Universidade do Minho
Escola de Engenharia

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Asymmetric PDLLA Membranes Containing Bioglass® for Guided Tissue Regeneration

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Professor Doutor João Filipe Colardelle da Luz Mano

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Abstract

In the treatment of periodontal defects, composite and asymmetric membranes might be applied to protect the injured area and simultaneously stimulate distinct tissue regeneration. This work describes the development and characterization of poly(D,L-lactic acid)/Bioglass® (PDLLA/BG) membranes with asymmetric bioactivity, prepared by an adjusted solvent casting method that promoted a non-uniform distribution of the inorganic component along the membrane thickness. We hypothesized that an improvement on structural and osteoconductive properties of the composite membranes would occur by the addition of BG, comparing to the pure PDLLA ones. To test this hypothesis a wide range of assays was performed.

In vitro asymmetric bioactive behavior was proved. SEM micrographs revealed the smoothness of pure PDLLA membranes surface contrasting to the homogeneous asperities distribution of BG on the composite membranes bottom side surface, in which was exhibited an apatite layer upon immersion in simulated body fluid. The detection of BG presence was complemented by FT-IR spectra analysis. Owing to the BG microparticles hydrophilicity, an enhancement on swelling ratio would be expected by their incorporation on the membranes. Such result was no significantly visible, which may have been influenced by the weight loss induced through BG dissolution in PBS, and which percentage was consequently statistically higher for PDLLA/BG membranes. Such process is consistent with the abovementioned event of the formation of an apatite layer. The mechanical properties of the membranes were not significantly compromised with the introduction of BG. Revealing that this formulation maintains the necessary integrity for the membranes function.

Human bone marrow stromal cells (hBMSC) and human periodontal ligament cells (hPDL) were seeded in osteogenic medium on the membranes surface, such as the ideally cell culture choice for the assessment of biological performance, respectively concerning to alveolar bone and periodontal ligament tissues. SEM observation, DNA content and metabolic activity quantification revealed an improved cell adhesion and proliferation for the PDLLA/BG membranes. A significant enhancement on cell differentiation was further detected by the measurement of APL activity, as well as a promoted mineralization, an extended extracellular matrix (ECM) and calcium nodule formation, suggesting the positive effect of the BG microparticles added. These last results were confirmed by both Ca content measurement and Von Kossa staining assays. Accordingly, from this formulation is expected a higher and even better regeneration of the abovementioned tissues. The results indicate that the proposed asymmetric PDLLA/BG membranes could have potential to be used in guided tissue regeneration therapies or in orthopaedic applications, with improved outcomes.

Resumo

No tratamento de defeitos periodontais, a utilização de membranas compósitas de *design* assimétrico deve ser aplicada de forma a proteger a área afectada e, simultaneamente, estimular a regeneração de tecidos distintos. Este estudo descreve o desenvolvimento e caracterização de membranas de poli(D,L-ácido láctico) (PDLLA) e biovidro (BG, do comercial Bioglass®) com bioactividade assimétrica, através de um método ajustado de evaporação de solvente que permitiu uma distribuição não-uniforme da componente inorgânica ao longa da espessura da membrana. Hipotetizou-se que um melhoramento das propriedades estruturais e osteoconductivas das membranas compósitas ocorreria graças à adição do BG, comparativamente às de PDLLA puro. Para testar esta hipótese, um alargado leque de testes foi aplicado.

O carácter bioactivo assimétrico foi comprovado *in vitro*. Micrografias SEM revelaram a suavidade da superfície das membranas de PDLLA puro, contrastante com a homogénea distribuição de asperidades do BG à superfície da face inferior das membranas compósitas, na qual foi exibida uma camada de apatite, após imersão em SBF. A detecção da presença de BG foi complementada por análise dos espectros FT-IR. Graças à hidrofilicidade do BG, seria de esperar um aumento da razão de dilatação pela sua incorporação nas membranas. Tal não foi visível significativamente, podendo ter sofrido influência da perda de peso que a dissolução do BG em PBS provoca e cuja percentagem, por conseguinte, se revelou estatisticamente superior para as membranas de PDLLA/BG. Este processo é consistente com o evento de formação da camada de apatite, acima mencionado. As propriedades mecânicas das membranas não foram significativamente comprometidas com a introdução do BG. Revelando esta ser uma formulação que mantém a integridade exigida à função da membrana.

Células humanas do estroma da medula óssea (hBMSC) e células humanas do ligamento periodontal (hPDL) foram cultivadas em meio osteogénico na superfície das membranas, como sendo a cultura celular ideal para a avaliação da performance biológica, no que respeita a tecidos como osso alveolar e ligamento periodontal, respectivamente. A observação SEM, bem como a quantificação do conteúdo em DNA e da actividade metabólica acusaram adesão e proliferação celular superiores nas membranas de PDLLA/BG. Um melhoramento significativo da diferenciação celular foi ulteriormente detectado por mensuração da actividade ALP, assim como uma promovida mineralização e extensa formação de matriz extra-celular e nódulos cálcicos, sugerindo o efeito positivo da adição do BG, confirmado inclusive por medição do conteúdo em Cálcio e procedimento *Von Kossa*. De acordo com estes resultados, desta formulação espera-se uma maior e melhor regeneração dos tecidos visados. Os resultados indicam que as membranas assimétricas de PDLLA/BG propostas podem ter potencial êxito se utilizadas em terapias de regeneração guiada de tecidos ou em aplicações ortopédicas.

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List of Abbreviations

3D – Three-dimensional

A

ALP – Alkaline Phosphatase

B

BF – Bottom Face

bFGF – Basic Fibroblast Growth factor

BG – Bioglass®

D

DNA – Dhesoxy Ribonuclease

E

ECM – Extracellular Matrix

ePTFE - Expanded Poly(Tetraluorethylene)

F

FBS – Fetal Bovine Serum

FDA – Food and Drugs Administration

FT-IR – Fourier Transform Infra-Red Spectroscopy

G

GBR – Guided Bone Regeneration

GTR – Guided Tissue Regeneration

H

HA - Hydroxyapatite

HCA – Hydroxy-carbonate apatite

hBMSC – Human Bone Marrow Stromal Cells

hPDL – Human Periodontal Ligament Cells

M

MEM – Minimal Essential Medium

O

OCPC - -cresolphthalein complexone

P

PBS – Phosphate Buffered Saline

PCL – Poly(ε – caprolactone)

PCR – Polymerase Chain Reaction

PDL – Periodontal Ligament

PDLA – Poly(D-lactic) Acid

PDLLA – Poly(D,L-lactic) Acid

PGA – Polyclycolic Acid

PLA – Poly(lactic) Acid

PLGA – Poly(lactic-*co*glycolic) Acid

PLLA – Poly(L-lactic) Acid

R

RT – Room Temperature

RT-PCR – Real Time Polymerase Chain Reaction

S

SBF – Simulated Body Fluid

SD – Standard Deviation

SEM – Scanning Electron Microscopy

T

TIPS – Thermally Induced Phase Separation

TMC – Trimethylene-Carbonate

TMS – Tetramethylsilane

U

UF – Upper Face

V

VEGF – Vascular Endothelial Growth Factor

W

WL - Weight Loss

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CHAPTER I. General Introduction

General Introduction

1 | Motivation and Outline

Periodontal defect exists when tooth-supporting tissues, including the alveolar bone, periodontal ligament and cementum destruction occurs, as a consequence of periodontitis. [1] Mechanical removing of the damaged structures is the first procedure applied [2], however it is usually followed by a surgical intervention. [3] These conventional therapies reveal to be efficient in halting the periodontal disease progression; even so they involve some drawbacks that restrict their efficiency, being the major limitation related to the promotion of tissue regeneration. It is crucial to repopulate the defect with viable specific cells which are able to promote the regeneration of periodontal ligament and alveolar bone, as well as do not allow the growth of undesirable tissues, which cells have a higher migration rate [4; 5]. Considering that, regenerative procedures appeared using physical barrier membranes to create a segregated space and reach the aforementioned cell manipulation, which is often referred to as guided tissue regeneration (GTR).

Periodontal defects can be a complex problem to treat and represents the main cause of tooth loss in adults. [1] Accordingly, although originally GTR membranes were used specifically for periodontal regeneration, they were afterwards introduced in the implants surgery field, to regenerate bone tissue and thus allowing the dental implants application and conferring the needed stability for the implants [6; 7]. This evolution has to be congruent with the emergent raise of implantology cases, nowadays, factor that more impulses the development of new membranes systems. In this case, the major challenge is the distinct tissue regeneration [8]. Combine bioactive ceramic or glasses with polymers, conferring an asymmetric bioactivity, seems to be a really promising option.

Poly(D,L-lactic acid), PDLLA, is a biodegradable and biocompatible polymer that could be adequate to be used as the matrix in the production of membranes. The polymer may be formulated with inorganic particles and processed into membranes using different techniques. Bioglass® is an approved osteoconductive biomaterial used in orthopedic and dental applications. Therefore, the aim of this work was to prepare a PDLLA membrane containing Bioglass® microparticles, hypothesizing that these microparticles could enhance structural and biological performance of PDLLA membranes, regarding to periodontal regeneration. Moreover, due to the distinct biological environment experimented *in vivo* by the two sides of an implanted membrane, it seems reasonable to produce membranes with distinct properties in each face. To test this hypothesis, asymmetric PDLLA/Bioglass® membranes were prepared, in a single step, by an adjusted solvent casting method.

This concrete chapter presents an overview of the guided tissue regeneration field, specifically applied to periodontal defects regeneration, as well as an additional analysis to the performance of poly(D,L-lactic acid) and Bioglass® in this area.

2| Periodontal Defects

Teeth anatomy involves a variety of different components, which can be classified as hard or soft tissues. We can identify three structural different layers in the tooth: enamel, dentine and pulp chamber. Enamel is a crystalline structure, extremely hard and highly mineralized, thus it is the outer layer and its function is to cover and protect the crown of the tooth. Dentine constitutes the core structure and pulp chamber composes the center of the tooth. Containing this center area, there are blood vessels and nerves, which connects the jaws vascular and the nervous supply through tooth apices. The tooth root is attached to the adjacent alveolar bone by the periodontal ligament. [9]

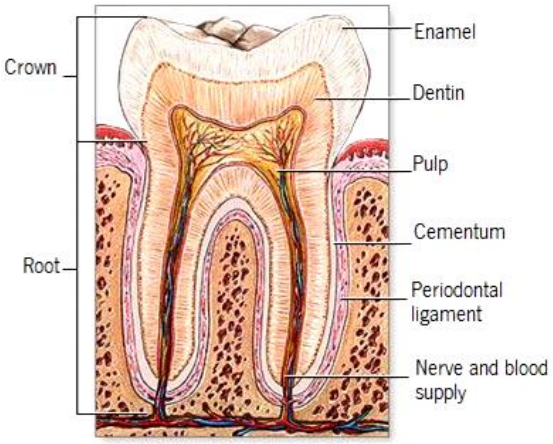

Figure 1.1 – Tooth anatomy and different distinguished structures. (Adapted from [9])

Periodontal ligament is the investing and supporting connective tissue structure that anchors a tooth within its alveolus. It is composed by collagen fibers that connect the cementum of the tooth to both gingival and alveolar bone and to the cementum of surrounding teeth. [10]

The etiology of periodontal diseases is related to the dental biofilm, of which evolution results in a progressive loss of dental insertion. [11] The presence of many bacteria in the supra and sub-gingival plaque was considered the major etiologic factor, and the accumulation of many microorganisms on it promotes the starting of periodontal destruction and consequent progression of the disease. [11; 12] Later on, the same author [13] revealed that inclusively some metabolic properties of dental biofilm assure the resistance of microorganisms to the natural body defenses. Logically, besides the bacterial intervention, aspects as genetic influences, immune host response and environmental factors are also included in the contribution for bacteria accumulation on gingival plaque and consequently in the progress of periodontitis.

Periodontitis is a disease, which destroys the tooth-supporting tissues, including the alveolar bone, periodontal ligament (PDL) and cementum, creating defects in the oral cavity. This is the major cause of tooth loss in adults. [1] The treatment of periodontal defects can be a complex process.

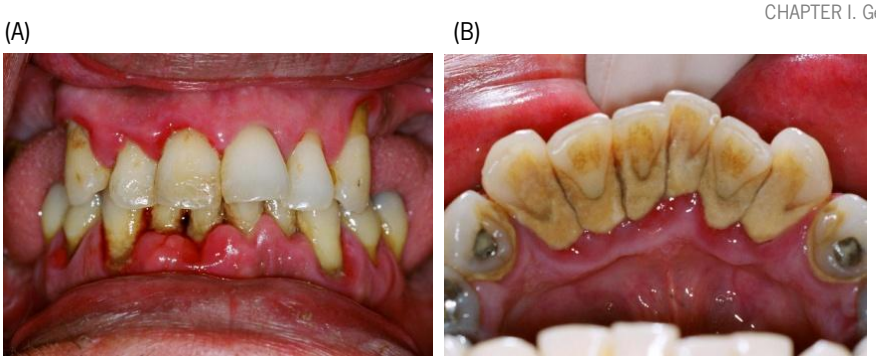

Figure 1.2 – Periodontal disease. (A) A case of advanced periodontitis, evidencing very swollen gums, loose teeth, staining and heavy plaque and calculus deposits on all the teeth. (B) Tartar deposits on the inside aspect of the lower incisor teeth in a patient with periodontitis. (Adapted from [14])

2.1 Current Therapies and Outcomes

Periodontal therapy mainly pretends to achieve reduction or even elimination of the tissue inflammation induced by the dental biofilm and correction of the anatomic defects, reestablishing the dental insertion integrity like before the disease. [15]

The mechanical regular removing of dental biofilm (as well as calculus, infected cementum, granulated tissue and crown portion of epithelium) is the first procedure applied. [2] After an evaluation of the initial phase outcomes, if the inflammation signs persist, the surgical treatment emerges as the best option. [3] Therefore, conventional periodontal therapy includes not just nonsurgical (debridement of root surfaces or root canals) but also surgical approaches (periodontal flap procedures, recessive surgeries). These last mentioned ones provide a better access for the cleaning of root surfaces and apical lesions, as well as for restoring the surrounding bone/root apex. [16]

Traditional recessive surgeries reveal to be efficient even for advanced disease stages, halting the periodontal disease progression, which is a good outcome once that allows and makes easy the removing of subgingival deposits, restituting the morphology of sustaining and recovering periodontal tissues. On the other hand, soft tissue recession, leading to poor esthetics in the anterior dentition, and residual pockets usually inaccessible to adequate cleaning, which negatively affect lon-term prognosis of the treated tooth, constitute the major drawbacks of this technique. [8] Nonetheless, the principal limitation of conventional therapies concerns to the promotion of periodontal regeneration. [17] Regeneration is though the most desirable but also the most difficult outcome for any therapy. [16]

Considering the overcoming of these limitations and difficult challenges, new approaches such as regenerative techniques have been proposed. The main goal is the inhibition of undesirable tissues growth, essentially epithelial tissue which migration and growth rate is higher (approximately 10 times faster) than conjunctive and bone tissues, dominating the initial healing phase. [18] According to that, it

is crucial to colonize the defect with viable specific cells which are able to promote the periodontal regeneration. [7; 18] This concept is named compartmentalization and was introduced by Melcher in 1976. [7] Thus, regenerative procedures used physical barrier mechanisms to reach the aforementioned cell manipulation, which is often referred to as guided tissue regeneration (GTR).

