Characterization and In Vitro Bioactivity of Chitosan/Hydroxyapatite Composite Membrane Prepared by Freeze-Gelation Method

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This work reports the properties of highly porous (>80%) membrane Chitosan/Hydroxyapatite (Cs/HA) composites obtained by the freeze-gelation processing route. These materials are of great interest for bone regeneration applications due to their ability to nucleate calcium phosphates in presence of simulated body fluid (SBF). The membranes porosity and bioactivity can be easily controlled by adding various amounts of hydroxyapatite to chitosan solution. The structural properties of the composite membrane of Cs/HA at various weight ratio (Cs/HA=70/30, 50/50 and 30/70) have been investigated by scanning electron microscopy (SEM), porosity measurements and FTIR spectroscopy. The surface of the composite membranes after immersion in SBF during more than 14 days shows a regular Ca-P layer as evidenced by FTIR spectroscopy and ICP analysis. These results suggest the potential interest of the Chitosan/hydroxyapatite composite membranes prepared by freeze-gelation process in bone regeneration and especially of the Cs/HA membrane with a ratio of 70/30.

Introduction

Extensive research has been undertaken to develop polymer/hydroxyapatite biomimetic composites as new bone substitute materials due to the presence of hydroxyapatite (HA) which is a bone inorganic component [1, 2, 3]. HA, [Ca_{10}(PO_4)_{6}(OH)_2], was used in various biomedical fields such as dental material, bone substitute and hard tissue. HA can accelerate the formation of bone like apatite on the surface of the implant [4]. However, the main limitation of the use of these HA particles lie in the difficulties that surgeons encounter to apply them and keep them in place after implantation [5]. To overcome this problem, one strategy consists to immobilize them in a polymeric matrix such as chitosan. This polymer attracts a particular attention in the development of biomaterial composites because this natural polysaccharide derived from chitin after N-deacetylation is biodegradable and biocompatible. This polymer has been largely used in biomedical applications especially as scaffolds. Ideally, the scaffolds should have a high porosity, a large specific area, a suitable pore size, and a highly interconnected pore structure to provide enough space for the tissue development and to promote neovascularization [6]. This porosity can also control inorganic crystal nucleation, growth, microstructure, and more generally the properties of such mineral-based materials.
Therefore, several preparation methods have been reported for porous scaffolds, including porogen leaching [7], saturation and release of CO₂ [8] and traditional phase separation technique [9]. A widely used method is freeze-drying (freezing at -80°C) which is a thermally induced phase separation (TIPS) [10, 5]. Recently, Ho and coworkers [11] have developed a novel method using freeze-gelation processing to prepare highly porous scaffolds without consuming time and energy. In fact, the freeze-gelation method requires only 12 hours of preparation whereas the freeze-drying method requires lengthy preparation (4 days) and more energy due to the lyophilisation of the samples. Besides, the formation of surface skin can be resolved and the limitation of the use of low boiling point solvents can be avoided by using freeze-gelation process instead of freeze-drying processing route. To our knowledge, the impact of the porous structure obtained by freeze-gelation method on biomineralization was never reported in the literature.

In the present work, porous composite membranes of chitosan/HA have been made by the freeze-gelation route. In this process, a frozen solution of chitosan in presence of HA particles is immersed in a gelation environment at a temperature lower than the freezing point of the polymer solution. By this way, the polymer matrix/HA is already gelled before the drying stage and the porous structure is kept in order to avoid the collapsing effect. This paper describes both the effect of the ratio of HA in Cs/HA on the porosity of the composite membranes and the in vitro bioactivity of the composite membranes in presence of simulated body fluid.

Materials & Methods

Chitosan (Cs) flakes from prawn shells (Pandalus borealis), with a degree of deacetylation (DD) of 88.4%, (nitrogen 7.30% determined by ICP, [12]) and molecular weight of 1.14 x 10⁵ (estimated by viscosimetry, [13]), were purchased from Marinard Biotech (Quebec, Canada). HA particles were prepared as reported earlier [14] by using CaCl₂ and K₂HPO₄ at 85°C. The growth medium (Simulated Body Fluid quoted SBF) was prepared according to the procedure described by Tas [15]. SBF is composed of 6,547 g/L NaCl, 2,268 g/L NaHCO₃, 0.373 g/L KCl, 0.178 g/L NaHPO₄·2H₂O, 0.305 g/L MgCl₂·6H₂O, 0.368 g/L CaCl₂·2H₂O, 0.071 g/ L Na₂SO₄, 6.057 g/L (CH₃OH)₃CNH₂. The pH of the solution was adjusted to 7.4 by adding 1M HCl at 37 °C. 1.5xSBF is a simulated body fluid which contains the same ions as the SBF solution but the ions concentration is multiplied by 1.5. All these chemicals of analytical grade were purchased from Sigma and used without further purification.

