Glycerylphytate crosslinker as a potential osteoinductor of chitosan-based systems for guided bone regeneration

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PII: S0144-8617(20)30443-4
DOI: https://doi.org/10.1016/j.carbpol.2020.116269
Reference: CARP 116269
To appear in: Carbohydrate Polymers

Received Date: 23 December 2019
Revised Date: 11 March 2020
Accepted Date: 7 April 2020

Please cite this article as: [ doi: https://doi.org/ ]
GLYCERYLPHYTATE CROSSLINKER AS A POTENTIAL OSTEOINDUCTOR OF CHITOSAN-BASED SYSTEMS FOR GUIDED BONE REGENERATION

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Highlights

- Glycerylphytate-crosslinked chitosan membranes were prepared for guided bone regeneration.
- Electrostatic interactions between glycerylphytate and chitosan provided highly stable systems.
- Swelling, crosslinking degree, and crosslinker release demonstrated a glycerylphytate-dependent content behavior.
- All the crosslinked-systems showed high biocompatibility.
- The incorporation of glycerylphytate to chitosan increased ALP activity and calcium deposition on hMSCs culture.

Abstract

Chitosan-based membranes are promising systems for guided bone regeneration. In this work, we used glycerylphytate as ionic crosslinker and osteinductor compound for the fabrication of chitosan membranes as supports for human mesenchymal stem cells. Three different glycerylphytate-crosslinked membranes were developed by changing the crosslinker concentration, from 2.5 to 10 wt-%, respect to chitosan. Physico-chemical characterization in terms of composition, morphology, and thermal behavior was further analyzed. Swelling degree, crosslinking density, and crosslinker release showed a glycerylphytate content-dependent behavior. Glycerylphytate suggested to improve osteointegration ability of chitosan surfaces by the formation of apatite-like aggregates after incubation in body simulated fluid. Stem cells cultured on the membranes increased their viability over time, and the incorporation of glycerylphytate improved osteogenic and osteoinductivity potential of chitosan by increasing calcium deposition and alkaline phosphatase (ALP) activity on cultured stem cells. These results demonstrated a potential application of glycerylphytate-crosslinked chitosan systems for promising bone tissue regeneration.

Keywords: glycerylphytate, chitosan, ionic crosslinking, osteogenesis, mesenchymal stem cells, guided bone regeneration
1. Introduction

Despite healthy bone possesses a powerful regenerative capacity, critical bone defects usually require from the application of reconstructive surgeries, which are mainly based on autologous bone grafts, allografts, and demineralized bone matrix. Bone regeneration is usually limited by the growth of connective tissue in the graft, which can evolve in a deficient osteointegration and loosening of the graft. Thus, scaffolds applied to bone repair processes should provide excellence osteoinductivity and osteoinduction properties (Greenwald et al., 2001; Stepniewski, Martynkiewicz, & Gosk, 2017). Currently, autographs are the most effective methodology regarding bone reconstruction process due to their high osteoconductivity and osteoinductivity. However, they exhibit severe limitations such as pain involved, possibility of infection, prolonged surgery time or the limited amount of tissue available (Stevens, Yang, Mohandas, Stucker, & Nguyen, 2008). Other bone substitutes like allographs does not possess the necessary osteoinduction activity which leads to a low osteointegration of the graph (Moore, Graves, & Bain, 2001). Finally, bone substitutes which are not based on natural bone are widely also applied. They can be classified in three generations: the first one is based on pure materials, the second one included the coating of the pure material with additional materials which prevent connective tissue formation, and the third one consists of materials closer to natural structure and characteristics of bone tissue. This last generation exhibits improved osteoconductivity and biodegradability properties in comparison to the other two (Amini, Laurencin, & Nukavarapu, 2012; Ribeiro et al., 2019; Stepniewski et al., 2017).

Guided bone regeneration (GBR) consists of the use of membranes with osteogenic and osteoinductive properties that can promote bone growth and avoid the migration of epithelial cells, which derives in ectopic osteogenesis (Luna-Domínguez et al., 2018). These membranes can contain inorganic phosphate components, osteogenic cations like Sr$^{2+}$, Zn$^{2+}$, or Mg$^{2+}$ (Lei et al., 2017; Luttrell, 1993; X. Wang, Li, Ito, & Sogo, 2011), or different bioactive compounds like growth factors (Yamano et al., 2014). Chitosan is a non-toxic natural polysaccharide that exhibits good biocompatibility properties (De La Mata et al., 2013; Khor & Lim, 2003) due to its similitude with the glycosaminoglycans present in the extracellular matrix (Jackson, Busch, & Cardin, 1991). Moreover, chitosan has demonstrated to exert an osteogenic effect similar to those provided by dexamethasone when it is used as an additive in mineralization medium (Amir, Suniarti, Utami, & Abbas, 2014). Taking together these beneficial properties, chitosan-based membranes have arisen as promising systems for GBR applications (Masoudi Rad et al., 2017; Tamburaci & Tihminlioglu, 2017; Q. Wang et al., 2017). Chitosan membranes are usually crosslinked with other compounds or mixed with synthetic components, like hydroxyapatite, to enhance its bone regeneration capacity (Anitha et al., 2014; Guzman et al., 2014; Lei et al., 2017; Luna-Domínguez et al., 2018). Herein, we propose the fabrication of chitosan membranes crosslinked with glycerylphytate (G$_3$Phy) to obtain systems with osteogenic properties and osteointegration potential. G$_3$Phy is a phytic acid derivative, which has previously demonstrated to improve cytocompatibility and osteogenic properties in comparison to its precursor (Mora-Boza et al., 2019). G$_3$Phy has
strong chelating activity against polyvalent cations such as Fe$^{2+}$ and Ca$^{2+}$. Its ability to form complexes with Fe$^{2+}$ confers it antioxidant properties and in vitro inhibition of lipid peroxidation of RAW267.4 macrophages (Mora-Boza et al., 2019). Thus, the use of G$_3$Phy is of great interest for the development of osteogenesis promoter materials. In particular, G$_3$Phy demonstrated excellent osteogenic activity against human mesenchymal stem cells (hMSCs), by the enhancement of ALP activity and osteogenic markers expression like ALPL and COLA1A (Mora-Boza et al., 2019). Chitosan-based materials that can support hMSCs proliferation and differentiation are of high interest because hMSCs possess the ability of self-renewal and differentiation towards osteogenic lineages when they are in presence of suitable osteoinductive compounds. Human MSCs can play a pivotal role in bone regeneration and repair in vivo to accelerating bone healing. (Lei et al., 2017; Tsai, Chen, Li, Lai, & Liu, 2012).

