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Preparation and characterization of a novel chitosan scaffold

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ABSTRACT

In this paper, a series of porous chitosan scaffolds were successfully prepared by freeze-drying of chitosan hydrogel (pre-gelled with dibasic sodium phosphate at 37 °C). Micro-structure, porosity, water adsorption and compressive strength were greatly affected by chitosan concentration. With the decrease of chitosan concentration, water adsorption and porosity of scaffold increased accordingly, while compressive strength of scaffold decreased. *In vitro* degradation test revealed that the chitosan scaffold was almost degraded by the lysozyme solution (1.5 μg/ml) after 28 day's incubation. *In vitro* cytotoxicity test showed the prepared chitosan scaffolds were non-cytotoxicity against NIH3T3 cell. The cell viability as in function with time with acridine orange (AO) staining also demonstrated that NIH3T3 cell were metabolically active and well distributed throughout the scaffold after 5 day's incubation. Scanning electron microscopy (SEM) also showed that NIH3T3 cell appeared to adhere well and exhibited a normal morphology on the surface of scaffold after 24 h cell culture.

1. Introduction

Nowadays, one of the popular tissue-engineering strategies is the transplantation of culture-expanded cells on a biodegradable scaffold (Hutmacher, 2000; Madihally & Matthew, 1999). An ideal scaffold should have following characterizations: non-toxicity, biocompatible, suitable mechanical strength, and its microenvironment could support cell attachment, growth, and differentiation to the desired phenotype (Hollister, 2005). Numerous natural and synthetic materials have been developed, characterized, and tailored as tissue scaffolds to specific applications (Freed et al., 1994). The micro-structure of these scaffolds span the range from hydrogels, to open-pore structures, to fibrous matrices (Hu, Liu, & Ma, 2008; Shapiro & Cohen, 1997; Tibbitt & Anseth, 2009). Since the wide applications of scaffold on the tissue engineering, there is a continuous ongoing search for materials which may have broad applicability and good biocompatibility suitable for the preparation of scaffolds (Choi, Xie, & Xia, 2009; Madihally & Matthew, 1999).

Among these materials, scaffolds-based chitosan and its derivatives have gained considerable attention (VandeVord et al., 2002). Chitosan, as the only cationic polysaccharide in nature, is composed of *N*-acetylglucosamine (GlcNAc) and glucosamine (GlcN) residues, which displays some favorable properties such as low-toxicity, good biocompatibility, biodegradability, mucoadhesive and, etc. (Jayakumar, Prabaharan, Reis, & Mano, 2005; Mourya & Inamdar, 2008). Chitosan is a crystalline polysaccharide and is normally insoluble as the pH is greater than 7. However, in dilute acids (pH < 6), the free amino groups on chitosan skeleton are protonated resulting in the soluble of molecule. The high charge density in dilute acidic solution allows chitosan to form complex by ion interaction with a wide variety of water soluble polyanionic species (Madihally & Matthew, 1999; Nettles, Elder, & Gilbert, 2002; VandeVord et al., 2002). Complex formulations such as film, scaffold, fibers, etc. based on chitosan and polyanionic polymer suitable for the tissue engineering have been documented (Bhattarai, Edmondson, Veiseh, Matsen, & Zhang, 2005; Cheng et al., 2003). Previous studies have revealed that the chitosan and its complex could be easily fabricated into porous three-dimensional (3D) scaffold for tissue engineering by the method of electrospinning and freeze-drying (Denkba, Seyyal, Pi kin, 2000; Deville, Saiz, & Tomsia, 2006; Landi, Valentini, & Tampieri, 2008). Chitosan scaffolds are usually prepared by the freeze-drying technology, because it is beneficial for those dissolved in acetic acid aqueous medium (O'Brien et al., 2004). In addition, properties of chitosan scaffold such as micro-structure, crystallinity, and mechanical strength can be modulated by changing chitosan concentration, freezing rate as well as the molecular weight and percent deacetylation of chitosan (Nettles et al., 2002). Chitosan scaffold also could be utilized for the encapsulation and controlled release of pharmacological agents, and has been shown to be an effective nonviral vector for gene delivery (Guo et al., 2006; Kim et al., 2003). Thus, the performance of chitosan scaffold could be enhanced by using them as the drug and plasmid carrier.

