CATÓLICA

ESCOLA SUPERIOR DE BIOTECNOLOGIA

PORTO

BIOFUNCTIONAL SILK-FIBROIN MEMBRANES WITH ANTIOXIDANT PROPERTIES FOR THE TREATMENT OF CHRONIC WOUNDS

by

Ana Sofia Ferreira Fernandes

January 2019

CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

BIOFUNCTIONAL SILK-FIBROIN MEMBRANES WITH ANTIOXIDANT PROPERTIES FOR THE TREATMENT OF CHRONIC WOUNDS

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Biomedical Engineering

by
Ana Sofia Ferreira Fernandes

Place: Escola Superior de Biotecnologia and Universidad de Vigo

Supervisor: Prof. Dr.ª Ana Leite Oliveira
Co-Supervisors: Dr.ª Cassilda Cunha Reis, Prof. Dr. Pío González

January 2019

RESUMO

Atualmente, as feridas crónicas são uma patologia cada vez mais recorrente, apresentando uma maior incidência na população idosa, principalmente quando existem fatores de co-morbilidade, tais como *Diabetes Mellitus* e doenças cardiovasculares. Nos últimos anos, têm sido propostos novos materiais de penso, incorporando uma diversidade de agentes bioativos para o tratamento de feridas crónicas. Entre os biomateriais naturais propostos, a seda ganhou atenção devido à sua biocompatibilidade, biodegradação, por ser facilmente modificada quimicamente e por possuir boas propriedades mecânicas. O objetivo deste estudo foi o desenvolvimento, otimização, e caracterização de membranas biofuncionais feitas à base de fibroína de seda (SF), com a incorporação de dois agentes antioxidantes (ácido cafeico – CA – e ácido tânico – TA) para o tratamento de feridas crónicas superficiais e consequente regeneração da pele. Através da técnica de *solvent casting*, seguida de um tratamento térmico, produziram-se membranas estáveis, que foram posteriormente caracterizadas relativamente à morfologia da superfície (SEM e perfilometria ótica), à composição química (FTIR-ATR), perfil de degradação, performance mecânica, análise térmica (TG-DTA), capacidade antioxidante e citocompatibilidade. A degradação foi avaliada pela percentagem de peso perdido pelas amostras. A atividade antioxidante foi avaliada pelo método ABTS. Os testes de citocompatibilidade foram realizados, utilizando a linha celular imortalizada de células de fibroblastos de rato (L929), através do método de contacto direto e dos extratos. Todas as membranas desenvolvidas demonstraram ser transparentes, inodoras, apresentando uma superfície homogénea, ainda que com espessura variável. As imagens de SEM mostraram que todas as membranas apresentavam uma superfície lisa e homogénea, sem rugosidade aparente à escala micrométrica. Através da perfilometria ótica concluiu-se que, à escala nanométrica, a adição de glicerol aumentou a rugosidade da superfície e, através dos ensaios mecânicos, verificou-se que este composto reduziu a rigidez das membranas, tornando-as mais maleáveis. Com as membranas no estado seco, os agentes antioxidantes potencializaram um efeito anti-plasticizante, enquanto, no estado hidratado, a água potencializou o efeito plasticizante do glicerol. As membranas de SF apresentaram uma massa constante ao longo de 15 dias, em PBS, enquanto que as restantes membranas apresentaram uma perda de massa inicial de aproximadamente 30%, após as primeiras 2 horas de incubação, maioritariamente relacionada com libertação de glicerol. Com a presença de Protease XIV, a mesma perda de massa inicial foi observada, aumentando drasticamente após 24 horas de incubação, evidenciando a suscetibilidade da seda para a degradação proteolítica. A resistência térmica da SF diminuiu com a introdução do glicerol nas formulações. À concentração de 0.5% de ambos os agentes antioxidantes essa propriedade foi aumentada e o oposto foi observado na concentração de 1%. As membranas de SF mostraram ter capacidade antioxidante, tendo a adição de 0.5% TA reforçado este efeito. Apenas o extrato puro de 1% TA revelou um efeito citotóxico para as células L929. Todas as restantes membranas não apresentaram citotoxicidade. Este sistema demonstrou ser bastante promissor, reconhecendo a capacidade das membranas de SF para incorporar moléculas antioxidantes e o seu possível potencial no tratamento de feridas crónicas.

Palavras – chave: antioxidantes, feridas crónicas, membranas de fibroína de seda, pensos para feridas.

ABSTRACT

Nowadays, chronic wounds are an increasingly recurrent pathology, presenting a higher incidence in the elderly population, especially when there are comorbidity factors such as *Diabetes Mellitus* and cardiovascular diseases. In recent years, new dressing materials incorporating a variety of bioactive agents have been proposed for the treatment of chronic wounds. Among the proposed natural biomaterials, silk gained attention due to its biocompatibility, biodegradation, for being easily chemically modified and due to its good mechanical properties. The objective of this study was the development, optimization, and characterization of biofunctional silk fibroin (SF) based membranes, with the incorporation of two antioxidants agents (AA) (caffeic acid - CA - and tannic acid - TA) for the treatment of superficial chronic wounds and consequent regeneration of the skin. Stable membranes were produced using the technique of solvent casting followed by a thermal treatment, which were characterized regarding surface morphology (SEM and optical profilometry), chemical structure (FTIR-ATR), degradation profile, mechanical performance, TG-DTA, antioxidant capacity and cytocompatibility. The degradation was assessed by obtaining the percentage of weight lost by samples. Antioxidant activity was evaluated by the ABTS method. Cytocompatibility tests were performed using the immortalized cell line of mouse fibroblast cells (L929) by direct contact and extracts tests. All the membranes were transparent, odorless and presented a homogeneous surface, although with variable thickness. SEM images showed that all membranes had a smooth and homogeneous surface, with no apparent roughness at the micrometer scale. Optical profilometry showed that, at the nanoscale, the introduction of glycerol (Gly) in the formulations increased the surface roughness and, the mechanical tests confirmed that this compound reduced the stiffness of the membranes, making them more malleable. With the membranes in the dry state, the AA potentiate an anti-plasticizer effect, while, in the hydrated state, water has potentiated the plasticizer effect of Gly. SF membranes exhibited a constant mass over 15 days in PBS, while the remaining membranes showed an initial weight loss of approximately 30%, after the first 2 hours of incubation, most likely related to Gly release. In the presence of Protease XIV, the same initial weight loss was observed, increasing drastically after 24 hours of incubation, evidencing the susceptibility of the silk to the proteolytic degradation. The thermal resistance of SF decreased with the introduction of Gly. At a concentration of 0.5%, the AA increased this property and the opposite was observed when the concentration raised to 1%. SF membranes showed to have antioxidant capacity, and the addition of 0.5% TA reinforced this effect. The pure 1% TA extract revealed a cytotoxic effect in L929 cells. All other membranes were non-cytotoxic. This system proved to be quite promising, recognizing the ability of SF membranes to incorporate antioxidant molecules and their potential in the treatment of chronic wounds.

Keywords: antioxidants, chronic wounds, silk fibroin membranes, wound dressings.

ACKNOWLEDGEMENTS

Nenhuma secção foi tão complexa de elaborar como esta. E nenhuma estará tão incompleta quanto esta. Muitas foram as pessoas que me acompanharam ao longo desta etapa da minha formação académica. E muitas foram as pessoas que me acompanharam, não só na minha formação académica, como na minha vida quotidiana e pessoal. Todas, sem exceção, contribuíram para o meu crescimento. A todas estas pessoas deixo, nesta secção, o meu maior e profundo agradecimento, e a maior gratidão que consigo expressar por palavras escritas.

À *Professora Doutora Ana Leite Oliveira*, pela incomparável orientação. Por todas as oportunidades que me deu ao longo destes quatro anos. Por nunca me ter deixado desistir. Por ter acreditado nas minhas capacidades. Por toda a paciência e disponibilidade, e por toda a ajuda nos momentos bons e menos bons. Foi um prazer enorme poder aprender com a professora e com toda a equipa durante estes anos. Ficar-lhe-ei eternamente agradecida por ter contribuído tanto no meu crescimento científico e profissional.

À *Doutora Cassilda Cunha Reis*, pela inigualável coorientação, por todos os ensinamentos, por toda a passagem de conhecimento científico, por todo o apoio em todos os momentos, principalmente nos mais difíceis; por todas as palavras de incentivo e por ter sempre acreditado nas minhas capacidades. Fico-te para sempre agradecida, Cassilda, por teres conseguido fazer com que todos os dias se transformasse em dias de esperança e alegria!

Ao *Professor Doutor Pío González,* à *Professora Doutora Júlia Serra* e a todo o grupo de investigação *Nuevos Materiales* da Universidade de Vigo, por tão bem me terem recebido e integrado, por toda a simpatia e amabilidade, pelas oportunidades que me deram e por todo o contributo na minha coorientação e no meu crescimento científico.

Ao *Professor Doutor João Paulo Ferreira*, coordenador do Mestrado em Engenharia Biomédica, pela oportunidade de frequentar este mestrado, que tanto contribuiu para o meu enriquecimento científico e académico, assim como por toda a disponibilidade que teve ao longo destes dois anos.

Aos meus *Colegas e Amigos de Laboratório – Ana Rita Pinto*, *Catarina Geão*, *Cassilda Reis*, *Gabriela Madanços*, *Gonçalo Soares*, *Marcus Vinícius*, *Nilza Ribeiro*, *Ricardo Serôdio*, *Sandra Borges*, *Sara Baptista* e *Sara Pérez*. Por terem deixado a vossa marca neste meu percurso científico. Por serem todos especiais *à vossa maneira*. Por cada um me ter passado novos ensinamentos. Por todo o companheirismo, por todo o apoio e por toda a alegria diária! Um agradecimento especial à *Doutora Sara Baptista da Silva* por todo o apoio, suporte e ajuda na realização do ensaio de ABTS.

À *Professora Doutora Goreti Sales*, à *Doutora Manuela Frasco* e ao *BioMark Research Group do Instituto Superior de Engenharia do Porto* pela realização do ensaio de TG-DTA, por toda a disponibilidade e integração.

Ás minhas *Colegas e Companheiras da Escola Superior de Biotecnologia – Beatriz Moreira*, *Francisca Ferreira* e *Juliana Aguiar* – por todos estes anos de amizade. Por todas as conversas. Por todo o apoio. Por todas as gargalhadas. Por todos os momentos que passamos juntas. Por todo o *stress partilhado* e por serem a melhor *equipa de trabalho* que poderia alguma vez ter! Sem vocês, esta conquista não teria o mesmo sabor!

Às minhas *Colegas e Companheiras de Faculdade – Catarina Loureiro*, *Carolina Magalhães*, *Marta Gonçalves* e *Sofia Sarmento* – por toda a amizade que desenvolvemos em cinco anos e que vai ficar para a vida toda! Por, mesmo estando longe, estarem sempre tão perto. Por fazerem a minha vida melhor!

A toda a *família dançante da Academia de Dança João Alves*, por me acompanharem durante estes cinco anos. Por me terem visto crescer e por terem acompanhado o progresso da minha formação académica. Por cada um de vós, à vossa maneira, sem exceção, ter contribuído, tanto na minha evolução profissional, como na minha evolução enquanto dançarina. Por terem sempre as palavras e os sorrisos mais doces para me dar. Pela vossa camaradagem e companheirismo. Por serem pessoas especiais. E por terem sido um dos motivos que me ajudou a *levar este barco a bom porto*. A todos vocês, sem exceção, o meu sincero obrigada!

À minha *Melhor Amiga – Mariana Silva*. Por ter sido o melhor que a faculdade me deu. Por ser um *anjo na Terra*. Por ser um dos maiores pilares da minha vida. Por ser a melhor amiga que poderia ter. Pelo ser humano lindo que é. Por toda a honestidade e humildade. Por ouvir os meus dramas diários durante *horas a fio*. Por estar sempre presente em todos os momentos da minha vida. Por nunca me deixar desistir daquilo que ambiciono. Por me mostrar que "*a distância não significa nada quando uma pessoa significa muito*". Por me fazer rir e sorrir quando menos espero. Por ter sempre o conselho certo para me dar. Por todos os momentos que já passamos. Por tudo o que ainda vamos passar juntas e por todas as histórias que vamos criar juntas. Por te teres tornado nas pessoas mais importantes da minha vida em tão pouco tempo. Por seres a irmã que nunca tive – o meu maior, mais sincero e eterno obrigada, minha *Marianocas*!

Aos meus *Vizinhos – Fernandinha* e *Sr. Manuel* – por me acompanharem nestes meus 23 anos de vida. Por me terem acompanhado nas diversas etapas da minha formação profissional e pessoal, por me verem crescer e evoluir, por acreditarem sempre em mim e por nunca me deixarem desistir de nada. Por me apoiarem em todos os meus projetos, por terem sempre os melhores conselhos e as palavras certas, e por acreditarem sempre no meu sucesso. Muito obrigada por serem os melhores vizinhos (e os melhores *avós emprestados*) do mundo!

À minha *Melhor Amiga de Quatro Patas – Laika* – por saber como me animar nos dias menos bons. Por me compreender sem saber falar português. Por me pôr a sorrir com brincadeiras simples. Pelo amor incondicional e por toda a proteção que me dá sem pedir nada em troca. Por ser a companheira mais fiel e mais leal que poderia ter. Por me ensinar que o melhor da vida está nas coisas mais simples e nos sentimentos mais puros, como o amor e a gratidão. Por ser a cadela mais bonita de sempre. Por me mostrar que os animais são das maiores dádivas que a natureza tem!

Aos meus *queridos Pais – Domingos Fernandes* e *Isaura Fernandes* – e aos meus *queridos Avós – Manuel Ferreira* e *Maria da Conceição Ferreira*. Os meus *anjos da guarda na Terra*. Os meus melhores amigos. Os meus heróis. Os meus guerreiros... Os meus exemplos de vida! Nunca hão de haver palavras suficientes para vos agradecer tudo o que fizeram por mim durante estes cinco anos. E muito menos palavras existirão para vos agradecer tudo o que fizeram e fazem por mim desde o meu primeiro segundo de vida. São o maior pilar da minha vida. A minha razão de viver. Os melhores amigos que alguma vez poderei ter e a melhor família que alguma vez poderia desejar ter. Grande parte do que sou a vocês devo. Grande parte do que conquistei e do que serei daqui para a frente a vocês devo. Ser-vos-ei grata para sempre. Por nunca me deixarem desistir dos meus sonhos. Por me apoiarem em tudo o que decido fazer. Por nunca me deixarem *baixar os braços*. Por fazerem os possíveis e os impossíveis para que nunca me falte nada. Por colocarem a minha felicidade e o meu bem-estar *à frente de tudo*. Por aturarem todas as minhas frustrações, toda a minha mimalhice e todos os momentos de palhaçadas! Por estarem sempre presentes em todos os momentos da minha vida. Por terem sempre as palavras certas para me dar, nos momentos certos. Por me darem os melhores conselhos. Por celebrarem comigo as minhas vitórias. Por nunca *me deixarem cair* e por me acompanharem sempre, de mãos dadas, em direção às *minhas metas*. Por serem a maior luz do meu caminho. Amo-vos com tudo o que tenho e com toda a força que tenho! Para SEMPRE! A vocês, amores lindos do meu coração... Fico-vos eternamente GRATA!

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

1. Introduction

In this chapter the concept of a chronic wound is introduced, as well as the current clinical approaches available, wound management products available in the market for the different types of wounds and the innovations that are being developed in an attempt to minimize this problem. Important topics, such as bioactive and multifunctional membranes to promote skin regeneration and wound healing will also be discussed.

1. Chronic Wounds

A wound results from the damage or disruption of the integrity of the skin, mucosal surfaces or organ tissues, which can compromise their normal anatomical structure and function (1,2). To restore the injured tissue, our body initiates a complex process of regeneration – the wound healing (2,3). This process is classically divided into four phases: haemostasis, inflammation, proliferation and tissue remodeling. Wounds can be classified according to the time this process takes to occur (1–5). The healing process can be conditioned by several factors, related to the patient: age, body type, patient's nutritional status, alcohol consumption, among others. In addition, the type of wound and some biological aspects may also influence the healing process. However, there are still some factors that condition the successful healing of these wounds which are not related to the patient, such as the lack of prevention, failure in treatment and strategies of management of these wounds by the health professionals (6,7).

Wounds can be classified according to several criteria, as previously mentioned, such as the healing time – this is one of the most fundamental criteria for the treatment and management of wounds (1,2,4). Wounds that pass through the four phases of the healing process quickly are designated acute wounds. However, wounds which have failed the normal reparative process of healing, over extended periods that range from 4 weeks to more than 3 months, often as a result of prolonged pathological inflammation, are classified as chronic wounds (3,8). This type of wound displays some specific characteristics, such as excessive levels of proinflammatory cytokines and reactive oxygen species (ROS), presence of senescent cells and they are stagnant in the inflammatory phase (8,9). In these wounds, the levels of proteases exceed their respective inhibitors, leading to the destruction of extracellular matrix and degradation of growth factors and their receptors. This not only prevents the wound from regenerating at a normal rate, but also attracts more inflammatory cells, amplifying the inflammation cycle. The immune cells of our body produce ROS, which in low concentrations, provide a defense against microorganisms (10). However, in chronic wounds, the predominant inflammatory environment increases ROS production, which causes cell damage. This, consequently, makes all the senescent cells present in chronic wounds – which have an impaired proliferative and secretory capacity – to lose the responsiveness they previously had to the regeneration process, during wound healing.

