Materials Science and Engineering C 79 (2017) 23–29

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

A novel bioactive vaterite-containing tricalcium silicate bone cement by self hydration synthesis and its biological properties

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ARTICLE INFO

Article history:
Received 1 July 2016
Received in revised form 16 March 2017
Accepted 4 May 2017
Available online 5 May 2017

Keywords:
C_3S bone cement
Carbonization
$Ca(OH)_2$
Vaterite
Bioactivity

ABSTRACT

A novel bioactive vaterite-containing tricalcium silicate bone cement (V5) was successfully synthesized through self hydration and carbonization, by introducing CO_2 into the hydration process of Ca_3SiO_5 (C_3S). The purpose of this work is to reduce the adverse effect of the hydration products $Ca(OH)_2$ on the C_3S bone cement, and improve further the bioactivity of the C_3S bone cement. XRD and FT-IR analysis indicated that vaterite was successfully formed in tricalcium silicate bone cement. The in vitro bioactivity of V5 was investigated by soaking in simulated body fluid (SBF) for various periods (1, 3, 7 days), in addition to setting time, compressive strength and cell behavior. The results showed that the V5 could rapidly induce hydroxyapatite (HA) formation. The bio-effects of V5 on MC3T3-E1 osteoblast-like cells were evaluated by studying cell viability, adhesion and proliferation. The CCK-8 assay shows that cell viability on the resulting V5 is improved obviously after through hydration and carbonization. The V5 cement enhanced the higher expression of cell attachment, proliferation and differentiation as compared to V50 cement. SEM results present significant improvement on the cell adhesion and proliferation for cells cultured on the V5. Experimental results demonstrated that, C_3S bone cement of containing vaterite has more excellent bioactivity and medium setting time too, suggesting their potential applications in areas such as MC3T3-E1 cell stimulation and bone tissue engineering.

1. Introduction

The demand for synthetic biomaterials to replace and repair bone tissue lost from injury or disease has significantly increased in the past ten years. Silicate bioceramics, as a new family of biomaterials, have received significant attention in their application to hard tissue regeneration [1]. A variety of calcium silicate-based materials, such as $CaSiO_3$, Ca_3SiO_5, Ca_2SiO_4, and bioactive glass, have been developed for use in the orthopedic and dental surgery. Some researchers developed a quick-setting calcium silicate bone cement with high bioactivity and enhanced osteogenesis after mixing with phosphate solution [2]. Therefore, calcium silicate bone cement opened up new possibilities in the field of self setting bioactive CaO-SiO_2-based bone graft and bone filling. Tricalcium silicate, which has drawn growing attention, is a potential biomaterial [3], in recent studies, the potential of tricalcium silicate cement as an injectable bone substitute has been investigated, and the results indicate that Ca_3SiO_5 pastes possess a good injectability and bioactivity, and a moderate in vitro biodegradability [4]. Tricalcium silicate is the main component of Portland cement [5], and can react with an aqueous phase at room or body temperature to form an injectable cement paste [6]. However, during the hydration of C_3S, it will generate $Ca(OH)_2$. The strength and chemical stability of $Ca(OH)_2$ are very poor. It is easily dissolved in soft water, and prone to acid corrosion or sulfate attacks, and the pH value of cement can be as high as 12.5 in the presence of $Ca(OH)_2$, which is suppressing further bone tissue growth. In conclusion, we urgently need to reduce the adverse effect caused by the hydration products $Ca(OH)_2$ in the C_3S bone cement.