GTR is specifically indicated for narrow intrabony and class II mandibular defects treatment, because the close proximity, between the defect and periodontal mesenchymal cell sources, allow their adequate migration, repopulation and differentiation into the defect. Based on the extensively proved concept that the ability to recreate the original periodontal attachment belongs only to the fibroblasts from the periodontal ligament or undifferentiated mesenchymal cells, GTR therapy was applied with success in the regeneration of periodontal defects. [8] Regarding to the furcation lesions, just for the treatment of class II mandibular furcations GTR can be effectively used, actually allowing the passage of class II into a class I mandibular furcation, easier to maintain overtime. However, on class II maxillary furcations it has a limited clinical effect (namely reduction in the horizontal furcation depth) doubtful clinical significant. [19] In respect to class III furcations, GTR efficacy is unpredictable. [19; 20] In the treatment of intrabony defects, apart from the high variability in clinical outcomes, GTR results show greater probing depth reductions and higher gain in attachment levels compared to conventional flap procedures. [21]

Behind the successful GTR strategy to regenerate periodontal defects, many factors can influence the clinical responses to GTR. For example, the majority of patient-related factors such as smoking and residual periodontal disease could be controlled trough behavioral and therapeutic interventions [8], respectively. Several patient and local factors, as well as factors related to the surgical treatment that should be evaluated are listed on Table 1.1 and contribute for the increasing of the predictability and success of outcoming results of GTR.

Table 1.1 – Factors that negatively influence guided tissue regeneration outcomes. (Adapted from [8; 16])

	FACTORS	INFLUENCE ON THE OUTCOMES
PATIENT	Smoking habit	Decreased vascular flow, altered neutrophil function, and impaired fibroblast function, increased prevalence of periodontal pathogens, decreased IgG production and lymphocyte proliferation.
	Poor plaque control Residual periodontal disease	Increased residual periodontal pockets and percentage of sites with bleeding. Higher risk of membrane contamination.

Table 1.1 (continuation) – Factors that negatively influence guided tissue regeneration outcomes. (Adapted from [8; 16])

	FACTORS	INFLUENCE ON THE OUTCOMES
PATIENT	Systemic compromised patient Occlusal trauma Poor oral hygiene/compliance Mechanical trauma (aggressive tooth brushing) Inflamed gingival tissues Immunosuppression Diabetes Stress	These ones were also cited as negative factors to the clinical outcomes of GTR, however the evidences are not enough.
LOCAL	Local anatomy and morphology strongly affect the predictability of GTR.	
	Case selection	Increased residual periodontal pockets and percentage of sites with bleeding represent a higher risk of membrane contamination.
	Gingival thickness (if less than 1 mm)	Increased prevalence and severity of flap dehiscence over GTR membranes.
	Calculus Overhanging restorations	Favored plaque accumulation.
	Shallow infrabony defects Wide defect angle > 45 degrees Horizontal bone loss < 3-wall defects Deep furcation involvement	Many studies have related significantly better results for GTR strategy than control groups (without GTR membranes) conjugated with the highest success rate for 3-wall defects with a deep infrabony component of ≥ 4 mm and defect angle < 45 degrees.
SURGERY	Excessive flap tension Early mechanical disruption Contamination during surgery Lengthy/traumatic surgery Inadequate wound closure Poorly designed incisions Membrane exposure	Basically, to achieve predictable tissue regeneration, 4 summarized factors are critical (the so-called PASS [22]): primary wound closure; angiogenesis as a blood supply and source of undifferentiated mesenchymal cells; space maintenance; and, finally, stability of the wound.

After the GTR procedures some postoperative care and maintenance are also required. Systemic antibiotics and nonsteroidal analgesics may be prescribed, either to reduce the risk of infection or to control pain. [8] Even the usual mechanical tooth cleaning should be avoided. If a non-resorbable membrane is used, it is needed a second surgical intervention, and, ultimately, if the postsurgical problems extend (e.g. membrane exposure as referred in Table 1.1) membrane has to be removed earlier. Membrane exposure frequency can vary between 50% and 100% and represents the major complication associated to the GTR strategy. [20; 23] To contour this drawback, the use of

bioabsorbable membranes has contributed and novel access flaps are introduced to preserve the interdental tissues, thus reducing the prevalence of membrane exposure. [8; 16] Actually, and unfortunately, GTR technique complications are frequent and play a negative effect on clinical outcomes, namely: bleeding, swelling, hematoma, erythema, suppuration, sloughing or perforation of the flap, membrane exfoliation and postoperative pain. [8]

In order to overcome some undesirable outcomes and complications, combining therapies appeared as a solution. The use of barrier membranes parallel to the placement of bone grafting materials came up and should be able to ensure clot stabilization and prevent the membrane collapse into the defect [24].

3| Periodontal Defects and Guided Tissue Regeneration

Nowadays, the main goals in the treatment of periodontal diseases are to regenerate periodontal tissue and to confer support to the bone tissue. Specific cells present in the periodontal tissue are able to produce a new periodontal ligament, cementum (tissue that involves the tooth root) and alveolar bone, if these components can migrate to the host local. The aim of GTR technique is to avoid the migration of epithelial cells to the lesion and, with the help of a membrane, create a physical space between the membrane and the bone. This space allows enough time for the formation of the new periodontal ligament, cementum and bone tissues, with the intention of the total affected area regeneration. The space created, specifically for the invasion of blood vessels and osteoprogenitor cells, protects the bone regeneration against the growth of non-osteogenic tissues, which have a higher migration rate. [4; 5; 25] Due to this dissimilarity on migration rates, soft tissues would grow in bone tissue's place, if the barrier would not exist. Thus, the employment of a barrier membrane is to guide the new bone growth where volume and dimension are not enough for its normal function.

Originally, GTR membranes were used specifically for periodontal regeneration, being afterwards introduced in the implants surgery field, to regenerate bone tissue in primary or secondary bone deficiencies. Primary bone insufficiency is prior to the implants integration and can follow two therapeutic procedures: insert simultaneously the implants and the membrane with or without grafts, or proceed in two steps, firstly getting a higher bone volume with the membranes action, and then put the implants. If the bone lack is posterior (secondary) to the implants inserting, such as peri-implant defects, fenestrations or dehiscence, membranes are implanted in order to restore the lost initial bone height. [26] These techniques are denominated as Guided Bone Regeneration (GBR).

GBR involves the GTR principles, being specifically directed to the alveolar bone reconstruction and thus allowing the dental implants application and conferring the needed stability for the implants. [6; 7] So, GBR is included in GTR, but is focused on the hard tissues regeneration (bone), instead of soft tissues. [27; 28] GBR has been extensively and fruitfully applied in the treatment of bone defects that are adjacent to the implants [29], once that the bone quantity is a crucial issue for the implant to succeed. In case of insufficient bone in the damaged area, implant cannot be totally engaged in the bone being in contact with soft tissues as gingival. It can lead to the soft tissue inflammation, which may result in the implant fail. These are the main reasons for the use of GBR in the dental implants application. [30; 31] Bone substitutes with osteoconductive properties [32] and growth factors [33; 34] were employed to promote bone growth, however, nowadays, the concept of bone regeneration is associated to membranes use. [35]

4| Membranes for Guided Tissue Regeneration

In this field the technique of GTR uses a membrane which acts as a physical barrier to protect the defect site and to prevent the epithelial cells, fibrous and gingival connective tissue to reach the injured area. This procedure favors the regeneration of lost and damaged tissue since it promotes cell repopulation of the periodontal ligament and adjacent alveolar bone. [36] Figure 1.2 illustrates the phenomena that occur during the progression of GTR membranes actuation in a periodontal defect, since its integration.

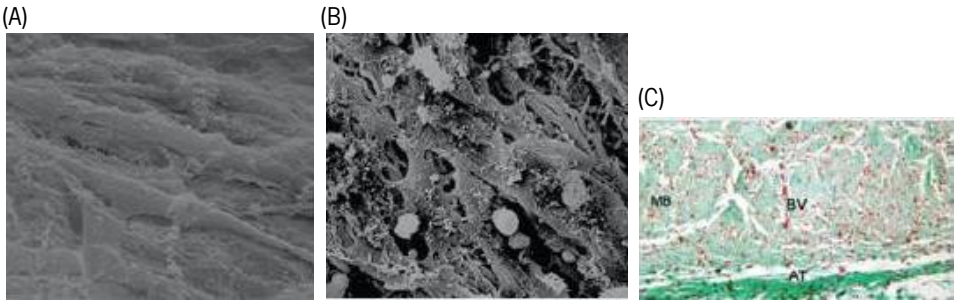

Figure 1.3 – Progressive action of one of the guided tissue regeneration marketed membranes. (A) Adhesion of PDL fibroblasts to the smooth upper face, promoting soft tissue healing. (B) Osteoblasts adhesion to the rough porous bottom face, allowing increased mineralization. (C) Complete vascularization and porous interconnectivity, at 2 weeks. (Adapted from [37; 38])

The membranes application respects a rigorous surgical protocol. In general lines, first of all, the injured tissue has to be removed in order to prevent possible infections. Once created the propitious environment, the membrane is positioned between the alveolar bone and the periodontal ligament. Hence, the pretended bone regeneration occurs during which the biodegradable membrane is absorbed by metabolic processes. The membrane acts not just as a physical barrier preventing the fibroblasts invasion into the injured area, but also as a buffer that just allows the osteogenic potential cells presence and avoids their migration to the exterior area. This way the osteogenesis occurs with no difficults, such as an inefficient bone bonding or an incomplete recuperation of the original bone hardness. [4]

Particularly in periodontal defects, the GTR technique is used to promote the conjunctive tissue adhesion to the tooth root surface, as well as to exclude the epithelial cells invasion from the injury. Additionally, it has been used to generate bone tissue around the implant, to avoid the fibrous encapsulation and to produce more bone tissue, thus enlarging the bone support. [4; 25] This space control, achieved by the use of membranes, permits also that the osteoblasts bone production occurs slowly with the aim of obtaining a better structural organization of the damaged tissue and, consequently, a more efficient healing. [25]

First membranes were used in the 80's. The real first ones were produced using expanded poly(tetrafluorethylene) (ePTFE). [39; 40] These membranes are not absorbed by metabolic processes. Still, ePTFE continued viable due to its biocompatibility and its use in vascular prosthesis. Although, this material presents many disadvantages such as: the regular exposition of the membrane after the implantation surgery, leading to the GTR fail and bacterial contamination; the need of a second surgical intervention to remove the membrane; and finally, after membrane removing, the exposure to the wear of new form bone. [6; 39] In order to overcome these limitations, biodegradable membranes have been introduced in the market [39]. Few characteristics are required to this kind of membranes. A description of membranes types, characteristics, materials and marketed products will be particularly described in the further sub-chapters.

4.1 Properties and Characteristics

GTR membranes should meet several properties to face the complex biological and sensitive system of the human body. Besides biocompatibility, barrier membranes should embrace physico-chemical and structural properties to guarantee basic, but complex and crucial conditions such as the ones described

in Table 1.2, in order to accomplish complete and perfect tissue integration. Non-toxicity, selective cellular occlusion and nutrients transfer are some of them.

Table 1.2 – Membrane requirements for guided tissue regeneration and its correspondent description (information collected and adapted from [41-52]).

		DESCRIPTION
MEMBRANES REQUIREMENTS	BIOCOMPATIBILITY NON-IMMUNOGENICITY NON-TOXICITY	Ability to prevent and do not generate adverse inflammatory or immune response and to resist to bacterial invasion and colonization.
	BIODEGRADABILITY TOTAL RESORBABILITY	To avoid second surgical procedure to remove the membrane. Absorption rate sufficient to maintain the physical barrier and at the same time compatible with the new bone formation. Total resorption of biomaterials, intended as a complete replacement of the foreign material by the regenerating tissues, appears still here as a perfect solution to interfacial problems.
	GOOD MECHANICAL INTEGRITY	To maintain the desired shape and configuration, namely to provide a secluded space for bone regeneration. Need to be sufficient to support or match surrounding native tissue at site of implantation, as well as mediate mechanical stimulus to cell during loading.
	EASY HANDLING, CUTTING AND CONTOURING	To allow the adaptation to the bone anatomy as well as an instant modeling by the professional (Dentist, Periodontologist, Implantologist) and consequent in site implantation of the membrane.
	MATERIAL ADHESIVENESS	Adhesiveness between membrane and surrounding bone tissues to prevent movement of membrane.
	CELL ADHESION	Optimization of cell seeding for retention of cells.
	SEMI-PERMEABLE	Adequate porosity to allow nutrient and oxygen supplies.
	BIOACTIVITY	To accelerate differentiation, mineralization and consequent bone formation. Eventually, to control the release of growth factors.

4.2 Types of Membranes

GTR membranes have been extensively studied and many materials have been proposed for their fabrication. They can be provenient from natural or synthetic source, and are either bioabsorbable or nonresorbable.

Both membranes classes reach the biological and mechanical aims defined for periodontal regeneration, inclusively above described on Table 1.2. Nevertheless, there is the risk of contamination, due to the membrane exposure after the first surgery. [53] Infections in the treated locals reduce the insertion gain and tissue regeneration, as well as accelerate the degradation process, though appeared the needing of a membrane not only with an ideal occlusion capacity but also with antimicrobial protection. [54] Facing that, resorbable membranes present a significant advantage, once they permit the incorporation of antimicrobian agents that can be released upon implantation.

Moreover, generally, non-resorbable membranes have to be removed by a secondary surgical procedure after the tissue had formed, increasing the risk of patient infection and other undesirable side effects. Also, they cannot be used to reconstruct large bone defects due to its bioinert properties constituting a significant disadvantage [55; 44]. In order to overcome such drawback, bioresorbable membranes have been proposed. In this case the degradation of the membrane should not interfere with bone healing and, before osseous regeneration has taken place, degradation should not be completed [56; 57].

4.3 Materials Employed and Current Marketed Membranes

Several membranes were studied and proposed for GTR. However, for a membrane to become commercially available there are many obstacles to overcome, such as the clinical evaluation and the approval by the competent regulatory agencies. After the membrane conception and *in vitro* characterization, preliminary studies in animals are performed; subsequently, the most trustable systems may be redirected to pre-clinical studies in humans. Clinical evaluation is such an important and exigent parameter. In consequence, only few have reached the stage of routine clinical application [58].

Expanded poly(tetrafluoroethylene) (e-PTFE), silicone rubber or titanium are the materials that constitute most non-resorbable membranes. The bioresorbable ones have been produced using collagen, collagen with elastin, poly(L-lactic acid) (PLA), poly(L-lactic acid)-blend-poly(D, L-lactic acid) (PDLLA), poly(D,L-lactide-*co*-glycolide) (PLGA) or trimethylene-carbonate (TMC). [56; 59; 60]

Natural collagen membranes have been the mostly used, not just because collagen is one of the components of the alveolar bone and periodontal ligament but also because this material performs almost all the criteria mentioned in the previous paragraphs. [61] For example BioGide®, Bicon®, BioMend®, BioSorb® and Ossix® are some of the current collagen membranes in the market. However collagen presents some drawbacks, including fast resorption rate, cytotoxicity, poor mechanical strength and fast biodegradation by enzymatic activity. [61; 62] To decrease this degradation rate and increase its hardness, collagen could be enhanced with cross-linking techniques [63] but this procedure (particularly with glutaraldehyde) can inhibit the attachment and proliferation of human PDL and human osteoblastic cells. [38] Adding the fact that its xenogenic origin presents a risk of disease transmission between animals and humans [64] and also that collagen tends to lose the ability to keep its own shape in wet conditions, as those existing in the oral cavity, [65; 66] these problems concur to the searching for new solutions.

Table 1.3 – Synthetic biodegradable marketed membranes, with respective constituent polymer, dimensions in mm, biodegradation time in weeks, price in Euros per unit and produced company.