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In the present study, a novel chitosan scaffold based on chitosan and disodium hydrogen phosphate was fabricated. Chitosan solution was first neutralized with disodium hydrogen phosphate and subsequently gelled at 37 °C for 24 h. Finally, the chitosan gel was lyophilized in a freeze dryer to obtain the chitosan scaffold. The objective of the study was to investigate the properties of this novel chitosan scaffold and *in vitro* degradation behavior. In addition, the *in vitro* biocompatibility and cell culturing of chitosan scaffold was also investigated. The results of this study may help us to understand and evaluate the possibility of using this novel chitosan scaffold for the tissue engineering.

2. Materials and methods

2.1. Materials

Chitosan (with 86% degree of deacetylation (DD)) with ~200 KD was supplied by Sigma–Aldrich (USA). Dibasic sodium phosphate and acetic acid were purchased from KeLong Chemicals (Chengdu, China). All other chemicals used in this paper were analytical grade. Ultrapure water from Milli-Q water system was used to prepare the aqueous solutions.

2.2. Preparation of chitosan scaffold

The wide pore spongy chitosan-based matrix of the scaffold was prepared in accordance with the procedure described by previous (Deville et al., 2006; Landi et al., 2008). Chitosan (1 g) was dissolved in 0.5% acetic acid solution under magnetic stirring for 48 h at room temperature. The resulting solution (pH ~ 5.6) was filtered and stored at 4 °C for further application. Autogelling solutions were prepared as follows: first, 4 ml of chitosan solution was placed in a glass vial and magnetically stirred in an ice bath. And then 0.4 ml of dibasic sodium phosphate (500 mg/ml) was added dropwise into chitosan solution with magnetic stirring. The pH value of the resulting mixture was found to be in the range of 7–7.2. Finally, the autogelling solutions were transferred into 24-well plate (2 ml per well) and incubated at 37 °C overnight to obtain the chitosan hydrogel. The obtained chitosan hydrogel was stored in a refrigerator at −80 °C for 24 h and lyophilized in a freeze dryer (FD-1A-50, Beijing Boyikang Co., Ltd., China) at −40 °C for 24 h. By changing the concentration of chitosan, a series of chitosan scaffolds were obtained, as shown in Table 1. The three-dimensional (3D) morphology of chitosan scaffold was recorded with a digital camera.

2.3. Characterization of chitosan scaffold

The morphological characterization of chitosan scaffold was performed by scanning electron microscopy (JSM-5900LV, JEOL, Japan). Scaffold samples were placed at cabinet drier for 24 h before observation. The cross section of scaffold was obtained by cutting scaffold after dealing with liquid nitrogen.

According to the description of Kuo, Yeh, & Yang (2009), porosity of the chitosan scaffold was evaluated using trimmed samples of 1.5 $cm^2 \times 0.9$ cm into ethanol. The porosity (P(%)) is calculated as the following formula:

$$P(\%) = \frac{V_c}{V_m} \times 100 = \frac{(W_{24} - W_0) \times \rho}{V_m} \times 100$$

where V_m is the total volume of chitosan scaffold (cm^3), V_c is the pore volume of the chitosan scaffold (cm^3), W_{24} is the weight of chitosan scaffold (g) after incubation with ethanol for 24 h, W_0 is the original weight of chitosan scaffold (g) and ρ is the density of the ethanol (0.789 g cm^{-3}).

Water absorption was evaluated by weighing the scaffolds before and after placing in water solution (Li et al., 2010). At specific time interval, the scaffolds were taken from the medium and weighed after removal of the surplus surface water using filter paper. The percentages of water absorption were calculated by following equation:

$$\text{Water absorption}(\%) = \frac{W_t - W_0}{W_0} \times 100$$

where W_t is the weight of scaffolds at time t and W_0 is the original scaffold weight at zero time, respectively. This experiment was performed in triplicate.

