Biodegradation behavior and cytotoxicity of the composite membrane composed of β-dicalcium pyrophosphate and glucose mediated (polyethylene glycol/chitosan)

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The purpose of this study is to prepare and evaluate the biodegradation behavior and cytotoxicity of a composite membrane, G-β-DCP, combining β-dicalcium pyrophosphate (β-DCP) ceramic particles and glucose mediated chitosan–polyethylene glycol (PEG) membrane. The cytotoxicity of the G-β-DCP was examined by the *in vitro* method of NIH 3T3 fibroblast cell culture. Extracts were obtained by soaking the G-β-DCP composite in lysozyme containing phosphate buffer solution for 2, 7, 14, 21 and 28 days, respectively. The substances released from the G-β-DCP composite were analyzed by gas chromatography–mass spectrometry (GC–MAS) and inductively coupled plasma atomic emission spectrometry (ICP-AES). The change in morphologies, chemical composition and crystal structure was examined by scanning electron microscopy (SEM) and X-ray diffraction pattern (XRD).

The results of extracts cocultured with fibroblasts show that the growth of fibroblasts would increase for the extracts obtained from different β-DCP feeding weight G-β-DCP composites after soaking for 7 days. After further increasing the soaking time, the cell number still increases. It is found that the glucose amine and calcium are gradually released from the G-β-DCP composites, which is considered to be nutritious for the growth of the fibroblast. The release rate of calcium ion and glucosamine concentration can be regulated by feeding the β-DCP. The degradation behavior of G-β-DCP composite is considered as an ''onion degradation model'' that the G-β-DCP degrades from outer layer to inner layer. The developed material should have a great potential as a cell substrate in the field of tissue engineering.

1. Introduction

The ultimate goal for implantation of biomaterials in skeletal tissue is to reach full integration of the non-living implant with living bone. Since infection and the risk of viral transmission are frequent problems, transplantation of living tissue by means of allogenic or xenogenic grafts is limited in their medical application. To overcome these problems of natural bone grafts, the attention has been focused on the development of artificial materials in the last decade. Chitosan, a cationic polysaccharide, is obtained by alkaline deacetylation of chitin, the principal exoskeletal component in crustaceans. It has been demonstrated to be biodegradable, homeostatic active, non-toxic, non-antigenic, and biocompatible [1]. In the past few years, chitosan and some of its modification types have been reported for use in biomedical applications, such as artificial skin and suture, drug carrier, and dietary fibers [2]. Recently, the use of chitosan and its derivatives as temporary scaffolds to activate the promotion of mineralization or stimuli endochondral ossification has received much attention [3, 4].

Although chitosan and its derivatives seem to have very excellent properties as biomaterial, the quite low solubility due to highly crystallized and rigid structure presents problems to its application. So, various studies were conducted to make water-soluble derivates of chitosan by chemical modification techniques. However, when chemical modifications change the fundamental skeleton of chitosan, they modified the chitosan's loses the original physicochemical and biochemical activities [5]. Poly (ethylene glycols) (PEGs) are water-soluble amphipathic polymers widely used as a pharmacological product of preferable

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hydrophilicity and biocompatibility. In addition, PEG can avoid the strong interaction between the constituents since it is an uncharged polymer [6]. In our primary study [7], chitosan–PEG polyblend was prepared by using PEG 6000 molecular weight. The result showed that by choosing PEG molecular length in appropriate range to blend with chitosan not only could improve the water affinity but also accelerate the degradation rate of polyblend.

However, chitosan is easily dissolved in weak acids, therefore crosslinking of chitosan to form a network is necessary. Conventional chitosan crosslinkers including bifunctional reagents, such as glutaraldehyde, epichlorohydrin, and cycloheptamylose are chemically synthesized [8–10]. They may lead to a toxic effect in physicological environment, due to the presence of residual crosslinker [11]. From this point of view, the search for naturally occurring crosslinker to obtain partially soluble devices is preferable. Reaction of amino group of chitosan with carbonyl compounds leading to the formation of Schiff bases (e.g. glutaraldehyde and glyoxal) has been reported [12]. Low-molecular weight sugars such as reduced native mono- (i.e. glucose, fructose) and non-reduced disaccharides (i.e. sucrose), are commonly used in human life and could be biocompatible crosslinkers. Otherwise, conformational ordering and intermolecular relationship of oxidized starch also can be stabilized by introducing sugars as cosolute [13]. It has been reported that the aldehyde or keto group of reduced sugar can react with the free amino groups of chitosan resulting in a partially soluble membrane in low pH environment [14]. Therefore, glucose solution was prepared as crosslinking solution in this study.

However, chitosan is non-bioactive and will be surrounded by fibrous tissue as implanted in organism. Furthermore, it is necessary to maintain the implant material structure integrity at early period of time and allows a direct bone growth onto the template. For this reason, to use the bioresorbable ceramic as a filler for chitosan–PEG polyblend would have distinct advantages, not only they may reinforce polyblend structure but also can facilitate the rate of integration of implant with bone and resorbed by human body finally.

