Original Research Article

Preparation and biomineralization of injectable hydrogel composite based chitosan-tetronic and biphasic calcium phosphate nanoparticles

Abstract

In this study, a hydrogel composite was enzymatically prepared with rapid gelation time from tyramine-tetronic-conjugated chitosan and biphasic calcium phosphate (BCP) nanoparticles. The compressive stress of the hydrogel composite was reached at 591 ± 20 KPa with 10wt% of BCP loading. Degradation study of the material showed 20% of weight loss after 4 weeks. *In vitro* study with MG 63 osteoblast cell evidenced that the cells were well-attached on hydrogel composite surfaces. Biomineralization on the hydrogel composites surfaces was well-observed after soaking for 14 days in simulated body fluid (SBF) solution. The obtained results indicated that the hydrogel composite could be an injectable potential material for bone regeneration.

Key words

Biomineralization, injectable, hydrogel composite, BCP nanoparticles, bone regeneration

Introduction

Recently, use of bioactive hydrogel scaffolds has been paid much attention in bone regeneration. The hydrogel scaffolds have highly porous 3D structure which creates a microenvironment for cell encapsulation, allowing nutrients and metabolites to diffuse to and from cells. However, most polymeric hydrogels do not occur a biomineralization which can be evaluated via formation of native apatite nanocrystals in SBF). Addition of an inorganic phase can produce a biomineralizable composites [1, 2]. Beside that the degradation of the hydrogel network also affects to the replacement by new bone formation, thus increases mechanically stability [3]. It is also well-known that the degradation times and mechanical properties of organic – inorganic composite materials are relevant and they can be controlled by the concentration of inorganic phase adding [4, 5]. In the presence of the loaded inorganic phase, the composites could promote nucleation and subsequent proliferation of calcium phosphate crystals that is often known as the capacity of a specific class of bone-substituting material to induce calcification [1]. This is an essential requirement for artificial material to generate to bonelike apatite and living bone. It also helps to neutralize pH caused by by-products, thus minimizing excessive inflammation around the implantation site [6, 7]. The

interactions between biological activity and its surrounding environment cause this precipitation [8]. Moreover, the loaded inorganic phase could provide cell adhesion sites for enable integration with surrounding bone tissue [9, 10]. There are many sources of inorganic phases, but one of the most commonly used calcium phosphate ceramic is BCP (mixture of hydroxyapatite and β-tricalcium phosphate) nanoparticles [11-13]. The nanoparticles have been utilized as inorganic phase for loading in several kinds of composite for bone regeneration.

This work focused on the preparation of hydrogel composite from chitosan derivative and BCP nanoparticles and evaluated its biomineralization via the formed apatite precipitate. Other features of hydrogel composite have also been studied to prove that this kind of material can be applied in bone regeneration.

Materials and methods

Chitosan (Low Mw), p-nitrophenyl chloroformate (NPC), tyramine (TA) were purchased from Acros Organics. HRP (type VI, 298) was purchased from Sigma-Aldrich. Calcium chloride and trisodium phosphate were purchased from Merck, Germany. Tetronic 1307 (Te, MW=18,000) was obtained from BASF. For the in vitro study, Fetal bovine serum (FBS), penicillin streptomycin antibiotic (PS), 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) solution, and trypsin-EDTA were purchased from Gibco, Carlsbad, CA. MG-63 osteoblast cells were derived from rabbit osteosarcomas.

Preparation of BCP

BCP NPs were synthesized by using an ultrasonic associated process as below formulation. Calcium chloride ($CaCl_2$) were dissolved in 1.5 L distilled water and trisodium phosphate (Na_3PO_4) were dissolved in 2.5 L distilled water with molar ratio of Ca/P = 1.57. $CaCl_2$ solution was put in an ultrasonic bath then adjusted pH 7 after that Na_3PO_4 solution was also put in the ultrasonic bath. The reaction was occurred in 12 hours at 50°C to obtain a white suspension. The precipitate was washed thoroughly with distilled water and filtered before it was dried in an oven at 70°C. Finally, the calcination was carried out at 750°C in air [11, 13].

Preparation of TTeC copolymer

TTeC copolymer was prepared as previously described [14]. The process to produce tetronic-grafted chitosan containing TA moieties is the combination of three synthetic

reactions without using any organic solvent to purify [14]. Briefly, the hydroxyl groups of tetronic were activated by NPC, then TA was partially added to conjugate into the activated product and the remaining moiety of tetronic-TA grafted onto chitosan to produce TTeC copolymer.