Compressive strength test of chitosan scaffold was detected as follows. The cylindrical samples 20 mm long and 18 mm in diameter were tested by a universal mechanical testing instrument (Instron-5567, Instron Corp., USA) at room temperature and relative humidity of 50%. Then the compressive strength of the samples along with longitudinal direction was determined at a compressing rate of 2 mm min^{-1}. All results were the mean values of five specimens.

2.4. In vitro degradation test

The *in vitro* degradation test of chitosan scaffolds (1.5 $cm^2 \times 0.9$ cm) was performed in 5 ml phosphate-buffered solution (PBS, pH = 7.4) at 37 °C containing 1.5 μg/ml lysozyme. The concentration of lysozyme was chosen to correspond to the concentration in human serum (Porstmann et al., 1989). Briefly, chitosan scaffolds with calculated weights were incubated in the lysozyme solution with gentle agitation for the period of study. The lysozyme solution was refreshed daily to ensure continuous enzyme activity (Masuda, Ueno, & Kitabatake, 2001). Samples were removed from the medium at predetermined time (7, 14, 21, and 28 days), and rinsed with distilled water, finally dried under vacuum and weighed. The degree of *in vitro* degradation was calculated by the weight loss:

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

where W_0 is the dry weight before degradation test and W_t is the dry weight at predetermined time t. To separate between degradation and dissolution, control samples were stored for 28 days under the same conditions as described above, but without the addition of lysozyme.

2.5. In vitro cell culture studies

2.5.1. Cytotoxicity screening

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test was performed to determine the cytotoxicity of chitosan scaffolds according to the previous studies (Chellat et al., 2000; Oliveira et al., 2006). The NIH3T3 cell was obtained from ACTT (USA) and cultured with DMEM medium at 37 °C and 5% CO_2. Briefly, cells were first seeded in 24-well plates with chitosan scaffold at a density of 1×10^5 cells/well in 1 ml growth medium in a humidified atmosphere with 5% CO_2, and the cells without a scaffold served as the negative control. At specific time point of 1, 3, and 5 days, 200 μl of MTT solution (5 mg/ml) was added to cor-

Table 1
Chitosan scaffolds obtained from various chitosan concentrations.

Samples	Chitosan (mg)	Disodium hydrogen phosphate (mg)
1	36.2	90.5
2	29.6	74.0
3	25.0	62.5

responding wells and cultured at 37 °C for 4 h to allow the formation of formazan crystals. Then the MTT solution was removed, scaffolds were washed with PBS and subsequently 750 μl of dimethylsulfoxide (DMSO) was added into each well. The well plate was left on a shaking platform for 10 min. Finally, the solution (150 μl) was collected and transferred into a 96-well plate. The absorbance was recorded on a Microplate reader (Bio-Rad, USA), using a test wavelength of 570 nm.

2.5.2. Cell adhesion and cell viability stained with acridine orange (AO)

Cell adhesion and cell viability as in function with time was observed with fluorescent staining. Briefly, the cell-construct was stained with 0.01% AO for 5 min; washed with PBS and examined under a fluorescence microscope (OLYMPUS, CKX41).

2.5.3. Evaluation of cell adhesion and morphology

Cell adhesion, morphology, and spatial distribution were observed by scanning electron microscopy (SEM). Cell-construct was washed with PBS (pH = 7.4) solution and fixed in 2.5% glutaraldehyde at 4 °C overnight. After that, the cell-constructs were dehydrated using a graded series of ethanol (30%, 50%, 70%, 80%, 90%, 95%, and 100%) for 10 min. Finally, the cell-construct was dried at a vacuum oven for 4 h. Afterward, the cell-construct was sputter coated with gold and observed by a scanning electron microscopy (JSM-5900LV, JEOL, Japan).