In this work, three different G$_3$Phy-crosslinked chitosan systems were fabricated by changing the G$_3$Phy weight concentration from 2.5 to 10% respect to chitosan. Physicochemical characterization of G$_3$Phy-crosslinked membranes was performed in terms of composition, morphology and thermal properties. In vitro behavior was investigated in terms of swelling, crosslinking density and crosslinker release. Moreover, in vitro deposition of apatite-like aggregates on G$_3$Phy-crosslinked chitosan surfaces was studied to evaluate the osteointegration potential of our membranes. Finally, in vitro biological properties regarding cell adhesion, viability, and osteogenic properties of G$_3$Phy-crosslinked chitosan surfaces were assessed on hMSCs. Osteoblastic differentiation of hMSCs seeded on the top of our systems was evaluated by ALP activity and alizarin red assay in absence of osteoinductor compounds. We envision that our systems based on G$_3$Phy and chitosan will have promising applications as osteogenic membranes for GBR.

2. Experimental section

2.1. Synthesis of G$_3$Phy

G$_3$Phy was synthesized as described by Mora-Boza et al. (Mora-Boza et al., 2019). Briefly, phytic acid sodium salt hydrate (Sigma-Aldrich) and glycerol (G, Sigma-Aldrich) were mixed in a molar ratio of 1:7, respectively, and heated at 120 ºC for 12 h to allow the condensation reaction under mechanical stirring. The reaction product was dissolved in water, precipitated twice in 2-propanol, dried under reduced pressure to remove solvent traces, and lyophilized.

2.2. Preparation of G$_3$Phy-crosslinked chitosan membranes

Chitosan of medical grade and endotoxin free (<100 EU/g) with a degree of deacetylation of 90% and M$_w$ = 300 kDa was purchased from Altakitin (São Julião do Tojal, Portugal) and was dissolved (2 wt-%) in acetic acid (1 % v/v) solution. 10 mL of chitosan solution were placed into a glass mold and heated in a humid chamber at 37 ºC until complete evaporation of the solvent. The obtained chitosan membranes were neutralized with NaOH solution (1 N) and washed with double distilled H$_2$O (ddH$_2$O) until neutral pH.
G₃Phy-crosslinked chitosan membranes also named as G₃Phy/chitosan were obtained by immersion of the previous membranes in aqueous solution of G₃Phy at different concentrations (2.5, 5 and 10 wt-% with respect to chitosan) and kept overnight at 37 °C. Nomenclature and composition of chitosan membranes crosslinked with G₃Phy are reported in Table 1.

Table 1. Nomenclature and composition of chitosan membranes synthetized in this work.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>[G₃Phy] (wt-% respect to chitosan)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ch</td>
<td>0</td>
</tr>
<tr>
<td>Ch/G₃Phy_2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Ch/G₃Phy_5</td>
<td>5</td>
</tr>
<tr>
<td>Ch/G₃Phy_10</td>
<td>10</td>
</tr>
</tbody>
</table>

2.3. Physicochemical characterization of G₃Phy-crosslinked chitosan membranes.

Crosslinking reaction was verified by Fourier Transform Infrared Spectroscopy (FTIR) using the technique of attenuated total internal reflectance (ATR-FTIR, Spectrum-One Spectrometer, Perkin-Elmer).

Elemental composition of the different membranes was analyzed by Energy Dispersive X-rays (EDX, Bruker XFlash model with detector 5030) and Induction Coupled Plasma Optical Emission spectroscopies (ICP-OES, 4300 DV Perkin-Elmer spectrophotometer with a Gemcone nebulizer). For ICP-OES analysis, membrane pieces of 12 mm (≈ 100 mg) were dissolved in 65 % v/v HNO₃ solution at 65 °C. Then, the samples were diluted 1:10 with ddH₂O. The G₃Phy amount incorporated in the membranes was calculated taking into consideration the phosphorus (P) content. The measurements were conducted in triplicate for each sample and the data obtained were expressed as mean values ± standard deviations (SD).

Surface morphology characterization was studied using scanning electron microscopy (SEM, HITACHI SU8000). Samples were platinum sputtered prior to analysis using a sputter coater Polaron SC7640 (Quorum Technologies).

Thermal degradation of the membranes was analyzed by thermogravimetric analysis (TGA) under nitrogen atmosphere using a thermogravimetric analyzer TGA Q500 (TA instruments). A heating rate of 10 °C/min from 40 to 800 °C was applied for the analysis.

2.4. Swelling degree and crosslinking density of G₃Phy-crosslinked chitosan membranes

G₃Phy-crosslinked chitosan membranes were cut in round pieces of 12 mm (≈ 100 mg) and were incubated in Phosphate-Buffered Saline (PBS, Sigma-Aldrich) solution (pH 7.4) at 37 °C. The swelling degree of the different membranes over time was calculated gravimetrically. At each time point, the excess of PBS was carefully removed with an
absorbent paper before weighing. Swelling degree percentage was calculated using equation 1:

$$Swelling\% = \frac{W_t - W_0}{W_0} \times 100$$ (1)

where \( W_0 \) is the initial weight of the dried sample and \( W_t \) is the mass at the specific time of incubation. The measurements were conducted in triplicate for each sample and the data obtained were expressed as mean values ± SD.

