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Strontium-modified chitosan/montmorillonite composites as bone tissue engineering scaffold

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ABSTRACT

The objective of this study is to develop chitosan/montmorillonite (C/MMT) composite scaffolds based on improved properties for bone tissue engineering applications. With the freeze-drying technique, strontium (Sr^{2+}) modified C/MMT composite scaffold with an interconnected porous structure was produced. X-ray diffraction, fourier transform infrared spectroscopy, thermal gravimetric analysis, and scanning electron microscopy (SEM) were employed to investigate the structural properties, surface morphology and porosity of the composite scaffold. One of the aims of this study was to document the release of Sr^{2+} from the non-modified and modified scaffolds into the cell culture medium. The biocompatibility of composite scaffolds was evaluated in cell cultures. Human osteoblasts (hOBs) were cultured, expanded and seeded on Sr^{2+}-modified and non-modified C/MMT scaffolds. In-vitro cell viability and proliferation were investigated using MTT (3-(4,5-dimethylthiasol-2-yl)-2,5-diphenyltetrazolium bromide) assay and DNA content analysis. Live/dead cell staining assay and SEM were used for evaluating the cell-laden constructs. In-vitro studies showed that C/MMT scaffolds had no negative effects on osteoblasts. Ions present in the MMT were released into the cell culture medium, to induce osteoblast activity in the C/MMT scaffold system. Findings indicate that Sr^{2+} modification of MMT-chitosan improves scaffold properties, suggesting Sr^{2+}-modified C/MMT composite may be a promising biomaterial for bone tissue engineering.

1. Introduction

The use of biomaterials for the treatment of bone tissue damage was extensively studied during the last decade [2,31]. For the successful regeneration of the damaged bone tissue, selecting the most suitable biodegradable three-dimensional (3D) temporary matrix has constituted a critical step [12,18]. The native bone consists of naturally occurring polymer and biological apatite. The combination of polymers and inorganic fillers to create an ideal biofunctional template for bone tissue engineering (BTE) and mimic the natural structure has been widely explored. Due to its improved osteoconductive and mechanical properties, it could be advantageous to use composite materials for supporting the growth, proliferation and activity of cells [5].

In recent years, clays have started to gain recognition as inorganic fillers over ceramics for the production of composite materials, due to their significant effects in improving mechanical and thermal properties of polymers [3]. The kaolinite group, the montmorillonite/smectite group, the illite group and the chlorite group are the four main groups of clay minerals. Among them, montmorillonite (MMT) is associated with implantable biomaterials due to its favorable properties, such as good cation-exchange capacity, high specific surface area, large adsorption capacity for polymer molecules, high swelling capacity, ease of modification and drug carrying capability [1]. Its price and availability are also important advantages for fabricating a suitable composite material [8]. MMT is a 2:1 clay mineral, meaning that it fundamentally consists of two silica tetrahedral sheets, sandwiching a central octahedral sheet. The isomorphous substitution of Al^{3+} for Si^{4+} in the tetrahedral sheets and Mg^{2+} for Al^{3+} in the octahedral layer gives each layer a net negative charge, which is compensated by exchangeable metal cations [32]. MMT is open to modification via cationic exchange and hydrogen-bonding processes between sodium ions in the layers and cations such as alkali earth cations, salts and cationic polymers [10,14].

As a natural polymer, chitosan, a copolymer of *N*-acetyl-D-glucosamine and D-glucosamine, offers a unique set of characteristics for developing advanced functions such as biocompatibility, biodegradability, hydrophilicity, and nontoxicity [13]. Under slightly acidic conditions, most of the amino groups of chitosan are protonated, and the positively charged chitosan can easily intercalate into the interlayers of MMT. Based on its properties, chitosan is blended with MMT to create composite materials, displaying some specific

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structural and functional properties that meet requirements for engineered bone tissue [17].

To date, the combination of chitosan with MMT has been widely employed to develop suitable composite scaffolds [34]. These studies have shown that C/MMT composites are suitable templates for meeting BTE application requirements. In this study, MMT was modified with strontium before incorporating with chitosan. To our knowledge, there has been no previous attempt to modify MMT with Sr^{2+} prior to blending with chitosan for fabricating composite scaffold in BTE applications. Sr^{2+} is capable of stimulating pre-osteoblast proliferation and activity, which build bone, and simultaneously has a down-regulating effect on the bone-resorbing osteoclasts [7,23]. Our aim was to develop strontium-containing C/MMT scaffolds by freeze-drying method, and to investigate their structural, thermal and morphological properties, as well as the response of human osteoblasts to these scaffolds.

2. Materials and methods

2.1. Materials

Medium molar mass chitosan (Mr ~400,000; > 85% deacetylation) was purchased from Fluka Chemical Company (Milwaukee, WI). Montmorillonite (MMT) used in this study was MMT K10 with a cation exchange capacity (CEC) of 70–100 meq/100 g from Aldrich Chemical Company (St. Louis, MO). Medium 199 with Earle's salts (M199), α-Modified Eagle's minimal essential medium (α-MEM), fetal bovine serum (FBS), ascorbic acid, β-glycerophosphate, dexamethasone, antibiotics, and all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO), except stated otherwise.

