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DEVELOPMENT OF NEW *BOMBYX MORI* SILK FIBROIN MEMBRANES FOR PERIODONTAL GUIDED TISSUE REGENERATION

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DEVELOPMENT OF NEW *BOMBYX MORI* SILK FIBROIN MEMBRANES FOR PERIODONTAL GUIDED TISSUE REGENERATION

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

by

Catarina Barros Geão

Place: 3B's Research Group – Biomaterials, Biodegradables and Biomimetics, Universidade do Minho, Caldas das Taipas, Portugal and CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Porto, Portugal

Supervision: Professora Doutora Ana Leite Oliveira, Professor Doutor Joaquim Miguel Oliveira

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RESUMO

A periodontite é uma das doenças inflamatórias mais comuns e uma das principais causas de perda dentária em adultos, sendo caracterizada por uma destruição progressiva das estruturas que suportam o dente. Procedimentos como a Regeneração Tecidular Guiada (RTG) e a Regeneração Óssea Guiada (ROG) foram estabelecidos como técnicas chave na medicina regenerativa periodontal devido aos seus resultados promissores. Estes procedimentos baseiam-se, tipicamente, numa membrana que é colocada entre a gengiva e o osso alveolar onde o processo regenerativo terá lugar. No entanto, as membranas comercialmente disponíveis apresentam limitações a nível estrutural, mecânico e biofuncional demonstrando a necessidade de se desenvolverem materiais clinicamente mais eficazes. Neste trabalho, propõe-se uma nova geração de membranas RTG/ROG utilizando um material de origem natural. Assim, desenvolveram-se membranas à base de fibroína de seda (FS), extraída de casulos *Bombyx mori*, plasticizadas com glicerol (GLY) e álcool polivinílico (PVA) de modo a melhorar a sua flexibilidade e estabilização. As membranas produzidas, desenhadas para estarem em contato com a gengiva, visam fornecer uma solução eficaz para a periodontite moderada (o tipo de periodontite mais prevalente no mundo), ajudando a restaurar a anatomia e a função dos tecidos periodontais perdidos ou lesados. Soluções purificadas de FS foram misturadas com GLY ou PVA em proporções de 0, 10 e 30% em peso. As membranas foram obtidas pelo método de "*solvent casting"* e secas a 85 °C durante 6 e 12 horas para posterior caracterização. Como controlo foi utilizada uma membrana de FS pura. As membranas foram caracterizadas em termos de morfologia, integridade física, estrutura química, propriedades mecânicas e térmicas, capacidade de inchamento e perfil de degradação *in vitro*. As membranas secas durante 6 e 12 horas apresentaram aspeto e microestrutura semelhante. As micrografias de microscopia eletrónica de varrimento (MEV) sugerem que o GLY está bem distribuído na matriz da FS enquanto o PVA apresenta separação de fases entre os dois polímeros. A microscopia de força atómica (MFA) revelou que a nanotopografia superficial e a rugosidade média das membranas são afetadas pelo tempo de exposição ao tratamento térmico e pela percentagem de polímero sintético. As medições de ângulo de contato mostraram uma diminuição do mesmo com a quantidade crescente de GLY e PVA, indicando que as superfícies se tornam mais hidrofílicas. As análises de espectroscopia de infravermelho com transformada de Fourier demonstraram que o tratamento térmico induziu a conformação "β-sheet" para ambos os períodos de exposição. Contudo, apenas as membranas secas por 12 horas apresentaram estabilidade por mais de 24 horas em água, atingindo o máximo grau de hidratação após 3 horas. Com a adição dos polímeros sintéticos, a capacidade de hidratação aumentou juntamente com a perda de peso tanto em PBS como em PBS com enzima. As propriedades mecânicas revelaram que o GLY e o PVA possuem um efeito plasticizante, tornando as membranas mais flexíveis e dúcteis sem comprometer a resistência mecânica necessária para esta aplicação. As membranas desenvolvidas demonstraram ser sistemas promissores para aplicação em regeneração guiada de tecido periodontal.

ABSTRACT

Periodontitis is one of the most common inflammatory diseases and a leading cause of tooth loss in adults, being characterized by progressive destruction of the tooth-supporting apparatus. Guided Tissue Regeneration (GTR) and Guided Bone Regeneration (GBR) procedures were established as basic techniques in periodontal regenerative medicine due to its promising results. These procedures are based, typically, on a membrane, that is placed between the gingiva and the alveolar bone in which the regenerative process will take place. However, the commercial available membranes present limitations at structural, mechanical and biofunctional level demonstrating the need for developing clinically effective materials. In this work a new generation of GTR/GBR membranes is presently being proposed using a natural-based material. *Bombyx mori* silk fibroin(SF)-based membranes have been developed using glycerol (GLY) and poly(vinyl alcohol) (PVA) as plasticizers to improve the flexibility and enhance SF stabilization. The developed membranes, designed to be in contact with the gingiva, aim at providing an effective solution for mild periodontitis (the most prevalent form worldwide) by helping to restore the anatomy and function of lost or damaged periodontal tissues. Purified SF solution was mixed with GLY or PVA at weight ratios of 0, 10, and 30%. The membranes were obtained by casting the final solutions into Petri dishes and dried at 85 ºC in the oven for 6 hours some and 12 hours others for further characterization. A comparative study was undertaken using the pure SF membrane as control. The SF-based membranes were characterized in terms of their morphology, physical integrity, chemical structure, mechanical and thermal properties, swelling capability and *in vitro* degradation behavior. Membranes dried for 6 and 12 hours presented similar aspect and microstructure. Scanning electron microscopy (SEM) micrographs suggested that GLY is well distributed in SF matrix while PVA presented phase separation between the two polymers. Atomic force microscopy (AFM) revealed that the surface nanotopography and the average roughness of the SF-based membranes are affected by the exposure time and by the percentage of additive content. Contact angle measurements showed a decrease of water contact angle with GLY and PVA increasing, indicating that the surfaces become more hydrophilic. Attenuated infrared spectroscopy (ATR-FTIR) analysis demonstrated that the thermal treatment induced SF β-sheet conformation for both exposure periods. However, only the membranes dried for 12 hours were water stable for more than 24 hours. These membranes reached equilibrium hydration degree after 3 hours. With the addition of the synthetic polymers, the hydration capacity increased together with the weight loss both in PBS and PBS with enzyme. The mechanical properties revealed that GLY and PVA had a plasticizing effect on the membrane which became more ductile without compromising its mechanical strength. The developed membranes demonstrated to be promising systems to be used in a guided tissue regeneration approach for periodontal regeneration.

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CHAPTER 1

INTRODUCTION

1.1. Periodontal disease and available therapies

The periodontium is a complex structure constituted by various tissues namely the cementum, the periodontal ligament (PDL), the alveolar bone and the dentogingival junction as it can be seen in figure 1.1 (Melcher 1976; Gillett et al. 1990; Polimeni et al. 2006). The periodontium anchors the teeth to the jaws (mandible and maxilla) and preserves their position providing nourishment to the teeth. Additionally, it guarantees proper function and dissipation of forces, preventing injury to teeth, mandible and maxilla (Bottino et al. 2012).

The cementum is an avascular and non-innervated calcified connective tissue that coats the roots of the teeth and fasten PDL fibers. The PDL, composed by collagen fibers, helps to support the teeth in their sockets and at the same time permits them to withstand the forces of mastication. It ranges in width from 0.15 to 0.38 mm, exhibiting a progressive decrease in thickness with age. Finally, the alveolar bone supports the teeth and the gingival tissues, absorbing and distributing the forces of mastication through the PDL (Nanci and Bosshardt 2006; Bottino et al. 2012).

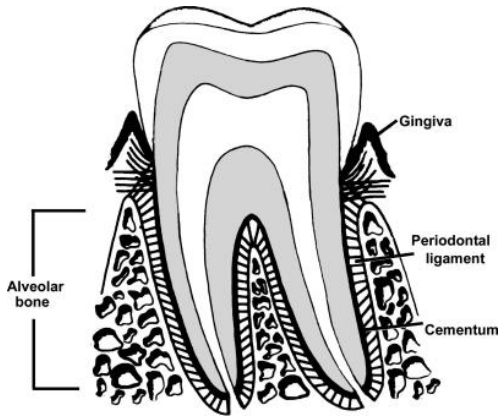

Figure 1.1 – Schematic representation of the tooth-supporting apparatus in normal periodontium (F.M. Chen and Jin 2010).

Periodontal disease (PD), which is mostly caused by pathogenic microflora that accumulates on teeth adjacent to the gingiva (also known as dental plaque), can be defined as any inherited or acquired disorder of the tissues surrounding and supporting the teeth (Pihlstrom, Michalowicz, and Johnson 2005; Akcali et al. 2013). Besides microbial origin, this disease can be caused by other factors like absence of oral hygiene and tobacco use, malocclusions and overcrowding, age, and genetic predisposition (Tatakis and Kumar 2005; Stabholz et al. 2010; Akcali et al. 2013).

PD has become an important public health problem due to its massive prevalence and consequences on systemic health (Kuo et al. 2008). Therefore, it has been described a correlation between this disease and common systemic diseases such as diabetes, adverse pregnancy outcomes

(preterm birth, low weight and neonatal morbidity), osteoarticular, respiratory and cardiovascular diseases (Wowern et al. 1994; Beck et al. 1996; Scannapieco et al. 1998; Soskolne and Klinger 2001; Taylor 2001; Azarpazhooh and Leake 2006; Kuo et al. 2008).

The periodontium is frequently affected by the PD which includes different inflammatory stages: an initial form called gingivitis, and an advanced one named periodontitis (mild and advanced) as shown in figure 1.2 (Pihlstrom, Michalowicz, and Johnson 2005; Kuo et al. 2008; Requicha et al. 2014).

Gingivitis is the earliest stage of gum disease and it is characterized by red and swollen gingiva that can easily bleed on contact. At this stage, probe depth is still minimal (approximately 3 mm) and it can be reversed since the bone and the connective tissue that hold the teeth in its place are not affected yet (Tariq et al. 2012; Akcali et al. 2013). When gingivitis is not treated, it can advance into periodontitis. In the moderate periodontitis further separation of the gingiva and teeth occurs leading to deeper gingival pockets (Daly, Seymour, and Kieser 1980; Akcali et al. 2013; Tariq et al. 2012). At this point, calculus formation and a probe depth of up to 6 mm are generally observed being difficult for the patients to effectively clean their teeth (Darveau 2010). In the final stage of periodontal disease (advanced periodontitis) there is significant alveolar bone resorption, cementum necrosis, and an excessive probe depth which most of the times requires teeth extraction (Darveau 2010; Tariq et al. 2012; Akcali et al. 2013). In this sense, Eke et al. conducted a study, published in 2015, to estimate the prevalence and severity of periodontitis in the adult US population using data collected from 2009-2012 provided by the National Health and Nutrition Examination Survey. Estimates were derived for dentate adults aged 30 and older from non-institutionalized population. The results showed that 46% of US adults representing 64.7 million people had periodontitis, with 8.9% having severe periodontitis. These results confirm that this is a very serious health problem that must be prevented and controlled (Eke et al. 2012; Eke et al. 2015).

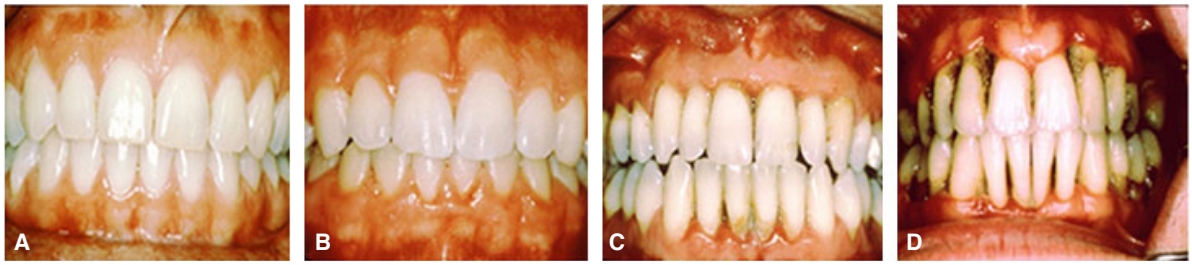

Figure 1.2 – Periodontal disease aspect. A: healthy gingiva; B: gingivitis; C: moderate periodontitis; and D: advanced periodontitis (adapted from Requicha et al. 2014).

During the last two decades, several regenerative procedures have been proposed, tested and evaluated in order to restore the tooth-supporting structures. This has included scaling and root planning, open flap debridement, autogenous bone grafting, implantation of biomaterials including bone derivatives and bone substitutes, the use of occlusive barrier membranes, and implantation of biological factors including enamel matrix proteins and platelet derivatives (Hämmerle and Jung 2003; Polimeni

et al. 2006; Hanes 2007; Habibovic and de Groot 2007; Hallman and Thor 2008; Chen and Jin 2010). These strategies aim to: (1) reduce and/or eliminate inflamed tissues caused by the deposition of anaerobic bacteria on the soft tissue pockets, (2) remodel defects or anatomical problems caused by the disease, and (3) regenerate new periodontal tissues (new cementum with PDL fibers connected to the new alveolar bone) (Bottino et al. 2012; Sam and Pillai 2014).

Typically, in a periodontal wound it's possible to observe a fast growing epithelial and gingival tissues that repopulate the empty space resulting in long junctional epithelium. Although the formation of this new epithelium may be compatible with a conventional clinical outcome, it doesn't regenerate the primitive PDL function (Wang et al. 2012). In this sense, guided tissue regeneration (GTR) and guided bone regeneration (GBR) were introduced into clinical dental practice, being hypothesized that if cells derived from the PDL and alveolar bone were the first to repopulate tooth root surface, the formation of a functional periodontium could be possible (Campbell et al. 1956; Hurley et al. 1959; Melcher 1976; J Gottlow et al. 1984)

The function of GTR and GBR (figure 1.3 and 1.4 respectively) is based on a tissue barrier, typically a membrane with appropriate shape, that is placed between the gingival connective tissue and the alveolar bone in which the regenerative process will take place. This membrane avoids the apical migration of epithelium and gingival connective tissues while promoting cellular growth from the preserved periodontal (Gottlow et al. 1986; Caffesse and Becker 1991; Bottino et al. 2012; Tal et al. 2012).

