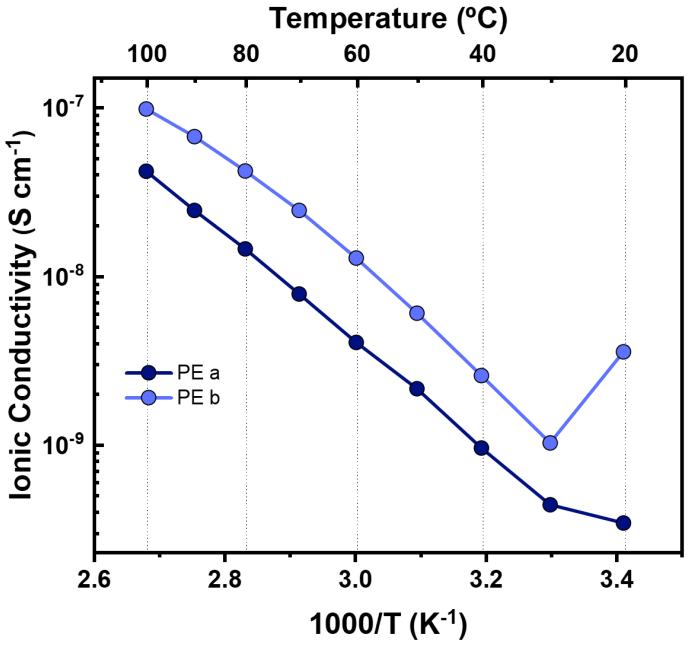
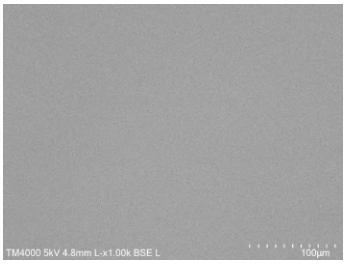


Figure 3.13: Eutectogel conductivity (2 repetitions of the membrane: 35% ChCl:PE + FP)

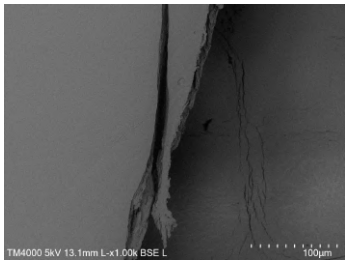
3.6 SEM analysis

SEM analysis revealed that all the membranes presented a smooth surface with minimal fractures and imperfections (Figures 3.14a,3.14d,3.14g,3.14j). These small imperfections might be the origin of the different contact angle results as theorized in Section 2.4.4; however, because of their negligible magnitude, it is most likely that these results varied for other reasons. In the images that focus on the border of the membrane (Figures 3.14c,3.14f,3.14i,3.14l) it is possible to see a higher number of imperfections (mostly due to the cutting method), and in the cases where a cross-section of the membranes is visible, the images present a more irregular structure that remains underneath the smooth surface.

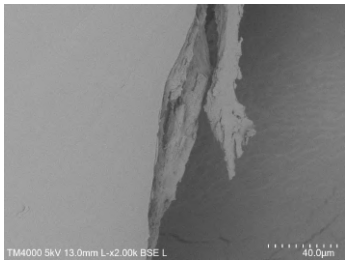
Additionally, the ChCl:Py and ChCl:GA membranes, although presenting small imperfection at their surface, also possess smoother edges, probably because they are more malleable and viscous than the other, more brittle membranes.



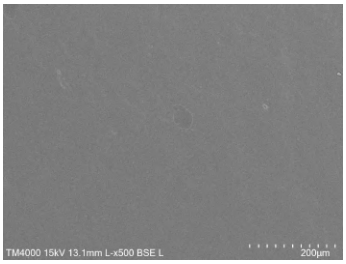
a)SEM image of a pure FucoPol membrane under 1000x magnification



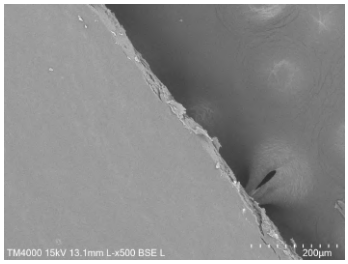
b)SEM image of a pure FucoPol membrane under 1000x magnification



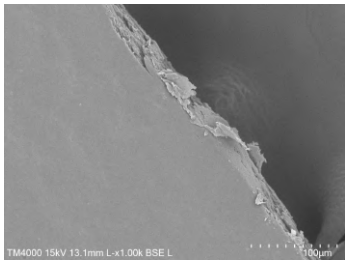
c)SEM image of a pure FucoPol membrane under 2000x magnification



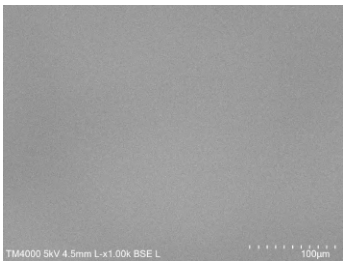
d)SEM image of a FucoPol and 35% ChCl:PE membrane under 500x magnification



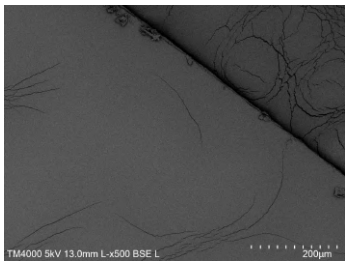
e)SEM image of a FucoPol and 35% ChCl:PE membrane under 500x magnification



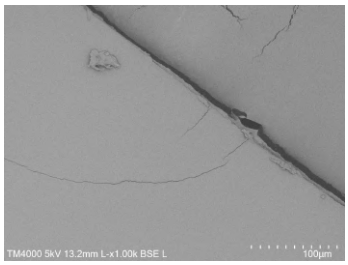
f)SEM image of a FucoPol and 35% ChCl:PE membrane under 1000x magnification