		POLYMER	DIMENSION (mm)	BIODEGRADATION TIME (weeks)[1]	PRICE (€/unit)	COMPANY
MARKETED MEMBRANES	*Artisorb®* [69]	PLA	-	9 – 12	-	Citagenix, Canadá
	BioCellect™ [70]	PLA	15x20 20x30	4 – 8	67 100	IMTEC Corporation, USA
	BioMesh® [71]	PGA PLGA PLLA	15x12 17x17 25x17 30X24 40X30 25X20	24	70 70 70 70 106 92	Samyang's, Daejeon, Coreia
	BioMesh-S® [71]	PGA PLGA PLLA		36	-	
	EpiGuide® [69]	D, D-L, L-PLA	18x30	56	46	Curasan Inc., USA
	Gore Resolut Adapt® [72]	PGA TMC	15x20 20x25 25x20	> 10	150	W. L. Gore & Associates, Inc., Arizona, USA
	Gore Resolut Adapt LT® [72]	PGA TMC	25x30	> 24	158	
	Inion® GTR™ [73]	TMC PLA PGA	30x40	> 12	96	Inion Ltd, UK e USA

[1] Until complete bioresorption

In order to avoid these undesirable characteristics, maintaining the desirable ones, synthetic materials have been more frequently used, predominantly the poly(α-hydroxyesters) family. [72] These materials are the gold standard in applications of biodegradables in medicine. The chemical properties of these polymers allow its hydrolytic degradation and removing by natural pathways. [68] Moreover their processing is easy compared to other polymers and the variety of existent molecular weights and copolymers permits a wide range of physical, mechanical and degradation rate related adjustments. Epi-Guide®, Gore Adapt®, Inion®, BioMesh® and BioCellect® are examples of current available marketed GTR membranes of this specific type, among many others described and analyzed in Table 1.3.

5| Biocomposite Materials

First generation of biomaterials was developed to achieve exclusively bioinert tissue response. The forward generation emerged as bioactive biomaterials. Bioactivity is described as the capacity of the material to elicit a controlled action and reaction in the physiological environment. [74]

Reinforcement of biodegradable polymers matrices to produce composites of tailored surface, chemical and mechanical properties is a desirable approach for potential biomedical applications, such as in the regeneration of hard or even soft tissues. [75] The increasing research efforts worldwide for bone tissue regeneration [73;76-88] are trying to fulfill as many requirements as possible. For example, inorganic phases of tricalcium phosphate [89], hydroxyapatite [90-92] or bioactive glasses [79; 93; 94] are included into biodegradable poly(α-hydroxyester)-based matrices, such as poly(lactic acid), poly(glycolic acid) and their copolymers, as a viable way to improve the abovementioned properties and enhance bioactivity. Actually, the possibility of counteracting the acidic degradation of biodegradable polymer by the use of bioactive glasses is another reason for the use of composites. [95-97]

Consequently, composite systems combining advantages of biodegradable polymers and ceramics seem to be a really promising choice.

5.1 Biodegradable Polymers, the Poly(α-hydroxy-esters)

There are two types of biodegradable polymers taking into account their origin. The natural-based materials are one category, including starch, chitosan, alginate, hyaluronic acid derivatives (common polysaccharides) or collagen, fibrin gels, silk and soy (proteins). [98; 99] Synthetic biodegradable polymers constitute the second category: in general they may be obtained with high purity and

exhibiting predictable and reproducible mechanical and physical properties (elastic modulus, compressive or tensile strength and degradation rate) due to their under controlled production. [100-103]

Poly-α-hydroxy-esters are the most widely used biodegradable polymers for tissue engineering and regeneration inclusively have been approved by the US Food and Drug Administration for several applications. In these range are included poly(lactic acid) (PLA) and poly(glycolic acid) (PGA), as well as poly(lactic-*co*-glycolide) (PLGA) copolymers. PLA exists in three forms: L-PLA (PLLA), D-PLA (PDLA), and racemic mixture D,L-PLA (PDLLA). [68; 100; 104; 105] Table 1.4 describes some of these parameters for the cited polymers, which may however vary with molecular weight and crystallinity [106]:

Table 1.4 – Physical properties of synthetic, biocompatible and biodegradable polymers from saturated aliphatic polyesters family used as scaffold materials. (Adapted from [75])

		T_m (°C)	T_g (°C)	BIODEGRADATION TIME (months)[1]	COMPRESSIVE*/TENSILE STRENGTH (MPa)	MODULUS (GPa)
POLYMERS	PDLLA	Amorphous	55-60	12-16	Pellet: 35-150* Film/disk: 29-35	Film/disk: 1.9-2.4
	PLLA	173-178	60-65	>24	Pellet: 40-120* Film/disk: 28-50 Fibre: 870-2300	Film/disk: 1.2-3.0 Fibre:10-16
	PGA	225-230	35-40	6-12	Fibre: 340-920	Fibre: 7-14
	PLGA	Amorphous	45-55	Adjustable:1-12	41.4-55.2	1.4-2.8
	PCL	58	-72	>24	-	-

[1] Until complete bioresorption

The chemical nature of these polymers allow hydrolytic degradation. Human body already contains highly regulated mechanisms for the complete removing of monomeric components of lactic and glycolic acids, i.e., once degraded, they are removed by natural pathways. PLA is eliminated through the tricarboxylic acid cycle, while PGA is converted in metabolites or cleared by other mechanisms. [68] The in vivo degradation of these poly(α-hydroxy esters) occurs by hydrolysis, releasing the lactic or glycolic acids which are metabolized through the Krebs cycle into carbon dioxide (CO_2) and water (H_2O), basic human body elements. [72] Even though, the degradation kinetics is affected by different factors, such as: chemical composition and configurational structure, molar mass (Mw), polydispersity (Mw/Mn), environmental conditions, stress and strain, crystallinity, morphology (e.g. porosity) and

chain orientation, distribution of chemically reactive compounds within the matrix, additives, presence of original monomers, overall hydrophilicity and their processing history. [95; 96]

In general, their processing is easy compared to other polymers and the various existent molecular weights and copolymers permit a wide range of adjustments, in respect to degradation rate, physical and mechanical properties. PLA is more hydrophobic than PGA due to the additional methyl group in the structure of PLA; therefore PGA degrades much more quickly (a few weeks [107; 108]) than PLA, which can remain stable for over 1 year [109], or more depending on its degree of crystallinity. Albeit, these polymers still have some drawbacks. Sometimes their bulk erosion can result in scaffolds premature fails and, for example, an abrupt release of these acidic degradation products can cause strong inflammatory responses, being the typical pH drop associated with PLA and PGA implants one of its major negative aspects. [110; 111]

5.1.1 Poly (D,L-Lactic Acid)

Poly (D,L-lactic acid), PDLLA, is the racemic polymer of the poly(lactic acid) family – see Figure 1.3, general chemical structure. It is originated by the chirality of carbon α that allows the synthesis of enantiomers composites: L and D. Due to the random distribution of L and D unities on the polymeric chain, PDLLA does not have crystalline domains, being an amorphous material with lower stiffness as compared to the semi-crystalline PLLA. Therefore, the hydrolysis of this amorphous polymer is faster due to the lack of crystalline regions. [112-114]

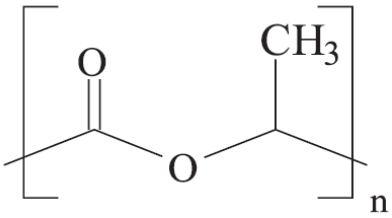

Figure 1.4 – General chemical structure of poly(lactic acid). (Adapted from [112])

Li *et al* [115] studied a series of PDLLA copolymers, concluding that after 12 weeks of *in vitro* degradation in PBS, the material bulk suffers significant mass decrease, being these results concomitants with the *in vivo* results among other studies. [115]

PDLLA shows excellent biocompatibility in vivo and high mechanical stability. [116; 117] Such excellent features of PDLLA with respect to implant performance have made itself an extensively investigated biomedical coating orthopedic material. [100; 118] Much more attention has been also

paid to PDLLA for applying it as a scaffold material for tissue engineering and regeneration. Desirable features such as the ability to combine with drugs like growth factors, antibiotics or thrombin inhibitor, establishing a locally acting drug-delivery system, are in the center of this interest. [118]

5.2 Bioactive Ceramic phases

Bioactive glasses and calcium phosphates have been largely applied in bioactive composite materials. Their excellent biocompatibility, as well as the possibility of counteracting the acidic degradation the ability of bone bonding are essential attributes for their choice. [119]

After implantation *in vivo* or contact with biological fluids, bioactive glasses and ceramics develop on the surface a biologically active hydroxy-carbonate apatite (HCA) layer which provides the bonding interface with the local tissue. The HCA phase is structural and chemically equivalent to the bone mineral phase, providing interfacial bonding (bridging host tissue with implants [120]). This procedure is achievable *in vitro* using a protein-free and acellular simulated body fluid (SBF), which nearly has the exact ion concentration of human blood plasma. [121; 122] This time-dependent kinetic modification of the surface is a bon-bonding behavior referred to as bioactivity, the main characteristic of this kind of materials. [119; 121; 123-126]

Table 1.5 – Mechanical properties of dense and highly porous 45S5 Bioglass®, hydroxyapatite, A/W glass-ceramic, and human cortical bone. (Adapted from [75])

		COMPRESSIVE STRENGTH (MPa)	TENSILE STRENGTH (MPa)	ELASTIC MODULUS (GPa)	FRACTURE TOUGHNESS (MPa $\sqrt{m}$)
CERAMICS	45S5Bioglass®	≈500	42	35	0.5 – 1
	Hydroxyapatite (HA)	>400	≈40	≈100	≈1.0
	Glass-ceramic A/W	1080	215	118	2.0
	Porous bioactive glass70S30C (82%)	2.25	-	-	-
	Porous Bioglass® - derived glass-ceramic (>90%)	0.2 – 0.4	-	-	-
	Porous HA (82-86%)	0.21 – 0.41	-	0.83 – 1.6 x 10^{-3}	-
	Cortical Bone	130 – 180	50 – 151	12 – 18	6 – 8
	Cancellous bone	4 – 12	-	0.1 – 0.5	-

Table 1.5 gives summarized information related to the typical mechanical properties of different bioactive glasses and ceramics. Bioactive glass-ceramics exhibit better mechanical performance compared to amorphous glass and calcium phosphate. Nonetheless their low fracture toughness and mechanical strength are still a drawback, specially comparing to cortical and cancellous bone (Table 1.5). [123; 127-130]

It is recognized that a layer of biologically active HCA must form to occur the bonding with the bone tissue; in fact, this is the only common characteristic of all the known bioactive implant materials. [74] Starting in 1967, Hench [121; 122] has extensively studied bioactive glasses as well as bioactive glass-ceramics. Although he could summarize the stages that are involved in the bone-bonding formation, some details remain yet indefinite. On the other hand, Hench was able to clearly define three classes of bioactive materials (A, B and C) characterized by the rate of bone regeneration and repair: materials that lead to both osteoconduction (the growth of bone along the bone-implant interface) and osteoproduction, as a result of the fast reactions on the implant surface, constitute the class A [131; 132]; alternatively, class B bioactivity takes place when just osteoconduction occurs [133; 134]; and, finally, materials that are resorbed within 10-30 days in tissue are included in class C. [121]

To emphasize the relevance of bioactive glasses there is the fact that they also have the ability to support enzyme activity [135-137], vascularization [138; 139], promote osteoblast adhesion, growth and differentiation, and induce mesenchymal cells differentiation into osteoblasts [85; 140; 141]. Furthermore, particularly for 45S5 Bioglass® composition, their dissolution products upregulate the gene expression that control osteogenesis and the growth factors production [142].

5.2.1 Bioglass®

Bioglass® is a proven osteoconductive material.[121] Since it belongs to the bioactive glasses family, it encloses a reactive silicate surface which, when in contact with biological fluids, forms a layer of carbonated HA, a strong and adherent bond with bone. Particularly for 45S5 Bioglass® composition, this complex multi-stage process occurs very rapidly. [143; 144] Additionally, Wilson *et al* [126] demonstrated that further than its excellent bone-bonding properties, Bioglass® also forms a bond with soft connective tissues.

In general, bioactive glasses contain SiO_2, Na_2O, CaO and P_2O_5. Specifically, for 45S5 Bioglass®, 45 represents 45 wt% SiO_2, S is the network former and 5 corresponds to the ratio of CaO to P_2O_5, with the rest weight percentage (around 24.5 wt%) correspondent to the Na_2O portion. [75; 143; 144] This was the original basis composition selected for the first investigations of Hench *et al.* [121] respecting the

ternary eutectic showed by the equilibrium phase diagram $Na_2O–CaO–SiO_2$. However, further researches have been trying different wt% proportions, in order to improve the bioactivity of these bioactive glasses. For example, 45S5 Bioglass® (see Figure 1.4), with 55% SiO_2, exhibit a high bioactivity index and bond to both soft and hard tissues. [75]

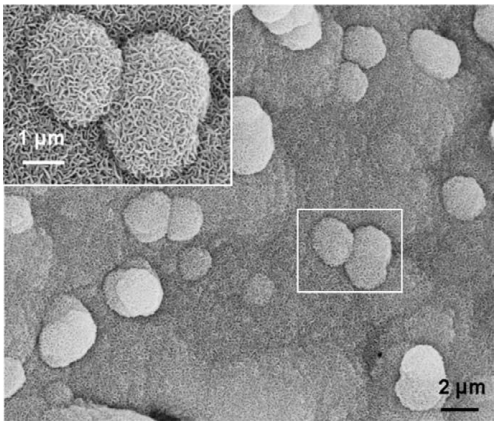

Figure 1.5 – SEM micrographs illustrating the typical "cauliflower" morphology of hydroxyapatite formed on the surface of a 45S5 Bioglass® based foam after immersion in SBF for 28 days. (Adapted from [151])

Bioglass® has been used as a reinforcing agent within polymer matrices, being expected to increase also mechanical properties of the composite. Stiffness and microhardness of high-density polyethylene composites was amplified with a reinforcement of filler volume fraction in Bioglass® particulates (melt blending, compounding, powdering and comprehension moulding techniques involved). [145] On the other hand, that cited positive reinforcing effect was not replicated in composites of poly(α-hydroxyesters). [93] The addition of bioactive glass (53 wt% SiO_2) as a filler for poly(D,L-lactide) composites may reduce its modulus and strength in specimens assessed, both dry and wet conditions. [80; 93] Rich *et al* [97] evaluated the influence of the incorporation of small particles of bioactive glass into poly(ε-caprolactone–*co*–D,L-lactide 96/4), concluding that it might accelerate the degradation of the composite. Although, it is important to state that high-temperature processing methods were involved, remaining to the possibility of the processing-temperature influence and consequent favor of the composite debilitation and faster degradation. Moreover, further studies have shown that the use of low-temperature processing for poly(α-hydroxyesters) with Bioglass® may circumvent these problems [79; 146], actually very recently, Blaker *et al* (2010) [144] proved it as well.

The addition of Bioglass® to bioresorbable polymers allows a rapid exchange of protons in water for alkali in the glass, providing a pH buffering effect at the polymer surface and consequently modifying the acidic polymer degradation. [83; 147] Thus it has been proved that bioactive glasses interfere on the polymer degradation behavior. [148] Actually, the pH is just one among other adjustable parameters controlling the degradation kinetics, by the integration of bioactive glasses in polymer scaffolds. These materials also modify the surface and inclusively the bulk properties of composite scaffolds, by increasing the hydrophilicity and water absorption of the hydrophobic polymer matrices. In particular, 45S5 Bioglass® particles were found to increase water absorption when included in PDLLA [149] and PLGA [83; 147] polymer foams, compared to the pure ones.