Vaterite is a mineral, a polymorph of calcium carbonate ($CaCO_3$) [7]. Vaterite is a promising candidate for practical biomedical applications due to its high specific surface area, high solubility, high dispersibility, and low specific gravity [8]. Vaterite particles can be applied in regenerative medicine, drug delivery and a broad range of personal care products [7]. Like other polymorphs of calcium carbonate, vaterite rapidly dissolves at acidic pH, and thus it can undergo degradation both in vivo and in vitro. Biodegradation of vaterite may occur in body fluids or some acidic environment, or upon the cellular phagocytosis and absorption. Body fluid contains a number of acidic metabolites, such as citrate, lactate and acid hydrolysis enzymes, which provide acidic environment for dissolution of the material. After entering cells (mainly macrophages) by phagocytosis, vaterite particles are split into ions under the effect of cytoplasmic and lysosomal enzymes, and then the degradation products, Ca^{2+} and CO_3^{2-}, can be transferred to extracell. Ca^{2+} can then participate

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http://dx.doi.org/10.1016/j.msec.2017.05.025
0928-4931/

in the formation of new tissue without causing organic damage and pathological tissue calcification [7]. Moreover, earlier studies have shown that trace amounts of silicon can stimulate collagen type I synthesis and osteoblastic differentiation in human osteoblast-like cells [9], also promoting bone formation [10].

In our previous studies [11], we had successfully turn the damage hydration products $Ca(OH)_2$ in the β-C_2S bone cement into vaterite. Furthermore, the results showed that vaterite-containing β-C_2S bone cement has much better HAp forming ability. Currently, in order to reduce the adverse effect of $Ca(OH)_2$, we similarly introduced CO_2 into the hydration process of C_3S cement. In the same way, we got another bioactive biomaterial that can be expected to further investigation as bone repairing material.

2. Material and methods

2.1. Preparation of C_3S powder

Reagent-grade calcium nitrate ($(Ca(NO_3)_2 \cdot 4H_2O$; 99.0%, XiLong Chemical Co, Ltd., China) and tetraethyl orthosilicate ($(Si(OC_2H_5)_4$; TEOS, SiO_2 content ≥ 28.4%, Sinopharm Chemical Reagent Co, Ltd., China) were used as precursor materials of calcium and silicon, respectively, to synthesize C_3S powders via a sol-gel method with an initial CaO/SiO_2 molar ratio of 3:1. Nitric acid was used as the catalyst. The synthesis process can be summarized as follows: At first, TEOS was hydrolyzed by the sequential addition of 2 mol/L HNO_3 and absolute ethanol. Each addition was followed by 1 h of stirring. Then, the required amount of $Ca(NO_3)_2 \cdot 4H_2O$ was added to the TEOS solution, and the mixture was stirred for an additional hour. The molar ratio of ($HNO_3 + H_2O$) to TEOS and to ethanol was 10:1:10. The solution was sealed and aged at 60 °C for 24 h until gelation occurred. Afterwards, the gel was dried at 120 °C and then calcined at 1450 °C for 8 h [12]. The resulting powders were ground and sieved through a 200 mesh grid for further experiments.

2.2. Preparation of vaterite-containing bone cement

To prepare vaterite-containing bone cement, using distilled water as the liquid phase, the synthesized C_3S powders and distilled water were added in the beaker, a powder-to-liquid ratio of 0.5 g/mL. According to the previous work, The vaterite were prepared by blowing CO_2 gas in the as-prepared cement for 1.5 h at a rate of 2000 mL/min. The resulting slurry was dried at 110 °C [11]. For this study, V5 denotes vaterite-containing tricalcium silicate bone cement synthesized using a powder-to-liquid ratio of 0.5 g/mL. For comparison, V50 denotes a reference material that was prepared following the same approach, except for the CO_2 introduction. The resultant vaterite-containing bone cement powders were ground and sieved to 200 mesh. The Na_2HPO_4 as liquid phase was used to mix vaterite-containing powders for preparing cement, and the liquid-to-powder (L/P) ratios of 0.8 mL/g was adopted [13]. After mixing, the cements were put into a cylindrical stainless steel mold (diameter, 6 mm and height, 12 mm) and incubated at 37 °C and 100% relative humidity and allowed to set for 1 day.