2.2. Modification of MMT with Sr^{2+}

To determine the optimal concentration of Sr^{2+} for incorporating into the MMT structure, the equivalent amount of Sr^{2+} was calculated according to CEC of MMT (1×; i.e. 9.1×10^{-4} g/mL). Firstly, MMT was dispersed in distilled water for 24 h at room temperature, using the magnetic stirrer to obtain clay suspension. The solutions of Sr^{2+} were introduced into MMT solution at different concentrations (1×, 2× and 4×; equivalent to 1, 2, and 4 times of the CEC of MMT) to determine the optimal Sr^{2+} concentration for MMT adsorption. For this, Sr^{2+} was dissolved in MMT solution and mixed overnight to allow the adsorption of strontium. The Sr-modified MMT was collected via centrifugation. The pellet was rinsed several times in distilled water to ensure the complete removal of chlorite ions, which were later tested by adding $AgNO_3$ to the suspension after the rinses. The product was crushed upon drying at 100 °C for two days. Then, X-ray diffraction (XRD) and X-ray fluorescence (XRF) spectroscopy were employed to compare the structures obtained by adding different concentrations of Sr^{2+}.

2.3. Fabrication of C/MMT scaffolds

Considering the XRD and XRF results, Sr^{2+}-modified MMT was prepared using the 2× Sr^{2+} concentration (1.82×10^{-3} g/mL) for bone scaffold preparation. C/MMT sponges were fabricated by using the freeze-drying technique. Briefly, a 2% solution of chitosan in 2% acetic acid was freshly prepared by mixing at room temperature for 30 min. The required amount of Sr^{2+}-modified MMT powder was then slowly added to the chitosan solution [C/MMT, 6/4 (w/w)]. To facilitate dispersion of MMT powder within the polymer matrix, the mixture was homogenized by mechanical stirring and ultrasonic agitation. Homogenous mixture was cast in a mold and transferred to a freezer at −80 °C which allowed a fast solidification of the solvent. Later, the frozen samples were lyophilized at −76 °C for 24 h using an Alpha 1–4 LD-plus model freeze dryer (Christ, Osterode am Harz, Germany), then neutralized by immersion in a 1 M NaOH solution, and then rinsed with double-distilled water and lyophilized again. Finally, the samples were cut into ~4 × 4 × 4 mm^3 size cubes using surgical blade and characterized. Non-modified C/MMT sponges were also prepared as the control sample. Scaffolds were sterilized with 70% ethanol, then washed with PBS.

2.4. Characterization of C/MMT scaffolds

2.4.1. XRD and XRF

The phase identification of the samples was carried out with an X-ray powder diffractometer system (D8 Advance; Bruker, Germany) using a monochromatic Cu-Kα radiation (λ = 1.5406 Å, applied voltage 40 kV, applied current 30 mA) at room temperature, over the 2Θ range of 5–50° at 2°min^{-1} scan rate. Samples were completely dried in a vacuum oven before the analysis.

The elemental analyses were carried out using a Spectro X–Lab 2000 model polarized energy dispersive XRF spectrometer (Kleve, Germany).

2.4.2. Fourier transform infrared spectroscopy (FT-IR)

The structural characterization of the scaffolds was carried out by FT-IR. The samples were dried overnight in vacuum, until they reached constant weight. Then, they were vacuum-dried, pressed into powder, mixed with KBr (1:10), and compressed into pellet form before examining with a Perkin Elmer FT-IR spectrometer (Spectrum 100; Waltham, MA). The spectra were measured in the region of 500–4000 cm^{-1} at 4 cm^{-1} resolution.

2.4.3. Thermal gravimetric analysis (TGA)

TGA was performed on the composite scaffolds using a Shimadzu DTG-60H model thermal analysis system (Kyoto, Japan). Approximately 20–24 mg of C/MMT scaffold, C and MMT were ground and placed in Pt sample pans. Thermal gravimetric analysis was performed at a sample heating rate of 20 °C/min, up to a maximum temperature of 600 °C. The analyses were performed at 20 °C/min scan rate and under an N_2 atmosphere.

2.4.4. Porosity measurement

A mercury intrusion porosimeter (Quantachrome, Hook, U.K.) was used to quantify the pore size distribution of the scaffolds. The pressures applied to the low-pressure and high-pressure domains were 0.1 to 33,000 psi, respectively. The contact angle was measured as 140° and surface tension of mercury, 480 mN/m for the samples.

2.4.5. Scanning electron microscopy (SEM)

SEM was used to examine the surface/cross-sectional pore morphology of composite scaffolds. The samples were placed through a series of graded ethanol dehydrations, immersed in hexamethyldisilazane for 5 min, and allowed to air-dry. Finally, samples were mounted on aluminum stubs and sputter-coated with palladium and analyzed under a SEM (Gemini 1525 FEG-SEM, Cambridge, U.K.).

2.4.6. Dissolution study in cell culture medium

The release of Sr^{2+} from the non-modified and modified scaffolds into cell culture medium at the end of each day was determined by inductive coupled plasma optical emission spectrometer (ICP-OES) (iCAP 6000, Thermo Scientific, Waltham, MA). Briefly, scaffolds were weighed and soaked in cell culture medium (1 mg/1 mL) in 24-well plates, later the samples were transferred into a cell incubator to mimic biological conditions for up to 7 days. The supernatants were filtered through a 0.2 μm filter to remove suspended particles prior to the ion concentration measurements.

2.5. Cell culture studies

2.5.1. Expansion and seeding of human primary osteoblasts

Human primary osteoblasts (hOBs) isolated from bone material