The widely investigated resorbable ceramic is β-tricalcium phosphate (β-whitlockite), but the data on biodegradability of it are controversial [15]. From X-ray analysis for human bone, β-dicalcium pyrophosphate (β-DCP), $Ca_2P_2O_7$, is one of the intermediate products in bone mineralization. Its biological reaction for a new bone development is similar to hydroxyaptite (HA), which is the major component of bone [16]. The β-DCP in block form can be dissolved in a biological environment and replaced by a new bone tissue [17], and also its Ca/P ratio is in the resorbable range (i.e. $Ca/P \leq 1$). In comparison with the resorption rate of hydroxyapatite and β-tricalcium phosphate (β-TCP), β-DCP has a remarkable potential as granular filler to reinforce the chitosan–PEG polyblend.

Therefore, a mixture of β-DCP powder and glucose mediated chitosan–PEG polymer matrix, denoted as G-β-DCP composite, was prepared. This study focuses on the cytotoxicity and biodegradation behavior of the G-β-DCP composite membrane. The NIH3T3 fibroblast was prepared and cultured for testing the cytotoxicity of the G-β-DCP composite. The degradation behavior was examined for (1) the change in composition, morphology and crystal structure in the G-β-DCP. (2) the concentration of releasing ion including calcium and glucose amine concentrations after incubating for a period of time. From these results, a degradation model is proposed to discuss the G-β-DCP composite degradation behavior.

2. Materials and methods

2.1. Materials

Chitosan(ch) with a high molecular weight (degree of deacetylation is 83%, $Mw \sim 4 \times 10^5$) was obtained in the form of flake from Fluka. PEG ($Mn = 6000$) was purchased from Ridel-dehaen., dextrose anhydrous was used as cross-linking agent and supplied by J. T. Baker Inc. All other reagents were extra pure grade and used as received.

2.2. Preparation of glucose mediated chitosan/PEG-calcium phosphate composite membrane

Chitosan solutions with concentrations of 3 wt % were prepared by dissolving in 2 wt % acetic acid. The mixture was stirred for 24 h to obtain a perfectly transparent solution. Chitosan–PEG blend was prepared by mechanical stirring the filtered chitosan and PEG flakes in a weight percentage of 70 : 30 at room temperature. The β-DCP powder was precalcined at 1100 °C and screened through an 120 mesh sieve. Then β-DCP granules with 20, 40, 50, 60, 70 wt % were added into the prepared solution, respectively, to make a polyblend/calcium phosphate mixture. After continuous stirring for a period of time, a homogeneous slurry was obtained. To avoid the phase separation for β-DCP in the polyblend solution, the mixture was rapidly transferred into an oven to make the composite membranes. After drying for 90 min at 68 °C, the membrane was neutralized with 10% (w/v) sodium hydroxide followed by rinsing with deionized distilled water (DDW) and dried at 30 °C for 60 min. The composite membrane was placed in a desiccator and allowed for further crosslinking. The crosslinking solution was prepared by dissolving 5 wt % glucose in DDW and adjusted pH value of the solution to 10 by 1 M sodium hydroxide and then thermal treated in an oven for 1 h at 120 °C. After drying, the membrane was soaked in the crosslinking solution for 2 days at 68 °C and the final glucose mediated composite membrane was obtained. After further rinsing twice by DDW, the membrane was dried at room temperature overnight and stored in desiccators.

2.3. Preparation of extracts from glucose mediated β-DCP composite membrane

The composite was cut into an $1 \times 1\,cm^2$ film with 0.2 g in weight and placed in a 20 ml scintillation vial with $4\,mg\,ml^{-1}$ lysozyme in pH 7.4 phosphate buffer solution (PBS) inside. All of the vials were kept in an incubator at

a temperature of 37 °C. After soaking for 2, 7, 14, 21 and 28 days, respectively, the extracts were collected for using in cell culture and examined by gas chromatography–mass spectrometry (GC–MAS) and inductively coupled plasma atomic emission spectrometry (ICP-AES).

2.4. Analysis of extracts from composite membrane

To measure the content of constituents in extracts, which were released from the series of the composite membrane, composite membrane was soaked in the phosphate buffer solution with lysozyme for a period of time. The calcium and phosphate contents in the extracts were determined by ICP-AES. The GC–MAS was used to detect the amount of glucose amine and schiff base by-products. GC–MS analyses were performed using a Agilent 5973 Network Mass Selective Detectors fitted with a Agilent 6890N GC gas chromatograph, an on column injector, and using helium as the carrier gas. Extracts were separated on a HP-5MS column (0.25 mm × 30 m (id), 0.25 μm film thickness). The GC oven was operated as follows: isothermal for 2 min at 50 °C; temperature programmed at 10 °Cmin^{-1} to 225 °C and then isothermal for 15 min. The MS was operated in full scan mode (47–400 a.u., 3 scans/s). Peaks were identified based on their mass spectral characteristics and GC retention indices, by comparison with commercial glucosamine (Aldrich, UK). After the composite membranes were taken out of the immersion solution, the microstructure and morphology of the crystals deposited on the surface were observed under SEM (JEOL 5120). The crystalline phases of specimens were determined by Rigaku X-ray powder diffractometry with CuK_{α} radiation and Ni filter. The scanning range of the samples was from 5° to 50° at a scanning speed of 4° min^{-1} with the accelerating voltage of 30 KV and current of 20 mA.

