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Fabrication and characterization of bioactive calcium silicate microspheres for drug delivery

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Abstract

The calcium silicate ($CaSiO_3$, CS) microspheres with diameter of 75–100 μm were fabricated by a spray-drying method. A new bone-like apatite layer fully covered the surface of the fabricated CS microspheres after soaking in simulated body fluid (SBF), suggesting the excellent activity of the material in inducing apatite deposition. The ionic extracts of CS microspheres promoted the proliferation of human osteoblast-like cells (MC3T3-E1). In addition, the porous structures of the CS microspheres resulted in favorable drug loading and sustained release property. Our study indicates that the fabricated multifunctional CS microspheres are a promising drug delivery system as an injectable bioactive filling material for bone-regeneration.

Keywords: Calcium silicate; Microspheres; Bioactivity; Cell proliferation; Drug delivery

1. Introduction

Recently, the applications of bioactive microspheres as injectable bone filling implants and drug/cell carriers have received considerable attention [1–3]. After implantation, the spaces among the microspheres are crucial for effective and functional bone regeneration as they allow for both bone and vascular ingrowths [3]. Furthermore, compared with the traditional macroporous block scaffolds, the main advantage of the microspheres is that they not only possess better injectable and drug-delivery capacities but also the ability to fill bone defects with irregular and complex shapes and sizes [3,4].

However, as for the microspheres to be applied as bone fillers for bone regeneration, there are three major issues that need to be considered: (1) bioactivity, (2) sustainable drug release ability, and (3) degradability. Currently available microspheres are made of bioceramics, biopolymers, and their composites. Biopolymer-based microspheres such as polylactic acid (PLA), poly(lactic-co-glycolic acid) (PLGA) and poly (hydroxybutyrate-polyhydroxyvalerate) (PHBV) are biodegradable but their bioactivity is unfavorable [5,6]. On the other hand, bioceramic microspheres such as hydroxyaptite bioceramics are bioactive, but lack adequate degradation [1].

Recently, Ca–Si-based silicate bioglasses and bioceramics have attracted considerable attention due to their excellent bioactivity and degradability [3,7–11]. They can quickly induce formation of a bone-like apatite layer on their surfaces after implantation *in vivo* [10]. This type of apatite layer plays an essential role in the formation of tight bone bonding between the bioactive materials and the host bone tissues [10–12]. The calcium silicate ($CaSiO_3$, CS) ceramics are biodegradable and possess excellent activity in inducing bone-like apatite layer formation *in vitro* and *in vivo* [10,13,14]. Our recent investigations demonstrated that the bioactive Si ions released from CS could provide a preferable extracellular environment for directing bone marrow mesenchymal stem cells (BMSCs) differentiation toward the osteogenic lineage, enhance human umbilical vein endothelial cells (HUVECs) proliferation and angiogenesis process even in the absence of extra osteogenic and angiogenic

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reagents, and significantly promote early bone formation compared with the traditional calcium phosphate bioceramics [15].

Many strategies have been developed to fabricate bioceramic microspheres, such as emulsion method [16], polymer mediated formation route [17], biomineralization technique [18], self-assembly approach [2,19], hard-template transformation process [20,21], and spray-drying method [22] among which the spray-drying method is particularly attractive because of its widespread use and relative ease of operation [23]. Moreover, the products with regulatable sizes from submicrometer to hundreds of micrometer-size, and polymorphic shapes, including mushroom-like and donut-like shapes, as well as spherical porous and hollow structures can be facilely controlled in a single step by modulation of the spraying parameters [22]. In addition, the products can be scaled up to ton quantities in one batch. In the present study, the bioactive CS microspheres as drug carriers have been developed using the spray-drying method for the potential application as injectable bone filling materials for bone regeneration. The antibiotic vancomycin model was selected in this study due to its broad spectrum of activity against both Gram-positive and Gram-negative bacteria that may induce bone infection. The fabrication method, morphology characterization, *in vitro* bioactivity, drug loading and delivery property, and the effect of the material extracts on cell proliferation are presented.

