Cell growth and function on calcium phosphate reinforced chitosan scaffolds

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Macroporous chitosan scaffolds reinforced by calcium phosphate powders such as hydroxyapatite (HA) or calcium phosphate invert glass were fabricated using a thermally induced phase separation technique. Human osteoblast-like MG63 cells were cultured on the composite scaffolds for up to 11 days, and the cell growth and function were analyzed. The cell growth is much faster on the chitosan/HA scaffolds incorporated with the glass (CHG) than on the chitosan/HA scaffold without the glass (CH). The total protein content of cells were quantified and increased over time on both composites (CH, CHG) but was significantly higher on CHG after 7 days of culture. The cells on CHG also expressed significantly higher amount of alkaline phosphatase at days 7 and 11 and osteocalcin at day 7 than those on CH. The results suggested that the addition of glass in chitosan/hydroxyapatite composite scaffolds might enhance the proliferation and osteoblastic phenotype expression of MG63 cells. However, the chitosan-matrix scaffolds did not show higher phenotype expression of MG63 cells, in comparison with the TCPS plate, probably due to the degradation of chitosan and release of acidic byproducts. Larger amount of soluble calcium phosphate invert glasses should be added into the scaffolds to prevent chitosan from fast degradation that may affect the differentiation of osteoblast cells.
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1. Introduction

Bone tissue engineering is an emerging interdisciplinary field that applies the principles of biology and engineering to the development of various substitutes that restore and maintain the function of human bone tissues. There are many approaches in bone tissue engineering. One of the most promising approaches involves seeding highly porous biodegradable matrices or scaffolds, in the shape of the desired bone, with cells and signaling molecules (e.g. protein growth factors), then culturing and implanting them into the defected bones to induce and direct the growth of new bone tissues. The cells attach to the scaffold, multiply, differentiate, and organize into healthy bone as the scaffold degrades. Biodegradable polymers have been widely recognized promising as scaffolding materials [1–3], among which, chitosan has recently drawn considerable interest due to its favorable biological properties over other polymers. Chitosan has been proven to be biologically renewable, biodegradable, biocompatible, non-antigenic, non-toxic, and biofunctional [4]. Chitosan surfaces are hydrophilic, which promotes cell attachment and proliferation. Chitosan and some of its complexes have been studied for use in a number of biomedical applications, including wound dressings [5], drug delivery [6–8] and space filling [9]. The use of porous chitosan scaffolds for tissue engineering has also been reported [10]. However, chitosan is mechanically weak and lack of bioactivity, which severely limits its use in bone tissue engineering. Furthermore, massive release of acidic degradation by-products by chitosan *in vivo* may result in inflammatory reactions, and cause biocompatibility problems.

It has been demonstrated that the incorporation of calcium phosphates with bone-bonding capability into a polymer matrix can render the polymer with sufficient bone-bonding ability and improve its mechanical properties [11, 12]. These calcium phosphates can also tailor the degradation and resorption kinetics of the polymer matrix, and improve its biocompatibility and hard tissue integration [13]. The basic resorption products of calcium phosphates can buffer the acidic resorption by-products of the polymer to avoid the formation of an unfavorable environment for the cells due to a decrease in pH [13, 14], and increase initial flash spread of serum proteins compared to the more hydrophobic polymer surface [15]. This eliminates long-term biocompatibility concerns and subsequent surgical intervention. Therefore, it is desirable to develop a calcium phosphate/chitosan composite scaffold suitable for bone repair.