2.5. Testing of cytotoxicity of extracts from glucose mediated chitosan/PEG/β-DCP composite membrane

In order to examine the influence of composite membrane extracts on the growth and morphological transformation of the 3T3 fibroblast, the extracts for use in cell cultures were sterilized by filtrating through 0.45 μm millipore filters and adding to cell cultures.

The NIH 3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% antibiotic–antimycotic (Gibco, USA). The cells were subcultured for about 3 days interval with trypsin-EDTA and maintained at 37 °C in a water-jacked incubator with a humidified 5% CO_2 atmosphere. The fibroblasts were placed in a six-welled tissue culture plate (1×10^5 cells per well) in a complete medium and incubated for 24 h. The medium was then replaced and the extracts from the series of glucose mediated β-DCP composite membrane were added in a ratio of 1 : 1 for the medium and extract, giving a final extract concentration of 50%. In the control group, the phosphate buffer solution (PBS) with lysozyme was mixed with the complete medium in a ratio of 1 : 1 for cell culture. After being cocultured for 2 days, the medium was removed and the cells were trypsinized resuspended, and then 1 ml trypan blue was added to make sure that the cells were still living and then were counted in a Neubauer counting-chamber under an optical microscope.

3. Results and discussion

3.1. Glucosamine molecules and calcium ions release

According to the GC–MAS analysis, the extracts contain lots of degradation products, including low-molecular chitosan, glucose amine and Schiff base product. The glucose amine, which is the basic unit of chitosan, is a nutritious element favorable to cell growth [18]. In addition, Martins *et al*. [19] pointed out that the reduced sugar such as glucose can react with primary amino group to produce Schiff's base product. By further reaction, the strecker degradation would occur and form heterecyclic compounds. These compounds such as furan, pyridine, pyrrole and oxazole still are controversial issues on their safety [20]. Fig. 1 shows the concentration of glucosamine molecules in the extracts of series of composites soaked in a PBS solution containing lysozyme enzyme (4 mg/ml). All the curves in Fig. 1 demonstrate that the glucose amine concentration in extracts increases slowly in the first 2 days from 20 to 60 ppm. It can be found that the more β-DCP content in the composite, the less glucose amine release. The glucosamine shows a regular release rate in the initial 2 days because the non-crosslinked chitosan/PEG is easy to be attacked by lysozyme then transforms to glucose amine during the period of soaking time. After 7 days, the G-β-DCP composite membranes contain 20 wt % β-DCP and glucose mediated chitosan–PEG membrane has the similar degradation behavior. Both of them all keep progressively in releasing glucosamine molecules in the extracts and increase to around 560–

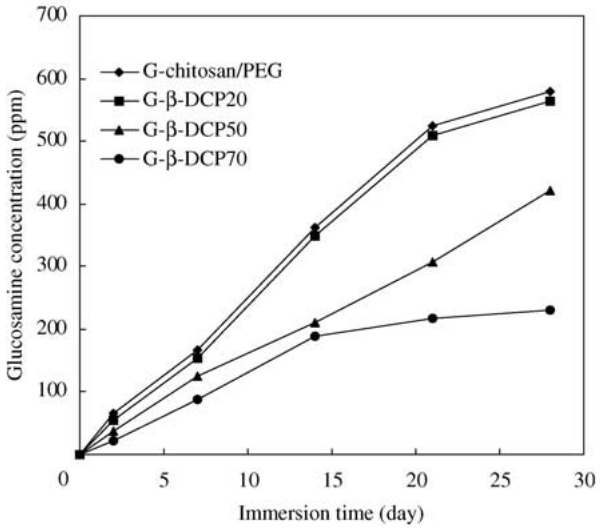

Figure 1 Relationship between glucosamine in the extraction and soaking periods of the series of glucose-mediated β-DCP chitosan/PEG composite in a PBS solution containing lysozyme enzyme (4 mg/ml).

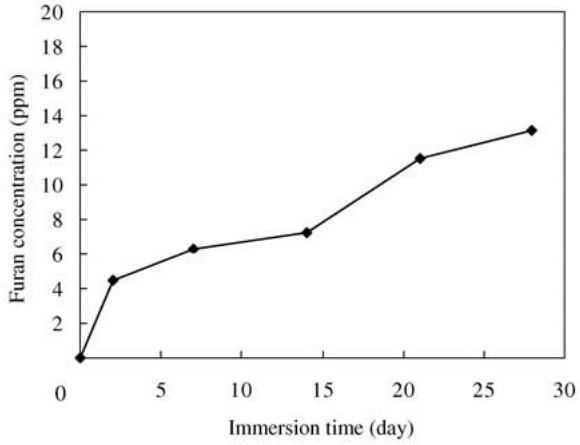

Figure 2 Relationship between furan in the extracts and soaking periods of the glucose-mediated β-DCP composite in a PBS solution containing lysozyme enzyme (4 mg/ml).

