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CHEMISTRY

Green functional biomaterials for wound healing

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ACKNOWLEDGMENTS

Agradeço à orientadora Raquel Viveiros, por ter sido a minha grande ajuda ao longo deste último ano e por me ter apoiado em todas as minhas decisões. Por ter estado lá nos bons e nos maus momentos e por me ter ajudado a crescer como pessoa e como estudante. Obrigada pelas conversas e pela boa disposição e pela forma bonita de ver a vida que vou levar comigo para sempre.

Queria também agradecer a todos do gabinete e do laboratório 510 por me terem integrado no grupo e por me terem ajudado ao longo do ano! Obrigada, também à professora Luísa Maia pela sua colaboração com o EPR.

Queria agradecer à Fundação Ciência e Tecnologia (FCT/MCTES) pelo suporte financeiro para o desenvolvimento desta tese através do projeto PTDC/EQUEQU/32473/2017, e ao Laboratório de investigação associado para a Química verde – Processos e tecnologias verdes – LAQV– REQUIMTE financiada pelos fundos nacionais da FCT/MCTES (UIDB/50006/2020 e UIDP/50006/2020) e co-financiada pela FEDER em acordo de parceria PT2020 (POCI-01-0145-FEDER – 007265).

Agradeço ao orientador Marco Araújo pelo que me ensinou ao longo deste ano e por me ter ajudado na realização de todos os projetos no âmbito desta tese.

Queria também agradecer a todos do laboratório de Bioengineered 3D Microenvironments por me terem acolhido e por me terem ajudado a finalizar a minha tese. Obrigada, também à professora Cláudia Monteiro e à Inês Cruz pela colaboração com os testes antibacterianos e à professora Susana Santos e à Carlota Sequeira pela colaboração nos testes com os macrófagos.

Rebeca, obrigada por seres como és e por teres estado sempre ao meu lado durante os 2 meses que estive no Porto.

Aos meus pais que me permitiram ter esta experiência de estudar fora de casa e que sempre fizeram de tudo por mim, obrigada é pouco, mas as palavras também não são suficientes para exprimir a minha gratidão. Mana, és e sempre serás o amor da minha vida.

Marta, Francisco, Sérgio e Pedro vocês são as minhas pessoas favoritas e a minha vida é bonita quando estou com vocês. A realização desta tese só foi possível por vossa causa, obrigada por tudo.

Obrigada a todos os meus amigos de Lisboa e de Aveiro e também a todos os meus familiares.

ABSTRACT

Wound care is one of the major concerns to public health and the global market is expected to expand at a Compound Annual Growth Rate of 6.2 % until 2029. The lengthy healing time of wounds raises the danger of infections which significantly decrease quality of life of the patients. High levels of inflammation, degradation of extracellular matrix, and bacterial infection are just some of the most important features to consider in the design of a biomaterial for wound healing. Moreover, the use of biomaterials in chronic wounds can regulate the wound microenvironment and encourage tissue regeneration.

Since the last years, there has been a significant interest in using alternative solvents/technologies, such as supercritical fluids, in particular supercritical CO₂ (scCO₂) to produce and process (bio)materials in several formats. Natural-derived and synthetic polymers have been widely applied in wound healing (WH) to achieve multifunctionality that would contribute to the healing progress. Herein, green, ready-to-use affordable biomaterial nano-coatings were developed by two different technologies (plasma activation and steglich esterification) that were further polymerized in scCO₂ with acrylic-based monomers, chosen from their biocompatible and antibacterial properties. Further, thymol (THY), a naturally monoterpenoid phenol with antibacterial, antioxidant, anti-inflammatory and cicatrizing activity was impregnated in the biomaterial using a similar technology.

The obtained biomaterials were characterized for their physico-chemical, morphological and mechanical properties. THY release profile, antibacterial and biocompatibility tests were investigated, returning very promising results.

The solvent-based functionalized membranes presented a more organized morphology than the plasma functionalized membrane, which was in accordance with the release profiles, in which the THY release was more controlled. The functionalized membranes showed to be biocompatible and to have antimicrobial activity, showing potential application in WH.

Keywords: Cellulose membranes; Acrylic Acid; Supercritical carbon dioxide; Thymol impregnation; Antibacterial tests

RESUMO

O tratamento de feridas é uma das maiores preocupações na saúde pública sendo expectável que o seu mercado global cresça a uma taxa anual de 6,2 % até 2029. O longo período de cicatrização das feridas aumenta o risco de infeções levando a uma diminuição significativa da qualidade de vida da população. Os elevados níveis de inflamação, a degradação da matriz extracelular e o aumento do pH da ferida provocado pela atividade bacteriana excessiva são as características mais importantes a ter em conta na conceção do material. Assim, o uso de biomateriais em feridas crónicas pode contribuir para o microambiente da ferida e promover a regeneração dos tecidos.

Desde os últimos anos, tem havido um interesse significativo na utilização de solventes/tecnologias alternativas, como fluidos supercríticos, em particular o CO₂ supercrítico (scCO₂) para produzir e processar (bio)materiais em diversos formatos. Os polímeros de origem natural e sintéticos têm sido amplamente aplicados na cicatrização de feridas (CF), e como tal, foram desenvolvidos nano-revestimentos verdes em biomateriais, prontos para usar, e acessíveis utilizando duas estratégias de funcionalização (ativação por plasma e esterificação por steglich) e posterior polimerização a partir de monómeros à base de ácido acrílico, devido às suas propriedades biocompatíveis e antibacterianas, para CF. O timol (THY), é um fenol monoterpenoide natural, com ação antibacteriana, antioxidante, anti-inflamatória e cicatrizante, e foi impregnado no biomaterial desenvolvido usando scCO₂.

Os biomateriais obtidos foram caracterizados quanto às suas propriedades físico-químicas, morfológicas e mecânicas. Foram investigados o perfil de liberação do timol, bem como testes antibacterianos e de biocompatibilidade, com resultados muito promissores.

As membranas funcionalizadas com processo à base de solvente apresentaram uma morfologia mais organizada que a membrana funcionalizada com plasma, consistente com a libertação mais controlada do THY. As membranas funcionalizadas demonstraram ser biocompatíveis e com atividade antimicrobiana, adequadas para processos de CF.

Palavas chave: Membranas de celulose; Ácido acrílico; Dióxido de carbono supercrítico; Impregnação do Timol; Testes antibacterianos

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ACRONYMS

AA	Acrylic Acid
CO₂	Carbon dioxide
CAGR	Compound annual growth rate
DCC	<i>N,N'</i> -Dicyclohexylcarbodiimide
DCM	Dichloromethane
DMAEMA	2-(Dimethylamino)ethyl methacrylate
DMAP	4-Dimethylaminopyridine
DMEM	Dulbecco's Modified Eagle's Medium
DPPH	2,2-diphenyl-1-picryl-hydrazyl
ECM	Extracellular matrix
EPR	Electron Paramagnetic Resonance
FBS	Fetal bovine serum
FTIR	Fourier-transform infrared spectroscopy
HEMA	(Hydroxyethyl)methyl methacrylate
IDF	International Diabetic Federation
ITA	Itatonic Acid
LPS	Lipopolysaccharide
MAA	Methacrylic acid
MHB	Mueller-Hinton Broth
MMP	Matrix metalloproteinases
NiPAAm	Poly(<i>N</i> -isopropylacrylamide)
PAA	Poly(acrylic acid)
PBS	Phosphate Buffered Saline
PDEAAm	Poly(<i>N,N</i> -diethylacrylamide)

RLU	Relative Light Unit
ROS	Reactive Oxygen Species
RT	Room temperature
SEM	Scanning Electron Microscopy
ScCO₂	Supercritical carbon dioxide
TSA	Tryptic Soy Agar
THY	Thymol
UV-Vis	Ultra-violet Visible
WH	Wound Healing

“The best way to predict the future is to create it.”

INTRODUCTION

1. Wound healing

Skin is the biggest organ in the body and one of its main roles is to work as a barrier against bacteria invasion, although it also presents other crucial roles such immune surveillance, sensory detection, and self-healing (1). To repair the tissue integrity and homeostasis after an injury in the skin, several cellular pathways must be turned on and coordinated, to protect against dehydration, exogenous material, and bacteria. The wound healing (WH) process involves vasoconstriction and coagulation, inflammation, cellular proliferation and tissue remodeling (2).

The healing process consists of transforming the formerly functioning tissue into a patch of cells and an unorganized extracellular matrix (ECM) known as a scar (3). The hemostatic, inflammatory, proliferative and remodeling phases of WH is shown in **Fig. 1** (4). The hemostasis phase starts as soon as a wound is caused by the production of fibrin clots and vascular constriction. Then, inflammatory cells begin to migrate into the wound. The inflammatory stage of WH is started by the recruitment of inflammatory cells such neutrophils, macrophages, and lymphocytes. This phase, during which epithelial cells multiply and move across the region of the wound, coincides with the proliferative phase. Fibroblasts and endothelial cells are the most noticeable cell types during the proliferative phase, which supports collagen production, blood vessel expansion, and granulation tissue creation at the injury site. The healing process moves towards tissue remodeling, which occurs after proliferation and the production of extracellular matrix have been finished (5). When the healing process doesn't occur in the expected timeframe chronic wounds are formed. Those wounds continue to linger after trauma or spontaneous rupture and do not go through the regular stages of healing remaining stagnant in the inflammatory phase. The biological variables that significantly affect the development of a chronic wound include persistent inflammation, ischemia-mediated loss of oxygen and nutrients, as well as bacterial coloniza-

tion or infection. Reactive oxygen species (ROS), matrix metalloproteinases (MMP), and pro-inflammatory cytokines that are overproduced in a common chronic wound environment cause epithelial cell loss and ECM degradation. The ECM is proteolytically broken down, which not only stops the wound healing process from progressing into the regeneration phase but also draws in additional inflammatory cells, so accelerating the inflammation cycle (6).

Every organ in the human body is vulnerable to bacterial infection and every species prefers infecting some organs over others. Numerous bacterial species are present in chronic wounds, and this creates a synergistic effect that makes previously nonvirulent bacterial species virulent and harmful to the host. It has been shown that bacteria in chronic wounds produce biofilms that slow the healing process. Bacteria grow slowly in a mature biofilm due to nutritional shortage, which causes antibiotic resistance in the bacteria (7). Most of the natural bacterial flora of this ecosystem is made up of *S. epidermidis*, which colonizes the skin and mucous membranes of humans. The major virulence factor for *S. epidermidis* infections is thought to be biofilm development. The extracellular material around the multilayered cell clusters is made up of bacterial and host products with varying chemical compositions (8).

This kind of wounds have an impact on tissue perfusion, resulting in diabetic foot ulcers, vascular ulcers and metabolic illnesses like diabetes (9).

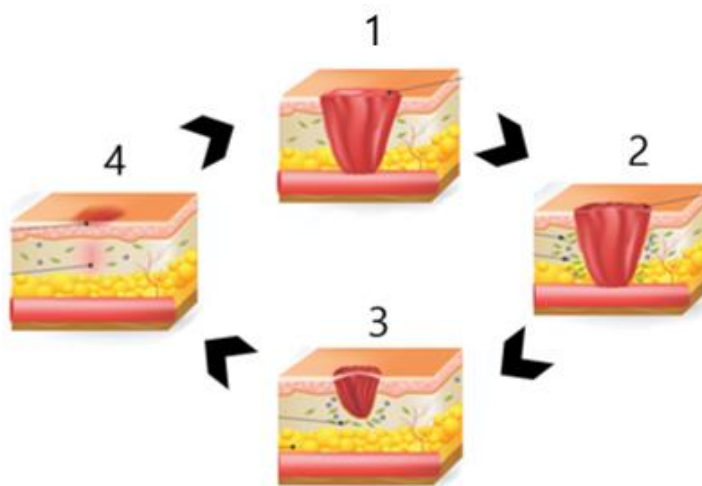


Figure 1 - The main phases of WH; 1-Hemostasis; 2-Inflammation; 3-Proliferation; and 4-Remodeling. Adapted from reference (4)

1.1 Wound care market

The prevalence of diabetic ulcers, chronic wounds, and an increase in surgical interventions worldwide are the main drivers of the global market for wound dressings. The International Diabetic Federation (IDF) estimates that by 2045 there will be 783 millions of people with diabetes, from which 40-60 million people worldwide suffering from diabetic foot ulcers.

