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Preparation, characterization and evaluation of chitosan macroporous for potential application in skin tissue engineering

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ABSTRACT

In this paper, chitosan macroporous membrane was developed by a selective dissolution technology using silica particles (SiO_2) as the porogen agent. Regarding of the weight ratio of silica particle/chitosan (1:2, 1:1 and 2:1; w/w), a series of chitosan membranes were developed. The initial SiO_2 content in the formulation was greatly influenced on the morphology of chitosan membrane. As the initial SiO_2/chitosan weight ratio was lower than 1:2, the developed chitosan membrane was dense absence of any porous structure. Meanwhile, the developed chitosan membranes were characterized by scanning electron microscopy (SEM), porosity, mechanical property, water adsorption as well as in vitro degradation. In vitro cytotoxicity study showed that the extractions of all developed chitosan membranes were non-cytotoxic against L-929 cells after 24 h of culturing. L-929 cells cultured with chitosan macroporous membrane (S3) revealed that the cells were flattened with effective spreading after 24 h and 48 h of culturing. All these results indicated that the developed chitosan macroporous membrane might have potential application in skin tissue engineering.

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1. Introduction

Skin, the largest organ of the body, is commonly damaged by wounding and burning. However, the large scale skin defects could not heal spontaneously, which still is the major problem in the surgical field [1]. Since the early 1980s, numerous skin substitutes including autografts, allografts, and xenografts have been developed to promote wound healing [2]. Due to the limited number of donator site and antigenicity, the application of these skin substitutes was greatly limited. More recently, numerous studies have been oriented to the wound dressings made from biodegradable polymers to promote wound healing and to reduce scar formation [2–5]. The ideal wound dressings needs to ensure that the wound remains moist with exudates, but not macerated, and free of infection as well as non-cytotoxicity, biocompatibility and with a certain strength [6–8].

Chitosan, poly(1,4-D-glucosamine), a semi-crystalline aminopolysaccharide obtained by deacetylation of chitin, has been widely used in the skin tissue engineering owing to its non-toxicity, well-biocompatibility, biodegradability, mucoadhesive, and permeation enhancing properties [5,9]. For the various skin tissue engineering applications of chitosan and its derivates, several formulations including membrane/films, microparticles, sponge, and so on have been fabricated and investigated. Nowadays, a commercial product (Tegaderm™ film) based chitosan membrane has been developed for wound healing [10,11]. As the potential scaffold for skin tissue engineering, the porous structure of scaffold was very important. In the past three decades, a lot of technologies have been employed to develop the microporous membrane/films of the chitosan matrix [12–14]. Phase inversion technique (polymer solution was cast and immersed into a coagulation bath) has been widely used to obtain the chitosan asymmetric membrane for the biomedical application and skin tissue engineering. However, only low mechanical strength of chitosan scaffold could be obtained using such a technology, which greatly limited its further in vivo applications [15]. Recently, the technique based on inverse porogen/polymer solubility and consisted of the dissolution of the porogen agent into the polymeric matrix, followed by solvent evaporation and porogen agent extraction by immersion on a suitable solvent, which has been employed to prepare chitosan macroporous membrane [16,17]. Santos et al. [14] employed such a technique to develop chitosan asymmetric membrane using silica as porogen agent for controllable drug permeability across the membrane. Many other applications of this membrane such as protein separation, gas separation, and so on have been performed.

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Besides the silica particles, many other porogen agents such as NaCl, $CaCO_3$, and so on were also used to develop chitosan/chitin macroporous membrane [18,19].

Herein, a series of chitosan macroporous membranes with various pore sizes were developed using silica particles as the porogen agent. The developed chitosan membrane was characterized by the morphological observation, water adsorption, porosity, mechanical properties, in vitro degradation behavior, cell cytotoxicity and cell attachment before its further in vivo applications.

2. Materials and methods

2.1. Materials

Chitosan (degree of deacetylation (DD): approximately 80%; viscosity: >400 mPa s, 1% in acetic acid (20 °C)) was supplied by Sigma–Aldrich (USA). Silica particles (40–70 μm) was supplied by KeLong Chemicals (Chengdu, China). Lysozyme was bought from the Amresco (USA). All other chemicals used in this paper were analytic grade. Distilled water from Milli-Q water system was used to prepare the aqueous solutions.

