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Synthesis and cytocompatibility of porous chitosan–silicate hybrids for tissue engineering scaffold application

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Abstract

Chitosan–silicate hybrids with 3D porous structures were prepared with freeze-drying precursor solutions derived from chitosan and γ-glycidoxypropyltrimethoxysilane (GPTMS). They were formed easily in any shape, such as sheets, pellets, disks, granules, and even roll-cakes. The pore size was strongly dependent on the freezing temperature: lower freezing temperature resulted smaller pores, about 110 μm for the hybrids frozen at −20 °C, and about 50 μm for those at −85 °C. The pore size was little dependent on the GPTMS content. In contrast, the GPTMS content affected porosity a littlie: ∼80% for chitosan, and ∼90% for the GPTMS-containing hybrids. Thus, their porous microstructure was controllable due to the freezing temperature and composition. MG63 osteoblastic cells were cultured up to 7 days on the porous hybrids. The cells adhered on the pore walls, proliferated, and migrated deep into the pore structure. It was thus concluded that the present chitosan–silicate hybrids were promising for tissue engineering scaffold applications.

Keywords: Chitosan; γ-Glycidoxypropyltrimethoxysilane (GPTMS); Organic–inorganic hybrid; Scaffold; Osteoblastic cell MG63; Cytocompatibility

1. Introduction

Tissue engineering approach to repairing, complementing, and regenerating damaged tissue depends on the materials that support and reinforce the regenerating tissue. Essential are three-dimensionally (3D) porous scaffolds that manipulate cell functions since they provide spaces and surface area enough for cell adhesion and proliferation, and to supply oxygen and nourishment. As Tateishi et al. pointed out [1], the scaffolds should not only promote cell adhesion, cell proliferation, and cell differentiation, but also be biocompatible, biodegradable, highly porous with a large surface to volume ratio, mechanically strong enough for handling, and capable of being formed into desired shapes. Those scaffolds mostly involve natural or synthetic polymers [2–5] as major ingredients. Among them, chitosan and some of its complexes have been frequently employed [6–10]. Ren et al. [11,12] already reported that porous hybrids of gelatin and γ-glycidoxypropyltrimethoxysilane (GPTMS) spontaneously deposited apatite under the body environment (being bioactive) and were cytocompatible to favor MC3T3-E1 cell culture. Shirosaki et al. [13] synthesized solid chitosan–silicate (chitosan–GPTMS) hybrid membranes and examined their in vitro osteocompatibility due to MG63 osteoblastic cell culture experiments. They found that the cells grew and were proliferated better on those chitosan–GPTMS hybrid membranes than on the chitosan [13]. GPTMS is one of the silane-coupling agents, which has an epoxy group and methoxysilane groups. The epoxy group is interacted with the amino groups of chitosan chains, while the methoxysilane groups are hydrolyzed and form silanol groups, and the silanol groups are subject to the construction of a siloxane network due to the condensation [12,13]. Therefore, the hybrids may

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be useful and promising materials for tissue engineering scaffolds.

In order to confirm the applicability, it should be further examined if pore structure (pore size and porosity) is controllable with ease, and if the porous body retains cytocompatibility that has been found for the corresponding solid hybrids. In this study, therefore, the porous chitosan–silicate hybrids were prepared using freeze-drying method, and the effects of compositions and synthesis conditions on the pore structure and water uptake were examined. In addition, their cytocompatibility was confirmed through culturing human osteoblastic cells, MG63.

