POROUS GELATIN HYDROGELS BASED ON CLAY PROMOTED BY CALCIUM MINERALIZATION

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A bioclean method was proposed to prepare porous gelatin-based hydrogels using clay as porogen, water as solvent and calcium as promoter: firstly, laponite clay and gelatin were individually dissolved in deionized water to get aqueous solutions; then the two solutions were blended and exposed to -20°C for 24h to form physically crosslinked hydrogel; finally the hydrogel was soaked in a calcium chloride solution at room temperature for 48h to obtain matrices with well-defined voids. The gelatin molecules intercalated into the clay slices gained uniform composites, which were confirmed by atomic force microscope, infrared spectrum, X-ray diffractomer and differential scanning calorimetry analyses. As proved by scanning electron microscope study, in the matrix containing 5% gelatin and 2% clay, the voids were interconnecting, and the size of pores focused on a range of 100-200 μm. Such matrix might serve as scaffold for tissue engineering because of its typical pore structure and size distribution. Additionally, such material could be used as wound dressings, for it was able to provide a fine moist environment for a surface.

Keywords: hydrogel, gelatin, clay.

INTRODUCTION

In recent years, considerable efforts have been put into natural macromolecule based hydrogels, since such materials hold incomparable biocompatibility, biodegradability and bioactivity which are precisely pre-requisite in a biosystem. Furthermore, the controlled porosity, pore size, degradation rate, as well as tissue matching mechanical properties and appropriate biological response obtained from biocompatible and biodegradable materials are essential for tissue engineering (Barbetta et al., 2010).

Gelatin, as a kind of natural polypeptide obtained from collagen, consists of 18 different amino acids of various active groups such as amino, carboxyl, hydroxyl and phenolic hydroxyl. These side groups endow gelatin with excellent physical and chemical characteristics like film-forming, surface activity, reversible sol-gel transformation, polyampholyte, and high reactivity (Bigi et al., 2004). In this article, two biocompatible materials, gelatin and clay, were proposed to prepare porous hydrogels in an aqueous medium. This technique, avoiding the use of organic solvent, may provide a simple and clean technique to fabricate macroporous scaffolds for tissue engineering applications or other related fields.

MATERIALS AND METHODS

Materials

Gelatin (type B, G9382, isoelectric point 4.7-5.2), derived from bovine hide with an alkaline hydrolysis process, was purchased from Sigma (USA). The clay (laponite, W233)
Porous Gelatin Hydrogels Based on Clay Promoted by Calcium Mineralization

with an average diameter of 25 nm was kindly supplied by Weipu Co. Ltd. (China) and its chemical compositions are: SiO$_2$ 55~57%, MgO 23.5~25.0%, Na$_2$O 2.8~3.8%, Li$_2$O 1.2~1.5%, F 5~5.8%. Calcium chloride and other reagents were analytical grade.

**Fabrication Process**

As schematically illustrated in Figure 1, the preparation for gelatin-based hydrogels consisted of three procedures: blending, gel-forming and mineralization. The operations were as follows. Firstly X g (X=0.2, 0.3, 0.4 and 0.5, respectively) gelatin was dissolved in 5ml deionized water at 50°C for 2h to get solution a, and 0.2g clay was dispersed in another 5ml deionized water at 25°C with rapid agitation to get solution b. Next, the two solutions were blended with agitation, and the blends turned into a translucent sol after high-speed shear-stirring for about 5min. Thereafter, the composite sol was poured into a cylinder mold (Ф3x4cm) and then exposed to a cryogenic environment at -20°C for 24h to obtain hydrogels with physical cross-linking. Finally, the hydrogel was soaked in a 0.5M CaCl$_2$ solution for 48h getting the final product.

**Characterization**

A SPM-9600 atomic force microscope (Shimadzu, Japan) was used to examine the microstructure change of the hydrogel at each procedure of fabrication. The IR spectra of lyophilized pure gelatin and final products were obtained on KBr pellet and performed on an MAGNA IR560 spectrophotometer (Nicolet, USA). The thermal stability of lyophilized pure gelatin and the hydrogel were assessed with DSC-60 (Shimadzu, Japan) over a temperature range of 30-200°C. X-ray diffraction curves for clay and hydrogels were recorded at 2°/min between 3° and 55° on a X’Pert Pro X-ray diffractometer (Philips, Netherlands) using CuKα radiation (λ=0.154 nm) at a voltage of 40kV and a current of 35mA. The cross-sections in matrixes were observed with a JSM-5900 scanning electron microscope (Philips, Netherlands) to get the microstructure of pores. Then an image analyzer program (Photoshop CS 8.0, Adobe) was used to obtain the top view of the voids in SEM images, in which at least 55 pores were measured to obtain the average diameter. The solvent replacement method was used for porosity measurements (Lai and Li, 2010). The sample was equilibrated in deionized water for 1h and weighed before being maintained at 37°C and 40% relative humidity in an
incubator (MJ-160B-II, Shanghai). The water evaporation rate was measured by weighing the sample at consecutive intervals. The percentage weight remaining (WR) of composite was calculated by 
\[
WR (\%) = \frac{W_r}{W_i} \times 100\%
\]
where \(W_i\) is the initial weight of wet gels, and \(W_r\) is the weight of material at each interval time.

