Enzyme-mediated \textit{in situ} preparation of biocompatible hydrogel composites from chitosan derivative and biphasic calcium phosphate nanoparticles for bone regeneration

To cite this article: Thi Phuong Nguyen et al 2014 \textit{Adv. Nat. Sci: Nanosci. Nanotechnol.} \textbf{5} 015012

View the article online for updates and enhancements.

Related content

- Organ Printing: Natural, synthetic and semi-synthetic polymers
  D-W Cho, J-S Lee, J Jang, J W Jung, J H Park and F Pati

- Enzymatic in situ formed hydrogel from gelatin-tyramine and chitosan-4-hydroxyphenylacetamide for the co-delivery of human adipose-derived stem cells and platelet-derived growth factor towards vascularization
  Nguyen Thuy Ba Linh, Celine D G Abueva and Byong-Taek Lee

- Biocompatible nanomaterials based on dendrimers, hydrogels and hydrogel nanocomposites for use in biomedicine
  Cuu Khoa Nguyen, Ngoc Quyen Tran, Thi Phuong Nguyen et al.

Recent citations

- Degradation Regulated Bioactive Hydrogel as the Bioink with Desirable Moldability for Microfluidic Biofabrication
  Xiaolu Liu et al

- I. Pereira et al

- Biocompatible nanomaterials based on dendrimers, hydrogels and hydrogel nanocomposites for use in biomedicine
  Cuu Khoa Nguyen et al
Enzyme-mediated in situ preparation of biocompatible hydrogel composites from chitosan derivative and biphasic calcium phosphate nanoparticles for bone regeneration

Thi Phuong Nguyen¹, Bach Hai Phuong Doan², Dinh Vu Dang³, Cuu Khoa Nguyen¹ and Ngoc Quyen Tran¹

¹ Institute of Applied Materials Science, Vietnam Academy Science and Technology, 1 Mac Dinh Chi, Ho Chi Minh City, Vietnam
² School of Biotechnology, International University, Quarter 6, Linh Trung Ward, Thu Duc District, Ho Chi Minh City, Vietnam
³ Graduate School, Can Tho University, Campus II, 3/2 Street, Ninh Kieu District, Can Tho City, Vietnam

E-mail: tnquyen@iams.vast.vn

Received 20 December 2013
Accepted for publication 13 January 2014
Published 31 January 2014

Abstract
Injectable chitosan-based hydrogels have been widely studied toward biomedical applications because of their potential performance in drug/cell delivery and tissue regeneration. In this study we introduce tetronic–grafted chitosan containing tyramine moieties which have been utilized for in situ enzyme-mediated hydrogel preparation. The hydrogel can be used to load nanoparticles (NPs) of biphasic calcium phosphate (BCP), mixture of hydroxyapatite (HAp) and tricalcium phosphate (TCP), forming injectable biocomposites. The grafted copolymers were well-characterized by ¹H NMR. BCP nanoparticles were prepared by precipitation method under ultrasonic irradiation and then characterized by using x-ray powder diffraction (XRD) and scanning electron microscopy (SEM). The suspension of the copolymer and BCP nanoparticles rapidly formed hydrogel biocomposite within a few seconds of the presence of horseradish peroxidase (HRP) and hydrogen peroxide (H₂O₂). The compressive stress failure of the wet hydrogel was at 591 ± 20 KPa with the composite 10 wt% BCP loading. In vitro study using mesenchymal stem cells showed that the composites were biocompatible and cells are well-attached on the surfaces.