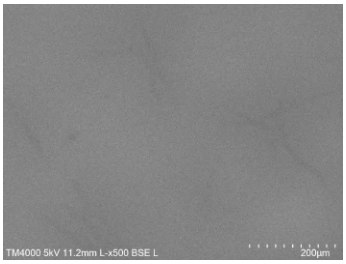
g)SEM image of a FucoPol and 35% ChCl:Pyro membrane under 1000x magnification



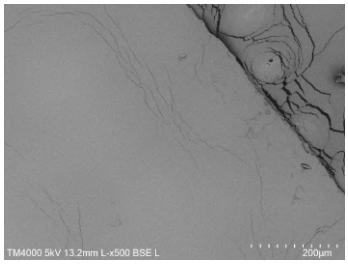
h)SEM image of a FucoPol and 35% ChCl:Pyro membrane under 500x magnification



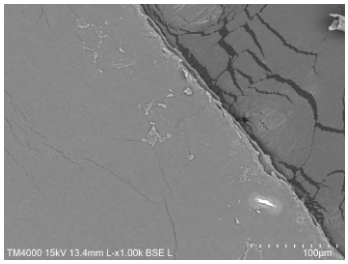
i)SEM image of a FucoPol and 35% ChCl:Pyro membrane under 1000x magnification



j)SEM image of a FucoPol and 35% ChCl:GA membrane under 500x magnification



k)SEM image of a FucoPol and 35% ChCl:GA membrane under 500x magnification



l)SEM image of a FucoPol and 35% ChCl:GA membrane under 1000x magnification

Figure 3.14: Surface SEM images of different membranes at 500x, 1000x, and 2000x magnifications

3.7 Antibacterial Activity

The antibacterial properties are crucial for evaluating the potential applications of FucoPol-based membranes. To assess the antimicrobial activities of the developed membranes and their eutectic solvent components, qualitative antibacterial tests were performed against selected bacterial strains (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*). These tests aimed to determine the inhibition capacity of different chloride-based eutectic solvents and to evaluate whether the membranes could effectively release their active compounds into the surrounding medium. However, certain physical properties of the tested materials, such as viscosity and structural integrity, influence the diffusion and overall effectiveness of the antibacterial agents. Qualitative antibacterial tests (figure 3.15) demonstrated that ChCl:Py exhibited activity against all tested bacteria, with *E. coli* showing the highest inhibition. Similarly, ChCl:PE showed antibacterial activity against all three bacterial strains, despite being lower than that of ChCl:Py. However, ChCl:GA showed only significant inhibitory activity against *S.aureus*, which differs from the expected behavior when compared to similar compounds. This discrepancy may be attributed to one of the limitations of this method - ChCl:GA's high viscosity at room temperature, which most likely restricted its diffusion through the agar medium. In comparison, other eutectic solvents, which were considerably more liquid, diffused more easily and created larger inhibition zones. Although the antibacterial activity of ChCl:GA was closer to that of ChCL:Py, its physical properties may have hindered its performance in this test.

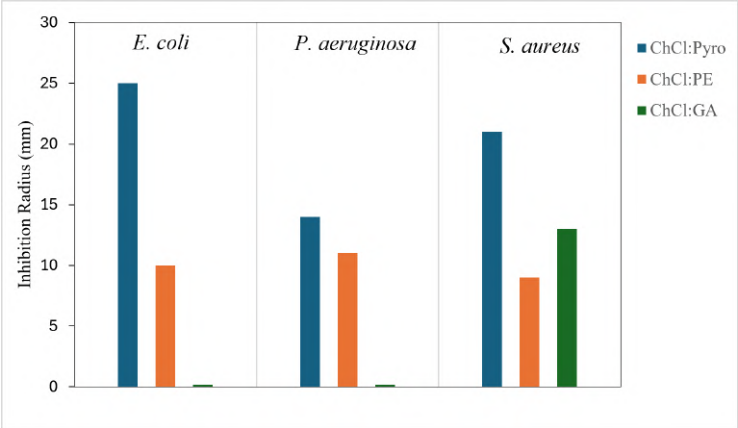


Figure 3.15: Antibacterial Activity of the different eutectic solvents

Additionally, the test made with the membranes themselves proved inconclusive. The membranes absorbed moisture from the agar medium, preventing the active components from diffusing effectively. No inhibition zones were observed around the membranes, and bacterial growth was suppressed directly beneath them. This suggests that, while the antibacterial agents remained active within the membranes, their release and diffusion into the surrounding medium were significantly limited.

Due to other disc diffusion tests being executed in different conditions, correlating

these results with other reported tests cannot be done with 100% exactitude, however it can be used as an approximate comparison to other eutectic solvents such as the ChCl:Urea (4:1), which presented slightly lower inhibition radius as the ChCl:Pyro for the *E.Coli* and *P.Aeruginosa*, and a higher radius for the S. Aureus, however, this last value has a very significant standard error[101]. When comparing our results to a reference standard such as Penicillin, both ChCl:Pyro and ChCl:PE seem to produce a higher inhibition radius of *E. Coli*, while with *S. Aureus*, only ChCl:Pyro has a higher radius, with the other two presenting similar inhibition radii (*E.Coli* - 7 mm, *S.Aureus* - 20 mm). [102]

3.8 Cytotoxicity

Eutectogels, formed by incorporating eutectic solvents into biopolymeric matrices, offer promising applications in biomedical and antimicrobial fields. However, their biocompatibility and safety must be evaluated thoroughly. In this study, we investigated the cytotoxicity of FucoPol-based eutectogels containing different eutectic solvents to determine whether FucoPol mitigates the potential toxicity of these compounds. The impact of each formulation and the potential of FucoPol as a protective agent in biomedical applications were assessed by evaluating cell viability at various concentrations.

