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Testing active membranes for bone regeneration: A review

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ABSTRACT

Objectives: Maxillofacial bone defects are the main hindering conditions for traditional dental implant strategies. Guided Bone Regeneration (GBR) is used to handle this situation. The principle of GBR is to use a membrane to prevent the colonization of soft tissue cells of the bone defect and favors the migration of osteogenic linages. Current membranes do not completely fulfill the requirements that an optimal membrane should have, sometimes resulting in non-predictable results. Thus, the need to develop an ideal membrane to perform this duty is clear. Recent developments in bio-manufacturing are driving innovations in membranes technology permitting the active participation of the membrane in the healing and regenerative process trough native tissue mimicking, drug-delivery and cells interaction, away from being a passive barrier. New membranes features need specific evaluation techniques, beyond the International Standard for membrane materials (last reviewed in 2004), being this the rationale for the present review. Nanotechnology application has completely shifted the way of analyzing structural characterization. New progresses on osteoimmmunomodulation have also switched the understanding of cells-membranes interaction.
Data and Sources: To propose an updated protocol for GBR membranes evaluation, critical reading of the relevant published literature was carried out after a MEDLINE/PubMed database search.
Conclusions: The main findings are that a potential active membrane should be assessed in its nanostructure, physicochemical and nanomechanical properties, bioactivity and antibacterial, osteoblasts proliferation, differentiation and mineralization. Immunomodulation testing for macrophages recruitment and M2 phenotype promotion in osteoblasts co-culture has to be achieved to completely analyze membranes/tissue interactions.

1. Introduction

Alveolar and maxilar bone defects are the major hindering conditions for traditional implant strategies. These defects are mainly caused by trauma, tumor or infection [1].

An efficient tool available to palliate this handicap is Guided Bone Regeneration (GBR). GBR is one of the most effective techniques to obtain osteogenesis. It is based on the necessity to isolate the bone defect from soft tissue, in order to prevent that epithelial and connective components migrate and colonize the hard tissue defect. For this reason, to achieve GBR it is indispensable the presence of a membrane, that will act as a barrier. According to Sanz et al. [2], and as one of the consensuses of the 15th European Workshop on Periodontology on Bone Regeneration, besides its occluding and isolation capacity, a membrane for GBR should meet some basic requirements: biocompatibility, biological activity, porosity and occlusive properties, mechanical properties, integration with tissues, exposure tolerance and biodegradability. Currently, there is not a commercialized membrane that meets the optimal characteristics. Recent developments in biomanufacturing are driving innovations in membranes technology to respond to this challenge. The major efforts in recent developments in membranes design are: i) the creation of nanostructured membranes mimicking the native tissue [3], ii) the active participation of the membrane in the healing and regenerative process trough drug-delivery and cells interaction, away

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from being a passive barrier [4,5]. These two new membranes features need specific evaluation techniques. Moreover, it should be considered that the International Standard -ISO- which gives the requirements for a technical evaluation of membrane materials was last reviewed in 2004 [6]. For these reasons, there is a need to develop a membrane for GBR to enable clinicians to accomplish more predictable regenerative surgeries [7], and to define how to examine new membranes characteristics trying to fulfill desired prerequisites of a potential membrane for GBR [2].

The main purpose of this review was to propose a specific and contrasted protocol for GBR membranes evaluation, from *in vitro* to *in vivo* testing and focusing on the most recent membranes evolution.

2. Surface characterization

2.1. Morphological analysis

Nanotechnology application in membranes manufacturing has completely shifted the way of analyzing structural characterization. Nanofibrous scaffolds are preferred as they possess unique properties: high surface area to volume ratio, porosity with interconnected pores, enhanced protein absorption, activation of specific gene expression and intracellular signaling, and promoted cellular reactions [3]. With larger surface to absorb proteins, nanoscaled scaffolds present more binding sites to cell receptors [3,8].

The use of Atomic Force Microscopy (AFM) would give the opportunity to observe membranes nanostructure at an atomic level, being able to access to the very scaffolding of matter, even at the chemical bond scale [9]. AFM can be used to assess nanoroughness, which is an important parameter that will promote protein non-specific adhesion and cellular attachment to the proposed matrices [10,11]. Fiber sizes, fiber to fiber distance and pore sizes can also be measured with Scanning Electron Microscopy (SEM) and AFM [12]. When using SEM for membranes structural characterization, the sample needs to be processed (specially, when analyzing natural polymers) whereas AFM measures accurately on the nanoscale, produces high-resolution images, requires little or no sample preparation and is able to work in humid conditions [13]. Much work has been done on the effect of pore dimension on osteoblast proliferation and differentiation. Scaffolds with interconnected pores usually enhance more bone growth compared with those with closed or non-existent pores [14]. This is because the delivery of osteoprogenitors to the scaffold is improved if the ingrowth of vasculature is facilitated [14,15]. It has been reported that nanometric porosity ranging from 50 to 500 nm selectively enhances protein adsorption (including fibronectin and vitronectin), contributing to cell attachment [11]. Cells growing on membranes containing pores between 5 and 8 μm showed increased osteogenic differentiation [16]. Mimicking collagen nanofiber diameters has been shown to enhance cell attachment on tissues about 1.7-fold [11]. Scaffold architecture greatly influences cell attachment and migration [16], so it is indisputably a fundamental part of tissue analysis. In addition, and to add importance to the fact of working at nanoscale, it has recently been described that nanofibrous materials provide high area-to-volume ratios, mimicking the extracellular matrix of native bone tissue, enhancing cellular adhesion and growth [17]. This fact has determined that novel artificial fibrillar membranes manufactured trough electrospinning are being developed (Fig. 1) [3,7].