Depending on the particle size and processing technique, the surface reactions on bioactive glasses can release critical concentrations of soluble Si, Ca, P and Na ions, inducing intra and extracellular responses. [142; 150] Ideally, the degradation and resorption of composite scaffolds are designed to allow cells to proliferate and secrete their own extracellular matrix, while the scaffolds gradually vanish, leaving space for new cell and tissue growth. [75] Stimulation of neo-vascularization is also appointed as an ability of 45S5 Bioglass®. In some studies, scaffolds containing controlled concentrations of Bioglass® showed to increase secretion of vascular endothelial growth factor (VEGF) *in vitro* and to enhance vascularization *in vivo*. [75; 138]

The entire aforementioned characteristics engaged by 45S5 Bioglass® made out it like a successful material for tissue regeneration. Their application extends since as scaffold materials, either as filler or coating of polymer structures [147; 151], or even as porous material involving melt-derived and sol-gel-derived glasses [125]. Bioglass® implants have been applied in the replacement of damaged middle ear bones and restoring hearing to patients [125]. Concretely, the main applied area is the Dentistry, in which there are already marketed products, such as Perioglass™ for clinical treatment of periodontal disease and Novabone™ used as bone filler. [121]

5.3 Bioactive Composites Processing Techniques

Bioactive composites can be processed by different techniques. Injection or compression moulding and twin-screw extrusion, are typical melt-based processes. To obtain porous composite scaffolds, molding with leachable particulates, sintering of composite microspheres and gas foaming are also melt-based techniques that can be used. Contrasting, there are also low-temperature processes, such as thermally induced phase separation (TIPS), combined solvent casting and porogen leaching, solid freeform methods and certain gas foaming methods. [144]

5.3.1 The Solvent Casting Method

Solvent casting is used to process biocomposite materials. This processing involves the dissolution of the polymer in a solvent and the consequent casting of the solution into a predefined mold. Particles may be added to the solution, with specific dimensions. The mixture is molded in accordance with the pretended final geometry, which means that this solution can be either converted in membranes using a flat glass plate, or in 3D structures using an appropriate mold. Subsequently, the solvent evaporates

and the final composite structure contains the polymer and the added particulates (e.g. ceramic granules) and will exhibit the shape of the mold. [152; 153]

This is an easy technique that does not need specialized equipment, which represents its main advantage. The porosity percentage and number of porous are parameters directly dependents on the particles quantity and dimension.[152] Other parameters involved are the polymer and solvent choosing [154] and mechanical properties, which are not very variable during the processing once that this technique does not use fusion process [155]. Nevertheless, solvent casting have some limitations: a) the variety of shapes, because, typically, just flat sheets are obtained; b) use of toxic solvents, possibiliting their retention within the polymer, and consequent interference on the quality and viability of the obtained samples; c) the denaturation of proteins and other molecules incorporated into the polymer by the use of some solvents that may decrease the activity of these bioinductive molecules. [153]

6| Asymmetric Biocomposite Membranes

Although many of membranes are commercially available, numerous studies continue to be developed in this area, in order to propose improved solutions.

The aim of tissue regeneration processes is intrinsic to the similarities between the new regenerated tissue and the original one. Acting as physical barriers is such a limitation on the clinical effects of the GTR membranes, since they provide no biologic effects on proliferation and differentiation of mesenchymal and PDL cells, respectively concerning to bone and periodontal ligament regeneration. [8] Basically, each side of the membrane is in contact with a distinct biological environment, and consequently it claims that the osteointegration should be ideally promoted just in one of the faces. The development of membranes with asymmetric properties may constitute a new direction for GTR in periodontal tissues.

GTR membranes with bioactivity properties have been studied in the last decade. In 2004, a poly(L-lactic acid)/calcium carbonate hybrid membrane was proposed, exhibiting an *in vitro* precipitation of hydroxycarbonated apatite [156]. Zhang *et al* prepared another bioactive composite membrane based on PLA including a bioactive glass [157]. Hydroxyapatite was also used as the ceramic phase in a nano-hydroxyapatite/collagen/poly(lactic acid)(PLA) membrane, where calcification was formed at the surface after immersion in simulated body fluid (SBF) [158]. A polycaprolactone/calcium-carbonate fibers composite membrane with osteoconductive properties was proposed [159]. Three layered nano-carbonated hydroxyapatite/collagen/PLGA composite membranes were also reported, with improved

bioactive, mechanical and biochemical properties [44]. Composite membrane of poly(ε-caprolactone-*co*-D,L-lactide), coated just in one side with bioactive S53P4 glass granules, induced the formation of a calcium-phosphate layer [160]. PCL reinforced with nanofibrous glass induced apatite-like precipitation on the surface, when immersed in SBF [161]. An electrospun PCL/nano-apatite composite membrane revealed the same bioactive potential [47]. A poly(lactic-*co*-glycolic acid) membrane grafted with hyaluronic acid bi-layer films demonstrated to promote angiogenesis due to its osteoconductive properties, acting distinctly in each face [162], among some other ones. Despite of all the work reported on asymmetric membranes, it is still necessary to develop new systems obtained by simple processes and characterized by a good integration between the layered constituents.

7 | References

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CHAPTER II. MATERIALS & METHODS

MATERIALS & METHODS

1| Materials

Poly(D,L-lactic acid) (PDLLA), (M_n= 31750 and M_w= 100000) with an inherent viscosity of 1.87 dL/g was purchased from Purasorb® (PURAC Biochem, The Netherlands) and was used as received. The 45S5 Bioglass®, with the composition: 45 SiO_2, 24.5 CaO, 24.5 Na_2O and 6.0 P_2O_5 in wt%, was supplied by US Biomaterials Corp. (Florida, USA). The particle size of the Bioglass® particles (BG), measured by laser scattering analysis (Coulter LS 100 particle size analyzer), was found to be lower than 20 μm. All the other reagents and solvents used were of reagent grade and were used without further purification.

2| Methods

2.1 Preparation of PDLLA and Bioglass® Membranes

All the membranes were prepared based on a solvent casting technique. The PDLLA films were prepared by dissolving 0.50 g of PDLLA in 30 mL of chloroform. After total dissolution, the solution was transferred to a Petri dish with 9 cm of diameter and covered with an aluminium sheet. The Petri dish was settled in a horizontal position to facilitate the formation of a cast film with uniform thickness. The assembly was kept in a hood for 24h, and chloroform was allowed to evaporate at a very slow rate. Then, the films were vacuum dried for 48h at 40°C.

The PDLLA/BG membranes were prepared in the exact same process as the pure PDLLA membranes. The PDLLA/BG dispersions were prepared by dissolving 0.40 g of PDLLA in 30 mL of chloroform. After total dissolution, 0.10 g of Bioglass® was dispersed in the above solution. During solvent evaporation the particles will preferentially deposit by gravity to the bottom side of the dispersion medium, creating in the end an asymmetric 80/20 of PDLLA/BG membrane along the thickness.

2.2 Bioactivity Tests

For the *in vitro* bioactivity tests an acellular simulated body fluid (SBF) (1.0x) with ions concentration nearly equal to human blood plasma was prepared [1]. Sample membranes of 20x15 mm^2 were cut from the original processed films for the bioactivity tests. Three replicates for each sample were immersed in 45 mL SBF for 2, 5, 7, 14 and 21 days at 37°C. After being removed from SBF the membranes were gently rinsed with distilled water and dried at room temperature.

2.3 Physico–chemical Characterization

2.3.1 Scanning Electron Microscopy (SEM)

Qualitative information of the morphology of PDLLA and PDLLA/BG membranes surfaces, after and before the immersion in SBF, was obtained using a Scanning Electron Microscope (Nova NanoSEM 200-FEI Company) at an accelerated voltage of 5 kV. Before being observed by SEM, the membranes were gold coated using a Hitachi coating unit IB-2 coater at 6 mA.

2.3.2 Fourier Transform Infrared Spectroscopy (FTIR)

The possible formation of a CaP layer onto the surface of polymeric membranes incorporating the microparticles after 14 days soaking in SBF, was analysed by infrared spectroscopy where the background noise was calibrated with pure KBr data. Also, each side of the composite membrane were analysed with the attenuated total reflection accessory. Spectra were recorded in an IR Prestige 21 FTIR spectrophotometer with the attenuated total reflection accessory (128 scans, resolution 4 cm^{-1}) in the spectral range 2000-400 cm^{-1}.

2.3.3 Swelling Properties

The water sorption capacity of the membranes was determined by swelling the samples in phosphate buffered saline solutions (PBS, Gibco) at pH 7.4 for up to 4 months at 37°C. The initial weight of each membrane (approximately 10×10 mm^2) was measured with an analytical balance (Scaltec, Germany) before immersion in the PBS for several pre-determined time intervals (5 min, 30 min, 1 h, 1 day, 7, 14, 22, 28 days, then 6, 8, 12 weeks and finally 4 months) and immediately weighted after the removal of excess of water by lying the surfaces on a filter paper (Whatman Pergamyn Paper, 100×100 mm^2). The swelling ratio (SR) was calculated using the following equation (1):

$$SR = \frac{W_w - W_d}{W_d} \qquad (1)$$

Where W_w and W_d are the weights of the samples at the swelling state and at the dry state, respectively.

2.3.4 Degradation Test

The degradation of both membranes (with or without BG microparticles) was evaluated by immersing samples (with known weights and approximately 10×10 mm^2) in PBS, at pH 7.4 for 4 months at 37°C. At pre-determined time intervals (5 min, 30 min, 1 h, 1 day, 7, 14, 22, 28 days, then 6, 8, 12 weeks and, finally, 4 months) samples were removed from the solution and washed with distilled water three times for the removal of salts. The samples were dried at room temperature and then weighted with an analytical balance (Scaltec, Germany). The following equation (2) was used to calculate the percentage weight loss (%WL) of the samples:

$$\%\, WL = \frac{W_i - W_f}{W_i} \times 100\% \qquad (2)$$

Where W_i is the initial dry weight of the sample and W_f is the weight of the dry sample after incubation in the PBS solution.

2.4 Mechanical Characterization

2.4.1 Mechanical properties

The tensile properties were determined using an INSTRON 4505 Universal Machine (Instron Int. Ltd., USA) equipped with a 1 kN load cell, with a loading rate of 5 $mm.min^{-1}$, up to 20% of strain, at room temperature. Samples were analyzed in the dry and wet conditions. For the wet condition, samples were immersed for 3 hours in PBS before being tested. The values reported represent an average of at least five testing specimens. Tensile force was taken as the maximum force in the force-deformation curve. Tensile modulus was estimated from the initial linear section of the stress-strain curve.

2.4.2 Nanoindentation Tests

Nanoindentation tests were carried out to evaluate both sides of the composite membranes using a Nano Test (Micro Materials Ltd.) at room temperature. A Berkovich diamond indenter was used, with a three-sided pyramid geometry with a cross-sectional area in terms of contact depth of $A(h_c) = 24.5h_c^2$.

A loading rate of 0.01mN/s was used until a maximum load of 10 mN was reached. At least 10 indents were made in random locations on each side of the membrane.

2.5 Cell culture studies using hBMSC and hPDL

Two types of cells were used in this study, namely human periodontal ligament cells (hPDL) and human bone marrow stromal cells (hBMSC). Both cells types were collected from 2 different donors.

PDL cells were obtained from human third molar according to the following procedure. After extraction, the teeth were washed three times for 10 minutes in PBS with 100units/mL penicillin and streptomycin. PDL tissue was scraped from the middle third of the root with a scalpel blade, to avoid contamination by epithelial or pulpal cells. The freed portions of the periodontal ligament were minced and transferred to a small culture flask, filled with 5 mL alpha minimal essential medium (α-MEM, Gibco) with 10% v/v fetal calf serum (FCS, Gibco), 50 mg/mL ascorbic acid (Sigma), 10-8 M dexamethasone (Sigma), 50 mg/mL gentamycin (Gibco) and 10 mM sodium β-glycerophosphate (Sigma). Medium was refreshed every 2 to 3 days. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and medium was replaced every 2 to 3 days. Upon reaching confluence, cells were released with trypsin/EDTA (0.25% w/v crude trypsin and 1 mM EDTA (pH 7.2)) and sub-cultured for 2 passages in standard culture flasks. The cells were then frozen in liquid nitrogen until used for the experiments.

Human BMSCs were isolated from bone blocks of human iliac crest biopsies of donors. The biopsies were discarded tissues during standard surgical procedures at Radboud University Nijmegen Medical Center (Nijmegen, The Netherlands). The bone blocks were cut into small pieces and subsequently placed in a 50mL tube to which 20mL alpha-minimal essential medium (α-MEM) was added. After that the tube was shaken vigorously and the medium with cells was collected. This procedure was repeated several times. The collected medium with cells was plated in culture flasks (T175; Greiner Bio-one) and expanded in Proliferation Medium. Cells were characterized and showed stem cells phenotype. Additionaly, a multipotential differentiation test was applied, demonstrating their stem cells capacity. Cells were cultured at 37°C in a humid atmosphere with 5% CO_2 and its passage was performed at 80% confluence using trypsin EDTA (Gibco). After the first generation, cells were plated at a density of 5000 cells/cm^2 in culture flasks (T175). The culture medium was changed twice a week.

Cells from passage 3 (hBMSC) and 5 (hPDL) were used in the biological experiments. The composition of both proliferation and osteogenic medium, for both cell types, is described in Table2.1:

Table 2.1 – Proliferative and osteogenic medium composition.

Medium	Cell type: hBMSC	Cell type: hPDL
PROLIFERATIVE	α-MEM (Gibco) 15% human FBS (Greiner Bio-one) 1% Penicillin Streptomycin (Gibco-BRL) 1% L-Glutamine (L-Glutamine) 1% Ascorbic acid (Sigma) (1% of volume added to each cell culture flask) bFGF	α-MEM (Gibco) 10% FBS (Greiner Bio-one) 1% Penicillin Streptomycin (Gibco-BRL)
OSTEOGENIC	α-MEM (Gibco) 15% human FBS (Greiner Bio-one) 1% Penicillin Streptomycin (Gibco-BRL 1% L-Glutamine (L-Glutamine) 1% Ascorbic acid (Sigma) 1% β-glycerolphosphate (Sigma) 1% Dexamethasone (Sigma)	α-MEM (Gibco) 10% FBS (Greiner Bio-one) 1% Penicillin Streptomycin (Gibco-BRL) 1% Ascorbic acid (Sigma) 1% β-glycerolphosphate (Sigma) 1% Dexamethasone (Sigma)

2.5.1 Cell Seeding

Metal rings (15 mm x 3 mm) were glued to the membrane samples to keep them in the solution, with RTV Silicone Adhesive (Nusil, Silicone Technology, USA, MED-1037). The gluing of the rings to the membranes arose from the need to firm the membranes to the well's bottom, do not allowing their winding neither fluctuation and, at the same time, guaranteeing an equal cell culture area for every sample. The cells were seeded directly on the membranes surface. For the BG group, cells were seeded on the side which contains BG particles. Once that the surface of the top side of PDLLA/BG membranes just contains pure polymer, is smooth and identical to the surface of PDLLA membranes (as it was already proved with previous characterization studies), they were considered as the same group in this experiment.

Prior to cell seeding, the samples were sterilized with 70% (v/v) ethanol for 60 minutes and then washed three times immersed in PBS. The samples were placed in 25-well plates and soaked in cell culture medium overnight. After removing the culture medium, 50 μL of a cell suspension with a 2.0 x 10^4/sample cell density, was seeded onto the surface of each sample. After incubation for 4 hours at 37°C in a 5% CO_2 atmosphere incubator, osteogenic medium (specific for each cell type) was added to

the seeded samples, according to the type of assay performed. On the control groups, cells were seeded directly on the well-plates and osteogenic medium was added immediately.

