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Bioactive gelatin-siloxane hybrids as tissue engineering scaffold

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Abstract

Ca^{2+}-containing porous gelatin-siloxane hybrids were prepared using sol-gel process, post-gelation soaking, and freeze-drying. The porosity and pore size of the hybrids could be well controlled by the freezing temperature and the pH value of the soaking solution. The pore characteristics were related to the structure change during the soaking treatment. A bone-like apatite layer was able to form in the Ca^{2+}-containing porous gelatin-siloxane hybrids upon soaking in a stimulated body fluid. The porous gelatin-siloxane hybrids could release gentamicin sulfate which is an antibiotic drug in bone chemotherapy. Thus, those hybrid materials are proposed to find application as novel bioactive and biodegradable scaffolds in bone tissue engineering.

1. Introduction

The expanding field of tissue engineering applications has accelerated the need of materials which are tissue compatible, biodegradable and with mechanical properties similar to the target tissues [1]. Biodegradable and biocompatible polymers, respectively, have been attractive candidates for scaffolding materials because they degrade as the new tissues are formed, eventually without inflammatory reactions or toxic degradation [2]. The scaffold material has an essential function concerning cell anchorage, proliferation and tissue formation in three dimensions [1-3]. Performance of these properties demands usually a porous scaffold structure, with the porosity characteristics being application specific. A number of synthetic and biological materials, such as PLA, PGA, collagen and chitin, for example, are currently being used as tissue scaffolds [3,4]. The microstructures of these systems range from hydrogels, to open-pore structures, to fibrous matrices. However, the current scaffold materials are not bioactive. Bioactive materials, such as Bioglass® [5] and A-W GC® [6], are able to spontaneously form a surface apatite layer under physiological conditions. This layer can act as a structural glue between the materials and body tissues [5,6]. Bioactivity was found to be favored by the co-operative behavior of Si-OH or Ti-OH groups on the material surface and the involved calcium ions, which may release from the implanted material into the body fluid [5,6]. Thus, hybridization among polymers and these inorganic species may yield biodegradable and bioactive scaffolds for tissue engineering.

Since they are porous, the scaffolds may incorporate some additives such as drugs, growth factors that have certain effects on cell growth, cell differentiation, and anti-inflammatory. Therefore, the combination of the scaffolds and antibiotics may be an important issue to be studied in the field of bone tissue engineering for the treatment and prevention of infection in orthopaedics, such as osteomyelitis. Osteomyelitis is a deep-seated infection of boon caused by the pyrogenic microorganism, *Staphylococcus aureus* [7]. The incorporation of antibiotics and their release is thought to yield a high concentration of antibiotics to the infected bone or tissue site without

systemic toxicity. Gentamicin sulfate has been the most widely used antibiotic in controlled release devices, due to its broad-spectrum antimicrobial activity, excellent solubility and stability at elevated temperatures [8].

In this paper, we synthesized a novel group of bioactive and porous Ca^{2+}-containing gelatin-siloxane hybrids, which were derived from the integration of gelatin, 3-(glycidoxypropyl) trimethoxysilane (GPSM), and calcium nitrate($Ca(NO_3)_2$) by a combined sol-gel processing, post-gelation soaking process, and freeze-drying. We also loaded gentamicin sulfate into the bioactive scaffolds and investigated the drug release behaviors from the gelatin-siloxane scaffolds.

2. Materials and methods

Appropriate amounts of GPSM, $Ca(NO_3)_2$, and gentamicin sulfate were added to a 15 mass% gelatin in 0.1 M HCl solution upon continuous stirring at 40°C. Although each mixture initially formed a two-phase emulsion, continuous stirring for 1h resulted in a clear homogeneous solution. 20 ml portions of the sols was poured into a polystyrene container (91 x 69 x 26.5 mm), capped and aged for 3-4 days until gelation at 40°C in an oven. The bulk gelatin-siloxane hybrids were obtained by drying wet gel at 60°C for 7 days. In order to get porous hybrids, the wet gels were soaked in 1M NH_4OH for 16 h, and then washed three times with distilled water. The obtained gels were frozen at -17°C, -80°C, and -196°C, respectively, and subsequently dried in a freeze-dryer.