2.2. Nanomechanical properties

Measuring nanomechanical properties has been demonstrated to be of great importance, since it has been proved that substrate stiffness can modify cell behavior and cells may probe and respond to mechanics in fibrillar matrices [18]. It has been described that native mesenchymal stem cells have extreme sensitivity to matrix-level elasticity, conditioning their differentiation to specific lineages, including osteogenic phenotypes [19]. However, measuring properties of individual nanofibers or even at a micrometric level is not completely reliable as it does not relate to the clinical use of these materials. These measurements performed on an individual fiber do not take into account the force dissipation due to molecular interactions within the fibers of the network and the force dissipation via interstitial spaces and flows [13]. This is why dynamic nanomechanical analysis is highly recommended instead of the classical static tests.

Polymers of long chains (*i.e.* cross-linked collagen) have unique viscoelastic properties, combining the characteristics of elastic solids and Newtonian fluids [20]. For this reason, specific viscoelastic parameters should be studied. Complex modulus (G*) reflects the contribution of both elastic and viscous components to the material's stiffness, the storage modulus (G') measures the stored energy and represents the elastic portion of the material, the loss modulus (G'') measures the energy dissipated as heat and the tan delta (δ) provides a measure of damping in the material, and it is the coefficient of loss and storage moduli (G'/G'').

To achieve biomimicking, the storage modulus and tan delta values

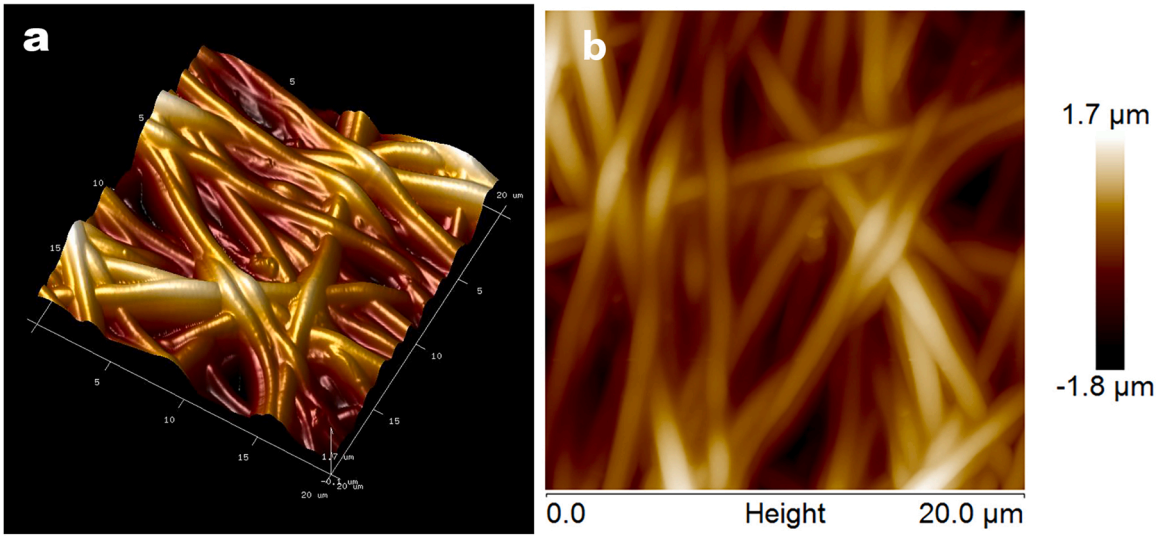

Fig. 1. AFM image of an electrospun nanostructured membrane surface manufactured by NanomyP® (Granada, Spain) using a novel polymer blend: $(MMA)_1$-co-$(HEMA)_1$/$(MA)_3$-co-$(HEA)_2$ doped with 5 % wt of SiO_2-NPs. Overlapped and randomly distributed nanofibers, mimicking collagen fibers, may be observed.

of the matrix should be similar to the calcified trabecular bone, which are around 15 GPa and 0,6; respectively [7,21].

2.3. Wettability

Several studies have used the Water Contact Angle (WCA) method, in order to stablish its hydrophobicity or hydrophilicity [22,23]. Normally, a high value of WCA indicates hydrophobicity, whereas a low value shows that a material is more hydrophilic. It is well known that improved surface hydrophilicity is necessary for cell adherence and growth [24]. Most synthetic biodegradable polymers are hydrophobic; thereby, extensive efforts have thus been devoted towards increasing the hydrophilicity of biomaterials. One convenient measure is to produce chemical modification of the membranes' surfaces introducing polarized groups such as hydroxyl, carboxyl, amino or sulfate groups on polymer surfaces using different techniques as may be plasma treatment [25].

2.4. Bioactivity

Aiming bone regeneration, the ability of a material to chelate calcium phosphate is of essential importance, since it would mean a step forward to obtain primary mineralization. Bioactivity and mineralization can be studied *in vitro*, following the International Standard ISO 23,317 [26], in which it is specified the method for detecting apatite formed on a surface of a material after immersion in simulated body fluid (SBF). SBF is a solution that mimics the blood serum in terms of ionic composition and pH [27]. This method is applicable for surfaces which are intended to be in direct contact with bone. After performing the previous mentioned procedure, the membranes could be analyzed with SEM and Elemental Diffraction Analysis (EDX) trying to look for calcium and phosphate deposits or X-Ray Diffraction Analysis (XRD) to directly detect crystals formation and its main components [8].