This decrease in proliferative capacity is directly correlated with the failure of wound healing (10–12).

Chronic wounds can be classified as vascular, diabetic and pressure ulcers, and the choice of the appropriate dressing will depend on the type of wound to be treated, mainly on its physiological characteristics (amount of exudate, location, among others) (9,10). This topic will be further discussed in the next chapter.

Chronic wounds have the highest incidence on the elderly population, since wound repair decreases as the body age increases, and the incidence of some diseases that promote the appearance of chronic wounds, as diabetes and cardiovascular diseases, increases with age. In 2015, diabetes was the direct cause of the death of 1.6 million people worldwide. In Portugal, in the same year, the prevalence of this disease was about 1 million people between the ages of 20 and 79 (13,14). Adding to this, cardiovascular diseases remains the largest cause of death worldwide (8).

The treatment of a patient with a chronic wound is an economically expensive procedure. According to the World Health Organization, in the United Kingdom, the number of patients developing new ulcers in a year was estimated to be more than 100,000 people, which represents an annual expenditure between £168 million to £198 million, excluding subsequent related problems such as anxiety, depression, among others (15). In this way, it is increasingly important to find sustainable, innovative and viable solutions which are capable of reducing the treatment time, accelerate healing and reduce the physical and psychological suffering of these patients. It is also important to increase the success of treatments, to reduce the costs associated with the current solutions.

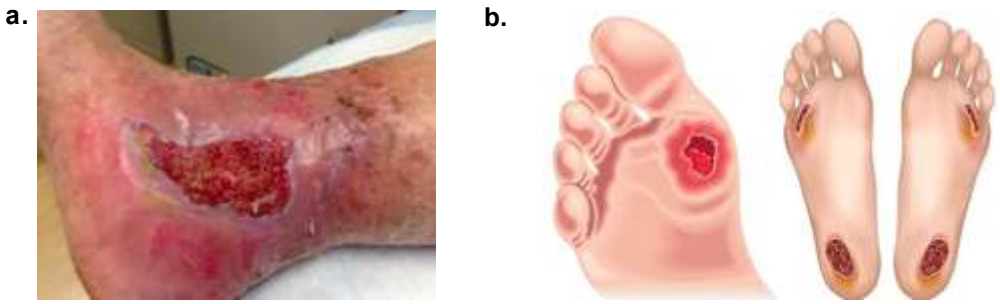

Figure 1.1 – Leg (a) and foot (b) chronic wounds examples (16,17).

2. Current clinical approaches to chronic wounds

Due to the rise of the morbidity associated to chronic wounds, wound care has become a topic increasingly important nowadays (18). Currently, the standard care for chronic wounds consists of swabbing for infection, leaning the wound, applying a dressing and, in some cases, debridement of the wound (18,19).

Generally, a wound dressing may be a single product or, in some cases, the combination of two or more layers of dressings, consisting of a primary contact layer and a secondary absorptive layer – not in direct contact with the wound. An ideal dressing is considered to be the one that maintains adequate humidity, remove the excess of wound exudate, permits thermal insulation, allows gaseous exchange, conforms to the wound surface, facilitates the debridement when necessary, minimizes the scar formation, is impermeable to extraneous bacteria, is non-fiber shedding/non-toxic, is non-adherent, comfortable and conforming (20). The use of wound dressings needs to be integrated into a general management plan, which must consider the different types of wounds and the problem that gave rise to them, and they should also be reviewed regularly with the progress of the treatment (20,21).

Dressings can be classified in several ways, according to their function in the wound, the type of material employed and the physical form of the dressing (22,23). Regarding their nature of action, dressings can be classified as passive, interactive and bioactive products (24). According to Willi Paul and Chandra Sharma (24), traditional primary and secondary wound dressings are included in the passive products classification, which include some components, such as gauze, lint, plasters, natural or synthetic bandages and cotton wool, and they're used mostly to protect the wound from contaminations. This type of dressings constitute the largest market segment (21,22). Interactive and bioactive products are included in the group of modern dressings and they are developed and designed not only for covering the wound, but also to facilitate their function and to deliver substances which are active in wound healing (24). This type of dressings and their classification is presented in Table 1, according to type, advantages, disadvantages and commercial name. (21).

Table 1. Types of modern dressings, their major functions and design.

Type of Dressing	Advantages	Wound type	Disadvantages	Commercial products	References
Alginate dressings	Can absorb 15 to 20 times their weight of fluid; suitable for highly exuding wounds; biodegradable; accelerate healing process by activating macrophages to produce TNF-α, which initiates inflammatory signals.	Useful in cavities and sinuses; undermining wounds; all wound types with high exudates.	Need for secondary dressing; need to be changed regularly; not suggested for dry wounds (adhere to healing wound surface, causing pain and damaging healthy tissue or removal), third degree burn wounds and severe wounds with exposed bones.	Algisite,Algosteril, Kaltostat,Melgisorb, SeaSorb, Sorbsan, Sorbsan SA, Tegagen,Urgosorb.	(21,25,26)
Antimicrobial dressings	-	Used in all locally colonized or infected wounds.	Drugs may not penetrate well into the wounds (due to poor blood flow and the presence of dead tissue); inappropriate use of systemic antibiotics can be associated with problems of allergy (due to the use of iodine), toxicity and the development of resistance in non-target organisms.	Acticoat, Actisorb Silver 200, Aquacel Ag, Arglaes, Avance, Inadine, Iodoflex, Iodosorb, Metrotop Gel.	(25–27)
Bioactive Dressings	Biocompatibility, biodegradability and non-toxic nature. Generally derived from natural tissue or artificial sources.	-	-	-	(21,25,26)
Foam dressings	Primary dressing for absorption; give degree of cushioning; transmit moisture vapour and oxygen and provide thermal insulation to the wound bed;	Flat, shallow wounds (control of exudate depending on type of foam); suitable for lower leg ulcers and	Need secondary dressing; not suitable for low exudating wounds, dry wounds and dry scars.	Allevyn Adhesive, Allevyn Cavity, Allevyn Lite Island, Allevyn Thin, Allevyn Plus Adhesive,	(21,25,26)

	highly absorbent; facilitate uniform dispersion of exudate throughout the absorbent layer; protect the area around the wound from further damage; may be left in place for two to three days.	moderate to highly exudating wounds; granulating wounds.		Allevyn Plus Cavity, Biatain Adhesive, Cavi-Care, Lyofoam Extra Adhesive, Tielle Plus, Tielle Lite, Tielle.	
Hydrocolloids	Can promote a moist wound healing; permeable to water vapour but impermeable to bacteria; absorbent; conformable; good in "difficult" areas – hell, elbow, sacrum; may be left in place for several days; useful debriding agent; highly absorbent; non-adherent; promote an autolytic debridement; reports to reduce wound pain; mostly used as a secondary dressing.	Cavity or flat shallow wounds with low to medium exudate (*e.g.* pressure sores, minor burn wounds and traumatic wounds); recommended for paediatric wound care management (don't cause pain removal).	May cause maceration; needs secondary dressing; not indicated for neuropatic ulcers.	Alione, Aquacel, CombiDERM, CombiDERMN, Comfeel Plus, Cutinova Thin, Duo DERM Extra Thin, Granuflex, GranuGel Paste, Tegasorb, Tegasorb Thin, Versiva.	(21,25,26)
Hydrogels	Supply moist environment to wounds with low to medium exudate; may be left in place several days; promote wound debridement by rehydration of non-viable tissue; soft elastic properties, provides easy application and removal after wound is healed without any damage; suitable for the four phases of wound healing, except	Suitable for sloughy or necrotic wounds; useful in flat wounds, cavities and sinuses; used for dry chronic wounds, pressure ulcers, burn wounds and chronic legs ulcers.	Need secondary dressing; may cause maceration; difficult to handle, because of their low mechanical strength.	Aquaform, Intrasite, GranuGel, Nu-Gel, Purilon, Sterigel.	(21,25,26)

	in infected and heavy drainage wounds; not irritant, no reactive, permeable to metabolites.					
Low adherent	Allow exudate to pass through into a secondary dressing while maintaining a moist wound bed; reduce adherence at the wound bed.	Useful for patients with sensitive or fragile skin.	-		-	(21,25,26)
Absorbent Membranes/Films	Absorb small amounts of wound exudate. Allow clinicians to observe a wound's progress without the necessity for removal due to their transparency.	-	-		OpSite ™ Post-Op Visible, Tegaderm Absorbent Clear Acrylic Dressing (3M).	(28)

Medicated Membranes/Films	Provides protection against infection and reduces bacterial load by a slowly release of iodine by the iodophor.	Applicable to cover and protect catheter sites and to secure devices to patient's skin.	-	Tegaderm Chlorhexidine Gluconate (CHG) IV Securement dressing (3M), Tegaderm ™ Plus (3M).	(28)
Polymeric Membranes/Films	Trap exudates, providing a moist environment. Impermeable to bacteria and liquid and permeable to moisture vapor and air.	Skin of surgical incisions.	Due to their non-absorbent characteristic, exudates can stay accumulate underneath the dressing. Seeping fluid pressure may cause a break in the environment maintained by the dressing.	Aluderm, Blister, Poly skin II, Silon-TSR, Tegaderm ™, Opsite.	(29)
Semipermeable Membranes/Films	Promote moist environment (permeable to air and water vapor); used mainly as a transparent primary cover; allow visual checks; may be left in the wound place several days; useful as secondary dressing (due to their high absorbancy and moisture vapour permeability); impermeable to fluids and bacteria.	Suitable for flat, shallow wounds with low to medium exudates; good for wounds in "difficult" anatomical sites (*e.g.* joints).	Adhere to healthy skin (but not to wound); cannot be used for infected or heavily exuding wounds; may cause maceration.	Bioclusive, Mefilm, OpSite Flexigrid, OpSite Plus, Tegaderm.	(21,25,26)

3. Membranes in wound healing

In the last decades, there have been some works proposing new membrane dressings, some of them, presented below, with particular attention for the application in chronic wounds. The first membrane developed was from a form of collagen obtained from the dried bladders of fish, in the 1880s (28). During the Second World War, Bloom (30) described the use of cellophane in burns patients (28). In 1948, Bull *et al.* (31) developed a nylon film dressing and, in 1950, Schilling *et al.* (28,32) conducted the first clinical trial with a film dressing. However, the most important assay was the one conducted by Winter and co-workers (33), in animals, in 1962. In 1963, Hinman and Maibach (24,28,34) have conducted a study in humans, which provided future support for the clinical use of membrane dressing. Both researches have demonstrated that a moist wound healing environment, created by film dressings, accelerated skin epithelialization twice as on wounds allowed to dry by exposure to air (24). From that year, membrane dressings were introduced under various brands (28).

According to Sussman *et al.* (28), a membrane dressing is indicated for the management of minor burns and simple wounds, such as scalds, abrasions, lacerations and lightly exudative wounds. The flexibility of these dressings gives them the ability to be applied to sutures and to continue to be used at the incision site, even after removal of the sutures or clips. Film dressings are especially good for reducing skin tension on flexor surfaces, to protect skin from shearing forces and to prevent and treat superficial pressure ulcers (28).

Ahmed and Boateng (35) developed antimicrobial membranes to deliver ciprofloxacin (CIP) for the treatment of bacterial infection in foot ulcers. Calcium alginate films loaded with ciprofloxacin were evaluated in their physico-chemical properties, such as porosity, swelling, equilibrium water content, water absorption, water vapor transmission, evaporative water loss, mechanical strength, adhesion, IR spectroscopy, scanning electron microscopy, X-rays diffraction, drug release, cytological and antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Different 1% w/v gels were dissolved in different solutions of sodium carbonate (0.005-0.028 M). In addition, different Glycerol (9.1, 20.0, 33.3, 42.8 and 50.0%), based on the total weight of the polymer (w/w), were added to the gels. After this, 20 g of each gel were dispensed into 86 mm diameter Petri dishes and dried (30°C) for 18 hours for obtaining the films. The drug was after loaded onto the optimized film containing 33.3% glycerol (w/w). The obtained films were soft, flexible and uniform, and their transparency was not affected by drug incorporation. They also verified that these membranes had showed potential tensile and bio-adhesive properties, which is required for an easy application of the dressing and to guarantee adherence to the wound bed. An optimal moisture environment and high biocompatibility with human keratinocyte cells was also observed. These results have confirmed that the design of biocompatible and effective dressings was successfully obtained, however, *in vivo* studies were required to be performed to confirm its effectiveness (35).

Chun-Hsu Yao *et al* (36) produced a bilayer membrane for wound dressing applications, with keratin extracted from human hair, blended with gelatin, sequentially electrospun onto a commercial polyurethane wound dressing. They verified that a gelatin/keratin blend solution can be successfully electrospun continuously, originating uniform and bead-free nanofibers, when appropriate

electrospinning parameters are used. The MTT cell viability assay showed that the residues released from the electrospun gelatin/keratin composite nanofibers enhanced cell proliferation. Fibroblasts showed a more favorable interaction with gelatin/keratin composite when compared to the gelatin mat. Animal studies revealed that the developed bilayer membranes promoted an earlier vascularization and a better skin wound healing. All of these results established the potential of the gelatin/keratin bilayer membranes as a wound dressing (36).

Ye Ma *et al* (37) proposed the production of a transparent flexible chitosan-based membrane dressing with antibacterial drugs by solvent casting method from suspension of chitosan floccules. Glycerol was used in different percentages in the matrix of the membranes to improve the mechanical properties and they verified that the introduction of this component as a plasticizer had a significant influence on the properties of the membranes. With the increasing of the concentration of glycerol, the swelling rate, water vapour permeability, wettability and tensile strength were improved significantly. The enzymatic degradation *in vitro* had showed that chitosan membranes had long-term stability and it was not compromised by the glycerol content. Tetracycline hydrochloride (TH) and silver sulfadiazine (AgSD) were added to improve the antibacterial properties of chitosan membranes and it was found that these membranes have a promising future in the treatment of bacterial infection – namely against *E. coli* and *S. aureus* – and, with the *in vitro* dermal fibroblasts seeded in both membranes, a significant higher viability during culture time of 1 to 3 days was observed. With this, it was possible to verify that the chitosan-based membranes with antibacterial agents could give a high therapeutic efficiency as a wound dressing. (37).

One of the demanding aspects when developing membranes for wound healing is the fact that there are no membrane dressings with the ability to combat the ROS produced in chronic wounds, combined with biocompatibility, biodegradability, good mechanical properties and non-cytotoxicity. In this sense, the development of novel multifunctional and bioactive dressings with the ability to aggregate all of these properties and respond to the challenges of a chronic wound is highly demanded.

3.1. Bioactive and multifunctional membranes

Recently, new strategies have been proposed to develop innovative wound dressings with the capability to enhance the healing process in chronic wounds. As explained in the previous sections, one of the aspects that characterizes this type of wounds is the excessive levels of ROS, which must be controlled by antioxidant agents, to guarantee the survival of the cells and, consequently, the regeneration of the wounds. Of all the types of dressings which have been investigated, bioactive and biofunctional membranes for wound healing applications are gaining interest.

Kavoosi *et al.* (38) investigated the improvement of the properties of a gelatin membrane incorporated with *Ferula assafoetida* essential oil (FAO) as a potential antioxidant and antibacterial wound dressing. These membranes were characterized regarding several physical-chemical properties, such as water solubility, swelling and water vapor permeability, mechanical behaviour and antioxidant and antibacterial activities. The results obtained in this study suggested that the incorporation of FAO in the gelatin membranes caused a significant decrease in swelling and an increase in the water vapor permeability and solubility. The tensile strength and elastic modulus decreased with the incorporation of FAO, while the elongation at break increased. The authors concluded that gelatin/FAO membranes could be used as active membranes for biomedical applications, including as a wound dressing material, since they showed exceptional antioxidant and antimicrobial characteristics, (38).

Rezvanian *et al.* (39) developed an alginate-based composite membrane for wound dressing applications, which had in its formulation simvastatin, a compound used in cardiovascular diseases for lipid lowering effects. These membranes were prepared and characterized based on their physical properties, as well as surface morphology, mechanical strength and rheology. The cytotoxicity and *in vitro* drug release results demonstrated that these membranes had appropriate wound dressing characteristics and high mechanical performance, as well as a controlled drug release profile and they were non-toxic for primary human dermal fibroblast cells, which made these membranes a good candidate for bioactive wound dressing. However, further *in vivo* investigations are required to prove the membrane toxicity and efficiency (39).