The morphology of the freeze-dried hybrids was imaged using scanning electron microscopy (SEM). The solid-state ^{29}Si cross-polarization/magic-angle spinning (CP-MAS) NMR spectra (59.6 MHz) were taken using a Varian INOVA300 instrument with: 3.5 kHz specimen spinning, 2.5 ms contact time, 5.0 μs pulse width, 10 s recycle delays and 10 ms dead time, accumulating the signals from about 8000 pulses. The chemical shift is represented in δ (ppm) by convention. Polydimethylsilane (PDMS: δ = -34.0 ppm against tetramethylsilane: δ = 0 ppm) was used as secondary external reference.

For the drug release studies, $1cm^3$ of gentamicin sulfate loaded porous hybrids were immersed in 10 ml phosphate buffer (pH 7.4), and left in a shaking water bath at 37°C. Samples were withdrawn at regular intervals and the release of gentamicin was estimated by using UV–Vis spectrophotometer.

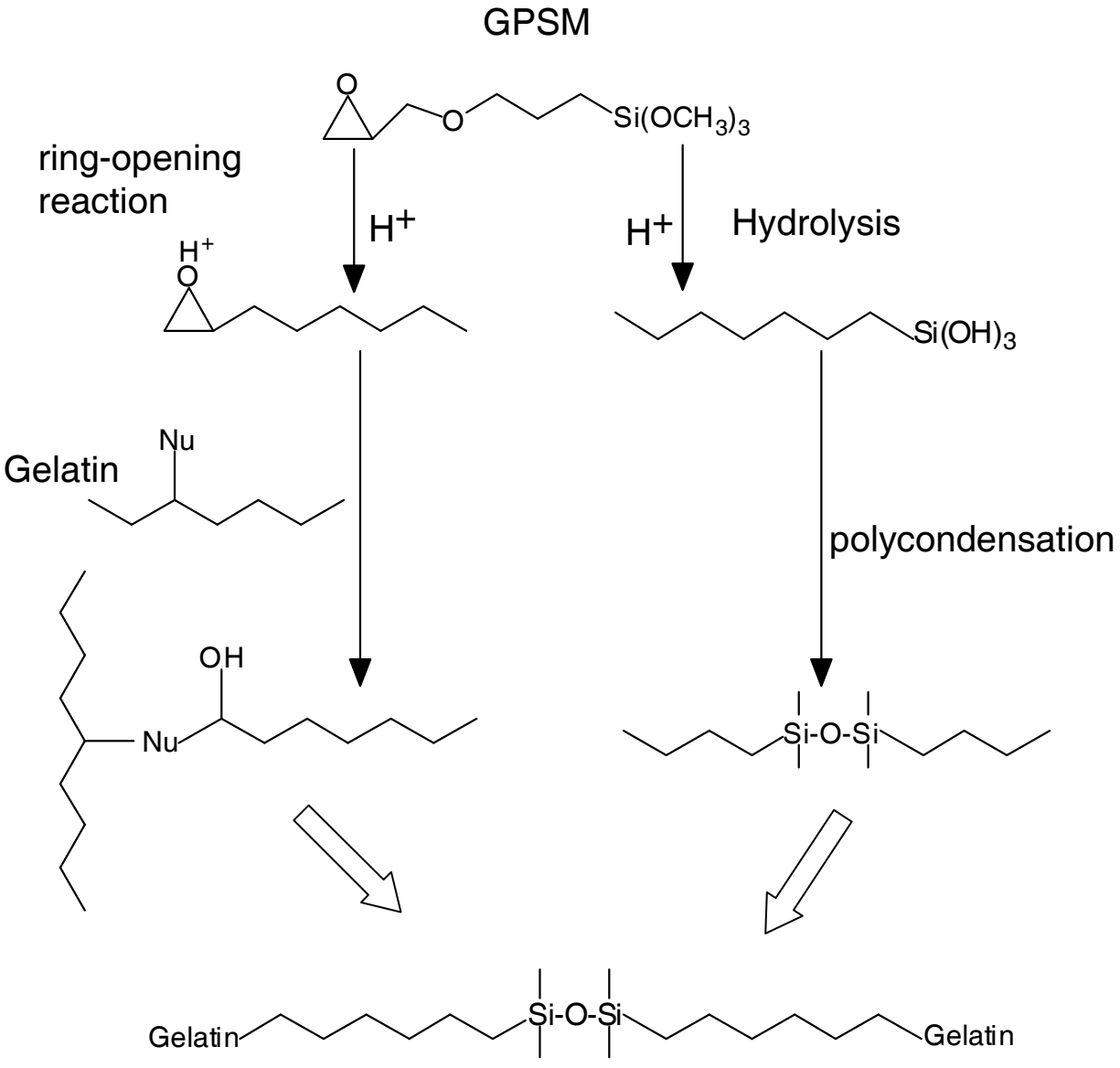
The degree of bioactivity was evaluated by examining the induction period for apatite deposition as the gelatin-siloxane hybrids were soaked in a simulated body fluid (SBF) up to 14 days. SBF contains the same inorganic ions having similar concentrations as human blood plasma (SBF in mM: Na^+ 142.0, K^+ 5.0, Ca^{2+} 2.5, Mg^{2+} 1.5, Cl^- 147.8, HCO_3^- 4.2, HPO_4^{2-} 1.0, SO_4^{2-} 0.5). SBF well reproduces *in vivo* behavior of implant materials in *in vitro* experiments [9]. SBF has been prepared as described in the literature, and buffered to pH7.40 with trishydroxymethylaminomethane (Tris) and HCl aqueous solutions, and kept at 36.5°C throughout the incubation periods. Apatite formed on the hybrid surfaces was detected using thin-film X-ray diffraction (TF-XRD; CuKα, 40KV, 20mA) with an angle of 1° to the direction of the incident X-ray, as well as by SEM observation.