2.5. Biodegradation

This section may only be applied to the resorbable membranes, and it is intended to find the average time that the biomaterial would remain with structural integrity. It has been previously described that in order to achieve a predictable GBR process, the membrane should remain physically and mechanically intact for at least an average time of 4–6 weeks [28–31]. This time period may vary depending on individual patients' conditions that negatively influence bone repair rates such as age, systemic and metabolic conditions or big defect size. These factors should be taken into account when selecting a membrane for GBR, opting for a delayed resorption or even a non-resorbable membrane. The main assay employed is a hydrolytic degradation test, achieved by immersing the membrane in a Phosphate Buffered Solution (PBS) at different time points [28,32]. Currently, it is known that the membrane contamination by periodontal pathogens such as *Porphyromonas gingivalis* and *Treponema denticola* (which are able to produce collagenases) and/or membrane exposure to the oral cavity during or after surgery is sometimes unavoidable. It has been assessed that collagen membranes' degradation is up to 80 % faster, when they are immersed in bacterial collagenase if compared to PBS [28]. This may explain the unpredictable clinical results, which is sometimes, attained by resorbable membranes. Moreover, it has been described that pores larger than 100 μm appeared during the degradation processes of membranes [28], which will jeopardize the soft tissue cells barrier effect required for a successful GBR therapy. Knowing the importance of biomaterial stability and maintaining the space in bone regenerations, these results would provide really important information about the membrane that is being tested and the possible clinical situations in which it could potentially be used.

3. Cytocompatibility

Cytocompatibility is defined as the property of a material or substance of not been toxic or harmful to a cell. It is normally tested by the use of cell viability assays. Cell viability is the quantification of the number of live cells and is usually expressed as a percentage of a control material [33]. The two tests which are used the most are: Cell Counting Kit-8 (CCK-8, Dojindo, Japan) and Live/Dead staining. In most of the studies both tests are used together in order to contrast ones results with the other. It has been recently reported [34] that Live/Dead dyes may not be used as an exact quantitative measurement of cell death. Red cells, stained with propidium iodide, have commonly been identified as dead cells, whereas they really represent cells that are injured, dead or starving viable cells. Therefore, red cells percentages should be taken with caution [35]. This may be an explanation for the need to contrast the results by two different methods.

4. Antibacterial effect of the material

After the surgical technique of GBR, the regenerative outcome of the surgery is sometimes compromised by bacterial colonization and infection [36]. This may occur in a more frequent way when the membrane is exposed to the oral cavity due to soft tissue collapse. This situation could be overcome if the membrane shows some bactericidal capacity. This effect can be achieved by modifying the membrane's structure or by adding components able to exert this function. Some of the components that have been described are metal, ions like zinc, copper or silver [1,17,37], or antibiotics, like metronidazole or doxycycline [38–40]. In order to assay the membrane's antibacterial effect, the protocol described by Bueno et al. [39], perfectly meets the required tests. The membranes need to be exposed to a periodontal multispecies biofilm in anaerobic conditions to simulate, as much as possible, the real conditions to which the membrane would be subjected subsequent to bacterial colonization. After culturing the bacteria, quantitative Polymerase Chain Reaction (qPCR) and Live/Dead assay should be conducted to quantify the surviving bacteria and to analyze the dynamics of the biofilm in the presence of the membrane. SEM would also be useful to observe the primary interactions between the initial colonizers and the biomaterial.

5. Other specific assays on membranes to cells interactions

5.1. Testing osteoblasts interactions with membranes

The osteoblast is a complex cell which actively participates in bone metabolism. Its main duties include being responsible for bone formation and regeneration and for the regulation of osteoclast activity. It also possesses immunologic functions, that include: the synthesis of cytokines, expression of antigens implicated in antigen presentation, allogenic stimulation, and phagocytic [41]. Several cell models have been used in *in vitro* studies, including primary human osteoblast cells, primary mouse osteoblast cells, primary bovine osteoblast cells, MG-63, MC3T3-E1 and SaOs-2. Primary human osteoblasts and MG-63 are the ones used the most. Primary human osteoblasts, are obtained from bone tissue of donors, and are the most relevant for clinical studies, but need long isolation procedures, limited accessibility and the cell phenotypes are sensitive to donor-related factors. On the other hand, in the case of MG-63 osteoblast-like cells, there are no interspecies differences with primary human osteoblasts, have a shorter isolation time and there is unlimited accessibly [42]. However, the results need to be extrapolated with caution, taking into account that a tumor line may have an alternative pattern of differentiation from primary human osteoblasts [41]. For this reason, it may be advisable to use at least, two different cell models in order to correctly understand the efficacy of the membrane. Several tests are proposed:

5.1.1. Osteoblasts proliferation

proliferation is defined as the cellular growth rate or as the quantified value for the daughter cell population [33]. Regarding cells

proliferation, the ability of the cells to replicate is the parameter being tested. For this reason, and differing from viability assays, the time points in which the tests are performed are longer (*i.e,* 1, 3, 10, 15 and 21 days); whereas in viability assays it is difficult to find an established time point longer than 48 h. Considering this, it is vital to provide the cells with nutrients (fresh media) during the assay, in order to avoid the entrance into an early stationary phase.

There are several methods to investigate osteoblasts proliferation. The most widely used is the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, in which the tetrazolium salt is metabolized by active mitochondria to form insoluble formazan crystals, detected by measuring the absorbance using a microplate reader. It has to be kept in mind that a false return of cell number would be encountered if the biomaterial affected the mitochondrial activity and cells survived via glycolysis. This is one of the reasons why it is recommended that proliferation is studied by different tests, in order to validate the results. Another commonly used method is the Alamar Blue assay. It is also a test which measures the metabolic action of the cells by the mitochondrial activity, in this case by oxidation of resazurin dye by means of a REDOX reaction. An absorbance microplate reader is also needed [7]. In addition, the calcein viability assay can also be used. It is a simple and extremely sensitive quantitative assay to measure the cell viability of adherent and suspension cells. It can detect as low as 50 viable cells in less than 30 min. Calcein is a non-fluorescent, hydrophobic compound that easily penetrates intact and live cells. During the Calcein assay, hydrolysis of Calcein AM by intracellular esterases produces a hydrophilic, strongly fluorescent compound that is retained in the cell cytoplasm and can be measured at Ex/Em = 485/530 nm. The measured fluorescence intensity is proportional to the number of viable cells [43]. Another used assay for cytotoxicity and cell viability with adherent cell cultures is Crystal Violet. The Crystal Violet assay is based on staining cells that are attached to cell culture plates. It relies on the detachment of adherent cells from cell culture plates during cell death. During the assay, dead detached cells are washed away. The remaining attached live cells are stained with Crystal Violet. After a wash step, the Crystal Violet dye is solubilized and measured by absorbance at 595 nm. The amount of Crystal Violet staining in the assay is directly proportional to the cell biomass that is attached to the plate [44].