3. Results and discussion

3.1. Preparation and characterization of chitosan scaffolds

Porous chitosan scaffolds were prepared by freeze-drying of pre-gelled chitosan solution at 37 °C. According to previous report of Nair's group (Nair, Starnes, Ko, & Laurencin, 2007), the addition of inorganic phosphate solutions to chitosan acetic acid solution could increase pH value of system to about 7 resulting in the gel formation at 37 °C. And they also demonstrated the potential of chitosan–inorganic phosphate solutions system as a cell carrier matrix. Here we prepared chitosan scaffold by freezing-drying technology with pre-gelling chitosan solution with disodium hydrogen phosphate. The appearance of prepared chitosan scaffold was presented in Fig. 1. Chitosan scaffold was white, although the chitosan film or chitosan powder is usually yellowish.

3.2. Effect of chitosan concentration on the chitosan scaffold

The effect of chitosan concentration on the morphology, porosity and water adsorption of chitosan scaffolds was investigated by using 1.8%, 1.5%, and 1.25% of chitosan solution with 0.1 g of dibasic sodium phosphate. Fig. 2 showed the effect of chitosan concentration on the porosity and water adsorption of the chitosan scaffolds. As depicted in Fig. 2, with the decrease of chitosan concentration, porosity of chitosan scaffolds increased accordingly. The reason is obvious, since the actual volume fraction occupied by the materials itself decreased when the chitosan concentration decreased (Tibbitt & Anseth, 2009; Wu et al., 2009). All the prepared chitosan scaffolds have high porosity >85%, up to 95%. Previous studies have revealed that the scaffolds with porosity higher than 85% can be appropriate for tissue engineering application (Hutmacher, 2000). This criterion arises from the demand for the cell ingrowth in sufficient space. Therefore, the prepared chitosan scaffold satisfied this criterion suitable for tissue engineering.

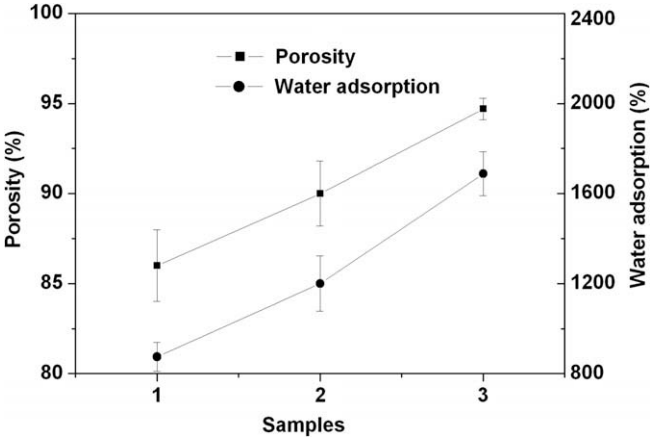

Fig. 2. Porosity and water adsorption of chitosan scaffold from various chitosan concentrations (1.8%, 1.5%, and 1.25%).

As a scaffold for tissue engineering, besides the porosity, the water adsorption of scaffold not only affects its morphology and structure but also affects the ingrowing cells (Hollister, 2005; Kim et al., 2003). When the chitosan scaffolds are applied in the tissue engineering, they have great water adsorption that can prevent the loss of body fluid and nutrients. As presented in Fig. 2, chitosan scaffold swelled more than about 10 times the dry scaffolds. The variations in the water adsorption of scaffold were similar to those in the porosity. It might be explained by that the chitosan scaffold made from low chitosan concentration had high porosity and large pore size, which offered more space for water storage, yielding the increase of water adsorption (Choi et al., 2009; Kim et al., 2003; Wu et al., 2009). In other words, chitosan containing primary amine ($-NH_2$) and hydroxyl group (–OH) cannot only increase its affinity to water but also form hydrogen bonds with water (Madihally & Matthew, 1999; Nettles et al., 2002). However, if the chitosan solution was not pre-gelled with dibasic sodium phosphate, the lyophilized chitosan scaffolds made from chitosan acetic acid