Preparation of hydrogel and gel composite

Preparation of hydrogel: 40mg TTeC was dissolved in 260 µL phosphate buffered saline (PBS) solution pH 7.4, and then, equally separated into two ependroff tubes. The PBS solutions of HRP (50 µL of 0.2 mg/ml) and H_2O_2 (50 µL of 0.2% wt/vol) were separately supplemented to each tube. TTeC hydrogel was immediately formed by mixing the solutions of 10% wt/wt polymer.

Preparation of hydrogel composite: Preparation of the TTeC/BCP hydrogel composites was done with same protocol in which BCP NPs (5 and 10% wt/wt) were added to two precursor copolymer solutions.

Gelation time of the hydrogel or hydrogel composite: The test tube inverting method was used to determine the gelation time. The solution was observed by inverting the vial and the gelation time was recorded when the solution stopped flowing. It was studied when the concentration of HRP was changed and the concentration of H_2O_2 was kept at constant.

Characterizations

The morphology and microstructure of the synthesized BCP powder was investigated by using FESEM (JSM-635F, JEOL). Compressive tests of the hydrogel composites were performed on a Universal Testing Machine (Unitech TM, R&B, Korea). To investigate the components of the hydrogel composite, the samples were analyzed via XRD (D8/Advance, Bruker, UK) with CuKα, (λ=1.5406 Å) as a radiation source over the 2θ range of 10 - 60º. Water uptake of the hydrogels was determined by using the gravimetric method. The hydrogel composites were lyophilized and weighed (W_0). These lyophilized hydrogels were immersed in 10 mL SBF solution at 37^0C for 2 days to reach equilibrium swelling. Surface water was removed and the samples were weighed (Ws). The water content in these lyophilized hydrogels was expressed by using the following equation: $\frac{W_s - W_0}{W_o}$ x 100%. The degradation of hydrogel composites was studied in PBS. The hydrogel composites were lyophilized and weighed (W_0). These lyophilized hydrogels were immersed in 10 mL PBS solution at 37^0C. After regular time intervals, surface water was removed from the samples

and washed with deionized water to remove the soluble inorganic salt then weighted (Wt) after lyophilization. The percentage of weight loss is calculated to evaluate the degradation of hydrogel and gel composite as following formula:

$$\text{Weight loss (\%)} = \frac{W_0 - W_t}{W_0} \times 100\%$$

Cell proliferation study

Firstly, MG-63 cells (5×10^4) seeded onto the UV-sterilized samples in 24-well plates for incubation, and came up with washing step by PBS for three times. The cell nuclei were counterstained with 20 mg/mL DAPI for 10 min at room temperature, the sample was then washed 3 times with 1X PBS. Finally, confocal laser scanning microscope (FV10i-W) was used to observe the stained cells on hydrogel composites after 5 days of cell seeding.

Biomineralization evaluation

To study the possible precipitate phase conversion, hydrogel composite samples immersed in a SBF buffer solution (pH 7.4). TTeC/BCP hydrogel composite was prepared and then lyophilized. Lyophilized hydrogel composite was cut to observe spongy surface then recorded its weight. Hydrogel composite was collected after 7 and 14 days of soaking in SBF and then washed with deionized water to remove the soluble ionorganic salt then weight (Wt) after lyophilization to confirm the decomposition of hydrogel composite. Finally, hydrogel composite was characterized by SEM, EDS and XRD.

Results and discussion

Characterizations of hydrogel composite

Figure 1 demonstrates in situ formation of hydrogel composite from two suspensions of BCP NPs and TTeC polymer in the presence of HRP enzyme. The gelation time of the TTeC/BCP hydrogel composites depended on concentration of HRP and H_2O_2 (Figure 2). A change in HRP concentration with minimal supply of H_2O_2 could result in reducing the gelation time due to the production of more phenolic radicals in the TTeC polymer solution. The minimal amount of the used H_2O_2 could be easily determined by evaluation phenolic content in the polymer solution. With the high concentration of HRP (0.1 mg/ml), it took about 3 seconds to form the hydrogel composite and when the concentration of HRP was 0.05 mg/ml, it took about 6 seconds to form hydrogel composite.