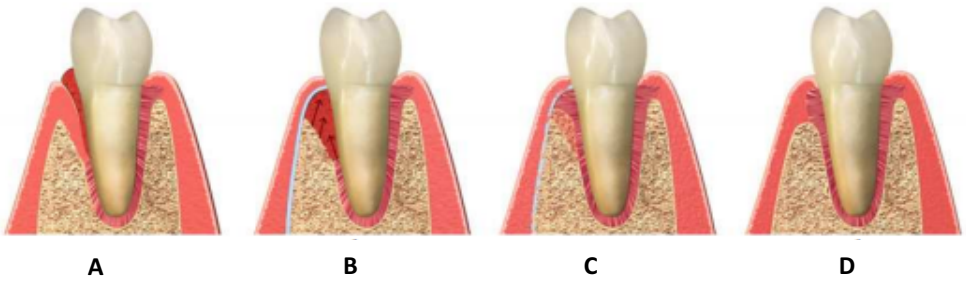

Figure 1.3 – GTR scheme describing the use of a resorbable barrier membrane. A: periodontal injurie in the jaw. B: resorbable membrane is stabilized over the debrided lesion and covered by the mucosal flap. C: the membrane starts to resorb; new bone, new periodontal ligament, and new cementum are visible. D: reestablishment of most of the periodontal attachment apparatus is completed (Tal et al. 2012).

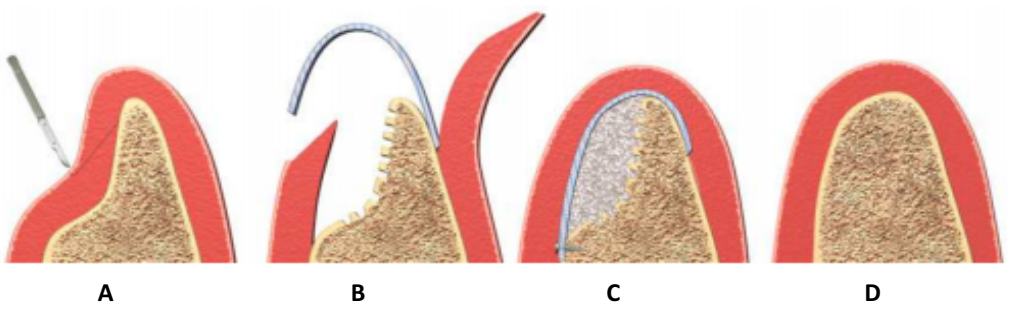

Figure 1.4 – GBR scheme describing the use of a resorbable barrier membrane. A: bone defect is diagnosed. B: the defect is debrided, bone cortex perforated and a membrane is placed. C: the membrane is stabilized and shaped to dictate the desired bone contours. D: bone regeneration is observed restoring the desired shape of the jaw (Tal et al. 2012).

Ideally, these materials should have the following characteristics (described for the first time by Scantlebury in 1993): (1) biocompatibility to allow integration with host tissues without inducing inflammatory responses, (2) cell-occlusiveness to exclude undesirable cells types from entering the isolated space adjacent to the root surface, (3) adequate degradation time matching the formation of new bone/tissue, and (4) proper mechanical and physical properties to allow its placement *in vivo* (Scantlebury 1993; Hardwick et al. 1995; Aurer et al. 2005).

1.2. The use of membrane systems in GTR/GBR applications

There are several GTR/GBR membrane systems available in the market. They are divided into two groups: non-resorbable and resorbable according to their degradation profile. The commercially available membranes are summarized in Table 1.1., although there are other companies which commercialize other products with the same components.

1.2.1. Non-resorbable membranes

The most used non-resorbable membranes are composed of expanded polytetrafluoroethylene (ePTFE), high-density polytetrafluoroethylene (dPTFE) or titanium meshes (Becker et al. 1987; Caffesse et al. 1990; Wang and Fenton 1996).

The ePTFE membranes *(e.g. GORE-TEX® membrane; W.L Gore & Associates, USA)* were extensively used for tissue/bone regeneration since they prevent the ingrowth of gingival connective tissue cells into the wound site (Simian et al. 1999; Marouf and El-Guindi 2000). They effectiveness was investigated in several clinical studies that confirmed their biocompatibility and a significant regeneration after 3-6 months of healing period (Simian et al. 1999; S. Zhao et al. 2000). Nowadays, these membranes have been discontinued and are not available for dental use due to bacterial infection reports (Buser et al. 1990; Becker et al. 1991; Tempro et al. 1993; Rakhmatia et al. 2013).

The dPTFE membranes *(e.g. Cytoplast® GBR-200 or TXT-200; Osteogenics Biomedical Inc., USA)* can be used alternatively. The first one was originally developed in 1993 and its success in bone and tissue regeneration is well documented (Bartee 1995; Bartee and Carr 1995). Contrarily to ePTFE membranes, some of them do not require a second surgery for their removal because they allow no primary closure, being removed easily with a gentle tug. Additionally, they are thinner and have a porosity of up to one hundred times lower than the ePTFE ones. Because of this small pore size, bacterial infiltration into the bone/tissue site is eliminated, which protects the underlying graft material and/or implant (Bartee and Carr 1995; Bartee 1995; Barber et al. 2007; Lee et al. 2010; Gentile et al. 2011; Rakhmatia et al. 2013).

Titanium membranes *(e.g. FRIOS® BoneShield; Friatec, Germany)* have been used in GBR dental applications, particularly for alveolar bone reconstruction prior to implant placement, due to its high mechanical properties, low density and consequent low weight, and finally its resistance to corrosion (Boyne et al. 1985; Zablotsky et al. 1991; Wang and Fenton 1996; Degidi et al. 2003). However, its stiffness can cause mucosal irritation that leads to exposure of the membrane, which represents the major surgical complication of this type of material (Louis et al. 2008).

One drawback in the use of non-resorbable membranes is the need of a second surgery for its removal that increases not only the risk of infection and site morbidity but also the discomfort and economic effort to the patient (Geurs et al. 2008; Tal et al. 2012; Zhang et al. 2013). Moreover, if the membrane is occlusive it can interrupt the adequate blood supply to the gingiva, causing ischemia followed by soft tissue dehiscence and subsequent membrane exposure (Wikesjö et al. 2003). These undesirable characteristics are often weighed with the positive effects of its use which encompasses their stability and easy manageability, biocompatibility, and more predictable profile during the healing process due to their adequate mechanical strength (Polimeni et al. 2006; Zhang et al. 2013; Rakhmatia et al. 2013).

1.2.2. Resorbable membranes

A variety of resorbable membranes that allow a single-step procedure, reduce patient discomfort and costs, and eliminate potential surgical complications due to exposure are currently available. The main limitation of these membranes can be an unpredictable resorption time or a low degree of degradation, which directly affects bone and tissue formation (Zellin et al. 1995; Aurer et al. 2005; Bottino, Thomas, and Janowski 2011; Gentile et al. 2011; Tal et al. 2012; Zhang et al. 2013; Zeng et al. 2014).

Resorbable membranes can be of synthetic or natural origin. Of these, aliphatic polyesters (e.g. PLA - poly(lactic acid), PGA - poly(glycolic acid), and PCL – poly(ε-caprolactone)) and collagen are the best known and well-documented for medical use (Hutmacher, et al. 1996; Owens and Yukna 2001; Hou et al. 2004; Aurer et al. 2005; Sculean et al. 2008; Geurs et al. 2008; Tal et al. 2008; Bottino et al. 2009; Coïc et al. 2009).

Synthetic resorbable membranes based on different variants of PLA – poly(lactic acid) and PGA – poly(glycolic acid) have gained interest during the last years since polyglycolide and polylactide can be easily produced in large quantities and are well-established for medical device production by FDA (Fields 2001; Y. Zhang et al. 2013). The biodegradation of these membranes is well-known and occurs by the breakdown of polymeric chain by hydrolysis, releasing glycolic acid and lactic acid respectively. These metabolites are eliminated through the Krebs cycle as carbon dioxide and water (Athanasiou et al. 1996).

Guidor® Matrix Barrier (Guidor AB., Sweden), a double layered resorbable membrane composed of poly(lactic acid) treated with acetyltributylcitrate, was the first to appear on the market for regenerate tissues in periodontology (Gottlow 1993). It has a matrix with two differently perforated layers: the

external layer presents rectangular pores (400-500/cm^2) to allow integration of the overlying gingival flap, and the inner layer has smaller circular pores (4000-5000/cm^2) in order to delay tissue penetration without interrupting nutrient permeation. According to the manufacturer, membrane resorption takes place 6-12 months after implantation (Gottlow 1993; Araujo et al. 1998; Gentile et al. 2011). Its efficacy on periodontal defects was proved in several clinical studies (Lundgren et al. 1994; Piattelli et al. 1996; Pontoriero et al. 1999; Garrett et al. 2002). Nevertheless, it was removed from the market for unknown reasons (Aurer et al. 2005).

Resolut LT® (W.L Gore & Associates, USA) is another double layered membrane composed by a poly(lactic-co-glycolic acid) sheet that prevents epithelial cell ingrowth, and a porous network of polyglycolide fibers that promotes tissue integration. Studies revealed a similar effectiveness to non-resorbable ones and a complete resorption 5-6 months after its placement (Pontoriero et al. 1999; Milella et al. 2001;Donos et al. 2002).

Vicryl Mesh® (Ethicon Inc., USA) is composed of fibers of polyglactin 910 (a copolymer of glycolide) and L-lactic. This membrane loses its structure after 2 weeks and its completely resorbed in 4 or more weeks. Unfortunately, some animal studies showed a lack of tissue integration in its presence (Gottlow 1993; De Sanctis and Zucchelli 1996; Araujo et al. 1998; Pontoriero et al. 1999).

Atrisorb® (Atrix Laboratories Inc., USA), an liquid product that is used directly at the surgical site, consists of poly(DL-lactic acid) dissolved in N-methyl-2-pyrrolidone. Clinical studies reported its efficacy in the treatment of periodontal defects and a complete resorption 6-12 months after implantation (Coonts et al. 1998; Hou et al. 2004).

Epi-Guide® (Kensey Nash Corporation, USA) is a three-layer porous membrane composed of poly(DL-lactic acid), that is used as an assistant to periodontal restorative surgery. It maintains its structure and function for 5 months after surgery, being completely resorbed after one year (Aurer et al. 2005). The porous side is in contact with the gingiva to promote fibroblast infiltration and attachment, and the other side is in contact with bone defect helping to support fluid uptake, to adherence to tooth surface and to inhibit fibroblast movement (Takata et al. 2001; Bilir et al. 2007).

Collagen is the most important constituent of natural extracellular matrix and it can be acquired from cadaveric human skin *(e.g. Alloderm ®, LifeCell Corporation, USA)*, bovine Achilles tendon *(e.g. BioMend Extend ®, Zimmer Dental Inc., USA)* or porcine skin *(e.g. Bio-Gide ®, Geistlich AG, Swiss)* (Felipe et al. 2007; Behring et al. 2008). In its native form collagen presents proteolytic resistance and high tensile strength due to its covalent crosslinks (Chattopadhyay and Raines 2014). Damage to such linkages upon extraction and over time weakens reconstituted forms of collagen (e.g. membranes or films, hydrogels or sponges). To overcome these problems, several crosslinking techniques have been developed, involving the multiplication of natural occurring connections among collagen molecules (Zellin et al. 1995; Hockers et al. 1999). The *BioMend Extend ®* is an example of a commercially available crosslinked membrane, that use glutaraldehyde combined with bovine type I collagen (Rothamel et al. 2005). This membrane showed *in vitro* and *in vivo* cytotoxicity that has been attributed to glutaraldehyde release during the degradation of collagen (Kodama et al. 1989). The main drawbacks of collagen-based membranes are their very shabby performance *in vivo* since they start to degrade at an early stage of

tissue regeneration and their high cost. Plus, the use of human- or animal-derived collagen may possess regulatory and other limitations such as religious belief (Döri et al. 2007; Behring et al. 2008; Bottino et al. 2012).

As mentioned above, the comercially non-resorbable and resorbabe membranes have limitations at structural, mechanical and biofunctional level. Owing to this fact, it is obvious that the “ideal” membrane has yet to be developed. In this sense, new generation of GTR/GBR membranes is presently being proposed using several natural-based materials such as as alginate, chitosan, gelatin, and silk (Ishikawa et al. 1999; Milella, Barra, et al. 2001; He et al. 2008; Kuo et al. 2009; S. Zhang et al. 2009; Ho et al. 2010; Fraga et al. 2011; Xue et al. 2014). The next two sections present examples of relevant studies using the former materials.

Table 1.1 – Examples of commercially available membranes for GTR/GBR applications.