The length of time needed for a wound to heal is increased when it develops problems such as prolonged inflammation, infection, and insufficient blood flow. The most prevalent chronic wounds are venous ulcers, including diabetic foot ulcers and pressure ulcers (10).

The market for wound dressings is expected to increase by a compound annual growth rate (CAGR) of 6.2 % until 2029, from 16.48 to 25.14 billion as shown in **Fig. 2** (11).



Figure 2 - Worldwide trend of wound dressings market (data from (11)).

It is crucial to properly care any wound, whether it is a small cut or a large incision. The use of a wound dressing is a key step in this procedure (12). The following conditions should be fulfilled by an ideal skin wound dressing:

- Sufficient physical and mechanical strength to ensure its integrity and prevent the intrusion of external bacteria caused by materials' breakage;
- Sufficient moisture retention to maintain the moist environment of the wound preventing dehydration (13).

There are several types of wound dressing, which were developed with the aim of treating different health problems. In **Table 1** are summarized some of the types that exist. Wound dressings having

antimicrobial properties are advantageous since chronic wounds have a propensity to generate infections and suffer from bacteria invasion. Antioxidant properties are important to neutralize ROS and prevent the epithelial cell loss and ECM degradation. Such attributes may be found on the coated-cellulose membranes to be developed during this thesis, which makes them a potential material over the existing therapeutic alternatives.

Table 1 - Wound dressing type and indications for their use (14).

<i>Product type</i>	<i>Indications for use</i>
<i>Semi-permeable films</i>	Dry, superficial wounds. Can be used as a secondary dressing. Cannot cope with exudates
<i>Semi-permeable film and pad</i>	Postoperative wounds
<i>Non-adherent contact</i>	Where there is a risk of adherence. Can be used as a transport layer for gels or ointments
<i>Hydrocolloid</i>	Low to moderately exuding wounds. They promote debridement of slough and necrosis. Can reduce pain through hydration of nerve endings
<i>Hydrofibre</i>	For moderate to heavy exuding wounds
<i>Foams</i>	Moderate to heavy exuding wounds. Can be used as a primary or secondary dressing. Can be used as a cavity filler
<i>Hydrogel</i>	Indicated for dry, necrotic, and thick sloughy wounds
<i>Antimicrobial</i>	Infected and critically colonized wounds. Can be used on wet and dry wounds
<i>Deodorizing</i>	Used to reduce or eliminate odor. Most dressings contain charcoal
<i>Larval therapy</i>	Used in the treatment of sloughy, infected wounds to debride devitalized tissue
<i>Topical negative pressure therapy</i>	Used to manage highly exuding wounds, complex wounds, and those where healing is compromised
<i>Protease-modulating matrix</i>	Indicated for chronic wounds to rebalance the wound environment

1.1.1 Materials for wound dressings

Biomaterials have been gaining attention as drug delivery devices, developed by loading of polymeric carriers with active pharmacological, biomedical and nutraceutical ingredients (15).

Due to their low cost, easy-to-handle and production, absorbent quality, air, and moisture permeability, non-allergenicity and biocompatibility, natural textile materials (cotton, silk, and linen) are an excellent alternative for treating both dry and wet wounds. However, these substances have the potential to dehydrate wounds and become mechanically anchored to the wound surface, making it difficult to remove the wound dressing. When they are used to treat wounds with high amount of exudates, they can also lead to skin maceration and bacterial development (16).

As it is difficult to have a single wound dressing to have all the qualities necessary to satisfy the general requirements for WH, some developed materials have gained attention in the last years, as presented in **Table 2**.

Table 2- Type of polymeric dressing and indications for their use (17).

<i>Type of polymeric dressing</i>	<i>Brand name</i>	<i>Use for</i>
<i>Foam</i>	Flexzan	Chronic wounds
<i>Hydrogels</i>	AlgiSite	Thickness burns
<i>Alginates</i>	Omiderm	Chronic ulcer
<i>Hydrocolloids</i>	Idosorb	Chronic ulcer
<i>Acrylate</i>	Mepore Film	Superficial wounds
<i>Acrylate</i>	Suprasorb F	Superficial wounds

Between the natural-derived polymers that could be applied in WH, cellulose is a naturally occurring glucose polymer, the most abundant in nature, being found as a component of the cotton and linen fibers or in plants, where they have a structural role. Its main component, α -cellulose, provides a lengthy and stiff molecular structure. The lengthy cellulose chain is made up of 1,4-glycosidic linkages connecting the β -1,4-D (+)-glucopyranose building units (**Fig.3**). Although cellulose from plants and bacteria have a similar chemical structure, their physical characteristics and macromolecular structure are different.

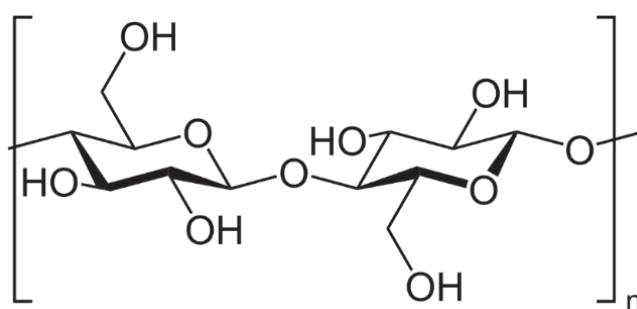


Figure 3 - Cellulose chemical structure.

Some cellulose-based textile fibers were functionalized to address these challenges, as it can be seen in **Table 3**.

Table 3- Conventional cellulose-modification for WH.

<i>Substrate</i>	<i>Monomer</i>	<i>Functionalization</i>	<i>Aim</i>	<i>Year</i>	<i>Ref</i>
<i>Cotton fabric</i>	AA	Grafting “from”	Drug delivery wound dressing	2014	(18)
<i>Cotton fabric</i>	NiPAAm	Grafting with Plasma	Wound dressing	2012	(19)
<i>Cotton fabric</i>	Acrylic/ Itaconic acid	Grafting “from”	Antimicrobial wound dressing	2013	(20)

Table 3 present the reported modifications of cellulose, where all of them include the activation of radicals on the membrane's surface and subsequent functionalization with various monomers.

Because they contain antibacterial properties that aid in the healing of wounds by limiting the destruction of the ECM, acrylates, namely acrylic acid, have the potential to be functionalized in cellulose.

1.2 Green Technologies

Green technologies are related to clean production and processing, the use of alternatives, and technologies that are less harmful to the environment. In this study 2 green technologies were used to produce membranes for WH: a) Plasma Activation, b) Supercritical carbon dioxide. Reported studies have been carried out in which cellulose was activated by plasma (21) and Steglich esterification (22).

1.2.1 Plasma Technology for material functionalization

The "*grafting from*" technique is one surface modification strategy that has been effectively used for membrane surface functionalization. In this methodology, the polymer chains grow at initiated sites on the membrane surface. By submerging the modified membranes in monomer solutions, the polymerization is then carried out from these initiator sites in standard "*grafting from*" way. In the end of the process, to remove completely any remaining traces of monomer that may have been retained in activated materials, several washes with organic solvents are conducted (23).

When cotton, is irradiated in plasma may lead to the creation of active sites on the fiber surface. Chemical reactions that are not generally displayed by non-modified material can be started by the newly generated active sites with substances that encounter the fiber. The benefits of plasma treatment are related with the fact that it enables the selective treatment of the material surface, and that the modification only affects the thin surface layer without affecting the bulk characteristics of the textile material (24).

In this study the cellulose membranes were functionalized by plasma technique with argon gas which according to the literature is the most used due to its low cost, and its inert which induce relatively low etching rates compared to oxidative and fluorinating plasma (25).

1.2.2 Steglich esterification for material functionalization

The polymerizable groups are attached to a surface in the "grafting-through" method. Then, a solution containing monomer and an initiator that serve as a crosslinker agent begins the polymerization process. In steglich esterification the AA monomer will be bound to the cellulose membrane, and the polymer will increase in scCO_2 from there.

In this study the cellulose membranes were functionalized with this technique to understand which method produces membranes with better characteristics for WH.

1.2.3 Supercritical carbon dioxide (scCO_2) technology

1.2.3.1 Properties and features of scCO_2

In the last few years, there has been an increasing interest in the use of supercritical fluids, due to their special and alternative features, in particular supercritical carbon dioxide (scCO_2).

Above its critical pressure of 73 bar and critical temperature of 31°C , CO_2 reaches a supercritical state (26). In **Fig. 4** the phase diagram of CO_2 is shown.

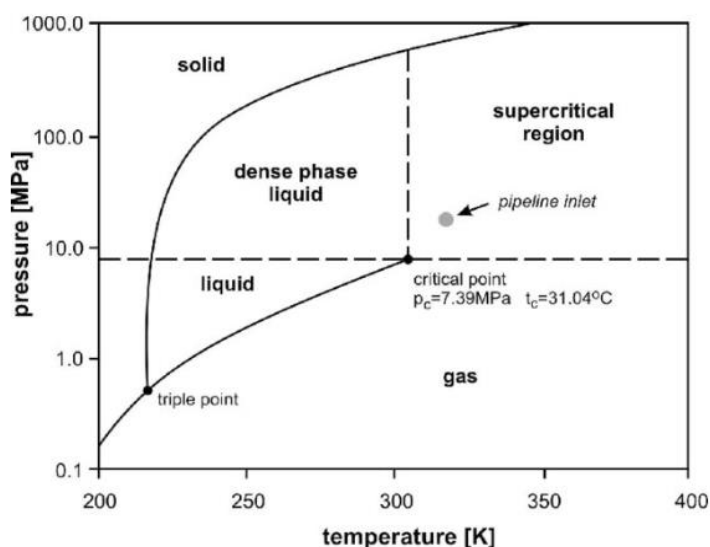


Figure 4 - Phase diagram of carbon dioxide (25).

ScCO₂ has a diffusivity comparable to a gas, and its density is like a liquid. It is a non-toxic, non-flammable, clean, affordable and inert solvent that in the supercritical state may be adjusted by slight changes in pressure and temperature (27).

Owing to these qualities sCO₂ is recognized as a green and alternative solvent/technology.

1.2.3.2 Synthesis and Processing under supercritical conditions

1.2.3.2.1 Polymeric materials in scCO₂

A green and sustainable strategy to produce polymers is the supercritical fluid technology. The polymers are synthesized in a ready-to-use state, without organic solvent residues, and with specific features that can meet a variety of applications. In **Table 4** is shown some polymers that have been synthesized by scCO₂.

Table 4 - Polymers synthesized using scCO₂ technology.

	<i>Polymers</i>	<i>Aim</i>	<i>Year</i>	<i>Ref</i>
<i>Polymerization using scCO₂ technology</i>	PDEAAm	Protein separation	2011	(28)
	DMAEMA-based polymers	Controlled drug delivery	2011	(29)
	MAA-based polymers	Removal of Bisphenol A from streams	2012	(30)
	ITA-based polymers	pH-triggered drug delivery	2018	(31)
	HEMA-based polymers	Pharmaceutical purification	2017	(32)
	PVA/chitosan-based polymers	Wound healing	2017	(33)
	Chitosan/ Cellulose/ Hyaluronic acid-based polymers	Wound dressing	2013	(34)

This technology can also be used for surface engineering, to coat polymers at surfaces for various applications as it shown in **Table 5**.

Table 5 - Polymer-coated surfaces using scCO₂ technology.