The initial cytotoxicity test results revealed low cell viability in most membrane wells, except for those containing ChCl:PE, which showed a survivability rate of over 67% at concentrations ≤ 10 mg/mL. Interestingly, pure ChCl:PE also had increased cell viability at lower concentrations. However, the concentration at which this occurred was more than three times lower than those of the corresponding eutectogel membranes. This suggests that FucoPol may reduce the cytotoxic effects of ChCl:PE, which prompted further investigation. To infer whether this protective effect extended to other eutectic solvents, additional tests were performed.

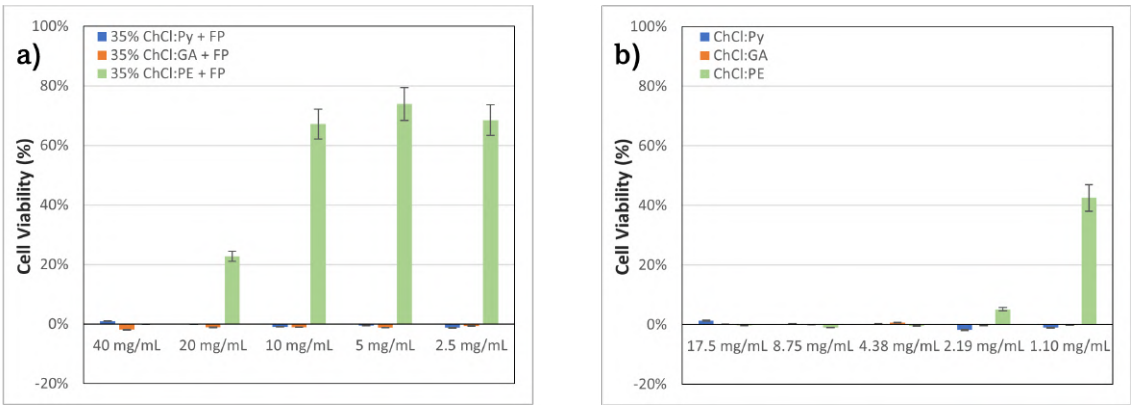


Figure 3.16: Cytotoxicity test 1 after 48h of incubation with the VERO cell line. a) Membranes containing 35% eutectic solvent; b) pure eutectic solvent.

The first cytotoxicity test indicated that the tested compounds exhibited higher toxicity than reported values for choline chloride (IC50 of 1.49 mg/mL [103]) and FucoPol (which

has no toxicity reported [104]). This implies that the primary source of cytotoxicity originated from the phenolic compounds present in the eutectic solvents. While there are no known cytotoxicity reports for 2-phenylethanol, pyrogallol and gallic acid have been reported to have an IC50 of 0.025± 0.004 mg/mL [105] and around 0.04 mg/mL [106, 107], respectively. The first test showed no clear dampening of cytotoxicity at these concentrations, which led to a second round of experiments using lower compound concentrations.

Additionally, inconsistencies in the first test results, such as unexpected stabilization of cellular viability at Ch:Cl:PE membrane concentrations below 10 mg/ml, suggested potential procedural errors. Specifically, excessive washing of the wells during the resazurin measurements step may have removed some cells, leading to lower observed viability. To prevent this, the same number of washes with the same intensity was executed in all wells in the other tests. Unfortunately, both the second and third membrane cytotoxicity tests produced unreliable results owing to procedural challenges, particularly when handling high-viscosity membrane solutions. In some cases, the incomplete removal of eutectogel residues may have interfered with the resazurin solution, causing artificial viability readings.

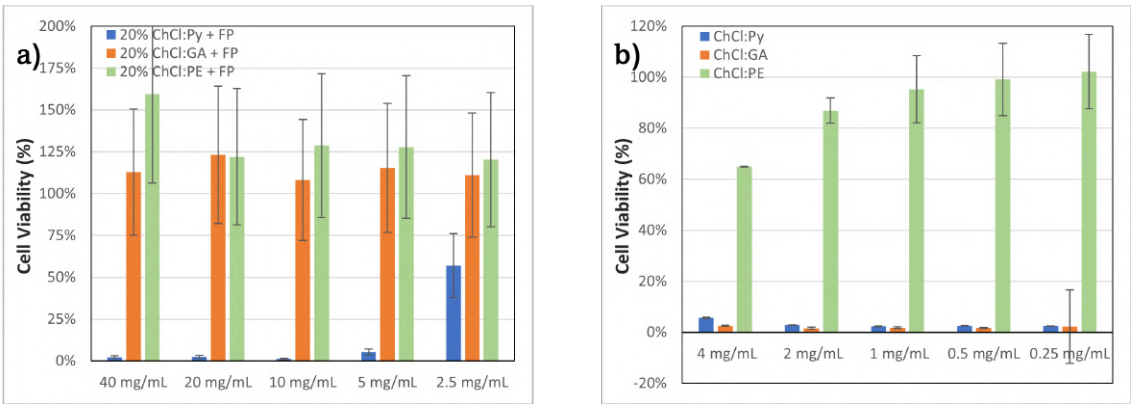


Figure 3.17: Cytotoxicity test 2 after 48h of incubation with the VERO cell line. a) Membranes containing 20% eutectic solvent; b) Pure eutectic solvent.

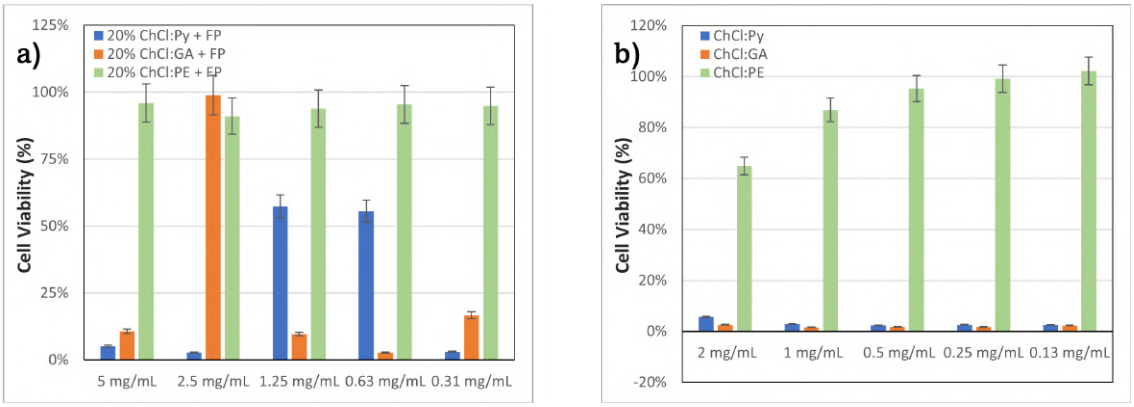


Figure 3.18: Cytotoxicity test 3 after 48h of incubation with the VERO cell line. a) Membranes containing 20% eutectic solvent; b) Pure eutectic solvent.