5.1.2. Osteoblasts differentiation

After testing cells proliferation, the differentiation potential of osteoblasts needs to be ascertained, since it is the stage in which they begin to play their role in matrix production and mineralization. Several methods to study osteoblasts differentiation can be found in literature:

a) Alkaline Phosphatase (ALP) activity. The differentiation and maturation of osteoblasts involves the synthesis of specific bone proteins which contribute to the synthesis of the extracellular matrix and its subsequent mineralization [41]. One of these mentioned proteins is ALP. ALP is one of the firsts functional proteins expressed in the process of calcification [45]. ALP activity is normally quantified using a colorimetric assay which determines early osteoblastic differentiation (*i.e.* 7 days of culturing) [46]. ALP activity is commonly expressed as a relative percentage, considering enzymatic activity of 100 % in a control group without biomaterial.

b) Sirius Red Staining. This colorimetric assay is used to quantitatively measure the amount of collagen (mainly types I and III) produced by osteoblasts. The results may provide a reliable imagen of osteoblasts proliferation, since 85–90 % of the organic extracellular matrix is composed of collagen. Sirius Red Staining, which is an anionic dye that binds to collagen, is diluted in saturated aqueous picric acid solution and added to the membrane. The cells are then washed with HCl to remove all the excess of dye and dissolved in a NaOH solution. Afterwards, the absorbance is measured at 550 nm [47].

c) Alizarin Red staining. Whilst ALP activity is able to measure mineralization indirectly, Alizarin red-S is the optimal assay to measure matrix mineralization in a direct way. This test is normally performed at different time points to evaluate the evolution of the mineralization nodules (*i.e.* 7, 15 and 21 days) [48]. At the different time points, Alizarin Red solution is added to the membrane and washed several times with deionized water in order to reduce non-specific staining. Calcium deposits present in the extracellular collagen matrix will be colored in red, revealing mineralization nodules [46]. These calcium deposits can be measured with a spectrophotometer after dissolving them with cetylpyridiniumchloride.

d) Quantitative Real Time Polymerase Chain Reaction (RT-qPCR). RT-qPCR is used to investigate the expression of genes encoding osteoblast differentiation markers. Before performing the RT-qPCR, the RNA needs to be extracted from the cells. The RNA obtained from the cells is measured by spectrophotometry. Then, the RNA must be transformed into complementary DNA (cDNA) by means of the reverse transcription, in order to avoid working with RNA, a molecule quite instable [49]. The chains of cDNA will then be amplified by PCR following the manufacturer's instructions of the commercialized kit used.

Primers need to be designed using NCBI-nucleotide library and Primer3-design in order to detect messenger RNA (mRNA) of the targeted genes. In Table 1 a list of the most useful primer sequences for the amplification of cDNA by real-time PCR is presented [50,51]. RT-qPCR can now be performed with the cDNA extracted from the cells and the designed primers. It has to be taken into account that each gene needs a specific annealing temperature, ranging from 60 to 65 °C [50]. Ubiquitin C (UBC), peptidylprolyl isomerase A (PPIA), and ribosomal protein S13 (RPS13) are commonly used as stable housekeeping genes in order to normalize final results [52,53].

e) Immunofluorescence staining. It has been used for a double purpose. Firstly, and more commonly exerted, to observe cytoskeleton organization. Cells are initially incubated with a Rhodamine-phalloidin dye; and afterwards, 4′,6-diamidino-2-phenylindole (DAPI) dye solution needs to be apply. These two dyes stain specifically actin filaments from the cytoskeleton and DNA from the nuclei of the cells, respectively [54, 55]. Analyzing the cells would give as an image of how well the cells spread over the biomaterial, the emission of filopodia and the shape they acquire when they differentiate. Tsai et al. [56], went a step further and, in addition to studying cytoskeleton and DNA, stained the membranes with osteoblast-specific marker proteins to study differentiation. They used osteocalcin (OCN) and bone sialoprotein (BSP) and primary antibody against osteoblast-specific marker protein OCN and BSP. This gave them the capacity of studying and quantifying the presence of these proteins under a Laser Scanning Confocal Microscope. BSP is a protein which is normally found in the bone matrix. It participates in the hydroxyapatite nucleation, so it has been proposed to be one of the initiators of mineralization of the extracellular matrix [57]. On the other hand, OCN is a specific protein produced by osteoblasts during the mineralization phase [58]. Therefore, BSP and OCN were used as markers of middle and mature stages of osteoblasts' differentiation, respectively [56].

f) Antigenic Phenotype. Osteoblasts maturation and differentiation may also be assessed by analyzing their antigenic phenotype, which is modified along the process of maturation of the cells and may be influenced by growth factors, cytokines, and hormones in the bone tissue, like CD54, CD80, CD86 and HLA-DR [59]. Osteoblasts should be exposed to the biomaterial that is being tested and after that, stained with anti-monoclonal antibodies (anti-Mabs), depending on the antibodies that need to be tested (*i.e*, to detect CD54, osteoblasts should be stained with anti-CD54 monoclonal antibody). After been incubated for approximately 30 min, aliquots are analyzed in a flow cytometer with diode laser at a wavelength of 488 nm to determine the percentage of fluorescent cells. Untreated cells need to be used as controls [46].

g) SEM and Energy Dispersive X-ray spectroscopy (EDX). Using SEM to detect cell differentiation may be useful. It has been described that osteoblasts' morphology is highly influenced by its differentiation stage. Spread morphology has been associated with the expression of differentiation markers and higher metabolic activity, whereas circularity has

Table 1
Primer sequences for the amplification of osteoblasts' cDNA by real-time PCR.

