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Properties and Cytocompatibility of Anti-Washout Calcium Phosphate Cement by Introducing Locust Bean Gum

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The washout resistance of injectable calcium phosphate cement (CPC) is highly requisite for more widely clinical applications. In this work, locust bean gum (LBG) was used as the anti-washout agent to improve the washout resistance of CPC. The results indicated that the washout resistance was greatly improved, and meanwhile the injectability, setting time and compressive strength slightly decreased when the content of LBG was no more than 1.0%. Additionally, the CPC with 1.0% LBG exhibited good cell compatibility of the mouse bone mesenchymal stem cells (mBMSCs). Therefore, the 1.0% LBG content was proposed to serve as a useful additive in CPC as a result of its ability to promote washout resistance, which may play an important role in clinical applications.

1. Introduction

Injectable calcium phosphate cement (CPC) has been considered as a very promising material that is applied to noninvasive or minimally invasive surgical treatments of bone defects due to its good biocompatibility, osteoconductivity, self-setting performance, excellent handling property, and capacity of drug delivery[1,2]. The washout resistance is an important performance for injectable bone repair materials, particularly for CPC. Conventional CPC is easily eroded by plasma or other body fluids during surgical procedure before the paste hardens, restricting its clinical application as an injectable artificial bone graft[3]. Thus, great efforts have been made to improve the washout resistance of CPC.

An effective approach to overcome this shortcoming is to incorporate with biocompatible biopolymers (e.g., sodium alginate[4], chitosan[5], hydroxypropyl methylcellulose[6], modified starch[7], hyaluronic acid[8]) to modify the cements by enhancing the cohesion of CPC paste. Another anti-washout CPC was the premixed cement by using the non-aqueous solution as the mixing liquid[3,9], but the water-immiscible liquids would restrain the setting process and the formation of hydroxyapatite (HA). Furthermore, the components of the mixing liquid were complex, leading to the complicated procedures. Previous studies evaluate the washout resistance of CPC pastes mainly by naked-eye observation, and the qualitative evaluation of the washout resistance usually is carried out for several minutes by injecting slurry into a liquid medium or shaking in an orbital shaker for hours using a shaped dough[3,10–12].

Locust bean gum (LBG) is a natural polysaccharide obtained from the seed endosperm of the Carob tree. It is a nonionic and linear galactomannan consisting of β-(1,4)-D-mannose units as the main chain and α-(1,6)-D-galactose units as the side chain, and the proportion of mannose and galactose is 4:1[13,14]. It has been extensively employed as food ingredient and pharmaceutical additive, because of its non-toxicity, biodegradability and biocompatibility[15,16]. The biopolymer LBG possesses high viscosity, hydrating properties and good bioadhesivity when it is dissolved in aqueous solution[17,18]. On account of the unique structure containing many hydroxyl groups, hydrophilic LBG is liable to combine with organic or inorganic constituent by hydrogen bonds network, and it possesses the water and saline retention capacity[19,20]. If the special network is introduced into CPC, the washout resistance of the cement might be improved.

In consideration of the merits of LBG, the incorporation of LBG into CPC shall be a good strategy to obtain injectable CPC with excellent washout resistance. In this study, the effects of LBG on the washout resistance, injectability, rheological properties, hardening behavior, compressive strength, and cytocompatibility of the CPC were investigated.

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2. Materials and Methods

2.1. Material preparation

The chemical raw materials, including dicalcium phosphate anhydrous (DCPA), calcium nitrate ($Ca(NO_3)_2{\cdot}4H_2O$), and diammonium phosphate ($(NH_4)_2HPO_4$), were commercially obtained from Shanghai No. 4 Reagent & H.V. Chemical Co. Ltd, China. The CPC used in this study consisted of partially crystallized calcium phosphate (PCCP) and DCPA at a weight ratio of 1:1, as described detailedly in our previous work[21]. Briefly, PCCP was synthesized from an aqueous solution of $Ca(NO_3)_2{\cdot}4H_2O$ (0.36 mol/L) and $(NH_4)_2HPO_4$ (0.15 mol/L) by chemical precipitation method and subsequently heat-treated at 460 °C. DCPA was dry-ground in a ball mill for 2 h. The anti-washout CPC was obtained by mixing PCCP, LBG (Sigma), and DCPA homogeneously, denoted as LBG-CPC. LBG-CPCs containing 0, 0.5, 1.0, 1.5, and 2.0 wt% of LBG were prepared. The blended CPC powders were homogeneously mixed with deionized water at a liquid to powder ratio of 0.4 mL/g to obtain injectable CPC pastes. The cement without LBG was used as the control group.