2. Experimental section

2.1. Fabrication and characterization of calcium silicate (CS) microspheres

The calcium silicate (CS) powders were prepared via a chemical precipitation method as previously described [7]. Briefly, 1000 mL of 0.5 mol $Na_2SiO_3 \cdot 9H_2O$ aqueous solution was added dropwise into 1000 mL of 0.5 mol $Ca(NO_3)_2 \cdot 4H_2O$ aqueous solution by vigorously stirring at room temperature to produce a white precipitate. After complete addition, the white precipitate was further stirred for 24 h followed by washing three times using distilled water, and then twice with 100% ethanol. After washing, the remaining liquid was removed by vacuum filtration, and the precipitate was dried at 120 °C for 24 h. The CS powders were obtained by calcining the dried precipitate at 900 °C for 2 h.

The CS microspheres were fabricated by the spray-drying method on a high-speed centrifugal spray-drying machine (LGZ-8, Wuxi Dongsheng, China). In brief, 100 g of CS powders was added into 500 g of aqueous solution containing 5 wt% polyvinyl alcohol (PVA) binders. Then the obtained CS suspension was atomized at a pressure of 1.5 MPa and a flow rate of 500 mL/h while the inlet and outlet temperatures of the nozzle were adjusted to around 180 °C and 80 °C, respectively. The spray-dried CS granules were collected and calcined at 900 °C for 3 h with a firing rate of 2 °C/min to burn out the PVA binders, and then cooled to room temperature in the furnace. Finally, the products were sieved to obtain the CS microspheres with 150–200 mesh. The hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$, HAp] microspheres were also fabricated by the spray-drying method using HAp nanopowders as raw materials. The HAp nanopowders were synthesized by the chemical precipitation method and then calcined at 800 °C for 2 h [24]. The obtained HAp microspheres were used as the control sample in the cell culture experiment.

The morphology and size of the fabricated CS microspheres were observed by scanning electron microscopy (SEM: JSM-6700F, JEOL, Japan). The phase of the microspheres was characterized by X-ray diffraction (XRD: D/max 2550 V, Rigaku, Japan) with mono-chromated Cu-Kα radiation.

2.2. Evaluation of bioactivity of CS microspheres in simulated body fluid (SBF)

The bioactivity of the fabricated CS microspheres was evaluated by examining the bone-like apatite layer formation on the surface of the microspheres in SBF [14], which was prepared as previously described by Kokubo and Takadama and had similar ion concentrations to those in human blood plasma [12]. Briefly, analytical reagent grade chemicals NaCl, $NaHCO_3$, KCl, K_2HPO_4, $MgCl_2$, $CaCl_2$, and Na_2SO_4 were dissolved in distilled water and the solution was buffered to pH 7.4 at 37 °C with tris(hydroxymethyl)aminomethane and hydrochloric acid (Tris–HCl).

The microspheres were soaked in SBF and vibrated at a constant speed of 240 rpm in a shaking air bath for 3 days at 37 °C with the ratio of mass (mg) to solution volume (mL) of 1.5. The SBF solutions were refreshed every 1 day. After soaking, the microspheres were removed from the SBF solution, gently rinsed with distilled water, and then dried at room temperature before further characterization. The formation of bone-like apatite layer on the surface of the microspheres was characterized by XRD, Fourier transform infrared spectroscopy (FTIR; A380 Nicolet Co., USA) and SEM.

2.3. Effect of ionic products from CS microspheres on MC3T3-E1 proliferation

The ionic extract method is a widely used international standard to evaluate the effect of chemical compositions on cell biological responses, which can effectively avoid the extra effects deriving from the material morphologies via direct incubation of materials with cells [2]. In this study, MC3T3-E1 mouse osteoblast cells were used, and the cells were cultured in Minimum Essential Medium α (MEM α, Invitrogen) containing 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin–streptomycin (PS; Gibco, USA). To prepare the extracts, a stock solution with a concentration of 200 mg/mL was first prepared by immersing the CS and HAp microspheres powders in MEM α culture medium. After incubation at 37 °C for 24 h, the mixtures were centrifuged and the supernatants were collected. The serial diluted extracts (100, 50, 25 and 12.5 mg/mL) were prepared by diluting the stock solutions with serum-free MEM α. Subsequently, these extracts were sterilized by filtration through 0.2 μm filter membranes for further cell culture experiments. The ion concentrations of the