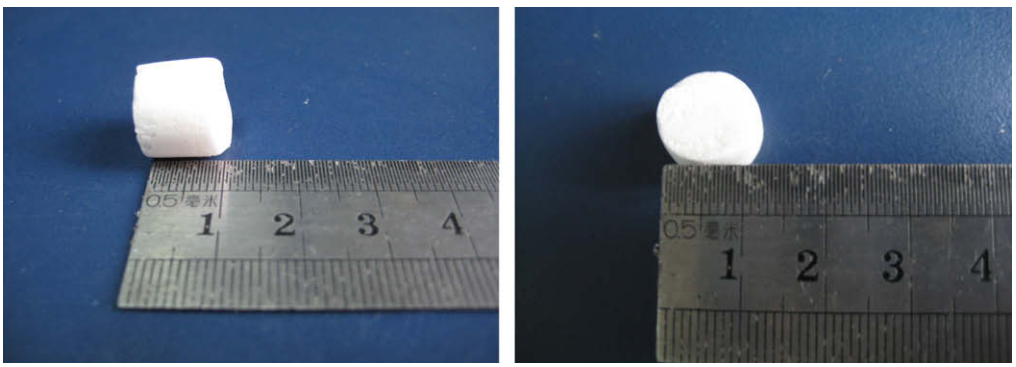

Fig. 1. The digital photographs of chitosan scaffold.

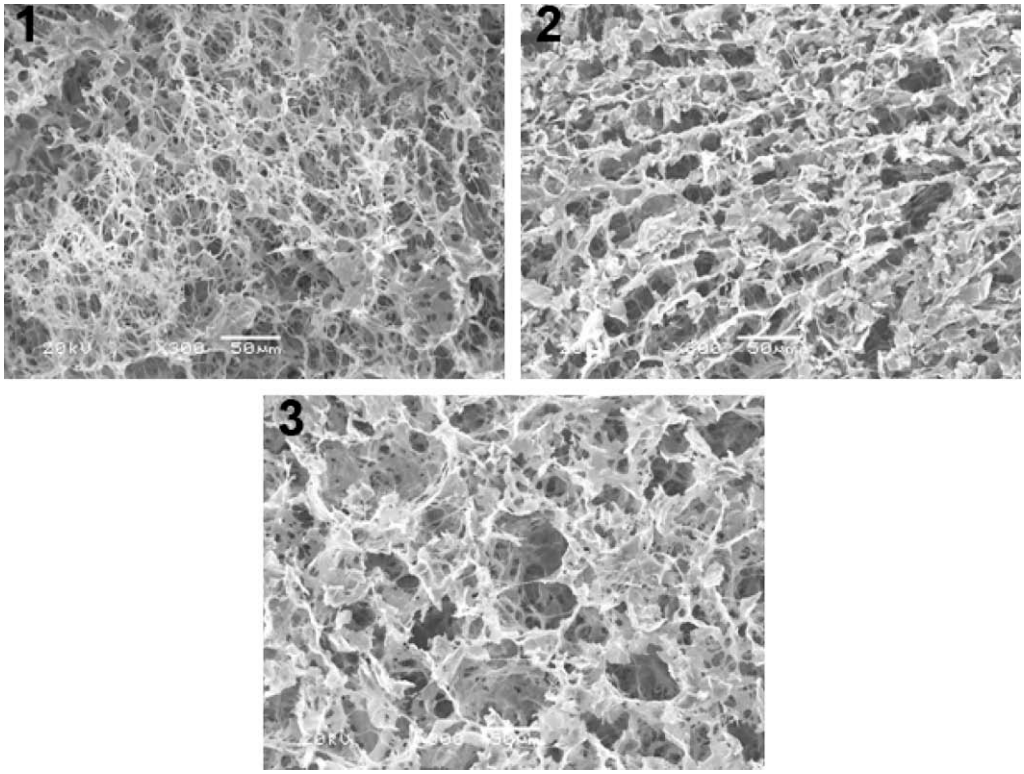

Fig. 3. SEM image of chitosan scaffold from various chitosan concentration: (1) 1.8%, (2) 1.5%, and (3) 1.25%.

solution exhibited rapid swelling and complete dissolution in water solution (data not shown). This indicated that the lyophilized chitosan scaffold was composed of soluble chitosan acetate, which is consistent with the report of Madihally et al. (1999). Hence, the obtained chitosan scaffold in this paper had large water adsorption suitable for the tissue engineering.