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The incorporation of tricalcium phosphate into chitosan scaffolds and use of these composite scaffolds in bone tissue engineering and drug delivery have been reported [16, 17]. However, tricalcium phosphate degrades very slowly, in comparison with chitosan, which makes it difficulty to control the degradation rate of the composite scaffolds. Bioactive HA and calcium phosphate glasses promoted a rapid new bone formation *in vivo* [18, 19]. Attempts have been made to combine the advantageous properties of HA with those of bioactive glasses [20, 21] by preparing their composites capable of undergoing different degrees of biodegradation, especially for bone grafting, filling or substitution [22, 23]. In our previous work, composite scaffolds were synthesized by incorporating HA and calcium phosphate invert glasses into chitosan matrix, and the experimental results demonstrated that the mechanical properties of the composites and their mineralization in simulated body fluid (SBF) have been significantly improved as compared to pure chitosan scaffolds [24]. In this study, the composite scaffolds were cultured with MG63 cells to examine their effect on cell growth and function. The cell morphology on the scaffolds was examined by scanning electron microscopy (SEM). Cell proliferation/ viability was estimated using a hemocytometer with trypan blue staining. Cell differentiation was evaluated by total protein expression, alkaline phosphatase (ALP) activity and osteocalcin (OC) release, which were measured spectroscopically using an ELISA reader.

2. Materials and methods

2.1. Preparation of composite scaffolds

The chemical composition of the invert calcium phosphate glass was 57.0CaO, $28.0P_2O_5$, $12.0TiO_2$ and $3.0Na_2O$ mol %. The procedure for preparation and characterization of the glass were previously reported [25]. The as-obtained calcium phosphate glass was ground down to about 30 μm in diameter, and HA powders (Sigma-Aldrich) were used as received. Chitosan solutions with concentrations of 2 wt % were prepared by dissolving chitosan in 0.2 M acetic acid. The glass and HA powders were added into the solution to make a polymer/calcium phosphate mixture. The mixture was then rapidly transferred into a freezer at a preset temperature of − 20 °C to solidify the solvent and induce a solid–liquid phase separation. The solidified mixture was maintained at − 20 °C for 8 h and the frozen mixture was then transferred into a freeze-dryer at a preset temperature of − 5 °C. The samples were freeze-dried under a vacuum of 0.5 mmHg for at least 4 days to completely remove the solvent. The compositions of the scaffolds were listed in Table I.

TABLE I Compositions of calcium phosphate/chitosan composite scaffolds

Sample	Chitosan/HA/glass (mol %)
CH	90/10/0
CHG	90/5/5

2.2. Cell culture

Osteoblast-like MG63 cell lines isolated from human osteosarcoma, which has a number of characteristic features of osteoblasts, were used in the experiments. The cells were routinely cultured in 75-cm^2 flasks at 37 °C under a humidified incubator with 5% CO_2. The flasks contain 10 ml Vitacell RPMI-1640 Medium (ATCC), 10% fetal bovine serum (FBS) (ATCC), 2 mML-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin (Gibco). The medium was changed every third day. When confluent, the cells were washed twice with PBS and incubated with trypsin-EDTA solution (0.25% trypsin, 1 mM EDTA) (Gibco) for 10 min at 37 °C to detach the cells, and the fresh medium was added at room temperature to inhibit the effect of trypsin. The cells were washed twice by centrifugation, and resuspended in the medium for reseeding.

2.3. Cell proliferation

Four replicates of each sample were placed into 24-well culture plates, and approximately 5×10^6 cells in 0.5 ml cell medium were seeded by dropping the cell suspension on the material. Then the material was incubated at 37 °C for 6 h to allow the cells to attach to the material, after which the medium was replaced with 1.5 ml fresh medium in each well. In the control cultures, the cells were placed directly into 24-well tissue culture polystyrene plates (TCPS) at the same density as placed onto the samples, and the medium was replaced after 6 h. After 1, 4 and 7 days of culture, the cells were extracted from the scaffolds by two trypsinizations, and the cell number and viability were determined by staining with Trypan Blue and counting using a hemocytometer.

2.4. Total protein content

Total protein content was measured by bicinchoninic acid (BCA) protein assay (Pierce Chemical Co., Rockford, IL, USA). The cell-seeded scaffolds were assayed after culture for 6 h, 1, 4, 7 and 11 days. The medium was removed from the cell cultures and frozen at − 70 °C. Cells were washed twice with PBS and lysed with 1 mL triton X-100 (0.2%). An aliquot of the triton lysate (150 μl) was added to 150 μl of BCA working reagent and the mixture was then incubated for 2 h at 37 °C. The protein concentration was determined from the absorbance at 570 nm wavelength read by an ELISA reader, using a standard protein concentration curve.