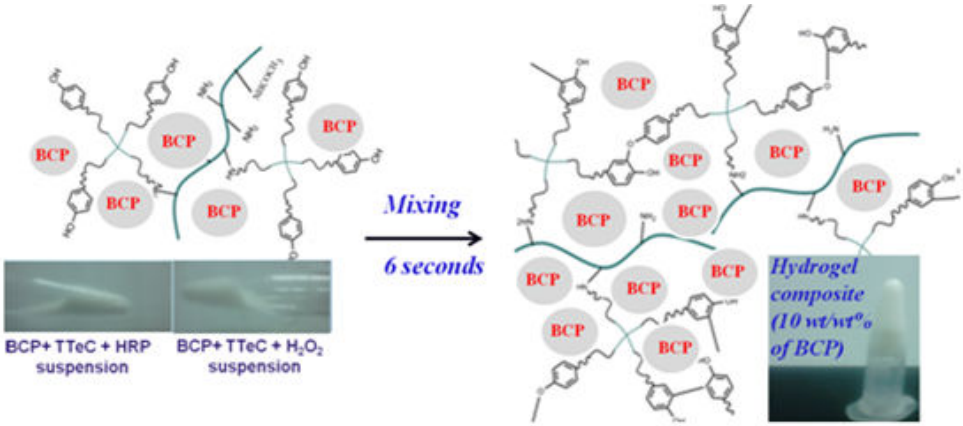

Figure 1. Formation of TTeC/BCP hydrogel composite

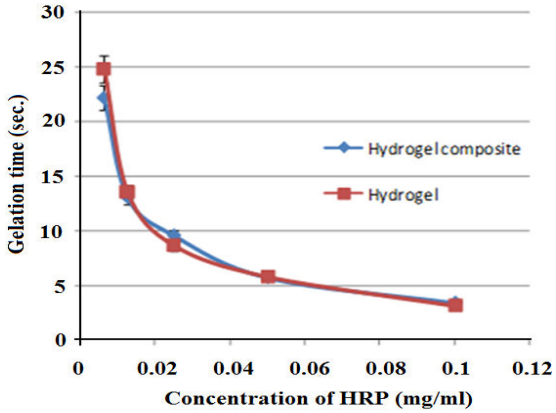

Figure 2. Effect of HRP concentration with feeded 0.05 wt% of H_2O_2 on gelation time of TTeC hydrogel and TTeC/BCP hydrogel composite at 10% wt/wt polymer in solution

Water uptake of scaffold is related to its (their) ability to absorb body fluid and transfer cell nutrients and metabolites and is one of the most important properties of biomaterials. Figure 3 shows the water utilization by hydogel composites. Both TeTC and their hydrogel composites could absorb water more than their own weight around 200%. The water uptake of TTeC-BCP hydrogel composite with 0, 5, and 10 wt% BCP was 472.28 ± 15, 331.48 ± 14, and 228.35 ± 14% respectively. The water uptake decreased with an increase in the content of BCP NPs, that means the water uptake increased with increasing the concentration of TTeC polymer matrix. It can be explained by the hydrophilicity of the chitosan polymer matrix for the absorption of the body fluid that could have resulted in the more concentration of polymer matrix, the higher water retention [15, 16]. Moreover, released phosphate ions from BCP play a role as a crosslinker for chitosan network that increased the crosslinking density resulting in reducing water uptake of the composite [17]

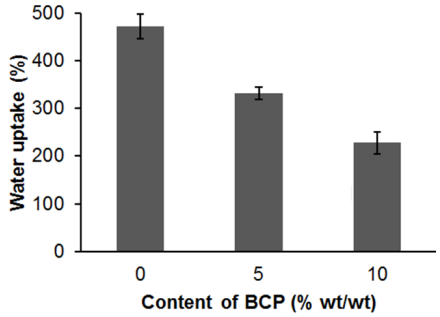

Figure 3. Water uptake of hydrogel composite at different content of BCP

Degradation behavior of scaffolds plays an important role in tissue regeneration because scaffolds create and maintain a space for cell attachment and proliferation. The hydrogel composites should be maintained for a suitable time to replacement of new tissue. The degradation of the hydrogel composites was investigated by examining the weight loss of the hydrogels as a function of incubation time in PBS at 37^0C. Figure 5 shows the degradation profiles of the TTeC/BCP hydrogel composites with different content of BCP NPs. Degradation of the hydrogel composites was maintained for over 1 month. The degradation of the TTeC/BCP hydrogel composites increased mass losses with increase in the polymeric matrix content. After 4 weeks, the weight loss ratios of the hydrogel composite containing 0 wt/wt%, 5 wt/wt% and 10 wt/wt% BCP NPs were 38%, 25% and 20% respectively. The degradation behavior of hydrogel composites was affected by adding of BCP NPs. It could be explained by the interaction between the released ions from BCP NPs and chitosan polymeric matrix. The NH_2. OH groups of chitosan and OH group of HAp in BCP NPs have hydrogen bonding as well as the chelation between NH_2 group and Ca^{2+} [17]. The degradation of hydrogel composite could be significant for growth of bone cells and new bone replacement [15, 16].