Silk-based membrane dressings have been proposed for wound healing. Srivastava *et al.* (40) developed a flexible silk fibroin membrane with dextrose (5-15% w/w) incorporated. Membranes were obtained by the solvent-casting method followed by crystallization with 80% methanol solution. It could be observed that the flexibility of the membranes increased with the increasing in the dextrose content and the elongation ate break increased from 3.2% to 40% with the increasing of this content in the membrane matrix. With this, it could be concluded that dextrose has acted as plasticizer for those membranes. FTIR and XRD studies showed that the dextrose content did not affect the crystalline structure of the silk fibroin membranes. SEM and AFM analysis showed that the surface roughness of these membranes also increased with increasing dextrose content and that this component enhanced the hydrophilicity and swelling capacity of the silk fibroin membranes. Degradation profile of the membranes was evaluated, showing a significantly higher mass loss than pure silk fibroin membranes after 50 days of incubation in Protease XIV. The adhesion, proliferation and viability of L929 fibroblast cells indicated that they had ability to support cell growth and proliferation when compared with pure silk

fibroin membranes. These dextrose modified films presented good potential to be used as dermal wound dressing material (40).

Karahaliloglu *et al.* (41) developed a nanostructured silk fibroin membrane for wound healing applications. For this, they started to modify the surface of silk fibroin membranes with NaOH alkaline treatment, in order to obtain a biological inspired nanofeatured surface morphology. Surface characteristics, such as roughness, energy and chemistry were evaluated. Skin-forming cells (keratinocytes) adhesion and proliferation were also studied to determine the promotion of an epidermal cover on the wound bed to form a new epidermal barrier, as well as dermal fibroblast adhesion and proliferation, in order to assess the capability of these membranes to replace injured dermal tissue in chronic wounds). The obtained results demonstrated that keratinocyte and fibroblast cell density was higher on the novel membranes compared with non-treated silk fibroin surfaces. The improvement in the cellular functions could be associated with a nanotopography induced by silk, wettability and a change in chemistry of this surface due to the NaOH treatment. With the obtained results, the developed nanofeatured silk fibroin membranes were considered a promising alternative for various skin reinforcement and wound dressing applications (41).

Xu *et al.* (42) improved the mechanical performance and the swelling ratio of chitosan membranes, properties that, until then, had limited their application in wound healing area. Thus, silk microfibers were incorporated in chitosan membranes, and its multiple physical properties were evaluated. By adding silk microfibers in the matrix, it was possible to verify that the mechanical properties were significantly improved, and the swelling ratio had decreased. SEM results have showed embedding of the microfibers and chitosan matrix, as well as connections among the silk microfibers. *In vitro* cytocompatibility was also evaluated with mouse fibroblasts of cell line L929, and it was showed significant cytocompatibility, demonstrated by cell proliferation and morphology. *In vivo* healing effects of these membranes were also evaluated on a fill-thickness skin wound rat model and it was possible to verify that the membranes containing silk microfibers exhibited increased wound healing efficiency when compared with pure chitosan membranes. By examining the histological changes, a higher level of epithelization and collagen formation in the chitosan membranes was verified with silk microfibers after 21-day repair period. These results indicated that the developed membranes might be a potential dressing for wound healing applications (42).

It is notorious the need to develop novel bioactive and biofunctional membranes with natural polymers. This strategy has been explored over the years but commercially, there are only few effective solutions. Being silk a natural biodegradable material with excellent mechanical properties, biocompatibility and antioxidant properties, it is expected to be a promising material to be applied on wound healing membranes. Thus, further studies are necessary for the development of this type of membranes, possibly able to combat the high levels of ROS of chronic wounds and facilitate healing, as well as to promote the regeneration of the damaged skin.

4. Main objective

The main aim of this work was the development, optimization and further characterization of new silk fibroin-based biofunctional membranes incorporating anti-oxidant agents to be applied in chronic wound healing, specifically in the stage of inflammation, to provide an efficient solution to combat the excessive levels of ROS, thus contributing to trigger the regeneration process.

A SF/glycerol (GLY) membrane system was produced, incorporating for the first time, caffeic and tannic acids (CA and TA, respectively), two well-known anti-oxidant agents with traditional application in the food sector.

CHAPTER 2

Materials and Methods

2.1. Materials and Reagents

2.1.1. Silk

Silk is a natural fibrous protein produced in specialized glands of various arthropods, that is spun into fibers during their metamorphosis phase. The silkworm *Bombyx mori* is the most extensively studied specie and the silk produced by this arthropod has been used commercially as sutures for biomedical applications and in textile industry for many years (43,44). Silk fibers are composed by two types of proteins: fibroin and sericin. Fibroin has a semi crystalline structure, providing strength and stiffness, and is the inner-core of the fiber. Sericin acts as an adhesive binder, forming an outer protective coating and maintaining the structural integrity of the fiber and cocoon (43,45). The structural components of *Bombyx mori* silk are illustrated in Figure 1.1.

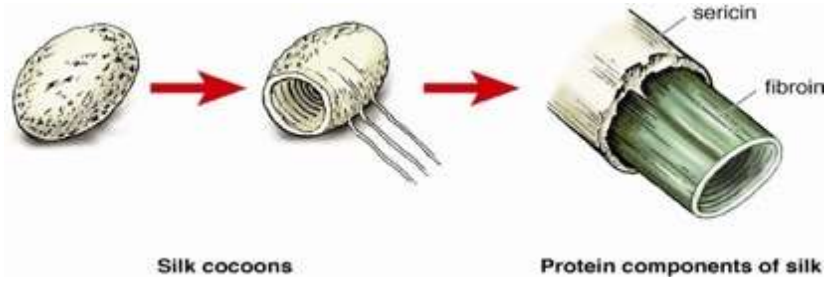

Figure 2.1.1. – Structural components of silk from *Bombyx mori* (43).

Silk fibers spun by silkworms hold excellent mechanical properties, such as high tensile strength, elongation at break and energy absorption (45). Silk is insoluble in most solvents (including water) and detailed structural analysis on silk proteins has yielded information on the orientation and organization of the small numerous β-sheet crystals in the fibers (43,46). This conformation of silk is commonly called Silk II – it's a mixture of noncrystalline and crystalline domains and provides a basis for the outstanding mechanical properties of silk. Nevertheless, the structure inside the middle silk glands is different and is named Silk I, which is the solid structure of silk fibroin (47).

In fact, the biological responses to the fibroin fibers appeared to be comparable to most other commonly used biomaterials (44). In the literature there is a clear evidence that silk is susceptible to proteolytic degradation *in vivo* and, over longer time periods, will slowly be absorbed (44). Due to the unique mechanical properties, biocompatibility and low immunogenicity advantages, these fibers have a large application in biomedical field, such as in the development of scaffolds for tissue engineering, coatings and drug delivery (45,48). Therefore, it constitutes a very interesting raw material for the development a functional wound healing dressing.

In the present work, cocoons from *Bombyx mori* were supplied by the Portuguese Association of Parents and Friends of Mentally Disabled Citizens (APPA-CDM, Portugal). All the remaining reagents were purchased from Sigma-Aldrich, unless otherwise stated. Lithium bromide was purchased from Honeywell, UK.

2.1.2. Glycerol

Glycerol, a derivative of natural and petrochemical raw materials. It is a viscous, colorless, odorless and water-soluble liquid with a wide range of applications, namely in medical and pharmaceutical preparations, due to its plasticizing action and low toxicity (49,50). Gly is systematically incorporated in film-forming solutions to prevent film brittleness, increase flexibility, workability and distensibility, and was used as a plasticizing agent in the developed formulations in this work (50,51).

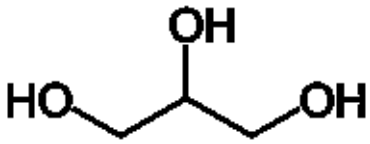

Figure 2.1.2. – Glycerol chemical structure (52).

2.1.3. Anti-oxidant molecules

The excessive level of ROS in chronic wounds is a serious problem that interferes with the healing process of these wounds. In this way, introducing antioxidant agents into these membranes may be favorable for the treatment of this type of wounds.

Tannic and Caffeic acids, two well-known molecules with anti-oxidant properties, were included in SF-based formulations. Tannic acid (TA) is a water-soluble polyphenolic compound which can be extracted from some fruits (*e.g.* grapes, pears and bananas), drinks (*e.g.* red wine, beer, coffee and black and green tea), lentils and chocolate (53,54). TA has shown antibacterial properties and UV-resistant activities, due to its polyphenolic structure. This compound has also shown high antioxidant activity, especially in the prevention of lipid oxidation, which makes it very useful in the biomedical area (55,56). Caffeic acid (CA), also a phenolic compound, is the result of the secondary metabolism of plants and is the main hydroxycinnamic acid present in the human diet. This acid is commonly found in various fruits and coffee beans, as well as in wine (57,58). Several studies have shown that CA has antioxidant capacity, as well as anti-inflammatory, antibacterial and anticancer properties which makes it attractive for use in treatment of chronic wounds (57).

Due to the natural origin and the high antioxidant potential of these two compounds, its introduction into the formulations of the membranes was seen as an added value.

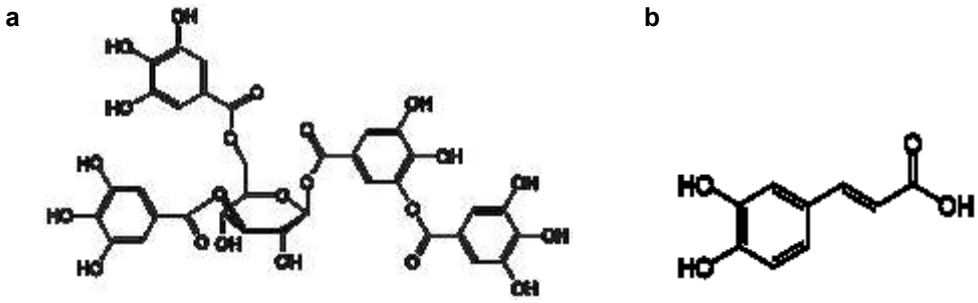

Figure 2.1.3. – Chemical structure of caffeic (a) and tannic (b) acids (59,60).

2.2. Preparation of silk fibroin aqueous solution

SF solution was prepared using a previously developed procedure (61). Briefly, for each extraction, 5 g of clean cocoons were cut into small pieces and boiled in 2 L of a 0.02 M sodium carbonate (Na_2CO_3) solution for 1 hour, under constant stirring. During this process, the outer layer of sericin was dissolved in the sodium carbonate solution, leaving a SF mesh available for further processing. In order to ensure that all the remnants of sericin were removed, the obtained fibroin was rinsed in 1 L of distilled water, under constant stirring, for 30 minutes. Lastly, the extracted fibroin mesh was dried, at room temperature, for 48 hours.

5 g of the dry pure silk-fibroin was then dissolved in 25 mL of a 9.3 M lithium bromide solution (LiBr) at 70ºC, with constant magnetic stirring, until complete dissolution. After that, the SF/LiBr solution was dialyzed for 48 hours against 5 L of distilled water, using a benzoylated dialysis tubing (length ~ 30 cm; molecular weight cut-off: 2000), in order to remove the salt. In the first day, the water was changed 1 hour, 2 hours and 4 hours, after starting the dialysis. In the second day, the water was changed 3 times, after regular time intervals. The purified SF solution was filtered using Whatman Filters (grade 1:11 μm – medium flow filter paper) and used in the same day. The concentration of SF after dialysis, using the described methodology, was previously determined by dry weight analysis (data not published) and is approximately 7% (w/w).

2.3. Preparation of SF-based membranes

Polymeric membranes have a huge applicability in different areas, especially in regenerative medicine (28,62). There are several techniques for producing membranes, such as solvent casting (63), layer-by-layer assembly (64) and electrospinning (65). However, solvent casting is the most used technique, namely for the production of silk fibroin membranes, because it enables the production of transparent films with uniform thickness distribution, at low cost and operational simplicity (63,66,67). This technique consists in dissolving a polymer on a solvent, casting this solution in a mold with the required geometry and then allowing for the solvent to evaporate under adequate conditions, leaving behind a membrane or a film with the same shape as the mold (63).

A control group of membranes containing exclusively SF was produced by casting 8 mL of the original solution into Petri dishes (100mm x 15mm) and drying at 85ºC, during 8h. Another control group of membranes, composed of 70% (w/w) SF and 30% (w/w) glycerol was prepared, by mixing the appropriate amount of both components, and leaving under constant stirring until the solution became homogenous. Then, 8 mL of that solution were casted onto Petri dishes and dried under the same conditions. Four experimental groups containing SF at 70% (w/w), glycerol at 29.5 % or 29 % (w/w) and either caffeic, or tannic at 0.5% and 1% (w/w) acid were produced following the same methodology. The respective formulations and sample designations are summarized in Table 2.

Table 2. List of the different formulations that were used and corresponding designation.

Sample Name	Formulation
SF	100% SF
SFGly	70% SF + 30% Gly
SF/Gly/CA05	70% SF + 29.5% Gly + 0.5% CA
SF/Gly/CA1	70% SF + 29% Gly + 1% CA
SF/Gly/TA05	70% SF + 29.5% Gly + 0.5% TA
SF/Gly/TA1	70% SF + 29% Gly + 1% TA

2.4. Organoleptic properties

The organoleptic properties of the membranes, such as color, transparency, smell, texture, and malleability, as well as their thickness and mass per unit of area, were evaluated. The organoleptic properties were evaluated by visual, olfactory and manual inspection. Mass and thickness were determined using an analytical scale (Mettler AE 200) and a digital micrometer (Adamel Lhomargy). Thickness was measured in 10 different points of the membrane, as suggested in Figure 2.4.1.

Figure 2.4.1 – Thickness measurement points in the samples.

2.5. Morphological characterization of the surface

2.5.1. Scanning Electron Microscopy (SEM)

The morphology of the films was analysed on a Leica Cambridge S360 (Wetzlar, Germany), by Scanning Electron Microscopy (SEM). The samples were fixed with mutual conductive adhesive tape on aluminium stubs and covered with gold/palladium using a sputter coater (Fisons Instruments, Sputter Coater SC502, UK) prior analysis and the micrographs were taken at an accelerating voltage of 15 kV at different magnifications (68).

2.5.2. Optical Profilometry

In order to assess and compare the surface roughness of the produced SF, SF/Gly, SF/CA and SF/TA membranes, the non-contact topographic characterization technique White Light Optical Interferometry (WLOI) was used. 3D surface maps were obtained using an optical profiling system Wyko-NT1100 (Massachusetts, USA), operating in Phase-Shifting Interferometry (PSI) mode and Vertical Scanning Interferometry (VSI) mode. PSI used a measurement range of 160 nm, while VSI used a measurement range of 2 mm. All images were analyzed using the WyconVision 32 software package and the average roughness (S_a) was obtained. The results were expressed as the average ± standard deviation of 3 samples.

2.6. Chemical characterization

2.6.1. Fourier Transformed Infra-Red spectroscopy with Attenuated Total Reflectance (FTIR-ATR)

In order to assess the chemical profile of the developed membranes, their spectra, as well as those of glycerol, caffeic acid and tannic acid were acquired using a FTIR spectrometer Nicolet 6700 (Thermo Scientific, United States of America) equipped with an Attenuated Total Reflectance (ATR) device. The software was programmed to record each spectrum between 4000 and 400 cm^{-1} at a resolution of 4 cm^{-1}. Samples and background (air) measurements were made by co-adding 32 scans. The analysis was performed in triplicate.

2.7. Degradation profile

The degradation profile of the SF-based membranes was conducted *in vitro*, for 15 days, based on the standard BS EN ISO10993-13:2009. Since chronic wounds are characterized by high levels of proteases and decreased protease inhibitors levels (69), two conditions were tested to assess the degradation of SF-based membranes: during incubation in PBS (negative control) and incubation in a solution of a protease in PBS, both at pH 7.4. Many proteolytic enzymes are used to digest silk-fibroin. However, protease XIV was contemplated to show high activity against beta-sheet structures in fibers, films and scaffolds of silk (70). Samples measuring 1.5 cm x 1.5 cm (n=3, weight ~ 14.86 mg) were weighted and immersed in either 1.5 mL of PBS, or in the same volume of a 0.01879 mg/mL of protease XIV solution in PBS (0.693 U mL^{-1}, derived from *Streptomyces griseus*), in order to obtain a ratio of 1U of protease/mg of silk (71). They were then incubated under orbital shaking (100 rpm) at 37°C, under sterile conditions, obtained by adding 0.2% of sodium azide (w/v) in the PBS solution. The samples were collected at predetermined time points (1, 3, 6, 8, 24, 48 and 72 hours, 7 and 15 days). The degradation medium was replaced every 2 days to ensure the activity of the enzyme. In each timepoint, the samples were removed from de solution, rinsed with distilled water and dried at 40°C overnight, until they reached a constant weight. After cooling at room temperature for 1 hour in a desiccator, the specimens were weighed (7). The degradation was expressed in terms of weight loss, and was determined following Equation 1:

$$(1) \quad \text{weight loss}\ (\%) = \frac{(w_i - w_f)}{w_i} \times 100$$

where, w_i is the initial weight of the sample and w_f is the weight of the sample after incubation in PBS or protease XIV solutions. The results were expressed as the average of three measurements ± standard deviation.