2. Materials and methods

2.1. Preparation of the porous hybrids

Chitosan (high molecular weight, deacetylation: 79.0%, Aldrich®, USA) was dissolved in 0.25 M acetic acid aqueous solution. Appropriate amounts of GPTMS (Chisso, Japan) were added to the chitosan solution so that the compositions described in Table 1 were obtained. The samples were coded as shown in Table 1 according to the precursor solution composition. Here, it was assumed that each of 79% chitosan cyclic units has an amino group (–NH_2), which force-opens the epoxy group of GPTMS to form a –NH–O– bond. Thus, the precursor solution for ChG05 involved 1/2 molar equivalent to the amino group that the chitosan held. After stirring at room temperature for 1 h, the resultant chitosan–GPTMS precursor solutions were poured into polystyrene containers, and kept in the refrigerator for 24 h at −20 °C or −85 °C. The frozen hybrids were subsequently transferred to a freeze-dryer (FDU-506, EYELA, Japan), and then, the samples were lyophilized for 12 h to complete dryness. Those porous hybrid xerogels were soaked in 0.25 M NaOH aqueous solution to neutralize remaining acetic acid, washed with distilled water, and lyophilized again in the freeze-dryer.

2.2. Characterization

The morphology or surface microstructure of the porous hybrids was observed under a scanning electron microscope (SEM, JSM-6300, JEOL, Japan). The mean pore diameter was obtained with the microscopic images using Image-Pro Plus software (Planetron, Tokyo, Japan). At least 20 pores were assessed from three different areas of the same samples, though the pores were similar in size for each sample, as shown later. Bulk density was derived from the average pore diameter, sample thickness, and true density of the hybrids, as well as apparent density and apparent porosity. The porosity was calculated as follows [14]

$$\text{porosity}\,(\%) = \frac{V_m - V_p}{V_m} \times 100 = \frac{D \times A - (m_m/\rho_p)}{D \times A} \times 100 \quad (1)$$

where V_m was the whole volume of the sample, V_p the polymer volume involved in the sample, ρ_p the density of the chitosan (0.858 g/cm^3), A the proportion of the sample, m_m the weight of the sample and D was the thickness of the sample.

The porous hybrids were soaked in phosphate-buffered saline solution (PBS, pH 7.4) to obtain water uptake, i.e., the amount of water adsorbed into the porous hybrids according to Eq. (2), where W_w and W_d stand for the weights after and before being soaked in PBS, respectively.

$$\text{water uptake}\,(\%) = \frac{W_w - W_d}{W_d} \times 100 \quad (2)$$

Note here that PBS causes no such effects as hydrolyzing or degrading the samples relevant to the present study. In addition, when the water uptake is presented in mass %, and 90% porosity is assumed for a scaffold with 0.05 g/cm^3 in bulk density, 1800% in water uptake corresponds to absorption of as much PBS volume as the pore volume.

2.3. In vitro cytocompatibility

Cytocompatibility of the porous hybrids was evaluated due to culturing osteoblastic cells MG63 (Dainippon Pharmaceutical Co., Tokyo, Japan). The cells are derived from human osteosarcoma and express a number of features characteristic of osteoblasts [15]. Two mm thick disks of the porous hybrids to be fit in the cell culture plate wells were prepared as described above. MG63 cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air, in Dulbecco's modified Eagle's medium (D-MEM, GIBCO, Japan), 10% fetal bovine serum (FBS), 1% penicillin and streptomycin solution (GIBCO, Japan), and 2.5 mg/ml fungizone and supplemented with 50mg/ml ascorbic acid, 1% MEM-non-essential amino acids (GIBCO, Japan) and 2.0 mM/ml L-glutamine. For the subculture, the cell monolayer was washed twice with phosphate buffered saline (PBS) and incubated with trypsin–EDTA solution (0.05% trypsin, 0.25% EDTA) for 10 min at 37 °C to detach the cells. The effect of trypsin was then inhibited by adding the culture medium at 37 °C. The cells were re-suspended in the culture medium for reseeding. The porous hybrids were soaked in PBS (pH 7.4) solution and sterilized due to autoclaving at 121 °C for 20 min. Then, the porous hybrids were placed into 24-well culture plates, and kept at 36.5 °C for 24 h after addition of D-MEM. The medium was removed from the porous hybrids. The cells prepared above were seeded by adding 200 ml of culture with 2.0×10^4 cells, onto the porous hybrids in the well. Then, the cells were incubated at 36.5 °C for 4 h to allow the cells to attach to the hybrids. Medium (1 ml) was then added to each well. The seeded specimens were evaluated in terms of total protein throughout the incubation time at days 1, 3, 5 and 7. The protein content was determined in 0.1N NaOH cell lysates according to Lowry's method, using bovine serum albumin as the standard. The results