2.6 Cell Adhesion, Proliferation and Metabolic Activity

2.6.1 DNA content

For DNA quantification and cell proliferation evaluation, Quant-iT™ PicoGreen® dsDNA reagent was chosen due to its simple reading, ultra sensitivity, high precision and accuracy. [2] PicoGreen is an ultrasensitive fluorescent nucleic acid which stains double stranded (ds) DNA in solution. 1xTris-EDTA-working solution consists of a 20x diluted stock with DNAse-free water. The PicoGreen stock had to be diluted 200x on the day of measuring with 1x Tris-EDTA to make a PicoGreen working solution. To generate a standard curve, serial dilutions of the dsDNA stock were made.

After the different experimental time points, medium was removed from the wells and the samples were washed twice with PBS. The analysis was performed on the supernatant of the substrates after day 1, 3, 7, 14 and 28 of culture. Cells were lysed using milliQ with subsequent sonification for 10 minutes between two cycles of freeze/thaw from -80°C. The supernatant was stored at -20°C until further analysis. A PicoGreen dsDNA Quantification Kit (Molecular Probes, Eugene, USA) was used according to manufacturer's instructions. The analysis was performed on the supernatant of the substrates on day 1, 3, 7 and 28. The standard DNA samples were prepared according to Table 2.2. To each 100 µL sample, 100 µL PicoGreen working solution was added. The samples must incubate for 2-5 minutes at room temperature, in the dark. After incubation, the fluorescence was measured on a fluorescence cuvette reader (microplate fluorescence reader, Bio-Tek, Winooski, USA) with a 485 nm excitation filter and a 530 nm emission filter.

Table 2.2 – Standard curve values for PicoGreen dsDNA Quantification, proliferation assay.

	ng/mL	Opl. A, µL	TE-buffer		ng/mL	Opl. B, µL	TE-buffer
1	2000	100	0	6	100	100	0
2	1000	50	50	7	50	50	50
3	500	25	75	8	25	25	75
4	250	12.5	87.5	9	12.5	12.5	87.5
5	125	6.25	93.75	10	6.25	6.25	93.75

2.6.2 Alamar Blue® staining

AlamarBlue® staining (Invitrogen) requires minimal handling and incorporates a nontoxic reagent allowing continuous monitoring of cell proliferation and metabolic activity on the same samples using fluorescence observation. [3] Cell metabolic activity was measured according to the instructions of the manufacturer. A solution was made with AlamarBlue and culture medium in a proportion 1:9 (v/v) and was placed at 37°C for 5 minutes. The medium was removed from wells and replaced with the solution. Plates were incubated (37°C and 5% CO_2) for 4 hours. After incubation, 200 µL of each sample solution was transferred to 96-well plates (Greiner Bio-one). Fluorescence was measured using a microplate reader (FL 600; Bio-Tek) at 570 nm. The assay was performed on day 1, 3, 7 and 28 of culture.

2.6.3 Scanning Electron Microscopy (SEM) observation

Adhesion of both cell types (hBMSC and hPDL) on membranes was analyzed by SEM (n=2). After day 3 and day 28 timepoints, cells were fixed in 2% v/v glutaraldehyde in 0.1 M sodium-cacodylate buffered solution, for 5 minutes. Cells were rinsed in cacodylate buffered solution, dehydrated in a series of ethanol dilutions in water (70%, 80%, 90%, 96% and 100% (v/v)), 1 hour in each, and dried in tetramethylsilane (TMS, Merck) to air. Finally, specimens were sputtercoated with a thin layer of gold, and examined in a JEOL 6310 scanning electron microscope.

2.7 Cell Differentiation and Mineralization

2.7.1 Alkaline Phosphatase Activity Measurements (ALP)

Alkaline phosphatase is a cell surface glycoprotein that functions as a marker in the osteoblastic differentiation *in vitro*. Appearing in the beginning of the process, this marker produces its peak levels (maximum reached in activity per cell basis) with osteoblast maturation [4] and starts to reduce its expression and activity in the last states, with the progress of mineralization phase. [5; 6] This transition is a positive indication of the transient character of a cell line osteoblastic differentiation. [7]

The same supernatants as used for PicoGreen assay were also used to measure alkaline phosphatase (ALP) activity (Sigma). To each 80 µL of the sample, 20 µL of 0.5M Alkaline Buffer (Sigma, cat#A9226) was added. Thereafter 100µL substrate solution 5mM paranitrophenylphosphate

(PNP, Sigma, cat#P5994) was added to each well. After 60 minutes of incubation at 37°C, 100 µL stop solution (0.3M NaOH) was added to each well. Finally, ALP activity was measured at 405 nm using an ELISA microplate reader (Bio-Tek Instruments Inc, USA).

Alkaline Phosphatase produced by the cells cleaves the phosphate ion from the substrate, p-nitrophenyl phosphate. The resulting p-nitrophenol can be measured colorimetrically by the addiction of an alkaline solution. The quantity of p-nitrophenol liberated from the substrate can be determined by comparison to a curve generated from known concentrations of p-nitrophenol standards. A standard curve was made (Table 2.3 and 2.4).

Table 2.3 – Needed solutions and respectively reagents and preparation instructions.

Solutions	Reagents and Instructions
Buffer solution	0.5M 2-amino-2-methyl-1-propanol(AMP); Stock 1.5M diluted to 0.5M
Substrate solution	5 mM paranitrophenylphosphate (PNP; MW=263.1): 1.315 mg paranitrophenylphosphate/1mL buffersolution. (= 50 mg/38mL)
Stop solution	0.3M NaOH (MW=40): 3 gram NaOH solute in 250 mlLMilliQ
Standard curve: 4-nitrophenol: (MW=139)	Stock 10 mM: 13.9 mg/10mL buffer Before use: add 25µL stock to 975µL buffer: 250µM 4- nitrophenol (NP)

Table 2.4 – Standard curve values for ALP assay.

nmol	250 µM 4-NP	Buffer	nmol	250 µM 4-NP	Buffer
25	100	-	7.5	30	70
20	80	20	5	20	80
15	60	40	2.5	10	90
10	40	60	Blank	-	-

2.7.2 Von Kossa Staining

Cells were fixed with 2% glutaraldehyde, stained with fresh 5% silver nitrate ($AgNO_3$), washed with distilled water, developed with 5% sodium carbonate (Na_2CO_3) in 25% formalin, and fixed with 5% sodium thiosulphate ($Na_2S_2O_3$). Stained samples were observed under a Leica MZ12 stereomicroscope and images were captured.

2.7.3 Ca Content

Calcium complexes with o-cresolphthalein complexone (OCPC). At pH 10-12 calcium yields a red complex with OCPC. The color intensity of the purple complex formed is directly proportional to the calcium concentration. The complex is stabilized by KCN, thereby eliminating interference from heavy metals. [8] Calcium content was assessed after 21 and 28 days of culture to obtain information about mineralized matrix formation. The samples were rinsed twice with milliQ. 1 mL of acetic acid was added to each sample. The samples were incubated overnight under vigorous constant shaking and the acetic acid with the diluted calcium was frozen and kept at -20°C, until further investigation. After thawing, the calcium content was determined using the OCPC method. Optic density was read with an ELISA reader (Bio-Tek Instruments Inc, USA) at a wavelength of 570 nm. Bare membranes were also assessed in order to further exactly quantify and distinguish cellular from acellular mineralization on the membranes.

2.8 Statistical Analysis

The biological tests were performed twice, with exception for Von Kossa. Each time with different donors, every sample was measured in triplicate. All the other procedures were performed just once, in duplicate for Von Kossa and SEM, in triplicate for the other ones. All results are presented as mean ± standard deviation. Statistical analysis of experimental data was performed using an unpaired ordinary ANOVA with standard parametric methods. Calculations were performed in InStat (v. 3.0 GraphPad Software Inc, San Diego, CA). Statistical significance was set to *p-value* ≤ 0.1 (*), to *p-value* ≤ 0.01 (**) and to *p-value* ≤ 0.001 (***).

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CHAPTER III. Asymmetric PDLLA Membranes Containing Bioglass® for Guided Tissue Regeneration: Characterization and *In Vitro* Biological Behavior

Asymmetric PDLLA Membranes Containing Bioglass® for Guided Tissue Regeneration: Characterization and *In vitro* Biological Behavior

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Abstract

In the treatment of periodontal defects, composite membranes might be applied to protect the injured area and simultaneously stimulate tissue regeneration. This work describes the development and characterization of poly(D,L-lactic acid)/Bioglass® (PDLLA/BG) membranes with asymmetric bioactivity, prepared by an adjusted solvent casting method that promoted a non-uniform distribution of the inorganic component along the membrane thickness. We hypothesized that the presence of BG microparticles could enhance structural and osteoconductivity performance of pure PDLLA membranes. *In vitro* asymmetric bioactive behavior (precipitation of an apatite layer upon immersion in simulated body fluid just on the BG rich face), SEM observation, FT-IR, swelling, weight loss and mechanical properties of the developed biomaterials were evaluated. Cell behavior on the membranes was assessed using both human bone marrow stromal cells and human periodontal ligament cells. SEM images, DNA content and metabolic activity quantification revealed an improved cell adhesion and proliferation on the composite membranes. Composite membranes also stimulated cell differentiation, mineralization, and production of extracellular matrix and calcium nodules, suggesting the positive effect of adding the bioactive microparticles in the PDLLA matrix. The results indicate that the proposed asymmetric PDLLA/BG membranes could have potential to be used in guided tissue regeneration therapies or in orthopaedic applications, with improved outcomes.

Keywords: PDLLA, Bioglass®, membrane, periodontal ligament, tissue engineering.

1 | Introduction

Periodontitis is a disease that destroys the tooth-supporting tissues, including the alveolar bone, periodontal ligament (PDL) and cementum. This is the major cause of tooth loss in human adults. [1] The treatment of periodontal defects can be a complex process and usually involves surgical intervention. However, periodontal defects, if left empty after open flap debridement, are filled with epithelial and fibroblasts, which are the first cell to reach the defect area, generating a core of fibro-epithelial tissues that does prevents the occurrence of an adequate regeneration process of the periodontal tissues. [2]

In this context, Guided Tissue Regeneration (GTR) strategies consist in the application of a membrane that acts as a physical barrier to protect the defect site, preventing the epithelial cells, fibrous and gingival connective tissues to reach the injured area. The creation of segregated space for the invasion of blood vessels and osteoprogenitor cells protects against the growth of non-osteogenic tissues. This procedure favors the regeneration of lost and damaged tissue since it promotes cell repopulation of the periodontal ligament and adjacent alveolar bone. However, acting solely as physical barriers is a limitation on the clinical effect of these membranes, since they provide no osteoconductive effects and their thus enabling only minor contributions for new cementum and bone formation, which, by definition, is not true periodontal tissue regeneration. [3] Each side of an implanted membrane is in contact with a distinct biological environment, in which the osteointegration should be ideally promoted just in one of the faces. Nevertheless, this asymmetric bioactive behaviour is almost inexistent in currently used GTR membranes and represents a possible challenge towards the development of innovative systems for the regeneration of periodontal tissues.

GTR membranes can be obtained from natural or synthetic materials, either bioabsorbable or nonresorbable. Degradability is one of the most important requirements for GTR membranes and intends to avoid second surgical removing procedure. Natural resorbable collagen membranes have been widely used, not just because collagen is concretely one of the components of the alveolar bone and periodontal ligament but also because this material meets almost all the criteria required. [4] Collagen, however, presents some drawbacks such as its fast resorption rate, cytoxicity and xenogenic origin, poor mechanical strength and fast biodegradation by enzymatic activity. [4; 5] In order to avoid these undesirable characteristics, maintaining the desirable ones, synthetic materials have been more frequently used, predominantly those from the poly(α-hydroxyesters) family. [6] The chemical properties of these polymers allow its hydrolytic degradation and the elimination of the resulting products by natural pathways. [7] Moreover their processing is easy compared to other polymers and the variety of existent molecular weights and copolymers permits a wide range of physical, mechanical and

degradation rate related adjustments. Poly(D,L-lactic acid), PDLLA, is an amorphous polymer, with interesting mechanical properties and with degradation times in the order of 12 to 16 months. [8] It exhibits excellent biocompatibility *in vivo*, high mechanical stability and the possibility to be combined with drugs. [9-11] Nevertheless, PDLLA is not osteoconductive. Among different strategies that could be used to improve bioactivity in polymeric systems [12], the combination of osteoconductive inorganic particles has been widely used [7]. Bioglass® is a well known bioactive ceramic and has the ability to enhance the osteoblast activity and attachment between the biomaterial and the surrounding bone tissue, possibiliting the bone growth on the materials surface. Furthermore its dissolution products can control the gene expression in order to control the osteogenesis and consequently the production of growth factors [8], as well as counteracting the acidic degradation of the poly(α-hydroxyesters) providing a pH buffering effect. [13; 14]

In this work, Bioglass® microparticles were compounded with a PDLLA membrane, using a solvent casting methodology. The conditions were optimized for the preparation of membranes exhibiting preferentially the BG in one of the sides of the membrane. It is envisioned that, upon implantation, the membrane side rich in BG could be faced to the defect side in which bone ingrowth should be stimulated while the more hydrophobic PDLLA rich side should act mainly as a barrier to avoid the invasion of soft tissue. Thus, this design would meet the current big challenge abovementioned for GTR in this specific area: the asymmetric bioactivity. Therefore, purpose of this work was to characterize some relevant properties of the developed membranes and to evaluate their biological performance, using two distinct cell types: human bone marrow stromal cells (hBMSC) and human periodontal ligament cells (hPDL).

2 | Materials and methods

2.1 Materials

Poly(D,L-lactic acid) (PDLLA), (M_n= 31750 and M_w= 100000) with an inherent viscosity of 1.87 dL/g was purchased from Purasorb® (PURAC Biochem, The Netherlands) and was used as received. The 45S5 Bioglass®, with the composition: 45 SiO_2, 24.5 CaO, 24.5 Na_2O and 6.0 P_2O_5 in wt%, was supplied by US Biomaterials Corp. (Florida, USA). The particle size of the Bioglass® particles (BG), measured by laser scattering analysis (Coulter LS 100 particle size analyzer), was found to be lower than 20 μm. All the other reagents and solvents used were of reagent grade and were used without further purification.

2.2 Preparation of PDLLA and PDLLA/Bioglass® Membranes

All the membranes were prepared based on a solvent casting technique. The PDLLA films were prepared by dissolving 0.50 g of PDLLA in 30 mL of chloroform. After total dissolution, the solution was transferred to a Petri dish with 9 cm of diameter and covered with an aluminium sheet. The Petri dish was settled in a horizontal position to facilitate the formation of a cast film with uniform thickness. The assembly was kept in a hood for 24h, and chloroform was allowed to evaporate at a very slow rate. Then, the films were vacuum dried for 48h at 40°C.

The PDLLA/BG membranes were prepared in the exact same process as the pure PDLLA membranes. The PDLLA/BG dispersions were prepared by dissolving 0.40 g of PDLLA in 30 mL of chloroform. After total dissolution, 0.10 g of Bioglass® was dispersed in the above solution. During solvent evaporation the particles were deposited by gravity to the bottom side, creating an asymmetric of 80/20 PDLLA/BG membrane along the thickness.

2.3 Bioactivity Tests

For the *in vitro* bioactivity tests an acellular simulated body fluid (SBF) (1.0x) with ions concentration nearly equal to human blood plasma was prepared [15]. Sample membranes of 20x15 mm^2 were cut from the original processed films for the bioactivity tests. Three replicates for each sample were immersed in 45 mL SBF for 2, 5, 7, 14 and 21 days at 37° C. After being removed from SBF the membranes were gently rinsed with distilled water and dried at room temperature.

2.4 Physico–chemical Characterization

2.4.1 Scanning Electron Microscopy (SEM)

Qualitative information of the morphology of PDLLA and PDLLA/BG membranes surfaces, before and after the immersion in SBF, was obtained using a Scanning Electron Microscope, SEM (Nova NanoSEM 200-FEI Company), at an accelerated voltage of 5 kV. Before being observed by SEM, the membranes were gold coated using a Hitachi coating unit IB-2 coater at 6 mA.