2.5. Alkaline phosphatase (ALP) activity

Quantitative ALP activity was determined by an assay based on the hydrolysis of *p*-nitrophenylphosphate (p-NPP) to p-nitrophenol (p-NP) [26]. An aliquot of the triton lysate (50 μl) was added to 50 μl of working reagent containing equal parts of 1.5 mol/l 2-amino-2-methyl-1-propanol, 20 mmol/l p-nitro-phenyl phosphate, and 1 mmol/l magnesium chloride (Sigma). The samples were then incubated at 37 °C for 30 min. After incubation, the reaction was stopped with 100 μl 1 N sodium hydroxide. ALP activity was determined from the absorbance of p-nitrophenol at 410 nm wavelength

using an ELISA reader and expressed as micromoles per milligram protein per minute.

2.6. Osteocalcin assay

After each period of culture time, the intact osteocalcin levels were determined by ELISA (Biomedical Technologies, Inc., Stoughton, MA, USA) with antibodies raised against bovine osteocalcin, which cross-reacts with human osteocalcin. The BTI intact osteocalcin ELISA kit measures only intact osteocalcin which is synthesized by the osteoblast and it eliminates any interference by circulating fragments. The osteocalcin level was determined from the absorbance of substrate at 450 nm wavelength and expressed in units of ng/μg protein.

2.7. Cell morphology by scanning electron microscopy (SEM)

The cells were fixed on scaffold samples with 2.5% glutaraldehyde in 0.14 M sodium cacodylate (pH 7.3) for 3 h at room temperature, and dehydrated in 70%, 80%, 90%, 95%, 100% ethanol, respectively for 10 min. The samples were then dried and gold-coated under an Argon atmosphere using a sputter coater set at 18 mA for a total of 120 s, before analyzed using a JEOL scanning microscope (JEOL 840A) equipped with energy dispersive X-ray (EDS) spectroscopy (Tracor Northern 5400 EDS System).

2.8. Statistical analysis

Data were presented from one of two replicate experiments which both yielded comparable results. Four replicates were used for each sample and data was expressed as the arithmetic mean $\pm$ standard deviation (SD). ANOVA test was carried out to determine the statistical significance, and the differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. SEM examinations

Both CH and CHG composite scaffolds were macroporous and had the same interconnected open pore microstructure with a pore size around 100 μm (Fig. 1). The high porosity of these composite scaffolds allowed more cells to adhere to the scaffold surface. After 4 days of culture, cells exhibited a fibroblastic spindle-shaped morphology on TCPS in the control culture, showing numerous, highly extended filipodia and rough dorsal surfaces characteristic of active cells, although they had more squamous cell bodies when adhered to the plate surface (Fig. 2(a)). Cells on both CH and CHG substrates were round and less flattened in appearance and appeared less spread out over the surface (Fig. 2(b), (c)).

3.2. Cell proliferation and viability

The number of cells on CHG was significantly higher than that on CH over the culture period, as shown in Fig. 3. At day 11, the number of cells on CHG and CH were $2.5 \times 10^6 \pm 5.1 \times 10^4$ and $2.2 \times 10^6 \pm 1.4 \times 10^5$ ($p < 0.05$), respectively. In comparison with the TCPS control, the cell number on CHG was significantly higher at 1 day and the same as that on TCPS after 4 days. The results of cell viability in Fig. 4 showed that more than 90% of cells were live on both CHG and CH substrates after 4 days of cell culture. However, a significant decrease in cell viability for CH was observed after 7 days, while the cell viability for CHG kept high, corresponding to the rates of living cells of 89.4% and 95.8%, respectively.