RESULTS AND DISCUSSION

Fabrication Precision

Figure 2. AFM images of the composites during the fabrication process (a, b and c for the composites containing 2% gelatin and 2% clay at the end of each step)

The composite hydrogels were prepared with three procedures including blending, gel-forming and mineralization. The micro-morphology of the composite at the end of each step was observed by AFM and shown in Figure 2. During the blending, gelatin molecules combined with clay molecules to form multi-dispersed amorphous matter (Figure 2a). While in the following two steps, the composite presented a more smoother and homogenous surface (Figures 2b, 2c), which suggested the gelatin molecules may interpenetrate into the interlayer region of clay molecules to obtain uniform composite.

The laponite clay has a layer structure, which, in dispersion in water, is in the form of disc-shaped crystals. The crystals become arranged into stacks which were held together electrostatically by sharing of sodium ions in the interlayer region between adjacent crystals. As indicated in Figure 3, although the 2θ had slight decrease from 28.35° to 23.83°, the XRD curve for the final composite had similar pattern to that for the clay of non-crystal properties. These data demonstrated that the gelatin molecules did not destroy the platelets of the clay except increasing their distance by intercalating into the interlayer space and forming a heterogeneous network.

Figure 3. XRD patterns of clay and the composite (both gelatin and clay content as 2%)

Figure 4. DSC thermographs of gelatin and the composite with 2% gelatin and 2% clay

Figure 5. IR spectra of gelatin and the composite (both gelatin and clay content as 2%)
Figure 4 shows the DSC thermographs of the gelatin and hydrogel. The two curves were similar with only one endothermic peak associated to the helix-coil transition of gelatin, which was the denaturation temperature (Td). In theory, crosslinking degree increases the thermal stability of gelatin, as shown by the shift of the Td to higher values. In addition, it is generally accepted that the endothermic process present in the DSC thermogram of collagenous materials involves rupture of hydrogen bonds and a rearrangement of the triple helix into a random configuration. On the other hand, the increased thermal stability exhibited by gelatin has been ascribed to the presence of crosslinking, which breaks exothermically. The Td of the composite rose by 18°C compared with the gelatin, which indicated that there was interaction between the gelatin and clay.

Gelatin is characterized by the amides of protein, IR spectra of which are located on two regions with amide I (anti-symmetric carboxylate absorbance or C=O stretching vibration) and amide II bands (C-N stretching vibration or N-H bending vibration). In this respect, the IR spectra for the hydrogel and gelatin were presented in Figure 5. Compared with gelatin, the amide peak of hydrogels holds a shift to higher wavenumber, specifically, the spectra of δ (NH₂) on 1537 and 1235 cm⁻¹ disappeared while the spectra of δ(NH₃⁺) on 1553 and 1251 cm⁻¹ emerged, indicating that the –NH₂ in the gelatin was protonized in the fabrication process. Besides another significant change from 1645 to 1632 cm⁻¹, attention was attracted by the 1460-1380 cm⁻¹ region, since certain differences can be seen in it: characteristic for the gelatin is at 1450 cm⁻¹ with a shoulder at 1390 cm⁻¹; for the hydrogel, a doublet at 1454 and 1417 cm⁻¹ with a shoulder at 1473 cm⁻¹. It is well known that, in the spectral region considered, the bands of the deformation vibrations of –C–H groups and the symmetrical stretching vibrations of –C–O groups of ionized –COO⁻ groups appeared, the latter of which might be helpful for the formation of coordinate bonding. Otherwise, the characteristic absorption peaks of Si-O tetrahedron at 1013 cm⁻¹ were found in the spectra of the composite, indicating the presence of silicate clay in the hydrogels.

As mentioned above, the formation of –NH₃⁺ and –COO⁻ was demonstrated by IR analyses. Also, the gelatin molecules interpenetrated into the interlayer region in the laponite clay forming a multiphase network. These results were available to elaborate the fabrication process of the gelatin-based hydrogels. The laponite clay consists of the platelets tightly bound together, which might be exfoliated and dispersed homogenously in a solution by absorbing water into interlayer region. In this study, the clay was firstly dispersed in water with rapid agitation, the interlayer surface of which had a negative charge. After high shear blending, the gelatin in the mixture (pH = 8.5, higher than pI of the gelatin) also had a negative charge, resulting in a polydispersed composite at the end of the first “blending” procedure. Because of small localized positive charges at the edges of the interlayer of the clay generated by absorption of hydroxyl groups, gelatin could interpenetrate into the interlayer region by electrostatic forces in the following cryogenic processing. In the final calcium mineralization step, some –NH₂ groups in the gelatin turned into –NH₃⁺ in the presence of calcium chloride, imparting gelatin partial cationic charge, which may be helpful for the electrostatic bonding between the platelets of the clay and the gelatin. Otherwise, calcium ion may suppress the electric double layer surrounding the platelets of the clay and cause the flocculation of the clay, which led to the strengthened interactions between the clay and the gelatin by shortening their distance. Furthermore, calcium ion could form coordinate or ionic linkage between gelatin and gelatin, as well as gelatin and clay, which ultimately intensified the crosslinking and stability of the whole system.
Effect of the Gelatin Content on the Micro-morphology