The pre-sterilized scaffolds were respectively placed in each well of a 24-well culture plate, and pre-wetted with 1 ml of α-MEM for 1 day. Then, the medium in each well was exchanged with 1 ml of α-MEM containing 1.0 x 10^4 MC3T3-E1 cells. The cells on each scaffold after culturing were fixed by soaking in a 0.1M phosphate-buffer (PB, pH 7.40) solution containing 2% glutaraldehyde for 2 hours at 4°C. After being dehydrated with the graded ethanol-water solutions of 50% to 100% for 15 min and 100% 3-butanol 3 times for 30 min at each step, the scaffolds were freeze-dried at 13.3 Pa and –5 °C. Finally, the scaffold surface was subject to SEM observation.

3. Results and Discussion

3.1 Synthesis of gelatin-siloxane hybrids

The cross-linked structure is a characteristic feature of the here presented hybrids, consisting of gelatin chains being cross-linked with siloxane bridges. GPSM content has a significant effect on the gelation of hybrids. Much lower or higher GPSM contents resulted in no gel formation, while moderate incorporation of GPSM into the gelatin solution favored gelation. The epoxy end group of a GPSM molecule reacted with one of the component amino acid residues of gelatin chain. The silanol groups derived from hydrolysis of the methoxysilane groups at the opposite end were condensed with another GPSM molecule grafted to the gelatin chain as illustrated in scheme 1.



Scheme 1 Gelatin-siloxane hybrids are suggested to be derived from the hydrolysis and polycondensation of GPSM with water and the ring-opening reaction of GPSM with gelatin.

The porous hybrid scaffolds were fabricated by freezing the swollen bulk hybrids and subsequently lyophilizing the frozen structures. Those scaffolds could be easily shaped into 3-dimensional scaffolds of any shape, such as orthorhombic, cubic, plate, or cylindrical ones. Freezing temperature affected the pore size and porosity (total and open) as illustrated in Fig. 1.