Table 1
Compositions of the precursor solutions and the sample codes. See text for GPTMS

Sample code	GPTMS/NH_2 group of chitosan (molar ratio)
Ch	0
ChG05	0.5
ChG10	1.0

are expressed in mg/cm^2. Some of the specimens were also observed under SEM after they were subject to a few more treatments: after the incubation, the medium in the dish was discarded and the cells were rinsed with 0.5 ml of 0.1 M PBS with neutral pH 7.4, and subsequently were dehydrated using graded ethanol–water solutions of 50–95% concentration for 15 min at each step. Then, The specimens were immersed in 100% ethanol for 15 min 3 times and in 100% *t*-butanol for 30 min three times. They were freeze-dried at 13.3 Pa (0.1 Torr) and −5 °C with a freeze-drying machine (JFD-310, EYELA, Japan). After coating with thin gold film, the fracture surface of the specimens at different depths from the top surface was observed with SEM at an accelerating voltage of 5 kV, with which it was intended to examine how deep the cells infiltrated.

2.4. Statistical analysis

Triplicate experiments were performed. The results are shown as the arithmetic means ± standard derivation (±S.D.). Analysis of the results was carried out using the *t*-test, with a significance level of $p < 0.05$.

3. Results

The lyophilized porous chitosan–silicate hybrids showed sponge-like elasticity, and were formed into three-dimensional (3D) scaffolds of any shape, such as sheet, pellet or disk, column, and bead or granule, as indicated in Fig. 1. The porous hybrids were so flexible that a sheet of the porous hybrids was even rolled up to look like a loaf of roll-cake (see the inset in Fig. 1). Table 2 summarizes the pore characteristics of the typical porous hybrids fabricated in the present study. Their porosity was almost 90%, regardless of composition, but the presence of GPTMS seemed to give a little larger porosity: ∼80% for Ch, and ∼90% for ChG05 and ChG10. In contrast, the pore size was strongly dependent on the freezing temperature, i.e., the size increased with the freezing temperature. The increase in the GPTMS content seemed little to increase pore size. Fig. 2 shows the fracture surfaces of two ChG10 hybrids prepared from the same precursor solutions but frozen at different temperatures: −20 °C and −85 °C. The former gave the pores with ∼110 μm and the latter gave ∼50 μm in size, respectively. Therefore, it is evident that the pore size of the hybrids was controllable in the range from a few tens of μm to a few hundreds of μm by adjusting the freezing temperature. Fig. 3 shows the effect of the composition on the pore size where the fracture surface of Ch and hybrids ChG05 and ChG10 frozen at −20 °C is indicated. Their pores were approximately 110 μm and almost independent of the compositions, while Ch seemed to have smaller pores than the others. Yet, chitosan itself is stiffer, and the pores sometimes are collapsed on fracturing even when a notch is given with a razor blade to the scaffold surface to ease fracturing. From these,

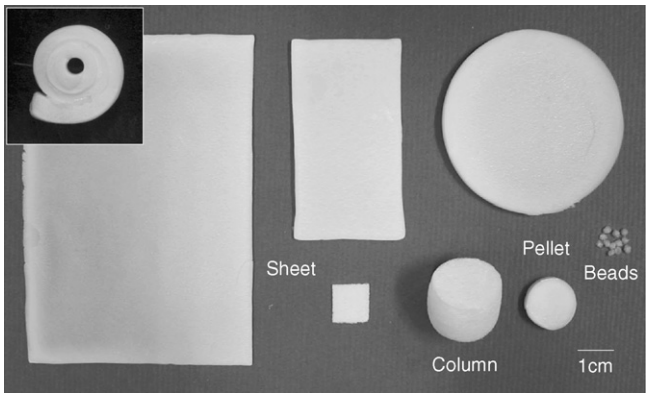

Fig. 1. The porous chitosan–silicate hybrids with sponge-like elasticity, in the shape of sheet, pellet/disk, column, and bead/granule, and even "roll-cake" (inset).

