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Thermal-crosslinked porous chitosan scaffolds for soft tissue engineering applications

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ABSTRACT

The aim of this study was to demonstrate the feasibility of using a steam autoclave process for sterilization and simultaneously thermal-crosslinking of lyophilized chitosan scaffolds. This process is of great interest in biomaterial development due to its simplicity and low toxicity. The steam autoclave process had no significant effect on the average pore diameter (~70 μm) and overall porosity (>80%) of the resultant chitosan scaffolds, while the sterilized scaffolds possessed more homogenous pore size distribution. The sterilized chitosan scaffolds exhibited an enhanced compressive modulus (109.8 kPa) and comparable equilibrium swelling ratio (23.3). The resultant chitosan scaffolds could be used directly for *in vitro* cell culture without extra sterilization. The data of *in vitro* studies demonstrated that the scaffolds facilitated cell attachment and proliferation, indicating great potential for soft tissue engineering applications.

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1. Introduction

Chitosan, a linear polysaccharide, holds great potential in tissue engineering applications due to its readily availability, unique physicochemical properties, biocompatibility and biodegradability [1–3]. Chitosan has been fabricated in the form of lyophilized porous scaffolds to support cell attachment and proliferation, and eventually to facilitate new tissue formation after chitosan degradation [3].

Sterilization of the porous scaffolds remains an issue for tissue engineering applications. Chitosan scaffolds are usually sterilized by ethanol rinse or by UV exposure before laboratory-level *in vitro* studies [4,5], while for *in vivo* and clinical studies, ethylene oxide (EtO) exposure or gamma ray (Cobalt-60) irradiation are compulsory and more efficient [6–8]. However, such sterilants are highly hazardous not only to the host tissues and organs, but also to the operators [9,10]. The complete evacuation of these reagents (especially for porous materials) usually takes weeks-to-months, which is time-intensive [7,11].

Steam autoclave is a common sterilization method for thermal-inert medical devices or buffered solution [12]. Compared with EtO exposure and gamma-ray irradiation, this process is simpler and more environmentally-friendly, while it is rarely applied for tissue engineering scaffolds given that most biomaterials have low thermal resistance. Our previous study reported that steam autoclave was used to sterilize and simultaneously induce chitosan-based hydrogel formation, and the resultant hydrogels are potent for drug delivery and tissue engineering applications [13].

In this study, we evaluated the feasibility of using steam autoclave to sterilize lyophilized chitosan scaffolds. The effect of sterilization on the scaffolds' performances such as porosity, swelling behavior and mechanical properties were demonstrated. Cells were statically seeded on the sterilized scaffolds and *in vitro* culture was subsequently undertaken. The cell attachment and proliferation within chitosan scaffolds were investigated.

2. Materials and methods

2.1. Materials

Chitosan (Catalog No. 448877, medium molecular weight and deacetylation degree of 75–85%), fluorescein diacetate (FDA), propidium iodide (PI), and Dulbecco's modified eagle medium (DMEM), were purchased from Sigma-Aldrich. MTS assay kit, fetal bovine serum (FBS) and penicillin–streptomycin solution (pen–strep) were purchased from Invitrogen. A 0.2 M acetic acid solution was prepared by diluting glacial acetic acid (Ajax Fine Chem) in MilliQ water. Chitosan solution (1.5 wt.%) was prepared by dissolving chitosan powder in 0.2 M acetic acid solution. Phosphate buffered saline (PBS, pH 7.2–7.4) was prepared by dissolving PBS tablets (Sigma) in MilliQ water.

2.2. Fabrication and sterilization of chitosan porous scaffolds

Chitosan solution as prepared above was poured into a custom-made mold and lyophilized for 30 h. The lyophilized scaffolds were collected and rinsed extensively by PBS to remove acidic residue, and eventually lyophilized again. The steam sterilization was held in a steam autoclave (Shenan Medical Devices) chamber for 30 min at 121 °C (Fig. 1A). Given the knowledge that chitosan possessed a melting temperature around 130–140 °C [14], the use of high temperature incubation 121 °C would not change the thermal behavior of chitosan.

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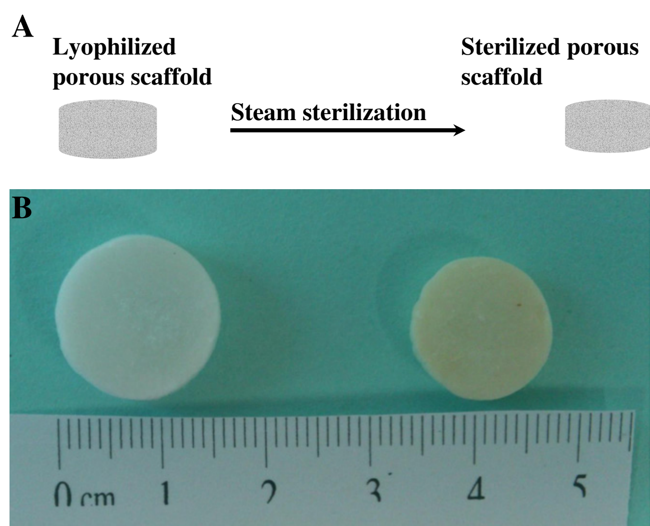

Fig. 1. A: Schematic diagram of chitosan scaffolds' fabrication and sterilization; and B: image of chitosan scaffolds before (left) and after (right) steam sterilization.