The effect of chitosan concentration on the morphology of chitosan scaffold was observed by SEM, as shown in Fig. 3. All the series of chitosan scaffolds had a porous structure, although the pore size was changeable. As indicated in Fig. 3, the pore diameter ranged between 50 and 150 μm. The pore size of chitosan scaffolds increased with the decrease of chitosan concentration. The result was similar to the results for the water/PVA system obtained by del Monte's group (Gutierrez et al., 2007). At low chitosan concentration (1.25%), the pores were irregular with size about 100 μm. In the contrast, the scaffold obtained from 1.8% chitosan solution had the smaller pores with size about 50 μm. This result indicated that the micro-structure of chitosan scaffold can be adjusted by modulating the concentration of chitosan during the preparation.

As a scaffold intending for the tissue engineering, chitosan scaffold should undergo various stresses during applications, so as to maintain the integrity of the scaffold and protect the encapsulated cells from being damaged. Fig. 4 depicts the effect of chitosan concentration on the mechanical properties of scaffold. The compressive strength of scaffold decreased from 0.07 to 0.038 MPa with the decrease of chitosan concentrations from 1.8% to 1.25%, which might be attributed to the higher porosity of scaffold made from lower chitosan concentration (1.25%) (Xu, Burguera, & Carey, 2007). Obviously, the chitosan concentration greatly effected on the compressive strength of scaffold. Though the compressive strength of scaffold is still not strong enough for tissue engineering, especially on the bone regeneration application, it might be improved by blending other polymers or addition of cross-linking agent (genipin ect.) (Muzzarelli, 2009; Yuan et al., 2007). And the further studies need to be performed to elevate the compressive strength of scaffold.

3.3. In vitro degradation test

It is well known that, in human serum, chitosan is mainly depolymerized enzymatically by lysozyme, and not by other enzymes or other depolymerization mechanisms (Oliveira et al., 2006; VandeVord et al., 2002). The enzyme biodegrades the polysaccharide by hydrolyzing the glycosidic bonds present in the chemical structure. Therefore, *in vitro* degradation of chitosan scaffold at physiological pH with lysozyme concentration corresponding to the concentration in human serum was performed. Fig. 5 shows the

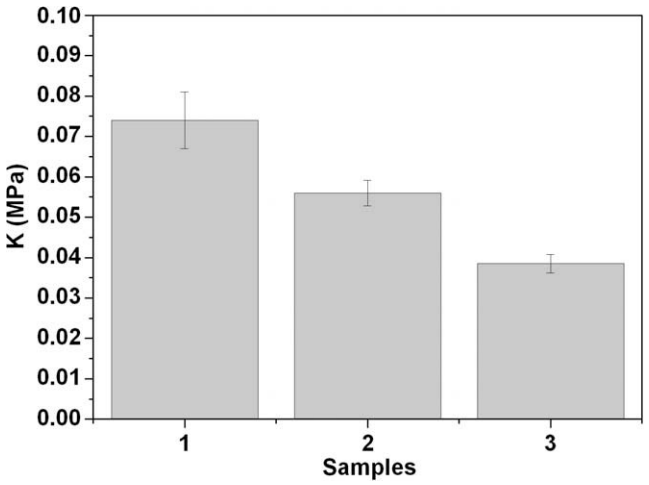

Fig. 4. Compressive strength of chitosan scaffolds made from various chitosan concentrations (1.8%, 1.5%, and 1.25%).