Keywords: chitosan, horseradish peroxidase, BCP nanoparticles, bone regeneration

1. Introduction

The autograft and allograft of bone tissue technique are widely known as treatment of bone loss and nonunion defect in the body. These approaches face several difficulties, such as insufficient material supply, donor site morbidity and contour irregularities [1]. There is an alternative approach which aids in bone regeneration via the use of several kinds of bioactive hydrogel scaffolds. The hydrogel scaffolds have highly porous 3D structure. They create a microenvironment for cell encapsulation allowing nutrients and metabolites to diffuse to and from the cells. An interesting approach using an enzyme-catalyzed reaction to prepare the hydrogels was recently reported. In the presence of the enzyme, solutions...
of phenolic moieties-conjugated polysaccharides rapidly formed several biocompatible hydrogels for biomedical applications [2–4]. Such hydrogel systems could also be formed in situ when a polymer solution is injected into the body and then forms a desired shape of hydrogel [3, 5]. These systems may be suitable to fill bone defects with minimally invasive surgical implantation. Following this approach, a group of hydrogel bio-composites for bone regeneration have recently been reported [6, 7]. The injectable and biocompatible hydrogel composite could be of great potential to be applied for minimally invasive surgical implantation.

As we know, chitosan is a biocompatible and biodegradable polymer. Several chitosan-based materials are widely applied in tissue regeneration treatment [2]. However, injectable chitosan-based hydrogels are generally highly biocompatible but have low mechanical properties [2, 4]. Calcium phosphates have been used in orthopedic applications because of their biocompatibility and osteoconductivity [8]. Biphasic calcium phosphate (BCP) has been reported as more efficient than hydroxyapatite (HAp) alone for repair of periodontal defects, and having better osteoinduction than single phasic HAp or tricalcium phosphate (TCP). The combination of HAp, TCP can induce better osteoinduction than single phasic HAp or tricalcium (HAp) alone for repair of periodontal defects, and having been reported as more efficient than hydroxyapatite osteoconductivity [9]. Calcium phosphate NPs have been also reported to improve the mechanical properties of the hydrogel-based material for bone regeneration.

In this study we introduced an injectable and biocompatible hydrogel composite based chitosan–tetronic and biphasic calcium phosphate nanoparticles (BCP–NPs) in which hydrogel network was formed in the presence of HRP enzyme. The injectable composite was characterized towards bone regeneration.

2. Materials and methods

2.1. Materials

Chitosan (low Mw), p-nitrophenyl chloroformate (NPC) and tyramine (TA), were purchased from Acros Organics. Horseradish peroxidase (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.

2.2. Preparation of BCP

BCP–NPs were synthesized using an ultrasonic assisted process. The calcium chloride reacted to tricalcium phosphate salts with molar ratio of Ca/P = 1.57 for 12 h at 50 °C under controlled pH 7 to obtain a white suspension. The precipitate was washed thoroughly with DI water and dried in an oven at 70 °C. Finally, the calcination was carried out at 750 °C in air.

2.3. Preparation of tyrosine–tetronic–grafted chitosan (TTeC) copolymer

Tetronic–grafted chitosan containing TA moieties was prepared in our previous publication in which three synthetic reactions were combined in one process without using any organic solvent to purify copolymers [5]. Briefly, four terminal hydroxyl groups of tetronic were activated with NPC, partial TA conjugated into the activated product and the remaining activated moiety of tetronic–TA grafted onto chitosan to obtain TTeC copolymer. The obtained copolymers were characterized by proton nuclear magnetic resonance (1H NMR) and thermogravimetric analysis (TGA).

2.4. Preparation of hydrogel and gel composite

TTeC (40 mg) was dissolved in phosphate buffered saline (PBS) solution (pH 7.4, 260 µl), and then, equally separated into two centrifuge tubes. The PBS solutions of HRP (50 µl of 0.2 mg ml⁻¹) and H₂O₂ (50 µl of 0.2% w/v) were separately added to each tube. TTeC hydrogel was rapidly formed by mixing the solutions of 10% w/w polymer. Preparation of the hydrogel composites was done with the same protocol in which BCP–NPs were added to two precursor copolymer solutions. Gelation time of the hydrogel or hydrogel composite is the time that took the gel to form (denoted by gelation time) which was determined using the vial tilting method. The time was determined when the solution did not flow for 1 min after inverting a vial.