Nevertheless, the first and third results for the ChCl:PE based eutectogels seem to produce high viability levels below 10 mg/mL. However, because the remaining data from these tests are clearly inaccurate, definitive conclusions cannot be reached with 100% assurance.

The second and third tests, showed that the 2-Phenylethanol-based eutectic solvent exhibited reduced cytotoxicity at approximately 2 mg/mL; however the results for ChCl:GA were inconclusive. This was not observed in the third test. This could have occurred because of the heterogeneity of the eutectic solvent solution and inaccuracies in the dilution steps which caused the theoretical concentration to be inaccurate to reality. This is a difficult problem to solve given the small sample quantities and, and sterility before its utilization.

Overall, the results suggest that FucoPol may mitigate the cytotoxicity of ChCl:PE, as 10 mg/mL of the 35% Eutectogel (containing 3.5 mg/mL of ChCl:PE) showed significantly higher cellular viability compared to lower concentrations of pure ChCl:PE (Figure 3.16). Although similar effects cannot be detected for other eutectic mixtures, this finding highlights a previously unreported ability of FucoPol to reduce the cytotoxicity of 2-Phenylethanol.

For a better understanding on how these results compare to other bio-active compounds, the toxic concentrations are comparable to a viability assay of gentamicin (an aminoglycoside antibiotic) with the same cell line (VERO cells)[108], proving that Phenylethanol not only has bio-active properties comparable to standardized compounds (such as Antibacterial activity), but also approximately the same level of cytotoxicity of other regulated compounds.

3.9 Franz Diffusion Cells

This section explores the findings related to the physicochemical and biological properties of the developed membranes. By analyzing the thermal stability, antibacterial activity, cytotoxicity, and permeability, we aimed to assess their potential applications

and limitations. The following results provide insights into how the incorporation of eutectic solvents influences membrane performance and functionality. Considering the high molecular weight of FucoPol, the filter used to mimic the skin barrier is expected to prevent the passage through the receptor solution. As a result, only limited amounts of 2-phenylethanol and choline chloride would be detected, with a maximum of approximately 0.4 mg of choline chloride and 0.16 mg of 2-phenylethanol, quantities corresponding to their contents in the membrane. These quantities were dissolved in 10 mL of PBS, resulting in nontoxic concentrations.

During the Franz Diffusion cell procedure, more specifically after the fourth sample was taken, a situational error caused the loss of a significant volume of the receptor solution, which resulted in the necessity to add excess water to the cell, significantly diluting the concentration values, as showen in figure 3.19. Additionally, this was most likely the reason why the samples of the other replica did not appear in the HPLC data.

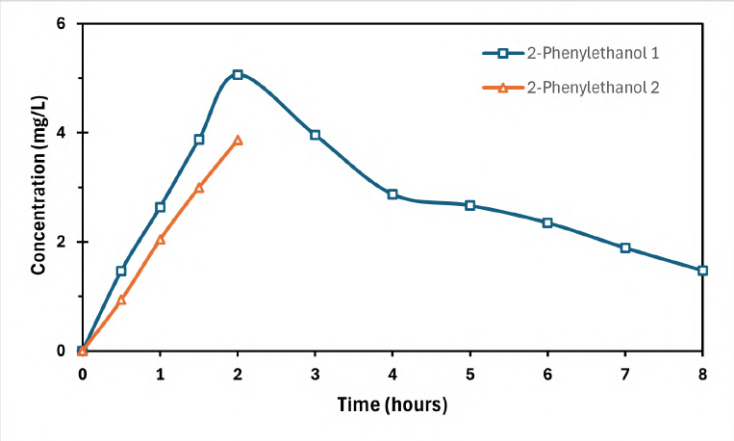


Figure 3.19: Franz Cell Diffusion Tests results: 2-Phenylethanol concentration in the receptor solution

Nevertheless, analyzing the obtained data is possible to infer a constant release of 2-Phenylethanol at a rate of 0.02-0.025 mg/hour during the first two hours, which at the maximum concentration of 4mg/L, is still 3 orders of magnitude lower than the concentration at which the cell viability in contact with the same compound, dropped to around 40% (Section 3.8)

These results demonstrate that, theoretically, the membranes composed of 65% FucoPol and 35% ChCl:PE, should be safe to use in contact with the epidermis and dermis, and on top of that, they can provide significant Antibacterial activity against at least three strains of bacteria, *E. Coli, P. Aeruginosa*, and *S.Aureus*.

4

Conclusion

4.1 Conclusions and future work

The eutectogel membranes based on the polysaccharide FucoPol and phenolic-based eutectic solvents, were successfully prepared. These eutectic mixtures were composed of choline chloride as a hydrogen bond acceptor (HBA), coupled with pyrogallol, gallic acid, and 2-Phenylethanol, each individually acting as a hydrogen bond donor (HBD), demonstrating distinct properties depending on the solvent used. The pyrogallol and gallic acid-based eutectogels formed softer and more viscous membranes with increasing solvent concentrations, while the 2-phenylethanol based membranes remained more rigid and brittle. ATR-FTIR analysis confirmed the integration of these eutectic solvents into the membranes, revealing the interactions between FucoPol and eutectic solvents.