Crosslinking density of the hydrogel was calculated following the Flory-Rehner equation (Khan & Ranjha, 2014; Peppas, Huang, Torres-Lugo, Ward, & Zhang, 2000; Ranjha, Ayub, Naseem, & Ansari, 2010; Reyes-Ortega et al., 2015):

$$M_c = \frac{V_p d_p [(V_p)^{1/3} - V_p/2]}{\ln(1 - V_p) + V_p + \chi(V_p)^2}$$ (2)

where \( M_c \) is the average molecular weight between crosslinks and \( 1/M_c \) is considered as the crosslinking density \( \rho_x \). \( \chi \) represents the polymer solvent interaction parameter and can be calculated by Flory-Huggins theory (Khan & Ranjha, 2014; Ranjha et al., 2010):

$$\chi = \frac{\ln(1 - V_p) + V_p}{V_p^2}$$ (3)

where \( V_p \) is the volume fraction of polymer in equilibrium state, calculated as follow:

$$V_p = \left[ 1 + \frac{d_p}{d_s} (Q - 1) \right]^{-1}$$ (4)

where \( d_p \) and \( d_s \) are the densities of the polymer (0.57±0.03 g/cm\(^3\), calculated with a Sartorius balance ME Series and a density Kit YDK-01) and the solvent (1 g/cm\(^3\)). \( V_s \) is the molar volume of solvent \( (V_s = 18.1 \text{ mL/mol}) \). \( Q \) refers to swelling ratio and it is defined as:

$$Q = \frac{W_f}{W_0}$$ (5)

where \( W_0 \) is the initial weight of the dried sample and \( W_f \) is the mass when swelling equilibrium is reached.

2.5. Rheological study of G3Phy-crosslinked membranes.

Rheological measurements were determined using an advanced rheometer from TA instruments, model AR-G2, equipped with a Peltier and a solvent trap. The last one allows leading the measurement in a water-saturated atmosphere by avoiding water evaporation from the membrane. Samples were previously stabilized by their immersion in 7.4 PBS for 24 hours at 37 °C. All tests were carried out using a 25 mm diameter steel sand blasted parallel plate. Frequency sweeping tests of membranes were conducted with a frequency scanning from 0.01 to 40 Hz at 0.1 % strain and 37 °C. Three replicates of each sample were evaluated.

2.6. Release of G3Phy from crosslinked-chitosan films
To study G3Phy release, round crosslinked-chitosan membranes pieces (12 mm, ≈ 100 mg) were incubated in 5 mL of 0.1 M Tris hydrochloride (Tris-HCl, pH 7.5, Mol. Biol., Fisher BioReagents) buffer solution at 37 °C. At different incubation times, the medium was taken and replaced with fresh one. The P amount of the medium was analyzed by ICP-OES.

2.7. In vitro biomimetic apatite deposition assay

The crosslinked membrane Ch/G3Phy_10 was used for biomineralization studies using simulated body fluid (SBF) at 1.5 concentration (1.5SBF). Three replicates of the sample were placed into containers with 100 mL of 1.5SBF solution, and incubated at 37 °C for 4, 7, and 14 days. The 1.5SBF solution was previously prepared according to the procedure previously described in the literature (Oliveira, Andrade, Parreira, Jacobovitz, & Pandolfelli, 2015). Membrane surfaces were dried at 37 °C and analyzed by SEM and EDX.

2.8. In vitro biological study

2.8.1. Cell Culture

The biological behavior of G3Phy-crosslinked chitosan membranes was assessed on human Mesenchymal Stem Cells–Bone Marrows (hMSCs, Innoprot). Cells were cultured in Mesenchymal Stem Cell Medium Kit (Innoprot) at 37 °C and 5 % CO₂. Cells were used from 4-8 passages for all the experiments. For subsequent experiments, hMSCs were cultured in Low Glucose-Dulbecco’s Modified Eagle Medium (LG-DMEM), supplemented with 20% fetal bovine serum, 200 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin (complete LG-DMEM).

G3Phy-crosslinked chitosan membranes were sterilized by immersion in 70 % v/v ethanol solution for 2 hours and washed with sterile culture medium to remove ethanol traces.

2.8.2. Cytotoxicity analysis.

Cytotoxicity was evaluated by Alamar Blue (Invitrogen) assay (Mosmann, 1983). The films were immersed in 5 mL of DMEM at 37 °C. The medium was removed after 1, 2, 7, and 14 days of incubation, and replaced with 5 mL of fresh medium. The toxicity of these extracts was evaluated by seeding hMSCs at a density of 9 × 10⁴ cells/mL in LG-DMEM in 96 well-plates. After 24 hours, the medium was replaced with the corresponding extracts and the cells were incubated for 24 hours. Human MSCs cultured in LG-DMEM without any membrane extracts were used as control for comparison. Cell viability was analyzed by adding Alamar Blue solution (10 % v/v) to LG-DMEM without phenol red to cultured cells and incubating them at 37 °C for 4 hours. Media were collected and, after laser excitation at 590 nm, emitted fluorescence at 530 nm was quantified using a plate reader (Biotek Synergy HT spectrophotometer). Cell viability (%) was calculated following the equation 5:

\[
\text{Cell viability} (\%) = 100 \times \frac{\text{OD}_S - \text{OD}_B}{\text{OD}_C - \text{OD}_B}
\]

(5)
where OD$_S$, OD$_B$, and OD$_C$ are the emitted fluorescence at 530 nm for the sample (S), blank (B, LG-DMEM without cells), and control (C), respectively. The experiments were conducted in triplicate for each sample and the data obtained were expressed as mean values ± SD.

2.8.3. Cell adhesion and proliferation assay

Alamar Blue assay was used to analyze cellular adhesion and proliferation hMSCs seeded on the films. Human MSCs were seeded on the films at a density of 10$^4$ cells/cm$^2$ in 24 well-plates. Human MSCs were also seeded at the same concentration in Thermanox™ coverslips (TMX, ThermoFisher Scientific), which were used as controls. Cells were cultured in complete LG-DMEM. Cell viability was analyzed after 2, 7, and 14 days of incubation by adding Alamar Blue solution (10 % v/v) to LG-DMEM without phenol red and incubating the cells at 37 ºC for 4 h. Medium was collected and, after laser excitation at 530-560 nm, emitted fluorescence at 590 nm was quantified using a Biotek Synergy HT plate reader. Cell viability percentages were calculated following the equation 5. The experiments were conducted in triplicate for each sample and the data obtained were expressed as mean values ± SD.

The cells adhered to the membrane surfaces were stained using Calcein AM (Sigma Aldrich) at different incubation times. Calcein was added in a concentration 1:1000 to the culture medium and incubated for 10 min. Fluorescent cells were visualized under fluorescence microscopy (Nikon Eclipse Microscopy model TE2000 equipped with a fluorescence light source CoolLED model CoolLED’s pE-300lite).