2.8. Mechanical Performance

A TA XT Plus Texture Analyzer (Stable Micro Systems Ltd., Surrey, United Kingdom) with tensile grips was used to determine the mechanical performance of SF-based membranes under uniaxial tensile stress. Films specimens were tested as suggested by Q. Sun et al. (2014) with some adaptations. SF, SF/Gly, SF/CA and SF/TA films were cut into strips (3 x 1 cm). Then, the thickness of each sample was measured with a Micrometer MI 20 (Adamel Lhomargy, France). The tests were conducted in dry and hydrated samples. In the latter case, the membranes were immersed in PBS for at least 3 hours, in order to assure that hydration equilibrium was achieved. In order to avoid the rupture of the membranes near the grips, or slippage, the edges of the samples were sheathed with two strips of paper held together with double sided adhesive tape and fixed between the grips. The distance between the grips was set to 10 mm and the crosshead speed was. 5 $mm.min^{-1}$. Young's Modulus (E) was calculated as the slope of the corresponding stress-strain curve. Ultimate tensile strength (UTS)

and elongation at break (ε) were obtained from the stress *vs* strain plots (72). The ultimate tensile strength (MPa) was calculated according to Equation 2:

$$(2) \quad \text{UTS (MPa)} = \frac{\text{Maximum load (N)}}{\text{Cross sectional area } (mm^2)}$$

The percentage of elongation at the break was obtained according to equation 3:

$$(3) \quad \varepsilon\ (\%) = \frac{\text{Sample length at break (mm) - Initial sample length (mm)}}{\text{Initial sample length (mm)}}$$

The results were expressed as the average of six measurements ± standard deviation.

2.9. Thermal properties

2.9.1. Thermogravimetry and Different Thermal Analysis (TG-DTA)

Thermogravimetry (TG) and Differential Thermal Analysis (DTA) were used to study the thermal properties of the films.

TG is a measurement of the rate of mass loss plotted against temperature and it is used for degradation evaluation, while DTA allows to measure the differences between the temperature of the sample and a reference, and it is used to ascertain phase changes in a sample. However, although these two techniques can be applied separately, each of the methods does not always give sufficient information. In this way, both techniques can also be applied simultaneously, in the same sample, at the same time. They work, in this way, as complementary techniques, but the optimal conditions may be different for both methods – *e.g.* the highest sensitivity for DTA experiment is achieved at high heating rates while, in TG, the best resolution is reached at low rates (73).

Simultaneous TG/DTA of each sample was carried out under a nitrogen flow rate of 50 mL/min, with the temperature ranging, from 30 to 350ºC, at a heating rate of 10ºC/min, using a Scansci-Hitachi TG/DT 7200 Exstar equipment. Each sample was cut so as to present a minimum volume that could fill the aluminum crucible (n = 3, weight ~ 3 mg). Three samples were analyzed for each condition under study.

2.10. Antioxidant capacity – ABTS assay

In order to determine the antioxidant activity of the produced films, an improved ABTS-based assay was performed. This method was adapted from Gião et al. (74). The ABTS solution was prepared by adding 7 mmol. L^{-1} of ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Sigma-Aldrich, Germany) to 2.45 mmol. L^{-1} potassium persulfate (Merck, Germany) solutions. The obtained solution was stored overnight in the dark for 16 hours, for the reaction to occur. This solution was subsequently diluted in ultrapure water to provide a final absorbance in the range of 0.700 ± 0.020. The absorbance of this solution was evaluated in a UV spectrophotometer.

For the analysis of the samples, a specific volume was added (v = 10 μL) to 1 mL of ABTS, in order to obtain an inhibition percentage between 20 and 80%, during 6 minutes of reaction. The average of 3 replicates was used. The total antioxidant capacity was obtained according to the following equation, expressed in percentage of inhibition (PIAC):

$$(5)\quad PIAC = \frac{Abs_{ABTS} - Abs_{sample}}{Abs_{ABTS}} \times 100$$

Where Abs_{ABTS} is the inicial absorbance of diluted ABTS and Abs_{sample} is the absorbance of the sample after the 6 minutes reaction. A calibration curve was made in the same day, with ascorbic acid (as a

standard) solutions with known concentrations. The results were expressed as equivalent concentration of ascorbic acid, I $g.L^{-1}$. From equation (5), the results were normalized relative to the membrane mass present in the 10 microliters.

2.11. Cytotoxicity tests

For the evaluation of the cytotoxicity of the produce film dressings, an assay based on the international standard ISO 10993-5 was conducted, *in vitro*, by direct contact and extract tests.

Cell culture and seeding

Immortalized mouse fibroblast cell line (L929), with 39 passages, were maintained at 37ºC in a humidified atmosphere containing 5% CO_2 in culture medium supplemented with 89% of MEM-alpha, 2mM of glutamine, 10% of fetal bovine serum and 1% of antibiotic. Culture medium was removed, and 5 mL of pre-warmed PBS was added to wash the cells, in order to remove dead cells and cellular debris. To detach the cells from the culture flaks, an enzymatic digestion with of trypsin was made. 2 mL of TrypLE Express (Gibco) was added, and the flask was incubated for 5 minutes at 37ºC in 5% CO_2 atmosphere. After this time, 5 mL of culture medium was added to neutralize the effect of trypsin. The flask was observed under the microscope to guarantee that cell detachment occurred. The obtained cell suspension was centrifuged (5 minutes, 1200 rpm) and the obtained pellet was resuspended in 10 mL of culture medium, adjusting cell density to $1x10^5$ cells/ml. The cells were subsequently seeded ($2x10^4$ cells/well) in a 96-well tissue culture plate and incubated for 24 hours (37ºC, 5% CO_2 atmosphere), to ensure cell recovery, adherence and progression to exponential growth phase. After 24 hours of incubation, culture medium was removed, and cells were washed twice with 200 μL of PBS, in order to remove dead cells and cellular debris. After this, direct contact and extract tests were performed at the same time.

Materials preparation

Membranes were sterilized by ethanol sterilization method. Samples were placed in ethanol solutions with different concentrations (90%, 50%, 10% and 0%) for 10 minutes in each solution. The use of a decreasing concentration of ethanol is to assure the elimination of traces of ethanol from the samples, in order to prevent cell death.

Extract tests

Extracts of each material were made based on ISO 10993-5. For films with irregular thickness, 6 cm^2 of each sample were added per mL of culture medium used. Samples were placed in falcon tubes containing culture medium and were introduced in an oven at 37ºC for 24 hours in a 5% CO_2 air atmosphere. A positive control, in which the cells were incubated with culture medium containing 20% of Dimethyl Sulfoxide (DMSO), a negative control in which the cells were incubated with regular culture medium and an extract control in which cells were incubated with regular culture medium heated for 24 hours were prepared. Three extract solutions with different concentrations were used: 100% extract, 50% and 30% (v/v) of the original extract diluted in culture medium original. The culture medium was removed, 200 μL of each extract were placed in each well and incubated for 24 hours at 37ºC in a 5% CO_2 air atmosphere.

Direct contact test

For the direct contact test, samples with 4 mm^2 area (10% of the well surface) were sterilized as previously described. The culture medium was removed and 200 μL of fresh culture medium were placed in each well. Samples of each membrane were carefully placed on the cell layer of each well and incubated for 24 hours at 37ºC in a 5% CO_2 air atmosphere.

MTT assay

For MTT assay, after 24 hours treatment, the culture medium was removed, and the cells were washed with 200 μL of PBS. 200 μL of MTT solution (0.5 mg/mL) were placed per well and the plate was incubated for 2 hours at 37ºC in a 5% CO_2 air atmosphere. After this time, the MTT solution was removed and 200 μL of pure DMSO was added per well. The samples were protected from light and absorbance was read using a microplate spectrophotometer at 570 nm and 630 nm. Three independent replicates were performed with n = 3 per replicate. The percentage of cell viability was calculated by normalizing the results relative to the negative control.

2.12. Statistical analysis

GraphPad Prism software was used for statistical analysis. The differences between the groups under study were analyzed for statistical significance by employing one-way ANOVA ($n > 6$), non-parametric tests ($n < 6$) and repeated measures ANOVA. A *p*-level inferior to 0.05 was considered to be significant.

CHAPTER 3

Results and Discussion

3.1. Organoleptic properties

Film wound dressings were designed to be in close contact with the injured tissue in superficial and shallow wounds with low exudate (62,75). When designing these devices, there are several requisites that need to be met in order to guarantee their good performance and safety. Along with specific chemical and physical attributes that will be discussed later in this chapter, the organoleptic properties play an important role on the applicability of this type of product. The first approach of clinicians and patients to a wound dressing will be through the senses, so, the general aspect of the product odor and its handling capability will have a significant contribution to its general acceptance (26).

In this study, SF-based membranes containing either caffeic or tannic acids were produced by solvent casting and thermally crosslinked at 85 °C for 8 hours. After that, the samples were cooled at room temperature until the equilibrium moisture content was reached, carefully removed from the molds and evaluated by visual, olfactory and manual inspection. The samples were then weighted, the thickness was measured, and the mass/area ratio was determined. The organoleptic properties of the membranes, as well as the thickness and the mass/area ratio are summarized in Table 3.

Table 3. Organoleptic properties and some physical aspects of SF-based membranes.

Formulation	Membrane	Color	Transparency	Texture	Malleability	Smell	Thickness (mm)	Membrane mass/Area of membrane (mg/mm²)
SF		Colorless	Transparent	Smooth and homogeneous	Not malleable (very brittle)	Odorless	0.180 ± 0.337	8.22
SF/Gly		Light yellow	Transparent	Smooth and homogeneous	Malleable	Odorless	0.063 ± 0.014	7.39
SF/Gly/CA05		Yellow	Transparent	Smooth and homogeneous	Malleable	Odorless	0.044 ± 0.031	6.87
SF/Gly/CA1		Brigth yellow	Transparent	Smooth and homogeneous	Malleable	Odorless	0.074 ± 0.043	7.88
SF/Gly/TA05		Light brown	Transparent	Smooth and homogeneous	Malleable	Odorless	0.039 ± 0.018	7.17
SF/Gly/TA1		Strong brown	Transparent	Smooth and homogeneous	Malleable	Odorless	0.079 ± 0.032	8.17

The developed membranes were transparent, odorless, with a soft coloration and presented a homogeneous surface with a smooth texture. These features are quite interesting for the proposed wound healing application. The transparency of the developed membranes will enable direct monitoring of the wound without dressing removal, which minimizes costs and patient discomfort. Wound malodor is a very important aspect for the clinical assessment of wounds, as it may be indicative of bacterial infection in the wound bed and can help to identify and manage the causing agents (76). Introducing odor to wound dressings may mask the wound odor and create an added difficulty in its assessment. Therefore, it is important that the developed membranes do not have odor, so as not to camouflage the odor of the wound. Colour is also an important aspect on the wound assessment, once it can indicate which process is occurring inside the wound. For instance, a red-coloured wound indicates the presence of granulation tissue, whereas black coloration shows the existence of necrotic tissue (26). Therefore, it is important that the dressings do not misrepresent the true colour of the wound, so that it is possible to more easily detect the wound stage without removing the dressing, in order to better match the treatment to be applied. SF membranes are colorless. However, SF/Gly membranes presented a very discrete light-yellow tonality. The incorporation of 0.5% and 1% CA led to a less subtle yellow tone and the incorporation of 0.5% and 1% TA led to membranes with a brown tonality. Although the incorporation of antioxidants caused the emergence of moderate levels of either yellow, or brown tones in the membranes, that coloration is discrete enough to guarantee proper color assessment of a wound without dressing removal (Table 3).

The handling capability is a crucial feature of a wound dressing. These devices must be easy to manipulate and adapt to the wound shape, for optimal functionality and patient comfort (26,77). SF membranes were very brittle and presented handling challenges, starting by the detachment from the molds. The remaining membranes were very pliable, easily detached from the molds and easily handled. With the addition of glycerol to the silk matrix, a remarkable improvement in the malleability of the dressing was promoted. Gly is a plasticizing agent and the main role of these additives in a material is to increase its flexibility (50). As a consequence, these substances reduce the deformation tension and hardness of the materials, and, at the same time, increase polymer chain flexibility and resistance to fracture (50). This plasticizer is commonly used in the manufacturing of polymeric membranes, in order to render them less brittle and it is recognized as a safe substance by Food & Drug Administration (50,78). This qualitative evaluation of membrane malleability was complemented with a quantitative assessment of the mechanical performance of the membranes, as presented in section 3.5.

Lastly, the thickness of the membranes was measured in the dry state, in 10 different points of each membrane, as described in section 2.4. The results are summarized in Figure 3.1.1 and Table 3.

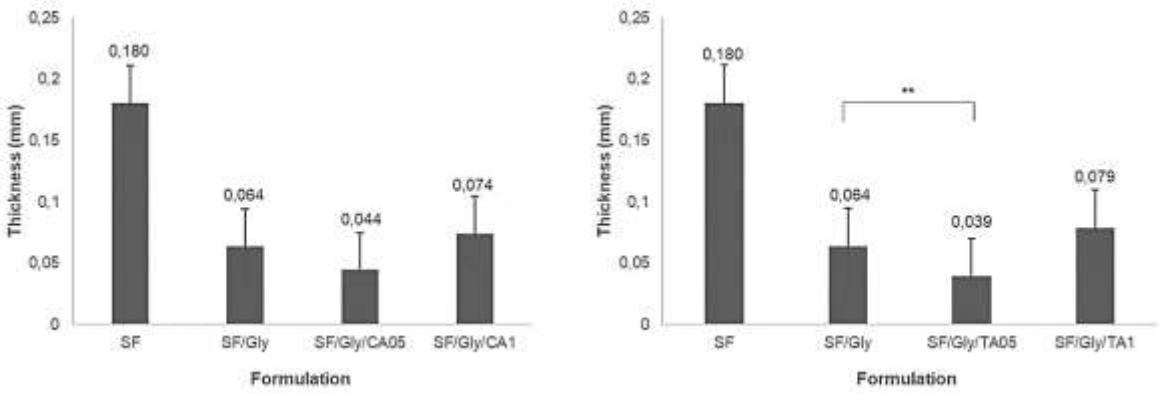

Figure 3.1.1 – Thickness of dry membranes measured in 10 different points of the silk-based membranes (n = 3, Kruskal-Wallis test, P = 0.0061, * – significant differences).

The thickness of the membranes, in the dry state, ranged between 0.039 and 0.180 mm. There were significant differences between SF/Gly and SF/Gly/TA05 membranes. Nevertheless, it should be noted that there is a wide variability in the thicknesses of the membranes. This may be related to the conditions they are subjected to the heat treatment (*e.g.* uncontrolled drying atmosphere, non-homogeneous molds), as well as the conditions in which the cooling process of these materials occurs (*e.g.* drying at room temperature in closed Petri dishes, protected from light). A possible approach to circumvent this variability could be to place the Petri dishes in a desiccator without the caps, during the cooling process, in order to avoid the condensation process on the surface of the membranes and thus better homogenize the surface thickness.

The appropriate thickness of the membranes for wound dressing application will depend on the type of wound and the amount of exudate present in the wound.

3.2. Morphologic characterization of the surface

As referred in the previous section, film wound dressings are designed to be in close contact with injured tissue, in shallow wounds with low exudate. Therefore, an in-depth analysis of the surface of the dressing is important to guarantee its adequate performance. The morphological characterization of the surface was performed by SEM analysis and Optical Profilometry, in order to assess the effect of the addition of glycerol and antioxidant agents (CA and TA) on the micro- and nanostructure of the SF membranes.

3.2.1. Scanning Electron Microscopy (SEM)

SEM analysis provided relevant information about the micro- and nanostructure of the membranes, as presented in Figure 3.2.1 and Figure 3.2.2.