2.4.2 Fourier Transform Infrared Spectroscopy (FTIR)

The possible formation of a CaP layer onto the surface of polymeric membranes incorporating the microparticles after 14 days soaking in SBF, was analysed by infrared spectroscopy where the background noise was calibrated with pure KBr data. Also, each side of the composite membrane were

analysed with the attenuated total reflection accessory. Spectra were recorded in an IR Prestige 21 FTIR spectrophotometer with the attenuated total reflection accessory (128 scans, resolution 4 cm^{-1}) in the spectral range 2000-400 cm^{-1}.

2.4.3 Swelling Properties

The water sorption capacity of the membranes was determined by swelling the samples in phosphate buffered saline solutions (PBS, Gibco) at pH 7.4 for upt to 4 months at 37°C. The initial weight of each membrane (approximately 10×10 mm^2) was measured with an analytical balance (Scaltec, Germany) before immersion in the PBS for several pre-determined time intervals (5 min, 30 min, 1 h, 1 day, 7, 14, 22, 28 days, then 6, 8, 12 weeks and finally 4 months) and immediately weighted after the removal of excess of water by lying the surfaces on a filter paper (Whatman Pergamyn Paper, 100×100 mm^2). The swelling ratio (SR) was calculated using the following equation (1):

$$SR = \frac{W_w - W_d}{W_d} \qquad (1)$$

Where W_w and W_d are the weights of the samples at the swelling state and at the dry state, respectively.

2.4.4 Degradation Test

The degradation of both membranes (with or without BG microparticles) was evaluated by immersing samples (with known weights and approximately 10×10 mm^2) in PBS, at pH 7.4 for 4 months at 37°C. At pre-determined time intervals (5 min, 30 min, 1 h, 1 day, 7, 14, 22, 28 days, then 6, 8, 12 weeks and, finally, 4 months) samples were removed from the solution and washed with distilled water three times for the removal of salts. The samples were dried at room temperature and then weighted with an analytical balance (Scaltec, Germany). The following equation (2) was used to calculate the percentage weight loss (%WL) of the samples:

$$\%\, WL = \frac{W_i - W_f}{W_i} \times 100\% \qquad (2)$$

Where W_i is the initial dry weight of the sample and W_f is the weight of the dry sample after incubation in the PBS solution.

2.5 Mechanical Characterization

2.5.1 Mechanical properties

The tensile properties were determined using an INSTRON 4505 Universal Machine (Instron Int. Ltd., USA) equipped with a 1 kN load cell, with a loading rate of 5 $mm.min^{-1}$ up to 20% of strain, at room temperature. Samples were analyzed in the dry and wet conditions. For the wet condition, samples were immersed for 3 hours in PBS before being tested. The values reported represent an average of at least five testing specimens. Tensile force was taken as the maximum force in the force-deformation curve. Tensile modulus was estimated from the initial linear section of the stress-strain curve.

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PDL cells were obtained from human third molar according to the following procedure. After extraction, the teeth were washed three times for 10 minutes in PBS with 100units/mL penicillin and streptomycin. PDL tissue was scraped from the middle third of the root with a scalpel blade, to avoid contamination by epithelial or pulpal cells. The freed portions of the periodontal ligament were minced and transferred to a small culture flask, filled with 5 mL alpha minimal essential medium (α-MEM, Gibco) with 10 % v/v fetal calf serum (FCS, Gibco), 50 mg/mL ascorbic acid (Sigma), 10-8 M dexamethasone (Sigma), 50 mg/mL gentamycin (Gibco) and 10 mM sodium β-glycerophosphate (Sigma). Medium was refreshed every 2 to 3 days. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and medium was replaced every 2 to 3 days. Upon reaching confluence, cells were released with trypsin/EDTA (0.25% w/v crude trypsin and 1 mM EDTA (pH 7.2)) and sub-cultured

for 2 passages in standard culture flasks. The cells were then frozen in liquid nitrogen until used for the experiments.

Human BMSCs were isolated from bone blocks of human iliac crest biopsies of donors. The biopsies were discarded tissues during standard surgical procedures at Radboud University Nijmegen Medical Center (Nijmegen, The Netherlands). The bone blocks were cut into small pieces and subsequently placed in a 50mL tube to which 20mL alpha-minimal essential medium (α-MEM) was added. After that the tube was shaken vigorously and the medium with cells was collected. This procedure was repeated several times. The collected medium with cells was plated in culture flasks (T175; Greiner Bio-one) and expanded in proliferation medium. Cells were characterized and showed stem cells phenotype. Additionaly, a multipotential differentiation test was applied, demonstrating their stem cells capacity. Cells were cultured at 37^{0}C in a humid atmosphere with 5% CO_2 and its passage was performed at 80% confluence using trypsin EDTA (Gibco). After the first generation, cells were plated at a density of 5000 cells/cm^2 in culture flasks (T175). The culture medium was changed twice a week.

Cells from passage 3 (hBMSC) and 5 (hPDL) were used in the biological experiments.

2.6.1 Cell seeding

Metal rings (15 mm x 3 mm) were glued to the membrane samples to keep them in the solution, with RTV Silicone Adhesive (Nusil, Silicone Technology, USA, MED-1037). Prior to cell seeding, the samples were sterilized with 70% (v/v) ethanol for 60 minutes and then washed three times immersed in PBS. The samples were placed in 25-well plates and soaked in cell culture medium overnight. After removing the culture medium, 50 μL of a cell suspension with a 2.0 x 10^4/sample cell density, was seeded onto the surface of each sample. After incubation for 4 hours at 37°C in a 5% CO_2 atmosphere incubator, osteogenic medium (specific for each cell type) was added to the seeded samples, according to the type of assay performed. On the control groups, cells were seeded directly on the well-plates and osteogenic medium was added immediately.

2.7 Cell Adhesion, Proliferation and Metabolic Activity

2.7.1 DNA content

After the different experimental time points, medium was removed from the wells and the samples were washed twice with PBS. The analysis was performed on the supernatant of the substrates after day 1, 3, 7, 14 and 28 of culture. Cells were lysed using milliQ with subsequent sonification for 10 minutes between two cycles of freeze/thaw from -80°C. The supernatant was stored at -20°C until

further analysis. A PicoGreen dsDNA Quantification Kit (Molecular Probes, Eugene, USA) was used according to manufacturer's instructions. To each 100 μL sample, 100 μL PicoGreen working solution was added. The samples must incubate for 2-5 minutes at room temperature, in the dark. After incubation, the fluorescence was measured on a fluorescence cuvette reader (microplate fluorescence reader, Bio-Tek, Winooski, USA) with a 485 nm excitation filter and a 530 nm emission filter.

2.7.2 Alamar Blue® staining

Cell metabolic activity was measured using AlamarBlue® staining (Invitrogen) according to the instructions of the manufacturer. A solution was made with AlamarBlue and culture medium in a proportion 1:9 (v/v) and was placed at 37°C for 5 minutes. The medium was removed from wells and replaced with the solution. Plates were incubated (37°C and 5% CO_2) for 4 hours. After incubation, 200 μL of each sample solution was transferred to 96-well plates (Greiner Bio-one). Fluorescence was measured using a microplate reader (FL 600; Bio-Tek) at 570 nm. The assay was performed on day 1, 3, 7, 14 and 28 of culture.

2.7.3 Scanning Electron Microscopy (SEM) observation

Adhesion of both cell types (hBMSC and hPDL) on membranes was analyzed by SEM (n=2). After day 3 and day 28 time points, cells were fixed in 2% v/v glutaraldehyde in 0.1 M sodium-cacodylate buffered solution, for 5 minutes. Cells were rinsed in cacodylate buffered solution, dehydrated in a series of ethanol dilutions in water (70%, 80%, 90%, 96% and 100% (v/v)), 1 hour in each, and dried in tetramethylsilane (TMS, Merck) to air. Finally, specimens were sputtercoated with a thin layer of gold, and examined in a JEOL 6310 scanning electron microscope.

2.8 Cell Differentiation and Mineralization

2.8.1 Alkaline Phosphatase Activity Measurements (ALP)

The same supernatants as used for PicoGreen assay were also used to measure alkaline phosphatase (ALP) activity (Sigma). To each 80 μL of the sample, 20 μL of 0.5M Alkaline Buffer (Sigma, cat#A9226) was added. Thereafter 100 μL substrate solution 5mM paranitrophenylphosphate (PNP, Sigma, cat#P5994) was added to each well. After 60 minutes of incubation at 37°C, 100 μL stop solution (0.3M NaOH) was added to each well. Finally, ALP activity was measured at 405 nm using an ELISA microplate reader (Bio-Tek Instruments Inc, USA).

2.8.2 Von Kossa Staining

Cells were fixed with 2% glutaraldehyde, stained with fresh 5% silver nitrate ($AgNO_3$), washed with distilled water, developed with 5% sodium carbonate (Na_2CO_3) in 25% formalin, and fixed with 5% sodium thiosulphate ($Na_2S_2O_3$). Stained samples were observed under a Leica MZ12 stereomicroscope and images were captured.

2.8.3 Ca Content

Calcium content was assessed after 21 and 28 days of culture to obtain information about mineralized matrix formation. The samples were rinsed twice with milliQ. 1 mL of acetic acid was added to each sample. The samples were incubated overnight under vigorous constant shaking and the acetic acid with the diluted calcium was frozen and kept at -20°C, until further investigation. After thawing, the calcium content was determined using the OCPC method. Optic density was read with an ELISA reader (Bio-Tek Instruments Inc, USA) at a wavelength of 570 nm. Bare membranes were also assessed in order to further exactly quantify and distinguish cellular from acellular mineralization on the membranes.

2.9 Statistical Analysis

All samples were measured in triplicate. Biological tests were performed twice, excepting Von Kossa. All results are presented as mean ± standard deviation. Statistical analysis of experimental data was performed using an unpaired ordinary ANOVA with standard parametric methods. Calculations were performed in InStat (v. 3.0 GraphPad Software Inc, San Diego, CA). Statistical significance was set to *p-value* ≤ 0.1 (*), to *p-value* ≤ 0.01 (**) and to *p-value* ≤ 0.001 (***).

3| Results and Discussion

3.1 Membranes characterization

3.1.1 Bioactivity, morphology and microstructure

The surface morphology of PDLLA and PDLLA/BG membranes were analyzed using SEM – see Fig. 3.1A. A flat, smooth, nonporous surface was observed on the PDLLA membranes with no evidence of surface irregularity (A_1). Both upper and bottom faces of the composite membrane (see scheme in Fig.

1B) were also analyzed. The upper face of the composite PDLLA/BG 80/20 membrane is also smooth (B_1) but the bottom face (C_1) presents some asperities, homogeneously distributed in the surface corresponding to the BG particles that were preferentially deposited in this side of the membrane. The image suggests that the particles are well incorporated in the polymeric matrix.

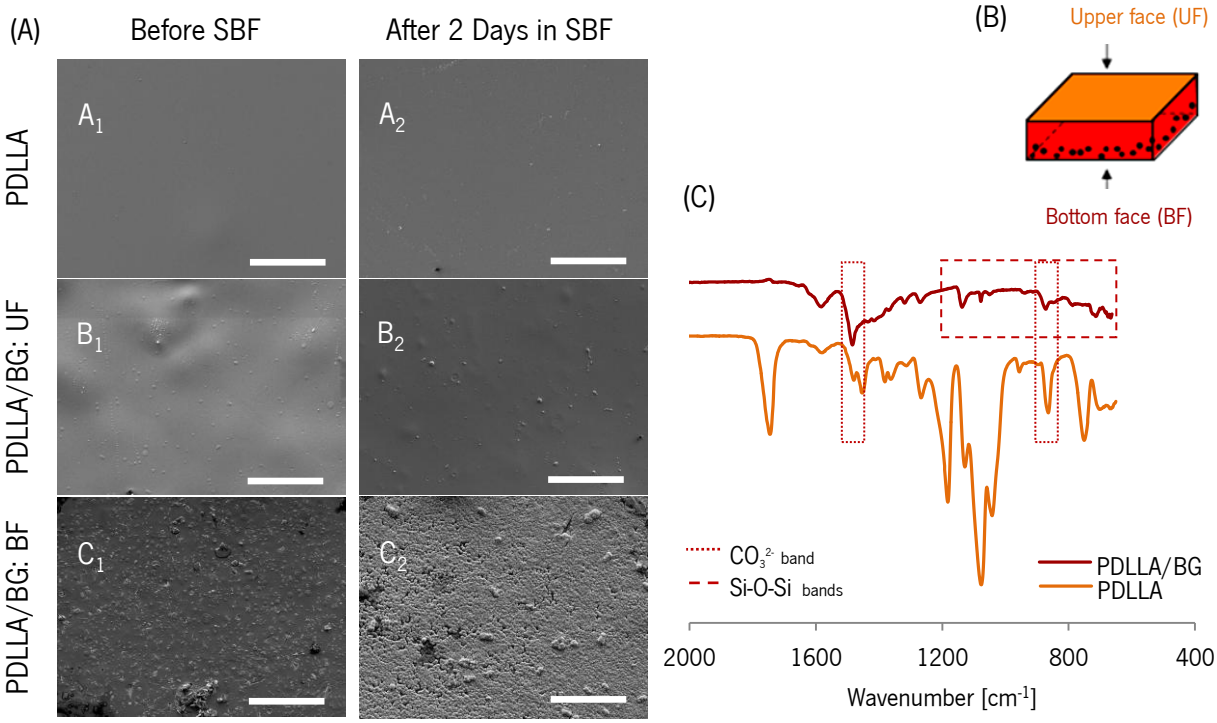

Figure 3.1 – *In vitro* bioactivity tests of the developed membranes: (A) SEM micrographs of the surfaces of the PDLLA (A_1) and PDLLA/BG membranes, from upper and bottom face (B_1 and C_1), before and after 2 days of immersion in SBF (A_2, B_2, C_2). The scale bar represents 50 μm. (B) Schematic design of the PDLLA/BG membrane, evidencing the distinct faces. (C) FT-IR spectra of both sides of the composite membrane: bottom face rich in BG (red line) and upper face exposing basically PDLLA (orange line).

The bioactive character of the produced composite membranes was tested *in vitro* by immersing the materials in SBF – see evolution of the morphology of the surfaces in Fig. 3.1A. No Ca-P layer was formed on the surface of pure PDLLA membrane (A_2) or of the upper face of the composite membrane (B_2), even after 21 days of immersion in SBF. Only the membranes face enriched with BG presented a bioactive character, where an uniform ceramic layer could be detected after only 2 days of immersion in SBF (C_2).

The morphological analysis was complemented with FTIR – see Fig. 3.1C. The characteristic FTIR bands of the upper face are located at 750 and 865 cm^{-1} (CH band); 1042, 1090, 1138, and 1186 cm^{-1} (=C–O stretch); 1375 cm^{-1} (CH_2 wag); 1452 cm^{-1} (CH_3 band) and 1751 cm^{-1} (C=O stretch, ester group) that are the characteristic bands of PDLLA [16]. The spectrum of the bottom face of the membrane

indicates the presence of BG particles: 867, 1454 cm^{-1} (CO_3^{2-} bands); 1200-700 cm^{-1} (Si-O-Si bands) [17; 18].

3.1.2 Material stability properties

In order to understand the behavior of the membranes in an aqueous environment, the weight loss – see Fig. 3.2 – and swelling ratio were measured up to 4 months.

