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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 $\rho$ 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 $1 \times 10^5$ 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 |