Membranes		*Commercial name*	*Manufacture and country*	*Mechanical strength*	*Degradation rate*	*References*
Non-resorbable	ePTFE	GORE-TEX®	W.L. Gore & Associates Inc., USA	Not available	–	(Marouf and El-Guindi 2000; S. Zhao et al. 2000; Toygar et al. 2009)
	dPTFE	Cytoplast® (GBR-200 or TXT-200)	Osteogenics Biomedical Inc., USA	Not available	–	(Barber et al. 2007)
		TefGen-FD®	American Custom Medical Inc., USA	Not available	–	(Marouf and El-Guindi 2000)
	Titanium	FRIOS® BoneShield	Friatec, Germany	Not available	–	(Louis et al. 2008)
Synthetic resorbable	Poly(lactic-co-glycolic acid)	Resolut LT®	W. L Gore & Associates Inc., USA	11.7 MPa	5 – 6 months	(Milella et al. 2001; Donos et al. 2002)
	Polyglactin 910 and L-lactic	Vicryl Mesh®	Ethicon Inc., USA	Not available	≥ 4 weeks	(De Sanctis and Zucchelli 1996)
	Poly(DL-lactic acid) and solvent (N-methyl-2-pyrrolidone)	Atrisorb®	Atrix Laboratories Inc., USA	Not available	6 – 12 months	(Coonts et al. 1998; Hou et al. 2004)
	Poly(DL-lactic acid)	Epi-Guide®	Kensey Nash Corporation, USA	Not available	~12 months	(Bilir et al. 2007)
Resorbable collagen-based	Collagen type-I derived from cadaveric human skin	AlloDerm®	LifeCell Corporation, USA	9.4 - 21.2 MPa	~16 weeks	(K. W. Owens and Yukna 2001; Bottino et al. 2009)
	Collagen type-I and type-III derived from porcine skin	Bio-Gide®	Geistlich AG, Swiss	7.75 MPa	24 weeks	(Bunyaratavej and Wang 2001; Coïc et al. 2009)
	Collagen type-I derived from bovine tendon	BioMend Extend®	Zimmer Dental Inc., USA	3.5 – 22.5 MPa	18 weeks	(Bunyaratavej and Wang 2001; Coïc et al. 2009)
		Cytoplast® RTM	Osteogenics Biomedical Inc., USA	Not available	26 – 38 weeks	(Vert 1989; Vert et al. 1992)

1.3. The next generation of GTR/GBR membranes

In the last decades, there have been some works proposing new bioresorbable materials (in this case membranes) for GTR/GBR applications using natural-based sources. Some of them, which as listed below, have drawn particular attention for interfacing the periodontal and bone tissue.

Chitosan, an alkaline linear and cationic polysaccharide, is obtained from the deacetylation of chitin that can be found in the exoskeleton of crustacean, insects, and some fungi. It has been studied for use in a number of biomedical applications that include wound dressing, drug delivery system, space filling implants, and periodontal tissue and bone regeneration (Miyazaki et al. 1981; Muzzarelli et al. 1989; Aiedehe et al. 1997; Madihally and Matthew 1999; Dash et al. 2011). Hong and collaborators in 2007 (Hong et al. 2007) developed an asymmetric porous chitosan membrane that was designed to be porous in the side of the injury and non-porous in the contrary side. This membrane was capable of maintaining its integrity for 5-6 weeks in the enzyme solution. *In vivo* studies using rabbits indicated that the membrane can prevent apical migration of gingival epithelial cells, and promoting growth of periodontal ligament cells. In 2009, Kuo et al. (Kuo et al. 2009) proposed chitosan and tricalcium phosphate membranes with weight ratios of 35:65, 67:33 and 90:10. *In vivo* tests performed with rabbits showed that the three membranes maintained their integrity after 4 weeks, providing as well good cell separation ability. Moreover, compared to pure chitosan membranes, the addition of tricalcium phosphate to the chitosan improved the formation of new bone. Other asymmetric porous chitosan membrane was projected by Ho et al. (Ho et al. 2010) in 2010 using a modified freeze-gelation method proposed elsewhere (Ho et al. 2004). The top surface of the membrane prevented the invasion of human GF cells and the bottom surface allowed the growth and the regeneration of bone tissues. *In vitro* studies revealed higher cellular activity and significant osteoblastic phenotypes, compared with the cells cultured on poly-L-lactic acid membranes. Additionally, the drug release experiment showed that the structure was appropriated for multi-staged drug delivery. Fraga and co-workers in 2011 (Fraga et al. 2011) created a chitosan membrane coated with hydroxyapatite. The membrane was immersed in a sodium silicate solution and then in a simulated body fluid solution. *In vitro* tests showed the formation of a homogeneous and semi-crystalline hydroxyapatite layer similar to the mineral phase of human bone. Moreover, it was observed that the longer the exposure time to simulated body fluid solution the more crystalline the coating and the lower its adhesion to the substrate. In 2012, Mota et al. (Mota et al. 2012) proposed a membrane combining chitosan with bioactive glass nanoparticles. These membranes exhibited lower mechanical properties when compared with pure chitosan ones, but improved bioactivity. Upon immersion in simulated body fluid they were able to induce the precipitation of a bone-like apatite layer. *In vitro* tests performed using human periodontal ligament cells and human bone marrow stromal cells indicated that the composite membranes promoted cell metabolic activity and mineralization.

Alginate is another natural polysaccharide that has been widely used in the pharmaceutical industry as an excipient for drugs, a dental impression material, and a wound dressing (Matthew et al. 1995; Ashley et al. 2005; Liew et al. 2006). Ishikawa et al. in 1999 (Ishikawa et al. 1999) developed an

alginate membrane for GBR applications that can be formed *in situ*. The bone defect was filled with sodium alginate aqueous solution that was ionically crosslinked in the surface by dropping a calcium chloride aqueous solution forming an alginate membrane. This membrane provided space for new bone ingrowth avoiding, at the same time, the growing of soft tissues. *In vivo* analysis using rats showed that 4 weeks after the implantation the bone defect was reconstructed whereas the bone defect was filled only with connective tissue when it had been kept open. In 2001, Milella and collaborators (Milella, Barra, et al. 2001) produced an asymmetric membrane combining poly(L-lactic) acid with alginate. *In vitro* studies revealed that the membrane preserved its mechanical and structural properties for more than 100 days in contact with culture medium. After that time, degradation occurred but the membrane continued to be stable and manageable for 6 months. It was also possible to conclude that the membrane could function as drug delivery vehicle by incorporating growth factors such as TGF-β. Additionally, the obtained membrane showed to promote lower bacterial adhesion when compared with commercial membranes namely Resolut ® and Biofix ®. He et al. in 2008 (He et al. 2008) proposed a calcium alginate film (CAF) for GTR or GBR purposes. Circular bone defects of five millimeters were created in the corners of the mandibles in 45 rabbits. The defects were covered with CAF served as the experimental group, or collagen membrane (CM) or left empty as controls. The results revealed significantly more and faster newly generated bone in CAF defects than that in CM defects or in empty defects at 2, 4, 6, and 8 weeks postsurgical.

Gelatin, a soluble protein derived from controlled hydrolysis of collagen, has received great attention owing to its availability, easy handling and cost efficiency. However, gelatin exhibits poor mechanical properties and fast degradation (Veis 1964). Therefore, it is seldom used alone to function as a GBR and GTR membrane. In 2009, Zhang et al. (Shen Zhang et al. 2009) proposed a gelatin nanofibrous membrane for GTR purposes produced by high temperature electrospinning. To improve the stability and mechanical properties, the films were chemically crosslinked by 1-ethyl-3-(dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxyl succinimide (NHS). The results indicated that the tensile properties of the films could be far enhanced by crosslinking with EDC/NHS and the water resistance could be improved with the increase of crosslinker concentration. *In vitro* tests showed that PDL cells cultured on the membranes exhibited good cell attachment, growth, and proliferation. Noritake et al. in 2011 (Noritake et al. 2011), fabricated a membrane combining β-TCP particles with gelatin hydrogel for GBR purposes. *In vitro* tests performed for 14 days, using rat bone-marrow cells, revealed time-dependent cell proliferation. Histological analysis indicated that more new bone volume was generated in symmetrical bone defects of rat calvariae covered by the gelatin-based membrane compared to the uncovered control defects at 4 and 8 weeks post operation. Xue and collaborators in 2014 (Xue et al. 2014) developed gelatin/PCL membranes blended with metronidazole (MNA) using the electrospun technique. The films presented good mechanical properties, appropriate biodegradation rate and good barrier function. Subcutaneous implantation in rabbits demonstrated that MNA-loaded membranes induced less inflammatory response than bare membranes until de MNA content reached 30%.

One of the critical aspects when developing bioresorbable membranes specifically for periodontal tissue regeneration is their shabby mechanical performance after implantation. In this sense, silk protein has been regarded as an important structural biomaterial.

1.4. Silk as new biomaterial

Silk is a fibrous protein produced in the glands of some arthropods (e.g. silkworms, spiders, scorpions, mites and bees) that is spun into fibers for instance during their metamorphosis (Vepari and Kaplan 2007; Omenetto and Kaplan 2010).

Bombyx mori fibers have been used as commercially-available sutures since the end of 19th century, and have proved to be an effective biomaterial (Moy, Lee, and Zalka 1991; Altman et al. 2003; Kearns and MacIntosh 2008). It is composed of two proteins: fibroin, a structural protein that is inside of the fiber, and sericin a globular protein that envelops silk fibroin forming a protective layer as it can be seen in figure 1.5 (Gulrajani 1988; Um et al. 2001; Lovett et al. 2007; Sobajo et al. 2008; Nogueira et al. 2010).

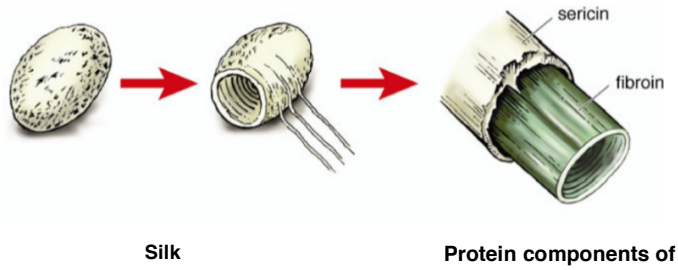

Figure 1.5 – Structural components of *Bombyx mori* silk (Sobajo et al. 2008).

During decades one of the biggest problems of these sutures, that limited its applications, was the biocompatibility of virgin silk (fibroin containing sericin coat). However, there is clear evidence in the literature, that in the absence of native silk sericin component, fibers demonstrate a minimal inflammatory tissue reaction which allows a successful *in vivo* implantation (Santin et al. 1999; Altman et al. 2002; Mondal 2007; Seo et al. 2007; Kearns and MacIntosh 2008). Besides that, silk have a genetically tailorable composition in order to moderate specific features such as molecular weight, solubility and crystallinity, and exhibits slow rates of degradation *in vitro* and *in vivo* which is very useful in cases in which slow tissue ingrowth is desirable (Santin et al. 1999). Additionally, being member of a fibrous protein family, silk demonstrates an impressive mechanical strength. These characteristics have led many researchers to try to use them in different biomedical applications as wound-repairing, drug release and, tissue engineering of bone, cartilage, tendon and ligament (Tsukada et al. 1994; Wang 2003; Meinel et al. 2005; Zhang, Reagan, and Kaplan 2009; Lammel et al. 2010; Guziewicz et al. 2011; Kundu et al. 2013).

Fibroin is the main filament of silk and is composed of a heavy chain (M_w ~ 390 kDa) and a light chain (M_w ~26 kDa) present in a 1:1 ratio and linked by a single disulfide bond (Zhou et al. 2000; Vepari and Kaplan 2007). The heavy chain consists in twelve domains that form the crystalline regions which

are organized by glycine-X repeats where X can be alanine (Ala), serine (Ser), threonine (Thr) or valine (Val) that can form anti-parallel β-sheets (Zhou et al. 2001). Fibroin fibers, which are dissolved into an alkaline aqueous solution, can be processed into different materials, as presented in figure 1.6 (Rockwood et al. 2011).

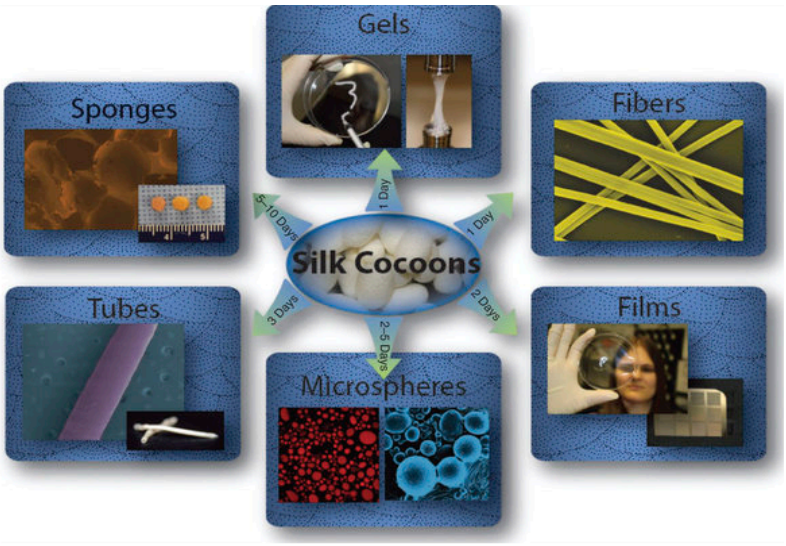

Figure 1.6 – Different types of materials which can be fabricated from silk fibroin (Rockwood et al. 2011)

The molecular conformation of silk fibroin (SF) is an important parameter that needs to be controlled since affects its physical and chemical properties (Moraes et al. 2010). SF has three types of molecular conformations, namely random coil, silk I (α-form), and silk II (β-sheet). Random coil and silk I are metastable forms of SF that are soluble in water and non-crystalline. Silk II is a highly stable and organized structure that is insoluble in water (Motta, Fambri, and Migliaresi 2002; Jin and Kaplan 2003).

Unfortunately, SF at solution does not present enough intermolecular hydrogen bonds to stabilize its structure. Thus, to induce water stability of SF, some researchers propose physical and chemical treatments using high temperature, high humidity and immersion in organic solvents such as ethanol or methanol (Rujiravanit et al. 2003; Moraes et al. 2010; Rockwood et al. 2011). However, with these treatments, SF materials (in this case membranes) tend to be brittle which limits their practical use (Kweon et al. 2001). To overcome this limitation, SF properties can be improved by blending with other natural or synthetic polymers.