	<i>Aim</i>	<i>Core material</i>	<i>Year</i>	<i>Ref</i>
<i>Polymer coating on surfaces using scCO₂ technology</i>	Release of bioactive molecules	Chitosan–Collagen	2014	(35)
	API purification	Silica beads	2017	(36)
	Wound care	Polyvinyl Acetate/ Polypyrrole Scaffolds	2022	(37)

Based on these two tables, it was decided to combine cellulose polymer which is not only bio-compatible but also it is a filter that is already made at the factory, with an acrylate, more precisely AA, because of its antibacterial properties.

1.3.2.2 ScCO₂-assisted impregnation

There has been a great interest in developing new and green strategies for processing (bio)materials in a variety of forms by alternative solvents and technologies, including scCO₂. The **Table 5** shows some drugs that has already been impregnated using scCO₂.

Table 6 - Impregnation of drugs using scCO₂.

	<i>Drug</i>	<i>Year</i>	<i>Ref</i>
<i>Impregnation of drugs using scCO₂</i>	Metronidazole	2014	(38)
	Flufenamic acid	2011	(39)
	Salicylic acid and acetylsalicylic acid	2006	(40)
	Thymol	2019	(41)

These impregnations were done to produce drug delivery systems but none of them were directly related with wound dressings.

1.3. Thymol (THY)

The physiologically active compounds found in medicinal plants have therapeutic benefits. Thymol (THY) is one of the significant components of thyme oil, having a phenolic structure (**Fig. 5**). The THY (2-isopropyl-5-methyl phenol) has been studied and reported due to its biological activity, including antioxidant, antibacterial, antifungal and antiparasitic activity. The great capacity of scCO_2 to diffuse through the (bio)materials can be advantageous to impregnate matrices with a wide range of bioactive molecules, such as natural antibacterial agents (42).



Figure 5 - Chemical structure of thymol.

1.4 Motivation and objective

Biopolymers like poly (acrylic acid) (PAA) can offer a very promising material for creating a protective and biologically friendly dressing.

The aim of this work consists of the development of green antibacterial coatings on cellulose membranes with PAA for WH, using two different technologies, namely plasma functionalization/Steglich esterification, followed by scCO_2 .

MATERIALS AND METHODS

2.1 Materials

Acrylic Acid (AA, 99 %) was purchased from Aldrich, Thymol (THY, ≥ 98.5 %) was purchased from Sigma-Aldrich, 2,2'-Azobis(2,4-dimethylvaleronitrile) (V-65, 98 %) was purchased from Wako Pure Chemical Industries and cellulose membranes with a pore size of 0.45 μm were provided by Sartorius Stedim Biotech. Ethanol (≥ 99.8 %) was purchased from Honeywell, Dichloromethane (DCC), *N,N'*-Dicyclohexylcarbodiimide (DCC, 99 %) and Phosphate Buffered saline (PBS) purchased from Sigma-Aldrich, 4-Dimethylaminopyridine (DMAP), was provided from MERCK-schuchardt. Argon gas was obtained from Alphagaz.

Carbon dioxide used for nano-coatings and impregnation in supercritical conditions was obtained from Air Liquide ($\geq 99.998\%$). All the compounds were used as received.

For the cytotoxic tests, L929 fibroblasts were kindly provided by Dr. Ângela Carvalho from the Biocomposites research group (i3S) and Dulbecco's Modified Eagle's Medium low glucose with 10 % v/v FBS, 1 % v/v Penicillin/Streptomycin (P/S) and 1 % v/v Amphotericin B (DMEM-LG-S) purchased by Gibco. Cytotoxic tests were performed using FBS and trypsin from gibco and DMSO and resazurin.

Antibacterial tests were performed using a *Staphylococcus epidermidis* (ATCC 35984). Tryptic Soy Agar (TSA) plate and Mueller-Hinton Broth (MHB) from Merck were used.

Radical scavenging ability was investigated using Reagent A (H_2O_2 in Tris buffer, Acridan Lumigen PS-3 kit, Lumigen), Reagent B (acridan solution in dioxane and ethanol, Acridan Lumigen PS-3 kit, Lumigen), constituting the ALPS-3 substrate. Kit (StemCell Technologies SARL, Grenoble, France), Histopaque-1077 (Sigma-Aldrich), fetal bovine serum (FBS, Biowest), culture medium (CM-RPMI 1640 + Glutamax, Invitrogen, Paisley, UK), LPS (Sigma-Aldrich Co., St Louis, MO, USA), BS-EDTA and RPMI were used for in-vitro anti-inflammatory assays.

2.2 Experimental Procedures

2.2.1 Cellulose functionalization

2.2.2.1 Plasma functionalization

In this study cellulose membranes were placed inside the plasma equipment presented in **Fig. 6**. and activated for 30 times using argon gas (gas flow rate was 10 scm, voltage was 230 V and power was 16 A) for further polymeric coating in scCO₂.

Several time periods for activation (10, 20, 30 and 60) were performed, to understand their impact in cellulose surface functionalization. The Electron Paramagnetic Resonance (EPR) spectroscopy technique was used to follow the process. The **Fig. 7** represents plasma functionalization.



Figure 6 -Functionalization of cellulose membranes with plasma (argon gas).

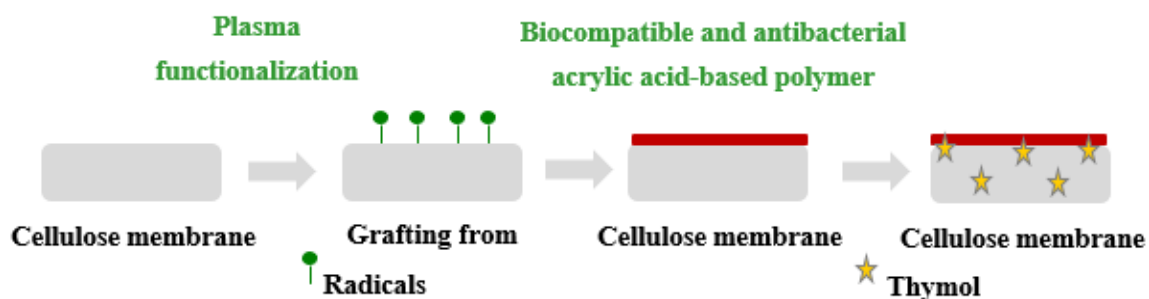


Figure 7 - Scheme of functionalization of cellulose membranes with plasma (argon gas).

2.2.2.2 Steglich esterification

A cellulose membrane (0.040 g, 2.7×10^{-3} mol, 2.5 cm diameter, Sartorius Biolab Products) was immersed into a round flask containing 100 mL of DCM, DCC (3g, 0.015 mol), DMAP (1.77 g, 0.015 mol) and 1 mL of AA. The physical appearance of the reaction mixture is shown in **Fig. 8**. The mixture was kept under stirring, at room temperature, in an orbital shaker equipment (IKA KS 4000 I) at 130 rpm for 48 h. At the end of the reaction, the samples were thoroughly washed with 30 mL of DCM and then 30 mL of ethanol on a glass for 15 min at 130 rpm. Finally, the sample was kept drying overnight under ventilation in the hood. In the following day, the functionalized membranes were collected dried.

The schematic in **Fig. 9** represents Steglich esterification.



Figure 8 - Steglich esterification: a) Beginning of the reaction and b) After 48 h.

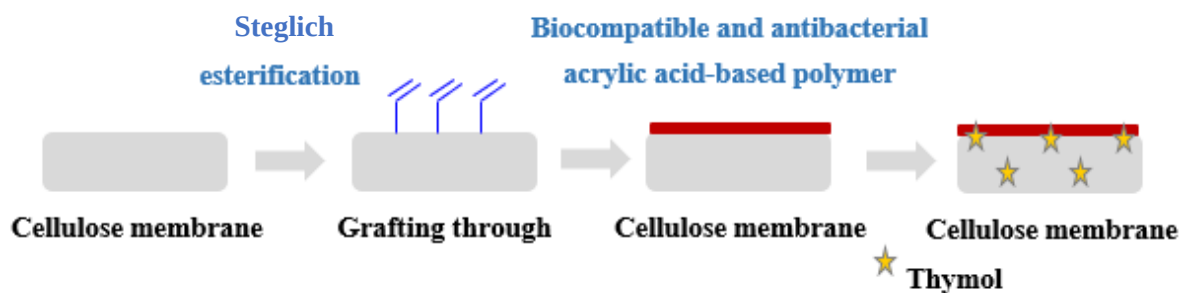


Figure 9 - Scheme of functionalization of cellulose membranes with Steglich esterification.

2.2.3 Fabrication of antibacterial cellulose under scCO₂

2.2.3.1 Development of poly(acrylic acid) coatings on cellulose membranes

The polymeric coatings were carried out in a stainless-steel high-pressure cell (33 mL) with a magnetic stirring bar submerged in a thermostatic water bath, at 65 °C. It was used a metallic net to separate physically the membranes from the reactants.

Pre-functionalized cellulose membranes (from Plasma technology or Steglich esterification) were added to the reactor with the initiator, 2,2'-Azobis (2,4-dimethylvaleronitrile) and 2 wt % AA (0.02 g, 2.7×10^{-4} mol) was added. Carbon dioxide was introduced in the high-pressure cell until reach the desired pressure, 20 MPa. In **Fig. 10**, the experimental apparatus is shown. At the end of the reaction (24h), the membranes were slowly washed with fresh high-pressure CO₂ for 1 h to remove any unreacted residues. The antibacterial polymeric coated cellulose membranes were then washed with 30 mL of ethanol for 2 hours at 130 rpm to remove the polymer that was physically bound to the membrane and kept drying overnight under ventilation.

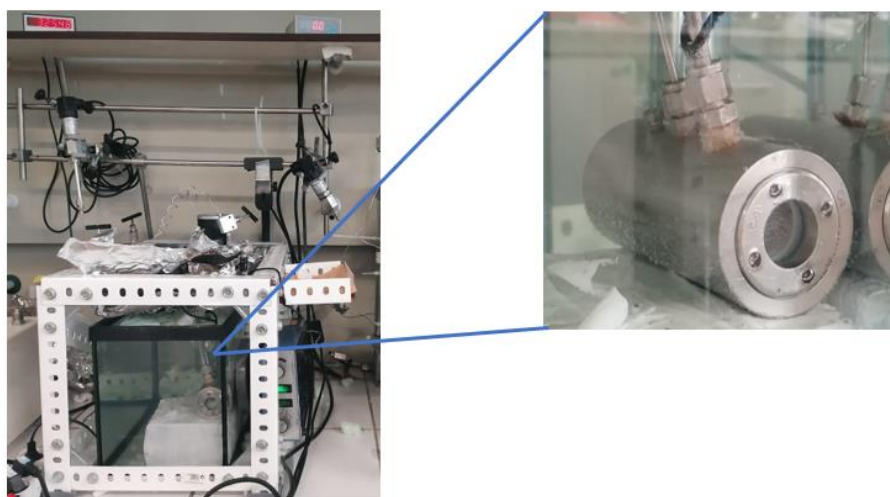


Figure 10 - Experimental apparatus of the high-pressure reactor used for the production of the polymeric coatings on cellulose.

2.2.3.2 Impregnation of THY on PAA coated-cellulose membranes

In a high-pressure cell (33 mL) with a magnetic stirring bar submerged in a thermostatic water bath, the impregnation of THY (0.5 g) in the PAA-coated cellulose membrane was carried out at 40 °C and 15 MPa for 24 h. It was used a metallic net to separate physically the PAA-coated membranes from the THY. At the end of the reaction, the valve of the reactor was opened very fast. The materials were collected and weighted.

2.3 Instrumentation /Membrane characterization

2.3.1 Plasma Technology

Plasma technology (Electronic diener, model FEMTO, using argon gas) was used for the green activation of the cellulose, producing the radicals at the cellulose membranes.