TGA and DSC revealed that the incorporation of eutectic solvents enhanced the thermal stability of the FucoPol membranes, which remained stable in the range of -50 to 150°C, with minimal degradation observed. The 50% concentration of eutectic solvent membranes presented the highest degradation peak temperature and no significant negative impact on the thermal properties of the membranes was observed, even with the highest eutectic solvent concentration tested.

The eutectogel membranes retained a water content of around 11 wt%, lower than the 15% of pure FucoPol membrane, which with the increase in contact angles with the addition of Eutectic solvents to the pure FucoPol, indicates a noticeable decrease in water affinity when comparing the eutectogel membranes with the pure FucoPol one.

To further infer the stability of eutectogel membranes, long-term stability tests should be conducted to determine the optimal storage conditions and shelf life of the materials. In terms of the cytotoxicity of the eutectogels, it was hypothesized that the phenolic compounds present in the eutectic solvent were the primary cause of toxicity, which was confirmed by our results. However, FucoPol can seemingly reduce this toxicity, as seen in 2-Phenylethanol, presenting higher cell viability at the same concentrations of ChCl:PE inside the membrane than when used alone

Overall, this study highlighted the ability of FucoPol to produce various eutectogel

membranes, demonstrating their ability to incorporate different phenolic-based eutectic solvents and their impact on the membrane properties. The membranes were successfully prepared using an optimized method, green, efficient, and cost-effective. The resulting membranes showed great potential for antibacterial activity, but also significantly reduced the cytotoxicity of the phenolic compounds, suggesting that FucoPol could mitigate the inherent toxicity of certain eutectic solvents while preserving their desired biological effects.

For future work, to enhance the understanding of their bioactivity, it would be valuable to measure their anti-inflammatory and antioxidant properties, especially since both FucoPol and phenolic compounds have been reported to exhibit such activities [50, 53, 74]. These properties are highly relevant, and conducting these tests at concentrations as low as 20% eutectogel, or even lower, would help minimize the cytotoxicity of these materials.

If these eutectogel membranes proved to possess beneficial biological properties at non-toxic concentrations, the next step would be to assess their mechanical properties, as this would help to determine the ideal application for these membranes, such as wound dressing, or as a drug delivery system. However, their water absorbance was significantly high, which could cause them to dissolve in a viscous gel. To address this limitation, crosslinking the FucoPol can create a stronger network, enabling the membrane to hold water without loosing its structural integrity.

The effect of eutectic systems on the toxic properties of phenolic compounds must also be further investigated. This could be achieved by testing membranes, eutectic solvents, and each compound individually until their first viable concentration is determined, which requires either using a different technique to measure cytotoxicity or adapting this one to the minimal concentration and characteristics of the tested compounds

Nonetheless, these eutectogels proved to be highly customizable and flexible, enabling them to be tailored to different necessities and applications. Moreover, this study indicates that these eutectogels, with their customizable properties and low toxicity profiles, could play a significant role in advancing sustainable and green technologies. As research into these materials continues, their potential for integration into bioelectronics, pharmaceutical applications, and soft robotics has become more evident. Continued optimization and exploration of their characteristics will be crucial in realizing the full potential of FucoPol-based eutectogels, allowing them to contribute to a range of emerging technologies and applications in the future.

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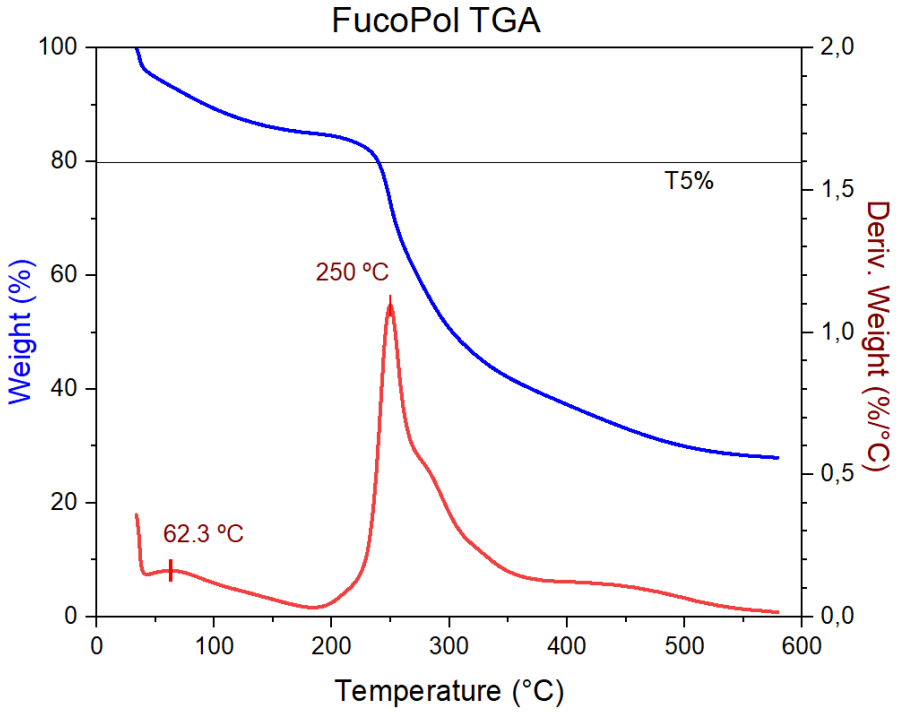