1.4.1. Silk fibroin membranes combined with different synthetic polymers

The development of SF blends has been proposed by several authors, as a strategy to increase its processability and final properties. Sun et al. in 1997 (Sun et al. 1997) reported the properties of blend fibers obtained by mixing SF with an acrylic polymer (PAC). The addition of up to 30% SF resulted in an increase of moisture absorption, while the mechanical properties decreased compared to pure PAC

fibers. Scanning electron microscopy studies revealed that the fibers have a sheath-core structure, with SF mainly in the sheath and PAC in the core. In 1999, Freddi et al. (Freddi et al. 1999) developed SF membranes blended with polyacrylamide (PAAm) by using conventional casting method for biomedical and pharmaceutical purposes. The blends showed increased thermal stability and slightly improved mechanical properties when compared with pure SF membranes. The most interesting one was the membrane with low content of PAAm (≤ 25%). These properties were attributed to the formation of specific molecular interactions between SF and PAAm. Poly(ethylene glycol) (PEG) diacrylate used as a macromere was polymerized in the presence of SF to formulate semi-interpenetrating polymer networks (SIPNs) by Kweon et al. in 2001 (Kweon et al. 2001). The tensile strength and elongation at the break of the SPINs were much higher than those of SF itself or the SF/PEG blend and increased with an increase of the PEG content. Additionally, the equilibrium water content of SF was increased by the formation of SPINs with PEG due to its hydrophilic property. Jin and co-workers in 2004 (Jin et al. 2004), produced SF films blended with poly(ethylene oxide) (PEO) using as well the conventional casting method for potential tissue engineering scaffolds. By blending PEO with SF, the surface of the films was more hydrophilic based on XPS and contact angle measurements. No macroscopic phase separation was observed, even after methanol treatment. However, porosity with uniform size and shape was observed microscopically due to phase separation which depends on the ratio of PEO to SF.

Many of the above-described studies do not emphasize a specific biomedical application for their developed formulations. However, it is extremely relevant to understand the targeted tissue to be regenerated in order to fabricate the best performing device. Combining on a membrane SF and synthetic polymers for periodontal guided tissue regeneration is an innovative approach, extremely important since the conventional therapies are not totally efficient, and also because of the prevalence of periodontal diseases worldwide.

1.5. Main goal of the project

The main goal of this work is the production, optimization, and further characterization of new *Bombyx mori* silk fibroin-based membranes using glycerol (GLY) or poly(vinyl alcohol) (PVA) as plasticizers in order to improve flexibility and enhance the rates of SF stabilization. The developed membranes, designed to be in contact with the gingiva, aim at providing an effective solution for mild periodontitis (the most prevalent form worldwide) by helping to restore the anatomy and function of lost or damaged periodontal tissues.

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CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Cocoons from *Bombyx mori* were supplied by the Portuguese Association of Parents and Friends of Mentally Disabled Citizens (APPA-CDM) from Castelo Branco, Portugal. The remaining reagents were purchased from Sigma-Aldrich, unless otherwise stated.

2.2. Preparation of silk fibroin aqueous solution

Silk fibroin solution was prepared using a previously developed procedure (Sofia et al. 2001). In brief, after cleaning and cutting the cocoons into small pieces (5 g for each extraction), they were boiled, in constant magnetic stirring, in 2 L of distilled water containing 4.24 g of sodium carbonate (Na_2CO_3, $M_w \sim 0.02$ M) for 1 hour. Then, the supernatant was rinsed thoroughly 1 L of distilled water for more 30 minutes to ensure the complete extraction of sericin. The extracted SF dried in the hood at least for 12 hours.

To dissolve the obtained SF, a 9.3 M lithium bromide (LiBr) stock solution was prepared. For each 5 g of SF, 25 mL of LiBr was added. The mixture was putted in the oven at 70 ºC for 1 hour. Then, the SF/LiBr solution was dialyzed against 5 L of distilled water, using a benzoylated dialysis tubing (molecular weight cut-off: 2000), for 48 hours in order to remove the salt. The water had to be changed at 1 hour, 2 hours and 4 hours in the first day and 3 times a day in the second one. The purified SF solution was stored at 4 °C until further use.

2.3. Preparation of SF/PVA and SF/GLY membranes

Poly(vinyl alcohol) is a synthetic polymer produced on an industrial scale by hydrolysis of poly(vinyl acetate), presenting improved mechanical properties and flexibility (Pal et al. 2007; Asran et al. 2010). Moreover, aa hydrophilic polymer, PVA exhibits excellent water retention properties, however, it requires water temperatures near 100 ºC for its total dissolution (Peppas and Merrill 1977). Glycerol applications range from its use in food and tobacco to its use in medical and pharmaceutical industry (Pagliaro and Rossi 2010). It is also a water-soluble synthetic polymer characterized by high tensile strength, flexibility, and capacity of improving materials smoothness (Pagliaro et al. 2007).

In this sense, purified SF solution was mixed with PVA or GLY at weight ratios of 0, 10 and 30% (w/v). To prepare a 10% (w/v) PVA stock solution, 1.56 g of dry PVA powder were dissolved in 50 mL of distilled water and heated in a water bath at 95 ± 2 °C for 2 hours to guarantee its complete dissolution. The 10% (w/v) GLY stock solution was prepared in the same conditions as mentioned before but without heating. The same procedure was used to prepare the 30% (w/v) PVA and GLY stock solutions.

The membranes were obtained by casting the final solution into Petri dishes and dried at 85 ºC in the oven for 6 hours some, and 12 hours others for further characterization. As control, pure SF membranes were prepared in the same conditions as described above.

2.4. Morphological characterization

Scanning Electron Microscopy (SEM)

The morphology of the membranes dried for 6 and 12 hours was observed by SEM using a Leica Cambridge S-360 (United Kingdom) operating at 15 kV accelerating voltage. All the samples were sputtered with a conductive gold layer, using a sputter coater SC502 (Fison instruments, United Kingdom).

Atomic Force Microscopy (AFM) Imaging

The produced SF membranes were imaged using a Dimension Icon microscope controlled by the NanoScope V from Bruker (France) operating in ScanAsyst mode. A ScanAsystAir cantilever (Bruker, France) with a resonance frequency of 70 kHz and a spring constant of 0.4 $N.m^{-1}$ was used. Substrate topographies were imaged with 512 X 512 $pixels^2$ at line rates of 1 Hz. For surface roughness analysis, 5 X 5 μm^2 AFM images were obtained and the roughness average (Ra) was calculated. At least three measurements were performed per sample.

2.5. Surface characterization

Contact angle and surface energy measurements

The wettability of the membranes was assessed by contact angle (θ). Static contact angle measurements were obtained by the sessile drop method using an OCA15+ contact angle meter with a high performance image processing system (DataPhysics Instruments, Germany). Two different liquids were used: ultrapure water (upH_2O; polar; 3 μL) and diiodomethane (CH_2I_2; non-polar; 3 μL), added using a motor driven syringe at room temperature. The presented data are the average of four measurements per sample. The surface free energy (γ) of the samples was calculated by Owens, Wendt, Rabel and Kaelble (OWRK) equation which is indicated for low energy surfaces such as polymers (D. K. Owens and Wendt 1969; Kaelble 1970).

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

All spectra were acquired using a spectrometer Perkin-Elmer (ABB, Switzerland) equipped with an attenuated total reflectance (ATR) device (PIKE Technologies, United States of America). The software was programmed to record each spectrum between 4000 and 450 cm^{-1} at a resolution of 4 cm^{-1}. Samples and background (air) measurements were made by co-adding 64 scans.

2.6. Hydration degree and degradation profile

Physical integrity

The solubility of the produced membranes was qualitatively estimated by their immersion in 20 mL of distilled water for 24 hours, at room temperature. After the incubation, the membranes were evaluated

regarding their physical integrity, as qualitatively soluble (S), partial soluble (PS) or non-soluble (NS) in water.

Hydration degree

The hydration degree of the samples was also studied in vitro. The samples (n=3, weight ~ 0.02 g) were immersed in 4 mL of PBS. The assay was performed at 37 ºC for 15 and 30 minutes, 1, 3, 6, 12 and 24 hours. At the end of each period, the samples were removed from the solution, gently blotted with a filter paper to remove the excess of liquid and weighted. The water uptake was calculated as follows:

$$\text{hydration degree } (\%) = [(m_w - m_f)/m_f] \times 100\% \quad (2.6.1)$$

where m_w is the weight of the sample after removal from the solution and m_f is the weight of the sample after drying.

Degradation studies

The degradation behaviour of the SF-based membranes was studied in vitro. The samples (n=3, weight ~ 0.02 g) were immersed in 4 mL of PBS containing 4.0 mg protease XIV mL^{-1}. Protease XIV was derived from *Streptomyces griseus* (Sigma, 3.5 $U.mg^{-1}$). As a control samples were incubated in PBS alone. The study was conducted at 37 ºC for 1, 3, 7, 14 and 30 days, under sterile conditions. The solutions were changed weekly. After each test period, the samples were removed from the containers, rinsed with deionized water, dried at 45 ºC in the oven overnight and weighted. The weight loss was determined as:

$$\text{weight loss } (\%) = [(m_i - m_d)/m_i] \times 100\% \quad (2.6.2)$$

where m_i is the initial weight of the sample and m_d is the weight of the sample after drying.

2.7. Mechanical properties

Mechanical Tests

Uniaxial tensile tests were performed using a universal electromechanical testing equipment (Instron 5543, USA) with a 1 kN load cell. SF-based membranes were previously cut and hydrated by immersion in a phosphate-buffer solution (PBS) for 3 hours, then their dimensions (thickness, width and length) were obtained from three measurements. For each condition, four hydrated samples with similar dimensions (10x35 mm) were tested under a crosshead speed of 5 $mm.min^{-1}$ until their rupture in the middle region. Based on the experimental stress-strain curves, the main tensile properties were determined: the tensile Young's modulus (E, MPa), the Ultimate Tensile Strength (UTS, MPa), and the Ultimate Strain (ε, %). Briefly, the Young's modulus was determined as the slope of the initial linear region of the curve, the ultimate tensile strength was the maximum stress value, and the ultimate strain

was the strain value presented for UTS value. For each mechanical parameter, mean and standard values were obtained using the experimental results from three samples.

2.8. Thermal properties

Differential scanning calorimetry (DSC)

The DSC experiments were performed in a TA Instrument model DSC Q100 (USA) under a nitrogen atmosphere as a purge gas (gas flux ~ 50 $mL.min^{-1}$). The samples were scanned in two continuous thermal cycles at the rate of 10 $°C.min^{-1}$: from 0 - 140 ºC in order to remove any water from the samples and from 0-350 ºC. Two samples were used per condition. The thermograms of the second thermal cycle were used for analysis.

CHAPTER 3

RESULTS

3.1. Surface morphology and topography

Morphological characterization was performed to assess the impact of the presence of GLY and PVA on the SF membranes' macro- and microstructure. Macroscopically, membranes dried for 6 and 12 hours at 85ºC present similar aspect and microstructure. In this way Figure 3.1 presents the macroscopic images and respective SEM micrographs for membranes dried for 12 hours as representative of the obtained morphology. As presented in Figures 3.1a, b and c, pure SF and SF/GLY membranes are similarly transparent while the presence of PVA in the SF/PVA membranes (Figures 3.1d and e) reduces transparency with increasing content. SEM micrograph of pure SF membrane (Figure 3.1f) reveals a smooth surface. When the GLY is incorporated (Figures 3.1g and h) it is possible to observe the formation of a microstructure that becomes more visible for concentrations of 30% (Figure 3.1h). These results may suggest that the glycerol is well distributed in the SF matrix. In the membranes with 10 and 30% PVA content (Figures 3.1i and j correspondingly) bubble-like formations with various sizes, immersed in a homogenous matrix are observed. These structures can indicate that a phase separation of the two polymers is occurring.

Figures 3.2 and 3.4 present the AFM images of the membranes' surfaces dried for 6 and 12 hours according to the polymer content. The correspondent average roughness is also presented at figures 3.3 and 3.5 correspondingly.

In brief, the surface nanotopography and the average roughness of the SF-based membranes are affected by the exposure time at 85ºC and by the percentage of additive content. Pure SF membranes (Figures 3.2a and 3.4a) and those containing 10% of GLY and PVA (Figures 3.2b and 3.4b respectively) dried for 6 hours present a smoother surface when compared with the 30% GLY (Figure 3.2c) and PVA membranes (Figure 3.4c). Pure SF membranes dried for 12 hours (Figures 3.2d and 3.4d) exhibit an increase in the topography when compared with similar membranes dried for 6 hours, possibly due to a self-assembly process during thermal treatment. When adding GLY and PVA the surface roughness increases being more evident for the highest percentage of PVA content (Figure 3.4f). Concerning the average roughness (R_a) as expected it is higher in the membranes dried for 12 hours which are the ones that exhibit a rougher surface for both type of membranes. Moreover, the addition of GLY (Figure 3.3) and PVA (Figure 3.5) to the membranes leads to an increase in the R_a values, especially in the membranes containing PVA (R_a values varying from 11.00 nm to 16.40 nm for the membrane with 30% PVA content). Those results are in agreement with the AFM images results.