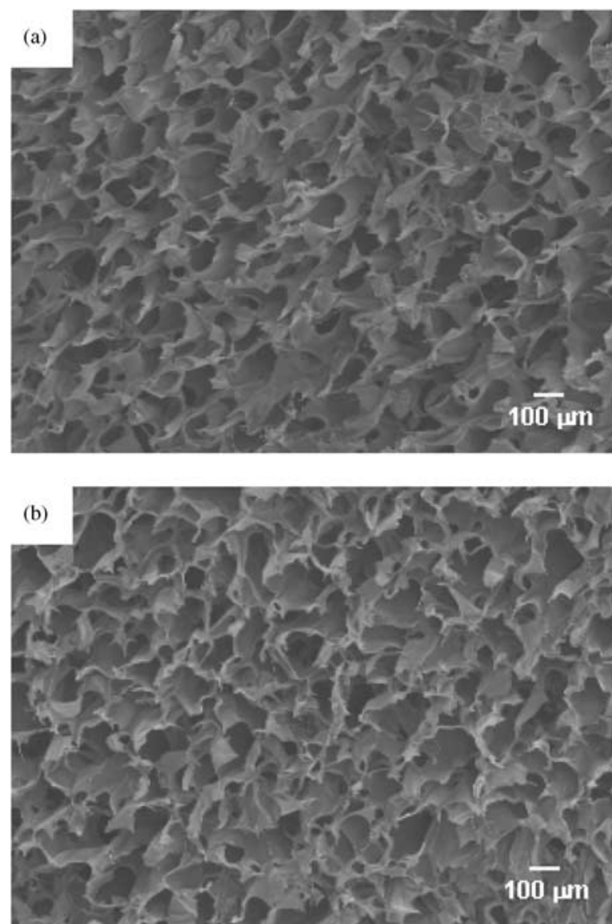
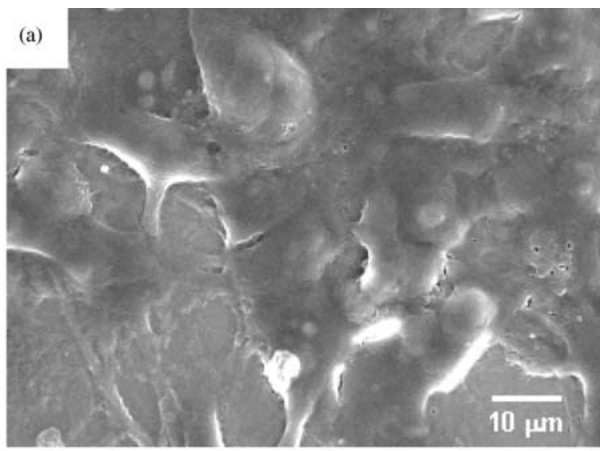
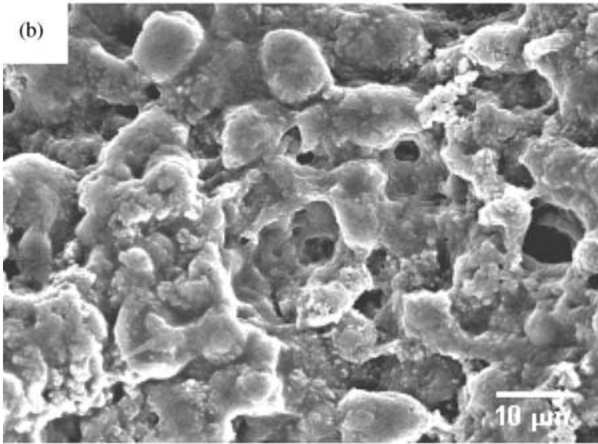


Figure 1 Scanning electron micrographs of the composite scaffolds. (a) CH, (b) CHG.