Table 2
Freezing temperature, bulk density, and pore characteristics of the scaffolds

Composition	Freezing temperature (°C)	Bulk density (10^{-2} g/cm^{-3})	Pore size (μm)	Porosity (%)
Ch	−20	9.9 ± 1.9	107 ± 14	83 ± 3
ChG05	−20	5.0 ± 0.6	111 ± 15	93 ± 1
ChG10	−20	7.4 ± 1.5	117 ± 12	89 ± 4
ChG10	−85	2.9 ± 1.0	53 ± 16	97 ± 1

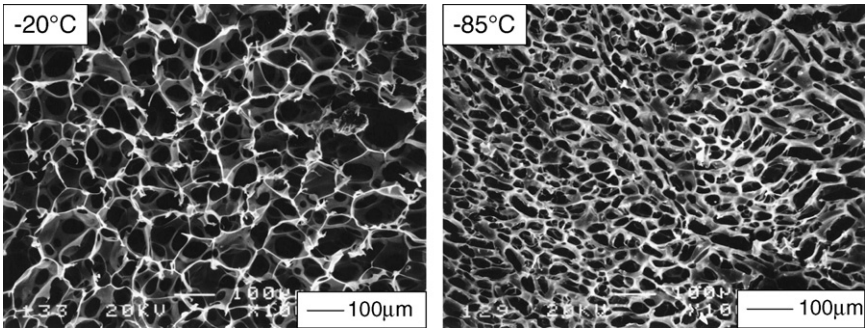

Fig. 2. SEM photographs of the fracture surface of porous hybrid ChG10, frozen at −20 °C (left) and −85 °C (right). The pore size was ∼110 μm and ∼50 μm, respectively.

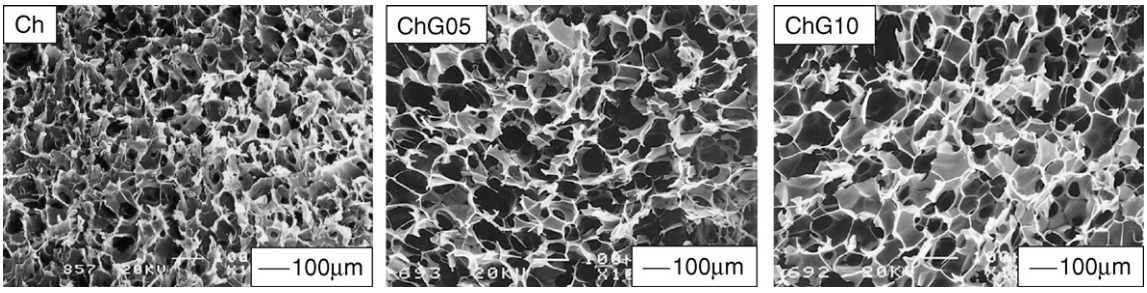

Fig. 3. SEM photographs of the fracture surface of porous scaffolds, Ch, ChG05, and ChG10, frozen at −20 °C. ChG05 and ChG10 had slightly larger pores than Ch.

the pore size of the porous hybrids depends only on the freezing temperature and little on the composition.

When soaked in water (PBS), porous bodies will be filled with PBS, sometimes accompanying degradation or swelling. Fig. 4 shows that all of the present scaffolds gave similar water uptake versus time curves, where each curve reached a plateau or an equilibrium value in 2 h. As Ch, ChG05 and ChG10 were similar in porosity, they were expected to be also similar in water uptake. Contrary to the expectation, Fig. 4 shows that ChG05 and ChG10 could absorb about two times as much water (PBS) as Ch. It means that the porous chitosan–silicate hybrids have more spaces for holding water inside of the hybrids than the porous chitosan. In addition, the volume or shape of all scaffolds scarcely changed during soaking in PBS, or the scaffolds absorbed PBS without collapsing of the 3D pore structure. Fig. 5 shows total protein content of osteoblastic cells MG63 cultured on the porous scaffolds. Total protein is a measure of cell proliferation. Total protein increased as the incubation time regardless of the specimens. At 7 days, total protein for ChG05 and ChG10 was greater than that for Ch though it was similar among those three within 5 days. It means, therefore, that the cells grew better on ChG10 and ChG05 than that on Ch when cultured for a longer period. Fig. 6 shows SEM photographs of the top surface of the samples after MG63 cells were cultured up to 7 days. The