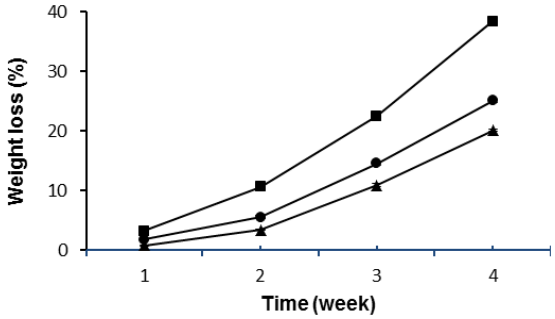

Figure 4. Weight ratios of TeTC/BCP hydrogel composites depending on content of BCP) ■ 0 wt/wt% BCP, ● 5 wt/wt% BCP, ▲ 10 wt/wt% BCP.

The compressive strength values of the TTeC/BCP hydrogel composites were determined 138.7 ± 15.9, 235.3 ± 15.3, and 591.7 ± 19.5 KPa for 0, 5, 10 % (wt) of the loaded BCP NPs, respectively (Figure 4). The compressive strength of the hydrogel composites was increased with increment in amount of the feed BCP NPs. This could be explained thatincorporation of an inorganic reinforcing phase and interface adhesion of BCP particles within the hydrogel resulting in reinforcing of the polymer matrix. Moreover, the chemical interaction between the NH_2 group of chitosan and OH group of HAp in BCP provided interfaces between polymer matrix and BCP particles [18-20]. Then, addition BCP into scaffolds improved the compressive strength of the composites.

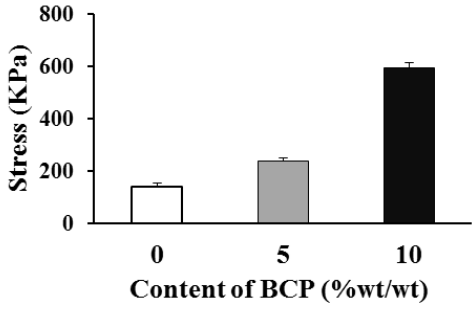

Figure 5. Compressive strength of the TTeC-BCP hydrogel composites.

Biocompatibiliy of the hydrogel composite

DAPI-stained cell attachment on hydrogel composites was observed via fluorescence microscopy. The nuclei of the cells were blue after staining. The immunostained images exhibited a well attachment and proliferation of the MG-63 osteoblast cells on the surface of the hydrogel composites. After 5 days of incubation, cells were well-adhered on surface of the composites, more cells were observed on the TeTC/BCP hydrogel composite with higher BCP content. This great surface adherence was due to a high biocompatibility of hydrogel combined with effective characteristics of BCP such as rough surface creation the roughness surface and positive influence of calcium phosphate on the behavior of osteoblast cells, leading to enhanced cellular attachment [21, 22]. Viability and cell adhesion data confirmed that hydrogel composites are biocompatible.

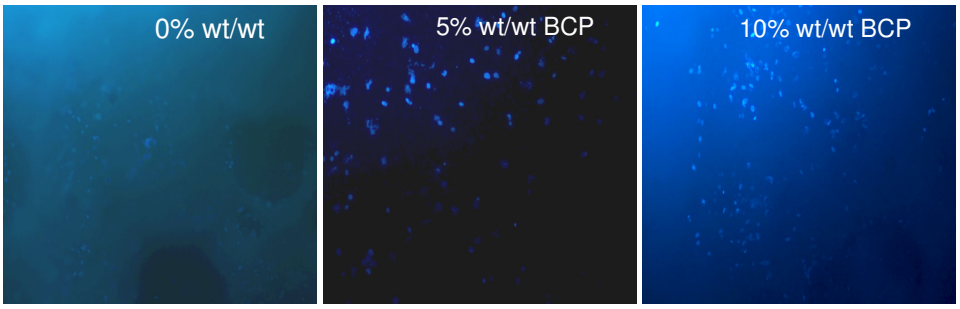

Figure 6. Confocal images of osteoblast cell MG-63 adhesion on TTeC/BCP hydrogel composites with different content of BCP after 5 days of incubation