Gene	Sense Primer	Antisense Primer
TGF- β1	5´-TGAACCGGCCTTTCCTGCTTCTCATG-3´	5´-GCGGAAGTCAATGTACAGCTGCCGC-3´
TGF- β R1	5´-ACTGGCAGCTGTCATTGCTGGACCAG-3´	5´-CTGAGCCAGAACCTGACGTTGTCATATCA-3´
TGF- β R2	5´-GGCTCAACCACCAGGGCATCCAGAT-3´	5´-CTCCCCGAGAGCCTGTCCAGATGCT-3´
TGF- β R3	5´-ACCGTGATGGGCATTGCGTTTGCA-3´	5´-GTGCTCTGCGTGCTGCCGA TGCTGT-3´
RUNX-2	5´-TGGTTAATCTCCGCAGGTCAC-3´	5´-ACTGTGCTGAAGAGGCTGTTTG-3´
VEGF	5´-CCTTGCTGCTCTACCTCCAC-3´	5´-CACACAGGATGGCTTGAAGA-3´
OSX	5´-TGCCTAGAAGCCCTGAGAAA-3´	5´-TTTAACTTGGGGCCTTGAGA-3´
BMP-2	5´-TCGAAATTCCCCGTGACCAG-3´	5´-CCACTTCCACCACGAATCCA-3´
BMP-7	5´-CTGGTCTTTGTCTGCAGTGG-3´	5´-GTACCCCTCAACAAGGCTTC-3´
ALP	5´-CCAACGTGGCTAAGAATGTCATC-3´	5´-TGGGCATTGGTGTTGTACGTC-3´
COL-I	5´-AGAACTGGTACATCAGCAAG-3´	5´-GAGTTTACAGGAAGCAGACA-3´
OSC	5´-CCATGAGAGCCCTCACACTCC-3´	5´-GGTCAGCCAACTCGTCACAGTC-3´
OPG	5´-ATGCAACACAGCACAACATA-3´	5´-GTTGCCGTTTT A TCCTCTCT-3´
RANKL	5´-ATACCCTGATGAAAGGAGGA-3´	5´-GGGGCTCAATCTATATCTCG-3´
UBC	5´-TGGGATGCAAATCTTCGTGAAGACCCTGAC-3´	5´-ACCAAGTGCAGAGTGGACTCTTTCTGGATG-3´
PPIA	5´-CCATGGCAAATGCTGGACCCAACACAAATG-3´	5´-TCCTGAGCTACAGAAGGAATGATCTGGTGG-3´
RPS13	5´-GGTGTTGCACAAGTACGTTTTGTGACAGGC-3´	5´-TCATATTTCCAATTGGGAGGGAGGACTCGC-3´

been associated with lower DNA concentrations [60]. In addition, attachment with the neighboring cells by means of extensions or filopodia may indicate cells differentiation [61]. Sometimes, rounded structures can be observed on the surface of osteoblasts which may correspond with mineral deposits, fact that can be confirmed by EDX analysis [61] (Fig. 2).

5.2. Testing macrophages interactions with membranes

Macrophages are cells of the innate immunity that are found nearly in all tissues. They derive from circulant monocites, which in turn, have their origin in hematopoietic stem cells (HSCs). Its main functions include phagocytosis of invading microorganisms, amplifying the inflammatory reaction and recruiting additional immune cells [62]. However, and although all mechanism are still not completely known nor understood, it has been shown that macrophages determine bone regeneration [63]. Macrophages can polarize into a pro-inflammatory phenotype M1 or pro-regenerative M2 phenotype in a context-dependent manner. After any situation which involves bone destruction (*i.e*, pathological fractures, implant placement, etc.), the M1