Figure 6. SEM images of the porous hydrogels (gelatin content as a, 3%; b, 4%; c, 5%; clay content as 2%)

Three types of hydrogels with different gelatin content were prepared and their SEM images and the pore size distribution were displayed in Figure 6. The average pore diameter were calculated and shown in Table 1. As indicated, both the porosity and the pore size decreased with an increasing content of gelatin in the hydrogels. There are two possible explanations for the observed findings. First, a higher gelatin concentration could result in an increasing growth rate of nucleation in the composites of gelatin and silicate and thus in a higher amount of pores. Nucleation depends on the instability of the liquor phase as well as the diffusion of atoms into clusters, the former increases with decreasing of temperature, and the latter increases with increasing of temperatures. Higher gelatin content resulted in a decrease of the freezing temperature of the solvent. As a result, the mobility of the atoms to diffuse into clusters was higher, and the nucleation rate increased. Second, by comparison of the gelatin content, the more concentrated materials could possess a decreased heat and protein transfer and thus result in smaller pores. A combination of both is likely the most plausible explanation.

Table 1. The morphological properties of porous gelatin-based hydrogels

<table>
<thead>
<tr>
<th>Sample</th>
<th>Clay content(w/v)</th>
<th>Gelatin content(w/v)</th>
<th>Porosity</th>
<th>Average pore diameter*</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>2%</td>
<td>3%</td>
<td>87.21±3.7%</td>
<td>249±16 μm</td>
</tr>
<tr>
<td>b</td>
<td>2%</td>
<td>4%</td>
<td>86.24±3.1%</td>
<td>192±21 μm</td>
</tr>
<tr>
<td>c</td>
<td>2%</td>
<td>5%</td>
<td>85.23±1.9%</td>
<td>146±10 μm</td>
</tr>
</tbody>
</table>

*At least 55 pores were assessed and the values were statistically analyzed and also expressed as the mean ± standard deviation.

Figure 6 also demonstrated the morphology of the pores in the gelatin-based hydrogels. The pores were interconnecting in the four hydrogels, and their average size decreased with the rising gelatin content in the gels. Furthermore, in this material containing 5% gelatin, the pore volume ranged from 50-350 μm, focusing on a range of 100-200 μm. Such pore size and matrix structure are coincidentally preferred by biomaterials (Vlierberghe et al., 2007), since gradient through this kind of matrix can help oxygen and nutrients diffuse to the cells, and waste products to drain out of the matrix, whereas the pore interconnectivity can promote cell migration and angiogenesis.

Effect of the Gelatin Content on the Solvent Evaporation Rate

The weight of the hydrogels was determined at different time intervals when exposed to a condition of 37°C and 40% relative humidity. The weight of the material
decreased linearly in the initial 2.5 h, while gently lowered with the prolonged time (Figure 7). Additionally, the evaporation rate of water slowed with the rising gelatin content. The total volume of the liquid absorbed by gel consists of the solvating solvent bound to polymer network and the capillary solvent filling macropores which can evaporate easier. There was more water absorbed in the large amount of microscopic subunits in the hydrogel with 5% gelatin, resulting in the slowest water evaporation. In addition, the water content in such matrix could be retained higher than 75% even if after 24h in this case. It is reported that a commercially available dressing loses about 50% of its bound water after 12 h and retains about 30% water after 24 h. Therefore the gelatin-based hydrogels could be considered as a potential wound dressing because it could provide a fine moist environment for healing wound surface (Mu et al., 2010).

CONCLUSIONS

An aqueous system was proposed and confirmed for preparing porous hydrogels with gelatin and laponite clay. The fabrication was identified as blending, gel-forming and mineralization three steps. The pore morphology, the pore size and the water evaporation rate could be easily controlled by varying the gelatin content. The hydrogel, consisting of 5% gelatin and 2% clay, had interconnecting pores with size focusing on a range of 100-200 μm, which would be available as scaffolds and wound dressings. This approach offers the advantage of fabricating the hierarchical organization of organic/inorganic components without using organic solvents and could be extended to develop new porous materials for a wide range of technological applications.

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REFERENCES