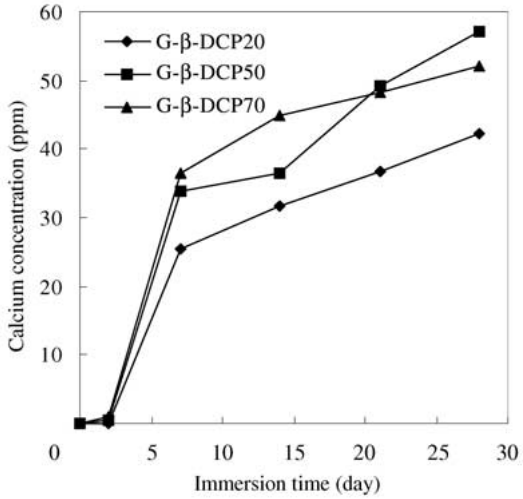

Figure 3 Relationship between calcium content in the extracts and soaking periods of the series of glucose-mediated β-DCP chitosan/PEG composite in a PBS solution containing lysozyme enzyme (4 mg/ml).

580 ppm. But as the β-DCP feeding weight increases to 50 wt %, the slowdown of glucosamine molecules releasing rate may be due to the attenuation of degradable rate of the G-β-DCP composite by the effects of chitosan/PEG cross-linked structure and the physical interlocking between chitosan/PEG and β-DCP particles. Therefore, the slowest glucose amine release rate is observed for 70 wt % G-β-DCP composite due to the high content β-DCP particles shielding the chitosan/PEG cross-linked structure. Fig. 2 shows the furan molecules in the extracts of 20 wt % G-β-DCP composites soaked in a PBS solution containing lysozyme enzyme (4 mg/ml). Most of the furan molecules release in the first 2 days then reaches a plateau after soaking for 7 days. In addition, the furan concentrations all are under 15 ppm during the soaking times. The tendency reflects a fact that the residual heterecyclic compounds in the G-β-DCP composite only liberate in the first few days and the releasing amount is low. Therefore, it may have a little adverse effect on cell growth during soaking.

According to Lin *et al*. [21], β-DCP ceramic could be dissolved in distilled water, and Ca^{2+} and PO_4^{3-} are continuously released from the ceramic. In the process of bone defect healing, the calcium ion is a required element in the ensuing ossification process because it is needed to reconstitute the mineral structure of regenerating bone. Fig. 3 shows the calcium ion concentration after the G-β-DCP composites are immersed in a PBS solution containing lysozyme enzyme (4 mg/ml) for 2–28 days. The curves in Fig. 3 demonstrate that the calcium ion concentration of 20 wt % β-DCP content composite extracts slightly increases in the initial 2 days and then increases with immersion time. It can be inferred that the presence of calcium ions in the extracts is attributed to the β-DCP particles dissolving in the PBS solution containing lysozyme enzyme (4 mg/ml). The dissolved β-DCP particles are thought to be on the free surface of the composite membrane and expose to the soaking solution. After the composite membrane has been soaked in soaking solution over 7 days, the chitosan/PEG decomposes and is released from the composite, which leads to more β-DCP particles coming into contact with solution. The end products of degradation, such as chitoolisacharide and glucosamine, are gradually released from composite and then β-DCP particles are continuously exposed and dissolved in the soaking solution, which would explain the continuously increasing calcium ion concentrations in the extracts. As the β-DCP content increases to 50 wt %, the calcium ion concentration also progressively increases in the period of 2–7 days but their releasing rate slows down in the period of 7–14 days. After that, the calcium ion-releasing rate increases until the end of soaking time. A large amount calcium ion increasing in the initial 2–7 days is due to much more β-DCP particles on the surface of the composite membrane and exposed to the PBS solution. Otherwise, after 2 days, chitosan/PEG also starts to decompose and the β-DCP particles exposed and dissolve in soaking solution in 2–7 days. After 7 days, owing to the physical interlocking between β-DCP and chitosan/PEG particles, the undissolved β-DCP retards the degradation of chitosan/PEG, which makes a lower calcium ion-releasing rate in the period of 7–14 days. After 14 days, chitosan/PEG decomposes again and the β-DCP particles dissolve in soaking solution. The above degradation behavior is also found in 70 wt % G-β-DCP composite membrane, but the time for each degradation stage is extended.

3.2. Microstructure and X-ray diffraction analysis

In order to estimate the biodegradability behavior of the G-β-DCP composite membrane, the crystal/chemical composition and structure was investigated. Figs. 4 and 5 show the XRD patterns of 20 and 50 wt % G-β-DCP composite membranes after immersion in PBS containing lysozyme enzyme (4 mg/ml) for 28 days. All characteristic peaks of the two patterns are in agreement with the X-ray diffracted JCPD data files of β-DCP and chitosan. The composite can be described to the β-DCP structure with the peaks detected at 27.6° for (022), 28.8° for (023) and 29.3° for (008). The peak found at 20° is assigned to chitosan chain aligned through intermolecular interactions. There is no phase transformation or reaction occurring and no other new phase appears

