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Preparation and characterization of cross-linked carboxymethyl chitin porous membrane scaffold for biomedical applications

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ABSTRACT

Porous dermal scaffold membrane (PDSM) was successfully prepared by using a so-called sol–gel freeze-drying method. In this method, the carboxymethyl chitin (CMC) hydrosol was first cross-linked by 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS), and then lyophilized to form the PDSM. For the first time, this research focused on the cross-linked CMC as the only component for three-dimensional PDSM. The effects of cross-linking conditions on the performance of the PDSM were investigated. And PDSM with optimal performance was obtained through 4-h cross-linking at 4 wt% of CMC concentration in the hydrosol, where the mass ratio of EDC to NHS to CMC was 5:3:10. The porosity of the optimized PDSM was more than 90% and the water swelling rate was above 4000%. The pore size was well distributed and was between 100 μm and 200 μm. And the tensile strength was above 0.09 MPa. The as-made PDSM could be degraded above 80% in 12 days in the presence of a 0.2 mg/mL lysozyme solution. Very importantly, the PDSM had no cytotoxicity and good biocompatibility from MTT assays. Our results showed the application possibility of the as-prepared PDSM as dermal scaffold for skin tissue engineering.

1. Introduction

As the largest organ of the body, skin comprises epidermis and dermis which provides complex nerve and blood, and has many essential functions, such as anti-infection protection, immune surveillance, tactile perception and temperature regulation and so on. As the barrier to maintain homeostasis and prevent microorganism infection, skin would usually be damaged or hurt from outside (Cirillo, Cirillo, & De Maria, 2014; Fuchs & Raghavan, 2002). In tissue engineering technology of repairing damaged dermis, biodegradable scaffold, seed cell and cell growth regulation factor play important roles. The tissue engineering dermal scaffold provides three-dimensional space and suitable environment which are beneficial for dermal cells' growth, adhesion and proliferation (Jayakumar, Chennazhi, & Srinivasan, 2011).

Different from other biological materials, scaffold materials are capable of combining with not only living cells but also biological systems, namely the cell compatibility and histocompatibility (referred as biocompatibility). Degradability is another important characteristic of tissue engineering scaffold. As cells' carrier, the scaffold will be constantly degraded and gradually replaced by the host tissue. Biocompatibility, biodegradability, and low cost of chitin and chitosan have drawn immense attention in many fields spanning from medicine, bioinspired material science, pharmaceuticals, to agriculture (Jayakumar, Prabaharan, Nair, Tokura, et al., 2010; Narayanan, Jayakumar, & Chennazhi, 2014). As a water-soluble derivative of chitin, carboxymethyl chitin (CMC) is biocompatible and biodegradable, and is more easily degraded in the human body by lysozyme comparing to chitin. CMC has the potential to be applied in the biomedical field, in particular to promote wound healing (Jayakumar, Prabaharan, Nair, & Tamura, 2010; Kumar, Ramya, & Jayakumar, 2013; Madhumathi, Kumar, & Kavya, 2009; Tokura & Tamura, 2001; Wongpanit, Tabata, & Rujiravanit, 2007).

The structure and biological properties of low deacetylation degree (DD) of CMC were most similar to glycosaminoglycan hyaluronic acid that exists in the extracellular matrix of higher organisms. It is more easily absorbed and degraded *in vivo* and could be used to prepare an ideal three-dimensional porous dermal scaffold through covalent cross-linking and freeze-drying, and the cytotoxicity and blood coagulation characteristics caused by the high charge density of chitosan are avoided. Heterogeneous collagen protein is unnecessary for the dermal scaffold because the

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autologous secretion of dermal fibroblasts collagen is easy which avoids the immune response caused by foreign proteins. Chitin and chitosan have poor mechanical property and its mechanical property can be improved with addition of biomaterials like hydroxyapatite (HAp), bioactive glass ceramic (BGC) (Jayakumar, Prabaharan, Nair, & Tamura, 2010). Carboxymethyl chitin (CMC) could be complex with poly(vinyl alcohol) (PVA) to form a novel fibrous membrane which supports cell adhesion/attachment and proliferation (Kavya, Jayakumar, Nair, & Chennazhi, 2013; Shalumon, Binulal, & Selvamurugan, 2009). Herein, this research for the first time focused on the cross-linked CMC as the only component for three-dimensional porous dermal scaffold membrane (PDSM). In this study, we successfully demonstrated the preparation of PDSM from cross-linked CMC with a sol–gel freeze-drying method. The effects of cross-linking conditions on the performance of the PDSM were investigated, that is, the surface morphology, porosity, swelling ratio, enzymatic degradation and cytotoxicity. The results clarified its structure and properties. Indeed, our results indicated the application possibility of the as-prepared PDSM as tissue engineering dermal scaffold.