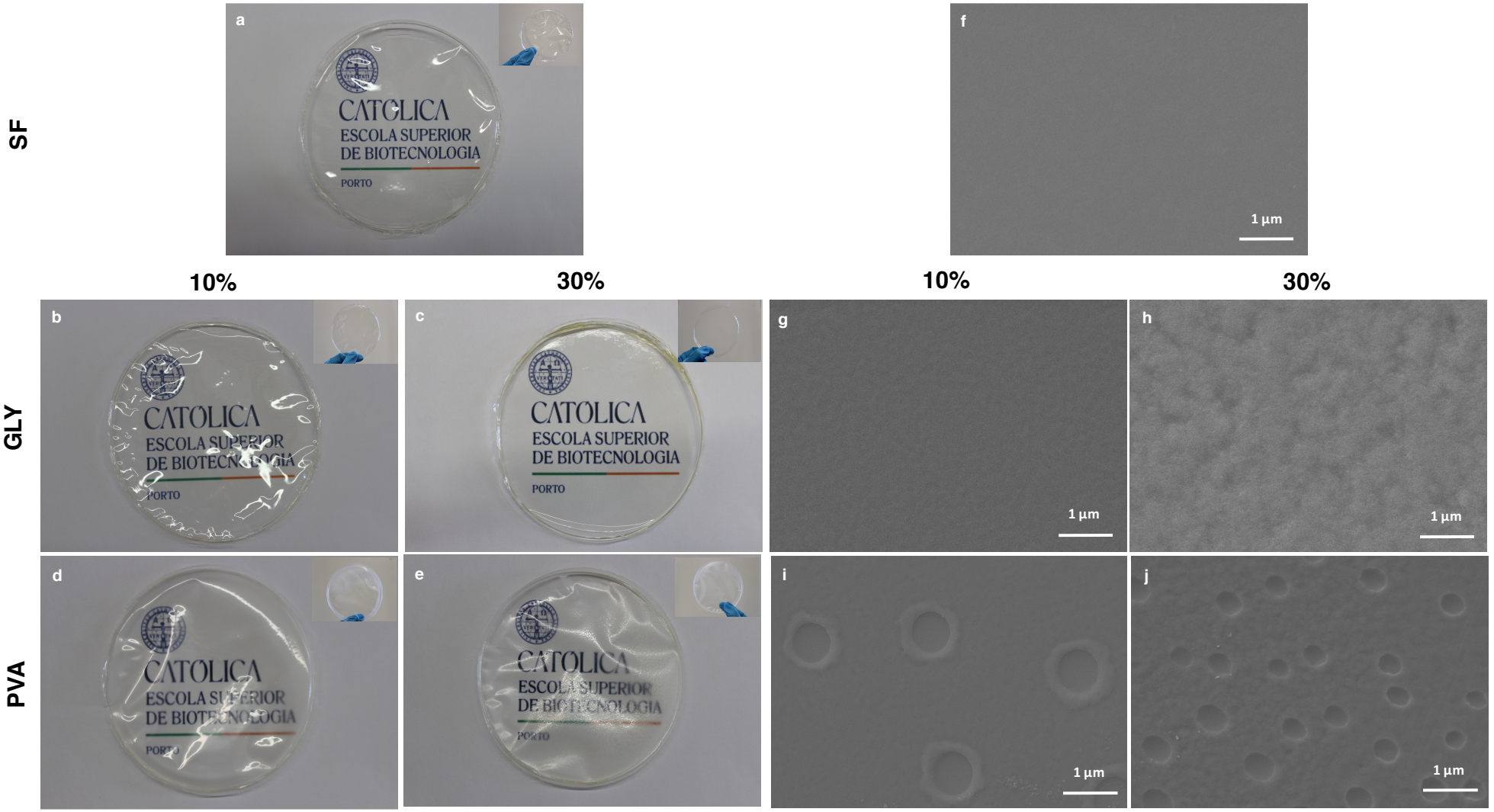


Figure 3.1 – Macroscopic images (a, b, c, d, e) and SEM micrographs (f, g, h, I, j) of the produced SF-based membranes: pure SF (a, f), 90SF:10GLY (b, g), 70SF:30GLY (c, h), 90SF:10PVA (d, i), and 70SF:30PVA (e, j) dried for 12 hours.

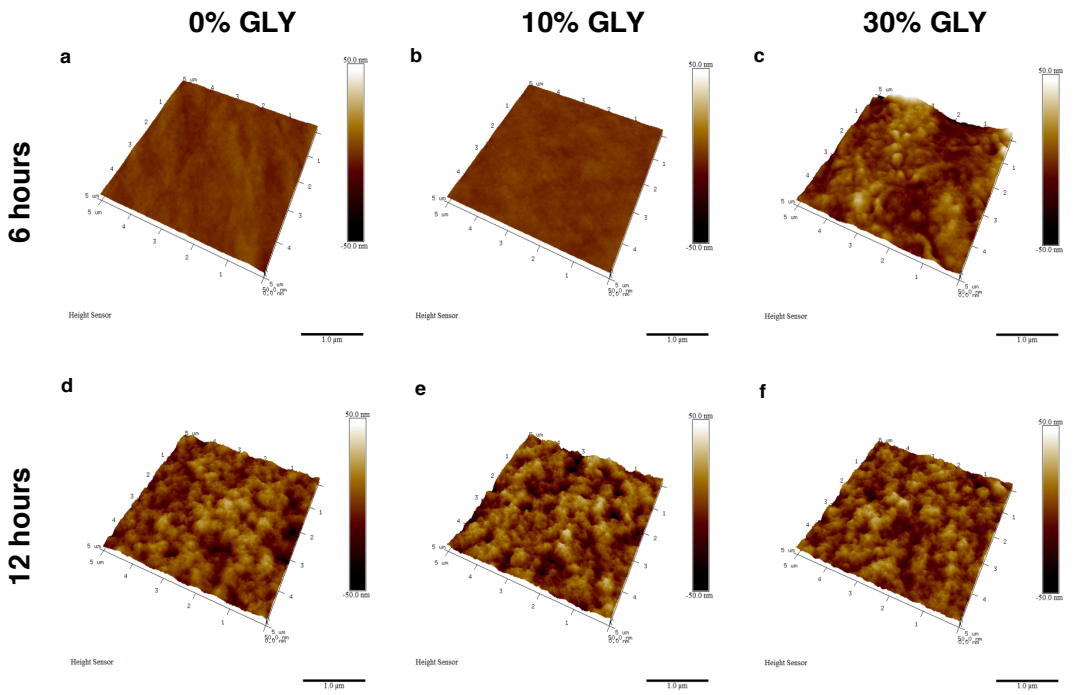


Figure 3.2 – AFM images (5×5 μm^2) of the SF/GLY membranes dried for 6 hours (a, b c) and 12 hours (d, e, f) according to the polymer content: 0% GLY (a, d), 10% GLY (b, e), and 30% GLY (c, f).

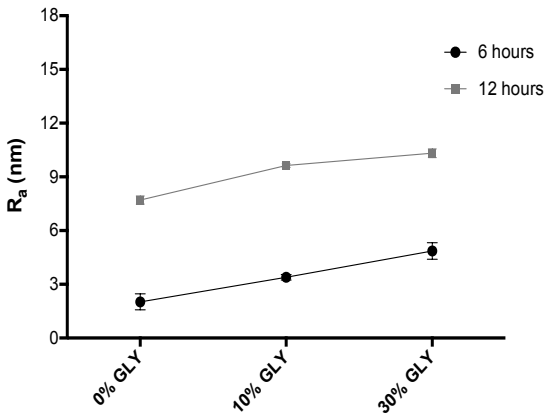


Figure 3.3 – Average roughness (R_a) of the SF-based membranes with increasing amount of GLY dried for 6 and 12 hours.

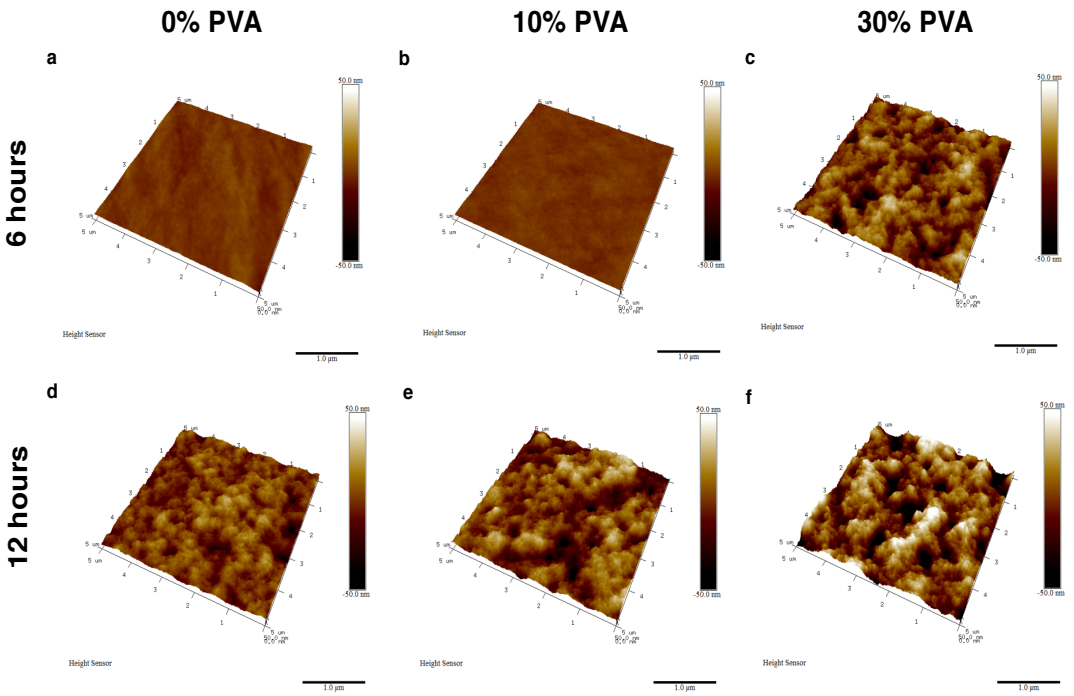


Figure 3.4 - AFM images (5×5 μm^2) of the SF/PVA membranes dried for 6 hours (a, b c) and 12 hours (d, e, f) according to the polymer content: 0% PVA (a, d), 10% PVA (b, e), and 30% PVA (c, f).

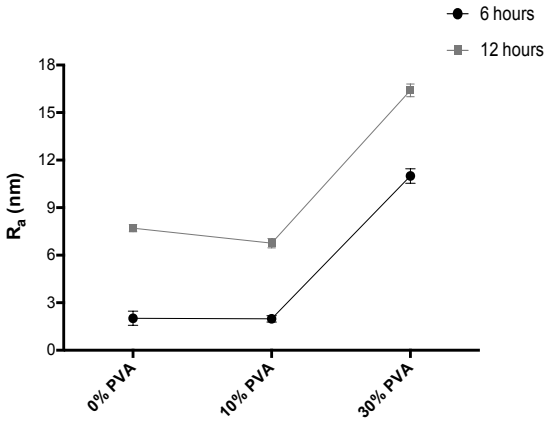


Figure 3.5 - Average roughness (R_a) of the SF-based membranes with increasing amount of PVA dried for 6 and 12 hours.

3.2. Surface wettability and chemical structure

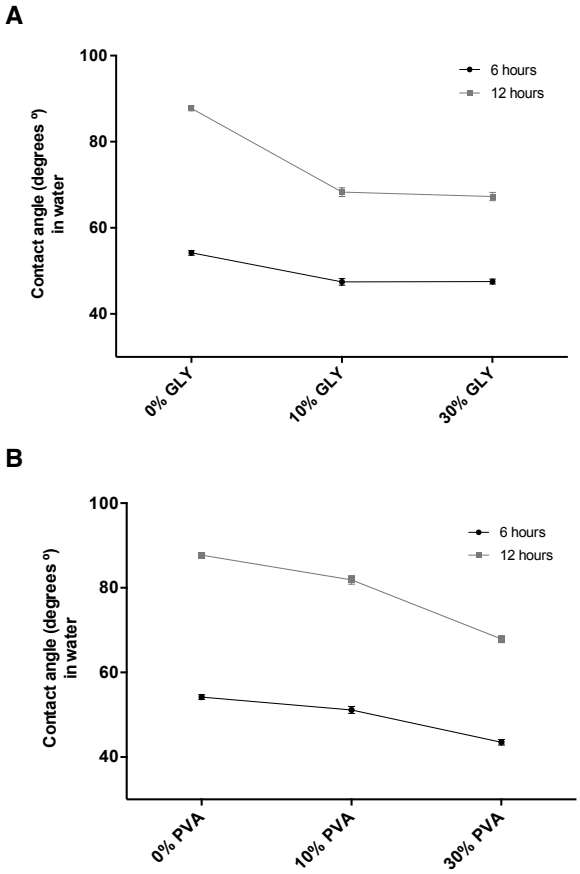


Figure 3.6 - Water contact angles obtained for the SF-based membranes with additive of GLY (A) and PVA (B) dried for 6 and 12 hours.

The water contact angle values obtained for the SF-based membranes under the two different drying periods are plotted in Figure 3.6 and the respective calculated surface energies are presented in Table 3.1.

In Figures 3.6A and B, it is possible to observe a general trend toward a decrease of the water contact angle with increasing amount of the synthetic polymer (GLY and PVA). Pure SF membranes present a contact angle of ~ 54.20º after 6 hours of thermal treatment which increase to ~ 87.00º after 12 hours, indicating that the surface becomes more hydrophobic for longer periods of thermal treatment. With the addition of GLY (Figure 3.6A) the membranes present a decrease in the contact angle value and consequently a decrease in the water contact angle when compared with the control membrane (0% GLY), especially in those dried for 12 hours, with a decrease from ~ 87.00º (control membrane) to ~ 68.3º and ~ 67.3º for the ones with 10% and 30% GLY content respectively. It is also possible to observe that the contact angle among the membranes with 10 and 30% GLY content does not present significant differences for both treatment periods. In the SF/PVA membranes (Figure 3.6B) the same results are observed comparing both periods of thermal treatment. Nevertheless, there are differences between the membranes with 10 and 30% PVA content, since the decrease in the contact angle is more

pronounced when the highest amount of PVA is present. Consequently, an increase in the surface energy is observed due to a general increase in the polar component more significant for the membranes dried for 6 hours, as it can be seen at Table 3.1.

Table 3.1 - Contact angle (θ), dispersive (γ_s^d) and polar (γ_s^p) components, and superficial energy (γ_s) of the membranes dried for 6 and 12 hours, calculated by the OWRK equation.