2.3.2 Electron Paramagnetic Resonance (EPR) spectroscopy

Electron paramagnetic resonance spectroscopy (EPR) was used to evaluate the presence and quantify the radical species produced by the plasma functionalization in the cellulose membranes. Then, after functionalization, each membrane (diameter 2.5 cm) was rolled and transferred to a 10 mm glass EPR tube; one membrane, not functionalized, was used as control experiment (zero time of functionalization). X-band EPR spectra were acquired at room temperature, in a Bruker EMX 6/1 spectrometer with an ER 4102ST cavity (Bruker), using a modulation frequency of 100 kHz, modulation amplitude of 0.3 mT and microwave power of 10 mW. Different modulation amplitude and microwave power values were tested to guarantee that the signals were not distorted/saturated, and the hypothetical fine structure is observable.

The intensity of each spectrum, that is a direct measure of the number of radical species present, was determined as the area of the integrated spectrum (spectrum double integration). To have a rough estimate of the number of radical species, solid 2,2,6,6-tetra-methyl-piperidin-nitroxide (TEMPO) was used as a standard (0.6 mg/cm in capillary tube, 3.85×10^{21} spin/g).

2.3.3 Contact Angle measurement

The measurement of the contact angle was carried out on coated and uncoated cellulose membranes (small samples), using a Goniometer OCA 15 plus, with an input power of 55 W and a line voltage of 12 V DC, from data physics (I3S). The method used to calculate the contact angle was the LaPlace-young fitting, and was used a Normal SD drop type of water with a volume of 4000 $\mu\text{L/s}$. The samples were glued with glue to a glass blade promoting their stability during the assay.

2.3.4 Fourier-Transform Infrared (FTIR) spectroscopy

Fourier-transform infrared (FTIR) spectroscopy was performed on developed materials, using a PerkinElmer Two spectrometer with 4 scans per second and a resolution of 1 cm^{-1} , from 4000 to 400 cm^{-1} . The samples were analyzed in the form of membranes measuring 2.5 cm in diameter and placed directly in the ATR-FTIR equipment.

2.3.5 Scanning Electron Microscopy (SEM)

The morphology of the membranes (cut into small sizes) was characterized through scanning electron microscopy (SEM) using a Hitachi S-2400 instrument, with an accelerating voltage set to 15 kV. Samples were mounted on aluminum stubs using carbon tape to stabilize the membranes. The magnifications used were x 400, x 1000 and x 1200.

2.3.6 Optical Microscopy

Microscope Images were characterized using Wireless EP50 camera-stereomicroscope Olympus CI1778 model SZX2-ILLT from Olympus corporation with a magnification of 0.63x.

2.3.7 Mechanical properties

The tensile properties of the cellulose membranes were tested with traction machine from manufacturer RheometricsScientific, Minimat Firmware 3.1 model, equipped with a 20 N load cell at room temperature. The length between the clamps was set at 10 mm and the speed of testing set of 5 mm/min. The Measurements were performed in all membranes. The Young Modulus was calculated from the slope of the linear portion of the of the stress strain curve.

2.3.8 Swelling and Degradation Tests

Swelling tests (equation *a*) were carried out placing a quarter of a entire cellulose membrane in 30 mL of PBS, for 1, 3 and 7 days at 37 °C under stirring (130 rpm). At the desired timepoints, the membranes were cleaned 5 times on each side with the aid of a paper and their weight was acquired. The membranes were washed with 30 mL of Milli-Q water at 100 rpm and room temperature, dried in the oven (50 °C) for one hour and their weight recorded again (equation *b*). All measurements were made using at least 3 replicates.

$$Swelling = \frac{W_{Wet} - W_{dry}}{W_{dry}} \times 100 \quad (a)$$

$$Degradation = W_{washed\ membrane} - W_{inicial\ membrane} \quad (b)$$

2.3.9 THY release

Triplicated samples of THY-coated and uncoated membranes were placed in 10 mL of PBS each, for a period between 2-145 minutes at 37 °C at 100 rpm. Samples (1 mL) were collected along the time and 1 mL of PBS was restored so that the volume remained the same. Then THY quantification was carried out in Perkin Elmer Lambda 25 UV/VIS Spectrometer at $\lambda = 274$ nm and at room temperature. The amount of THY released was quantified (*c*) from the calibration curve..

$$Thymol\ release = \frac{W_{thymol}}{W_{cellulose\ membrane}} \quad (c)$$

2.3.10 Water vapor permeability

The water vapor permeability evaluation was measured from three replicates of the coated cellulose membranes. PBS solution was placed in glass bottles of the same size and type, and coated cellulose membranes were glued to their apertures.

The thickness of the membranes was measured in three different parts using a micrometer. To measure the amount of water evaporated, each set (bottles + PBS) was weighted and stored at 37 °C for 24 hours. Then, the weight of each set was recorded again.

$$Water\ vapor\ permeability = \frac{W_{Set\ after\ 24h} - W_{Inicial\ set}}{Membrane\ area} \quad (d)$$

2.3.11 Radical-scavenging ability

The free-radical scavenging ability of the functionalized cellulose membranes was evaluated through the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) antioxidant assay. The membrane (4 mm in diameter) was placed in a 48-well plate and soaked in a 0.1 M solution (750 μ L) in ethanol (96 %) at 70 rpm and room temperature. The samples were protected from the light and 65 μ L of the incubated medium were taken at room temperature at different timepoints (0, 2.5, 5, 10, 20, 30, 40, 60, 120, 1440 minutes) and analyzed through UV-Vis (Lambda 35, PerkinElmer, USA) to measure the absorbance at $\lambda = 517$ nm. The amount of scavenged DPPH was retrieved from the calibration curve, obtained from different DPPH solutions (0.2, 0.125, 0.1, 0.008, 0.05, 0.02, 0.01, 0.002 mM). Three replicates were used to determine the DPPH scavenging activity.

2.3.12 *In vitro* tests

2.3.12.1 Cell Culture

The fibroblasts L929 were put in a culture flask in contact with culture medium - Dulbecco's Modified Eagle's Medium (DMEM) low glucose supplement with 10 % FBS and 1 % of pen strep, to let them grow. L929 grew until they reached 80% confluency and were subsequently used to carry out the assay.

2.3.12.2 Metabolic activity

Metabolic activity tests were performed using triplicated samples of coated-cellulose membranes and native membranes. An indirect contact test was performed according to ISO10993-5. Briefly, the membranes with 4 mm of diameter were sterilized with the UV lamp from the laminar flow chamber and placed into 96-well plates. They were maintained in contact with the medium (450mL) for 24 h inside the incubator (37 °C, 5% CO₂).

After the necessary growth of fibroblasts, the medium was discarded, leaving them clinging to the bottom and 4 mL of trypsin was added to remove them. After being incubated for 5 min at 37 °C, 10 mL of DMEM-LG-S was added to neutralize the cells.

The 14 mL (V_f) cell suspension were collected and 20 μ L of it was put in a Neubauer chamber to count the number of cells using the equation (d) below:

$$Number\ of\ cells = \frac{Number\ on\ the\ Neubauer\ chamber}{4} \times V_f \times 10^4 \quad (e)$$

The cell suspension was centrifuged for 3 minutes at 200 *g* and DMEM-LG-S was subsequently added until achieving a concentration of 1×10^5 cells/mL. Then, 100 μ L of the cell suspension (1×10^4 cells) was seeded in each well of a 96-well plate and incubated for 24 h at 37 °C.

The following day, dilutions of the medium that was in contact with the coated-cellulose membranes (100 %, 75 %, 50 % and 25 %) were performed, and these (100 μ L) were subsequently added to the cells in the 96-well plate, from where the medium had been previously aspirated, and maintained in culture conditions for more 24 h (37 °C, 5 % CO₂). For each concentration, 4 wells were used. DMEM-LG-S and DMSO were used as a negative/positive control for cell death, respectively.

In the last day, resazurin test was performed to assess the metabolic activity of the L929 cells.

The medium that was in contact with the cells was removed and a solution containing 20 % resazurine + 80 % DMEM-LG-S (150 μ L), were added to be in contact with them for 2 h in the incubator (37 °C, 5 % CO₂). Then the solutions were transferred to a black cell plate and analyzed at the plate-reader (530/590 nm) (Synergy Mx, BioTek). The metabolic activity of cells was subsequently calculated using the equation (e) below:

$$\text{Metabolic Activity (\%)} = \frac{\text{Indirect contact samples}}{\text{Negative control samples}} \times 100 \quad (f)$$

2.3.12.3 Anti-inflammatory activity

Primary human monocyte isolation and macrophage differentiation

Buffy coats were centrifuged for 20 minutes at room temperature, with an acceleration of 5, no brake, and 1200 rcf (xg). The Peripheral Blood Mononuclear Cell (PBMC) layer was obtained, and it was then incubated with RosetteSep human monocyte enrichment isolation kit (StemCell Technologies SARL, Grenoble, France), 66.7 μ L per 1 mL of PBMC, for 20 min on a roller shaker. The mixture was diluted and layered over an equivalent amount of Histopaque-1077 (Sigma-Aldrich) in sterile PBS containing 2 % fetal bovine serum (FBS, Biowest). The mixture was then centrifuged at 1200 rcf (xg) for 20 min at room temperature with acceleration 2 and without brake, using an Eppendorf Centrifuge 5810R from VWR International LLC. The monocyte layer was gently removed and cleaned three times with PBS.

The cell pellet was then resuspended in full culture medium (CM-RPMI 1640 + Glutamax, Invitrogen, Paisley, UK), which was also supplemented with 1 % penicillin G-streptomycin, and 10 %

FBS. A Neubauer chamber was used to count the cells, which were plated on a tissue culture plate and incubated at 37 °C/5 % at a density of 1×10^6 cells/mL per well.

Indirect Contact:

After 10 days, macrophages were stimulated with 10 ng/mL LPS (Sigma-Aldrich Co., St Louis, MO, USA). Supernatants from unstimulated and LPS stimulated macrophages were collected after 1 h under sterile conditions, centrifuged at 400 xg for 5 min at 4 °C, and stored at -20 °C.

Direct Contact:

After 10 days, the cells were washed with PBS and BS-EDTA (5mM) was added for 10 min in the incubator. After 10 min, the cell scraper helped to detach the remaining cells. The cells were counted and centrifuged at 1200 rpm for 5min at RT. After that they were resuspended in RPMI and plated for 2 h incubation with cellulose membranes (4 mm) and 1×10^6 cells/mL per well. After the incubation period, macrophages were stimulated with 10 ng/mL LPS (Sigma-Aldrich Co., St Louis, MO, USA). Supernatants from unstimulated and LPS stimulated macrophages were collected after 1 h under sterile conditions, centrifuged at 400 xg for 5 mins at 4 °C and stored at -20 °C.

Reactive oxygen species (ROS) measurement:

Reagent A (H₂O₂ in Tris buffer, Acridan Lumigen PS-3 kit, Lumigen) was mixed with reagent B (acridan solution in dioxane and ethanol, Acridan Lumigen PS-3 kit, Lumigen) in a 40:1 volume ratio (ALPS-3 substrate). Then, 10 µL of conditioned media (CM) from macrophages preconditioned media was placed in a 96 well plate (43). The final volume of each sample was adjusted to 50 µL with PBS, followed by the addition of 25 µL of ALPS-3 substrate. The plate was incubated in the dark for 5 min at room temperature RT and the spectra were recorded using a BioTek synergy 2 plate reader in luminescence mode ($\lambda = 450\text{--}455$ nm) using the Gen5 software.