2.2. Preparation of chitosan macroporous membrane

According to the previous studies, the chitosan macroporous membrane was successfully developed by a selected dissolution technology using silica particles as the porogen agent [14]. Briefly, 2 g of chitosan was dissolved into 1% acetic acid aqueous solution to form 2% (w/v) chitosan acetic aqueous solution. Thereafter, different amounts of silica particles, regarding of weight ratio of silica particle/chitosan (1:2; 1:1; 2:1; w/w) were added to the chitosan acetic aqueous solution with continuous stirring to form the homogeneous suspension. Finally, the resultant suspension was poured into a glass plate (8 cm × 8 cm) followed by drying in oven at 60 °C for 1 day to obtain a series of chitosan/silica particle membrane. To develop chitosan macroporous membrane, the obtained chitosan/silica particle membranes were immersed into 1 M NaOH solution at 80 °C for 6 h to dissolve the silica particles component. The obtained chitosan macroporous membrane were washed with distilled water for three times to neutral (pH = 7) followed by drying at room temperature. The obtained chitosan macroporous membranes were coded as S1, S2 and S3 for silica particles/chitosan weight ratios of 1:2, 1:1 and 2:1, respectively.

2.3. Scanning electron microscopy (SEM) observation

The top-surface and cross-section of S1, S2 and S3 were observed by a scanning electron microscopy (JSM-7500F, JEOL, Japan). The cross-section of samples was obtained by treatment of membrane with liquid nitrogen. And all the samples were sputtered with gold before SEM observation.

2.4. Porosity measurement

The porosity of chitosan macroporous membranes was measured by using Archimedes principle as previous study [13]. The samples were divided into pieces (2 cm × 2 cm) and weighted (W_0), followed by immersion into an absolute ethanol solution for 24 h to achieve the saturation. After that, the samples were withdrawn from absolute ethanol and weighted (W_{24}). And the porosity of the samples was calculated by following formula:

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

where ρ is a constant of the density of ethanol (0.789 g cm^{-3}). Porosity was expressed as mean ± SD ($n = 3$).

2.5. Water adsorption study

Each chitosan macroporous membranes (2 cm × 2 cm) were immersed into 20 ml of phosphate buffer solution (PBS; pH = 7.4). After soaking in PBS solution at 37 °C for 24 h, the samples were withdrawn from the PBS solution followed by the removing the surplus surface water using filter paper and weighted. The percentages of water absorption were calculated by following formula:

$$\text{Water absorption } (\%) = \frac{W_{24} - W_0}{W_0} \times 100$$

where W_{24} is the weight of sample after soaking for 24 h and W_0 is the original weight before the test, respectively. The values are given as mean ± standard deviation ($n = 6$).

2.6. Mechanical properties

The mechanical properties of chitosan macroporous membranes were detected by a universal mechanical testing instrument (Instron-5567, Instron Corp., USA) at room temperature and relative humidity of 50% [20]. The specimens with dimensions of 40 mm × 5 mm × 0.06–0.10 mm were used for test. The tensile strength and the elongation were evaluated at a displacement rate of 20 mm/min with 20-mm gauge length. All results were mean values of six specimens.

2.7. In vitro degradation test

In vitro degradation test of chitosan macroporous membrane were carried out in 5 ml phosphate-buffered solution (PBS, pH = 7.4) with 1.5 μg/ml of lysozyme at 37 °C as the previous report with little modification [20]. Briefly, each chitosan macroporous membrane was divided into pieces (2 cm × 2 cm) and then placed into 25 ml BD tube followed by addition of 5 ml PBS solution with 1.5 μg/ml of lysozyme. The lysozyme solution was refreshed daily to ensure continuous enzyme activity. At predetermined time point (7 days, 14 days, 21 days and 27 days), the samples were withdrawn from medium and rinsed with distilled water solution for three times followed by lyophilization and weighted. The weight loss percentage (%; w/w) at each time point were calculated using the following formula:

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

where W_0 is the original weight of samples before the test and W_t is the dry weight of samples at predetermined time t. All results were mean values of three specimens.

2.8. In vitro cell culture studies

2.8.1. In vitro cytotoxicity test

According to the previous study of Oliveira et al. [21], MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test was used to evaluate the in vitro *cytotoxicity* of chitosan macroporous membranes. Following the sterilization with ^{60}Co gamma ray irradiation at a dosage of 15 kGy, each chitosan macroporous membranes (2 cm × 2 cm) were placed in a 6-wells plate followed by addition of 5 ml DMEM solution for culturing at 37 °C. After soaking for 24 h, 4 ml extractions of samples were collected for cytotoxicity test. Rat fibroblast (L-929 cell) was used for evaluation of in vitro cytotoxicity of extractions. First of all, L-929 cells were cultured at DMEM to achieve with cell concentration of 1×10^5 cells ml^{-1}, and then seeded onto 96-wells plates overnight. After that, a series

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