2.8.4. ALP activity.

For these experiments, hMSCs were seeded in the same conditions as previous assay. ALP activity was evaluated by measuring p-nitrophenol absorption at 405 nm after 14 days culture. ALP activity per cell was normalized by DNA quantification. Thus, ALP/DNA ratios indicate the amount of ALP activity per cell. Total DNA amount was measured using a PicoGreen dsDNA quantitation kit and following the manufacturer instructions (Molecular Probes, 231 P-7589). The experiments were conducted in triplicate for each sample and the data obtained were expressed as mean values ± SD.

2.8.5. Alizarin red assay.

Calcium deposition on the membrane surfaces was evaluated by Alizarin red assay after 7 and 14 days of incubation. For this experiment, hMSCs were seeded on the films at a density of 10$^4$ cells/cm$^2$ in complete LG-DMEM. Cells were fixed with 70% v/v ethanol at 4 ºC for 1 h. Then, the films were rinsed twice with ddH$_2$O, and subsequently stained with alizarin red staining solution (pH = 4.2, 40 mM) for 30 min at room temperature. Then, alizarin red solution was removed, and the membranes were washed five times with ddH$_2$O. Finally, alizarin red dye was extracted from the cell monolayer by incubating the membranes in 1 mL cetylpyridinium chloride (CPC, Sigma-Aldrich) buffer (10 wt%, 10 mM Na$_2$PO$_4$) for 15 min (Gregory, Gunn, Peister, & Prockop, 2004). Absorbance values were read at 550 nm on a UV Biotek Synergy HT detector. The dye concentration
in the membranes was calculated by a calibration curve of alizarin red solution staining in the same CPC solution previously used for dye extraction. The experiments were conducted in triplicate for each sample and the data obtained were expressed as mean values ± SD.

3. Results and discussion

The synthesis of \( G_3 \text{Phy} \)-crosslinked chitosan membranes resulted in stable systems at physiological conditions. According to the molecular structures of chitosan and \( G_3 \text{Phy} \) (Figure 1a), the main interactions that take place during the crosslinking process are the electrostatics attractions between protonated amino groups of chitosan and the phosphate groups present in \( G_3 \text{Phy} \). In addition, weak interactions consist of hydrogen bonds between hydroxyl groups of \( G_3 \text{Phy} \) and hydroxyl/amino groups of chitosan, hydrogen bonds between intermolecular hydroxyl groups of chitosan, and hydrogen bonds between hydroxyl groups of chitosan and water molecules (Figure 1b). SEM micrographs of uncrosslinked chitosan and \( G_3 \text{Phy} \)-crosslinked membranes are illustrated in Figure 1c. All images showed smooth and uniform surfaces without signs of phase segregation.

3.1 Physicochemical characterization of \( G_3 \text{Phy} \)-crosslinked chitosan membranes.

\( G_3 \text{Phy} \)-crosslinked chitosan membranes were subjected to ATR-FTIR spectroscopy to observe the differences in bond structure due to \( G_3 \text{Phy} \) incorporation into the polymer matrix. The infrared spectra of chitosan and \( G_3 \text{Phy} \)/chitosan membranes are presented in Figure 1d. The main characteristic peaks of chitosan in uncrosslinked membrane spectra were: peaks at 2867 and 2925 cm\(^{-1}\) corresponded to \( \text{CH}_2 \) stretching; 1647-1587 cm\(^{-1}\) peaks indicated the presence of amide I and II respectively; and the band at 1378 cm\(^{-1}\) was assigned to \( \text{N-CH}_3 \) stretching. Finally, typical signals of saccharide structures appeared between 1160 and 900 cm\(^{-1}\) (Tamburaci & Tihminioglu, 2017; Yao, Peng, Goosen, Min, & He, 1993). Bands between 3600 and 3000 cm\(^{-1}\), which are typically assigned to associated \( \nu \text{ O-H and N-H} \), (Tamburaci & Tihminioglu, 2017) became narrower when \( G_3 \text{Phy} \) amount increased from 2.5 to 10 %. After \( G_3 \text{Phy} \) crosslinking process, analysis of ATR-FTIR spectra of these samples revealed other differences compared to the chitosan membrane, mainly the appearance of a new peak at 2972 cm\(^{-1}\), whose intensity increased proportionally to the incorporated amount of \( G_3 \text{Phy} \). This signal corresponded to the asymmetric and symmetric stretching vibrations of C-H bonds of the inositol rings of the crosslinker (Mora-Boza et al., 2019). Another feature that showed the successful \( G_3 \text{Phy} \) crosslinking of chitosan membranes was the displacement of the band at 1587 cm\(^{-1}\) to 1582 cm\(^{-1}\), which indicated the presence of \( \text{NH}_3^+ \) groups due to the ionic interaction between the amino groups of chitosan and phosphate groups of \( G_3 \text{Phy} \). Finally, changes in the intensities and displacements of different signals in the region of the saccharide structure (1160 to 900 cm\(^{-1}\)) confirmed the reorganization of polymeric matrix due to the ionic crosslinking. Our ATR-FTIR results were consistent with those previously reported for other phytic acid-crosslinked chitosan systems (Ravichandran et al., 2013; Shu & Zhu, 2002a, 2002b).
Figure 1. a) Molecular structure of chitosan and G₃Phy; b) Schematic illustration of interactions that take place between chitosan and G₃Phy: (1) electrostatic and (2) hydrogen bond interactions; c) SEM micrographs of uncrosslinked and G₃Phy-crosslinked chitosan membranes; d) ATR-FTIR spectra of uncrosslinked and G₃Phy-crosslinked chitosan membranes.