The results showed that the higher the freezing temperature (T_f), the larger the pore size. Therefore, it is evident that the pore size of the hybrids could be controlled in the range from a few μm to several hundreds μm, by varying the freezing temperature. The fact that the fast freezing at –196 °C produced smaller pores than the slow freezing process at –17 °C can be interpreted by the faster rate of nucleation and generation of more ice nuclei due to the greater super-cooling effect. The pH value of the soaking solutions also affected the pore size and pore volumes of the hybrids. Frozen at -17°C, and only soaking in the basic solutions (1M NH_4OH and Tris pH 10.0 buffer) yielded porous hybrids. No pores were observed with SEM for hybrids soaked in neutral or acidic solutions. The macroporous architecture may provide not only channels for improving mass transport and neovascularization after being implanted *in vivo*, but also better environment for cell distribution, adhesion, growth, and differentiated function [3].

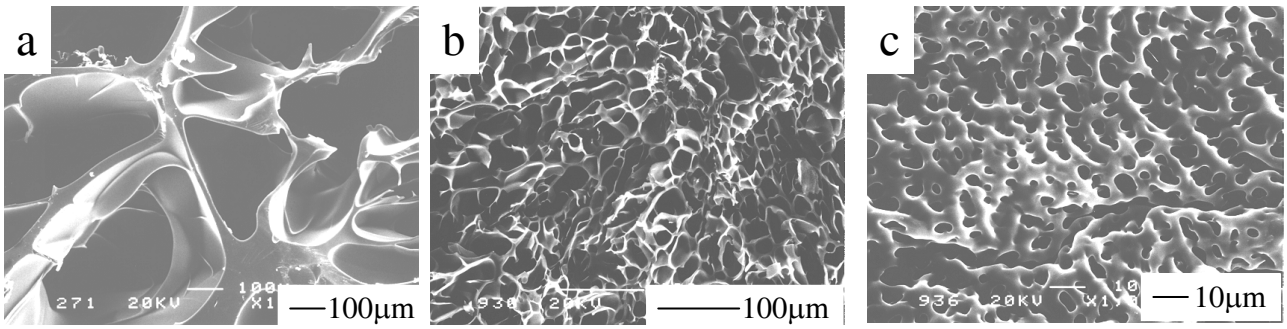


Fig.1 The fracture surface of porous hybrids obtained at (a) T_f = -17°C, (b)T_f = -80°C, and (c) T_f = -196°C.

Figure 2 shows the ^{29}Si CP-MAS-NMR spectra for the GPSM monomer, the bulk hybrid, and the porous hybrid. In the present solution system of GPSM - 0.1 M HCl – gelatin, the methoxysilane groups (Si-$(OCH_3)_3$) of GPSM were hydrolyzed to give silanol groups resulting in T^2 or T^3 bridging bonds, with T^2 and T^3 denoting R-Si(-OSi)$_2$(OCH_3, OH) and R-Si(-OSi)$_3$ (R is the organic backbone from GPSM), respectively [10]. This indicates all of the Si atoms of GPSM are condensed to yield two or three bridging bonds. However, it is not certain only from the NMR data if all of the methoxy silane groups were hydrolyzed or remained. The peak intensity at δ= -57 ppm (T^2) and doublet at -63 and -66 ppm (T^3) for the bulk gel changed significantly after freeze-drying. The peak T^2 almost has the same intensity as peak T^3 in the porous hybrid, indicating the presence of more -Si-OH groups, due to dissociation of the Si-O-Si bridging bonds during soaking in the ammoniac solution. Basic solutions may cause decomposition of the peptide bonds constructing the gelatin chains or the Si-O-Si bonds due to hydrolysis. Hence, treatment with ammonia solution resulted in an even larger increase in porosity. The ^{29}Si MAS NMR spectra in Fig. 2 nicely demonstrate structural rearrangements at the Si-O-Si bridged sites. It follows that a fraction of the Si-O-Si bonds have been hydrolyzed to yield a larger number of -Si-OH groups and a smaller number of bridged bonds, favoring the structural rearrangements as the ice crystals grew.