Figure A.1: TGA analysis of the pure FucoPol membrane with derivative

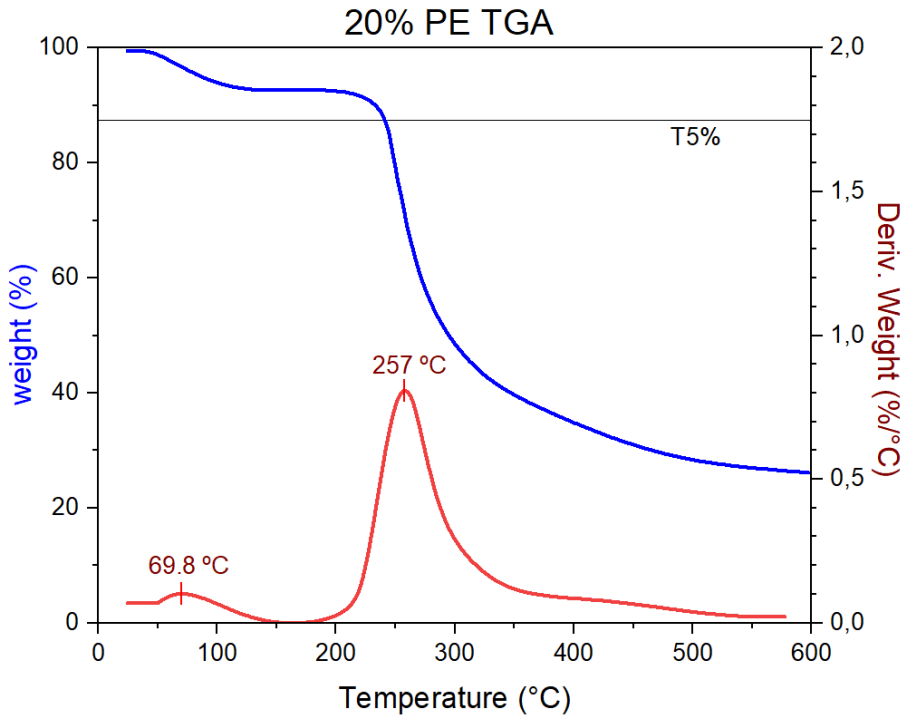

Figure A.2: TGA analysis of the 20% ChCl:PE eutectogel membrane with derivative

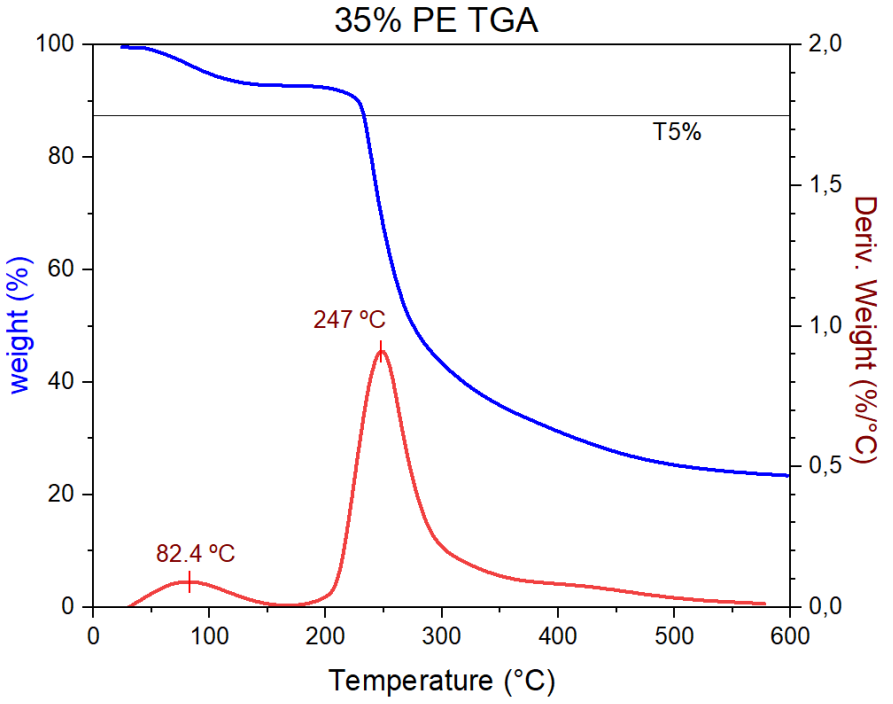

Figure A.3: TGA analysis of the 35% ChCl:PE eutectogel membrane with derivative

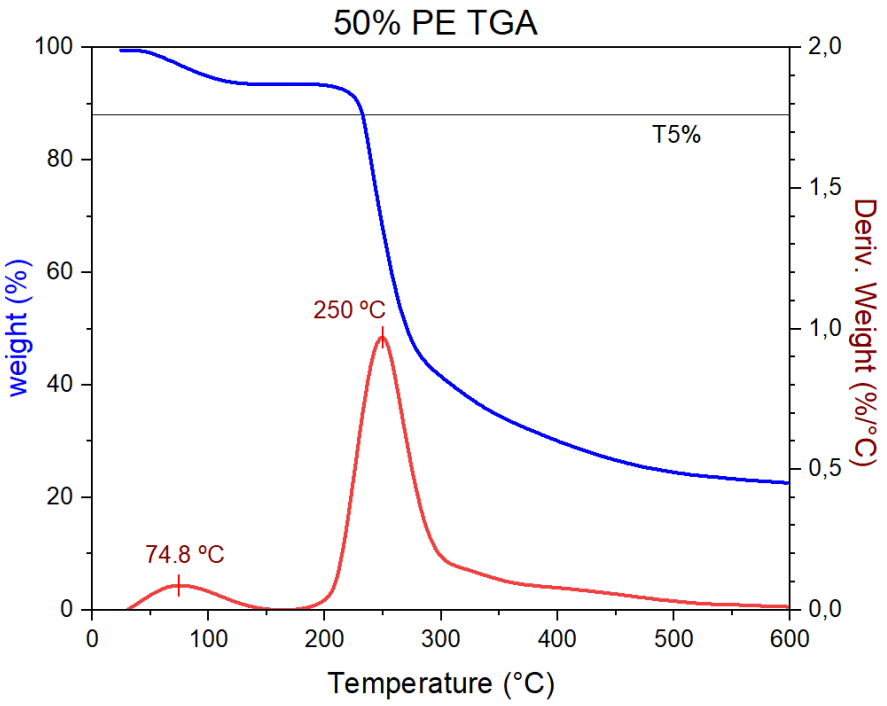

Figure A.4: TGA analysis of the 50% ChCl:PE eutectogel membrane with derivative