Elemental composition of G₃Phy-crosslinked chitosan membranes was evaluated by EDX and ICP-spectroscopies (Figure 2). EDX spectra of Ch/G₃Phy membranes (Figure 2a) showed the peaks of the main elements C, O, and N at their characteristic energy levels of 0.27, 0.52, and 0.39 keV, respectively. In addition, a peak at the energy level of P (2.01 keV), coming from the G₃Phy polyanions, was also observed and confirmed the ionic crosslinking reaction. The amounts of P incorporated to the membranes after ionic crosslinking process were measured by ICP-OES and are also displayed in Figure 2c.
Figure 2. a) EDX spectra of a representative sample of each membrane composition; b) Linear regression adjustment curve for percentage of phosphorus incorporated in the membrane (determined by ICP-OES) over percentage of initial amount of $G_3\text{Phy}$ used during crosslinking reaction; c) EDX results and $P$ content measured by ICP-OES for the different $G_3\text{Phy}$-crosslinked membranes. $P_{\text{memb}}$: Amount of phosphorous found in the membranes. $P_{\text{theor}}$: Theoretical amount of phosphorous should be found in the membranes.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>EDX</th>
<th>ICP-OES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C/P</td>
<td>N/P</td>
</tr>
<tr>
<td>Ch/G$<em>3$Phy$</em>{2.5}$</td>
<td>361.5</td>
<td>78.5</td>
</tr>
<tr>
<td>Ch/G$<em>3$Phy$</em>{5}$</td>
<td>126.7</td>
<td>30.0</td>
</tr>
<tr>
<td>Ch/G$<em>3$Phy$</em>{10}$</td>
<td>104.8</td>
<td>25.8</td>
</tr>
</tbody>
</table>

Figures 2b and 2c illustrate that the concentration of $P$ in the membranes increased with the amount of initial $G_3\text{Phy}$, showing a linear regression adjustment with a $R = 0.9998$ (Figure 2b). In addition, the ratio between the final incorporated amount of $P$ in the membranes ($P_{\text{memb}}$) and the initial amount of $P$ used in crosslinking reaction ($P_{\text{theor}}$) was constant for all the systems, independently to the initial $G_3\text{Phy}$ concentration. This result indicated that an average of $27 ± 0.01$ % of the total used $G_3\text{Phy}$ was involved in the ionic interactions with amino groups of chitosan.
Taking EDX and ICP-OES results altogether, Figure 2c shows that both C/P and N/P ratios notably decreased as the content of G₃Phy increased in the membrane, demonstrating incorporation of P in the respective polymeric matrixes. These results confirmed the ionic crosslinking of chitosan membranes with G₃Phy at all the studied concentrations.

Thermal behavior of G₃Phy/chitosan membranes was studied by thermogravimetric analysis. Thermal degradation profiles (TGA and DTGA) and representative thermal parameters of all the studied films are represented in Table 2 and Figure S1 respectively.

**Table 2. Maximum temperature values (DTGA curves), weight loss percentages (TGA curves) of the two main stages of thermal degradation and residue percentage values at 800 °C for all the crosslinked systems.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>First Stage</th>
<th>Second Stage</th>
<th>Remaining weight at 800 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T&lt;sub&gt;max&lt;/sub&gt; (°C)</td>
<td>Weight loss (%)</td>
<td>T&lt;sub&gt;max&lt;/sub&gt; (°C)</td>
</tr>
<tr>
<td>Ch</td>
<td>127.2</td>
<td>8.7</td>
<td>302.0</td>
</tr>
<tr>
<td>Ch/G₃Phy_2.5</td>
<td>147.3</td>
<td>8.5</td>
<td>295.6</td>
</tr>
<tr>
<td>Ch/G₃Phy_5</td>
<td>142.5</td>
<td>8.1</td>
<td>297.6</td>
</tr>
<tr>
<td>Ch/G₃Phy_10</td>
<td>128.4</td>
<td>8.0</td>
<td>290.4</td>
</tr>
</tbody>
</table>

Thermograms of uncrosslinked and G₃Phy-crosslinked membranes exhibited a similar thermogravimetric trend since thermal degradation occurred in two main stages in all the systems (Figure S1). Initial weight loss started at 60 °C in all the samples and corresponded to vaporization of absorbed water. Considering the weight loss percentage values of the first degradation stage for chitosan and G₃Phy/chitosan systems, the water holding capacity differed in somewhat between the films. These results indicated a different water-polymer interaction as a function of the incorporated G₃Phy (Neto et al., 2005), indicating that the membranes with higher concentration of G₃Phy crosslinker contained less water in their structure. The capacity of chitosan to retain water in its structure is based on the interaction of water with the hydroxyl and amino groups (Rueda, Secall, & Bayer, 1999). G₃Phy interacts ionically with these groups which can limit the water absorption capacity of chitosan from the surrounding medium. This behavior in water-polymer interaction will play a key role in other physicochemical properties, such as swelling, degradation or crosslinker release.

The second stage (between 302 and 290 °C) corresponded to the thermal degradation of the polymer structure and indicated the thermal stability of the system since it is associated to chitosan pyranose ring degradation (Pawlak & Mucha, 2003). Similar values for chitosan depolymerization temperature have been reported by previous studies (Nieto, Peniche-Covas, & Padro’n, 1991; Tamburaci & Tihminlioglu, 2017). In our systems, we observed that thermal stability of chitosan decreased as G₃Phy concentration increased. At low crosslinker amounts, the formation of intra-crosslinking reactions between polysaccharide chains that interfered with the formation of hydrogen
bonds between structures, is favored reducing the thermal stability of the systems (Neto et al., 2005). Finally, residue percentages (remaining weight % at 800 ºC) increased from 35 %, for uncrosslinked chitosan, up to 40 %, for Ch/G3Phy_10 sample, which confirmed the incorporation of G3Phy in the membranes due to the presence of phosphate moieties that remained without degradation at high temperature.

3.2 Swelling degree and crosslinking density.

Water adsorption capacity of a biomaterial is an essential feature to be studied because it will determine its ability to interact to body fluids. Consequently, swelling properties and crosslinking density due to the G3Phy incorporation in the chitosan membranes will be decisive to discern their biological properties. Swelling degree of uncrosslinked and G3Phy-crosslinked membranes were studied in PBS at 37 ºC (Figure 3).

![Swelling degree (%) profiles of the different chitosan-based membranes in PBS (pH 7.4) at 37 ºC.](image)

Swelling results showed that water-uptake equilibrium was reached after 8 h of incubation for all the systems. However, swelling degree depended on G3Phy content (147% for Ch; 139% for G3Phy_2.5; 128% for G3Phy_5 and 93% for G3Phy_10). These results demonstrated that increasing G3Phy contents leaded to higher crosslinking ratio and lower swelling degrees. As it can be observed in Figure 3, swelling degree of Ch/G3Phy_10 sample was the lowest in comparison to the rest of samples. For membranes crosslinked with 2.5 and 5 % of G3Phy, swelling degrees were closer to those found for uncrosslinked chitosan film. These results correlated with thermal degradation analysis which showed that the membranes with increasing G3Phy content exhibited less water content in their structures. Then, it can be assumed that higher
G₃Phy concentrations led to reduction of chitosan amino groups to interact with water molecules, and hence, the system exhibited less water adsorption capacity. Although swelling properties are important for nutrient diffusion in the scaffolds, high swelling degrees can compromise scaffold integrity. In our systems, swelling degree was reduced by half with respect to chitosan for the membrane with higher content of G₃Phy. Recent studies claim that high initial swelling degrees are desirable for ensuring a suitable supply of nutrients to the whole scaffold, but moderate swelling degrees at equilibrium can enhance long-term mechanical stability of the membranes at physiological conditions, and even cellular adhesion and viability (Francolini et al., 2019; Unnithan, Park, & Kim, 2016).