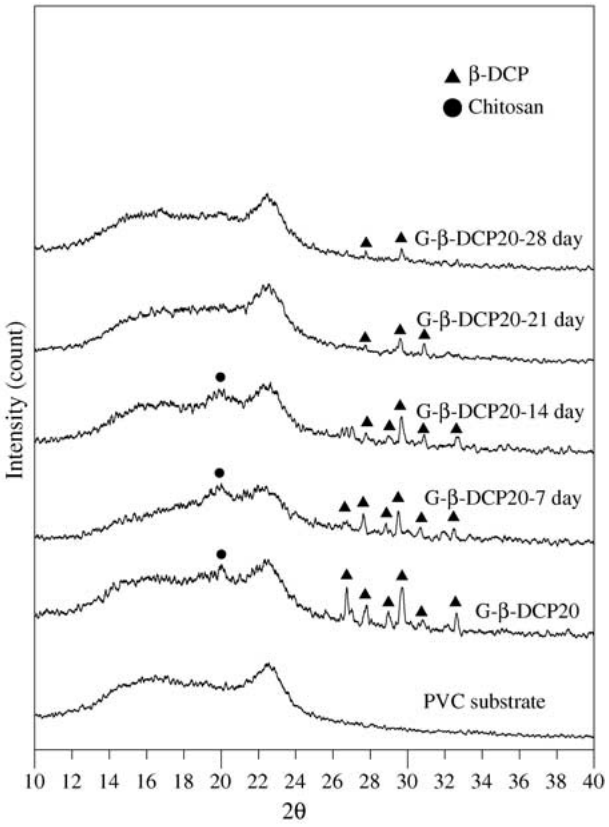

Figure 4 X-ray diffraction patterns of 20 wt % G-β-DCP chitosan/PEG composite after immersion in a PBS solution containing lysozyme enzyme (4 mg/ml) for 7–28 days.

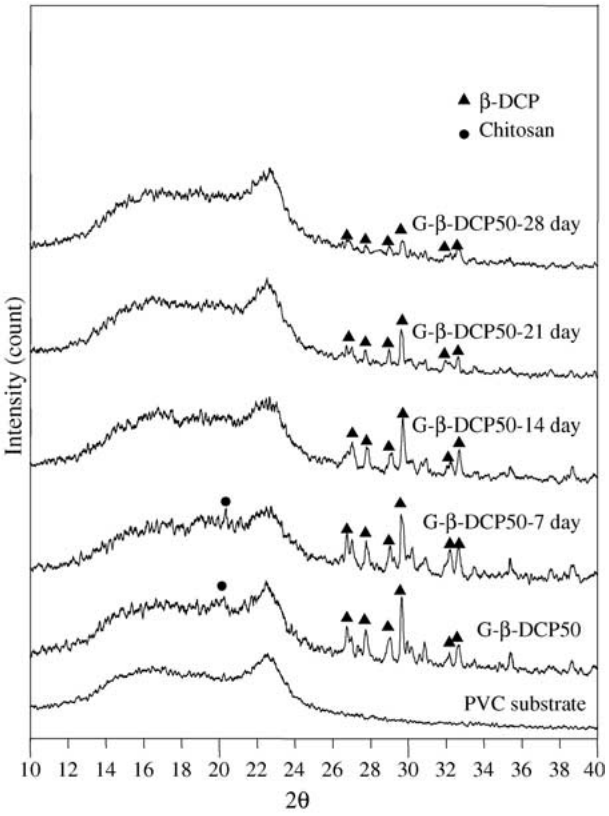

Figure 5 X-ray diffraction patterns of 50 wt % G-β-DCP chitosan/PEG composite after immersion in a PBS solution containing lysozyme enzyme (4 mg/ml) for 7–28 days.

during the immersion period. For 20 wt % G-β-DCP composite, the presence of the diffused peak at 20° indicates some degree of ordered alignment for the chitosan chain in composite membrane during the 7–14 days. But as the soaking time is over 14 days, a broader peak is shown at 20° and the crystalline peaks lose their prominence suggesting that the crystal structure of chitosan/PEG is distorted. On the other hand, all of characteristic peaks of the β-DCP gradually decrease and disappear with immersion time increasing. It indicates that β phase DCP could be dissolved in PBS solution, leading to DCP disintegration. For a high β-DCP content membrane such as 50 wt % G-β-DCP composite, the similar phenomena also occurs in the characteristic peaks of the chitosan, but the intensity of β-DCP peaks is more stable during 7–14 days then gradually decreases and finally disappears after 14 days. It conforms that the β-DCP degradation rate slows down during 7–14 days resulting that the calcium ion concentration slightly increases and then β-DCP degradation rate increases resulting in the calcium concentration increasing after 14 days.

Fig. 6 shows the weight loss of the different β-DCP feeds as immersed in soaking solution for 28 days. For 20 wt % G-β-DCP composite, the weight loss continuously increases from 2% to 36% during 28 days. Previous study has shown that chitosan is susceptible to enzymatic hydrolysis mediated by lysozyme, which is ubiquitous in the human body [22]. In respect to chitosan, the bulk form of β-DCP degradation rate is slow [21]. Our preliminary study shows that the glucose-mediated chitosan/PEG is susceptible to hydrolysis by lysozyme at pH 7.4 PBS solution and the weight loss reaches to 19% after soaking for 7 days. Thus, compared to the weight loss of the 20 wt % G-β-DCP composite, the main degradation mechanism of the low β-DCP feeding weight of the G-β-DCP composite is mainly controlled by the chitosan/PEG degradation rate. But as the β-DCP content is higher than 40 wt %, the site for lysozyme attacking decreases. Therefore, the high β-DCP feeding weight of the G-β-DCP composite degradation behavior