2.5. Biocompatibility of hydrogel composite

Cell proliferation on the hydrogel composites was evaluated with Mesenchymal stem cell (MSC) from bone marrow of rabbit. 5 × 10³ MSC cells were seeded onto the UV-sterilized samples in 24-well plates. After incubation, these cells were washed with PBS three times, cell nuclei were counterstained with 0.5 mg ml⁻¹ of 4’,6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature, and then samples were washed three times with PBS. Finally, the stained cells on hydrogel composites after 1, 3 and 5 days of cell seeding were observed by confocal laser scanning microscope (FV10i-W). The nuclei of cells fluoresce blue light.

2.6. Characterization

The phase analysis of the samples was identified using an x-ray diffractometer (XRD) D8/Advance, Bruker, UK, using CuKα (λ = 1.5406 Å) as a radiation source over the 2θ range of 10–70° at 25 °C. The morphology and microstructure of the synthesized powders were investigated using field-emission
Figure 2. Formation of hydrogel composite.

Figure 3. Dependence of gelation time of these hydrogel composites on catalytic concentrations. (a) Effect of HRP concentration on gelation time with 0.005 wt% of H$_2$O$_2$; (b) effect of H$_2$O$_2$ concentration on gelation time with 0.025 mg ml$^{-1}$ of HRP.

Figure 4. XRD profiles of hydrogel (a), composites with 5 wt% BCP (b), 10 wt% BCP (c) and BCP (d).

Figure 5. Compressive strength of the hydrogel composites. Individual compressive strengths were obtained from the load–displacement curve at break.

3. Results and discussion

3.1. Morphology of BCP nanoparticles

Figure 1 shows the FESEM images of BCP nano powders which were synthesized using ultrasound irradiation. The scanning electron microscope (FESEM) JSM-635F, JEOL. Compressive tests of the hydrogel composites were performed on a universal testing machine (Unitech TM, R&B, Korea). Hydrogel composites were prepared in teflon mold with uniform rectangular shapes and then placed on the metal plate, where they were pressed at a crosshead speed of 1 mm min$^{-1}$. The
shows that crystalline phases of BCP still
shows that the MSC cells were well-adhered and
4
5
2
6
5
3
2
[48x353]◦
3
10
260
300
(co)polymers exhibited a weight loss with two stages when
well-performed proton signals of the tetronic–grafted chitosan–CH–CH δ
shown in figure
3.3. Charaterizations of hydrogel composite
3.2. Characterizations of the TTeC copolymer

1H NMR spectra of TTeC copolymer indicated some peaks corresponding to chemical shift of −CH3 (polypropylene oxide block, δ = 1.08 ppm), −CH3 (chitosan, δ = 1.96 ppm), −CH2−CH− (polyethylene glycol block, δ = 3.62 ppm) and −CH=CH− (tyramine moiety δ = 6.78 and 7.02). The well-performed proton signals of the tetronic–grafted chitosan confirmed success of the grafting method. Thermograms of (co)polymers exhibited a weight loss with two stages when heated in inert atmosphere. The first weight-losing stage of chitosan and TTeC was, respectively, below 260 and 300 ◦C. The second stage started from 300 to 600 ◦C, due to the degradation of chitosan and TTeC. Tetronic exhibited a weight loss from 320 to 420 ◦C. The results indicated that TTeC is more thermostable than chitosan [5].

3.3. Charaterizations of hydrogel composite

Our previous study indicated that the TTeC hydrogel could be rapidly formed within a couple of seconds after mixing two polymer solutions in the presence of HRP and H2O2. The TTeC hydrogel are highly biocompatible in vitro and in vivo [5]. In the current study, upon adding BCP–NPs to the TTeC polymer solutions, it took several seconds to form the enzyme-mediated hydrogel composite when two suspensions were mixed, as shown in figure 2.

The gelation time of the hydrogel composites were dependent on the used concentration of HRP and H2O2, as shown in figure 3.