2.3.11.4 Antibacterial Tests

Antibacterial tests were carried out using *S. epidermidis* and three replicates of coated-cellulose membranes (4 mm diameter). Firstly, 10 µL of the *S. epidermidis* strain was incubated in a Tryptic Soy Agar (TSA) medium plate (solid medium) and left overnight at 37 °C. The next day, 2 bacterial colonies were placed in 5 mL of MHB (Liquid Medium) and allowed to incubate overnight at 37 °C. Subsequently the bacteria were centrifuged at 2700 rpm for 10 min and the supernatant was discarded. Sterile PBS (5 mL) was added, and a new centrifugation was performed under the same conditions. Then the pellet was resuspended in 5 mL of sterile PBS and a 1:10 dilution was performed to measure the optical

density at 600 nm. The inoculum concentration was adjusted to 1×10^5 CFU/mL in MHB resulting in a microorganism density of 4773 per mm².

Dilutions were made up to 10^{-3} and then 200 µL of the adjusted inoculum were added to the coated-cellulose membranes, leaving them to incubate overnight at 37 °C. Finally, 10^{-3} dilutions of the microorganisms that were put in contact with the coated-cellulose membranes, and these were plated on a TSA plate. After 18 hours at 37 °C, the colonies were counted manually.

RESULTS AND DISCUSSION

3.1 Development of antibacterial cellulose membranes

3.1.1. Cellulose functionalization (Plasma Technology vs Steglich Esterification)

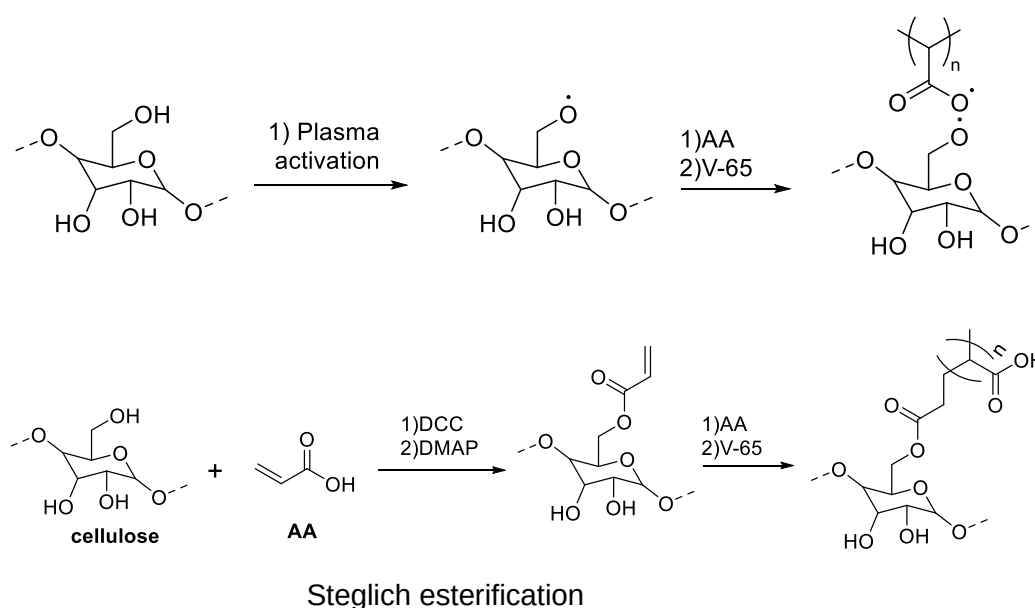


Figure 11 - General scheme of the functionalization of cellulose membranes by plasma activation and steglich esterification.

Two distinct methodologies were used to functionalize cellulose membranes. While in a “*grafting from*” strategy radicals that will subsequently attach to AA are generated during plasma activation (**Fig. 11**), this is not the case during Steglich esterification, which involves an esterification reaction between the hydroxyl group of cellulose and the carboxyl group of AA constituting a “*grafting through*” approach (**Fig.11**). In “*grafting-from*” the polymer chains grows *in-situ* from an initiator that has already been produced at the surface of the cellulose membrane. It is reported the use of plasma (nitrogen) to

activate cellulose to target cellulose modification, which is a green technique, however in this case, it was used argon to functionalize these cellulose membranes.

The "*grafting-through*" polymerization occurs from the monomer (acrylic acid) that was anchored through esterification to the membrane's surface.

After plasma activation and the steglich esterification procedure, the scCO₂ was used to produce a polymeric layer of PAA. As a result, the final membranes had a higher mass than the original ones.

EPR

The effectiveness of Plasma functionalization strategy was assessed by Electron Paramagnetic Resonance (EPR) spectroscopy, which provided insights on the presence of radicals in the cellulose membranes. The native cellulose membrane does not have radical species, as indicated by the absence of EPR signals (**Fig. 12**, bottom spectra). After plasma activation, a complex EPR spectra emerges, characterized by broad signals (main peak-to-peak > 2mT), centered around $g \approx 2.004$, with no identifiable hyperfine interactions. The EPR spectra does not significantly change their format with the time of plasma functionalization, but its intensity increases with increasing plasma functionalization time, from 10 to 60 min (**Fig. 12**). The signals observed are consistent with carbon-centered radicals with adjacent oxygen atoms (that typically have $g \approx 2.003-2.004$), as would be expected for cellulose; the presence of oxygen-centered radicals (typically $g > 2.004$) is also supported by these spectra (notice the low field feature at 339.8 mT). The random orientations of the radical species would greatly contribute to unresolved, not equivalent, inhomogeneous hyperfine interactions and, thus, to the large signal broadening observed.

The EPR spectrum does not change significantly with the time of plasma activation. The radicals were not activated for 60 min since the research group's procedure called for 30 minutes as the maximum duration for activation and longer than that was deemed excessive at the manufacturing level.

The quantification of the radical species revealed to be a highly challenging task. It requires one standard that gives rise to a similar signal, must be in the same matrix, have the same shape and positioning (geometry) in the EPR cavity and be in the same type of sample tube. In the absence of such ideal standard, the spin quantification was roughly estimated using solid TEMPO, which also gives rise to a single intense symmetrical line (peak-to-peak ≈ 1.3 mT). Using this standard, it can be suggested that one hour of plasma activation yields the formation of 2.7×10^{16} spin/g (corresponds to 10 au intensity in **Fig.12**).

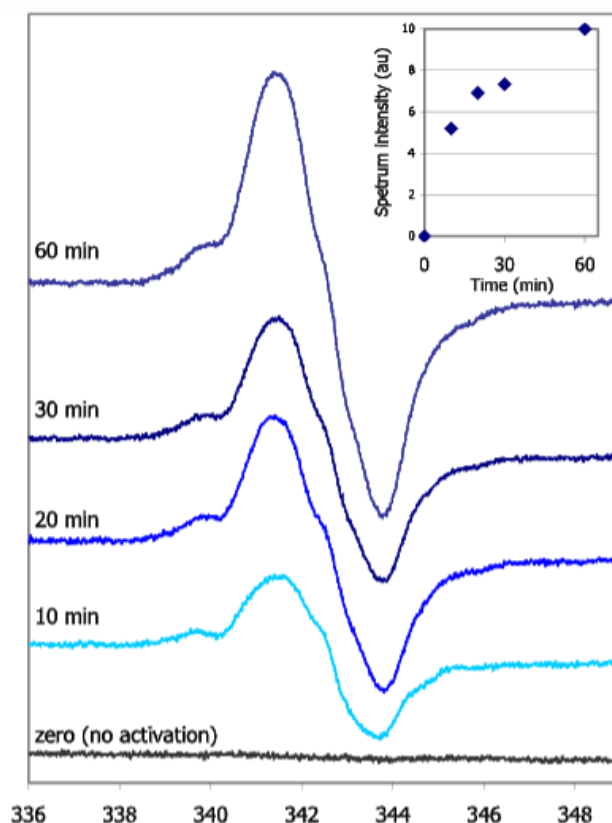


Figure 12 - EPR spectra of plasma functionalized cellulose membranes for zero (no activation), 10, 20, 30 and 60 minutes as indicated. Inset shows the spectrum intensity (double integration), in arbitrary units (au), as a function of the time of activation.

Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra of untreated cellulose membranes, coated membranes (plasma functionalized cellulose membranes and solvent-based functionalized cellulose membranes) are shown in **Fig. 13**. For the untreated cellulose membrane, the absorption peaks at 3300 cm^{-1} and 2900 cm^{-1} are attributed to the stretching vibrations of -OH and -CH_2 , the peaks at 1420 cm^{-1} and 1370 cm^{-1} are mainly due to bending vibrations of -CH_2 (44).

Regarding the functionalized membranes, the peaks present at 1700 cm^{-1} are attributed to C=O stretching vibrations and 1200 cm^{-1} to C-O stretching vibrations from COOH functional group (45).

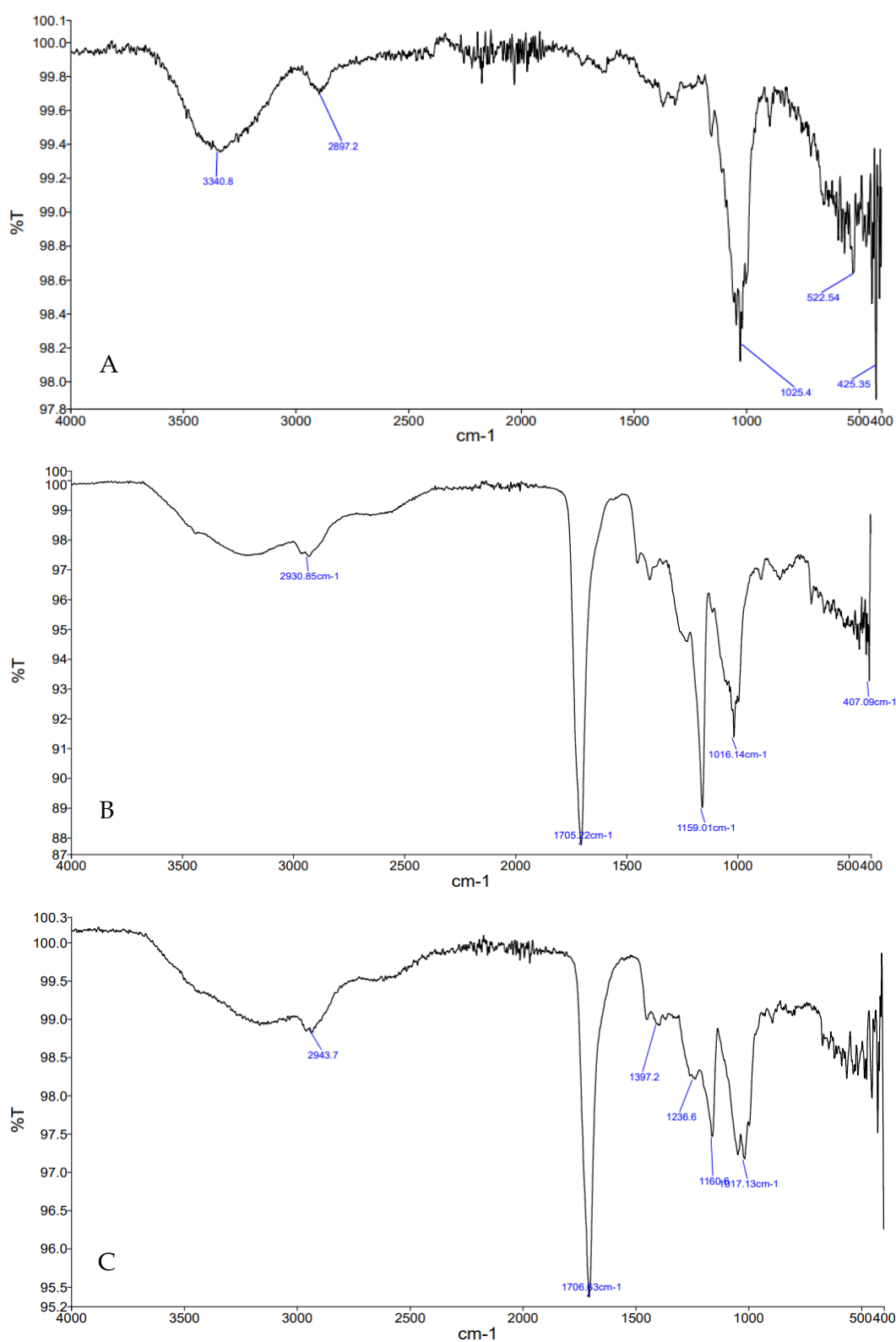


Figure 13 - FTIR spectra of: A) native cellulose membrane B) cellulose membrane (plasma functionalization); C) Solvent-based functionalized cellulose membrane.