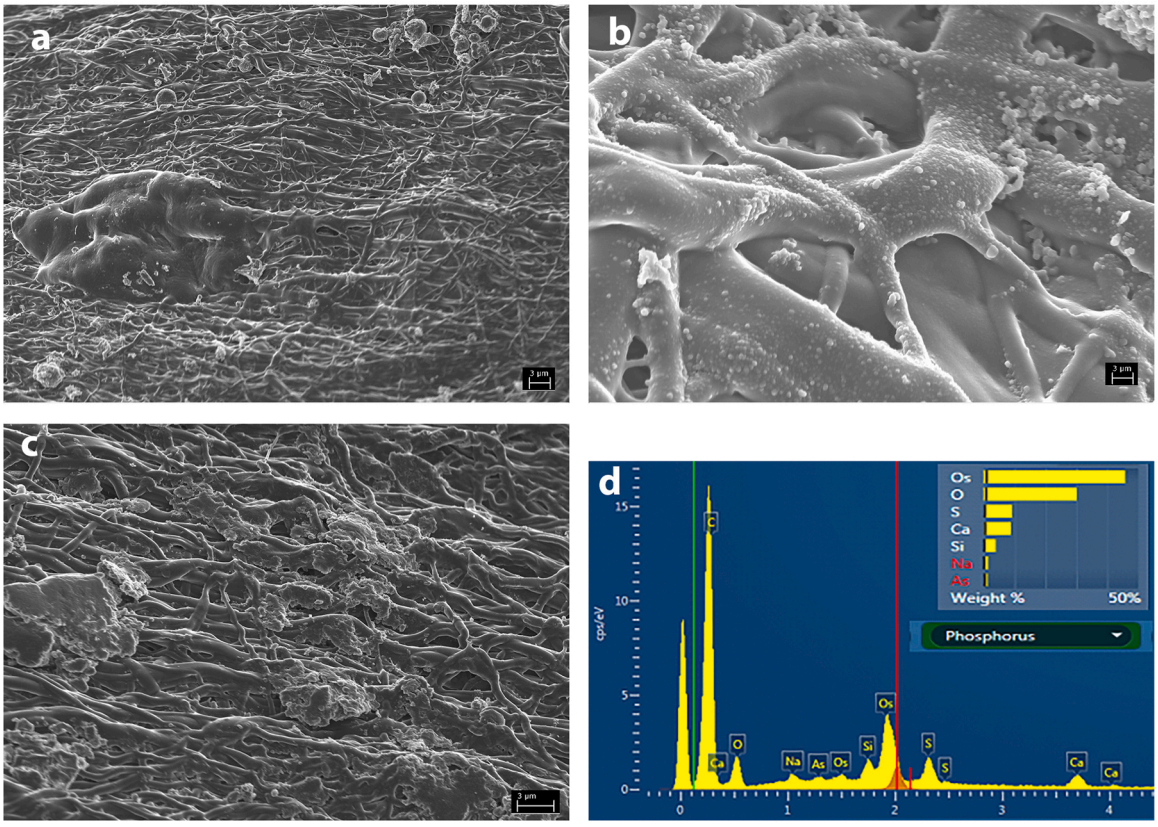

Fig. 2. Surface FESEM images of a non-resorbable polymeric electrospun membrane seeded with osteoblasts cells. a) A flat and elongated osteoblast cell is noticed on the membrane. Long osteoblasts' filapodia may be observed crossing over the membranes' surfaces. b) At higher magnification filapodia are intermingled with membranes fibers and covered by extracellular substance. c) Numerous filapodia are detected on the surface and are difficult to distinguish from nanofibres of the electrospun membrane, mineral deposits are also observed. **d)** Calcium and phosphorous were identified after EDX analysis. Phosphorous is in orange overlapped on the osmium spectrum peak. Silicon is also present at the EDX spectra.

phenotype is needed in order to begin the immune response by producing and releasing pro-inflammatory cytokines and chemokines (IL-1, IL-6, TNF-α, inter alia) that recruit other immune cells. A prolonged time in M1 phase, would lead to fibrous encapsulation and failure of bone regeneration or implant osseointegration [64]. In contrast, if a switch from M1 to M2 phenotype is achieved, it would result in bone regeneration and anti-inflammatory environment. It has been recently ascertained that nanostructured surfaces, hydrophilicity, several chemical radicals as hydroxyl or carboxyl groups or the presence of certain cations (Zn^{2+}, Ca^{2+}, Si^{2+}) at biomaterials may facilitate M2 macrophage polarization [65,66] (Fig. 3). Hence, biomaterials which are capable of controlling and modifying the M1/M2 polarization at tissue-biomaterial interaction locations, will be highly promising for bone regeneration strategies [67].

In addition to this, the role of macrophages in bone regeneration is crucial since it has been recently discovered that there is a cross-talk between macrophages and bone forming cells [63], participating in the process known as osteoimmunomodulation. Luo et al. [68], demonstrated, *in vitro,* that the inclusion of macrophages in an osteoblast culture enhanced osteoblasts differentiation and mineralization, measured by ALP, Alizarin Red Staining and RT-qPCR (through expression of the genes RUNX2, ALP, OCN and BMP2). They even showed that all these parameters where improved just by adding media derived from a 24 h cultured macrophage colony to their osteoblasts cultures [68]. It is speculated that this improvement in mineralization and differentiation could be, partially, due to the ability of the macrophages to diminish Reactive Oxygen Species (ROS) from the osteoblasts [68].

In the light of this information, it would be of utmost importance to investigate the behavior of macrophages when put in direct contact with the biomaterial in order to be able to design a membrane which enables their polarization into M2 phenotype. Studying the impact of macrophages on osteoblasts' activity would help to develop a material with osteoimmunomodulation ability. The analysis of this interaction osteoblast-macrophage-biomaterial could be best achieved by means of a co-culture of macrophages and osteoblasts on the studied membrane [68].

5.2.1. *Macrophages polarization testing*

Firstly, in order to obtain macrophages (M0), THP-1 cells need to be stimulated with the presence of phorbol-12-myristate-13-acetate (PMA) [67]. After this process, M0 macrophages can be cultured on the membranes to observe their polarization pattern when exposed to the biomaterial. After 24−48 h of culture there are different techniques that may be undertaken. RT-qPCR can be used in order to detect differences in the expressions of M1 and M2 markers. For M1 profile; IL-1, IL-6 and TNF-α are normally explored; whereas for M2 markers IL-10, Arg1, CD206 and TGF-β are the selected genes [51,67]. Recommended genes' sequences are presented in Table 2 [51,67]. Immunofluorescence staining can also be used to quantify the M1/M2 ratio. It can be done by

Table 2
Primer sequences for the amplification of macrophages' cDNA by real-time PCR.

Gene	Sense Primer	Antisense Primer
IL-1	5′-GGTTGAGTTTAAGCCAATCCA-3′	5′-TGCTGACCTAGGCTTGATGA-3′
IL-6	5′-GAAAGGAGACATGTAACAAGAGT-3′	5′-GATTTTCACCAGGCAAGTCT-3′
TNF-α	5′-CAGCCTCTTCTCCTTCCTGAT-3′	5′-GCCAGAGGGCTGATTAGAGA-3′
IL-10	5′-GAGGCTACGGCGCTGTCA-3′	5′-TCCACGGCCTTGCTCTTG-3′
Arg1	5′-ACGGAAGAATCAGCCTGGTG-3′	5′-GTCCACGTCTCTCAAGCCAA-3′
CD206	5′-GGGTTGCTATCACTCTCTATGC-3′	5′-TTTCTTGTCTGTTGCCGTAGTT-3′
TGF-β	5′-ACTACTACGCCAAGGAGGTCA-3′	5′-TGCTTGAACTTGTCATAGATTTCG-3′