Preparation of chitosan/HA composite membranes

Solutions of chitosan and HA were obtained by dissolving 1% wt chitosan flakes in aqueous solutions of acetic acid (1 vol %) in presence of different amounts of HA (0.00; 0.43; 1.00; 2.33 wt %). After gas evolution under ultrasonication, the solutions were set in glass Petri dishes and cooled at 4°C for ½ hour before being frozen at -22°C for 3 hours. The frozen chitosan solutions were immersed in NaOH/ethanol aqueous solutions pre-cooled at -22°C and the pH was adjusted to observe the gelation of chitosan. The wet membranes such obtained were cautionary removed from their solutions then thoroughly rinsed with deionized water and dried at room temperature.

Immersion in SBF solutions

Samples (Cs and Cs/HA composite membranes) (2x2) cm² were placed in plastic jars with 20ml of 1.5xSBF. The jars were covered with an airtight cap and put into an air oven thermostated at 37°C. The SBF solutions were replaced each day. Samples were retrieved after 7, 14, 21 and 28 days in contact with SBF in order to perform various characterizations after thoroughly rinsing them with distilled water and after drying them at room temperature.

Characterization

The composite and chitosan morphology were observed by scanning electron microscopy (Philips Quanta 200) without further preparation. FTIR spectra of HA, Cs and their composites were recorded on FTIR-8400 (SHIMADZU) Fourier transform infrared spectrometer between 400-4000 cm⁻¹ in KBr pellets. ICP analysis of phosphorus and calcium in the membranes were carried out with a
Thermoelectron ARL3580. The dissolution of the samples was realized by the following procedure: (i) 100 mg of the sample was mixed with 500 mg of lithium metaborate (Aldrich, 99.9%); (ii) the mixture was heated at 1100 °C in a graphite vessel during 1 hour and (iii) the resulting viscous product was dissolved in nitric acid (1M). Analyses of nitrogen were realized by “Service Central d’Analyse” (ECHANGEUR DE SOLAIZE - BP 22 - 69390 – VERNAISON, France).

The phosphorus contents in SBF solutions were determined with a UV mini 1240 spectrophotometer (SHIMADZU) as reported by Murphy and Riley [16], and the calcium concentration was determined by volumetric titration using NaOH, Murexide and EDTA (from Aldrich).

Results and Discussion

Membranes morphology before immersion

Figure 1a displays the porous structure of Chitosan obtained by the freeze-gelation processing method. This typical morphology is characterized by a three-dimensional porous and sponge-like network structure with random pore. This morphology indicates that the porous structure is generated after the phase separation of the homogeneous chitosan solution during the freezing stage. The gelation of chitosan by NaOH/ethanol solution cooled at -22°C avoids the collapse of the pores during the removal of the solvent due to the formation of a rigid polymer which retains its porous structure. Consequently, the space originally occupied by the solvent is used to increase the porosity in the membranes. The porosity of the polymer is made up of interconnected macropores. The diameter of the macropores ranges between 6 and 100 µm. The average distance between each pore has been estimated at about 50 µm by means of the SEM micrographs. The walls of the macropores are constituted of micropores whose diameter varied from 0.5 to 3 µm (not shown in fig. 1a).

The gelation processing route can also be used with chitosan in presence of ceramic such as HA. Figure 1b shows a SEM micrograph of Cs/HA composite membrane obtained by the gelation processing method. A good adhesion between HA particles and polymer has been obtained as evidenced in Fig. 1b. The HA particles are dispersed on the entire surface but are also encapsulated in the polymer.