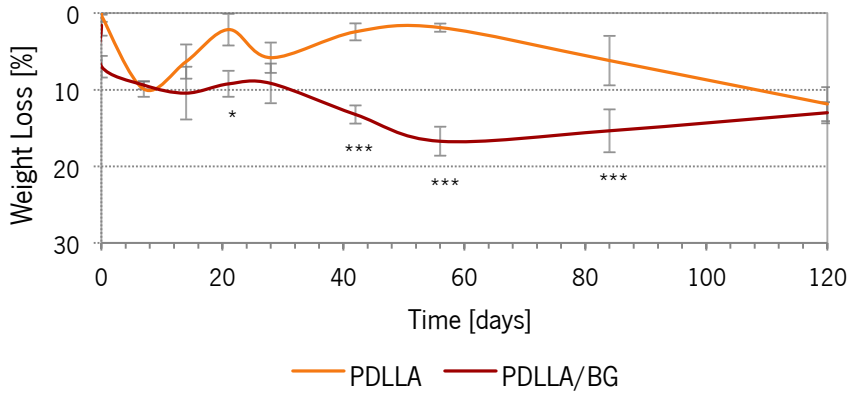

Figure 3.2 – Weight loss percentage of PDLLA and PDLLA/BG membranes, in PBS solution, at 37°C up to 120 days. Values are reported as mean ± SD (n=3). Statistically significant differences in the weight loss values of the PDLLA/BG membranes when compared to those of the pure PDLLA are indicated by (*) for $p < 0.1$, (**) for $p < 0.01$ and (***) for $p < 0.001$.

The incorporation of BG particles has been reported to play an important role in polymer surface wettability [19]. Its introduction in polymer matrices can modify the surface and inclusively the bulk properties of the composite, by enhancing both hydrophilicity and water absorption [20]. In the system studied in this work, no significant swelling was observed in the membranes even when BG is incorporateds (data not shown).

Within the time period analyzed the weight loss of the PDLLA membrane was not significant: after 120 days of immersion in SBF, the weight loss was about 12%. For the case of the composite membrane we could detect a faster weight loss up to 50 days that could be related to the slow dissolution of the inorganic component. Such process is consistent with the event of the formation of an apatite layer upon immersion in SBF, discussed before.

3.1.3 Mechanical properties

Adequate mechanical integrity is known as an important requirement for membranes in guided tissue regeneration. Either to adapt the desired shape or to support the stresses of the surrounding native tissue at the site of implantation, membranes should be strong but flexible. [3] Tensile

mechanical tests were performed for the PDLLA and PDLLA/BG membranes. Both dry and wet specimen conditions were assessed; the last condition simulate better the behavior of these materials in the oral cavity environment. Representative stress-strain curves are shown in Fig. 3.3. Table 3.1 shows the maximum strain and stress obtained in these experiments as well as the tensile or Young modulus of the membranes. The addition of BG in the PDLLA membranes significantly reduced the maximum strain and a small reduction of the maximum stress could be also detected. Previous works reported also a reduction of the strength upon reinforcement in composites of poly(α-hydroxyesters). [21; 22] The behavior observed in the membranes may be due to the increase in pore size by introducing Bioglass® in the pure PDLLA matrix. Young modulus values were very similar between both types of membranes, in both dry and wet conditions.

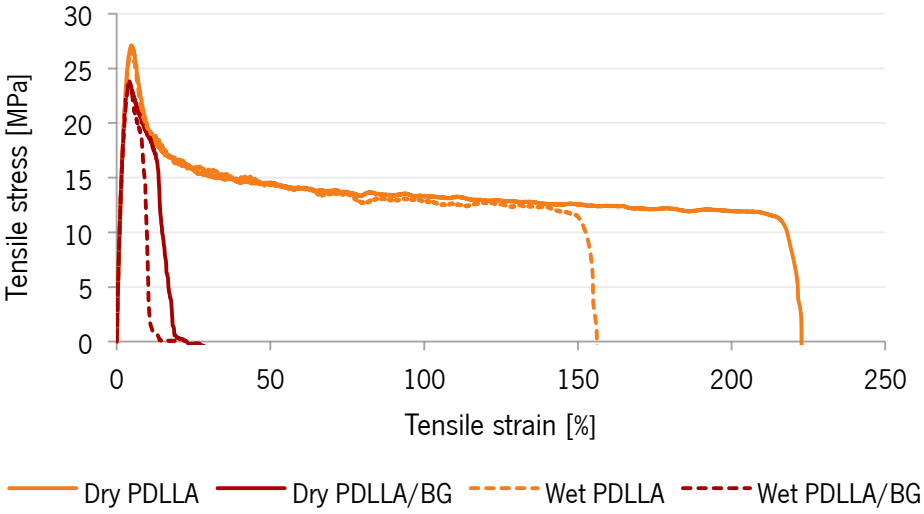

Figure 3.3 – Representative stress *versus* strain curves obtained for PDLLA and PDLLA/BG membranes in dry (solid lines) and wet (dashed lines) conditions.

Table 3.1 – Young modulus, maximum strain and maximum stress of the PDLLA and PDLLA/BG membranes obtained in dry and wet conditions. Values are reported as mean ± SD. Statistically significant differences in the mechanical properties of the PDLLA/BG membranes when compared to those of the pure PDLLA are indicated by (*) for $p < 0.1$ and (**) for $p < 0.01$.

		YOUNG MODULUS (MPa)		MAXIMUM STRAIN (%)		MAXIMUM STRESS (MPa)	
	CONDITION	DRY	WET	DRY	WET	DRY	WET
SAMPLE	PDLLA	713.7 ± 77.9	656.9 ± 38.1	166.2 ± 81.3	141.0 ± 94.1	26.2 ± 1.56	27.4 ± 1.38
	PDLLA/BG	743.3 ± 30.3	617.9 ± 38.8	21.9 ± 5.5 *	21.6 ± 11.4 *	17.7 ± 5.21**	23.1 ± 1.43

No significant differences were observed between the dry and wet samples. Water can affect strongly the molecular wettability and the viscoelastic properties of poly(lactic acid) [23]. However, in the

membranes prepared in this work swelling was almost inexistent and no significant plasticization effect of water toke place, as reflected in the tensile mechanical properties at 37°C.

Nanoindentation experiments were also performed in order to have more insights about the mechanical properties of both membranes surfaces. A series of load (P)-displacement (h) curves are shown in Fig. 3.4A. The hardness (*H*) was calculated by dividing the maximum load by the contact area $H = \frac{P_{max}}{A}$ where P_{max} is the maximum load applied during the indentation and *A* is the projected area of contact between the indenter and the sample. The slope of the unloading curve, d*P*/d*h*, provides a measure of the elastic moduli, *E* [24; 25]. Repeated experiments performed on the upper face of the membrane present load-depth curves very superimposed. The bottom face of the membrane presents a more heterogeneous surface that could explain the much more scattered "bottom face" plots of Fig. 3.4A.

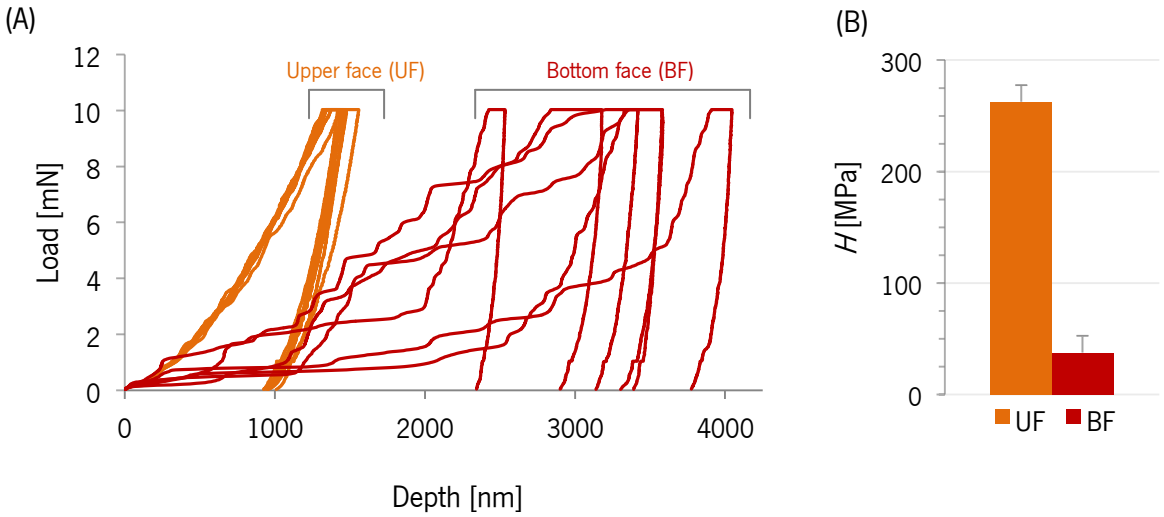

Figure 3.4 – Nanoindentation results obtained by cycled experiment: (A) Six different indents either for the upper face (UF) and the bottom face (BF) of the membrane and (B) hardness values for both sides of the membranes. There were no statistically significant differences ($p > 0.05$) observed between the parameters of the analyzed materials.

The nanoindentation curves show a greater resistance to deformation in the upper face than in the bottom face as indicated by how far the indenter was able to penetrate on each side of the membrane for the same maximum force. This resulted in different values of hardness (*H*) (Fig. 3.4B) that were higher for the upper side (≈250 MPa) than for the bottom side (≈5 MPa) of the composite membranes. Such difference could be ascribed to the more porous structure of the bottom side of the composite membrane.

3.2 *In vitro* hBMSC and hPDL cells culture

3.2.1 Cell Adhesion, Proliferation and Metabolic Activity

DNA content was quantified at different culture time points for both membranes – see Fig. 3.5A. A clear proliferation of both cell types onto the PDLLA and PDLLA/BG membranes was observed until 28 days of culture. Significantly higher cell content was found in BG containing membranes seeded with hBMSC after 28 days of culture.

Complementarily, cell metabolic activity was measured. As the values between different days for fluorescence intensity of Alamar Blue are not comparable, it was calculated a ratio between the composite and PDLLA membrane samples, in each time point – see Fig. 3.5B. Therefore, values above 1 means higher metabolic activity for samples with BG than the ones without BG. Average cell growth and their metabolic activity was higher in the PDLLA/BG group than in the PDLLA group, for hBMSC.

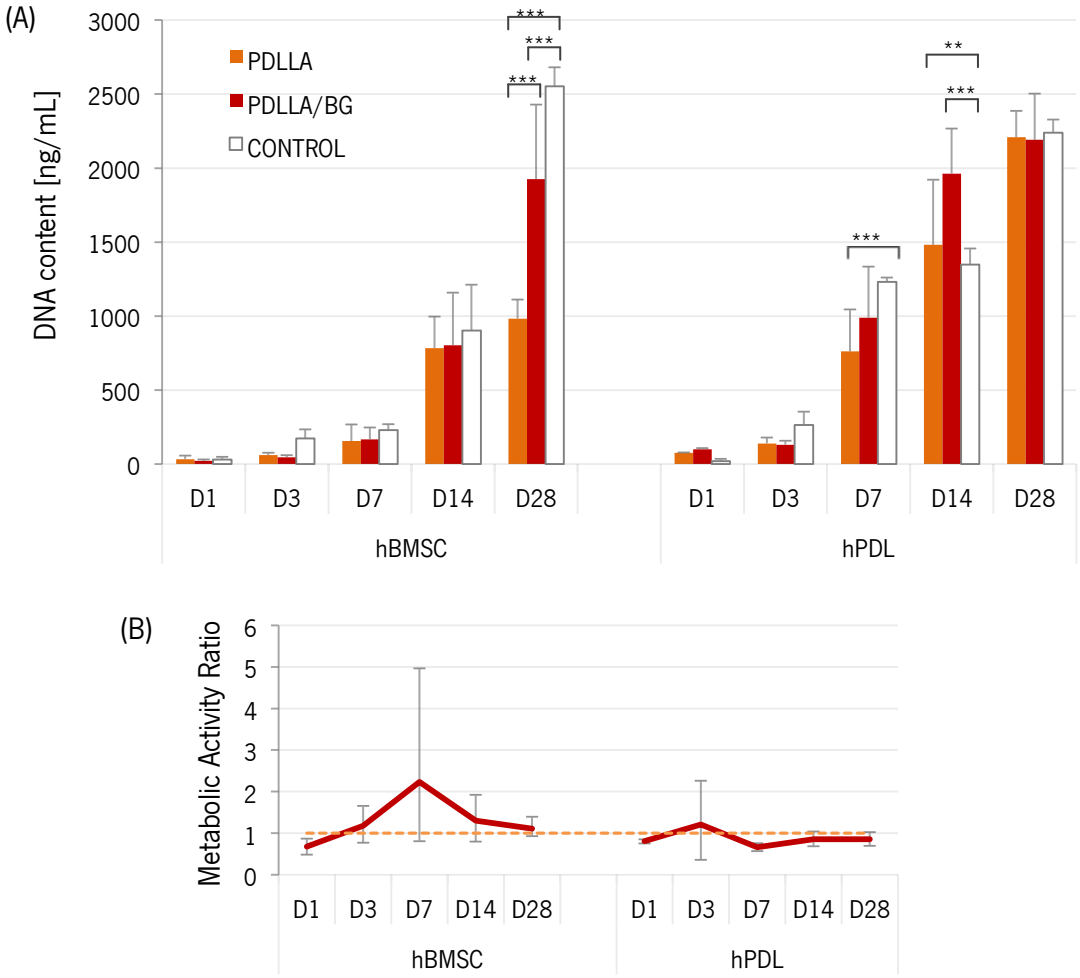

Figure 3.5 – Results obtained from DNA and Alamar Blue assays at 1, 3, 7, 14 and 28 days of culture: (A) DNA content, in ng/mL, of hBMSC and hPDL cells seeded onto each membrane type and controls (plastic cell culture in polystyrene), (B) metabolic activity ratio between the samples with and without BG (solid lines) for both cell types, where the border line (dashed orange line) is y=1. Values are reported as mean ± SD (n=3). (*) shows significant differences for $p < 0.1$, (**) for $p < 0.01$ and (***) for $p < 0.001$.

These enhanced hBMSC cells proliferation and metabolic activity showed by the PDLLA/BG membranes is in accordance with some previous studies [26; 27; 28] that reported the promotion of cell proliferation by Si and other released products from bioactive glasses. Sun *et al.* showed an enhanced metabolic activity of hBMSC on akermite ceramics verified trough Alamar Blue staining, which has a composition very similar to BG [29]. Other studies [30; 31; 32; 33; 34] reported the enhancement of osteoblast cell proliferation by the influence of BG particles corroborating the results herein obtained for PDLLA/BG membranes, using hBMSC. Higher concentrations of DNA were observed in BMSC's cultured when 45S5 Bioglass® was added to a new ceramic based on hydroxyapatite compared to the pure material.[35] High contents of bioactive component may lead to a negative effect on cell behavior. For example, cell studies with scaffolds of PDLLA, containing different contents of BG (0.5 and 40 wt%) showed enhanced proliferation and ALP activity for the 5% ones [36]. Due to the subsequent augmented and prolonged ion release and increase of pH, the materials with the largest BG concentration appeared to fail. Exceptions have been also reported. For example, Wilda *et al.* obtained better results with PDLLA/BG 30 wt% comparing to 0 or even 5 wt% [37].