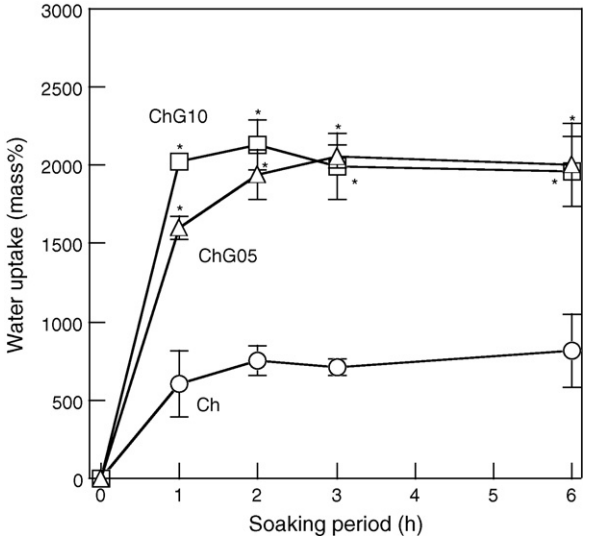

Fig. 4. Water uptake of the scaffolds (frozen at −20 °C) when soaked in PBS up to 7days. *Significant statistical difference between Ch and the ChG hybrids.

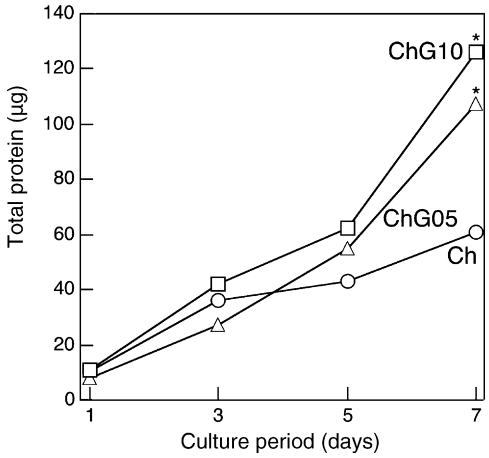

Fig. 5. Total protein content of MG63 cells cultured on the scaffolds. *Significant statistical difference between Ch and the ChG hybrids.

cells attached, proliferated and formed layers covered the mouth of almost all the pores of the hybrids surfaces after culturing 7 days. Fig. 7 shows SEM photographs of the cross-sections of ChG10 after cultured 7 days at different depths from the surface. The cells were infiltrated and, it is to be emphasized, they grew in the pores deep inside of the porous hybrids. They had many pseudopodia and formed the layer on the wall of the pores.

4. Discussion

4.1. Controllable porous structure

The present chitosan–silicate porous hybrids have shown many advantages as described above. Most important aspect is that the present hybrids are controllable in the pore size due to the freezing temperature, that is, the higher freezing temperature yields the larger pores. Such an effect of the freezing temperature agreed with Ren et al. [11,12], Kang et al. [16], and Ho et al. [17]. The GPTMS content, in contrast, caused negligible increase in the pore size as far as the same freezing conditions were employed. In the present study, the entity of the chitosan or chitosan and GPTMS was almost constant in the precursor solutions, or the precursor solutions involved the same volume of dilute acetic acid solution as the solvent. Then, the same pore volume should be obtained on freeze-drying, since the pore volume is basically equivalent to the water volume in the systems. In