The hydrogels were obtained by enzymatic cross-linking under physiological conditions using HRP as a catalyst and H2O2 as an oxidant. Coupling of phenols can take place either via a carbon–carbon at the ortho positions or via a carbon–oxygen bond between the carbon atom at the ortho position and the phenoxy oxygen [10]. The enzymatic cross-linking TTeC is carried out under mild reaction conditions containing room temperature, neutral pH and aqueous solution. The mixed solutions formed an opaque solid state by adding HRP and hydrogen peroxide. At the polymer concentration of 10% (w/v) and BCP 10 wt%, the mixed suspensions were opaque because the suspensions contained nano BCP particles, resulting in opaque hydrogel phases after cross-linking. The gelation time was very fast and changed at the wide ranges from three to twenty five of seconds.

In figure 3(a), the gelation times decreased from ∼25 to ∼3 s as the ratio of HRP increased from 0.01 to 0.1 mg ml−1 at a constant polymer concentration of 10% (w/v) and H2O2 0.05 wt%. This is presumably ascribed to increases in the rate of the decomposition of hydrogen peroxide and the production of phenoxy radicals by HRP.

In figure 3(b), the gelation times decreased from 20 to 5 s as the concentration of H2O2 increased from 0.01 to 0.1 mg ml−1 under the same conditions containing a polymer concentration of 10% (w/v), BCP 10 wt% and the concentration of HRP is 0.025 mg ml−1. The faster gelation time could be explained by the fact that there is a higher concentration of H2O2 which would increase the production of phenoxy radicals and facilitate gel formation [2].

Figure 4 shows that crystalline phases of BCP still remain in the hydrogel composites. XRD data of the hydrogel composite also shows two diffraction peaks at 19.10° and 23.30° that are similar to the crystalline phase of polyethylene glycol. These peaks could be explained by interaction of polyethylene glycol-b-propylene glycol copolymer and BCP–NPs resulting in increasing crystalline phase of the copolymer [10]. These results confirmed that BCP loaded in hydrogel composites.

The compressive strength of the hydrogel composites were determined to be 138.7 ± 15.9, 235.3 ± 15.3 and 591.7 ± 19.5 KPa for 0, 5 and 10 wt% of the loaded BCP–NPs, respectively (figure 5). The compressive strength of the hydrogel composites increase with increment in amount of loaded BCP–NPs. This could be explained that the incorporation of an inorganic reinforcing phase and interface adhesion of BCP particles within the hydrogel resulting in reinforcing of the polymer matrix.

Figure 6 shows that the MSC cells were well-adhered and proliferated well on the hydrogel composite surfaces when

Figure 6. Confocal images of MSCs adhering and proliferating on hydrogel composite with 10 wt% BCP after 1 day (a), 3 days (b) and 5 days (c) incubation.
the samples were immunostained with DAPI. When culture incubation time subsequently increased from 1 to 5 days, the density of cells seemed to be increased on the surface of the hydrogel composite. High cell adhesion on the hydrogel composite is responsible for a strongly cellular affinity of the chitosan-based materials. Our previous study indicated that the TTeC hydrogel is highly biocompatible. On incorporation of the active BCP–NPs to the hydrogel, BCP–NPs created the rough surface of the composite guiding cells to be well-attached on the surfaces, leading to enhanced cellular attachment. Moreover, a high serum protein adsorption of BCP–NPs is the positive influence on the behaviors of cells [11]. Therefore, high attachment and proliferation of MSC on the hydrogel composites could be seen in the study. With a preliminary obtained result, hydrogel composite systems could be a promising material for tissue engineering applications.

4. Conclusion

By incorporation of BCP to injectable and biocompatible copolymer–grafted chitosan hydrogel, the hydrogel composite showed a high mechanical property and well-attached and proliferated MSCs on the composite. These results have offered great potential of the injectable and biocompatible hydrogel composite for bone regeneration.

Acknowledgment

This research is funded by the Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 104.04-2011.49.

References