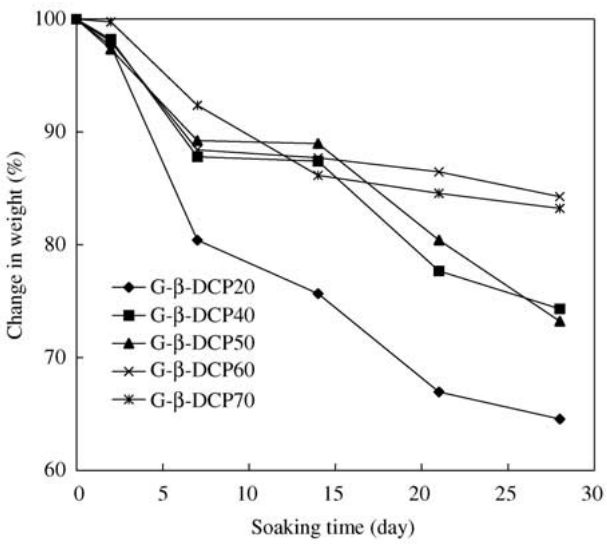

Figure 6 Weight loss curves of the series of glucose mediated β-DCP chitosan/PEG composite soaking in a PBS solution containing lysozyme enzyme (4 mg/ml) for 28 days.

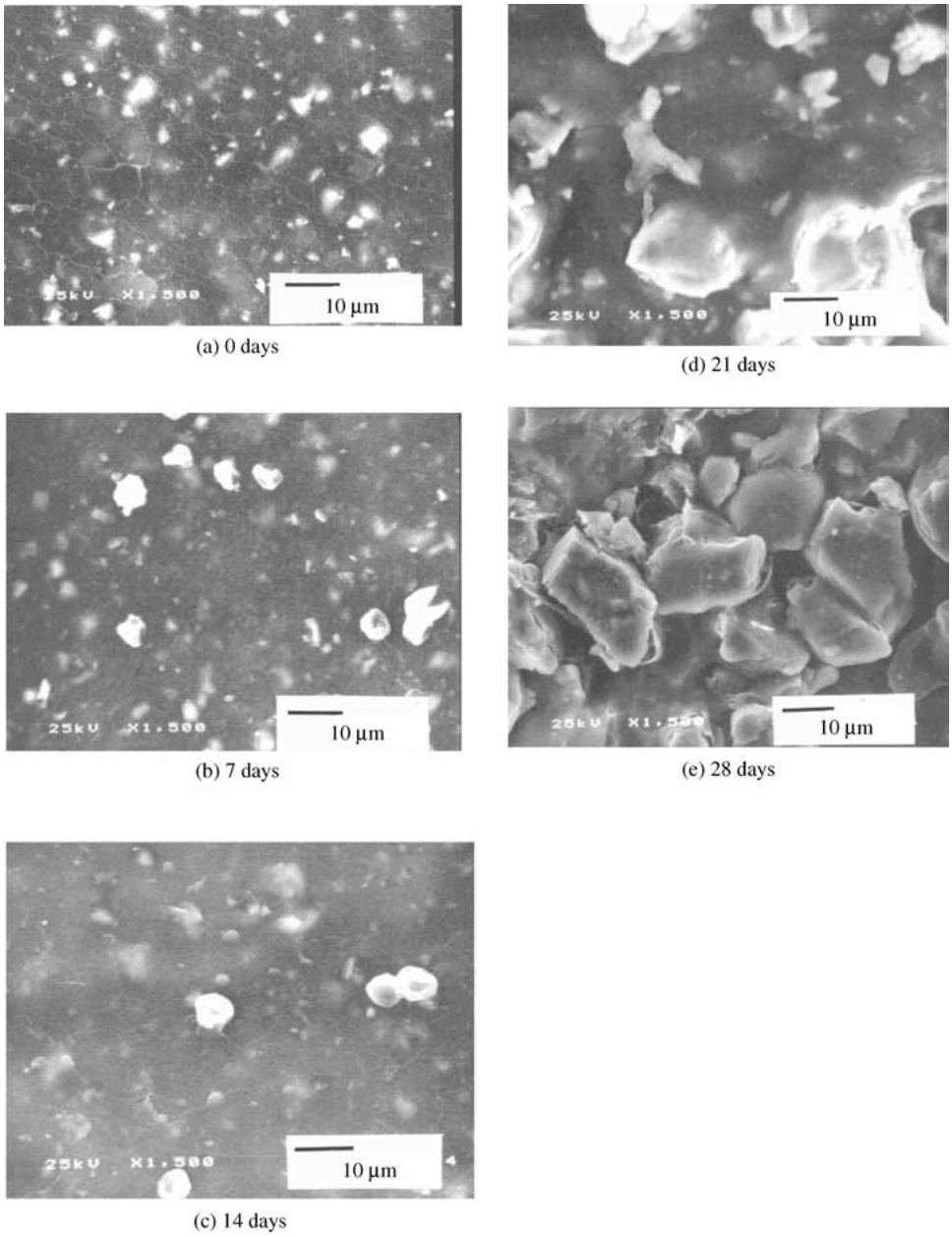

Figure 7 Surface morphology change of the 50 wt % glucose mediated β-DCP composite after soaking in a PBS solution containing lysozyme enzyme (4 mg/ml) for (a) 0 day (b) 7 days (c) 14 days (d) 21 days (e) 28 days.

is mainly controlled by the slow degradation rate of β-DCP particle.