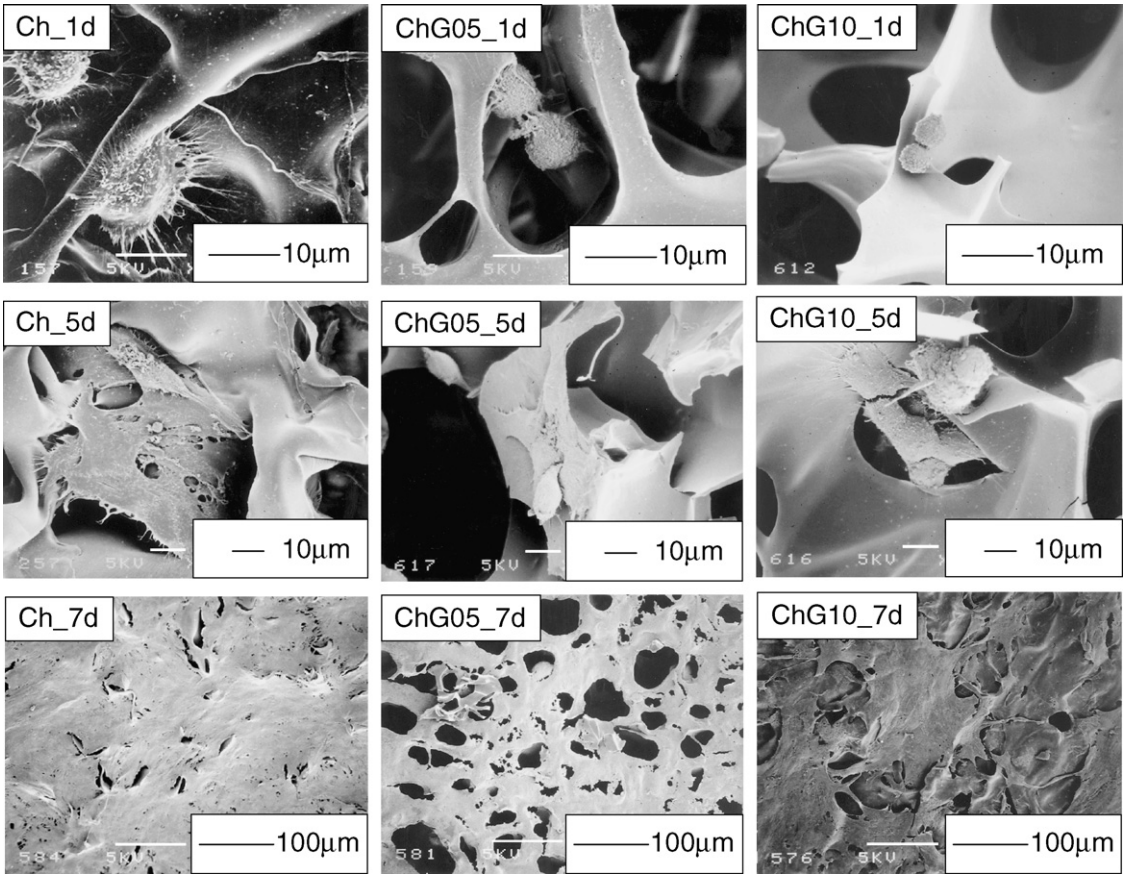

Fig. 6. SEM photographs of osteoblastic cell MG63 cultured on the porous hybrids (ChG05 and ChG10) and porous chitosan (Ch) for 1, 5, and 7 days.

other words, the total pore volume is controllable due to the total concentration of the source materials. In this respect, it is natural that similar total porosity was attained in the present systems.