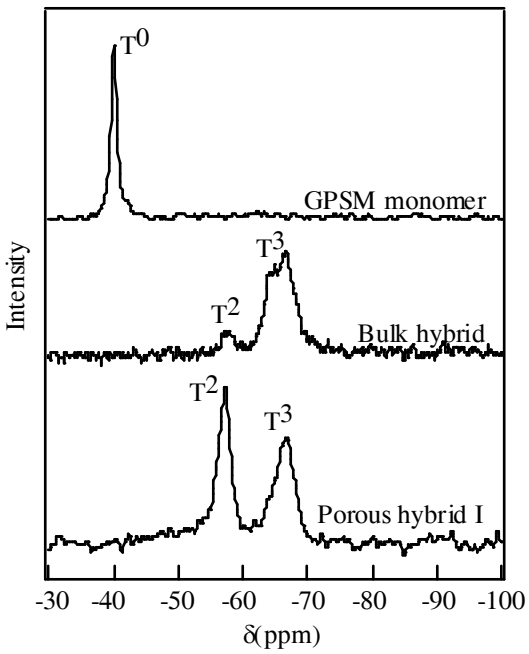


Fig. 2. The ^{29}Si MAS NMR spectra of porous hybrid, bulk hybrid gel, and the GPSM monomer.

3.2 Biomimetic apatite deposition in SBF and in vitro bioactivity

Fig. 3 shows the SEM photographs of the cross sections of porous Ca^{2+}-containing gelatin-siloxane hybrid after soaking in SBF for 3 days. It is indicated that apatite particles covered the whole inner wall of the pores, and the morphology of the deposited apatite was namely semi-spherical agglomerates consisting of flake-like crystallites. The TF-XRD patterns and FTIR spectra (data not shown here) also demonstrated that Ca^{2+}-containing hybrid could deposit apatite after soaking in SBF for 1 day, while Ca^{2+}-free hybrids did not even after soaking in SBF for 14 days. Thus, only the Ca^{2+}-containing hybrids showed *in vitro* bioactivity. The incorporation of Ca^{2+} ions is found to be a key factor to provide the porous hybrids with bioactivity *in vitro*. A number of studies on bioactive ceramics have indicated that *in vivo* formation of Ca-P-rich layer and surface bone-like apatite is a key step for formation of direct bond between bone and in the bone-bonding behavior of these materials, and that SBF well reproduces *in vivo* reaction of the materials [11]. Thus, the present Ca^{2+}-containing porous hybrids may also show bioactivity *in vivo*.

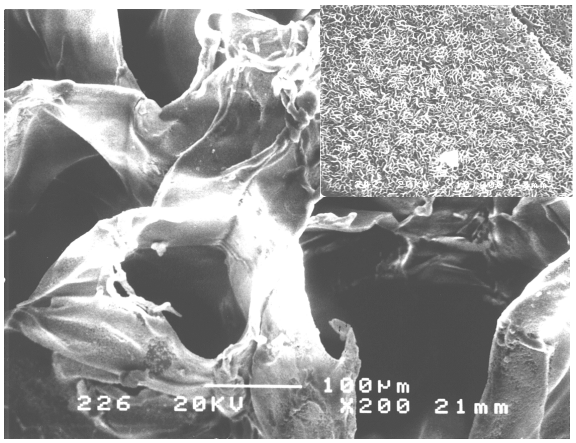


Fig.3 Apatite formed on the pore walls of Ca^{2+}-containing porous gelatin-siloxane hybrid after soaking in SBF up for 3 days.

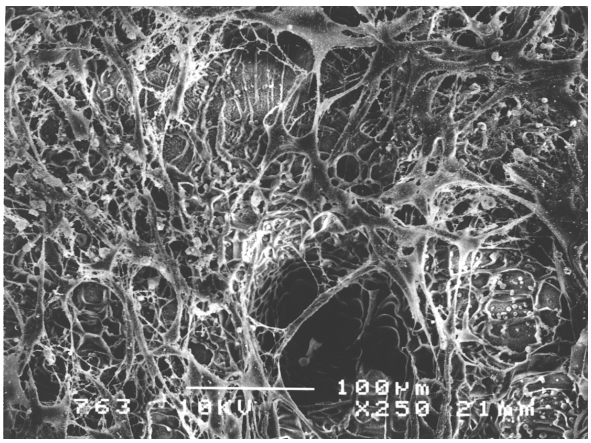


Fig.4 The SEM photographs of MC3T3-E1 cells cultured on Ca^{2+}-containing porous gelatin-siloxane hybrid for 7 days.