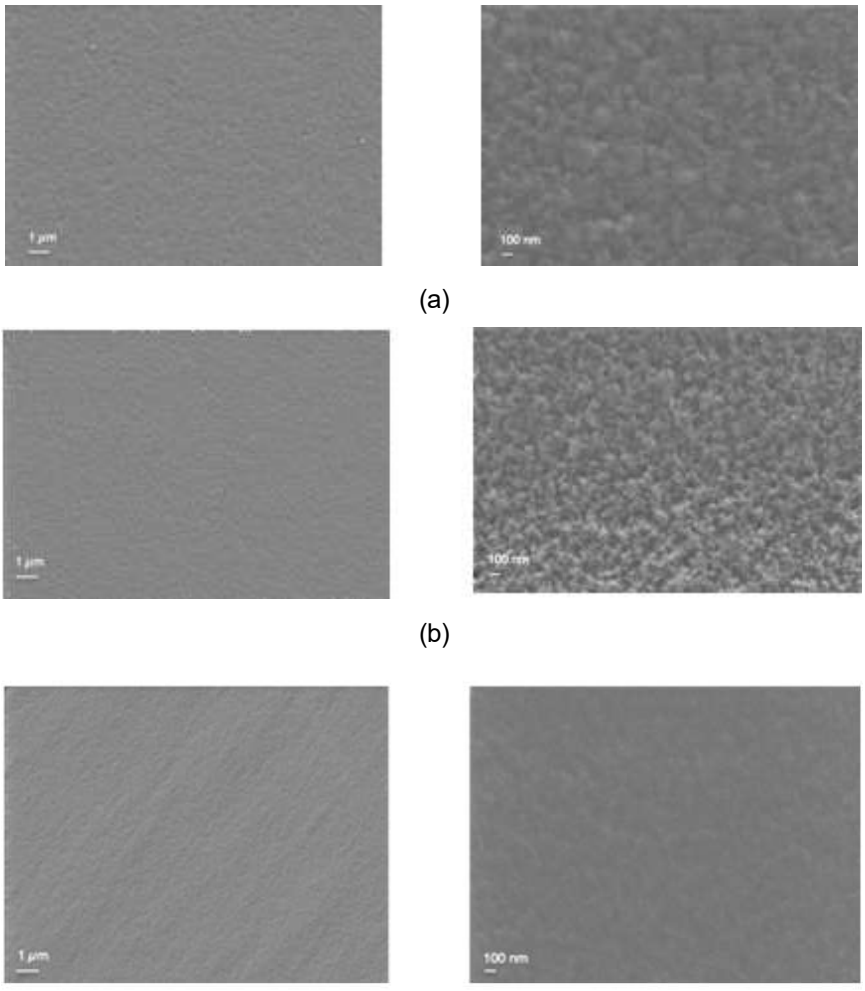

Figure 3.2.1 – SEM images of (a) SF, (b) SF/Gly and (c) SF/Gly/CA05 membranes (Magnifications of 500x and 20000x).

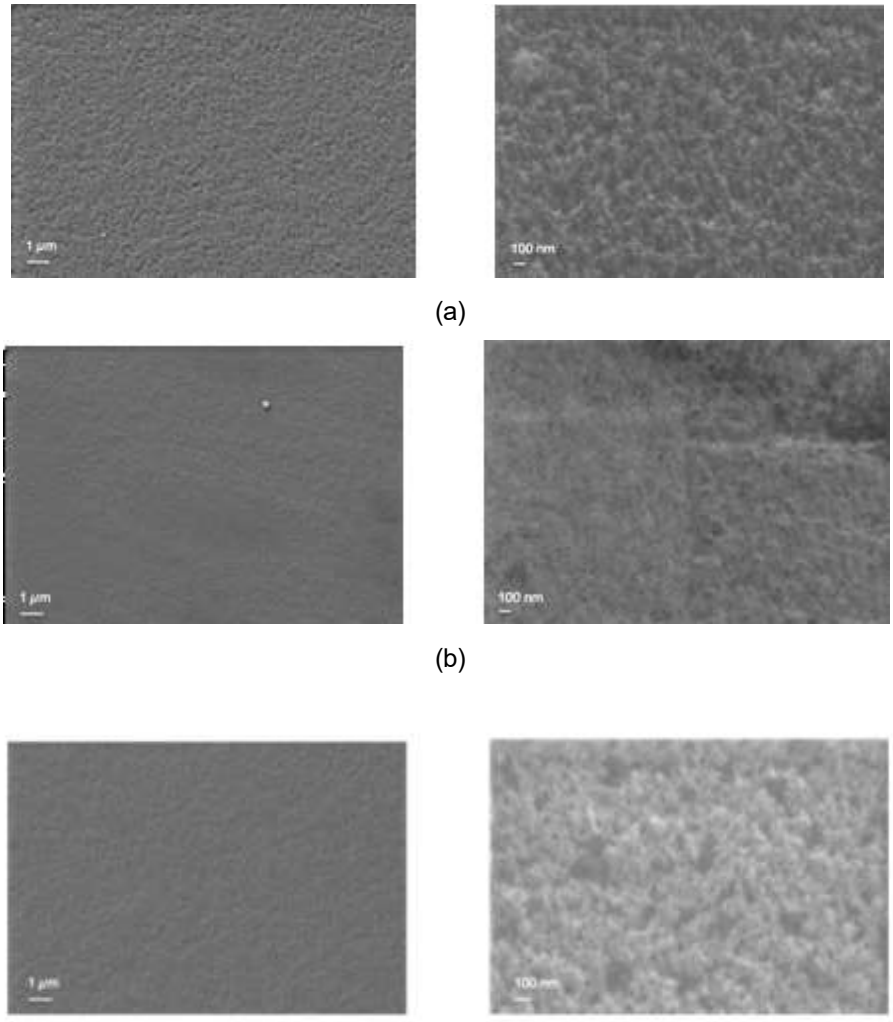

Figure 3.2.2 – SEM images of (a) SF/Gly/CA1, (b) SF/Gly/TA05 and (c) SF/Gly/TA1 membranes (Magnifications of 500x and 20000x).

The use of a low magnification (500x) enabled to assess the microtopography of the samples. All the membranes display a homogeneous surface, with no apparent roughness in the micrometric scale, which corroborates the macroscopic analysis performed on the organoleptic properties. The use of a high magnification (20 000x) allowed to visualize the nanotopography of the membranes surface, which shows a small roughness. Although extremely important, this analysis is merely qualitative and does not allow to assess for any nanotopographic differences in the evaluated samples. In order to do so, a quantitative analysis was performed by optical profilometry, as presented in the next section.

3.2.2. Optical Profilometry (OP)

Representative 3D surface maps showing the nanostructure of the surface of the membranes are presented in Figure 3.2.3. The images corroborate the results obtained by SEM and further suggest that introducing glycerol in the membrane formulations leads to an increase in the surface roughness, characterized by the presence of more red regions in the respective maps.

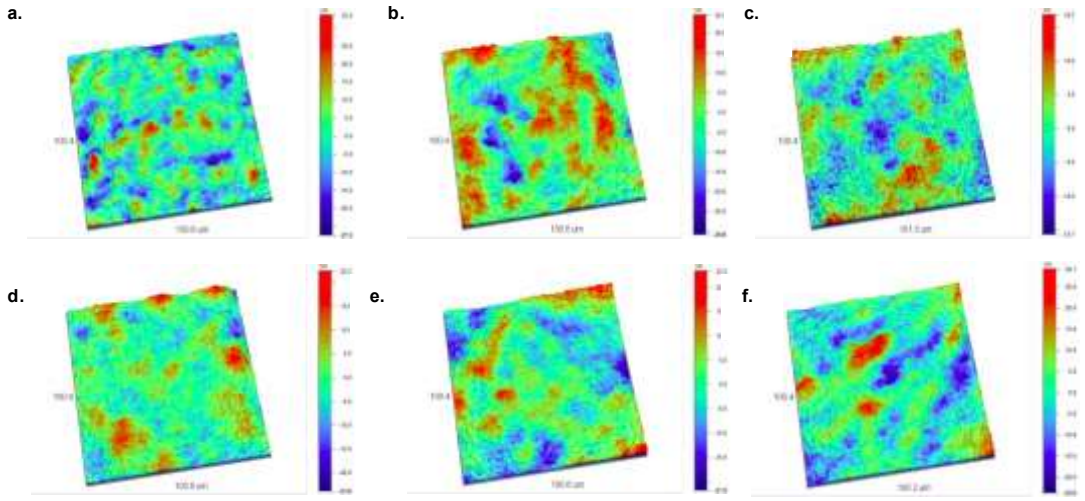

Figure 3.2.3. – OP nanostructure images of SF (a), SF/Gly (b), SF/Gly/CA05 (c), SF/Gly/CA1 (d), SF/Gly/TA05 (e) and SF/Gly/TA1 (f) membranes.

This observation is supported by the quantitative analysis of the average roughness (S_a), as presented in Figure 3.2.4.

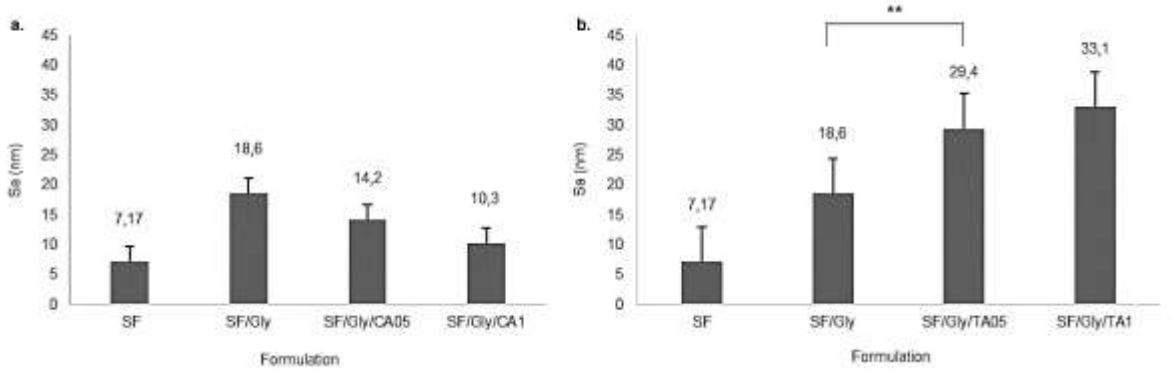

Figure 3.2.4. – Average roughness of the SF and SF/GLY membranes incorporating 0.5 and 1% of caffeic acid (a), tannic acid (b) obtained by optical profilometry (n = 3, Kruskal-Wallis test, P = 0.0078, * – significant differences).

The average roughness ranged from 7 nm in SF membranes, to 33 nm in SF/Gly/TA1 membranes. Despite the evident roughness increase in the formulations containing glycerol, statistical differences were only observed between the values obtained for SF and SF/Gly/TA05 films ($P = 0.0078$). Most likely, a higher sample number would have enabled to discriminate statistical differences between other experimental groups and the control group. It has been reported that protein-based films require the addition of plasticizers to reduce its fragility, and that the use of plasticizing agents like glycerol in the production of thin membranes leads to an increase on surface roughness (79). Other studies have demonstrated that nanotopographic features in silk-based membranes similar to the ones obtained in this work are adequate for the development of natural-based wound dressings (80–82).

3.3. Mechanical Performance

The evaluation of the mechanical properties of a wound dressing is of extreme importance in order to predict its mechanical behavior in the clinical application context, as well as to help to define how the clinical team can handle safely these dressings. It is also important to know its ability to resist the stresses to which it will be exposed to during handling and in the physiological wound healing environment. In this study, the mechanical properties of the obtained silk fibroin-based membranes were investigated by performing uniaxial tensile tests. Figure 3.3.1 demonstrates distinct mechanical behaviors for all membranes developed. Young's Modulus (E), Ultimate Tensile Strength (UTS) and Elongation at Break (ε) parameters were investigated.

The mechanical performance of the membranes was evaluated both in dry and hydrated states. The distinction in the physical state of the membranes was made to predict their behavior when stored and manipulated by clinicians (dry state), as well as when applied to a wound (hydrated state).

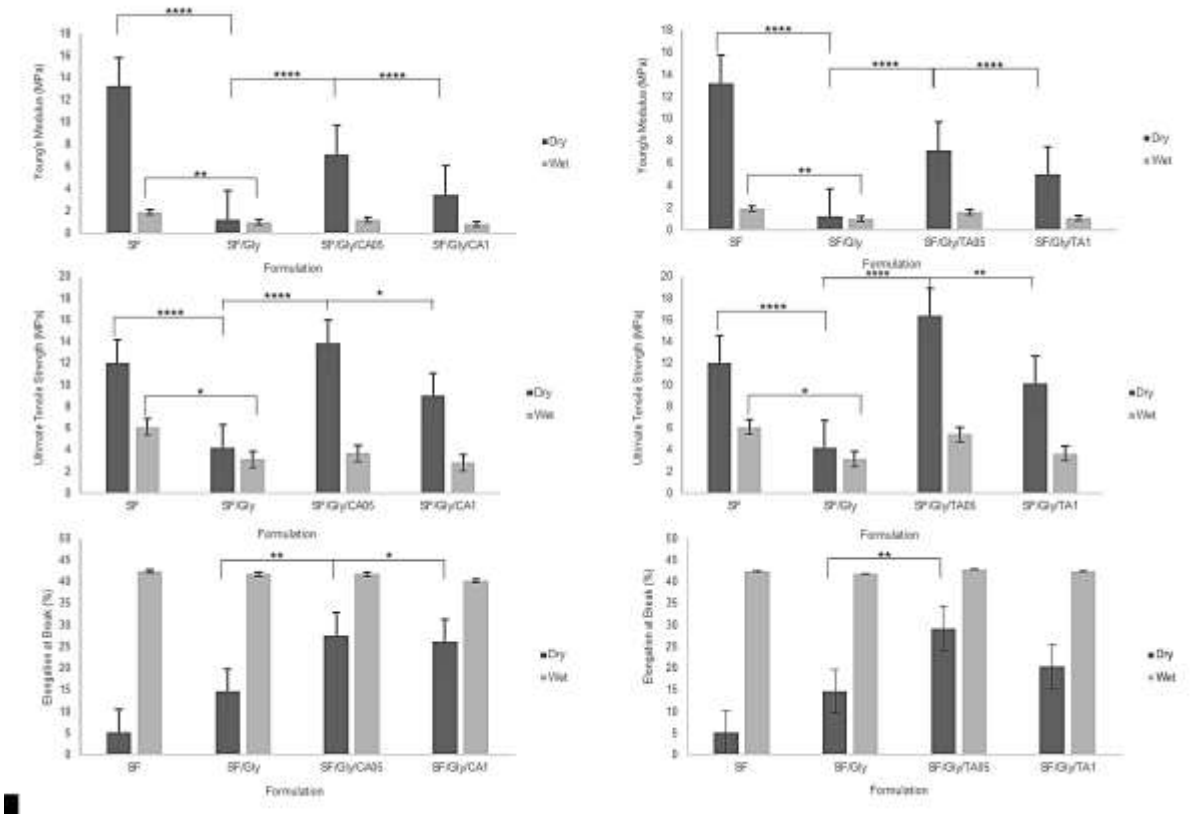

Figure 3.3.1 – Young's Modulus (E), Ultimate Tensile Strength (UTS) and Elongation at Break (ε) parameters of all membranes, in dry and wet state (n = 10, One-way ANOVA test, P = 0.0078, * – significant differences).

In the dry state, SF membranes demonstrated a significant difference in the Young's Modulus, which decreased with the introduction of glycerol in the membrane's matrix. The UTS decreased while an increase was observed for the ε. The introduction of Gly into the matrix of SF membranes has reduced its stiffness, which makes them more malleable, flexible and resistant to fracture, as described in section 3.1. According to Sobral (83), the mechanical properties of films produced by solvent-casting method depends strongly on the type and concentration of plasticizers, as glycerol (83,84). It has been reported that Gly makes SF membranes more ductile than membranes of pure silk (85). This phenomenon occurs, because when Gly is added into SF solution before the thermal treatment, hydroxyl groups in glycerol form hydrogen bonds with hydrophilic polar groups in SF macromolecules and water molecules. With these interactions, a network structure between SF and glycerol is created and the elongation at break of the films increases (86).

When antioxidant agents were incorporated in the SF/Gly membranes matrix, significant differences were observed between E, UTS and ε of both CA and TA. However, there were no significant differences in ε between SF/Gly/TA1 and SF/Gly membranes. CA membranes showed an increase in all parameters when compared with SF/Gly membranes. Although, 1% CA membranes showed lower values when compared with 0.5% ones. TA membranes showed the same behavior as CA ones, with the exception of ε parameter in 1% membranes. In this case it can be supposed that the antioxidants added to the matrix of SF/Gly membranes exerted an anti-plasticizing effect, opposing to what was

observed to glycerol, since they made the membranes more rigid and resistant. In section 3.1, it could be verified an increase in the thickness of the membranes when the incorporation of the antioxidant agents in the SF/Gly matrix. However, 0.5% TA membranes showed lower thickness when compared with the SF/Gly membranes. Thicker membranes may be more rigid. Though, membranes that have a lower thickness may have a more fragile behavior due to higher the contribution of the surface properties, which corroborates the mechanical results obtained.