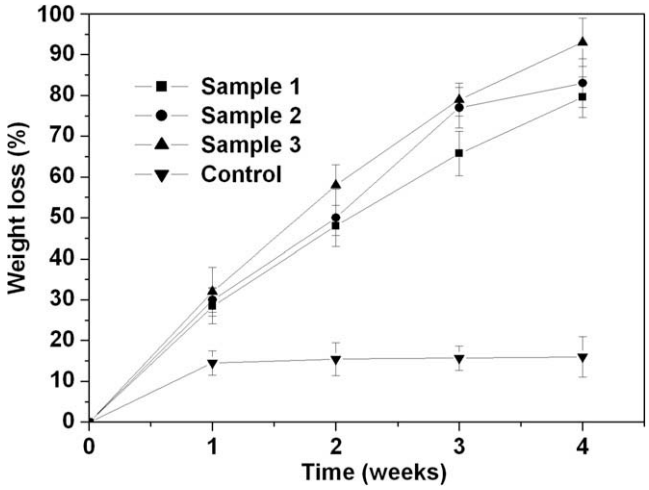

Fig. 5. *In vitro* degradation behavior of various chitosan scaffold in PBS solution at 37 °C with lysozyme concentration at 1.5 μg/ml.

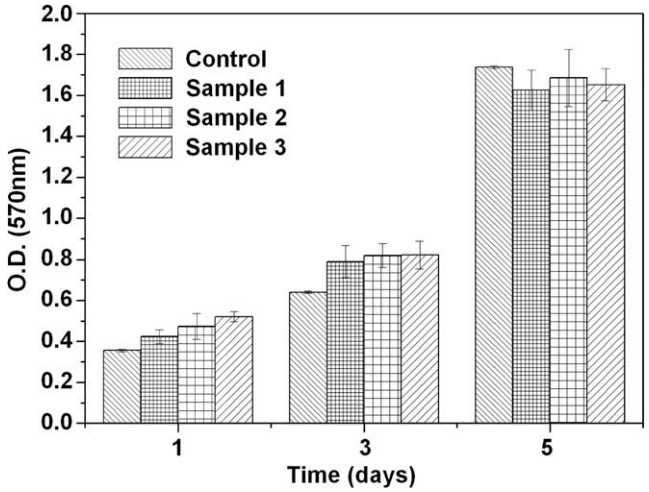

Fig. 6. MTT viability assay of chitosan scaffold following 1, 3, and 5 days after cell seeding.

enzymatic degradation profiles of chitosan scaffolds made from various chitosan concentrations. There is very little weight loss of chitosan scaffold (about 15% weight loss) in 4 weeks incubation as exposing in only PBS solution absence of lysozyme. Meanwhile, we observed that the weight loss of scaffold was mainly occurred in the first week of the test, which might be explained by that the redundant dibasic sodium phosphate was leakage from the scaffold resulting in the quick weight loss of scaffold. For chitosan scaffolds incubation in PBS solution containing 1.5 μg/ml lysozyme, the degree of degradation of chitosan scaffold increased with time. And the higher chitosan concentration, the lower the percentage of weight loss could be obtained. For example, scaffold made from 1.25% chitosan solution had a percentage weight loss of 93.5% after 28 day's degradation, indicating that the chitosan scaffold was almost completely degradaded, while, for the scaffold made from 1.8% chitosan solution only had a percentage weight loss of 78.9%. This might be explained by that chitosan scaffold made from 1.25% chitosan concentration had the larger pore structure than that of scaffold made from 1.8% chitosan concentration, resulting in the more lysozyme solution was absorbed into the scaffold. The experimental results implied that the degradation behavior of chitosan scaffold could be modulated by changing the chitosan concentration during the preparation.