3.3. Total protein content

The total protein content of the MG63 cells cultured on CH and CHG as a function of incubation time was given in Fig. 5. For cells grown on the both substrates incubated for up to 11 days, the total protein content increased with the incubation time. There was no significant difference in the total protein content between CH and CHG during the first 4 days. However, the total protein contents of cells after 7 and 11 days of culture were in the sequence: CHG > CH > TCPS. The results showed that alterations in total protein content were observed in the presence of the calcium phosphate glass in the scaffolds, with the significance $p < 0.05$.





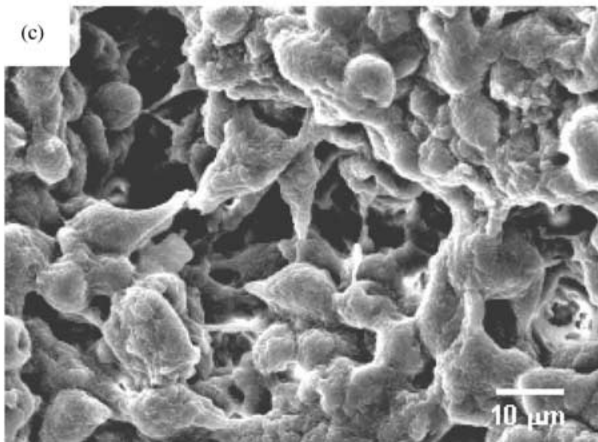


Figure 2 Scanning electron micrographs of MG63 cells after 7 days of culture on (a) TCPS, (b) CH and (c) CHG.

3.4. Alkaline phosphatase activity

The identification and characterization of osteoblast-like cells are based on the assays of specific metabolites. For this purpose, the activity of ALP, a membrane enzyme routinely used in *in vitro* experiments as a relative marker of osteoblastic differentiation [27], was determined and the results are shown in Fig. 6. ALP produced by MG63 cells propagated on CH and CHG was normalized to total protein content. Results relative to the ALP activity were expressed in nmol/mg protein/min. The ALP level of MG63 cells grown on CHG was higher than that on CH after 7 and 11 days of culture, and apparently, the scaffolds containing the glass (CHG) resulted in a significant increase in ALP activity, by 23% and 12.6% ($p < 0.05$), respectively.

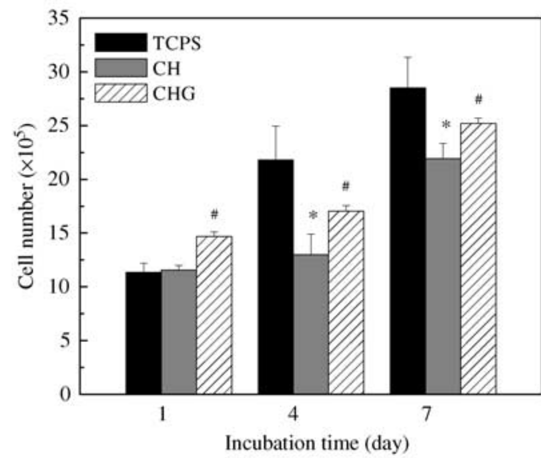


Figure 3 Number of MG63 cells after culture on TCPS, CH and CHG. Values are the mean ± SD of four cultures. * $p < 0.05$, composite scaffolds versus TCPS; # $p < 0.05$, CHG versus CH.

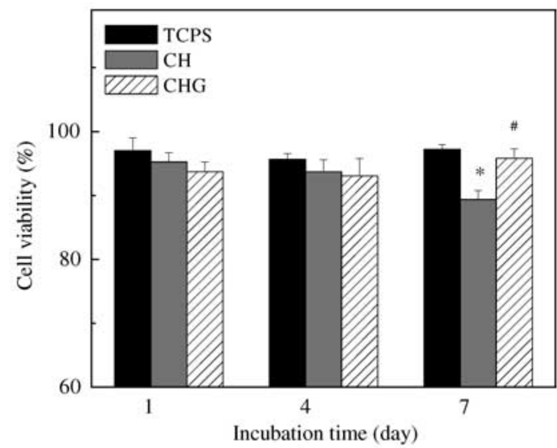


Figure 4 Viability of MG63 cells after culture on TCPS, CH and CHG. Values are the mean ± SD of four cultures. * $p < 0.05$, composite scaffolds versus TCPS; # $p < 0.05$, CHG versus CH.