In the wet state, SF membranes demonstrated a decrease in E and UTS values with significant differences when compared with SF/Gly membranes. The ε value increased, but without statistical significance. With hydration, the membranes became more flexible and resistant. It has been reported that water acts as a mobility enhancer (87). Gly and water molecules create hydrogen bonding interactions frequently (84). However, during silk protein conformational shift toward β-sheet formation, a disruption in the hydrogen bonding occurs and hydrogen bonding sites become available for interaction by water molecules in aqueous media (84). Therefore, active and available hydration sites are reactive with water molecules and it has been reported that this result has significant implications for the formation of less brittle silk materials (84). Minoura *et al.* (88) described an increase in softness of silk-fibroin based membranes when in presence of water, which suggested the plasticizer effect of water in these membranes. In that study it was also suggested that silk fibroin membranes become more elastic with the increase of absorbed water by the membranes (88). Results similar to the ones obtained in this study for the hydrated SF/Gly membranes were described by *Shenzhou et al.* (89). The addition of the antioxidant agents in the SF/Gly membranes, in wet state, did not show significant differences on their mechanical performance when compared with the SF/Gly membranes.

Healthy skin is known to have a high degree of elasticity, as compared to other biological tissues. In this way, Young's Modulus of the skin is an important parameter to evaluate the skin characteristics (90). It has been reported that, for tensile tests, E values for healthy skin are between 25 kPa and 140 MPa. Despite E values of the membranes developed in this work being lower than skin's values, when they are compared with the developed non-commercial dressings, they are in the same range. Is important to note that, although the developed membranes are not a skin substitute, it is essential that their properties are similar to the skin properties. If this is verified, the dressing may be better adapted to the wound. It is also important to ensure that the properties of the developed membranes are also similar to the ones of the commercial dressings.

UTS and ε values obtained in this work are similar to the ones reported in other studies of non-commercial wound dressings, which can be a possible validation for these membranes to be applied as a skin wound dressing (37,39,79). These membranes more malleable than skin which can be an advantage to allow for a better manipulation during the clinical application (42,91).

3.4. Fourier transform infrared attenuated total reflectance (FTIR-ATR) spectroscopy

In this study, Fourier Transformed Infrared Spectroscopy (FTIR) was performed to understand the chemical structure of SF membranes and to evaluate possible effects from the blending with Gly and further incorporation of CA (Figure 3.4.1 (a)) and TA (Figure 3.4.1 (b)).

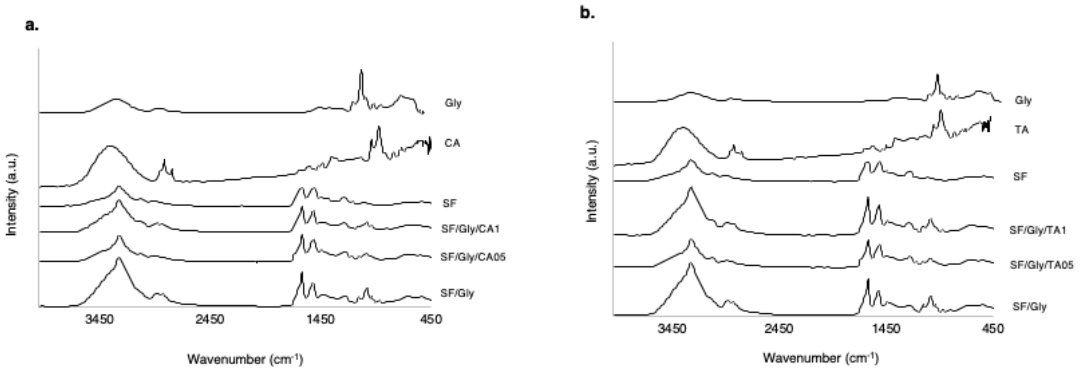

Figure 3.4.1. – FTIR spectra of the SF and SF/GLY membranes incorporating 0.5 and 1% of caffeic acid (a), tannic acid (b).

Glycerol contains an O-H bending at 920 cm^{-1}, C-O and C-H stretching at 1000 and 1100 cm^{-1}, respectively, a C-O-H bending around 1400-1460 cm^{-1}, C-H stretching between 2880 and 2930 cm^{-1} and a —OH band at 3000-3500 cm^{-1} (92,93). Caffeic acid is characterized by the presence of hydroxyl and carbonyl functional groups. The vibration of the —OH groups attached to benzene ring are located at 1200-1270 cm^{-1} and the C=O stretching peaks are generally observed at 1600-1700 cm^{-1} (94). Tannic acid presents a strong absorption around 3450 and 3000 cm^{-1}. This band is due to a varied hydrogen bonding between OH which are consigned to the stretching vibrations of the —OH groups (95).

The amide bands are the more informative infrared bands to analyze proteins. Therefore, Amide I vibrations are associated to C-O stretching (1600-1700 cm^{-1}), amide II vibrations describe the bending of N-H bond related with C-N stretching (1520-1540 cm^{-1}), and amide III vibrations characterize the combination of C-N stretching vibration with the N-H deformation (1220-1300 cm^{-1}) (96). In addition, the molecular conformations of protein materials are described by random coil absorption peaks at 1650 cm^{-1} (amide I), 1540 cm^{-1} (amide II), and 1230 cm^{-1} (amide III) and β-sheet absorption peaks around 1630 cm^{-1} (amide I), 1520 cm^{-1} (amide II), 1270 cm^{-1} (amide III) (97). In SF membranes, the presence of β-sheet conformation was confirmed due to the peaks detected at 1625 cm^{-1} and 1521 cm^{-1}. In caffeic and tannic acid membranes β-sheet conformation was also present. Therefore, the incorporation of the acids in the silk-based matrix and further thermal crosslinking did not affect the expected chemical structure, i.e., it was not possible to observe any shift in the amide I, II and III.

3.5. Degradation profile

Some studies have focused on the characterization of acute and chronic wounds, in order to understand the processes that transform an acute cicatricial wound into a chronic wound (11). These studies have led to the hypothesis that diabetic chronic wounds (*e.g.* diabetic foot wounds) often cannot heal due to high concentrations of pro-inflammatory cytokines in the wound that induce high concentrations of proteases (such as matrix metalloproteinases), which degrade multiple growth factors, receptors and matrix proteins that are essential for wound healing process, when they are not in a balanced concentration (11,98).

Matrix metalloproteinases are the enzymes that naturally degrade extracellular matrix proteins and they are classified into different classes based on their *in vivo* substrate specificity (99). Within each class, these metalloproteinases recognize specific peptide sequences. Protease XIV, a serine protease, recognizes some of the sequences which lie within the amino acid profile of the silk fibroin heavy chain and it can degrade both crystalline and non-crystalline domain of silk, and these was the main reason that it was applied in this assay (85,100,101).

Macroscopic evaluation of the degradation of the samples incubated in PBS and Protease XIV media, up to 15 days, is presented in Figure 3.5.1.

Figure 3.5.1 – Macroscopic evaluation of the samples before and after incubation in either PBS, or protease XIV (derived from *Streptomyces griseus*, 0.693 $U.mL^{-1}$), up to 15 days.

In Figure 3.5.1 it is possible to observe that the integrity of all the membranes was preserved throughout the 15 days, in PBS. The same is not observed in membranes that have been exposed to the action of Protease XIV. SF and SF/Gly/CA05 membranes maintained their integrity throughout the 15 days. However, this observation does not mean that degradation did not occur, since they may have undergone erosion degradation (102), which does not immediately lead to membrane disintegration. The remaining membranes suffered disintegration at the end of different timepoints. 1% CA membranes disintegrated after 24 hours. SF/Gly and 0.5% TA membranes have disintegrated after 72 hours. 1% TA membranes have disintegrated after 7 days, which made them more resistant to the action of this enzyme when compared with the previous ones. With the analysis of Figure 3.5.2, the results obtained in the degradation tests can be better understood.

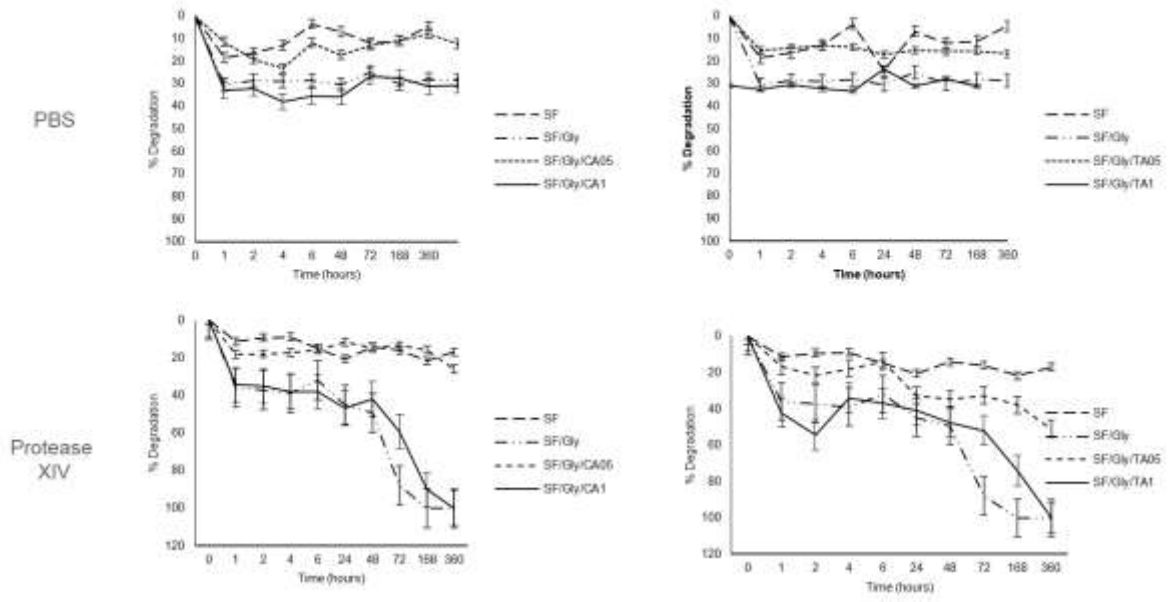

Figure 3.5.2 – Degradation profile of the samples incubated in either Phosphate Buffered Saline (PBS), or protease XIV (derived from *Streptomyces griseus*) (0.693 $U.mL^{-1}$), up to 15 days.

It is possible to observe that the mass of SF membranes remained constant throughout the 15 days of incubation in PBS. However, SF/Gly, SF/Gly/CA05, SF/Gly/CA1, SF/Gly/TA05 and SF/Gly/TA1 membranes have shown a decrease in the initial mass of approximately 30%, after the first 2 hours of incubation. In order to assess the significance of the observed differences, a repeated measures ANOVA was performed, and it revealed statistical differences ($P < 0.0001$) between the different timepoints of each membrane. After that initial loss, the weight of the samples remained constant until the end of the study. This may be related to the release of the Gly to the degradation medium, since the lost mass corresponds to the percentage of Gly present in the formulations of the films. This is in agreement with other study were it was reported that the silk membranes lost the majority of the mass corresponding to the percentage of glycerol in the first hour of incubation, during the degradation assay (85). It has been reported that polar solvents displace glycerol from the silk materials and almost all of the glycerol in the silk blended materials is extracted when incubated in water which corroborates the

obtained results (84). With the release of Gly from the membrane's matrix, its mechanical properties modify. Gly reduces film fragility, confers some plastic properties and reduces the mechanical strength of materials, as previously mentioned (50). However, this is not verified in the mechanical tests. Therefore, the results obtained in this assay corroborate the fact that water may be acting as a plasticizer when the 30% Gly is exited after 1 hour of incubation. In order to better evaluate the interaction of Gly with the SF matrix, it would be important to carry out release studies with the resulting solutions from the different timepoints of the degradation assay. The introduction of 0.5% of TA in the matrix of the membranes has shown to confer some resistance to degradation. Studies related with the structure and mechanisms of silk degradation and with the *in vitro* degradation of silk fibroin have confirmed the stability of SF membranes in PBS, which corroborates the results obtained in this study (102,103).

With the addition of Protease XIV, it is possible to observe the same initial mass loss of approximately 30%, followed by a large loss after 24 hours of incubation. In order to assess the significance of the observed differences, a repeated measure ANOVA was also performed, and it revealed statistical differences ($P < 0.0001$) between the different timepoints of each membrane. Due to the existence of β-sheet conformation in the silk membranes, they become more rigid, which makes their degradation difficult to occur, which makes SF films more stable (100). With the introduction of Gly and TA in the matrix, the membranes become more susceptible to degradation by Protease XIV. The mechanical tests demonstrated that the introduction of TA increased the resistance of the membranes. However, the introduction of 0.5% TA in the matrix of the SF/Gly membranes made them less thick, which may interfere with their physical resistance. Less thickened membranes may be subject to faster degradation, which corroborates the results obtained. Possibly due to the release of the antioxidant agents from the matrix, this output could function as an easier way for the degradation mechanism to occur, because it creates more space for the enzyme to penetrate the sample. It should be noted that although there was a large degradation observed with Protease XIV, this behavior does not illustrate what necessarily happens *in vivo.* Horan *et al.* (102) demonstrated that the complex *in vivo* environment specific for a type and location of the wound is essential to determine the rate of degradation (102). However, Numata *et al.* (70) reported that the degradation by the Protease XIV is faster than other types of enzymes present on wound exudates (70). The authors had also demonstrated that the fast degradation observed in beta-sheet crystals of silk by Protease XIV is not present in the human body, which leads to believe that the results obtained in this study of degradation do not invalidate the use of the developed silk membranes as wound dressings for periods of 4 days, or more (70).

3.6. Thermal properties

3.6.1. Thermogravimetry and Different Thermal Analysis (TG – DTA)

TG – DTA is an important method for the determination of the decomposition temperature/steps and kinetics parameters for solid materials (104).

The results obtained are presented in Figure 3.6.1.

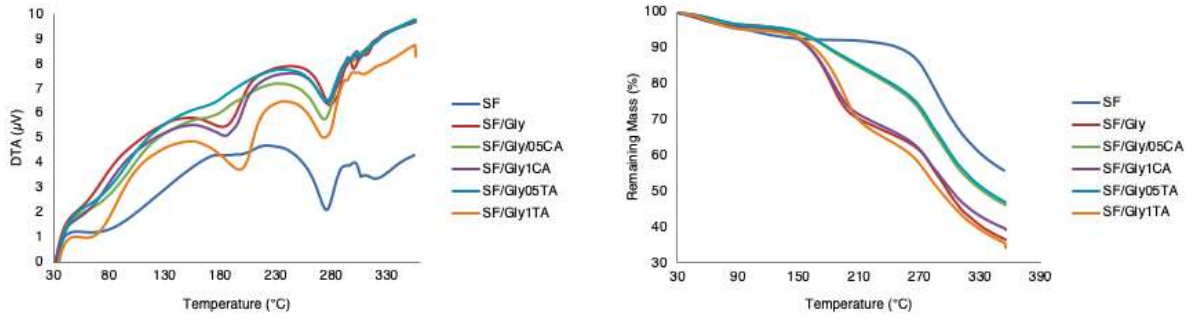

Figure 3.6.1. – TG and DTA curves for SF, SF/Gly, SF/Gly/CA05, SF/Gly/CA1, SF/Gly/TA05 and SF/Gly/TA1 membranes, from 30 to 330ºC.

With the analysis of DTA and TG curves, an inflection point around 95ºC can be observed which can be associated to the evaporation of water (105). Around 280ºC, an endothermic peak is observed, which corresponds to the thermal decomposition of silk (105). The mass loss due to the evaporation of Gly starts at 120ºC (106) and its evaporation starts around 211ºC (84). In the region of 180ºC – 250ºC, a gradual decrease in mass is observed with the increasing of the temperature. It has been hypothesized that a variety of chemical interactions that glycerol may establish with silk matrix, resulting in a gradual evaporation (84). A decrease in thermal resistance of SF is observed with the introduction of Gly in the membrane matrix. Gly seems to be the major contribution to the differences observed in the thermal profile of SF membranes. With the addition of 0.5% of the antioxidant agents, it is verified an increase in thermal resistance and the opposite is observed in the presence of 1% of these compounds. These results can be related to the roughness and the mechanical performance of the membranes. There is an increase in roughness for greater amounts of antioxidants, with a loss of mechanical properties, which may be related to the formation of some internal microporosity, which may have some consequences in the thermal behavior of the samples. With a high surface area (due to an increase of roughness), greater will be the thermal degradation. The analysis of cross section by SEM would have been useful to verify the porosity of the samples.

SF/Gly/TA05 membranes, when in the presence of PBS solution, increase the resistance to degradation of the SF matrix. With an increasing of the resistant to degradation, these membranes have gained thermal resistance. On the other hand, at the mechanical level, membranes with 0.5% of CA and TA revealed to have a higher stiffness, which can also confer a higher thermal resistance.

3.7. Antioxidant properties – ABTS assay

The obtained values for the antioxidant activity of the different produced membranes are plotted in Figure 3.7.1, ranging from 0.08 mg Ascorbic Acid Eq/mg membrane in SF films, to 0.13 mg Ascorbic Acid Eq/mg membrane in SF/Gly/TA05 membranes. SF membranes showed antioxidant capability Other studies have also demonstrated the antioxidant capacity of silk fibroin (107,108). Kruskal-Wallis multicomparisons test only revealed statistical differences between the values obtained for SF and SF/Gly/TA05 membranes ($P = 0.0369$). With the results obtained in this study, it was found that the addition of antioxidants, in particular of TA at 0.5% w/w, to the silk-fibroin matrix helped to improve the antioxidant effect of the SF membranes.