Fig. 7(a)–(e) shows the SEM observation of 50 wt % G-β-DCP composite after being immersed in the PBS solution containing lysozyme enzyme (4 mg/ml) for 0, 7, 14, 21 and 28 days, respectively. As seen in Fig. 7(a), the β-DCP particles are well dispersed in the glucose mediated chitosan/PEG matrix before soaking. After 7 days, the surface of 50 wt % G-β-DCP is partly dissolved and appears to have round pores on the surface, which are caused by the chitosan/PEG degradation and following release of the β-DCP particles. Interesting, after 14 days, the surface of 50 wt % G-β-DCP does not change a lot, there are still some round voids and fragments spreading on the surface, which is coincident with the result of 50 wt % G-β-DCP weight loss during the 7–14 days. The apparent surface change occurs after soaking for 21 days. As shown in Fig. 7(d), lots of large β-DCP particles are exposed on the surface, that is considered to be original from the subsurface of the composite. It indicates that the outer surface of glucose mediated chitosan/PEG matrix is dissolved after immersing for 21 days. After 28 days, many large cavities are presented on the surface and the composite structure starts to disintegrate due to the enzymatic degradation of glucose mediated chitosan/PEG and the β-DCP decomposes to calcium ion and phosphate ion.

3.3. Effects of glucose mediated β-DCP composite membrane (G-β-DCP) extracts on NIH 3T3 cell culture

Fig. 8 shows the relationship between cell number and soaking periods after series of G-β-DCP composite membrane extracts being cocultured with 1×10^5 cells/ml NIH 3T3 fibroblasts in each well of 6-well tissue plate for 2 days. The cell number increases with the extractive period in the initial 2 days, thereafter the cell numbers are higher than those of the control group where the 1×10^5 fibroblast is cocultured with blank PBS

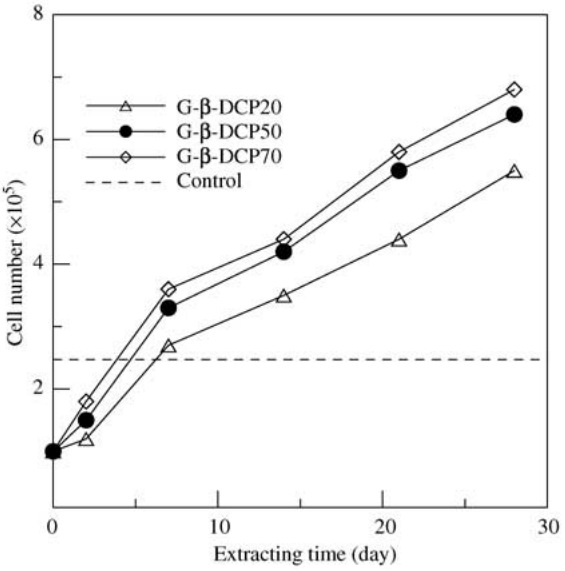

Figure 8 Relationship between cell number and extractive periods as the series of glucose mediated β-DCP chitosan/PEG composite extract cocultured with 1×10^5 NIH 3T3 fibroblast for 2 days.

instead of glucose mediated composite extract for 1 week extractive period. The fibroblasts are increasing up to 2.5×10^5 over the original amount (1×10^5) after coculturing with blank PBS for two days. In addition, the cell numbers increase as the β-DCP feed in weight increases. According to Yao *et al*. [23], the calcium ion content is beneficial to the growth of the myoblast and would promote the premature growth of cell fusion. In the present study, series of solution with different in calcium contents in the range of 20–50 ppm with an increment of 5 ppm were prepared and then cocultured with the 1×10^5 cells/ml fibroblast for 2 days. Fig. 9 shows that the cell number increases with the calcium content and the control group has the lowest value. It infers that the calcium ion in the extracts would be beneficial to the growth of the NIH 3T3 fibroblast.

In order to investigate the effect of glucose amine and furan concentration on the growth of fibroblast, series of solution were prepared, which are differing in glucose amine and furan concentrations in the range of 50–550 and 15 ppm, respectively, then cocultured with 1×10^5 cells/ml fibroblast for 2 days. Fig. 10 shows the

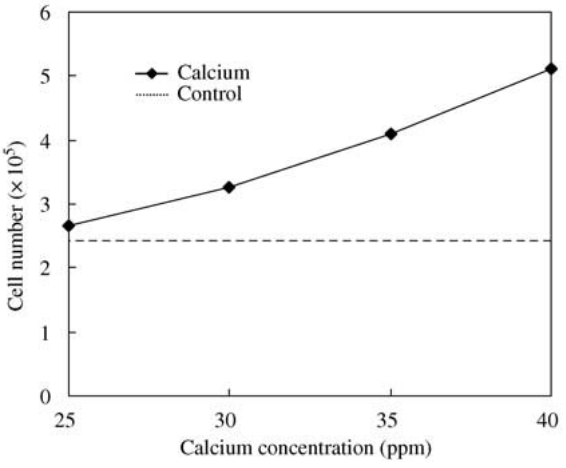

Figure 9 Effect of calcium content in solution on NIH 3T3 fibroblast growth as cocultured for 2 days.