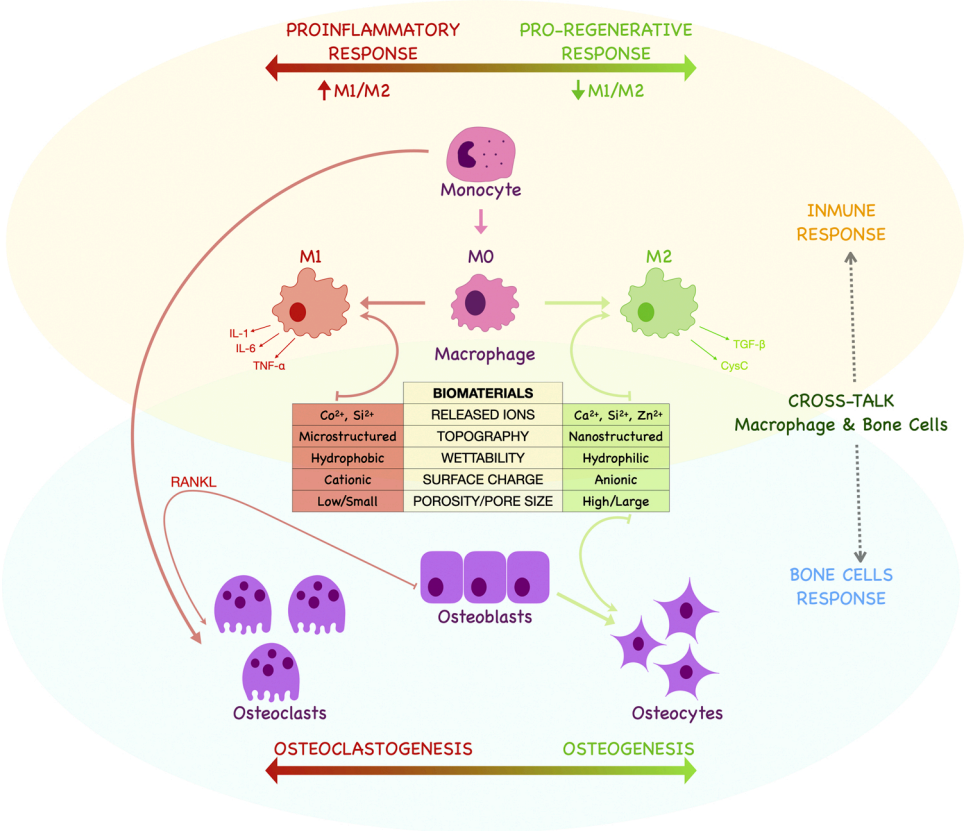

Fig. 3. Biomaterials properties modulate the immune response and the osteoblasts osteogenic potential.

staining the macrophages, in a first step, with specific primary antibodies for M1 and M2, which could be rabbit anti-iNOS and rabbit anti-CD163, respectively; and then a common secondary antibody Alexa Fluor 488-conjugated goat anti-rabbit. Before being analyzed with confocal fluorescence microscope, they should be washed with PBS and the macrophages' nuclei should be dyed with DAPI. The ratio of positive cells (in each case of M1 and M2) needs to be compared with the total cells using a software with an available tool for this purpose [67]. These tests would provide the researchers with enough information to be able to know which profile of macrophages is potentiated with the proposed biomaterial, favoring inflammatory or anti-inflammatory cellular reactions.

5.3. *Macrophages and osteoblasts co-cultures*

The second approach regarding macrophages is focused on investigating how the potential of the osteoblasts is modified, when they are cultured in presence of macrophages. A control group of monoculture of osteoblasts without macrophages is needed in order to compare the results. There are mainly three models of co-culture [68]: a) Conditioned media: after culturing macrophages for 24 h, the media is collected, centrifuged and the supernatant is added to a separate osteoblasts culture; b) indirect co-culture: macrophages are cultured on a specific hanging cells, which are inside the main osteoblasts culture, and c) direct co-culture: osteoblasts and macrophages are cultured together in the same flasks. The first type of co-culture is the one which differs the most from *in vivo* conditions but would be a good way to approach co-culture since the results are more easily interpreted. On the other hand, in direct co-culture, more factors can influence the results, but cell-to-cell contact is achieved leading to more clinically relevant results.

After the co-culture, osteoblasts proliferation and differentiation should be analyzed with some of the techniques described in previous sections, in order to encounter the effects that macrophages may exert on osteoblasts activity.

6. *In-vivo* bone regeneration analysis

The ultimate and most clinically relevant challenge before using a membrane in humans is the *in vivo* testing in an animal model. Animal experimentation should be carried out following the local directives. In Europe, experiments need to be developed following the US National Institute of Health (NIH for Care and Use of Laboratory Animals) and the European Directive 86/609/EEC guidelines concerning animals care and use for experimentation. They should also fulfill the European Directive 2010/63/EU about the animals' protection for scientific purposes and be in accordance with all local laws and regulations [66]. It should be taken into account that, for ethical reasons, the minimum number of animals should be utilized [69]. Therefore, the importance of previously select the correct experimental membrane prototype trough the above described tests is clear. For the present purpose, the most used animal model is the New Zealand calvaria rabbits [8,70–75]. In this model, several calvaria defects are surgically created and occluded with the tested material, leaving always a defect without treatment which would act as negative control. The main difference among studies is the diameter of the defect. The defects can be classified as critical size defects (CSD) or non-critical size defects. CSD were defined by Schmitz and Hollinger in 1986 [76] as "the smallest size intraosseous wound in a particular bone and species of animal that will not heal spontaneously during the lifetime of the animal". The size of the CSD defect varies depending on the animal that is chosen for the experiment. In New Zealand rabbits is considered as CSD when the diameter of the defect is above 7 mm [66,70]. After the stablished time, the animals are euthanized. The most common ways of analyzing the effect of the applied biomaterials are Micro-CT and different histological techniques. It should be noted that this animal model is not the only one described in the literature. When GBR need to be tested, especially in the case of studying GBR together with osseointegration of dental implants or periodontal regeneration, Beagle dogs [77–79] or Minipigs [80] models are normally used, completely mimicking membranes clinical applications.