Since wounds dressings are designed to cover a determined area of wound, and the interactions between the dressing and the wound will be governed by the area of exposure, besides the antioxidant capacity per mass unit, it is also important to assess the antioxidant capacity per unit of area. Therefore, the ratio between the values of mass of Ascorbic Acid Equivalent per area of membrane was calculated. The values are plotted in Figure 3.7.1, ranging from 0,010 mg Ascorbic Acid Eq/cm^2 membrane in SF films to 0,019 mg Ascorbic Acid Eq/cm^2 membrane in SF/Gly/CA05 membranes. A one-way ANOVA test only revealed statistical differences between SF and SF/Gly membranes, and SF/Gly and SF/Gly/CA05 membranes ($P < 0.0001$). This result may be related to the higher thickness present in SF membranes when compared to SF/Gly membranes, which increase the surface area of the films. With the addition of 0.5% CA in SF/Gly matrix, the antioxidant capability per area of membrane also increased. With the obtained results, it was found that the addition of Gly and 0.5% CA to the matrix of SF membranes also helped to improve the antioxidant effect of the silk-based membranes.

Other studies have confirmed the antioxidant activity of SF and TA by the ABTS method (53,107,108). However, the results of these studies were not related to equivalents of ascorbic acid, therefore it is not possible to compare the final results and it will not be possible to compare the antioxidant activity of the developed membranes with others already produced. However, in order to verify if the obtained results for the antioxidant activity changes with the effect of the thermal treatment to which the membranes are subjected, it would be interesting to test the antioxidant activity of the preparation solutions of the membranes in their liquid state by the ABTS method. Another possible solution would be quantifying the antioxidant activity through other method, more commonly used, such as ORAC.

Although it is not possible to compare the antioxidant activity obtained in the developed membranes with others already available, the fact that these membranes have an antioxidant potential is a characteristic that makes them possible to be used as wound dressings, since they can have great benefits in the healing process of chronic wounds.

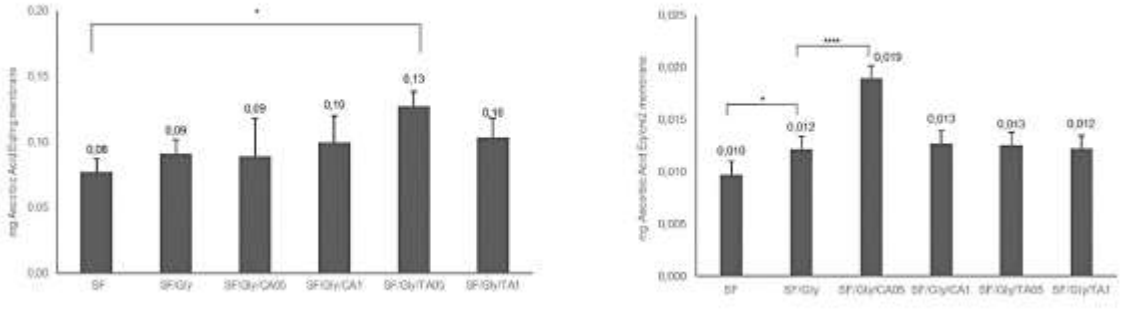

Figure 3.7.1 –Results of (a) mg Ascorbic Acid Eq/mg membrane (n = 6, Kruskal-Wallis test, P = 0.0369, * – significant differences) and (b) mg Ascorbic Acid Eq/cm^2 membrane (n = 15, One-way ANOVA test, P < 0.0001, * – significant differences) obtained by the ABTS assay.

3.8. Cytotoxicity tests

Assessing the potential cytotoxic effects of biomedical devices is crucial. Potentially hazardous effects arising from the biomaterial itself or from any additives used during film manufacturing have to be dismissed before pre-clinical studies may be considered. Figure 3.8.1 shows the results obtained when both films extracts, and film samples were incubated with L929 cells for 24h.

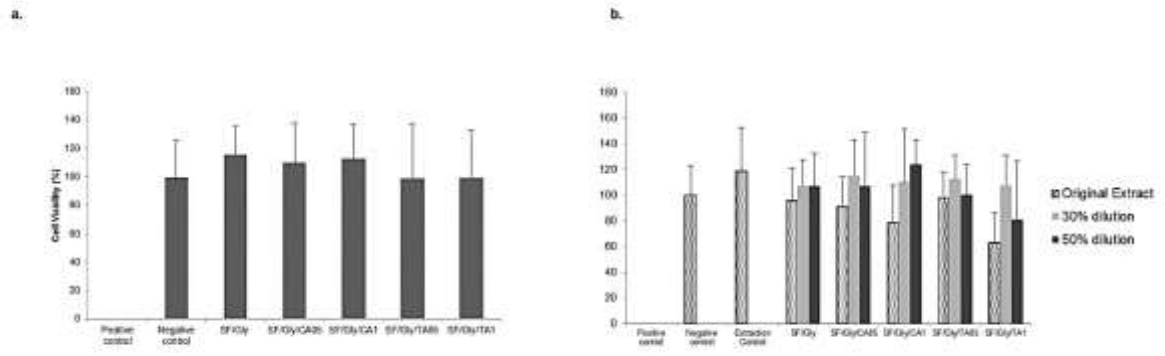

Figure 3.8.1 – Cell viability results for the membranes (a) and for the extracts (b) in L929 fibroblasts after the 24 hours of incubation.

For both direct and extracts tests, statistical analysis revealed no significant changes between the cell viability of the negative control and all the other tested conditions for the membranes (One-way ANOVA, P < 0.0001). No cytotoxic effects were observed during the period of study for the film samples

of all the formulations. For extract tests, a large decrease in cell viability of the pure 1% TA extract was observed. According to the international standard ISO 10993-5, a cell viability lower than 70% indicates that there is cytotoxicity, which makes this concentration of TA cytotoxic to the cell type used in this study. Sahiner *et al.* (109) reported the cytotoxic behavior of TA particles. When applied for 24 hours at a concentration of 75 $\mu g.mL^{-1}$, this compound has shown to be cytotoxic to L929 cells. The authors also concluded that the cytotoxic effect of this compound is dependent on the concentration of the particles. This leads to believe that the pure 1% TA extract may have revealed a cytotoxic effect for L929 cells, because of the high concentration of this compound in the extract. Despite the results obtained for 1% TA, all the other tested extracts, regardless of the used concentration, did not cause a decrease in cell viability, evidencing no toxic effects towards L929 cells.

The obtained results in both tests are quite encouraging and establish a first validation regarding the safety of these films, and their application as wound dressings. Once film wound dressings can stay in place for a week (28), this study should be repeated for a period of 7 days to evaluate the effects of longer exposure.

CHAPTER 4

Conclusions

In the present study, the development of novel SF-based stable membranes incorporating caffeic and tannic acids for the treatment of superficial chronic wounds was successfully accomplished. The results obtained for the organoleptic and mechanical properties are suitable for the function for which these membranes were designed to and comparable with the results found in the literature for the dressings available commercially. The malleability of the membranes can facilitate membrane application by the health professionals, in a clinical context. The transparency of the films allows the evaluation and the analysis of the wound without removing the dressing, which can promote the patient comfort and reduce the costs associated with the treatment. Since odor is an important characteristic in the evaluation of chronic wounds, the fact that the membranes were odorless is an advantage, since the odors emanated from chronic wounds are not misrepresented. The increase in the concentration of the antioxidant agents in the SF/Gly membranes caused a change in the mechanical properties of the membranes, because an anti-plasticizing effect was observed, which made the membranes more rigid and resistant. The degradation profile with Protease XIV showed that SF membranes were the more resistant to degradation. With the presence of Gly and the increasing in the concentration of the antioxidant agents, the SF-based membranes became more susceptible to degradation with Protease XIV. Gly decreased the thermal resistance of the membranes. 0.5% of CA and TA increased this property while the presence of 1% of these compounds showed the opposite. SF membranes showed antioxidant capability and the addition of Gly, 0.5% CA and 0.5% TA helped to improve the antioxidant effect. The antioxidant activity also decreases with the increase to 1% of the antioxidant agents in the matrix of the SF-based membranes. This behavior leads to hypothesize that the introduction of increasing concentrations of the antioxidant agents in SF-based membranes may induce nanoporosity, and this feature may be compromising the physicochemical properties of the membranes, in general. The antioxidant capacity observed in the membranes is a promising feature in combating the high levels of ROS present in chronic wounds, as it helps to prevent cell death and consequent wound regeneration. The cytocompatibility of the membranes is also a promising aspect in the application of these membranes as wound dressings. Despite pure 1% TA extract revealed a cytotoxic effect for L929 cells, the membrane samples of all the formulations and the remaining extracts revealed no cytotoxic effects, which is a quite encouraging result and can establish a first validation for the safety and application of these films as wound dressings. The developed membranes presented characteristics that make them interesting for the application in superficial chronic wounds, suitable to be used as a wound dressing. However, other physico – chemical parameters (described in the following section) need to be evaluated until they can be proceeded to preclinical tests.

Future work

In order to assess the full functionality of the developed materials, more physical and biological tests should be performed. Within the physical tests, the study of the water vapor permeability of the membranes, hydration degree with wounds exudates, adhesion tests using pig skin models and release studies in wound exudates should be performed. Further structural and morphologic characterization tests should be performed, such as contact angle, AFM, XPS and NMR to have a better surface characterization of the dressings. Within the biological tests, an *in vitro* oxidative stress assay should be performed in order to evaluate the antioxidant capacity of these membranes in cells exposed to oxidative stress, as well as cytotoxicity tests with longer timepoints (3 to 7 days). *In vivo* tests in mice models should be performed for a better evaluation of the applicability of these films as a possible wound dressing.

COMUNICATIONS

Fernandes, A. (2018, June). *Antioxidant membrane dressings for the treatment of chronic wounds*. Oral communication presented at the Congress "IV Jornada Científica de IBEROS", Lugo, Spain.

Fernandes, A., Baptista-Silva, S., Borges, S., San Román, J., Oliveira, A.L., (2017, May). *Antioxidant Silk Fibroin/Tannic Acid Dressing for the Treatment of Chronic Wounds.* Poster presented at the Congress "Frontiers in Polymer Science", Seville, Spain.

Borges, S., Fernandes, A., Baptista-Silva, S., Alves, P., Oliveira, A.L., (2016, November). *Novel biofunctional membranes with antioxidant and antimicrobial potential for the treatment of infected chronic wounds.* Poster presented at the Congress "APTFeridas 2016", Porto, Portugal.

Borges, S., Geão, C., Fernandes, A., Baptista-Silva, S., Alves, P., Oliveira, A.L., (2016, September). *Development of new biofunctional membrane systems for tissue healing and regeneration*. Poster presented at the Congress "2nd Symposium on Science and Technology of Life and Health" of the Universidade Católica Portuguesa, Porto, Portugal.

REFERENCES

1. Robson, M. C., Steed, D. L., & Franz, M. G. (2001). Wound healing: biologic features and approaches to maximize healing trajectories. Current problems in surgery, 38(2), 72-140.
2. Velnar, T., Bailey, T., & Smrkolj, V. (2009). The wound healing process: an overview of the cellular and molecular mechanisms. Journal of International Medical Research, 37(5), 1528-1542.
3. Singh, S., Young, A., & McNaught, C. E. (2017). The physiology of wound healing. Surgery (Oxford), 35(9), 473-477.
4. Lazarus, G. S., Cooper, D. M., Knighton, D. R., Margolis, D. J., Percoraro, R. E., Rodeheaver, G., & Robson, M. C. (1994). Definitions and guidelines for assessment of wounds and evaluation of healing. Wound Repair and Regeneration, 2(3), 165-170.
5. Robson, M. C. (1997). Wound infection: a failure of wound healing caused by an imbalance of bacteria. Surgical Clinics of North America, 77(3), 637-650.
6. Stacey M. Why don't wounds heal? Wounds Int. 2016;7(1):16–21.
7. Järbrink, K., Ni, G., Sönnergren, H., Schmidtchen, A., Pang, C., Bajpai, R., & Car, J. (2017). The humanistic and economic burden of chronic wounds: a protocol for a systematic review. Systematic reviews, 6(1), 15.
8. Järbrink, K., Ni, G., Sönnergren, H., Schmidtchen, A., Pang, C., Bajpai, R., & Car, J. (2016). Prevalence and incidence of chronic wounds and related complications: a protocol for a systematic review. Systematic reviews, 5(1), 152.
9. Nunan, R., Harding, K. G., & Martin, P. (2014). Clinical challenges of chronic wounds: searching for an optimal animal model to recapitulate their complexity. Disease models & mechanisms, 7(11), 1205-1213.
10. Frykberg, R. G., & Banks, J. (2015). Challenges in the treatment of chronic wounds. Advances in wound care, 4(9), 560-582.
11. Lobmann, R., Ambrosch, A., Schultz, G., Waldmann, K., Schiweck, S., & Lehnert, H. (2002). Expression of matrix-metalloproteinases and their inhibitors in the wounds of diabetic and non-diabetic patients. Diabetologia, 45(7), 1011-1016.
12. Stanley, A., & Osler, T. (2001). Senescence and the healing rates of venous ulcers. Journal of vascular surgery, 33(6), 1206-1211.
13. Sociedade Portuguesa de Diabetologia (2016). Factos e Números–O Ano de 2015− Relatório Anual do Observatório Nacional da Diabetes 2016. Lisboa: SPD.
14. World Health Organization. Diabetes [Internet]. 2017. Available from: http://www.who.int/news-room/fact-sheets/detail/diabetes
15. Phillips, C. J., Humphreys, I., Fletcher, J., Harding, K., Chamberlain, G., & Macey, S. (2016). Estimating the costs associated with the management of patients with chronic wounds using linked routine data. International wound journal, 13(6), 1193-1197.

16. Couture, M. (2015). Does continuous diffusion of oxygen have potential in chronic diabetic foot ulcers. Podiatry Today, 28(12).
17. Ramasamy LD. Diabetic Foot & Chronic Wounds. [cited 2018 Nov 21]. Available from: http://www.srishticosmeticsurgery.com/Chronic-wounds.php
18. Han, G., & Ceilley, R. (2017). Chronic wound healing: a review of current management and treatments. Advances in therapy, 34(3), 599-610.
19. Dreifke, M. B., Jayasuriya, A. A., & Jayasuriya, A. C. (2015). Current wound healing procedures and potential care. Materials Science and Engineering: C, 48, 651-662.
20. Vowden, K., & Vowden, P. (2017). Wound dressings: principles and practice. Surgery (Oxford), 35(9), 489-494.
21. Medeni, Ö. Ç. (2015). Acute Effect of Kinesiotape Muscle Technique on Hamstring Flexibility and Pain During Stretching. Türk Fizyoterapi ve Rehabilitasyon Dergisi, 26(2).
22. Boateng, J. S., Matthews, K. H., Stevens, H. N., & Eccleston, G. M. (2008). Wound healing dressings and drug delivery systems: a review. Journal of pharmaceutical sciences, 97(8), 2892-2923.
23. Falabella, A. F. (2006). Debridement and wound bed preparation. Dermatologic therapy, 19(6), 317-325.
24. Paul, W., & Sharma, C. P. (2004). Chitosan and alginate wound dressings: a short review. Trends Biomater Artif Organs, 18(1), 18-23.
25. Medeni, Ö. Ç. (2015). Acute Effect of Kinesiotape Muscle Technique on Hamstring Flexibility and Pain During Stretching. Türk Fizyoterapi ve Rehabilitasyon Dergisi, 26(2).
26. Borda, L. J., Macquhae, F. E., & Kirsner, R. S. (2016). Wound dressings: a comprehensive review. Current Dermatology Reports, 5(4), 287-297.
27. Halstead, F. D., Rauf, M., Bamford, A., Wearn, C. M., Bishop, J. R., Burt, R., Webber, M. A. (2015). Antimicrobial dressings: Comparison of the ability of a panel of dressings to prevent biofilm formation by key burn wound pathogens. Burns, 41(8), 1683-1694.
28. Queen, D. (2010). Technology update: Understanding Hydrofiber® Technology. Wounds international, 1(5), 29-32.
29. Mir, M., Ali, M. N., Barakullah, A., Gulzar, A., Arshad, M., Fatima, S., & Asad, M. (2018). Synthetic polymeric biomaterials for wound healing: a review. Progress in biomaterials, 1-21.
30. Bloom, H. (1945). 'CELLOPHANE'DRESSING FOR SECOND-DEGREE BURNS. The Lancet, 246(6375), 559.
31. Bull, J. P., Squire, J. R., & Topley, E. (1948). Experiments with occlusive dressings of a new plastic. The Lancet, 252(6519), 213-215.
32. Schilling, R. S. F., Roberts, M., & Goodman, N. (1950). Clinical trial of occlusive plastic dressings. Lancet, 258, 293-296.
33. Winter, G. D. (1962). Formation of the scab and the rate of epithelization of superficial wounds in the skin of the young domestic pig. Nature, 193(4812), 293.
34. Goldman L, Franke E, Kindel D, Blaney D, Richfield D. © 1963 Nature Publishing Group. Nature. 1963;197(487):912–4.