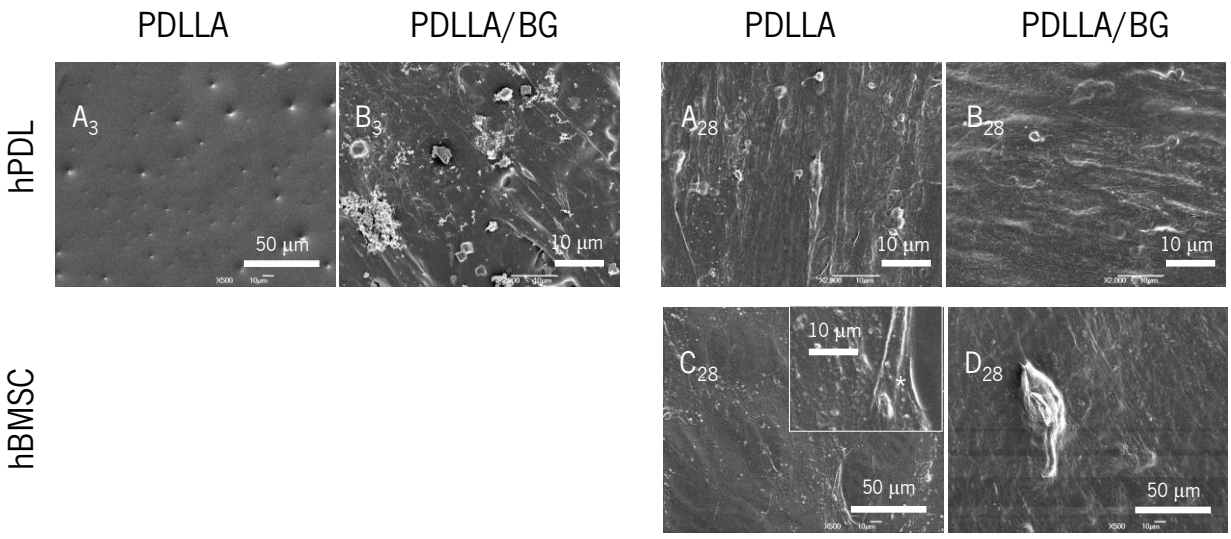

Figure 3.6 – SEM micrographs of PDLLA and PDLLA/BG membranes after cultured with hPDL and hBMSC cells. The subscripts indicate the incubation time.

PDLLA and PDLLA/BG membranes cultured with hBMSCs and hPDL cells were further studied using SEM – see Fig. 3.6. After 3 days of culture, hPDL cells spread perfectly on the PDLLA/BG membranes (B_3). On day 28, there was a very dense extracellular matrix (ECM) deposition for both hBMSC and hPDL cells on PDLLA/BG membranes (B_{28} and D_{28}), which hampered the distinction of the cells morphology and the examination of surface roughness in the material. Once that osteoblasts mature and start depositing ECM, this extended ECM is a signal of cell differentiation [38]. Still at this

time point, (C_{28}) the adhesion of hBMSC could be detected, either spread in an elongated shape, polarized with lamellipodia (see (*) on inset image of C_{28}) or in some cell agglomerates. Many studies have examined the ability of BG to enhance not just cell proliferation [39; 40] but also the ECM production [41], which is in accordance with these results.

3.2.2 Cell Differentiation and Mineralization

Alkaline phosphatase activity was measured to assess the osteogenic differentiation potential of the cells cultured in the developed membranes. The results obtained from this assay (Fig. 3.7) were normalized by the DNA content measured for the same sample. No detectable ALP activity could be seen during the first time points, being consistent with the undifferentiated state of the cells. After day 14, and specially day 28, we can see significant ALP activity of the cells in the membranes.

While some authors [26; 27] claim that the addition of BG particles has no effect on ALP activity of rat primary culture osteoblasts and murine osteoblasts, others state that this addition has stimulatory effects on the ALP activity of human primary osteoblasts [42], BAF cells [43; 37], and hBMSC [29]. At 28 days of culture, higher ALP activity was detected for the composite membranes cultured with hBMSC, compared to the pure PDLLA membranes and even to the control group. This provides a clear indication of a more extended osteogenic differentiation of hBMSC on the PDLLA/BG composite membranes.

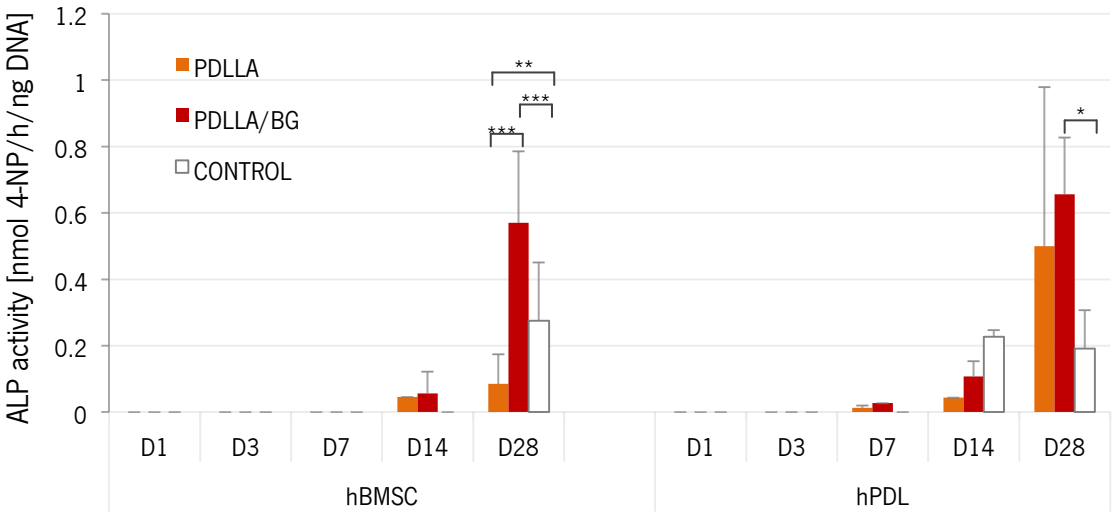

Figure 3.7 – Alkaline phosphatase activity, normalized by the DNA content, of hBMSC and hPDL cells on PDLLA and PDLLA/BG membranes and controls(plastic cell culture in polystyrene) at 1, 3, 7, 14 and 28 days of culture. Values are reported as mean ± SD (n=3). (*) shows significant differences for $p < 0.1$, (**) for $p < 0.01$ and (***) for $p < 0.001$.

In alignment with the ALP results, statistically significantly higher calcium content was found in the PDLLA/BG membranes (Fig. 3.8A). Von Kossa staining allowed observing the formation of mineralization nodules by hPDL cells on composite membranes, after 28 days of culture (Fig. 3.8B). No nodule formation was detected on PDLLA membranes (data not shown). It is also to note the fact that there was an increasing calcium deposition, over time (21 to 28th day), statistically significant for the hBMSc cells on composite membranes.

These results are in accordance with other related studies. For example, a stronger and earlier calcium phosphate mineral formation in bioactive composites was observed for rat bone marrow cells [44]; faster nodule formation in porous bioactive glass scaffolds, as compared with control cultures [28] using human primary osteoblasts, among others previous studies [45; 46], demonstrating that BG causes an increase of calcium in the medium, which is a modulator of intracellular events.

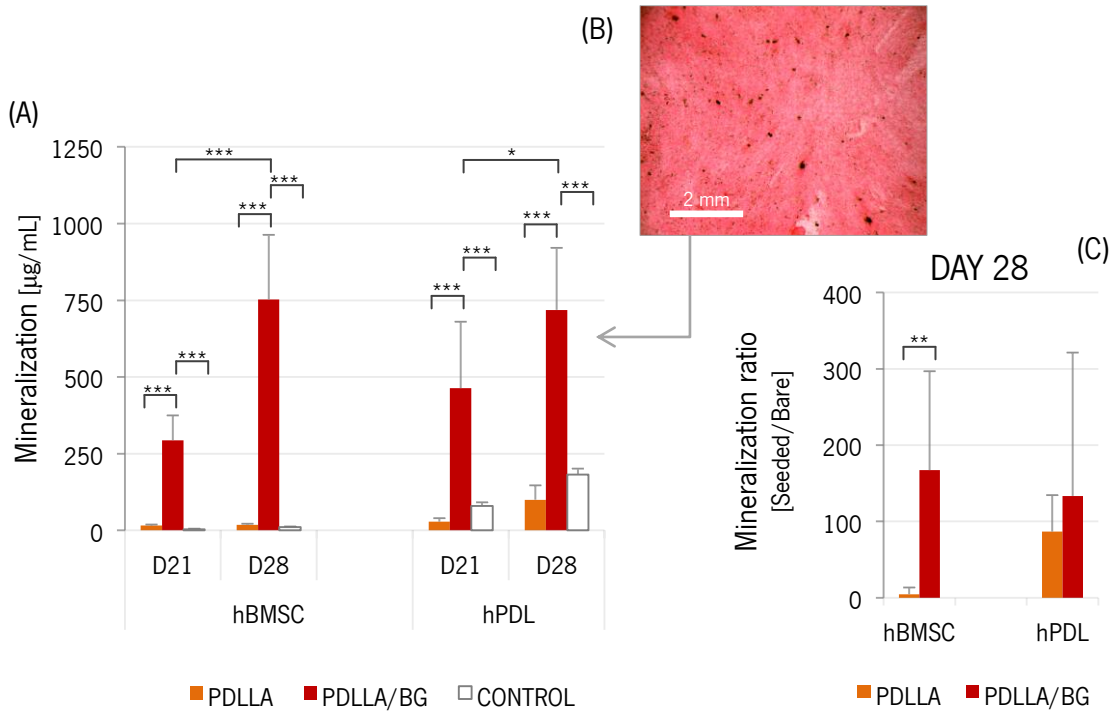

Figure 3.8 – Evaluation of the mineralization of hBMSC and hPDL cells on PDLLA and PDLLA/BG membranes and controls (plastic cell culture in polystyrene), after 21 and 28 days of culture: (A) Calcium content quantification and comparison between 21 and 28 days of incubation. (B) Calcium nodules formation, verified by Von Kossa staining at day 28 of incubation, on PDLLA/BG membrane cultured with hPDL cells. (C) Ratio of mineralization between seeded and bare membranes. Values are reported as mean ± SD (n=3). (*) shows significant differences for $p < 0.1$, (**) for $p < 0.01$ and (***) for $p < 0.001$.

It has been reported that bone nodule formation occurs when human bone-derived cells are cultured for extended periods of time in the presence of ascorbate and/or β-glycerophosphate (components present in the osteogenic medium used in this study) [47]. Bone nodules consist of differentiated osteoblasts, extracellular matrix, and associated minerals, and their formation characterizes a late stage of osteoblast differentiation [47], being a good index of osteogenesis *in vitro* [47; 48; 49]. However, it was previously demonstrated that calcified bone nodule formation can be detected as early as day 6 in culture on the bioactive glass, without either of the above supplements in the culture medium. [42] Both of these mineralization assays indicated that BG improved mineralization, complementing each other information.

4| Conclusions

The incorporation of BG in PDLLA membranes modified their physico-chemical and biological properties. The mechanical properties of the membranes were not significantly compromised with the introduction of BG. The asymmetric distribution of the BG particles along the thickness in the composite membrane permitted to induce a bioactive character in one of the sides of the membrane. The inorganic component had a positive impact in the adhesion, proliferation, differentiation and mineralization of hBMSC cells on PDLLA/BG membranes. Therefore, our results suggest that the obtained asymmetric bioactive PDLLA/BG 80/20 membrane, with osteoconductive properties in just one of the faces, could have potential use in the regeneration of distinct tissues, namely periodontal ligament and bone.

5| References

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CHAPTER IV. General Conclusions & Future Research

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1 | General Conclusions and Future Research

A novel biocompatible and biodegradable membrane was obtained by combining poly(D,L-lactic acid), PDLLA, and microparticles of Bioglass®, BG, featuring an asymmetric bioactivity and a good integration, between the polymeric and inorganic fractions, in a 80/20 reason. The membranes were prepared in a single step, by a simple and viable adjusted solvent casting methodology. The asymmetric distribution of the bioactive BG particles was conferred during the processing of the membrane, in which, while solvent was evaporating, the particles were preferentially depositing by gravity to the bottom side. The incorporation of BG in the PDLLA membranes influenced their physico-chemical properties and, consequently, their biological properties. These properties were further evaluated, contributing to the understanding of the biological applicability of asymmetric composite membranes.

Only the inorganic-rich face could promote the deposition of a bone-like apatite layer immersing the membrane in simulated body fluid, which occurred after 2 days just on the face containing BG, proving the asymmetric bioactivity of the PDLLA/BG membranes. The good impregnation of the BG particles on the membranes conferred a homogeneous roughness to their surface and improved porosity. As a result, the particles were more exposed and could be more easily dissolved accelerating the degradation rate, and simultaneously the weight loss. No significant differences between PDLLLA and PDLLA/BG membranes were found for swelling and mechanical tensile behaviors, revealing this to be a stable combination. These parameters are passive of being tailored, once that polymer/bioglass ratio could be optimized according to specific applications.

Attending to the distinct biological environments in which the two sides of the membranes are in contact upon implantation, two different cell cultures were selected for the biological assessment in this study. Human bone marrow stromal cells, hBMSC, and human periodontal ligament cells, hPDL, were seeded in osteogenic medium on the membranes surface. Bioactive character of composite membranes seemed to significantly favor cell adhesion, which was reflected in higher cell proliferation and metabolic activity values. An enhancement on cell differentiation was further detected for both cell types, as well as a promoted mineralization, an extended extracellular matrix and calcium nodule formation, due to the bioactive character of the BG added. All the biological properties were improved with significant differences comparing to the controls (plastic cell culture) and with a higher differential for hBMSC.

The new asymmetric composite PDLLA/BG membrane is expected to have potential applications in guided tissue regeneration. Structural and biological performances presented in this study by this membrane showed to be advantageous and possibly profitable applicable to a wide range of periodontal defects and additionally bone deficiencies that restrain dental implants application. Considering that

periodontal diseases represent the major cause of tooth loss in adults and that, subsequently, the Implantology cases are exponentially increasing, such new approaches are essential to supply the mentioned needing.

In vitro, osteoblast differentiation is a gradual process represented by a temporal expression of genes and characterized by three principle periods: proliferation, extracellular matrix production/maturation, and mineralization, with mRNA peak levels defining the transition between periods. [1] Bone tissue contains several non-collagenous proteins, such as osteocalcin (OCN) and alkaline phosphatase (ALP), which distinguish it from other types of tissues. ALP is the most widely recognized marker for osteoblast activity, expressed in culture by the osteogenic cells, namely hBMSC, [2; 3] and its metabolic activity was assessed in the current study. However, a further evaluation of gene expression would be interesting and valuable to investigate the differentiation progress on the PDLLA/BG membranes, mainly, since OCN is a marker that appears late during osteoblast differentiation and characterizes mature cells of the osteoblastic lineage (e.g. osteocytes), actively producing mineralized tissue. [1] A RT-PCR (real-time polymerase chain reaction) procedure could be a smart choice to help in the diagnosis of differentiation evolution.

Another interesting feature to explore would be the PDLLA excellent ability to combine with drugs like growth and differentiation factors, establishing a locally acting drug-delivery system. [4] Moreover the Bioglass® dissolution products (particularly the 45S5 composition) could upregulate the gene expression that control osteogenesis and the growth factors production [5]. Such gene expression control and the use of growth factors are indicated as promising approaches in periodontal therapy. [6] Cleverly combined with the PDLLA/BG asymmetric membranes, these approaches might positively manipulate the osteoblastic differentiation, inducing selective cellular repopulation of periodontal defects, consequently resulting in a substantial increase on bone formation. Prospecting better clinical outcomes, this combination could contribute with an enhancement in long-term complete periodontal regeneration.

The data demonstrated in this study derived from an *in vitro* experimental model, which constitutes by itself a limitation. Therefore, the biocompatibility, both *in situ* and *ex vivo*, of the current membranes material is still unclear in human oral cavity. In order to clarify these points, supplementary and complementary, the development of animal experiment studies should be further employed. Despite of the referred limitation, the results obtained suggests the PDLLA/BG membranes as a great promising biomaterial for guided tissue regeneration.

2 | References

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