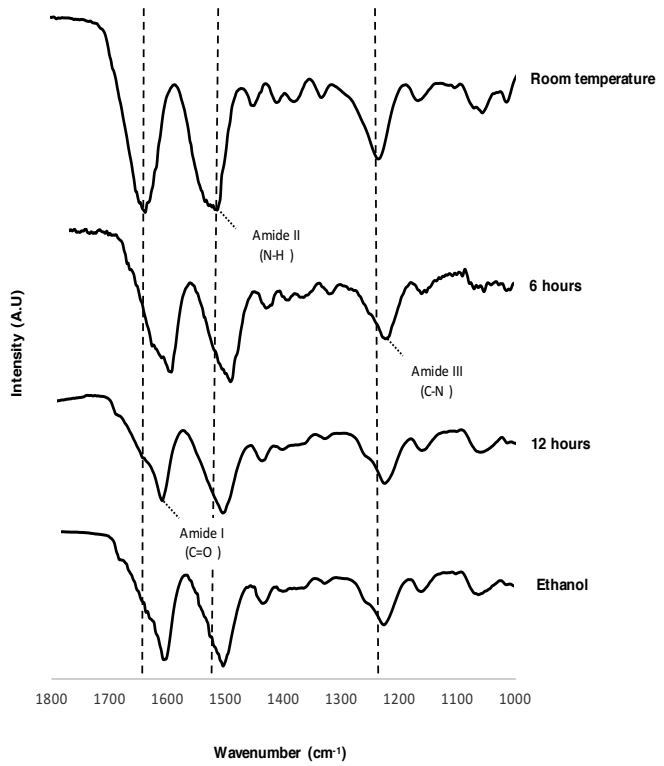
Membranes	θ_{water} (°)		γ_s (mN m^{-1})		γ_s^d (mN m^{-1})		γ_s^p (mN m^{-1})	
	6 h	12 h	6 h	12 h	6 h	12 h	6 h	12 h
Pure SF	54.2 ± 0.56	87.0 ± 0.55	28.46	48.57	23.22	24.57	5.24	24.00
10 % GLY	47.4 ± 0.77	68.3 ± 1.01	39.71	55.33	25.32	29.75	14.39	25.58
30 % GLY	47.5 ± 0.55	67.3 ± 0.96	40.86	55.41	26.26	29.09	14.60	26.32
10 % PVA	51.1 ± 0.82	81.9 ± 1.02	31.49	53.02	23.94	29.94	7.55	23.08
30 % PVA	43.5 ± 0.65	67.9 ± 0.83	39.11	57.70	23.77	29.52	15.34	28.18

B. mori SF has three major conformations in the solid state namely random coil, silk I (α-form), and β-sheet (or silk II) which is the most stable one (Asakura et al. 1985; Canetti et al. 1989; Silva et al. 2008). The most common method to convert SF from random coil and/or silk I to β-sheet is too add low dielectric constant organic solvents such as methanol, ethanol, or dioxane to the samples (Canetti et al. 1989; Yoshimizu 1990; Tsukada, Freddi, Gotoh, et al. 1994).

Conformational characterization of pure and blended membranes was carried out by means of attenuated infrared spectroscopy, as presented in Figure 3.7. To characterize the structure of the polypeptide, the amide I, amide II and amide III region of pure SF membranes was examined, before and after methanol and thermal treatments. SF membrane dried at room temperature displays an amide I band at 1638 cm^{-1} which is characteristic of random coil and/or silk I formation (Surewicz and Mantsch 1988; Ayub et al. 1994).

According to the literature, a pure SF membrane immersed into ethanol shows intense bands around 1615-1620 cm^{-1} for amide I, 1510-1515 cm^{-1} for amide II, and 1230-1235 cm^{-1} for amide III as it can be seen in Figure 3.7, by a shift to the right of these bands when compared with untreated silk (Asakura et al. 1985; Chen et al. 1997). The pure SF membranes dried for 6 and 12 hours present similar structural conformation which also indicates the predominance of the silk II structure. These results suggest that the thermal treatment induces the transition of random coil and/or silk I structure to β-sheet conformation.

A



B

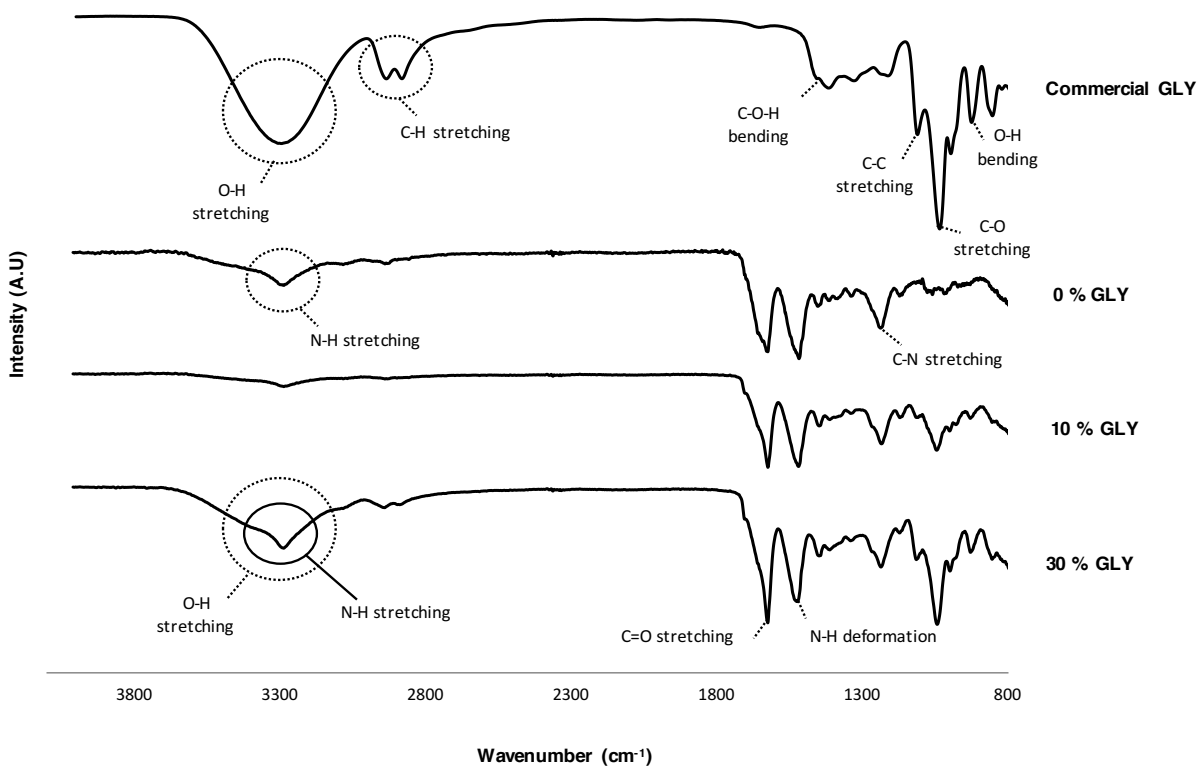
	Amide I	Amide II	Amide III
Room temperature	1638	1515	1233
6 hours	1618	1512	1230
12 hours	1619	1513	1231
Ethanol	1620	1512	1231

Figure 3.7 – (A) ATR-FTIR spectra of pure SF membranes dried: at room temperature; for 6 hours; 12 hours and immersed into ethanol. (B) Obtained peaks for amide band (cm^{-1}) in each tested condition.

Figure 3.8 shows the ATR-FTIR spectra of SF-based membranes with different GLY contents. The main groups of commercial glycerol include O-H stretching at peak range of 3000-3500 cm^{-1}, C-H stretching around 2880 and 2930 cm^{-1}, C-O-H bending at 1400-1460 cm^{-1}, C-O and C-H stretching about 1000 and 1110 cm^{-1} respectively, and finally O-H bending at 920 cm^{-1} (Kongjao et al. 2010; Indran et al. 2014). As presented above, the pure SF membranes dried for 6 and 12 hours present characteristic peaks derived from amide I (C=O stretching), amide II (N-H deformation), and amide III (C-N stretching). Moreover, a broad peak assignable to the N-H stretching of amide bonds appears between 3100-3600 cm^{-1}. In the membranes with 10% and 30% GLY content it is possible to observe the characteristics peaks of pure SF although there is an overlapping at 3000-3600 cm^{-1} between de O-H stretching of GLY

and the N-H stretching of SF. Regarding the amide I, II and III bands it is possible to see through the table below the ATR-FTIR spectra that there are no significant differences among the treatment periods.

A



B

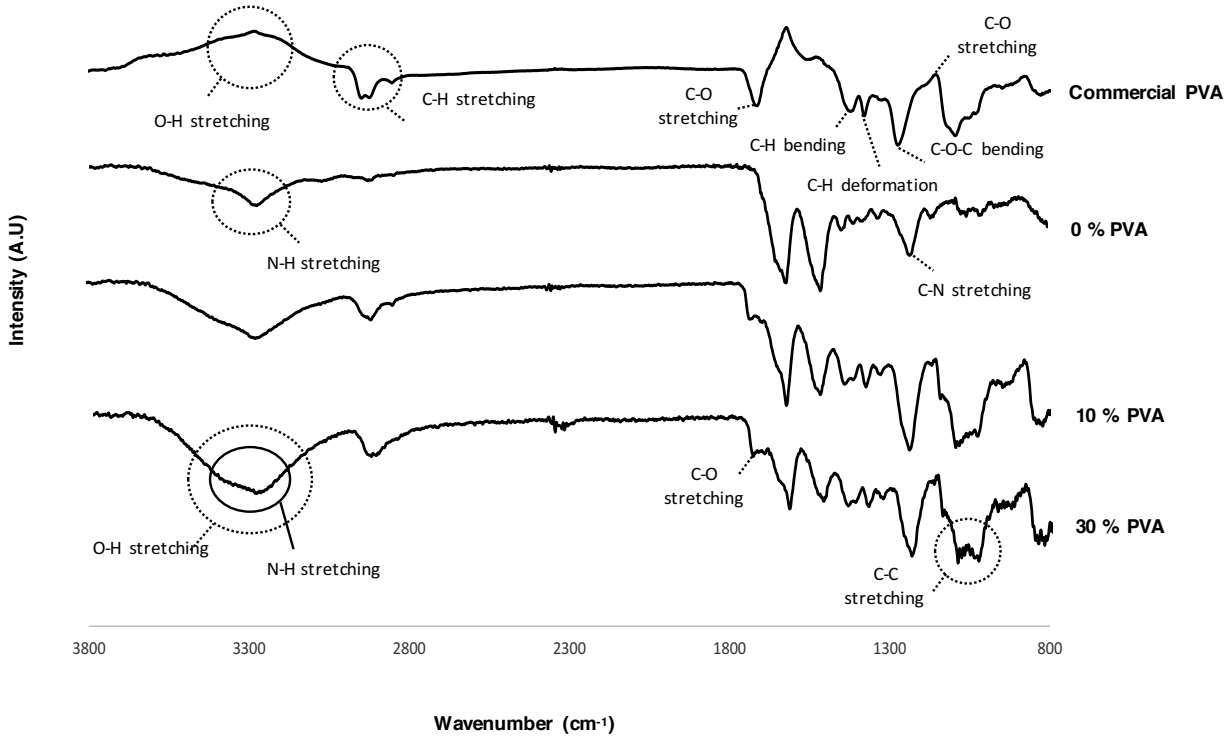
	Amide I		Amide II		Amide III	
	6 h	12 h	6 h	12 h	6 h	12 h
0% GLY	1618	1619	1512	1513	1230	1231
10% GLY	1618	1619	1515	1515	1229	1230
30% GLY	1620	1620	1514	1515	1229	1230

Figure 3.8 – (A) ATR-FTIR spectra of commercial GLY, and of the produced SF-based membranes with 0%, 10%, and 30% GLY content dried for 12 hours. (B) Obtained peaks for the amide bands (cm^{-1}) in each tested condition.

Figure 3.9 presents the ATR-FTIR spectra of SF-based membranes with different PVA content. The main peaks of commercial PVA are observed around 3278, 2935 and 2914, 1711, 1418, 1324, 1241, 1143 and finally 1087 cm^{-1}. These peaks are assigned to the O-H stretching vibration of the hydroxyl group, C-H stretching, C-O stretching, C-H bending, C-H deformation, C-O-C bending, C-O

stretching, and C-C stretching, accordingly (Sudhamani, Prasad, and Sankar 2003; Han, Chen, and Hu 2009). In the membranes with 10% and 30% PVA content it is possible to identify the characteristics peaks of pure SF, that have already been described above, and some characteristic peaks of PVA namely the C-H stretching around 2935 and 2914 cm^{-1}, the C-O stretching at 1711 cm^{-1}, C-C stretching near 1087 cm^{-1}, and an overlapping at 3200 - 3600 cm^{-1} between O-H of commercial PVA and N-H of pure SF that are the most pronounced ones. Regarding the amide I, II and III bands the same results were obtained.

A



B

	Amide I		Amide II		Amide III	
	6 h	12 h	6 h	12 h	6 h	12 h
0% PVA	1618	1619	1512	1513	1230	1231
10% PVA	1618	1619	1514	1515	1230	1230
30% PVA	1619	1620	1514	1515	1230	1230

Figure 3.9 – (A) ATR-FTIR spectra of commercial PVA, and of the produced SF-based membranes with 0%, 10%, and 30% PVA content dried for 12 hours. (B) Obtained peaks for the amide bands (cm^{-1}) in each tested condition.

3.3. *In vitro* stability

According to the literature, the behaviour of polymeric materials in the living environment can result in degradation caused by enzymatically and chemically induced cleavage, involving water, enzymes, metabolites and ions that interact with the material (Almeida et al. 2013). In this sense, the solubility of the pure and blend SF membranes was evaluated and the results are shown in Table 3.2.

Table 3.2 – Results of physical integrity test of the produced membranes being S = soluble, PS = Partial soluble, and NS = Non-soluble.

Membranes	Physical integrity	
	6 h	12 h
Pure SF	PS	NS
10 % GLY	S	NS
30 % GLY	S	NS
10 % PVA	S	NS
30 % PVA	S	NS

It is possible to observe that, except for the pure SF membrane, the blends dried for 6 hours are water-soluble after 24 hours. The ATR-FTIR spectra suggest that thermal treatment induced SF β-sheet conformation for both exposure times, which seems contradictory. Thus, these results may suggest that β-sheet domains need time to create an insoluble network. On the other hand, as expected, pure and blended membranes dried for 12 hours are not water-soluble revealing a formation of a stable structure. Regarding these results, further studies were conducted using the membranes dried for 12 hours.

The hydration degree was studied for the SF-based membranes after immersion in a buffer solution for 24 hours at 37 ºC (Figures 3.10A and B). In both cases the membranes reached equilibrium hydration after 3 hours and started to present a weight loss at 24 hours (data not shown). It is possible to observe a trend towards an increase in the hydration capacity with the addition of the synthetic polymers. These results show that the membranes are able to uptake a maximum of ~ 68 wt.% which is registered for the membrane with 30% PVA content (Figure 3.10B) as compared with the pure SF membranes (control) which present a lower hydration degree (~ 48 wt.%).