4.2. Water uptake and hydrophilicity of the scaffolds

Water up take showed a strange behavior: in spite of similarity in porosity among Ch, ChG05 and ChG10, the equilibrium uptake for ChG05 and ChG10 (∼2000%) was more than two times as large as that for Ch (∼800%). Note here that chitosan is insoluble into water, and that water occupies the atomic level space in the matrix as well as the pores. Thus, no other reasons explaining the difference in water up take between Ch and the GPTMS-containing hybrids could be proposed than the difference in the ability of the matrix to hold water. That is, the difference is only interpreted in terms of better PBS affinity of the pore wall or hybrid matrix. How much water can be held in the walls depends on the matrix microstructure? The porosity and bulk density data for Ch frozen at −20 °C yield 830% according to Eq. (1) when all pores are assumed to be filled with water. Here, such assumption is rationalized because it reproduced the empirical water uptake data for Ch. Similarly applying Eq. (1) to the data for ChG05 and ChG10 in Table 2 gives 1220% and 1860%, respectively. Yet, the experiment gave ∼2000% for both scaffolds. Therefore, the respective differences 140% and 780% for ChG05 and ChG10 are attributed to water involvement in the wall matrix with or without swelling. The bulk density data derive such extra volumes of water as 7% and 58% of the original bulk volumes for ChG05 and ChG10, respectively, if no water is assumed to be involved in the wall matrix. Those values seem rather large, but only 2% and 20% increase in the size of the specimen, i.e., swelling can reproduce those extra volumes even if full hydrophobicity of the matrix is assumed. However, when squeezed to drive out water held in the pores, both scaffolds still felt wet, indicating that they held water in the walls. It means that far less swelling rate than 20% is suggested, if any. Difficulty of precisely evaluating the pore characteristics of highly porous materials prevents from deducing definite conclusions in the present case, too. Therefore, although observation with naked eyes did not detect distinct swelling of the scaffolds, it is highly probable that hybrid scaffolds ChG05 and ChG10 did swell a little when soaked in PBS as they hold water in the wall matrix.

The larger swelling rate for ChG10 suggested above implies that the larger content of Si–O species favors more water to be held in the matrix. The larger content of GPTMS apparently introduces many chitosan–chitosan-bridging bonds. If so, ChG10 has more rigid matrix than ChG05, and would deduce a lower degree of swelling. This contradicts with the discussion above. The key to solve this paradox resides in the preparation procedure. After the precursor solutions were first freeze-dried, they were subject to be washed with 0.1 M NaOH solution to get rid of remaining acetic acid from the scaffold pore wall matrices. The matrices involve bridging skeletons like (chitosan)–(GPTMS skeleton)–Si–O–Si–(GPTMS