The water absorption capacity (ABS) of the Cs/HA membranes at different ratio has been reported in Table 1. The equation (eq.1) used in our study to estimate ABS values is that used by Yang [17] to determine porosity.
Characterization and In Vitro Bioactivity of Chitosan/Hydroxyapatite Composite Membrane

Table 1: Water absorption capacity of membranes prepared by freeze-gelation process at different Cs/HA ratio

<table>
<thead>
<tr>
<th>Cs/HA weight ratio</th>
<th>ABS (%)</th>
<th>100/0</th>
<th>70/30</th>
<th>50/50</th>
<th>30/70</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>240.63</td>
<td>111.13</td>
<td>100.2</td>
<td>93.00</td>
</tr>
</tbody>
</table>

\[
P(\%) = \frac{m_1 - m_0}{d_{\text{water}}} \times \frac{100}{V} \tag{1}
\]

where \( m_0 \) represents the weight of the dry membrane, \( m_1 \), the weight of the wet membrane, \( d_{\text{water}} \) the water density and \( V \) the effective volume of the wet membrane determined with a digital caliper rule.

In fact, this equation express a fraction of water volume contained in the material compared to total volume of the wet material: \( m_1 - m_0 \) is the water weight retained, \( (m_1 - m_0) / d_{\text{water}} \) is water volume and consequently the ratio of this value and total volume of the wet material can express empties fraction occupied by water. In these conditions, found values are greater than normal porosity (100%). This indicate that, for microspongy structure of material, a complementary quantity of water was probably fixed by various mechanisms such as interactions of Van der Waals and absorb greater than it’s weight. In addition, the membrane prepared by freeze-gelation is very spongy (fig. 1 a), the congelation cause the crystallization of water in membrane structure and space the polymeric chains of chitosan witch led to increasing the effective volume of membrane. In this fact, porosity values seem to be very high.

We note that porosity (determined by using equation 1) of membrane prepared by traditional phase separation technique is about 50%

Indeed, the increase of the hydroxyapatite content decreases the ABS and then porosity but all the membranes have a suitable porosity (>80%) for tissue engineering applications [18] and the structure of the interconnected pores should be able to provide enough space for possible neovascularization when being implanted.

**Bioactivity tests in SBF**

The variation of the membrane ABS versus the immersion time in SBF are summarized in Table 2 for different weight ratio of Cs/HA. Examination of this table shows that the ABS decreases with the immersion time which indicates a degradation of the structure. The decrease of the ABS may be attributed to calcium phosphate deposition in the porosity of the membrane or the biodegradation of the polymer in presence of SBF which is responsible of the collapse of the membrane structure. The both phenomena may also occur simultaneously. The proportion of chitosan in the Cs/HA membrane has a strong influence on the degradation rate of the polymer. The degradation is more important as the ratio of chitosan, in Cs/HA membranes, is high. A significant decrease of the porosity is observed

<table>
<thead>
<tr>
<th>Cs/HA ratio</th>
<th>Days</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>100/0</td>
</tr>
<tr>
<td>0</td>
<td>240.63</td>
</tr>
<tr>
<td>7</td>
<td>106.2</td>
</tr>
<tr>
<td>14</td>
<td>90.12</td>
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<tr>
<td>21</td>
<td>87.80</td>
</tr>
<tr>
<td>28</td>
<td>56.66</td>
</tr>
</tbody>
</table>

Table 2: Water absorption capacity of Cs/HA membranes prepared by freeze-gelation process at different ratio after immersion in 1,5xSBF
after 28 days of immersion. The decrease of the porosity of the Cs/HA membranes during immersion in SBF can be explained by the growth of apatite layers. Consequently, the porosity decreases down to a constant value close to the porosity of HA layer.

The kinetics of degradation of the composite membranes in presence of SBF has been determined by studying the variation of the weight of the membrane versus the time of contact with SBF solution:

\[ W(\%) = \frac{m_a - m_b}{m_b} \times 100 \]  

(2)

where \( m_a \) is the weight of the membrane after immersion in SBF and \( m_b \) is the weight of the dry membrane before immersion in SBF.

Figure 2 displays the variation of Cs/HA composite membranes weight versus the immersion time in SBF for Cs/HA equal to 30/70, 70/30 and 100/0 respectively. A loss of weight is observed for the chitosan membrane in absence of HA (Cs/HA=100/0) during the first week of contact of the membranes in SBF. Silva et al. [5] reported that the highest weight loss of a chitosan membrane obtained by freeze-drying route was obtained after 30 days of immersion in SBF. This decrease of the chitosan membrane weight in absence of HA particles can be explained by an important degradation of chitosan. Indeed, the high porosity of this membrane enhances the penetration of the SBF solution within the porous structure of the polymer and makes polymer degradation easier. The physicochemical degradation of the membrane comes from (i) the breaking of the electrostatic interactions and the intermolecular bonds, (ii) the depolymerization of the membrane, and (iii) the hydrolysis of the membrane which is responsible of the formation of harmless compounds such as carbohydrates, carbon dioxide and water.