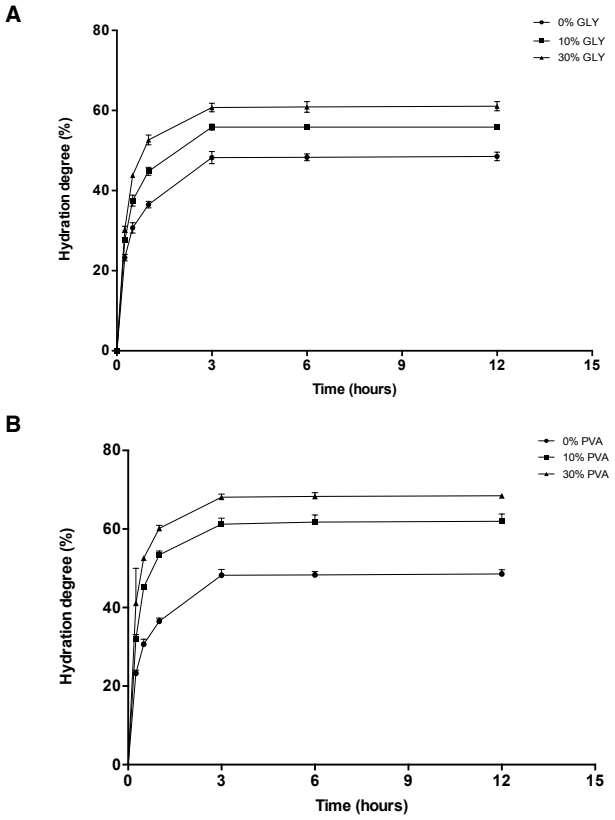


Figure 3.10 - Hydration degree of SF-based membranes dried for 12 hours, after immersion in buffer solution for 24 hours (% weight).

Additionally, the degradation behaviour was plotted for SF-based membranes after immersion in buffer solution with and without enzyme up to 30 days at 37 ºC (Figure 3.11). It is also possible to observe a tendency regarding an increase in the weight loss with the addition of synthetic polymer both in PBS (Figures 3.11A and B) and in PBS with Protease XIV (Figures 3.11C and D). This increase is, as expected, higher in the membranes immersed in buffer solution containing the enzyme, and higher in the membrane with 30% PVA content (30% induces a weight loss at 30 days ≈ 83%). These results are in agreement with those obtained for the wettability where the membranes with PVA addition showed an increase in the hydrophilicity when compared with the control membrane (pure SF one) having, consequentially, a higher capacity of absorbing water-based solutions.

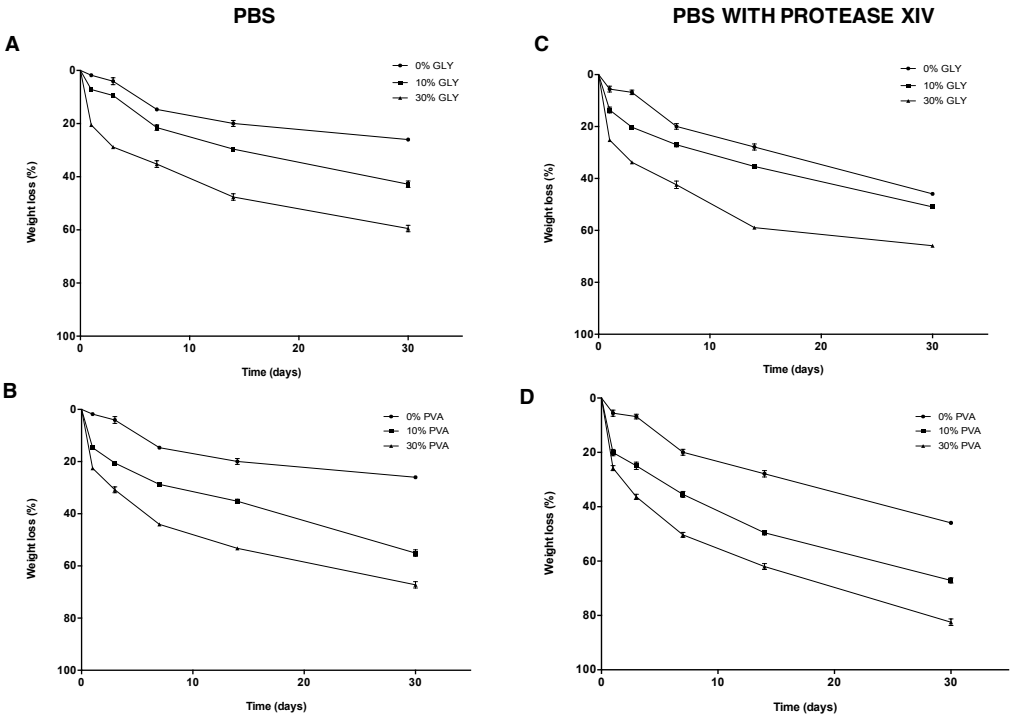


Figure 3.11 - Degradation behavior of SF-based membranes dried for 12 hours, after immersion in buffer solution without (A and B) and with (C and D) protease XIV for up to 30 days.

3.4. Mechanical properties

The study of the mechanical properties is of primary importance for determining the performance of a material that is expected to undergo various kinds of stresses during use (Freddi et al. 1999). In the present study, the effect of the presence of GLY and PVA on the mechanical performance of the SF membranes was evaluated. Therefore, quasi-static tensile tests were performed and the results are plotted at Table 3.2 and 3.3. Figures 3.12 and 3.13 show representative stress-strain curves for all formulations.

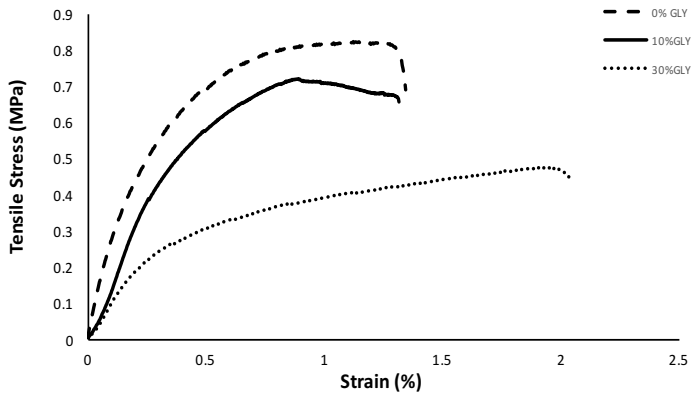


Figure 3.12 - Representative tensile stress (σ) – strain (ε) curves of the membranes with different GLY content, after immersion in PBS solution.

Table 3.3 - Young's Modulus (MPa), Ultimate Tensile Stress (MPa), and Ultimate Strain (%) of the membranes with different GLY content.

Membranes	Young's Modulus, E (MPa)	Ultimate tensile stress, UTS, σ_{ULT} (MPa)	Ultimate strain, ε_{ULT} (%)
0% GLY	248.52 ± 19.18	0.79 ± 0.08	0.90 ± 0.20
10% GLY	142.52 ± 17.09	0.59 ± 0.11	0.63 ± 0.42
30% GLY	111.75 ± 14.88	0.44 ± 0.06	1.53 ± 0.54

As expected, the pure SF membranes (control membrane) present a considerable higher strength and stiffness compared with the blended membranes (tensile modulus ~ 249 MPa). Concerning the blend membranes, the results show a decrease of both Young's modulus and the ultimate tensile strength with the increasing of GLY content (Table 3.2). The ultimate strain or maximum extension should increase with the addition of the synthetic polymer although in the one with 10% GLY content this mechanical property presents a decrease from 0.90% (0% GLY) to 0.63%. In general, the results suggest that with the addition of GLY the membranes became more flexible.

Regarding the SF/PVA membranes, the results also reveal a marked decrease in the Young's modulus and in the ultimate tensile strength with the increasing of PVA content (from ~ 249 MPa to ~ 66 MPa for 10% PVA content and ~ 57 MPa for 30% PVA in the E, and from ~ 0.79 MPa to ~ 0.32 MPa and ~ 0.26 MPa in the UTS accordingly) as it can be seen in Figure 3.13 and at Table 3.3.

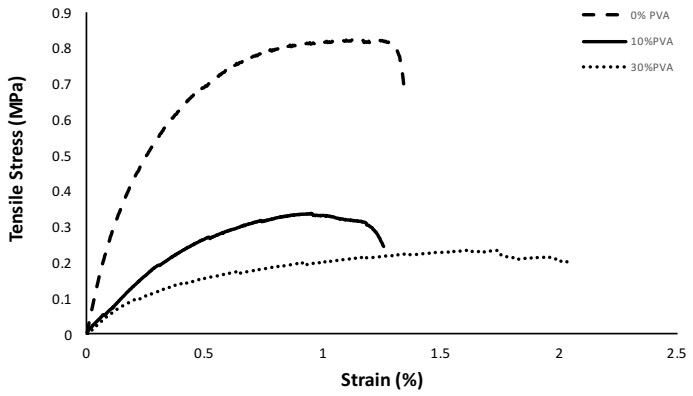


Figure 3.13 - Representative tensile stress (σ) – strain (ε) curves of the membranes with different PVA content, after immersion in PBS solution.

Table 3.4 - Young's Modulus (MPa), Ultimate Tensile Stress (MPa), and Ultimate Strain (%) of the membranes with different PVA content.

Membranes	Young's Modulus, E (MPa)	Ultimate tensile stress, UTS, σ_{ULT} (MPa)	Ultimate strain, ε_{ULT} (%)
0% PVA	248.52 ± 19.18	0.79 ± 0.08	0.90 ± 0.20
10% PVA	66.09 ±13.63	0.32 ± 0.01	1.20 ± 0.24
30% PVA	57.18 ± 7.15	0.26 ± 0.03	1.58 ± 0.23

As expected, the ultimate strain value is higher in the blended membranes, especially in the one with 30% PVA content (ε = 1.58 %). Overall, the addition of PVA leads to flexible and less brittle structures. The structures become again more ductile, and with higher capacity to deform as compared with SF/GLY membranes.

3.5. Thermal behaviour

DSC measurements can provide valuable data on phase transitions inside materials. Glass transition and melting are two important phenomena in polymers which are affected by processing conditions and additives in the materials (Sakurai, Maegawa, and Takahashi 2000). Figure 3.14 shows the DSC thermograms of commercial GLY, pure SF and SF/GLY membranes.

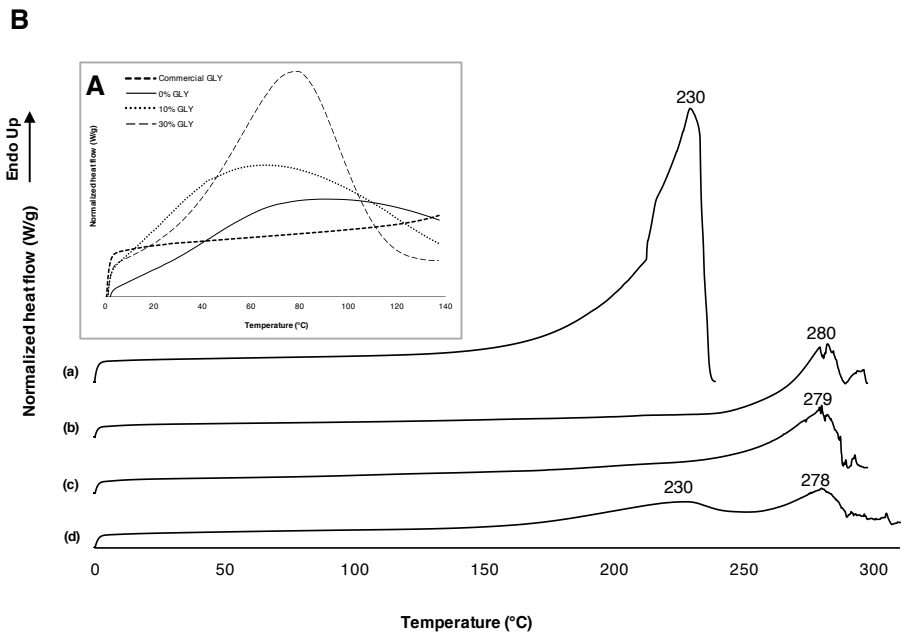


Figure 3.14 - DSC thermograms of the first heating cycle (A) and of the second heating cycle (B) that includes commercial GLY (a), and the produced SF-based membranes, dried for 12 hours, with 0% (b), 10% (c), and 30% (d) GLY content (scan rate of 10 ºC min^{-1}).

The first thermal cycle (Figure 3.14A) was only used to remove the water of the samples. Regarding the second thermal cycle (Figure 3.14B) commercial glycerol (a) reveals an endothermic peak nearby 230 ºC (L. Zhao et al. 2016). Pure SF membrane (b) also presents an endothermic peak around 280 ºC attributed, accordingly with literature, to the thermal decomposition of β-sheet SF (Magoshi and Nakamura 1975). The same peak is observed in the membranes with 10% (c) and 30% (d) GLY content although this peak shift to lower temperatures, 279 ºC and 278 ºC respectively, with the increasing of synthetic polymer. These results suggest that the crystallinity of SF membranes decreased with the increase of GLY content, and consequently thermal stability decreased. Additionally, it is also possible to observe the GLY thermal degradation peak in the membrane with higher synthetic polymer content contrarily to what happens in the one with 10% GLY.

Considering the SF/PVA membranes the DSC results are similar (Figure 3.15).