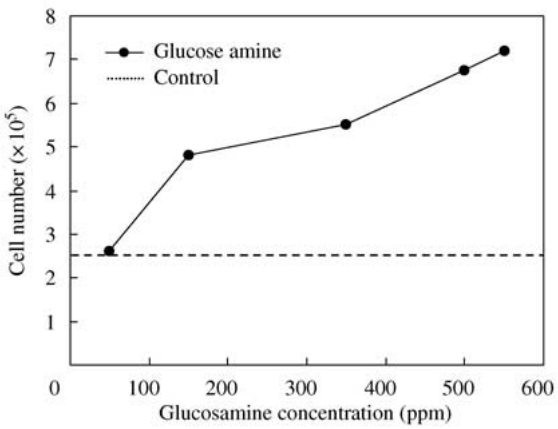

Figure 10 Effect of glucosamine content in solution on the NIH 3T3 fibroblast growth as cocultured for 2 days.

relationship between cell number and concentration of glucose amine solution. As the glucose amine concentration is lower than 50 ppm, the cell numbers of the fibroblast almost maintain a constant value of about 2.6×10^5 cells/ml, which is a little greater than that of the control group. It infers that the glucose amine solution below 50 ppm is considered to be too low to accelerate the growth of fibroblast. But as the glucose amine concentration is over 50 ppm, the cell numbers have a positive tendency with the increase of glucose amine concentration. The result implies that the glucose amine solution with concentration higher than 50 ppm is a nutritious element for the fibroblast growth. When the G-β-DCP composites are soaked in the PBS solution containing lysozyme enzyme (4 mg/ml) for over 7 days, the glucose amine concentrations in the extracts are over 50 ppm as shown in Fig. 1. The results demonstrate that the cell number exhibits an increasing tendency as the soaking time is over 7 days. On the other hand, for the furan with 15 ppm concentration, the cell number of the fibroblast maintains at a constant value about 2.5×10^5 cells/ml, which has no inhibition effect on cell growth.

3.4. Possible degradation mechanism for G-β-DCP composite membrane

According to the above analyses, a possible degradation mechanism of the G-β-DCP composite membrane (e.g., β-DCP feeding weight of 50 wt %) could be proposed as following: (1) Stage I: At the first day, the glucose mediated chitosan/PEG starts to swell and β-DCP granules still hydrate on the composite surface. (2) Stage II: At the second day, the unchained chitosan, low molecular chitosan, PEG, heterecyclic compounds and glucosamine gradually are released from G-β-DCP composite but the β-DCP is still in the hydrated state. (3) Stage III: After seventh day, the glucose amine is progressively released from the composite and the β-DCP granules leave from the composite surface and dissolve in PBS solution resulting in disintegration of the first composite surface layer. (4) Stage IV: During 7–14 days, the second composite surface layer swells and the β-DCP granules are in hydrated state. (5) Stage V: During 14–28 days, the glucose amine is progressively

released from the second layer of composite and the β-DCP granules are exposed then dissolve in PBS solution resulting in disintegration of the second layer composite surface. (6) degrade by repeating the stages I–V. The above degradation behavior is named as ''onion degradation model'' because the composite degrades layer by layer. In this model, the degradation rate G-β-DCP composite is controlled by the decomposition of glucose-mediated chitosan/PEG. Therefore, in a low β-DCP content (20 wt %), the release of chitosan/PEG is not easy to impede by the β-DCP granules resulting in a higher degradation rate. But for a high β-DCP content (70 wt %), the lower chitosan/PEG content makes the first layer degradation time prolonging to 14th day and extending the second layer swelling time. Based on this onion degradation model, it could be a feasible way to predict the degradation behavior of resorbable ceramic (such as β-TCP, α-TCP)/chitosan based composite.

4. Conclusion

The 5 wt % glucose solution used as a cross-linking agent and added to the developed β-DCP/chitosan/PEG composite has no adverse effect on the growth of fibroblast. The cell numbers rapidly increase after the fibroblasts are cocultured with the extracts of G-β-DCP composite membranes as soaked for 7 days. This is due to the release of calcium ions and glucose amine from the G-β-DCP composite membranes. The glucose amine concentration in the range of 50–580 ppm, and the calcium ion show as positive factors for the growth of fibroblast. By adjusting the β-DCP feeding content, the release rate of calcium ion and glucosamine concentrations are changed. For the 20 wt % G-β-DCP composite membrane, the degradation behavior is mostly controlled by the dissolution of chitosan/PEG matrix during the soaking periods and results in a higher glucose amine and a lower calcium ion concentration in the initial 7 days. With the β-DCP content increasing to 50 wt %, the degradation behavior is dominated by the low degradation rate of β-DCP granules and physical interlocking structure of the composite. By investigating the degradation behavior of G-β-DCP composite, a possible model named as ''onion degradation model'' is proposed, which is considered to be useful in discussing the degradation behavior of resorbable ceramic (such as β-TCP, α-TCP)/chitosan based composite.

Based on this study, the developed material will be dissolved in the physico-chemical environment but not harmful to the cell growth. It is thought that the materials developed have a great potential as a cell substrate in the field of tissue engineering.

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