35. Ahmed, A., & Boateng, J. (2018). Calcium alginate-based antimicrobial film dressings for potential healing of infected foot ulcers. Therapeutic delivery, 9(3), 185-204.
36. Yao, C. H., Lee, C. Y., Huang, C. H., Chen, Y. S., & Chen, K. Y. (2017). Novel bilayer wound dressing based on electrospun gelatin/keratin nanofibrous mats for skin wound repair. Materials Science and Engineering: C, 79, 533-540.
37. Ma, Y., Xin, L., Tan, H., Fan, M., Li, J., Jia, Y., ... & Hu, X. (2017). Chitosan membrane dressings toughened by glycerol to load antibacterial drugs for wound healing. Materials Science and Engineering: C, 81, 522-531.
38. Kavoosi, G., Shakiba, A., Ghorbani, M., Dadfar, S. M. M., & Purfard, A. M. (2015). Antioxidant, antibacterial, water binding capacity and mechanical behavior of gelatin-ferula oil film as a wound dressing material. Galen Medical Journal, 4(2), 103-14.
39. Rezvanian, M., Amin, M. C. I. M., & Ng, S. F. (2016). Development and physicochemical characterization of alginate composite film loaded with simvastatin as a potential wound dressing. Carbohydrate polymers, 137, 295-304.
40. Srivastava, C. M., Purwar, R., Kannaujia, R., & Sharma, D. (2015). Flexible silk fibroin films for wound dressing. Fibers and Polymers, 16(5), 1020-1030.
41. Karahaliloğlu, Z., Ercan, B., Denkbaş, E. B., & Webster, T. J. (2015). Nanofeatured silk fibroin membranes for dermal wound healing applications. Journal of Biomedical Materials Research Part A, 103(1), 135-144.
42. Xu, Z., Shi, L., Yang, M., Zhang, H., & Zhu, L. (2015). Fabrication of a novel blended membrane with chitosan and silk microfibers for wound healing: characterization, in vitro and in vivo studies. Journal of Materials Chemistry B, 3(17), 3634-3642.
43. Sobajo, C., Behzad, F., Yuan, X. F., & Bayat, A. (2008). Silk: a potential medium for tissue engineering. Eplasty, 8.
44. Altman, G. H., Diaz, F., Jakuba, C., Calabro, T., Horan, R. L., Chen, J., ... & Kaplan, D. L. (2003). Silk-based biomaterials. Biomaterials, 24(3), 401-416.
45. Ude, A. U., Eshkoor, R. A., Zulkifili, R., Ariffin, A. K., Dzuraidah, A. W., & Azhari, C. H. (2014). Bombyx mori silk fibre and its composite: a review of contemporary developments. Materials & Design, 57, 298-305.
46. Kaplan, D., Adams, W. W., Farmer, B., & Viney, C. (1994). Silk: biology, structure, properties, and genetics. In ACS symposium series (USA).
47. Asakura, T., Okushita, K., & Williamson, M. P. (2015). Analysis of the structure of Bombyx mori silk fibroin by NMR. Macromolecules, 48(8), 2345-2357.
48. Hardy, J. G., Römer, L. M., & Scheibel, T. R. (2008). Polymeric materials based on silk proteins. Polymer, 49(20), 4309-4327.
49. Pagliaro, M., & Rossi, M. (2010). Glycerol: properties and production. The future of glycerol, 7.
50. Vieira, M. G. A., da Silva, M. A., dos Santos, L. O., & Beppu, M. M. (2011). Natural-based plasticizers and biopolymer films: A review. European Polymer Journal, 47(3), 254-263.

51. Karbowiak, T., Hervet, H., Léger, L., Champion, D., Debeaufort, F., & Voilley, A. (2006). Effect of plasticizers (water and glycerol) on the diffusion of a small molecule in iota-carrageenan biopolymer films for edible coating application. Biomacromolecules, 7(6), 2011-2019.
52. PubChem (National Center of Biotechnology Information). Glycerin. [cited 2018 Nov 22]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/glycerol#section=Top
53. Gülçin, İ., Huyut, Z., Elmastaş, M., & Aboul-Enein, H. Y. (2010). Radical scavenging and antioxidant activity of tannic acid. Arabian Journal of Chemistry, 3(1), 43-53.
54. King, A. M. Y., & Young, G. (1999). Characteristics and occurrence of phenolic phytochemicals. Journal of the American Dietetic Association, 99(2), 213-218.
55. Yang, X., Huang, P., Wang, H., Cai, S., Liao, Y., Mo, Z., ... & Li, J. (2017). Antibacterial and anti-biofouling coating on hydroxyapatite surface based on peptide-modified tannic acid. Colloids and Surfaces B: Biointerfaces, 160, 136-143.
56. Lopes, G. K., Schulman, H. M., & Hermes-Lima, M. (1999). Polyphenol tannic acid inhibits hydroxyl radical formation from Fenton reaction by complexing ferrous ions1. Biochimica et Biophysica Acta (BBA)-General Subjects, 1472(1-2), 142-152.
57. Genaro-Mattos, T. C., Maurício, Â. Q., Rettori, D., Alonso, A., & Hermes-Lima, M. (2015). Antioxidant activity of caffeic acid against iron-induced free radical generation—A chemical approach. PloS one, 10(6), e0129963.
58. Gülçin, İ. (2006). Antioxidant activity of caffeic acid (3, 4-dihydroxycinnamic acid). Toxicology, 217(2-3), 213-220.
59. PubChem (National Center of Biotechnology Information). Caffeic Acid. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/caffeic_acid
60. PubChem (National Center of Biotechnology Information). Tannic Acid. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/16129778
61. Sofia, S., McCarthy, M. B., Gronowicz, G., & Kaplan, D. L. (2001). Functionalized silk- based biomaterials for bone formation. Journal of Biomedical Materials Research: An Official Journal of The Society for Biomaterials and The Japanese Society for Biomaterials, 54(1), 139-148.
62. Mir, M., Ali, M. N., Barakullah, A., Gulzar, A., Arshad, M., Fatima, S., & Asad, M. (2018). Synthetic polymeric biomaterials for wound healing: a review. Progress in biomaterials, 1-21.
63. Vepari, C., & Kaplan, D. L. (2007). Silk as a biomaterial. Progress in polymer science, 32(8-9), 991-1007.
64. Wang, X., Kim, H. J., Wong, C., Vepari, C., Matsumoto, A., & Kaplan, D. L. (2006). Fibrous proteins and tissue engineering. Materials today, 9(12), 44-53.
65. Chen, Y., Lin, J., Fei, Y., Wang, H., & Gao, W. (2010). Preparation and characterization of electrospinning PLA/curcumin composite membranes. Fibers and Polymers, 11(8), 1128-1131.
66. Lu, Q., Hu, X., Wang, X., Kluge, J. A., Lu, S., Cebe, P., & Kaplan, D. L. (2010). Water-insoluble silk films with silk I structure. Acta biomaterialia, 6(4), 1380-1387.
67. Siemann, U. (2005). Solvent cast technology–a versatile tool for thin film production. In Scattering methods and the properties of polymer materials (pp. 1-14). Springer, Berlin, Heidelberg.

68. Ribeiro, V. P., Silva-Correia, J., Nascimento, A. I., da Silva Morais, A., Marques, A. P., Ribeiro, A. S. & Oliveira, A. L. (2017). Silk-based anisotropical 3D biotextiles for bone regeneration. Biomaterials, 123, 92-106.
69. McCarty, S. M., & Percival, S. L. (2013). Proteases and delayed wound healing. Advances in wound care, 2(8), 438-447.
70. Numata, K., Cebe, P., & Kaplan, D. L. (2010). Mechanism of enzymatic degradation of beta-sheet crystals. Biomaterials, 31(10), 2926-2933.
71. Zhou, J., Cao, C., Ma, X., Hu, L., Chen, L., & Wang, C. (2010). In vitro and in vivo degradation behavior of aqueous-derived electrospun silk fibroin scaffolds. Polymer Degradation and Stability, 95(9), 1679-1685.
72. Sun, Q., Xi, T., Li, Y., & Xiong, L. (2014). Characterization of corn starch films reinforced with CaCO3 nanoparticles. PloS one, 9(9), e106727.
73. Haines, P. J. (2012). Thermal methods of analysis: principles, applications and problems. Springer Science & Business Media.
74. Gião, M. S., González- Sanjosé, M. L., Rivero- Pérez, M. D., Pereira, C. I., Pintado, M. E., & Malcata, F. X. (2007). Infusions of Portuguese medicinal plants: Dependence of final antioxidant capacity and phenol content on extraction features. Journal of the Science of Food and Agriculture, 87(14), 2638-2647.
75. Dabiri, G., Damstetter, E., & Phillips, T. (2016). Choosing a wound dressing based on common wound characteristics. Advances in wound care, 5(1), 32-41.
76. Akhmetova, A., Saliev, T., Allan, I. U., Illsley, M. J., Nurgozhin, T., & Mikhalovsky, S. (2016). A comprehensive review of topical odor-controlling treatment options for chronic wounds. Journal of Wound, Ostomy, and Continence Nursing, 43(6), 598.
77. Jones, V., Grey, J. E., & Harding, K. G. (2006). ABC of wound healing: wound dressings. BMJ: British Medical Journal, 332(7544), 777.
78. Food & Drug Administration. Glycerol. CFR - Code of Federal Regulations Title 21. 2018.
79. Jafarzadeh, S., Alias, A. K., Ariffin, F., & Mahmud, S. (2018). Physico-mechanical and microstructural properties of semolina flour films as influenced by different sorbitol/glycerol concentrations. International Journal of Food Properties, 21(1), 983-995.
80. Silva, S. S., Popa, E. G., Gomes, M. E., Cerqueira, M., Marques, A. P., Caridade, S. G., ... & Reis, R. L. (2013). An investigation of the potential application of chitosan/aloe-based membranes for regenerative medicine. Acta biomaterialia, 9(6), 6790-6797.
81. Silva, S. S., Popa, E. G., Gomes, M. E., Oliveira, M. B., Nayak, S., Subia, B. & Reis, R. L. (2013). Silk hydrogels from non-mulberry and mulberry silkworm cocoons processed with ionic liquids. Acta biomaterialia, 9(11), 8972-8982.
82. Boyan, B. D., Hummert, T. W., Dean, D. D., & Schwartz, Z. (1996). Role of material surfaces in regulating bone and cartilage cell response. Biomaterials, 17(2), 137-146.
83. Sobral, P. D. A. (2000). Influencia da espessura de biofilmes feitos a base de proteinas miofibrilares sobre suas propriedades funcionais. Área de Informação da Sede-Artigo em periódico indexado (ALICE).

84. Brown, J. E., Davidowski, S. K., Xu, D., Cebe, P., Onofrei, D., Holland, G. P., & Kaplan, D. L. (2016). Thermal and Structural Properties of Silk Biomaterials Plasticized by Glycerol. Biomacromolecules, 17(12), 3911-3921.
85. Allardyce, B. J., Rajkhowa, R., Dilley, R. J., Redmond, S. L., Atlas, M. D., & Wang, X. (2017). Glycerol-plasticised silk membranes made using formic acid are ductile, transparent and degradation-resistant. Materials Science and Engineering: C, 80, 165-173.
86. Xie, R. J., & Zhang, M. (2013). Effect of glycerol on structure and properties of silk fibroin/pearl powder blend films. In Advanced Materials Research (Vol. 796, pp. 126-131). Trans Tech Publications.
87. Mali, S., Sakanaka, L. S., Yamashita, F., & Grossmann, M. V. E. (2005). Water sorption and mechanical properties of cassava starch films and their relation to plasticizing effect. Carbohydrate Polymers, 60(3), 283-289.
88. Minoura, N., Tsukada, M., & Nagura, M. (1990). Physico-chemical properties of silk fibroin membrane as a biomaterial. Biomaterials, 11(6), 430-434.
89. Lu, S., Wang, X., Lu, Q., Zhang, X., Kluge, J. A., Uppal, N. & Kaplan, D. L. (2009). Insoluble and flexible silk films containing glycerol. Biomacromolecules, 11(1), 143-150.
90. Kalra, A., Lowe, A., & Al-Jumaily, A. M. (2016). Mechanical behaviour of skin: a review. J Mater Sci Eng, 5(4), 254-260.
91. Wang, L., Khor, E., Wee, A., & Lim, L. Y. (2002). Chitosan- alginate PEC membrane as a wound dressing: Assessment of incisional wound healing. Journal of Biomedical Materials Research: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials, 63(5), 610-618.
92. Indran, V. P., Zuhaimi, N. A. S., Deraman, M. A., Maniam, G. P., Yusoff, M. M., Hin, T. Y. Y., & Rahim, M. H. A. (2014). An accelerated route of glycerol carbonate formation from glycerol using waste boiler ash as catalyst. RSC Advances, 4(48), 25257-25267.
93. Kongjao, S., Damronglerd, S., & Hunsom, M. (2010). Purification of crude glycerol derived from waste used-oil methyl ester plant. Korean Journal of Chemical Engineering, 27(3), 944-949.
94. Belay, A., Kim, H. K., & Hwang, Y. H. (2016). Binding of caffeine with caffeic acid and chlorogenic acid using fluorescence quenching, UV/vis and FTIR spectroscopic techniques. Luminescence, 31(2), 565-572.
95. Pantoja-Castro, M. A., & González-Rodríguez, H. (2011). Study by infrared spectroscopy and thermogravimetric analysis of tannins and tannic acid. Revista latinoamericana de química, 39(3), 107-112.
96. Nogueira, G. M., Rodas, A. C., Leite, C. A., Giles, C., Higa, O. Z., Polakiewicz, B., & Beppu, M. M. (2010). Preparation and characterization of ethanol-treated silk fibroin dense membranes for biomaterials application using waste silk fibers as raw material. Bioresource technology, 101(21), 8446-8451.
97. Vasconcelos, A., Freddi, G., & Cavaco-Paulo, A. (2008). Biodegradable materials based on silk fibroin and keratin. Biomacromolecules, 9(4), 1299-1305.

98. Greener, B., Hughes, A. A., Bannister, N. P., & Douglass, J. (2005). Proteases and pH in chronic wounds. Journal of wound care, 14(2), 59-61.
99. Curran, S., & Murray, G. I. (1999). Matrix metalloproteinases in tumour invasion and metastasis. The Journal of pathology, 189(3), 300-308.
100. Brown, J., Lu, C. L., Coburn, J., & Kaplan, D. L. (2015). Impact of silk biomaterial structure on proteolysis. Acta biomaterialia, 11, 212-221.
101. Talukdar, S., Mandal, M., Hutmacher, D. W., Russell, P. J., Soekmadji, C., & Kundu, S. C. (2011). Engineered silk fibroin protein 3D matrices for in vitro tumor model. Biomaterials, 32(8), 2149-2159.
102. Horan, R. L., Antle, K., Collette, A. L., Wang, Y., Huang, J., Moreau, J. E. & Altman, G. H. (2005). In vitro degradation of silk fibroin. Biomaterials, 26(17), 3385-3393.
103. Li, M., Ogiso, M., & Minoura, N. (2003). Enzymatic degradation behavior of porous silk fibroin sheets. Biomaterials, 24(2), 357-365.
104. Ahmad, N., Alam, M., Naushad, M., Ansari, A. A., Alrayes, B. F., & Alotaibe, M. A. (2018). Thermal Decomposition And Kinetic Studies Of Tannic Acid Using Model Free-Methods. Journal of the Chilean Chemical Society, 63(1), 3824-3828.
105. Zhang, H., Magoshi, J., Becker, M., Chen, J. Y., & Matsunaga, R. (2002). Thermal properties of Bombyx mori silk fibers. Journal of applied polymer science, 86(8), 1817-1820.
106. Castelló, M., Dweck, J., & Aranda, D. (2009). Thermal stability and water content determination of glycerol by thermogravimetry. Journal of thermal analysis and calorimetry, 97(2), 627-630.
107. Baycın, D. E. N. I. Z., Altıok, E. V. R. E. N., Ülkü, S., & Bayraktar, O. (2007). Adsorption Of Olive Leaf (Olea Europaea L.) Antioxidants On Silk Fibroin. Journal Of Agricultural And Food Chemistry, 55(4), 1227-1236.
108. Sheng, X., Fan, L., He, C., Zhang, K., Mo, X., & Wang, H. (2013). Vitamin E-loaded silk fibroin nanofibrous mats fabricated by green process for skin care application. International journal of biological macromolecules, 56, 49-56.
109. Sahiner, N., Sagbas, S., & Aktas, N. (2015). Single step natural poly (tannic acid) particle preparation as multitalented biomaterial. Materials Science and Engineering: C, 49, 824-834.