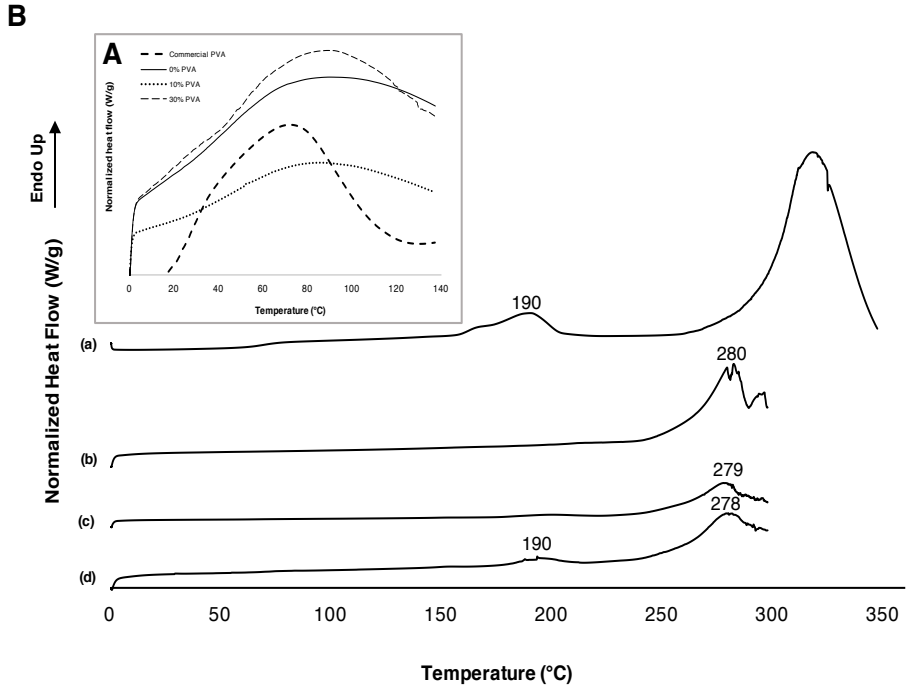


Figure 3.15 - DSC thermograms of the first heating cycle (A) and of the second heating cycle (B) that includes commercial PVA (a), and the produced SF-based membranes, dried for 12 hours, with 0% (b), 10% (c), and 30% (d) PVA content (scan rate of 10 ºC min^{-1}).

The first heating cycle (Figure 3.15A) was also used to remove the water of the samples and at the second heating cycle (Figure 3.15B) the melting temperature of PVA can be found around 190 ºC (Koosha et al. 2015). The addition of PVA to the SF membranes also contribute to see lower melting peaks, when compared to the pure SF (endothermic peak at 280 ºC). Moreover, it is possible to see the melting peak of PVA in the membrane with 30% PVA content (d).

CHAPTER 4

DISCUSSION

Periodontitis, the major cause of tooth loss in adults, is a globally prevalent inflammatory condition that leads to a progressive destruction of periodontal tissues (Daly, Seymour, and Kieser 1980; Beck et al. 1996; Tariq et al. 2012). In the last decades, a broad range of treatment options have been tested in order to reconstruct and restore the form and the function of the lost structures (Hämmerle and Jung 2003; Polimeni et al. 2006; Hanes 2007; Chen and Jin 2010). However, periodontal regeneration so far has not been entirely successful in humans. The poor ability of damaged tissues to regenerate and the constant microbial challenge in the oral cavity demonstrates the need of developing clinically effective materials to renew healthy periodontal tissues (Tariq et al. 2012; Zeng et al. 2014).

4.1. Design of SF membranes through blending

Guided Tissue Regeneration and Guided Bone Regeneration procedures were established as basic techniques in periodontal regenerative medicine due to its promising results (Campbell et al. 1956; Hurley et al. 1959; Melcher 1976; Sam and Madhavan Pillai 2014). Thus, the employed barrier membrane has to meet certain essential design criteria emphasizing their clinical manageability to allow its placement *in vivo* (Gottlow 1993). Natural-based materials, as silk fibroin obtained from *Bombyx mori* coccons, have gained popularity owing the fact of presenting excellent biological properties, such as cell adhesiveness, biocompatibility and biodegradability, and notably low effective cost (Santin et al. 1999). Although extracted SF is characterized by high mechanical strength, after processing it is brittleness resulting in consequent low manageability (Rockwood et al. 2011). As a strategy to increase ductility and therefore ensure its adequate manipulation and adaptability to the periodontal defect the development of SF membranes blended with different synthetic polymers has been proposed by several authors in the past few years (Sun et al. 1997; Freddi et al. 1999; Kweon et al. 2001; Jin et al. 2004).

The present work proposed two different strategies to produce SF-based membranes by blending with poly(vinyl alcohol) and glycerol, two well-known synthetic polymers used as plasticizers to improve flexibility of SF (Tanaka, Tanigami, and Yamaura 1998; Kawahara, Furukawa, and Yamamoto 2006). Both polymers have been widely used in a broad range of biomedical applications including wound healing and drug delivery system due to their biological properties, especially their non-cytotoxicity. In this sense, Lu and co-workers (Lu et al. 2009) prepared SF/GLY films for biomedical applications. In a preliminary study, they concluded that 30% glycerol-blending (which was the condition with suitable mechanical properties) supported fibroblast attachment and growth, proving that at least 30% GLY content is nontoxic for this type of cells. Additionally, Yeo et al. (Yeo et al. 2000) studied the effect of PVA/chitosan/silk fibroin-blended sponge in rats wound healing. Histopathological inspection

12 days after the implantation showed an increase of vascular ingrowth and the absence of inflammatory cells.

The miscibility of GLY in SF was higher than in PVA, as confirmed by SEM where for SF/PVA it was possible to detect a phase separation between the two polymers, leading to a decrease in transparency. Jin et al. (Jin et al. 2004) found out that by blending SF with polyethylene glycol (PEG) similar results were obtained. Moreover, the bubble-like formations detected in the SEM micrographs of SF/PVA membrane are in agreement with an earlier study performed by Tsukada et al. (Tsukada, Freddi, and Crighton 1994) in which the presence of nucleating structures at different dimensional levels are caused by this phase separation. To further investigate the presence of one or both components at the nucleating structures, transmission electron microscopy (TEM) could be used in future work.

4.2. Effect of thermal treatment on the stabilization of the membranes

It has been described that extracted SF does not present enough intermolecular hydrogen bonds to stabilize its structure. Consequently, to induce water stability of SF-based materials (in this case membranes), physical and chemical treatments such as the presence of water vapor (Hu et al. 2011), high temperature (Tsukada et al. 1992; H. Kweon, Woo, and Park 2001), and immersion in organic solvents (Tsukada, Gotoh, et al. 1994) have been suggested (Rujiravanit et al. 2003; Rockwood et al. 2011). According to Tsukada et al. (Tsukada et al. 1992) to understand the effects of thermal treatment at the structural level may contribute to better control and fasten processing conditions as well as to give the hint for producing limited modifications to enhance material performance and consequently expand their use to other applications. Based on this principle we selected thermal treatment as a viable processing route, easy to be controllable and industrialized, for stabilizing our blended SF membranes using the lowest temperature possible and minimal time of exposure to ensure water stability of the membranes processed by solvent casting. After preliminary stability screening, two exposure times, 6 and 12 hours and the temperature of 85 ºC were defined for the present study.

The AFM images showed that the thermal treatment induced a modification in the surface nanotopography and a subsequent increase in the surface roughness, especially in the membranes treated for 12 hours. These results can be explained by SF self-assembly during the thermal treatment. Self-assembly, by definition, is the spontaneous organization of molecules under thermodynamic equilibrium conditions intro structurally well-defined and stable arrangements through a number of noncovalent interactions (typically hydrogen bonds, ionic bonds, and Van der Waals forces) (Brinker et al. 1999; Shuguang Zhang 2002). The thermal treatment induced, as expected, the evaporation of membranes' water and allowed sufficient molecular mobility for the arrangement of SF molecules into more a stable conformation as β-sheet. However, at an initial stage of 6 hours these forming structures, which are not yet in sufficient number to create a stable intermolecular network, will suffer rearrangements so as to create topographical changes. Therefore, these membranes were still water unstable and had smoother surfaces similar to the untreated ones. This similarity is corroborated by Hofmann et al. (Hofmann et al. 2006) that directed a study in which a smother surface for unthreaded

silk film and a rougher surface for methanol treated ones are observed, demonstrating that the treatments influenced the surface nanotopography.

ATR-FTIR spectra of the produced SF-based membranes dried out for 6 and 12 hours confirmed the transition of SF from random coil/silk I to β-sheet conformation during the thermal treatment. These fallouts were corroborated by Kweon et al. (H. Kweon, Woo, and Park 2001) who study the effect of heat treatment at structural and conformational level of *Antheraea pernyi* silk fibroin films. This study settled that the formation of β-sheet domains occurred as a consequence of random coil/silk I disruption and fibroin chain rearrangements caused by the applied heat treatment. Therefore, it was possible to confirm that the temperature and treatment time greatly influenced the conformation of the produced films. No significant differences regarding the amide I, II and III bands among the treatment periods were observed, indicating that from 6 hours treatment the β-sheet conformation is already formed, as compared with samples without thermal treatment. In order to test the efficiency of the thermal treatment to stabilize the structure of the membranes a solubility test was performed in water for 24 hours, for the samples treated for 6 and 12 hours. Regardless of presenting similar β-sheet content as presented at ATR-FTIR results, after 6 hours the samples dissolved whereas after 12 hours of thermal treatment the samples remained stable. The fact that after 6 hours of thermal treatment the samples are still water soluble, might be explained with the fact that β-sheet domains need time to be spatially arranged to create an insoluble network created mainly by hydrogen bonds. Therefore, the samples treated for 12 hours were selected for further studies. In this study the quantification of β-sheet domains present in the structures was not determined, being possible that the materials treated for 6 hours presented less amount of β-sheet as compared to the amorphous structures such as random coil and silk I. Fourier transform self-deconvolution could be used to determine the fractional contributions of the FTIR amides absorbance spectrum according to the applied treatment, as proposed by Hu and co-workers (Hu, Kaplan, and Cebe 2006).

4.3. Physicochemical performance of the developed membranes

GLY and PVA are two water-soluble polymers characterized by a large band around 3000 – 3500 cm^{-1} typical of hydroxyl (OH) group that leads to an increase of water affinity of the blend membranes according to their polymer content, as confirmed by the contact angle results and hydration degree profile. These outcomes resulted in molecular interactions with SF chain via intermolecular forces, most likely hydrogen bonds, between hydroxyl groups of the synthetic polymers and the amide groups of silk as previously described by Dai et al. (Dai, Li, and Yamada 2002).

The DSC curves of SF-based membranes presented a decrease in their endothermic peak with synthetic polymer content addition. The same decrease has been observed by H. Zhang et al. (H. Zhang et al. 2011) who conducted a study on sericin films blended with different GLY content. In this case these results were attributed to a decrease in crystallinity of sericin. As stated in Chapter 1, the β-sheet conformation of SF (also known by crystalline structure) in insoluble water. Therefore, extrapolating the obtained fallouts of H. Zhang et al. the addition of GLY and PVA content to the SF membranes may

validate the results obtained for the degradation behavior where it was possible to observe that the degradation of the materials in PBS solution with and without enzyme was higher in the blended membranes than in the pure SF one (control membrane). Regarding the degradation studies, it is important to consider the presence of enzymes when developing *in vitro* studies, to better replicate the physiological environment. Thus, protease XIV is an enzyme that is usually used for this purpose. Horan et al. (Horan et al. 2005) also used this type of protease, changing the solution on a daily basis due to a significant loss in its activity when incubated at 37 ºC for periods longer than 24 hours. In our case the solution was changed weekly, which could be the reason for the maintenance of up to 30 days of all membranes. These results demonstrate that the produced materials are susceptible of being biodegradable which can eliminate the 2nd surgical intervention.

The mechanical performance is in the range of what is expected for a biomaterial for this application, since the blended membranes exhibit higher flexibility and ductility when compared with the pure SF membrane. Table 3.1 at chapter 1 summarizes some of the most commercialized membranes for GTR/GBR applications. Coïc and collaborators (Coïc et al. 2009) tested the mechanical properties of BioMend Extend® membrane, derived from collagen type I of bovine tendon, in wet and dry state. The mechanical strength of BioMend Extend® membrane (22.5 MPa) in wet state is pointedly lower than the produced SF-based membranes with mechanical strength ranging from 57.8 – 248.5 MPa. AlloDerm® membrane was also tested in terms of mechanical properties by K.W. Owens et al. (K. W. Owens and Yukna 2001) presenting a mechanical strenght in wet state near 21.2 MPa which is also lower than the obtained results for SF pure and blended membranes. For the other membranes, namely BioGide® and Resolut LT, the discrepant results may be explained by the undergo conditions of the mechanical tests, suggesting that the evaluation of commercial membranes was performed in dry conditions.

CHAPTER 5

CONCLUSIONS

The results reported in this study show that SF/GLY and SF/PVA membranes could be easily prepared by using a conventional casting methodology followed by a thermal treatment. It is noteworthy that blend films exhibited increased mechanical properties as compared to the pure SF membrane, being suitable in terms of ductility and flexibility to be manipulated by the surgeon and to adapt to the periodontal defect site. This behavior can be attributed to the formation of specific molecular interactions between de SF and the synthetic polymers.

Regarding the obtained results and the primary aim of the work, the development of a biodegradable membrane with sufficient clinical manageability, it is possible to conclude that the best approach is to blend silk fibroin with 30% glycerol content. With this additive the resulting membrane presents less deformation and higher percentage of elongation than the membrane with 30% poly(vinyl alcohol) content. Additionally, it shows less degradation behavior in 30 days which is attractive for being used in periodontal tissue regeneration since the membrane degradability needs to time match with the formation of new tissue. Furthermore, it was demonstrated that the processing versatility allows, by varying the additive polymer content or the thermal treatment period, to modulate the membrane performance in an *in vivo* scenario for a periodontal tissue regeneration approach.

FUTURE WORK

To demonstrate the biocompatible behavior of the developed membrane systems the future step of this work will be to do *in vitro* biological studies by performing cell culture tests using primary periodontal ligament cells (PDLs), so as to evaluate cell adhesion, proliferation, and viability over time of study. The selected conditions that will be tested are the ones with the most adequate mechanical properties, namely the blended membranes with the highest percentage of synthetic polymer.

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