2.2. Washout resistance evaluation

The as-prepared CPC paste was manually injected into simulated body fluid (SBF, formulated as reported[22]) at 37 °C immediately. Then the samples were put into a shaker and vibrated at the speed of 120 r/min at 37 °C. The cements were considered to pass the washout resistance test if the specimens did not visibly disintegrate in SBF for 60 min. The mass of the as-prepared paste was represented as M_0. After 60 min, the largest mass of the solid residues was collected and weighed as M_1. Then the washout resistance was evaluated according to Eq. (1). Each test was repeated for four times and the average value was calculated.

$$\text{weight loss}(\%) = (M_0 - M_1)/M_0 \times 100\% \quad (1)$$

2.3. Injectability and rheological properties testing

After mixing CPC powder with deionized water for 1 min, the as-prepared paste was poured into a 5 mL syringe with a needle of 1.6 mm inner diameter. The paste was extruded from the syringe at a crosshead speed of 15 mm/min until a maximum force of 100 N was reached[23]. The injectability was calculated as the percentage of the mass of the paste extruded from the syringe divided by the original mass of the paste inside the syringe. Each test was performed six times, and the average value was calculated.

The rheological properties, including viscosity and yield stress, of the pastes were tested with a rheometer (R/S Rheometer, Brookfield, USA).

2.4. Setting time and mechanical strength measurement

The setting time of the cements was measured according to the method of Gilmore needle[24].

The samples with an inner diameter of 6 mm and a height of 12 mm were prepared by curing in an incubator at 37 °C and 97% humidity for 72 h. The compressive strength of the cement specimens were measured using a universal material testing machine (Instron 5567, Instron, USA) at a crosshead speed of 0.5 mm/min. Each measurement was repeated 6 times and the average value was calculated.

2.5. Phase composition analysis and microstructure observation

The hydrated samples were milled into powders and analyzed by using X-ray diffraction (XRD; X'Pert PRO, PANalytical, the Netherlands) to obtain their phase composition. IR transmittance spectra were recorded by Fourier transform infrared spectroscopy (FTIR; Avatar 360, Nicolet, USA) in the 4000–400 cm^{-1} range. The microstructure of the hydrated cement specimens was observed by a scanning electron microscope (SEM; Nova NanoSEM 430, FEI, USA).

2.6. Cytocompatibility assessment

The hydrated CPC samples with diameter of 6 mm and height of 2 mm were sterilized by gamma radiation at 15 kGy, and then the disks were put into 48-well plates and pre-wetted with Dulbecco's Modified Eagle Medium (DMEM, Gibco). Mouse bone marrow mesenchymal stem cells (mBMSCs, ATCC, CRL-12424) were seeded on the samples at a density of 1.5×10^4 cells/well. The cells were cultured in DMEM (Gibco) containing 10% fetal bovine serum (FBS, Gibco) and incubated at 37 °C in a humidified incubator with 5% of CO_2. The culture medium was refreshed every other day. After 1 day of incubation, the samples were gently washed by phosphate buffer solution (PBS) and immobilized with 2.5% glutaraldehyde solution for 4 h, then dehydrated with graded ethanol. The morphology of cells on the cements was observed by SEM.

The viability of mBMSCs on the samples was evaluated after 1 and 3 days of incubation using a Live/Dead kit (Biotium, USA). The specimens were observed using a fluorescence microscope (Zeiss Axioskop, Germany). The proliferation of mBMSCs on the samples was evaluated after 1, 3, and 7 days of incubation using CCK-8 assay (Dojindo Laboratories, Japan) and measured with a microplate reader (Thermo 3001, USA).

Alkaline phosphatase (ALP) activity of mBMSCs on the cements was tested after 7 and 10 days of culture, using the osteoblast inducing conditional media (10 mmol/L sodium β-glycerophosphate, 0.1 μmol/L dexamethasone and 50 mg/L vitamin C), which served as the culture media. After incubation, the specimens were gently rinsed with cold PBS and 250 μL of lysis buffer was added to each well at 4 °C for 2 h. The lysate was collected for measuring the total protein content and ALP content. The total protein content was assayed by a BCA protein assay kit (Thermo Scientific, USA). The ALP content was tested using *p*-nitrophenylphosphate (p-NPP, Sigma, USA) assay. 20 μL of the lysate with 200 μL of 5 mmol/L p-NPP solution was incubated at 37 °C for 15 min, then 100 μL of 1 mol/L sodium hydroxide solution was added to terminate the reaction. The concentration of *p*-nitrophenol (p-NP) was calculated by measuring the absorbance at 405 nm. ALP activity was estimated as enzyme activity unit per milligram of total protein content.

2.7. Statistical analysis

All the data are given as the means ± standard deviations. Statistical difference was analyzed by using one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered to be statistically significant.

3. Results and Discussion

3.1. Chemical structure of LBG

Fig. 1 shows the FTIR spectra of LBG. In the spectrum of LBG, the absorption peaks are observed at about 3433 cm^{-1} for hydroxyl (═OH) stretching, 2925 cm^{-1} for methoxy-CH stretching, 1653 cm^{-1} for hydroxyl bending, 1431 and 1378 cm^{-1} for methylene ($=CH_2$) bending, 1322 cm^{-1} for methyne (≡CH) bending. The bands