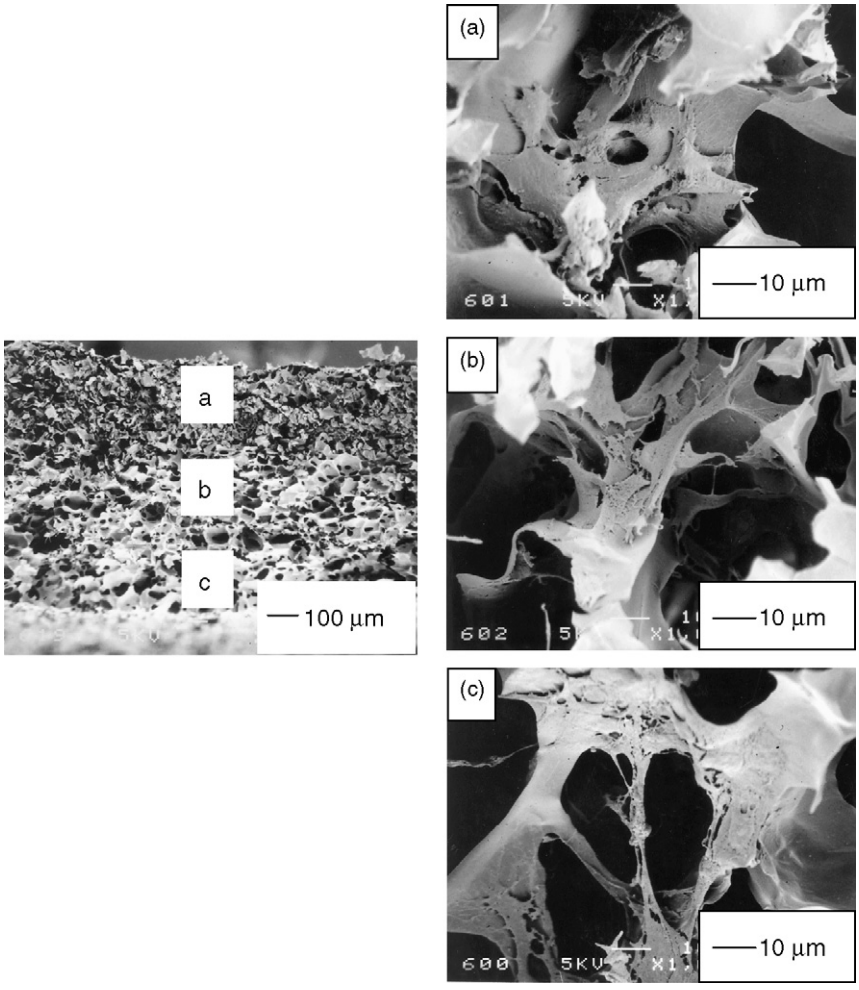

Fig. 7. (Left) SEM photographs of cross-section of ChG10 scaffold, frozen at −20 °C, on which the cells were cultured for 7days. (Right) The larger magnification photographs for cross-section regions (a–c), indicating MG63 cells infiltrated into the scaffold. Depth from the top: region (a) 0–200 μm, region (b) 400–600 μm, and region (c) 800 μm–1000 μm.

skeleton)–(chitosan). The bridging bonds enlarge intermolecular distance of the chitosan. Those –Si–O–Si– bonds in the center of the bridging bonds were prone to be hydrolyzed by the NaOH solution into (chitosan)–(GPTMS skeleton)–Si–OH bonds. Then, the hydrolysis would reduce the tight intermolecular bridging bonds and leave many hydrophilic groups, and hence the matrix would be allowed more to swell when soaked in PBS. In consequence, the large water uptake can be correlated with the presence of GPTMS that improves hydrophilicity and expands the chitosan intermolecular distance, as far as the GPTMS content exceeds 5 mass%. This is another significant advantage of the present porous hybrids: the pore walls keep wet with the body fluid and, when necessary, supply it to the cells attached on the walls, and the pores themselves let the fluid circulating and subsequently induce infiltration and immigration of cells, which favor tissue generation. Indeed, Fig. 7 has confirmed that cell infiltration and migration.

4.3. Biocompatibility

The present hybrids have excellent biocompatibility in terms of osteoblastic cell MG63 culture. The cells adhered and proliferated well on the hybrids. The cells have many pseudopodia and connected with each other. At culture 7 days, most of the pores were covered with the cell layer. It is commonly difficult to disperse the cells into the pores in the scaffolds with 3D porous microstructure and to provide nourishment for the cells. The present chitosan–silicate hybrids not only have greatest porosity and can uptake water in a shorter period but also have many interconnected pores. Therefore, the cell suspension would infiltrate and bring the cells well into the pores. The MG63 cells hence were dispersed well and grew on the walls of the pores. It is indicated that the porous structure, i.e., their size and interconnectivity, in the chitosan–silicate hybrid 3D scaffolds greatly affect the growth of the osteoblastic cells.

5. Conclusions

Precursor solutions derived from chitosan and γ-glycidoxypropyltrimethoxysilane (GPTMS) were frozen at −20 °C and −85 °C before dried to yield 3D porous chitosan–silicate hybrid scaffolds of any shape, such as sheets, beads, or even rollcakes. Porosity was a little dependent on the GPTMS content: ∼83% for chitosan (Ch), and ∼90% for the GPTMS-containing

hybrids, ChG05 and ChG10, while the pore size was independent of the composition but depended on the freezing temperature: 110 μm for −20 °C and ∼50 μm for −85 °C. Thus, the pore size was controllable by the freezing temperature. The introducing GPTMS greatly increased water uptake in mass basis: ∼800% for Ch and 2000% for ChG05 and ChG10. Analysis of total protein for osteoblastic cell MG63 incubating up to 7 days indicated that the presence of GPTMS favored cell proliferation: the cells were not only attached, proliferated and grew on the surface but also migrated deep into the pores, attached, and proliferated on the pore walls. It was thus concluded that these chitosan–silicate porous hybrids were promising for tissue engineering scaffolds.

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