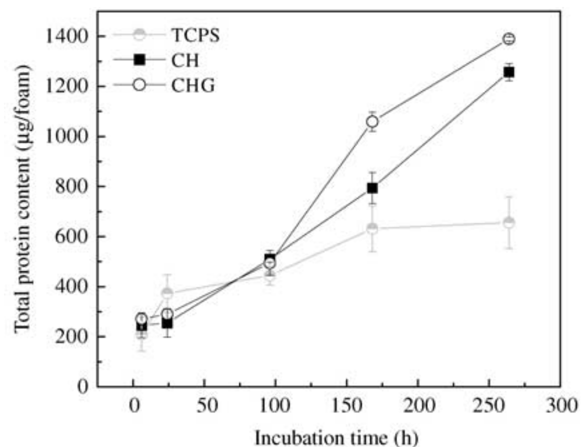


Figure 5 Total protein contents of MG63 cells extracted from the composite scaffolds over time. Values are the mean ± SD of four cultures.

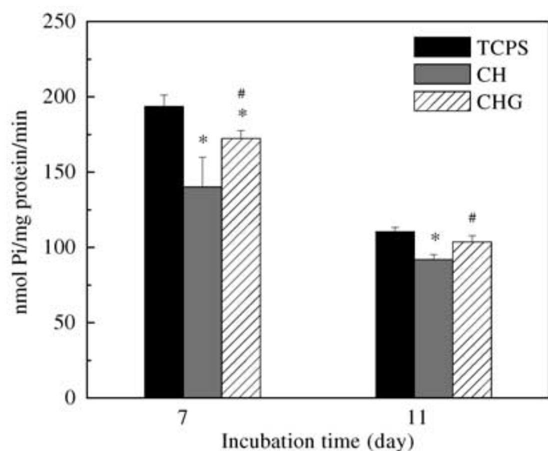


Figure 6 Alkaline phosphatase (ALP) activity of MG63 cells on the composite scaffolds after 7 and 11 days of culture. Values are the mean $\pm$ SD of four cultures. * $p < 0.05$, composite scaffolds versus TCPS; # $p < 0.05$, CHG versus CH.

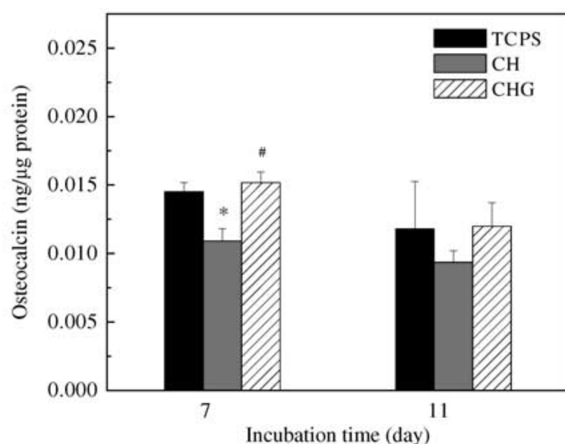


Figure 7 Osteocalcin production of MG63 cells on the composite scaffolds after 7 and 11 days of culture. Values are the mean $\pm$ SD of four cultures. Values are the mean $\pm$ SD of four cultures. * $p < 0.05$, composite scaffolds versus TCPS; # $p < 0.05$, CHG versus CH.

3.5. Osteocalcin secretion

Osteocalcin is another osteoblastic marker and plays a role in bone mineralization. It is primarily deposited into the ECM of bone and only a small amount of newly synthesized osteocalcin is released directly into the circulation. Osteocalcin produced by MG63 cells propagated on CH and CHG was normalized to total protein content (Fig. 7). Similar to the increase in ALP activity induced by the glass in MG63 cell culture, results indicated that osteocalcin levels secreted by the cells cultured on CHG were significantly higher at day 7 (39%, $p < 0.05$), compared to that of cells cultured on CH. Osteocalcin (OC) secretion was upregulated by the glass.