Crosslinking densities for chitosan-based systems were calculated by applying the Flory-Rehner theory and using equations (2) to (5) described in subsection 2.4 taking the Q values at equilibrium state that are displayed in Table 3.

**Table 3.** Values of swelling ratio (Q), average molecular weight between nodes (Mₑ), crosslinking density (ρₓ), and polymer solvent interaction (χ), of chitosan-based membranes.

<table>
<thead>
<tr>
<th></th>
<th>Q</th>
<th>Mₑ (g/mol)</th>
<th>ρₓ (g/cm³)</th>
<th>χ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ch</td>
<td>2.448 ± 0.008</td>
<td>11.416 ± 0.073</td>
<td>0.088 ± 0.001</td>
<td>0.819 ± 0.001</td>
</tr>
<tr>
<td>Ch/G₃Phy_2.5</td>
<td>2.402 ± 0.065</td>
<td>10.977 ± 0.613</td>
<td>0.091 ± 0.005</td>
<td>0.828 ± 0.012</td>
</tr>
<tr>
<td>Ch/G₃Phy_5</td>
<td>2.343 ± 0.075</td>
<td>10.425 ± 0.702</td>
<td>0.096 ± 0.006</td>
<td>0.839 ± 0.015</td>
</tr>
<tr>
<td>Ch/G₃Phy_10</td>
<td>1.965 ± 0.204</td>
<td>7.145 ± 1.635</td>
<td>0.140 ± 0.034</td>
<td>0.939 ± 0.075</td>
</tr>
</tbody>
</table>

As it was expected from swelling study, molecular weight between nodes (Mₑ) decreased as crosslinker concentration increased whereas crosslinking density (ρₓ) increased as G₃Phy content increased in the membranes. These results illustrated the hydrophilicity of the polymer systems in the sense that higher values of Mₑ resulted in higher swelling ratios in the films. Finally, solvent interaction parameters are related to the interaction between polymer and solvent. It is reported that high values of χ are indicative of weak interactions between polymers and solvent (Kali, Vavra, László, & Iván, 2013). In our systems, high concentrations of G₃Phy resulted in less hydrophilic films due to the lower film-solvent interaction, as it was observed in swelling analysis.

Finally, mechanical properties of the Ch/G₃Phy_10 membranes were analyzed by rheology (Figure S2). Storage and loss moduli of uncrosslinked and G₃Phy-crosslinked samples exhibited a plateau in the studied frequency range, which indicated the stability of the polymeric networks. This plateau also evinced a solid-like behavior of the membranes, since storage moduli was independent on the applied frequency (Oatway, Vasanthan, & Helm, 2001). Finally, crosslinked membranes showed lower storage moduli values in comparison to uncrosslinked chitosan, which indicated that G₃Phy crosslinker provided softer surfaces.

### 3.3 G₃Phy release from chitosan-based membranes
G₃Phy release from the G₃Phy-crosslinked membranes was analyzed by following P content of aliquots obtained by ICP-OES taken at different incubation times (Figure 4).

![Graph](image)

**Figure 4.** P release from G₃Phy-based membranes incubated in Tris-HCl buffer (pH 7.4) at 37 °C.

P release was observed in all the G₃Phy-crosslinked chitosan membranes. As it was expected, P release achieved higher values as G₃Phy content increased in the membranes. A burst release at the first 8-12 hours took place in all the G₃Phy-crosslinked membranes. This period agreed with the necessary time to achieve water-adsorption equilibrium in the membranes, as it was shown in swelling analysis (Figure 3). P release for all the studied systems achieved a plateau from 24 hours to the end of incubation period (7 days), giving P release values of 0.6, 1.9 and 2.5 µg P/mg membrane for Ch/G₃Phy_2.5, 5, and 10, respectively. Taking into consideration the final P release and the corresponding amount of P in the membrane (Figure 2c), it was estimated that an average of 80 % of the total crosslinking agent was released during the first 24 hours independently on the incorporated G₃Phy amount in the membranes.

### 3.4. In vitro 1.5SBF biomimetic study

*In vitro* biomimetic studies were carried out by immersion of Ch/G₃Phy_10 membrane in 1.5SBF solution (Yokogawa et al., 1997). Ch/G₃Phy_10 system was chosen because of its higher G₃Phy content in comparison to Ch/G₃Phy_2.5 and 5 samples. The formation of aggregates with apatite-like structure and composition was assessed by SEM and EDX after 4, 7, and 14 days of incubation (Figure 5).
Some disperse aggregates concentrated in localized zones, were observed on the membrane surfaces after 4 days of incubation. After 7 days, the deposits were more disseminated, and they colonized almost the whole surface after 14 days of incubation. At higher magnification (2000x) deposits resembled apatite-like phosphates. The morphology of these deposits agree with that found by Yokoi et al. corresponding to apatite deposits (Yokoi, Kawashita, Kikuta, & Ohtsuki, 2010). Yokio reported a granular apatite formed at high phosphate concentration on the surface of a polyacrylamide hydrogel. Elemental composition of surface aggregates at 4, 7, and 14 days was analyzed by EDX (Figure 5). EDX spectra of the precipitates showed the characteristic peaks of Ca and P at any incubation time, obtaining Ca/P ratios of 1.92, 1.45, and 1.66 for 4, 7, and 14 days, respectively. These results confirmed that the aggregates formed in the Ch/G3Phy_10 surface after 1.5SBF immersion exhibited a chemical composition close to that of biological apatite (Ca/P = 1.66).