Contact Angle

The hydrophobicity of the materials was measured by contact angle at room temperature using deionized water as shown in **Fig. 14**. Although the same quantity of AA was used in the coating reactions, the hydrophobicity of the membranes showed to be dependent on the type of functionalization performed.

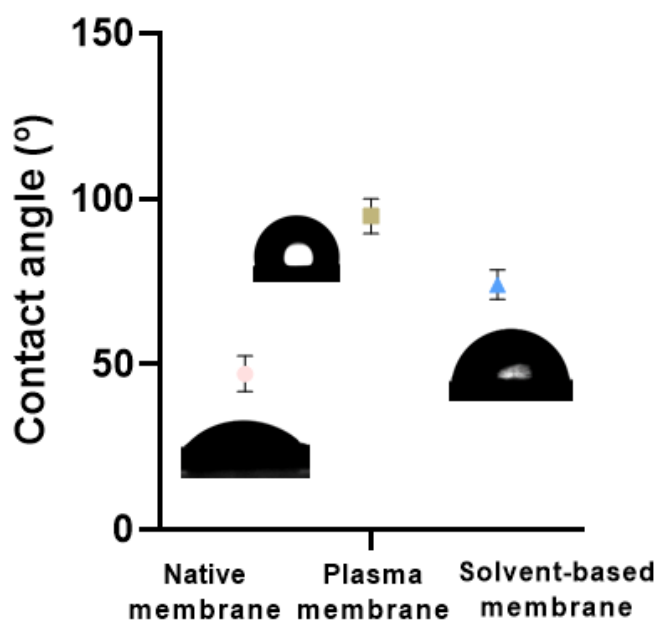


Figure 14- Contact angle of the cellulose membranes using water droplets.

Native membranes have poor stability to moisture because the water molecules swell the polymer and disrupt its barrier properties. Coating membranes with PAA improved their contact angle due to the formation structural composition of the polymer in ethylene and carboxylic moieties.

The obtained results revealed that the functionalization process had impact in the way that polymer grows at the cellulose surface, since the solvent-based functionalized membranes had lower contact angle than plasma activation membranes. This may have occurred because of the plasma membrane's surface seems to form a polymer coating with higher thickness.

The hydrophobicity of wound dressings and the removal or adsorption of microorganisms are not directly correlated. When the wound dressing is overly hydrophobic, it either fails to absorb wound fluids, which increases the risk of infection, or it breaks down and loses its characteristics, consequently, it's essential to have a balance, with solvent-based membrane being the most suitable.

The hydrophilic-hydrophobic character of a good wound dressing should be enough to allow the interaction with the body fluids released by the wounds without absorbing them all at once.

Mechanical properties

Tensile tests were used to assess the membranes' strength and elasticity, two important variables to consider in wound dressings.

Young modulus data are compiled in **Table 7**, showing that native membranes are the ones with less strength since they lack functionalized AA monomer. Plasma membranes exhibited higher mechanical resistance when compared to solvent-based membranes, likely because of the thickness of the coating. However, both strategies increased the mechanical strength of the membranes in comparison with the native samples.

Table 7 - Young modulus of functionalized cellulose membranes.

<i>Membrane</i>	<i>Young modulus (MPa)</i>
<i>Native</i>	10.5
<i>Plasma</i>	17.1
<i>Solvent-based</i>	13.1

Membranes for wound dressings should be mechanically resistant and flexible to better adapt to clinical demands and patient handling, to effectively speed up the healing process. The values obtained were in-line with reported systems (46).

Swelling and Degradation

The swelling ratio, which measures the ability of the membranes to absorb the surrounding medium was investigated as well as the degradation profile (**Fig. 15**) of the cellulose membranes. In a situation of chronic wound, it is crucial that the dressing remains time enough to perform its functional role contributing to the regulation of wound environment, promoting healing.

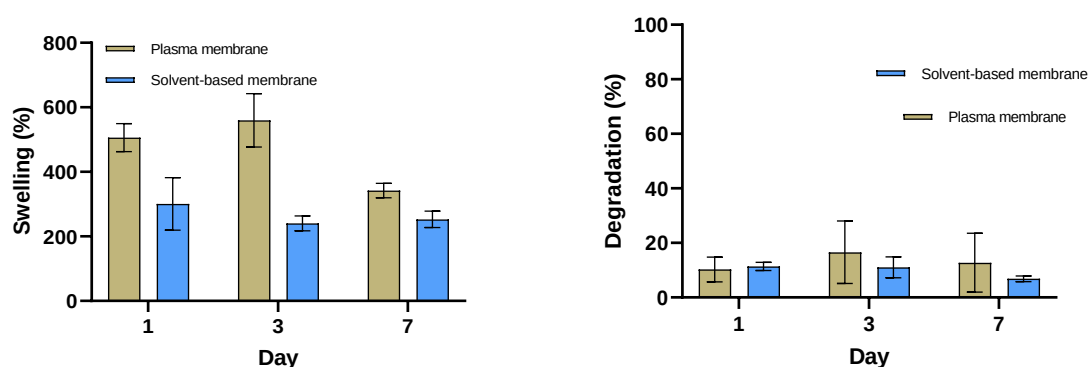


Figure 15- A) Swelling (%) at 37 °C; B) Degradation (%).

Swelling of plasma membranes and solvent-based membranes are presented in **Fig. 15**. Both membranes demonstrated capacity to absorb the surrounding medium, with the plasma-functionalized

membrane presenting a higher swelling ratio through the 7 days of incubation probably due to the higher polymer thickness in plasma membranes compared to solvent-based membranes.

Fig. 15 A demonstrates that samples swelled by at least 200 %, which is common in wound dressings (47). As plasma membranes are more hydrophobic than solvent-based functionalized membranes (**Fig. 14**), it would be expected a lower swelling ratio. The main difference between the two systems was probably the disorganized morphology (**Fig. 17**), which may have more space to accommodate water, or even the higher thickness of the polymeric coating.

PBS was employed in this instance to mimic the physiological conditions and cellulose membranes must controllably swell to collect tissue fluid effusion while avoiding excessive absorption of normal tissue fluid, which would be harmful to wound healing with the solvent-based membrane being the most suitable.

Regarding the degradation ratio presented in **Fig. 15 B** it could be seen that both membranes showed a residual degradation over the 7 days of incubation, which is a positive impact since it indicates that the wound dressing is stable.

Water Vapor Permeability

The water loss and evaporation should be optimally controlled by a wound dressing. The membrane should be permeable to keep the area wet and prevent wound dryness. As a result, one of the key properties of wound dressings is their capacity to allow the passage of water vapor (48). A wound in a dry environment result in tissue desiccation, which causes the tissue matrix to become dehydrated, the cells to die, and a hard scab to develop (49). The keratinocytes must then go through this scab to reach viable tissue, which requires time and energy and slows the healing process. It is crucial to stress that a moist wound environment is different from a wet one since excess exudates can cause macerated peri wound skin and hyper granulation tissue to grow in the wound bed in patients (50). Therefore, choosing a dressing that can keep the wound surface wet while also removing exudates is crucial when providing topical wound care. A rate of 2500 g/m² per day is suggested for an optimum wound dressing to give an acceptable quantity of moisture without running the danger of wound dehydration (49).

In **Fig. 16** the evaporation of PBS through the membranes is higher than the suggested rate for a wound dressing, which maintain the wound more dehydrated than it should, after 24 hours at 37 °C.

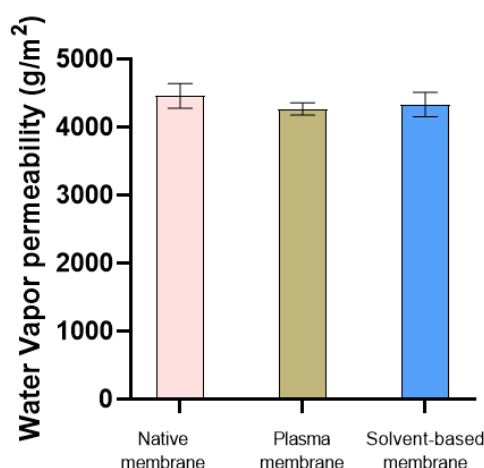


Figure 16 - Water Vapor Permeability (g/m²) per day.

The PAA coating and the THY impregnation did not significantly affect the permeability of the membrane, considering that the deviations of the error bars intersect.

SEM

SEM analyses were used to assess the morphology of cellulose membranes (**Fig. 17**). The analyses revealed the success of the polymeric coating and the impregnation of THY using scCO₂ technology.

The native membrane in **Fig. 17 A** has a disordered porous network and there is a considerable empty space but on the other hand in **Fig. 17 C** it can be observed that the solvent-based functionalized membrane (Steglich esterification) have a more homogeneous and organized porous network (**Fig. 17 B**) than the plasma functionalized membrane, which may be related with a more ordered polymerization provided by the "*grafting through*" strategy, where it occurs from the monomer moieties previously anchored to the membrane surface. In the "*grafting from*" strategy, polymerization occurs from the radicals generated at membrane surface which can result in a more heterogeneous polymeric network (**Fig. 17**).

The polymerization process occur in the presence of pre-functionalized cellulose membrane, monomer and initiator (51).

Since thymol is known to have antibacterial and cicatrizing ability, having potential properties for WH, then coated membranes were impregnated in scCO₂. In **Fig. 17 D** and **E**, it can be seen a structural arrangement because of thymol loading in scCO₂.

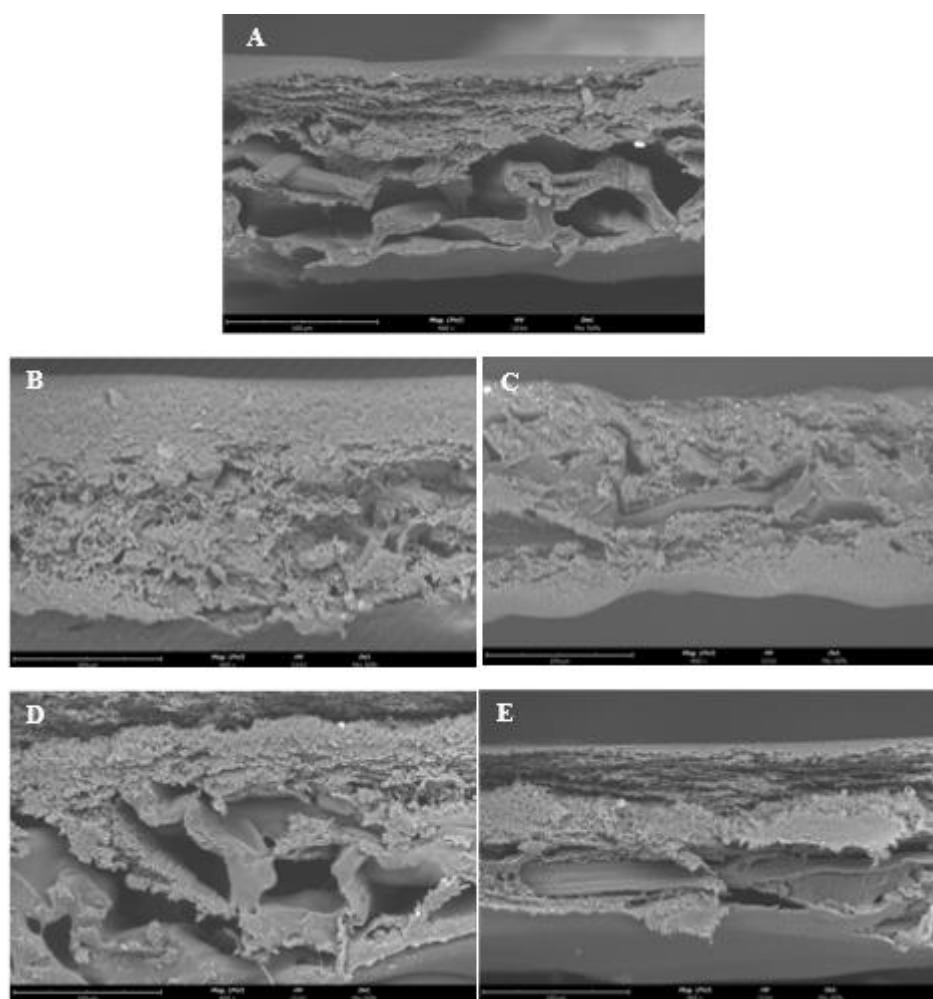


Figure 17 - SEM images obtained for (A) Native membrane, poly(acrylic acid) coatings on: (B) Plasma-functionalized membrane and C) Solvent-based membrane; THY-loaded cellulose membranes on: (D) Plasma functionalized membrane and (E) Solvent-based functionalized membrane, x 400 amplification.