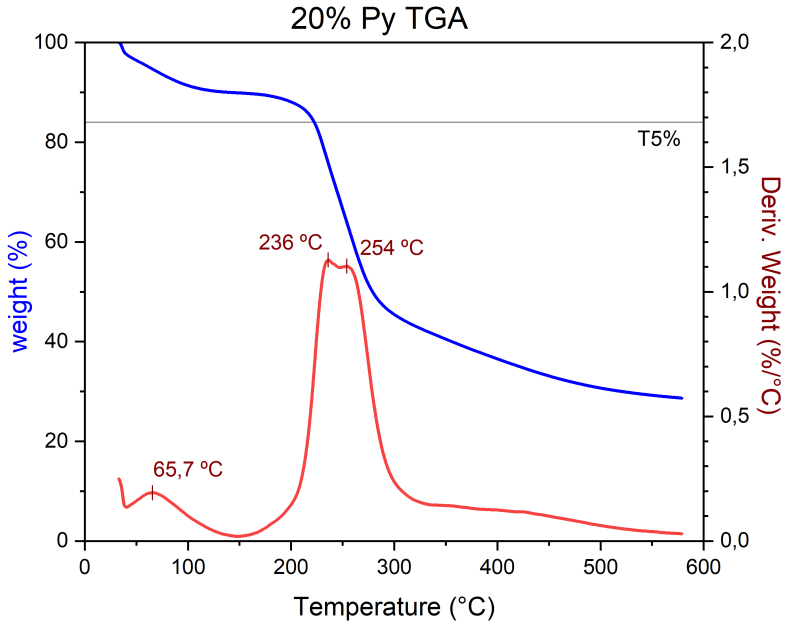

Figure A.5: TGA analysis of the 20% ChCl:Pyro eutectogel membrane with derivative

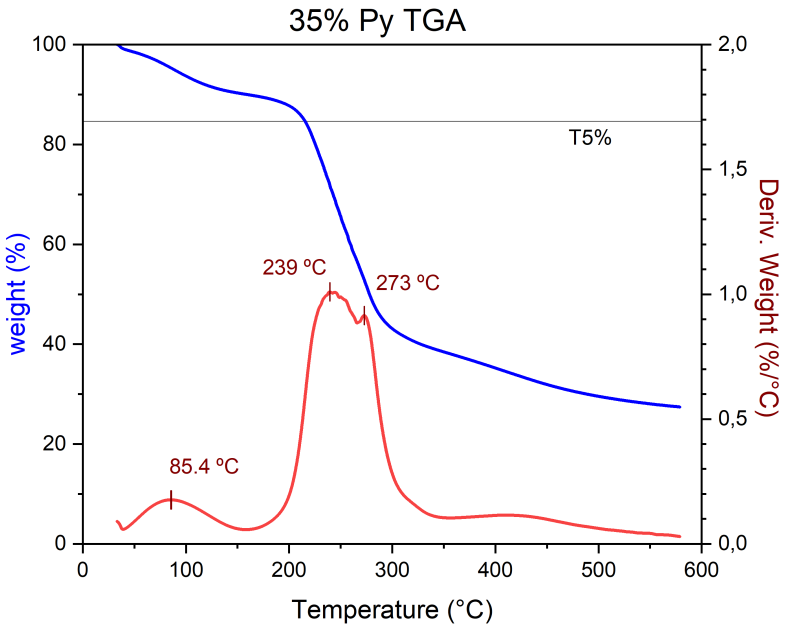

Figure A.6: TGA analysis of the 35% ChCl:Pyro eutectogel membrane with derivative

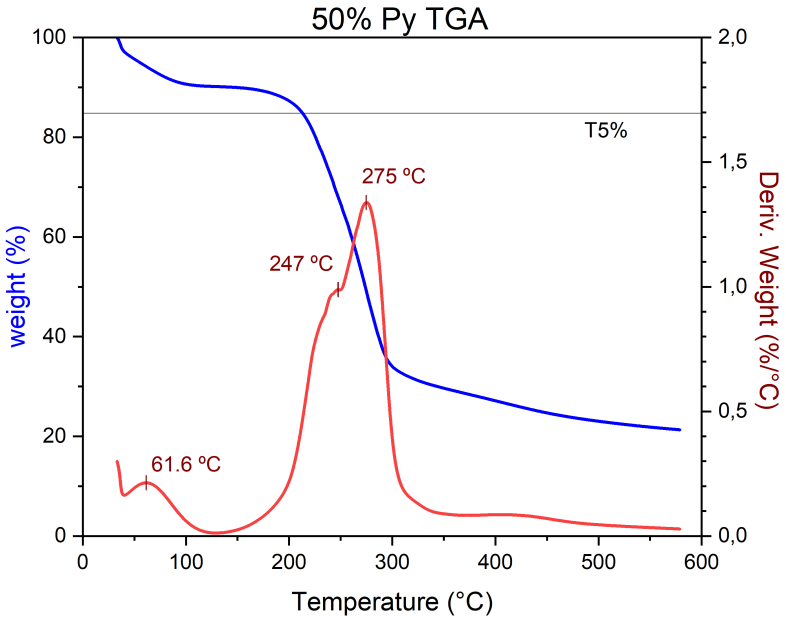

Figure A.7: TGA analysis of the 50% ChCl:Pyro eutectogel membrane with derivative