3.4 Cell seeding in scaffolds

Cellular behavior on the scaffolds is an important factor in determining evaluating their biocompatibility [12]. Our experiments indicated that the incorporation of Ca^{2+} ions in scaffolds evidently enhanced MC3T3-E1 osteoblast cell proliferation and differentiation on the gelatin-siloxane hybrid scaffolds. MC3T3-E1 cells attached to the surfaces after culture for 1 day (data not shown here), and began to form an interconnecting network within a week on Ca^{2+}-containing porous gelatin-siloxane hybrids (Fig. 4). It is also found from Fig. 4 that MC3T3-E1 cells retained their characteristic polygonal morphology, suggesting that Ca^{2+}-containing porous gelatin-siloxane hybrids are suitable for osteoblast growth.

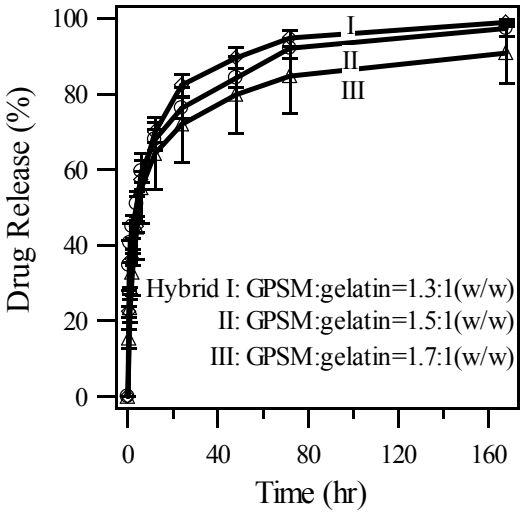


Fig. 5 The effect of GPSM content on the release of gentamicin sulphate from porous gelatin-siloxane hybrids.

3.5 Drug delivery behavior

A preliminary experiment indicated that gentamicin sulfate fully released from the porous gelatin scaffolds within 2 hr. Fig. 5 depicts the effect of GPSM content release of gentamicin sulphate from gelatin-siloxane hybrids in phosphate buffer at 37^{o}C. Each point in the graph represents the mean ± standard deviation of the four experiments. Study was carried out for a period of 7 days. The porous gelatin-siloxane hybrid demonstrated a very high burst effect and almost 70% of loaded drug was released within first day of experiment from scaffolds. The isoelectric point (IEP) of the gelatin is in the range of 4.9-5.2 [13] and at pH above IEP, the gelatin chain contain negative charge which may hinder the acid-base interaction between amino acids of gelatin and gentamicin sulphate. It is also shown in Fig.5 that the drug release became slow with increasing the GPSM content. This phenomenon could be explained on the basis of lower degree of swelling due to the interaction between GPSM and gelatin as illustrated in Scheme 1. After initial burst effect, the porous gelatin-siloxane hybrid scaffold gave a steady drug release up to 3 days as shown in Fig.5. Gelatin-siloxane hybrids may highly swelled in PBS buffer, it is thus suggested that the scaffold serve as a diffusion barrier and the drug was released mainly by diffusion mechanism [14].

4. Summary

Bulk gelatin-siloxane hybrids have been prepared by using a sol-gel process. The porosity was introduced to these bulk gels by post-gelation soaking and by a subsequent freeze-drying. Pore characteristics have been related to the structural changes due to soaking and freezing. The freezing temperature and pH value of the soaking solutions controlled the porosity and the pore size of the hybrids. The Ca^{2+} containing porous hybrids showed an *in vitro* bioactivity as they biomimetically deposited apatite. The porous gelatin-siloxane hybrid could release gentamicin sulfate which is an antibiotic drug in bone chemotherapy, and GPSM content may affect the release rate. Thus, the here presented Ca^{2+}-containing scaffolds may find applications in bone tissue engineering.

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