4. Discussion

An ideal scaffolding material for bone tissue engineering should be able to promote the osteoblast proliferation and expression of the osteoblastic phenotype. Recent work has demonstrated that tricalcium phosphate/chitosan sponges support the proliferation and differentiation of seeded osteoblastic cells, indicated by a high ALP activity and the deposition of mineralized matrices by the cells [16]. This work concluded that the tricalcium phosphate/chitosan sponges were excellent candidates to serve as scaffolding materials for tissue-engineered bone formation *in vitro*. However, these sponges do not have controlled biodegradation due to very slow resorption of tricalcium phosphate. In this study, we examined the use of HA/glass/chitosan composite scaffolds as template materials for osteoblast-like cell growth and *in vitro* expression of osteoblastic phenotype, and studied the effect of calcium phosphate glass on the cell proliferation and differentiation. Our results showed that the glass promoted the growth and maturation of osteoblast-like MG63 cells on the composite scaffolds.

The MG63 cell population on both CH and CHG substrates increased consistently with the cell culture time, and the number of cells grown on CHG increased faster than that on CH. This suggested that the incorporation of the calcium phosphate glass into chitosan matrix improved the growth of the cells. The analysis on the cell viability showed that, after 4 days of culture, there were more viable cells growing on CHG than that on CH, indicating the possibility of improving long term biocompatibility of the composite scaffolds by the addition of the glass. The slow rate of cell proliferation and relatively low protein content of the MG63 cells seeded on CH may be attributed to the difference in the physicochemical characteristics of the surface of the CH and CHG substrates, which not only affects the protein adsorption but also the subsequent protein-mediated cell function. The glass containing sodium, calcium and phosphate as its main components undergoes corrosion in the culture media with a leaching of alkali ions, resulting in the formation of a calcium phosphate layer on the scaffold material surface. One possible function of this layer is to selectively adsorb serum glycoproteins, such as fibronectin [28] and other factors that may serve to enhance cell growth and function.

Previous work has shown that even though a biomaterial surface supports the growth and division of osteoblast cells, they often alter their phenotypic expression, leading to a loss of their key characteristics, such as bone matrix formation and bonding [29]. Two parameters are typically used as markers of osteoblastic differentiation: alkaline phosphatase (ALP) activity and osteocalcin production. Osteoblasts usually exhibit higher basal levels of alkaline phosphatase, a relatively early differentiation marker, than cells that do not mineralize their matrix, such as fibroblasts [30]. At the same time, the osteoblasts begin to produce mRNA for osteocalcin, the most abundant noncollagenous protein of the mineralized extracellular bone matrix. Osteocalcin binding calcium plays a role in matrix mineralization, and represents the later state of osteoblastic differentiation. MG63 cells grown on CHG showed both higher ALP activity at day 7 and 11 and higher osteocalcin production at day 7 than the cells on CH. The significant increase in ALP activity and osteocalcin production was induced by the addition of the glass. It is worth noting that although bioactive HA is incorporated into chitosan matrix, the produced enhancement on osteoblastic activity by HA is significantly less than that by calcium phosphate invert glass. The chitosan-matrix scaffolds did

not show higher phenotype expression of MG63 cells, in comparison with the TCPS plate, probably due to the degradation of chitosan and release of acidic byproducts. Larger amount of soluble calcium phosphate invert glasses should be added into the scaffolds to prevent chitosan from fast degradation that may affect the differentiation of osteoblast cells.

5. Conclusion

In summary, macroporous chitosan scaffolds incorporated with hydroxyapatite (HA) and calcium phosphate invert glass were prepared to study the cell growth and function on these composite scaffolds. Our study showed that the incorporation of the glass into chitosan matrix led to enhanced cell growth, ALP activity and osteocalcin production, in comparison with HA.

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