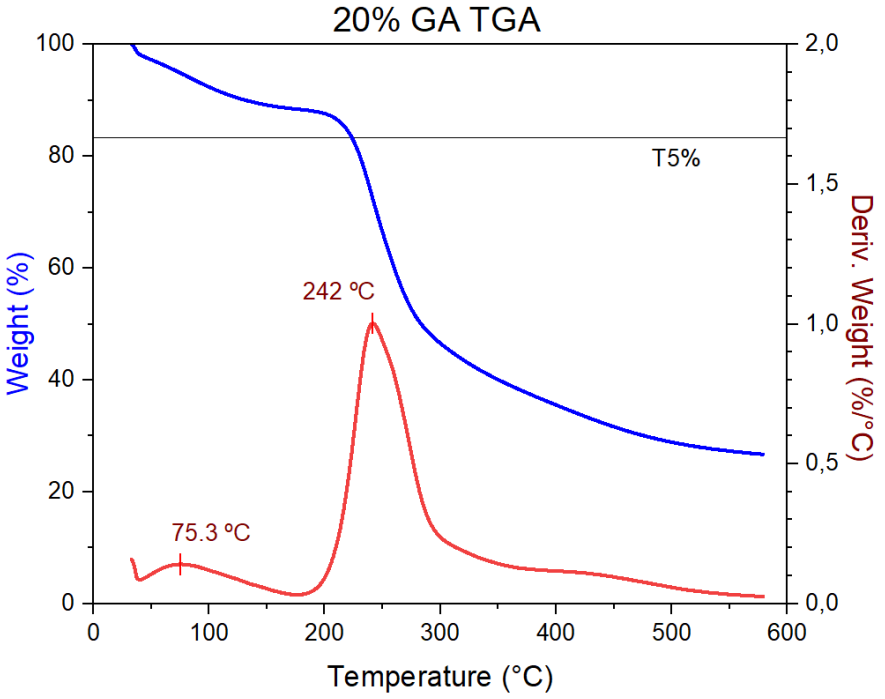

Figure A.8: TGA analysis of the 20% ChCl:GA eutectogel membrane with derivative

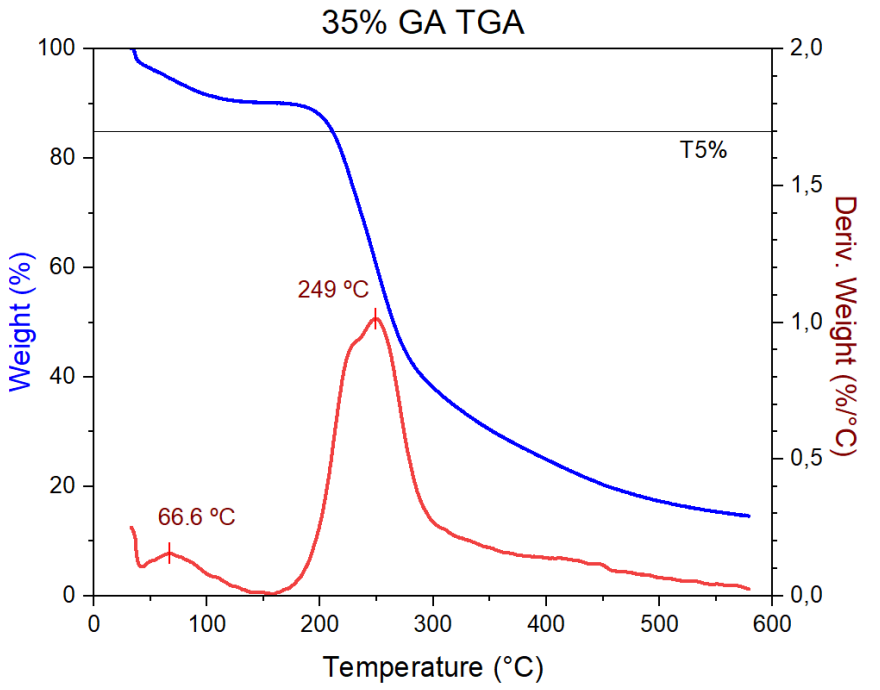

Figure A.9: TGA analysis of the 35% ChCl:GA eutectogel membrane with derivative

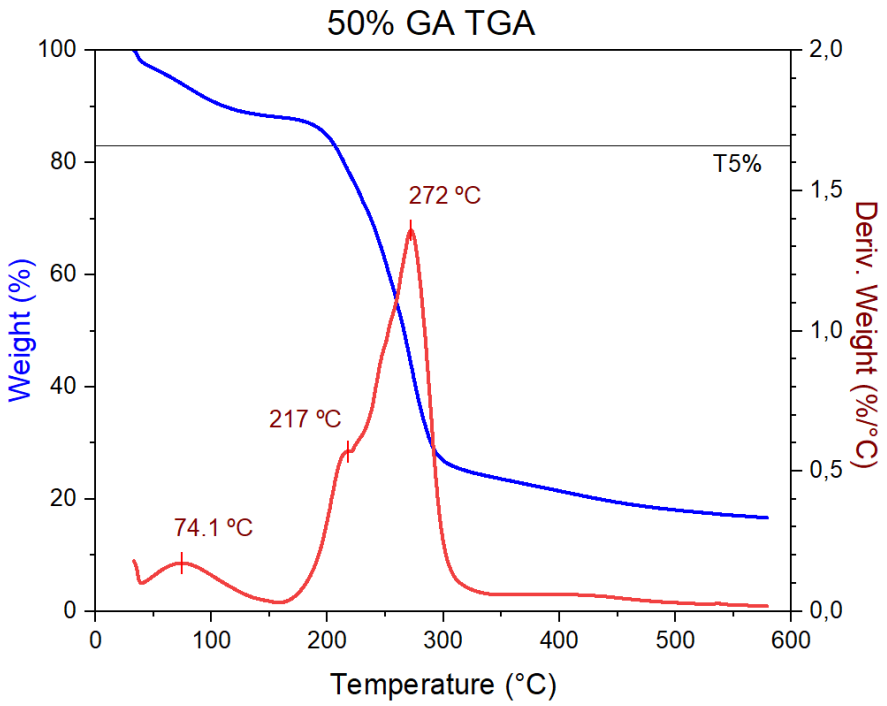

Figure A.10: TGA analysis of the 50% ChCl:GA eutectogel membrane with derivative