THY release

The release profile (**Fig. 18**) of THY from the coated cellulose membranes was measured to evaluate their potential as a drug delivery device. The ability of the membrane to release THY into the surrounding medium under physiological conditions (PBS, pH = 7.4, 37 °C) was evaluated.

By SEM analyses (**Fig. 17**) it could be observed that the different morphologies of the membranes are dependent on the functionalization methodology. The morphology of the coating was highly influenced by the functionalization strategy as well as the THY release into the medium. However, replicate membranes obtained through the same functionalization methodology released substantially different amounts of THY, indicating that the impregnation was not homogeneous between different batches.

THY release in the same batch

Variability between several batches

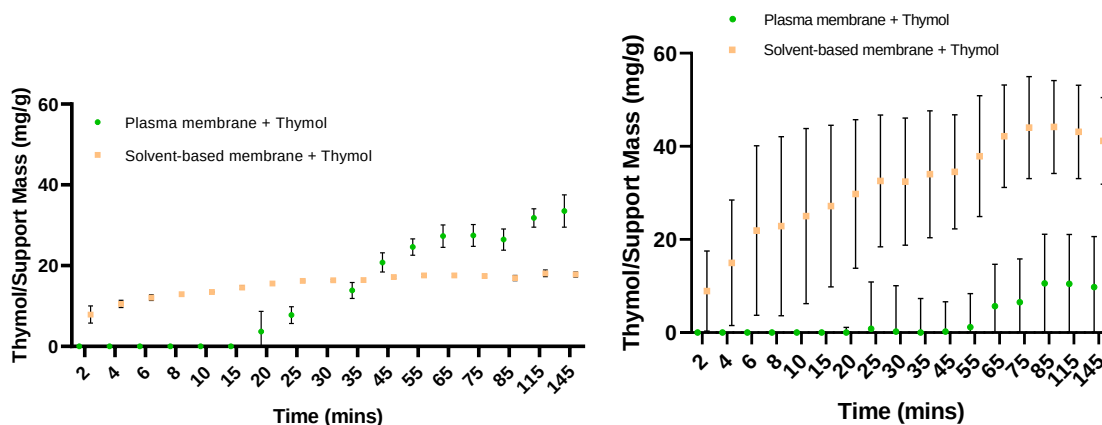


Figure 18 - Release ratio (Thymol/Support Mass (g)) of the coated cellulose membranes.

Reported studies have demonstrated a similar release profile of thymol as the solvent-based membrane in wound dressings (52), and for clinical application, they should be preferable due to their more organized structure which allows a more controlled release over time.

3.3 Radical-scavenging ability

The radical-scavenging ability of PAA coated-cellulose membranes was assessed using the DPPH assay to determine its capacity to neutralize ROS, commonly present in high excess in chronic wounds (**Fig. 19**). The maximum absorbance of DPPH, a persistent free radical with a purple colour and an unpaired valence electron at one nitrogen atom, occurs at $\lambda = 517$ nm. It can be converted to its most stable form, diphenyl-picrylhydrazine, in the presence of antioxidants, and the capacity to scavenge free radicals may be assessed by looking at the decrease of absorbance at this wavelength (43).

To carry out this test, all membranes in contact with the DPPH solution and the solution itself without the presence of membranes (blank) were analyzed.

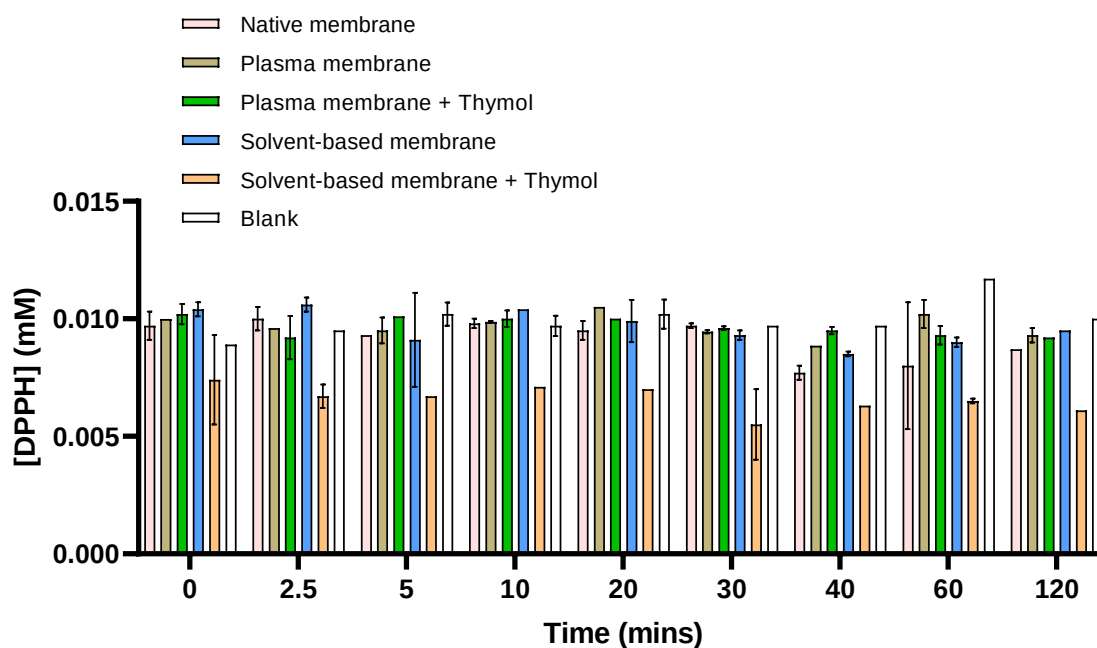


Figure 19 - Free radical-scavenging ability of native and PAA coated-cellulose membranes.

In the first two hours, all the membranes appeared to have identical radical scavenging kinetics. **Fig. 19** shows that no free radical neutralization is seen in native or coated membranes, demonstrating that this happens because of thymol release. The Steglich-derived membranes revealed ability to neutralize DPPH, which stabilizes between 30 and 40 minutes, agreeing with the point at which the thymol release reaches a plateau (**Fig.18**). However, a replicate of this experiment using only plasma-functionalized membranes loaded with THY (**Fig. 23 – Annex A**) revealed that. these membranes were also able to neutralize most DPPH free radicals in the first minutes. These findings suggest that THY impregnation and functionalization may not have occurred in a homogenous way between different membranes, providing some inconsistent results between samples with similar treatment as in the release tests where thymol release was substantially different for membranes with the same functionalization.

3.4 Biological behaviour

The cytotoxicity of the PAA-coated cellulose membranes was assessed through indirect contact following ISO10993-5 and using a resazurin-based test (**Fig. 20**).

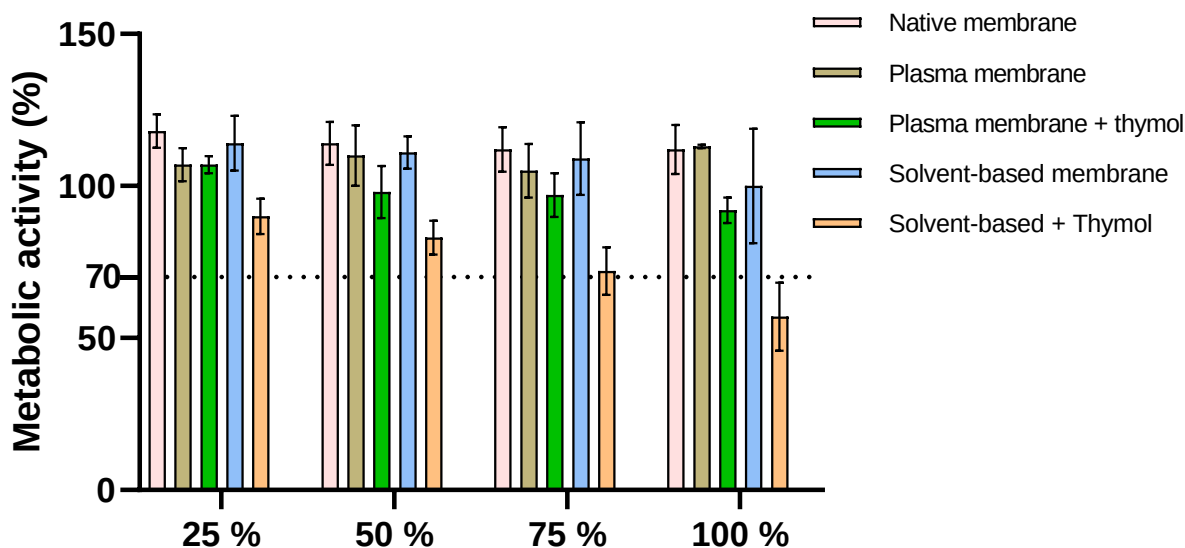


Figure 20 - Metabolic Activity of L929 cells after being in indirect contact with PAA-coated cellulose membranes. Concentrations of 25 %, 50 %,75 % and 100 % of the medium that were in contact with the membranes were studied.

The native membrane is entirely made of cellulose, therefore it is completely biocompatible with all concentrations. The functionalization of the cellulose membranes with a synthetic polymer poly (acrylic acid) (PAA) at their surface either through plasma or following a solvent-based methodology maintained their biocompatibility, since PAA is also known to be biocompatible (51).

Fibroblasts remained metabolically active in all membranes except for the solvent-based membranes impregnated with THY, which induced a general decrease in metabolic activity in comparison with the non-impregnated Plasma and solvent-based membranes. Such cytotoxicity was more evident at higher medium concentration (100 %). Although THY, possesses desirable pharmacological properties like antibacterial, antioxidant, anti-inflammatory and healing actions (52) some tests with other THY concentration should be performed in order to better investigate its effects.

3.5 *In vitro* radical scavenging ability of coated cellulose membranes

The *in vitro* ability of the coated cellulose membranes to capture extracellular ROS produced by macrophages in unstimulated or LPS-stimulated pro-inflammatory conditions is shown in **Fig. 21**. The amount of ROS was detected using the Acridan Lumigen PS-3 kit and this was measured in luminescence mode. It is well known that LPS is an effective stimulator of monocytes and macrophages, causing

a burst of ROS production and consequently an inflammatory-like environment (53). Time-course studies of direct and indirect contact exposure during 1 h of LPS for inducing higher ROS production in monocyte-derived macrophages were performed.

While in indirect contact the medium in which the membranes were in was put with the macrophages, direct contact involved placing the membranes directly in contact with them. Verifying the neutralization of ROS was the aim of both.