On the contrary, the weight of the membrane containing 70% of HA remains constant during 7 days and increases afterward. The SEM pictures of this membrane after 7 days of immersion show a deposit of calcium phosphate (Figs. 3a and 3b). The formation of apatite layer arises from the presence of hydroxyl and amine groups in chitosan as showed by FTIR and ICP analysis (vide infra). The loss of weight resulting from the degradation of the membrane is counterbalanced by the formation of calcium phosphate as evidenced by SEM pictures realized after 7 days of immersion in SBF (Figs. 3a and 3b). The increase of weight of the composite membrane containing 30% of HA from start of the immersion can be explained by
Fig. 3 Scanning electron micrograph of Cs/Ha: 70/30 after immersion in 1, 5xSBF solution, (a, b) 7 days, (c) 14 days, (d, e) 21 days.
a more important deposit phenomenon compared to the degradation of the polymer.

In the case of the investigated composite membranes, the particles of HA were accessible to the nucleation making it possible to reach the supersaturation of SBF and thus the precipitation of calcium phosphate. The composite membrane containing 30% of HA has a strong power of fixation of the mineral.

**Morphology after immersion in SBF**

*In vitro* bioactive tests (immersion in SBF solutions) have shown that the formation of an apatite-like layer at the surface of the porous composite structures is induced by the presence of HA particles. After 7 days, the surface is almost covered by calcium phosphate particles and a secondary nucleation appears over the initial layer (Figs. 3a and 3b). After 14 days, (Fig. 3c), the entire surface is coated by a porous calcium phosphate layer. After 21 days of immersion in SBF, the composite porous materials were covered by dense layers (Fig. 3e) and the reactant particles may appear as homogeneously nucleated over the surface (fig. 3d).

Figure 4 shows the comparison between the composite membranes containing 30% and 70% of HA (quoted respectively Cs/HA: 70/30 and Cs/HA: 30/70) after 14 days of immersion. Cs/HA: 30/70 shows the presence of numerous Ca-P particles distributed irregularly onto the membrane surface without modifying significantly the porosity of the composite membrane after an immersion in SBF from 7 to 28 days (table 2).

**FTIR**

Figure 5 shows a comparison between FTIR spectra of (a) chitosan, (b) Cs/HA composite and (c) HA before immersion in SBF solution. The spectrum of chitosan (Fig. 5a) exhibits characteristic bands such as: a strong and broad absorption band between 1080 and 1030 cm\(^{-1}\) and a peak at 1252 cm\(^{-1}\) attributed to the free primary amino group (-NH\(_2\)) at C\(_2\) position of glucosamine and others organic bonds in chitosan [19]. The Peak at 1651 cm\(^{-1}\) (amide I) with a minor shoulder at 1596.2 cm\(^{-1}\) (amide II) are attributed to acetylated amino group and confirms that the sample is not fully deacetylated. The peak at 1380.9 cm\(^{-1}\) was assigned to the -C-O stretching of primary alcoholic group (\(-\text{CH}_2\text{-OH}\)) and the peak at 894.9 cm\(^{-1}\) is attributed to the C-O stretching.

The FTIR spectrum of HA (Fig. 5c) shows the presence of bands at 1998, 1030-1033, 960, 620 and 560 cm\(^{-1}\) which are attributed to orthophosphate. The bands located at 1030-1033 and 560 cm\(^{-1}\) are characteristic of

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**Fig.4 scanning electron micrographs of Cs/HA membranes with different ratios after 14 days of immersion in 1,5xSBF: (a) 70/30, (b) 30/70**
phosphate bending vibration, while the band at 960 cm\(^{-1}\) is attributed to phosphate stretching vibration. The FTIR spectrum of HA (Fig. 5c) is in fair agreement with the infrared spectra reported by Ramay and Zhang [20].