3.4. Cell culture studies

3.4.1. In vitro cytotoxicity test

An ideal scaffold suitable for tissue engineering should not release toxic products or produce adverse reactions, which could

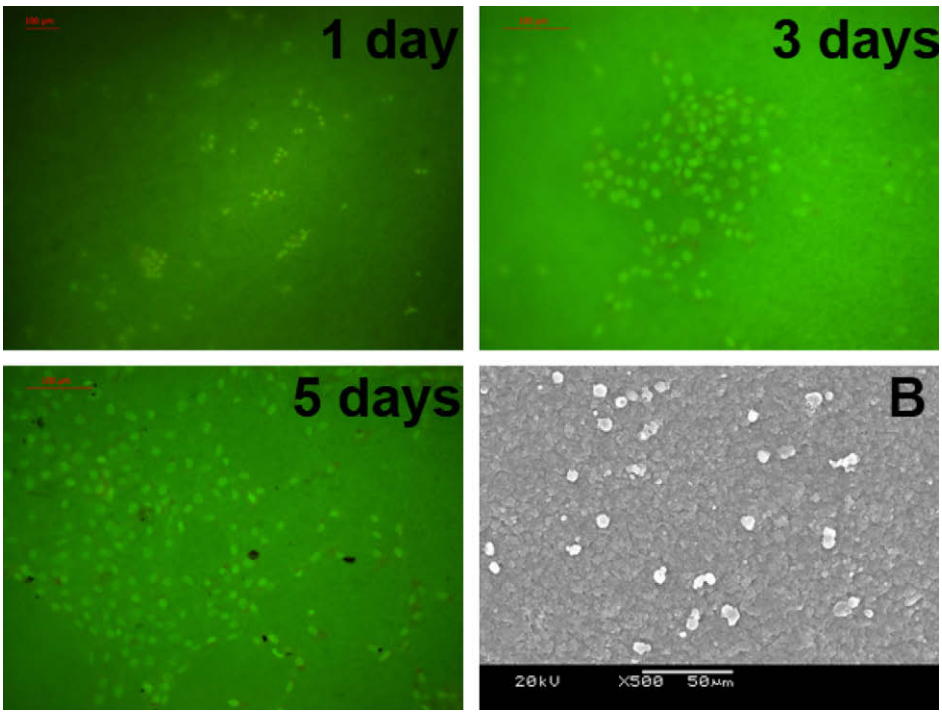

Fig. 7. Cell adhesion after 1, 3, and 5 days of cell-culture in the chitosan scaffold analyzed by acridine orange (AO) staining and SEM micrograph after 1 day's cell-culture on the surface of chitosan scaffold.

be evaluated through *in vitro* cytotoxicity tests. For *in vitro* cytotoxicity test, NIH3T3 cell was used as reference. In MTT test, as shown in Fig. 6, the prepared chitosan scaffolds were noncytotoxicity to the NIH3T3 cell after 24 h's culture. Cells cultured with chitosan scaffold have metabolic activities similar to those obtained by cells grown in DMEM (negative control).

3.4.2. Cell adhesion and stained with acridine orange (AO)

Cell adhesion on the chitosan scaffolds was monitored by microscopy. As depicted in Fig. 6, the tested NIH3T3 cell had high metabolic rates as a function of time, denoting a high viability and proliferation profile. Moreover, a cell viability as function with time with acridine orange (AO) staining (Fig. 7), also demonstrated that NIH3T3 cell were metabolically active and well distributed throughout the scaffold. As revealed in Fig. 7, the longer culture time, the increased cell density on the dish could be obtained.

3.4.3. Cell adhesion and morphology

The chitosan scaffold (1.5%) was used to evaluate the cell adhesion, spreading and interaction. Fig. 7(B) showed SEM images of NIH3T3 cell grown on the chitosan scaffold after 24 h cell culture. It could be found that, NIH3T3 cell appeared to adhere well and exhibited a normal morphology on the surface, which was due to the large surface area available for cell attachment.

4. Conclusion

In this paper, a series of porous chitosan scaffolds were successfully prepared by freeze-drying of chitosan hydrogel (pre-gelled with dibasic sodium phosphate at 37 °C). Porosity, water adsorption as well as compressive strength of chitosan scaffold could be regulated by the chitosan concentration. And the prepared chitosan could be degraded by lysozyme solution after 28 day's incubation. *In vitro* cell test showed that the NIH3T3 cell were metabolically active and well distributed throughout the scaffold after 5 day's incubation. All these experimental results indicated that the prepared chitosan scaffolds might have great potential in the application of tissue engineering.

Acknowledgments

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