2.3. In vitro cell culture

In vitro cell culture was performed on sterilized chitosan scaffolds under a biosafety cabinet. Each scaffold (~10 mm diameter and 3 mm thickness) was immersed in culture media (DMEM, 10% FBS, 1% pen–strep) at 37 °C for at least 2 h. The cells (human skin fibroblast cells GM3348) were then seeded (pipetted) onto the scaffold at a concentration of 1×10^5 cells/sample (for MTS assay, a cell concentration of 1×10^4 cells/sample was used). The cell-seeded scaffolds were kept in a CO_2 incubator (Thermo Fisher HERAcell 150i) at 37 °C for further characterizations. The media was refreshed every two days.

2.4. Characterizations

2.4.1. Fourier transform infrared (FTIR) spectroscopy

The molecular interactions of chitosan scaffolds upon steam sterilization were determined by using Fourier transform infrared (FTIR) spectroscopy (Varian 660-IR) with a resolution of 4 cm^{-1}, averaging for 32 scans.

2.4.2. Gel permeation chromatography (GPC)

The molecular weight of fresh and steam sterilized lyophilized chitosan scaffold was determined by using GPC as previously described [15]. In brief, each chitosan scaffold was dissolved in an acetic acid (0.3 M)/sodium acetate (0.2 M) buffer with a pH of 4.5 to achieve a concentration 0.5 mg/mL. Magnetically stirring and a moderate heating (~40 °C) were helpful for complete dissolution of sterilized chitosan. The chitosan solution was eventually filtered through a 0.45 μm membrane (Millipore) before GPC (Perkin Elmer Series 200) analysis. The molecular weight was calculated from a calibration curve using a series of poly ethylene glycol (PEG) with known molecular weights.

2.4.3. Surface morphology

The porous structure on the surface of resultant scaffolds was visualized by using a phase contrast microscope (Leica). The porous structure on the cross section of resultant scaffolds was examined after cryo-fracture as previously described [16]. In brief, each fabricated scaffold was immersed in liquid nitrogen for 45 s, and cut by using a pre-cooled single edge razor blade. The fractured cross section was then mounted on glass slide for observation under a light microscope (Leica). The equivalent circle diameter (ECD) was then calculated by using ImageJ software. At least 300 pores were analyzed for each condition.

2.4.4. Mechanical strength

Uniaxial compression tests were performed in a hydrated state (in PBS) at room temperature by using an Instron (Model 5543) with a 500 N load cell. Prior to mechanical testing, the scaffolds were immersed in PBS for at least 2 h; The compression (mm) and load (N) were collected at a crosshead speed of 30 μm/s until 60% compression was achieved. The compressive moduli were then calculated as the tangent slope of the stress–strain curves within linear regions (10–20% strain rate).

2.4.5. Swelling behavior

The swelling behaviors of the scaffolds were evaluated at 37 °C in PBS. The initial dry weights were recorded (W_0). After immersion in excessive PBS for different time intervals (1–24 h), the swollen chitosan scaffolds were weighted (W_t). The swelling ratio was subsequently calculated as $(W_t - W_0) / W_0$.

2.4.6. Overall porosity

The overall porosities of resultant chitosan scaffolds were evaluated as previously described [17]. In brief, each scaffold was submerged in absolute ethanol of a known volume (V_1), and a series of vacuum-release cycles was performed to force the liquid into the pores of the scaffold. After these cycles, the volume of the liquid and liquid-impregnated scaffold was recorded as V_2. When the liquid-impregnated scaffold was removed, the remaining liquid volume was recorded as V_3. The overall porosity was given as $[(V_1 - V_3) / (V_2 - V_3)] \times 100\%$ (n = 9).

2.4.7. Live/dead staining

Cell proliferation in the resultant scaffolds was examined by live/dead staining. The cell-seeded scaffolds were stained with FDA and PI (both 1 μg/mL in PBS) for 5 min. The stained samples were assessed by using a fluorescence microscope (Eclipse E800, Nikon). Live cells were stained with fluorescent green due to intracellular esterase activity that de-acetylated FDA to a green florescent product. Dead cells were stained with fluorescent red as their compromised membranes were permeable to nucleic acid stain (PI). Percent cell viability values were calculated by counting the number of live (green) cells and the number of dead (red) cells on images (10× magnification). The values were obtained by dividing the number of live cells by the number of total cells (live cells + dead cells). A statistical significance level of 99.5% ($p < 0.005$) was considered to avoid potential human error in cell counting (n = 9).

2.4.8. In vitro proliferation assay

In vitro cell proliferation was examined by using MTS assay. The cell-seeded scaffolds (1×10^4 cells/sample) were immersed in culture medium within 48 well-plates (n = 10). At different time intervals (3 h, 1 day, 4 and 7 days), the samples were rinsed by PBS three times; 250 μL fresh medium and 50 μL MTS was subsequently added into each well. The samples were then kept in a CO_2 (5% CO_2 and 95% humidity) incubator at 37 °C for 1 h; allowing MTS to react with metabolically active cells and subsequently result in water-soluble formazan product quantifiable by the optical density (OD) at 490 nm by using a microplate reader (Bio Rad 680).

2.5. Statistical analysis

Each test was repeated three times except elsewhere mentioned. The statistical significance was determined at each condition by an independent Student's *t*-test for two groups of data using SPSS statistical software (PASW Statistics 18). Data are represented as mean ± standard deviation (SD). Confidence level of 95% ($p < 0.05$) was considered as statistically significant except elsewhere mentioned.

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