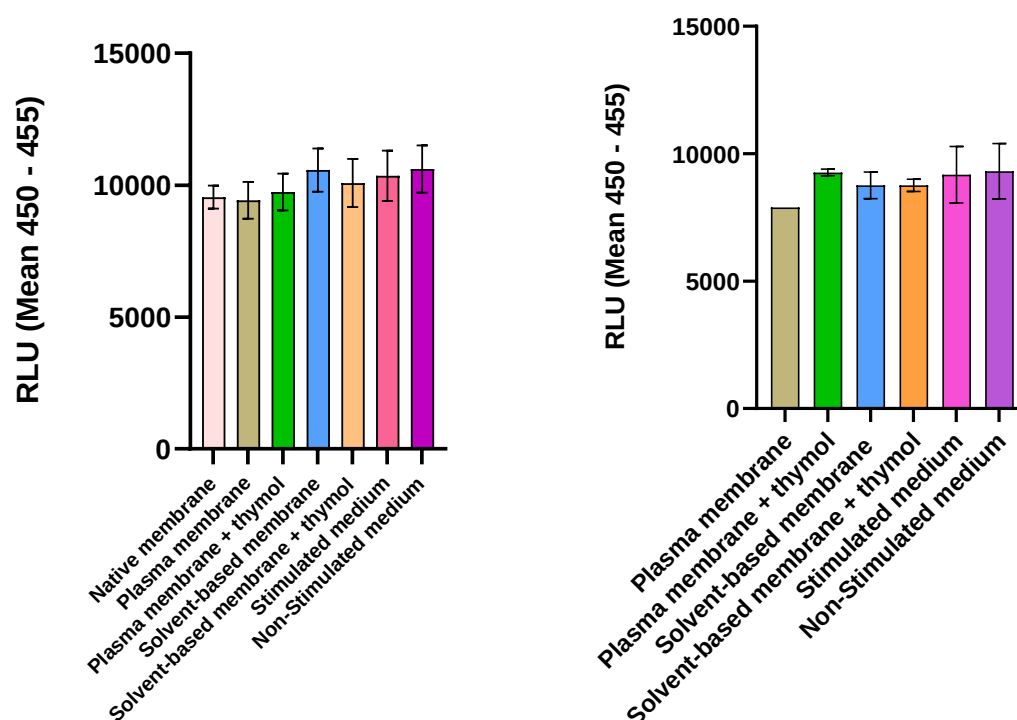


Figure 21 - LPS-induced ROS formation and in vitro radical scavenging ability: A) Indirect Contact; B) Direct Contact.

The luminescence of stimulated media (which contains LPS) is like the non-stimulated medium, as shown by the graphs of indirect and direct contact. This was not expected, since ROS are produced by macrophages after LPS stimulation, meaning that the stimulated medium should have a higher RLU value. Given that stimulation was not effective, it could not be observed any difference in the amount of ROS between PAA-coated membranes with/without THY, providing an inconclusive assay.

This allowed to conclude that probably a stimulation time of 1 hour was insufficient, and thus this time should be more extended to ensure the production of higher amount of ROS by the macrophages.

3.6 Antibacterial activity

The antibacterial activity of the membranes produced through this work was determined against *S. epidermidis* (Fig. 22).

The native membrane did not prevent bacterial growth, as it was predicted, and the CFU/mL number was the same of the bacterial growth control. Due to the presence of PAA, which possesses antibacterial ability, the development of *S. epidermidis* was suppressed by both coated membranes (plasma and solvent-based functionalized membrane). This happens due to the electrostatic repulsion between the bacterium, the solvent, and the surface.

Since radicals in plasma membranes can extract an electron from other substances, they can oxidize them and make them toxic to bacteria, preventing bacterial development more than solvent-based membranes (54).

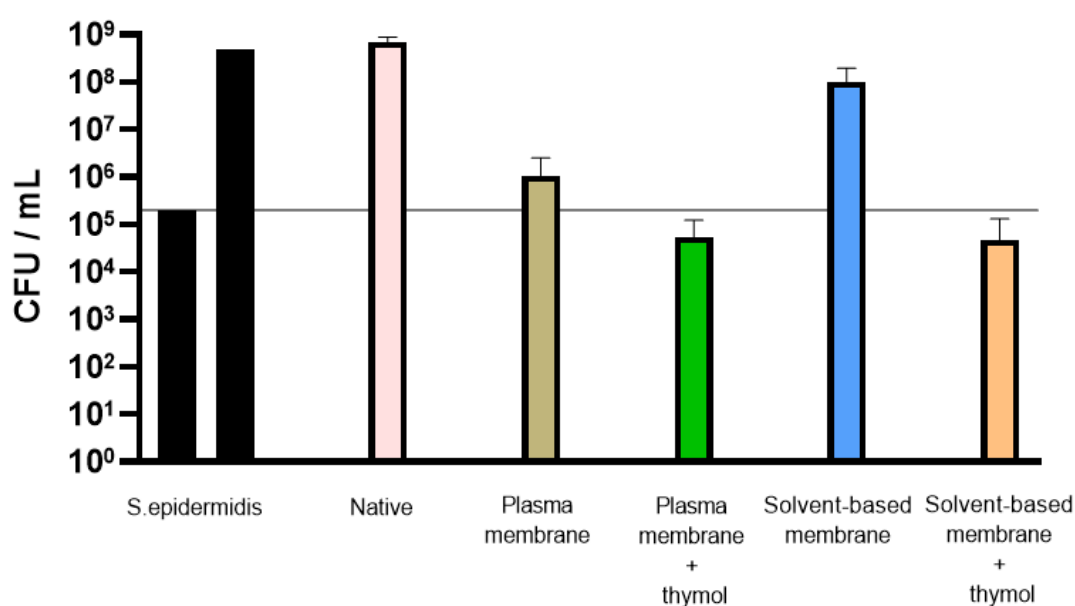


Figure 22 - *S. epidermidis* growth during 1 day for all the membranes conditions.

THY-impregnated membranes not only prevented bacterial growth but also exhibited lower CFU/mL values compared with the control material, suggesting that some bacteria died as a result of the thymol's controlled release into the medium and its antibacterial, antifungal and antiparasitic abilities {Formatting Citation}. All the developed membranes successfully inhibited the bacterial growth.

CONCLUSIONS AND FUTURE WORK

In this work, the surface engineering of cellulose using green technologies were successfully achieved. It was evaluated two functionalization strategies (green "*grafting from*" and conventional "*grafting through*"). Further, the scCO₂-assisted cellulose membrane coatings with poly(acrylic acid) PAA and impregnation with THY were performed.

The EPR analyses demonstrated that the presence of free radicals at the surface of cellulose membranes is influenced by plasma technique and the number of radicals increases as the number of activations increases.

PAA was successfully grafted at the surface of the cellulose membranes as confirmed by FTIR analysis, turning the membranes more hydrophobic. The solvent-based membranes presented a more organized morphology than the plasma membrane which agrees with the more controlled release profile of THY. Plasma membranes exhibited higher resistance when compared to solvent-based membranes.

Both native and functionalized membranes showed high values of water vapor permeability and swelling and a slow degradation ratio, which may be advantageous in situations of chronic wound healing.

The functionalized membranes were found to be biocompatible and demonstrated antimicrobial activity, although THY decreased cell metabolic activity while totally inhibiting bacterial growth. To avoid affecting the biocompatibility while preserving the antibacterial characteristics of the membranes, the amount of THY impregnated should be investigated.

The results are very promising, although the antioxidant and the release tests indicated that THY impregnation and membrane's functionalization might not have happened in a homogenous way requiring protocol optimization and further experiments to assure both product and process reproducibility.

More research should be done in the future using different concentrations of thymol and AA to confirm their effects on biocompatibility.

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APPENDIX

A.1

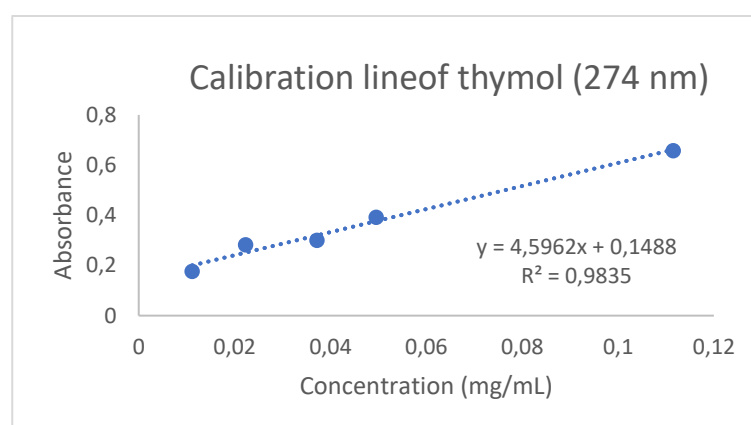


Figure 23 - Calibration line of thymol Dilutions of 4, 9, 12, 20 and 40.

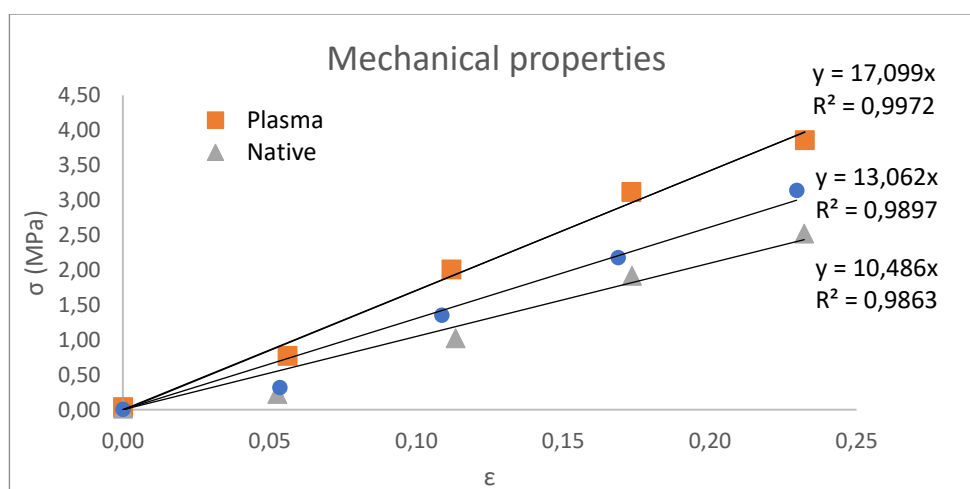


Figure 24 - Young modulus of cellulose membranes.

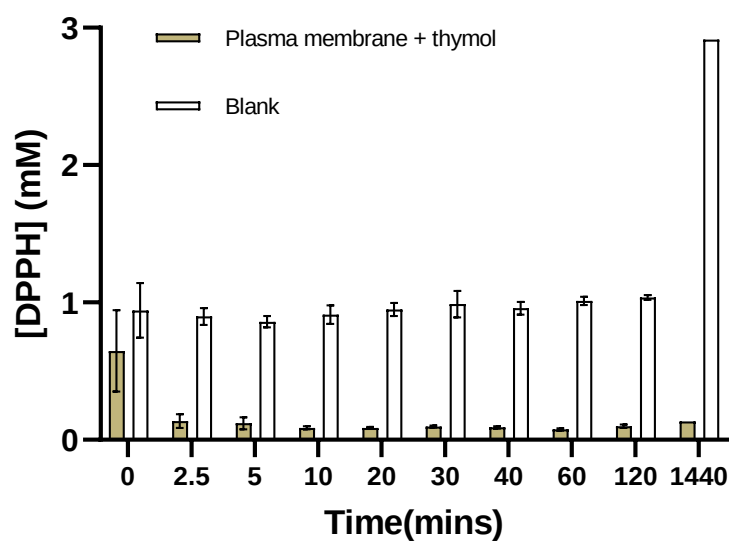


Figure 25 - Free radical-scavenging ability of cellulose membranes functionalized by plasma and impregnated with thymol.



<2023>

Matilde Silva

Green functional biomaterials for wound healing