Osteointegration, which consists of the direct bonding between bone substitute and new living bone, is one of the key properties an ideal bone-substitute must exhibit. This bonding should be sufficiently fast to avoid the generation and deposition of connective tissue between the bone and the implanted material. The formation of a fibrous layer will limit osteointegration and can result in loosening of the graft (Stepniewski et al., 2017). The rapid generation of an apatite-like layer in a bone-substitute material is an excellent indicator of an appropriate osteointegration (Miyazaki, Ishikawa, Shirosaki, & Ohtsukid, 2013; Yokogawa et al., 1997). Some studies have been reported about the generation of Ca/P layers on chitosan-based materials. For example, Yokogawa et al. (Yokogawa et al., 1997) performed a profound study of calcium phosphate layer growth on phosphorylated chitin fibers when they were soaked in 1.5SBF. Other studies have claimed the deposition of spherical Ca/P particles on titanium surfaces that were

Figure 5. SEM micrographs of Ch/G3Phy_10 membranes surfaces after different times (4, 7, 14 days) of soaking in 1.5SBF at different magnifications. EDX spectra of surface deposits of Ch/G3Phy_10 films with their calculated Ca/P ratios.
decorated with phytic acid and calcium hydroxide (Liu et al., 2019). In this case, phytic acid seemed to act as the bridge between Ca ions and titanium surfaces due to its strong chelating capacity of phytic acid against divalent ions such as Ca$^{2+}$ (Luttrell, 1993). Because of the claimed stability of calcium-phytate complexes under \textit{in vitro} neutral pH (Meininger et al., 2017), phytic acid has been proposed as an inhibitor of bone resorption in animal models of osteoporosis (Arriero Mdel, Ramis, Perello, & Monjo, 2012; Cordoba et al., 2016; López-González et al., 2013). In addition, phytic acid has been also applied as novel component in calcium phosphate cements due to its excellent Ca$^{2+}$ chelating properties (T. Konishi et al., 2017; Toshiisa Konishi et al., 2013; Meininger et al., 2017). Meininger \textit{et al.} (Meininger et al., 2017) used phytic acid in their work as an alternative setting retarder, and these calcium phosphate cements demonstrated enhanced cells activity and proliferation, as well as, good \textit{in vitro} stability of the formed Ca-Phytate complexes. $G_3$Phy is a hydroxylic derivative of phytic acid which demonstrated a tunable chelating ability against Ca$^{2+}$ ion in comparison to phytic acid due to the incorporation of glyceryl moieties to the phytic acid structure (Mora-Boza et al., 2019). In this sense, the present work demonstrated that $G_3$Phy chelating capacity is enough to promote \textit{in vitro} biomineralization of chitosan membranes in a short incubation time (4 days). These results highlight the potential of the developed $G_3$Phy-crosslinked systems as promising candidates for direct bone-biomaterial bonding.

3.5. \textit{In vitro} effect of $G_3$Phy-crosslinked membranes on hMSCs culture.

3.5.1. Membrane cytotoxicity

The toxicity of lixiviates coming from different membranes was measured on hMSCs to evaluate the possible cytotoxic effects of the systems. Figure 6a shows that cell viability was not affected by lixiviates coming from any of the $G_3$Phy-crosslinked membranes as cell viability percentages were higher than 80% in all the systems. In addition, hMSCs exposed to extracts of Ch/$G_3$Phy_10 membranes taken at short incubation times (1 and 2 days) showed a significantly higher cell viability in comparison to TMX and chitosan membrane. This feature would be related to the highest P release, at short times (Figure 4) in comparison with the other membranes, since $G_3$Phy was claimed to exhibit excellent cytocompatibility and osteogenic properties against hMSCs culture (Mora-Boza et al., 2019).
3.5.2. Cell adhesion and proliferation.

Human MSCs proliferation on uncrosslinked and G3Phy-crosslinked membranes was evaluated by Alamar Blue and fluorescence microscopy assay at 2, 7 and 14 days (Figure 6b and S3). Human MSCs proliferation increased over time for all the systems. At short times (2 days) cell proliferation was significantly reduced in chitosan and Ch/G3Phy membranes in comparison to TMX. However, cell proliferation in G3Phy-crosslinked membranes was similar to those of chitosan membranes (no significant differences were found). For prolonged incubation times (7 days), cell proliferation significantly decreased in Ch/G3Phy_5 system in comparison to chitosan membrane. The reduced cell proliferation in G3Phy-crosslinked systems could be claimed to the G3Phy release events that take place in these systems (Figure 4) and to the osteogenic differentiation processes that G3Phy should lead on hMSCs (Mora-Boza et al., 2019), which could slow down cell proliferation at this incubation time. Finally, no significant differences on hMSCs proliferation were found between chitosan and Ch/G3Phy_2.5 and _5 membranes, while Ch/G3Phy_10 film demonstrated a significantly higher cell proliferation at 14 days. This behavior was attributed to the higher G3Phy content in the system which notably improved cell adhesion and proliferation at prolonged incubation times. Collectively, these results confirmed that the incorporation of G3Phy to chitosan at concentrations up to 10 wt-% with respect to chitosan improved cell cytocompatibility and proliferation. Previous studies have shown that phytic acid improved the proliferation

Figure 6. a) Cell viability (%) of hMSCs cultured with extracts of chitosan and G3Phy/chitosan membrane taken at 1, 2, 7, and 14 days of incubation. Significant differences with TMX samples at each time are marked with * (**p<0.05, ***p<0.001) and with chitosan membranes are marked with # (#p<0.05, ##p<0.01, ###p<0.001). b) Cell proliferation values of hMSCs seeded on the crosslinked membrane surfaces measured by Alamar Blue assay. Significant differences with respect to TMX sample at each time are marked with * (**p<0.05, ***p<0.01, ****p<0.001) and with chitosan membranes are marked with # (#p<0.05, ##p<0.01, ###p<0.001).
of stem cells in different models (T. Konishi et al., 2017; Liu et al., 2019; Sun et al., 2016). For example, Sun et al. (Sun et al., 2016) demonstrated enhanced cell adhesion and proliferation in phytic acid polyaniline hydrogels. Konishi et al. (T. Konishi et al., 2017), who developed a novel injectable cement using chitosan and phytic acid among other compounds, also observed that cell viability was improved at prolonged cell culture times in the systems containing phytic acid.

3.5.3. Osteogenic effect of G₃Phy-crosslinked membranes on hMSCs culture.

The ALP activity and calcium deposition of hMSCs seeded on chitosan and Ch/G₃Phy membrane surfaces were evaluated to assess osteogenesis capacity of the systems (Figure 7).