Biomineralization of the hydrogel composite

Biomineralization study was used to predict the bioactivity of biomaterial in vivo study such as bone-bonding ability of bioactive materials. This mean, the apatite layer bond the biomaterial and the living bone [22, 23]. For hydrogel composite, mineralization behavior could be observed the apatite forming ability when samples were immersed in SBF for various periods of time. Figure 7 showsSEM micrographs of the surface morphology of hydrogel composites on which nucleation and growth of the precipitated-phase nanocrystals are visible on the surface of samples after 7 and 14 days. Figure 7(a) and (d) showed no apatite formation on surface of the TeTC hydrogel after 7 and 14 days of immersion in SBF. In contrast, the TeTC/BCP hydrogel composites were able to form the apatite layer when were being soaked in SBF. After incubation in SBF for 7 days, numerous tiny granular apatite particles were deposited on the hydrogel composite surfaces. Increasing the BCP content could result in forming more apatite crystals (Figure 7 (b), (c) and (e), (f)). The mineralization also increased with increasing immersion time. The results indicate that BCP enhanced the apatite forming ability on composites. BCP particles act as apatite nucleation sites, and then the apatite nuclei could grow by consuming the calcium and phosphate ions in the surrounding environment. Hence, the formation of apatite is more efficiently on the composite with higher content of BCP. The apatite precipitation through SBF test is a consequence of a dissolution and precipitation process. BCP in the composite gradually dissolves and releases calcium and phosphate ions which are beneficial to apatite formation. Moreover, the hydroxyl and phosphate unit in HAp, TCP crystal structure reveal negative charge of BCP particle surface when immersed in SBF. This negative charge attracted the positive calcium ions in SBF to form rich calcium surface, which interacts with the negative phosphate ions in SBF. As consequence, calcium and phosphate ions migrated to surface of hydrogel composite that induced the apatite precipitation [24-26]. EDS results in Figure 7

indicate that the precipitates on surface of TeTC/BCP hydrogel composite are calcium, phosphorus and oxygen due to the composing element of apatite, which could be further confirmed by XRD analysis (data not shown here). No new peaks appeared after immersion in SBF was observed in XRD data. Hence, the precipitation on the surfaces of hydrogel composites is apatite.

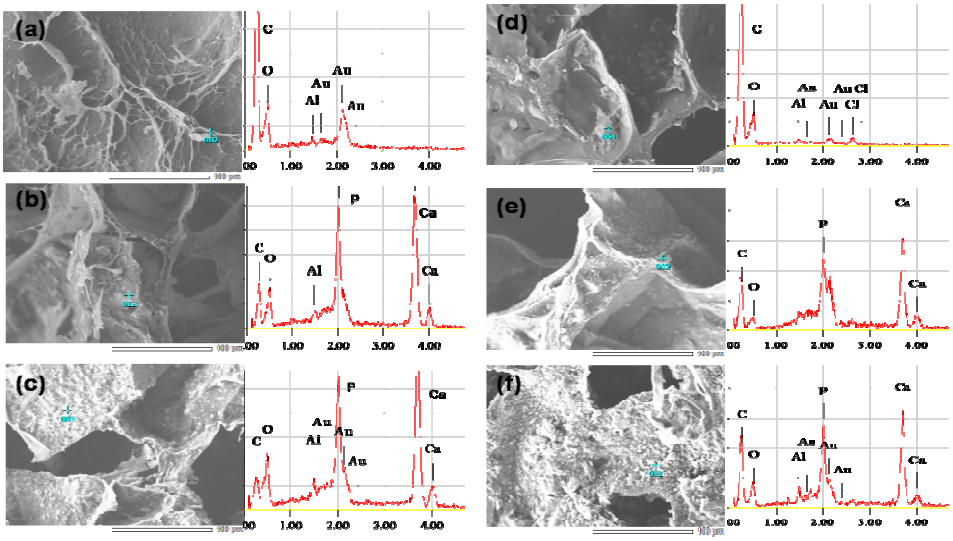

Figure 7. SEM micrograph and EDS profile of the composites without BCP NPs (a, d), with 5% BCP NPs (b, e) and with 10% BCP NPs (c, f) after soaking in SBF 7 and 14days.

Conclusions

Injectable polymer-grafted TeTC-BCP hydrogel composites prepared successfully. The obtained results indicated that water absorbance, degradation behavior, compressive strength and cell attachment as well as biomineralization of the hydrogel gels are dependent on the content of BCP. Especially, the ability of forming apatite as well as cell proliferation of hydrogel increased with higher content of BCP because BCP has positively influences on the behavior of osteoblast cells and biomineralization process. These composite hydrogels have the advantages of large amount of water absorbance, good mechanical strength, and suitable degradation time, all of which are important to the regeneration of new bone tissue. TTeC-BCP hydrogel composites could be promising materials for bone regeneration.

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