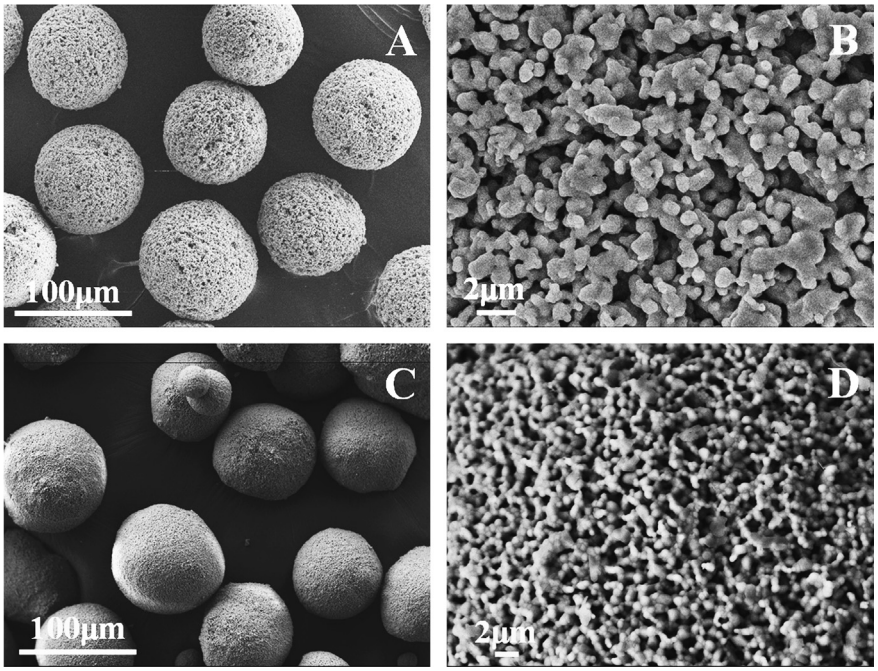

Fig. 1. FESEM morphology of the fabricated CS (A, B) and HAp (C, D) microspheres: low (A, C) and high (B, D) magnification photomicrographs.

extracts were measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES; VISTA AX, Varian Co., USA).

The MC3T3-E1 cells were seeded in the 96-well tissue culture plates (Corning, USA) at a density of 4000 cells/well in 100 μL of extracted medium and then cultured for 1, 3 and 7 days by incubation at 37 °C with 5% CO_2 and 95% air at 100% RH. The medium was replaced by the prepared extracts every 2 days. The samples used were removed at designated time points for further biological analyses. After cell culture, 100 μL (0.5 mg/mL) of (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) was added into each well. After an additional incubation for 4 h, the MTT solution was removed and replaced with 100 μL of dimethylsulfoxide (DMSO). After 10 min of slow shaking (Vibramax 100, Metrohm, USA), the absorbance was read at 590 nm on a microplate reader (Epoch™ microplate spectrophotometer, BioTek Instruments, USA), and the results were expressed as optical density (OD). All experiments were done in triplicate to obtain the average data.

2.4. In vitro study of drug loading and release

The antibiotic vancomycin model was applied to characterize the drug loading and release properties of the fabricated CS microspheres. The antibiotic vancomycin powder was provided by New Drug Research and Development Co., Ltd., North China Pharmaceutical Group. The vancomycin release measurements were carried out using UV/vis spectroscopy at a wavelength of 280 nm with correlation coefficient of $R^2 = 0.9999$ for the calibration curve between the absorbance and vancomycin concentration in the range of 0–1.0 mg/mL. Three samples from each group were measured to obtain the average data of the drug loading amount and release amount.

2.4.1. Drug loading procedures

Typically, 0.25 g of CS microspheres was added into the vials with 1.0 mL of aqueous solution with a vancomycin concentration of 10 mg/mL. The vials were sealed to prevent the evaporation of aqueous solution. After vibration at a constant speed of 240 rpm in a horizontally shaking air bath at 37 °C for 24 h, the vancomycin-loaded CS samples were separated by centrifugation, and then dried in vacuum at 37 °C for 24 h. The amount of loaded vancomycin was measured by the depletion method that determines the difference of vancomycin concentration in the loading media before and after loading. The amount of drug loading was calculated according to the following formula:

$$\text{amount of drug loaded} = (A - B)/A \times 100\%$$

where A and B represented the drug concentrations before and after loading, respectively.