![Figure 7. a) ALP activity of hMSCs at 14 days seeded on chitosan and G₃Phy/chitosan membrane surfaces incubated in non-osteogenic medium. b) Alizarin Red content (mM) on hMSCs cultured on the surface of different chitosan membranes. Significant differences with respect to chitosan sample at each time are marked with * (*p < 0.05, **p < 0.01, ***p < 0.001).](image)

The ALP activity of the different Ch/G₃Phy membranes was evaluated after 14 days of incubation in non-osteogenic medium (Figure 7a). All G₃Phy-crosslinked membranes showed significantly higher ALP activity in comparison to uncrosslinked chitosan membrane. Although ALP activity decreased as G₃Phy content increased, no significant differences were found when Ch/G₃Phy compositions were compared one to each other (p > 0.1). These results indicated that even low contents of G₃Phy in the systems enhanced ALP activity of hMSCs seeded on membrane surfaces since ALP activity of Ch/G₃Phy_2.5 system increased 1-fold with respect to chitosan. Chitosan has been previously claimed to stimulate early osteogenic differentiation of dental pulp stem cells (DPSCs). Amir et al. (Amir et al., 2014) demonstrated in their work that the addition of chitosan to DPSCs cultured in mineralization medium increased ALP activity and chitosan presence had an osteogenic effect similar to that of dexamethasone. G₃Phy was previously demonstrated to exhibit osteogenic properties by increasing ALP activity.
respect to phytic acid after 14 days of incubation and upregulating early osteogenic markers such as COLA1A and ALPL at basal conditions (non-osteogenic medium) (Mora-Boza et al., 2019). ALP results showed in this work, demonstrated that the combination of chitosan and G3Phy led to a positive synergistic effect in the osteogenic differentiation of hMSCs seeded on membrane surfaces even in non-osteogenic stimulating conditions. Osteogenic activity has been observed in other systems based on phytic acid (Cordoba et al., 2016; Cui et al., 2017; Liu et al., 2019). For example, Liu et al. (Liu et al., 2019) demonstrated that calcium and phytic acid-decorated titanium surfaces enhanced ALP activity and early osteogenic markers, which could lead to new bone formation and osteointegration. Likewise, Córdoba et al. (Cordoba et al., 2016), who developed a similar grafting of phytic acid molecules on titanium surfaces, also observed higher expression levels of osteogenic markers on MC3T3-E1 cells.

Biomineralization of cultured hMSCs on membrane surfaces was assessed by alizarin red assay at 7 and 14 days of incubation. Alizarin red stains the calcium deposits containing in hMSCs. Figure 7b shows that alizarin red concentration increased over time for Ch/G3Phy systems, while it remained stable in uncrosslinked chitosan membrane from 7 to 14 days of incubation. Amir et al. demonstrated in their work that although chitosan upregulated osteogenic differentiation of DPSCs, no significant stimulation on mineral deposition was found (Amir et al., 2014). At 7 days of incubation no significant differences were found between G3Phy-crosslinked membranes and chitosan membranes. Nevertheless, after 14 days, alizarin red content was significantly higher in all Ch/G3Phy systems in comparison to uncrosslinked chitosan. In addition, biomineralization increased with the G3Phy content in the membranes. Thus, Ch/G3Phy_10 sample exhibited the highest biomineralization capacity in comparison to the other G3Phy-crosslinked compositions. These results correlated with the in vitro 1.5SBF biomineralization analysis (Figure 4). Other phytic acid-based systems have claimed to improve biomineralization (Cordoba et al., 2016; Meininger et al., 2017). For example, high mineralization of MC3T3-E1 cells after 21 days was observed on titanium surfaces grafted with phytic acid molecules (Cordoba et al., 2016).

Taking the biological results in overall, we can claim that the G3Phy crosslinking to chitosan membranes and its further release improved the osteogenic properties of chitosan. In this context, we would like to highlight that all the in vitro experiments were conducted without osteogenic inductors typical for osteogenic differentiation of hMSCs (Hanna, Mir, & Andre, 2018). We previously reported that G3Phy exhibited osteogenic properties by its own and it has been proposed as promising candidate in bone-tissue engineering applications (Mora-Boza et al., 2019). Here, we demonstrated that the use of G3Phy as a polysaccharide crosslinker results in an osteogenic biomaterial that has great potential in the bone-tissue engineering field.

4. Conclusions

In this study, G3Phy-crosslinked chitosan membranes as supports of hMSCs were developed for bone tissue engineering applications. Crosslinking reaction was confirmed by ATR-FTIR, EDAX, ICP-OES, and TGA analysis. Swelling and G3Phy release
exhibited a crosslinker content-dependent behavior. *In vitro* incubation of Ch/G₃Phy_10 system in SBF showed the deposition of apatite-like aggregates, which demonstrated the osteointegration potential of the chitosan surfaces crosslinked with G₃Phy. Human MSCs cultured on crosslinked-chitosan surfaces increased viability over time, and improved osteogenesis features in comparison to uncrosslinked chitosan. In particular, G₃Phy incorporation to chitosan membranes enhanced ALP activity and mineralization in absence of any typical osteogenic inducer. Taken together, these results evinced that G₃Phy-crosslinked chitosan membranes provide a suitable environment for hMSCs culture and differentiation into osteoblastic lineage. In conclusion, the developed systems could be applied as promising substrates for guided bone regeneration.

**CRediT author statement**

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**Luis García-Fernández:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Visualization

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**Funding:** This work was supported by the Ministry of Science, Innovation and Universities (Spain) [MAT2017-2017-84277-R], Instituto Salud Carlos III (ISCIII)-Fondo Europeo de Desarrollo Regional (FEDER), Centro de Investigación Biomédica en Red en Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Fundación para a Ciência e a Tecnologia [UID/Multi/50016/2019], Post-Doc grant to S. Baptista-Silva [SFRH/BPD/116024/2016]. Interreg V-A POCTEP Programme through FEDER European Union [0245_IBEROS_1_E.C.A.] and "Biotherapies: Bioengineered Therapies for Infectious Diseases and Tissue Regeneration" [NORTE-01-0145-FEDER-000012].

Ana Mora-Boza is supported by “La Caixa” Foundation [ID 100010434, scholarship code LCF/BQ/ES16/11570018].
5. References