The FTIR spectrum of Cs/HA composite (Figure 5b) gathers all the characteristic absorption peaks of HA and Cs. A broad band appears from 3470 and 2885 cm\(^{-1}\) corresponding to the broad – OH stretching absorption band between 3100-3470 cm\(^{-1}\) and the aliphatic C-H stretching bands between 2885 and 2990 cm\(^{-1}\). As it appears that there is no shift of peaks of any group in the composite membrane spectrum, Cs/HA composite membrane may be considered as only a mixture of Cs and HA, i.e. no chemical reaction has taken place between the individual components.

After immersion of the Cs/HA: 70/30 composite membrane in SBF solutions, the absorption bands located at 1596 cm\(^{-1}\) and 1380 cm\(^{-1}\) have disappeared (Fig. 6). These absorption bands characterize -C-O primary alcohol and secondary amide and their disappearance indicates that both amide and alcoholic groups contribute to the formation of Ca-P layer at the surface of the membranes. The nature of the apatite at the membrane surface is a carbonate substituted one as a predominant band located at 1419.5 cm\(^{-1}\) is observed after immersion in SBF (Fig. 6). The deposit of apatite-layer at the membrane surface is confirmed by SEM (Figs. 3a to 3e).

**Analysis of Ca and P contents of membranes after immersion**

Volumetric titration of Ca and colorimetric titration of P in SBF solutions have revealed a decrease in Ca and P concentration in SBF solution during the immersion period confirming that the materials have uptaken calcium and phosphate from the SBF solution to form the Ca-P layer (Figs. 7 and 8).

The variation of the Ca/P molar ratio vs. the immersion time in SBF solution (fig. 9) indicates that during the SBF immersion step, a first Ca/P layer characterized by a Ca/P molar ratio less than 1.5 is formed followed by a coating with a
Fig. 6 FTIR spectra of Cs/HA: 70/30 ratio prepared by freeze-gelation after immersion in 1.5xSBF solution: (a) 7 days, (b) 14 days and (c) 21 days

Fig. 7 Variation of Ca²⁺ concentration in SBF solution vs. immersion time of the composite membranes with Cs/HA = (a) 70/30; (b) 50/50; and (c) 30/70
calcium-deficient apatite because the Ca/P ratio increases all along the immersion in SBF solution. Similar results were observed in previous works by Varma et al. [1] as they evidence by EDAX the presence of calcium phosphate particles with a Ca/P ratio lower than pure hydroxyapatite (<1.67).

ICP analyses confirm the results obtained by volumetric and colorimetric titration of calcium and phosphate as show in fig. 10 which displays the variation of calcium, phosphate and nitrogen content in the Cs/HA: 70/30 composite membrane during the immersion stage in SBF solution. The increase of calcium and phosphate contents in the composites has been confirmed by SEM observations (Figs. 3a-e). The decrease in nitrogen content in the membrane during the soaking step confirms the degradation of chitosan ever evidenced previously by weight measurements.

Fig.8 Variation of phosphate concentration in SBF solution vs. the immersion time of the composite membranes with Cs/HA = (a) 70/30; (b) 50/50/ and (c) 30/70

Fig.9 kinetics of molar ratios Ca/P fixed by Cs/HA membranes soaked in 1,5x SBF: (a) 70/30, (b) 30/70 and (c) 50/50
All of these results confirm that the materials uptakes Ca and P ions from the SBF solution leading to the precipitation and growth of the Ca-P layer.

**Conclusion**

A material for bone regeneration applications constituted of a highly porous composite membrane (porosity>80%) containing chitosan (Cs) and hydroxyapatite (HA) has been prepared by the freeze-gelation process. The original characteristics of Cs and HA have been preserved as no chemical reaction occurs between the individual components. SEM analysis reveals that the chitosan membrane is structured in a 3D macroporous network that offers a good accessibility to a simulated body fluid (SBF) and a good adhesion between the ceramic (HA) and the polymer (Cs). The presence of HA within the composite involves the formation of an effective preliminary layer which promotes local supersaturation conditions for the precipitation of Ca-P during the immersion of the composite membrane in SBF. Indeed, the composite membranes surface has been almost covered with an apatite-like layer after 7 days and can be controlled by altering the amount of HA in the composite membrane. FTIR spectroscopy, porosity measurements, ICP analysis and SEM micrographs carried out on composite membranes prepared by freeze-gelation process with various ratios of hydroxyapatite shows that the best composite scaffold for a potential application in bone regeneration may be a chitosan-hydroxyapatite membrane containing 30% of hydroxyapatite.

**References**