It has to be noted that for a first approach of a biomaterial to an *in vivo* testing, the calvarial model might be the most predictable, since primary closing of the incision is always achieved, reducing the risk of infection and influences from saliva or bacterial biofilms prevailing in intraoral models [81]. In contrast, in Beagle dogs and minipigs models, the surgeries are performed on the jaw, increasing the clinical relevance of the results. However, in these animal models there are more factors that can negatively influence the regeneration. These intraoral animal models should be implemented at a second stage, in order to study the behavior of the membranes in a jaw model, when the innate osteogenic properties of the biomaterial have already been stablished.

Regardless of the employed animal model, after the selected healing periods and animals' euthanasia, the following techniques are the most employed to analyze bone regeneration around tested membranes:

6.1. *Micro-CT*

Micro Computer Tomography (Micro-CT) makes possible to analyze the defects' average bone density in the so-called Hounsfield Units (HU). It is interesting to divide the defects in spherical volumes of interest (VOIs) in a rosette arrangement, presenting a central VOI and peripheral VOIs in contact with bone edges (Fig. 4). Using this distribution, the analysis can be carried out from two different approaches, assessing the whole defect or just the central VOI. The central VOI is the most critical part of the defect since it is not in contact with the edges of the defect. Consequently, analyzing this area makes it easier to discriminate between different osteoinductive potential of the tested biomaterials [66].

6.2. *Histomorphometric analyses*

The first step should be to retrieve the blocks from the regenerated bone defect using an oscillating autopsy saw. The obtained bone specimens need to be fixed and dehydrated before they can be included in blocks of acrylic resin and prepared for ground sectioning [66].

Afterwards, the subsequent processing of the samples depends on the staining that is going to be carried out. The most frequent ones include Haematoxylin and eosin (HE) and Masson's trichrome staining [55,82,83] but it needs to be kept in mind that there are some more specific techniques to be performed, allowing the researcher to directly study several specific bone regeneration parameters:

a) Von Kossa (VK) silver nitrate can be applied in order to visualize the mineralized bone. From the images obtained with VK staining, the following structural indexes can be calculates and studied: Bone surface (BS), percentage of bone area [BS/total surface (TS)], bone perimeter (BPm), and bone thickness (BTh) [70].

b) Immunofluorescence staining can also be performed in order to detect M2 markers like CD206, pan-macrophage markers like F4/80, or osteoblasts differentiation markers like VEGF, BMP, RUNX2, etc. [84]. In order to be able to perform this assay, the samples should be embedded in paraffin [84].

c) Calcein may be located and quantified trough fluorescence and it may help researchers to detect the calcium deposited in the last 7 days, in order to differentiate osteoid (newly deposited bone matrix) from mineralized tissue [66].

7. Conclusions and trend to the future

The need of using a membrane in GBR in order to exclude soft tissue's cells for colonizing the bone defect is unquestionable and has been incredibly implemented by oral surgeons. However, it is recognized that the membrane able to compile all desired properties is not still in the

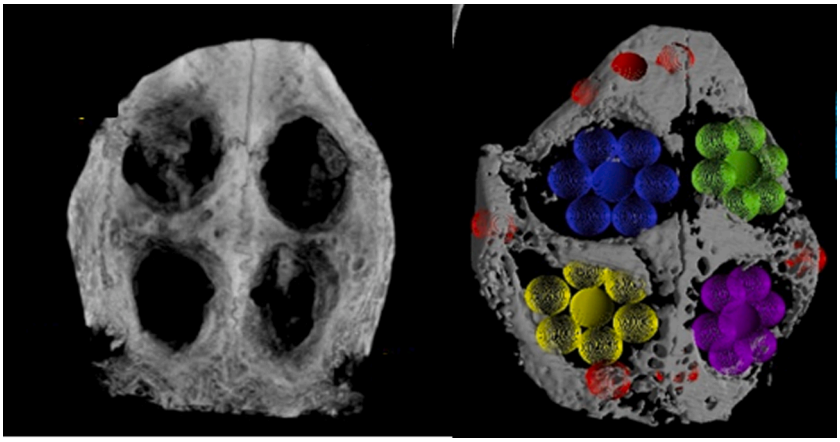

Fig. 4. Micro Computer Tomography (Micro-CT) image of four critical bone defects in a calvarial rabbit (left). At the right image, it is shown that each defect is divided in spherical volumes of interest (VOIs) in a rosette arrangement, with a central VOI and peripheral VOIs in contact with bone edges. VOIs may be analyzed for bone density together (assessing the whole defect) or just the central VOI in each defect.

market, and clinical outcomes are not always predictable. With the actual research and developed technology, the creation of an improved membrane according to the functional and biological requirements is possible. Following the structure of the present review, a potential active membrane should be assessed in its nanostructure. Physicochemical and nanomechanical properties, bioactivity and antibacterial, osteoblasts proliferation, differentiation and mineralization should also be determined. Finally, immunomodulation testing for macrophages recruitment and M2 phenotype promotion in osteoblasts co-culture needs to be achieved in order to completely analyze membranes-host tissue interactions. Membranes which successfully accomplish all these parameters may be prone to create a perfect bone-healing environment and successfully achieve GBR. Hence, appropriately controlled human studies in a clinical scenario are always necessary.

From scientific and clinical perspectives, the challenge of developing an active membrane has been potentiated by new scientific data regarding the mechanisms of GBR, tissue engineering and drug delivery approaches. All this boosts new research questions and may broaden future clinical opportunities for GBR [85].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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