2.4.2. In vitro drug release

0.25 g of drug-loaded CS microspheres was placed into 1.0 mL of phosphate buffer solution (PBS) at pH 7.4 and vibrated in the air bath at 37 °C. At the pre-determined time intervals, the solutions were centrifuged, and then the release medium was withdrawn and replaced with fresh release medium (0.50 mL). The amount of drug release was determined by UV/vis spectroscopy.

2.5. Statistical analysis

Data were analyzed for statistical significance using an analysis of variance. Differences at p values < 0.05 were considered significant.

3. Results and discussion

3.1. Characterization of CS microspheres

Fig. 1 shows the morphologies of the obtained products. Low magnification SEM images show that the obtained products had uniformly sphere-like morphology with a diameter of 75–100 μm. High magnification SEM images show that the obtained microspheres were composed of microparticles with a diameter of 0.3–0.6 μm, and micro-pores having a size range of 0.4–2 μm were uniformly located and distributed among the CS microspheres. Fig. 2(A) reveals that the fabricated CS microspheres could be well identified as pure crystalline β-$CaSiO_3$ (β-CS) phase (JCPDS card: NO. 84-0655), and no other phases were observed.

Fig. 3 shows the SEM photomicrographs of CS microspheres after soaking in SBF for 3 days. It is clearly seen that the surfaces of the CS microspheres were completely covered by a dense layer after soaking in SBF (Fig. 3(B)), compared with the images before soaking. The high magnification image inserted in Fig. 3(B) shows that newly formed dense layers consisted of a fine structure of worm-like particles with a diameter around 50 nm and a length up to 150 nm. Fig. 2(B) shows the XRD pattern of the CS microspheres after soaking in SBF for 3 days. It can be seen that, compared with Fig. 2(A), the intensity of the CS peaks decreased remarkably after soaking in SBF while the typical diffraction peaks of HAp (JCPDS card: NO. 09-0432) were observed, which revealed that a bone-like apatite layer was formed and covered the surface of the CS microspheres.

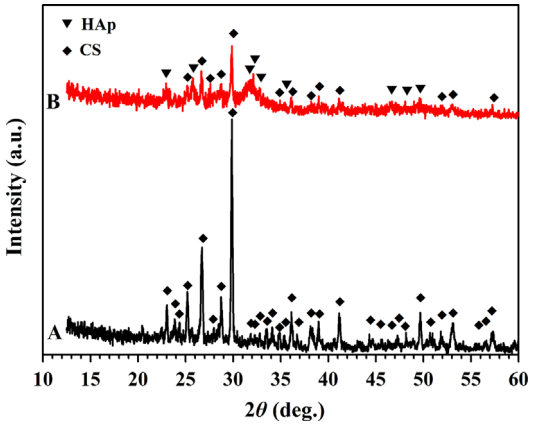

Fig. 2. XRD patterns of the fabricated CS microspheres (A) and the CS microspheres (B) after soaking in SBF for 3 days.

FTIR was applied to evaluate further the surface properties of materials. Fig. 4 presents the FTIR spectra of the CS microspheres before and after soaking in SBF for 3 days. Before soaking, the intense peaks between 901 and 1062 cm^{-1} were assigned to the absorption peaks for silicate (Si–O–Si), and the peaks at 683, 644, 566, and 473 cm^{-1} were assigned to the Si–O–Si bending mode [13,14]. After soaking in SBF for 3 days, the intensity of silicate (Si–O–Si) absorption peaks decreased remarkably. The newly appeared peaks at 1093 and 1036 cm^{-1} could be assigned to the phosphate group (PO_4^{3-}) of HAp, those at 1483 and 1419 cm^{-1} to the carbonate group (CO_3^{2-}), and those at 3448 and 1644 cm^{-1} to the adsorbed water molecules [13,14]. The FTIR spectra further confirmed that the bone-like hydroxycarbonate apatite (HCA) covered on the surfaces of the CS microspheres after soaking in SBF. The bone-like HCA layer, which precipitates on the surface of a bioactive material, is considered to play an essential role in the formation of tight chemical bonds between the bioactive materials and the neighboring tissues [10,12]. In this study, our results showed that the fabricated CS microspheres could induce fast the precipitation of bone-like HCA layer on their surfaces in SBF, suggesting the excellent bioactivity of the CS microspheres.