2. Experimental details

2.1. Preparation of the PDSM

The purified CMC (DD was 0.49, substitution degree of carboxymethyl (DS) was 0.95, supplied by Shenzhen Bright Way Novel Bio-material Tech. Co., Ltd.) was dissolved in deionized water to a mass percentage concentration. A certain proportion of N-Hydroxysuccinimide (NHS, Alfa Company) and 4-MES (Alfa Company, as dispersing agent) were added and fully mixed to pH between 5 and 7. Then the sol–gel and the prepared 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC, Alfa Company) solution were both pre-frozen at −20 °C for 5 min. These two solutions were rapidly mixed and stirred for 1 min and then injected to the mold. The cross-linking CMC was taken out at room temperature for some time and then pre-frozen at −20 °C for 12 h. The frozen gel was lyophilized for 12 h to get PDSM in the lyophilizer (LGJ-10, Ningbo Scientz Biotechnology Co., Ltd.). The PDSM was soaked in 0.1 mol/L of Na_2HPO_4 and 0.9% of NaCl aqueous solution for 24 h and 72 h, respectively. The PDSM cleaned with deionized water was finally lyophilized for another 12 h. The as-prepared PDSM was stored for further test.

2.2. Characterization of the PDSM

2.2.1. Scanning electron microscope

The micromorphology of PDSM surface was scanned by Hitachi S-3400N scanning electron microscope (SEM). PDSM was tested after spraying process and the acceleration voltage was 15 kV. The pore size was directly determined by SEM images.

2.2.2. Porosity

Ethanol was selected as the solvent and certain amount of PDSM (W_1) was immersed into the volumetric flask with ethanol and the flask was circulated by a vacuum pump until no bubbles were observed. The weight of volumetric flask containing ethanol and PDSM was W_2. Taking out the PDSM containing alcohol, the weight of volumetric flask containing residual ethanol was W_3, the porosity of PDSM was calculated according to formula:

$$\text{Porosity}\,(\%) = \frac{V_{\text{pore}}}{V_{\text{CM-chitin}} + V_{\text{pore}}} \times 100 = \frac{W_2 - W_3 - W_1}{W_2 - W_3} \times 100$$

V_{pore} was the pore volume of the sample; $V_{\text{CM-chitin}}$ was the volume of CMC in the sample; $V_{\text{C-chitin}} + V_{\text{pore}}$ represented the total volume of sample. $W_2 - W_3 - W_1$ was the mass of ethanol that had the same volume as the pores in PDSM; $W_2 - W_3$ expressed the mass of ethanol that had the same volume as PDSM.

2.2.3. Swelling rate

PDSM was immersed into phosphate buffer (pH 7.4) and taken out after every 1 h. The excess water on the membrane surface was adsorbed by the filter paper.

$$\text{Swelling rate}\,(\%) = \frac{W_t - W_0}{W_0} \times 100$$

W_t (g) was the stable membrane mass after immersed in buffer and W_0 (g) was the membrane mass before immersed in buffer.

2.2.4. Mechanical properties

PDSM was cut into the sample of size 20 mm × 10 mm and the sample thickness was tested with the vernier caliper. The sample was fixed in the TP-219A universal material testing machine (Tuo Feng Instrument Technology (Shanghai) Co., Ltd., China) for the determination of load-shift curve. The tensile strength and elongation at break of PDSM sample were calculated. The stretching rate was 10 mm/min and clamping length was 10 mm, and the sample width was 10 mm.

2.3. In vitro *enzymatic degradation of the PDSM*

In vitro enzymatic degradation of the PDSM was evaluated in lysozyme solution. After every PDSM sample had been weighed, they were individually immersed into 10 mL of phosphate buffer (pH 7.4) containing 0.2 mg/mL of lysozyme (U/mg ≥ 20,000) at 37 °C in an incubator. Three samples were taken out every day, washed with deionized water and lyophilized for weighing. The degradation rate was calculated as the following equation:

$$\text{Remaining rate}\,(\%) = 1 - \text{degradation rate}\,(\%) = \frac{W_t}{W_0} \times 100$$

W_t (g) was the sample mass after degradation and W_0 (g) was the sample mass before degradation.

2.4. Cytotoxicity

The PDSM was first sterilized by Cobalt (Co_{60}) irradiation and then immerged into the cell culture medium (DMEM, membrane surface/media was 6 cm^2/mL) for 3 days in 37 °C in a 5% CO_2 incubator. The PDSM extraction was diluted to 100%, 50%, 25% and 12.5% with DMEM medium. DMEM medium was the blank control and PE extraction solution was the negative control. Each test had 8 parallel experiments. The MTT assays for L929 fibroblasts were conducted as reference (Pagliacci, Spinozzi, & Migliorati, 1993).

3. Results and discussion

3.1. Surface morphology of the PDSM

When the mass percentage concentration of CMC in the hydrosol was 4%, the mass ratio of EDC to NHS to CMC was 5:3:10 and the hydrosol was cross-linked for 8 h, the performance of PDSM was optimal and its surface SEM image was shown in Fig. 1. In this freeze-drying method, water acted as a pore-forming role. When the pre-freezing temperature was below 0 °C, water in gel formed ice nuclei through the stage of nucleation and growth, two-phase separation between ice and cross-linked material appeared, and pores formed in the original ice location by vacuum sublimation of ice.

These pores of the scaffold were connected with each other, pore distribution was uniform, and their stability was increased by cross-linking. By measuring the pore size of a number of SEM