2.3. Property of vaterite-containing bone cement soaking in SBF

To evaluate the in vitro physiochemical activity, the 24-h-set cylindrical specimen (Φ6 mm × 3 mm) was soaked in a 11-mL SBF (pH value = 7.40) at 37 °C, and the ratio of specimen surface area to solution volume of SBF was 0.1 cm^2/mL [14]. The SBF was prepared according to the procedure described by Kokubo and Takadama [15]. After soaking for various periods (0, 1, 3, 7 days), specimens were removed from the centrifuge tube and gently rinsed three times with deionized water and acetone, and then dried for 24 h in an oven at 60 °C for analysis of phase composition and morphology.

2.4. Test and characterizations

XRD (Geigerflex, Rigaku, Japan) analysis was performed to investigate the phase structure, and the structure and chemical composition of the samples were investigated by SEM (S-4800, JEOL, Tokyo, Japan) coupled with EDS using an accelerating voltage of 15 kV. All samples were coated with Au prior to their examination by SEM. The FT-IR spectra were recorded on a NEXUS 670 FT-IR spectrophotometer to further analyze the chemical composition and identify the chemical bonds. The setting times of the pastes were measured using a 400-g Gillmore needle with a 1-mm diameter according to international standard ISO 9917-1. The moment when the needle weighing 400 g, with 1.0 mm diameter, could only penetrate no more than 1 mm into three different areas of the cement cylinder was considered as the setting time. All of the samples were maintained at 37 °C with 100% relative humidity environment during the testing process. A total of six specimens were used for testing Compressive strength. Testing was conducted on an electronic universal material testing machine (CMT6203, Shenzhen Shiji Tianyuan Instrument Co., Ltd. Guangdong, china) at a loading rate of 0.5 mm/min. The pH values of SBF solution before and after soaking with the samples for different time point were obtained from the pH meter (PHSJ-3F, Shanghai Jie Sheng Scientific Instrument Co., Ltd.).

2.5. Cell culture

MC3T3-E1 cells were cultured in α-MEM medium (Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 100 IU/mL penicillin-streptomycin (Invitrogen, Burlington, Canada). Cell cultures were maintained in humidified incubator at 37 °C in a 5% CO_2 atmosphere.

The extracts were prepared by adding the powders of V50 and V5 to α-MEM medium at 0.2 mg/mL and incubated at 4 °C for 3 day. After centrifugation at 1500 rpm for 5 min at room temperature, the supernatant was collected.

2.5.1. CCK-8 cell proliferation assay

CCK-8 test was performed to determine the proliferation of MC3T3-E1 cells treated with different extract. Cells were seeded into 96-well plates at density of 2000 cells per well in 100 μL fresh medium. After 24 h, they were treated with different extracts for 24 h, 48 h and 72 h, respectively. 10 μL CCK-8 reagent (Beyotime, Jiangsu, China) was added to the cells and the cells were incubated for 2 h at 37 °C. Then, the absorbance at 450 nm was measured using a microplate reader (Bio-Rad 680, USA). All the results were demonstrated as optical density (OD) values minus the absorbance of blank wells. Cell distribution was observed under a fluorescence microscope (Zeiss Axioskop 40, Germany).

2.5.2. Lactate dehydrogenase assay

The relative amounts of lactate dehydrogenase (LDH) released into the medium were used as an index of cytotoxicity. MC3T3-E1 cells were seeded at a density of 10^4 cells per well in 100 μL fresh medium into 96-well plates and cultured at 37 °C in a 5% CO_2 atmosphere for 24 h before treatment. Then, 100 μL extracts or 50 μL fresh medium and 50 μL extracts were added per well. For the vehicle control group and maximum LDH release control group, 100 μL fresh medium were added. After 24 h, 48 h and 72 h incubation, the supernatants were harvested. For the maximum LDH release control group, first, the cells were washed with phosphate buffered saline (PBS), then, lysis buffer were added and after 1 h incubation at 37 °C, the supernatants were collected. After then, LDH were measured using LDH assay kit (Beyotime, Jiangsu, China) according to the manufacturer's instructions.

2.5.3. Cell morphology

The specimens were cut into discs with a diameter of 6 mm and a height of 2 mm and sterilized by gamma radiation at 15 kGy [16]. MC3T3-E1 cells were seeded on the discs which were previously placed