3.2. Effect of ionic products from CS microspheres on MC3T3-E1 proliferation

Compared with the extracts from the control HAp microspheres, a stimulatory effect of the ionic products from CS microspheres in a wide concentration range on MC3T3-E1 proliferation was observed (Fig. 5). The extracts of CS microspheres at concentrations between 12.5 and 200 mg/mL

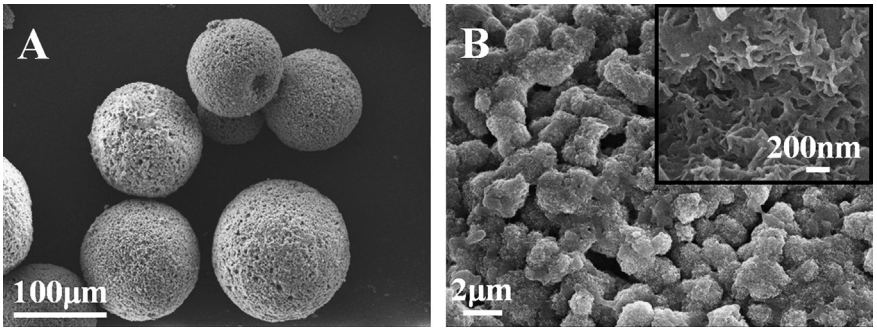

Fig. 3. FESEM morphology of the CS microspheres after soaking in SBF for 3 days: low (A) and high (B) magnification photomicrographs.

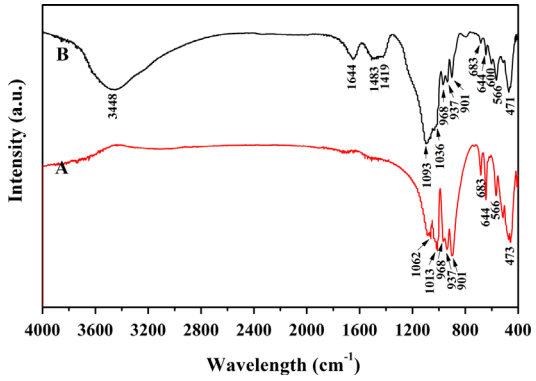

Fig. 4. FTIR spectra of the CS microspheres before (A) and after (B) soaking in SBF for 3 days.

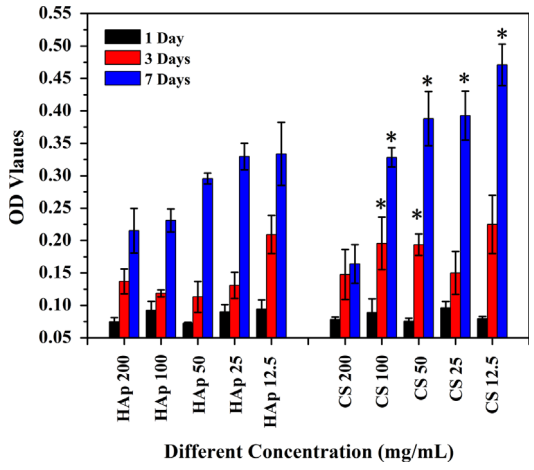

Fig. 5. The effect of ionic products from the control HAp microspheres and CS microspheres on proliferation of MC3T3-E1 after culture for 1, 3, and 7 days; * indicates significant difference between CS microspheres group and HAp microspheres group at the same concentration and culture time, $^{*}p < 0.05$.

exhibited no cytotoxicity. The cell proliferation was evident for all samples over the duration of the culture time. The stimulatory effects of the ionic products from CS microspheres on MC3T3-E1 proliferation were apparently higher than those from HAp materials in the extract concentration range of 50–100 and 12.5–100 mg/mL after 3 and 7 days of culture, respectively. The results might indicate the better therapeutic potential of CS microspheres for bone regeneration, compared with the traditional hydroxyapatite bioceramics.

It is well known that the Si ion plays the critical role in biological performances, and the function of Si ion on bone cells is dose-dependent. In our study, the ICP-OES analysis showed that the Si ion concentration of the CS microsphere extracts (12.5–200 mg/mL) for cell culture was 0.40–6.42 mmol/L. The previous studies have confirmed that the Si ion released from Si-containing biomaterials could significantly stimulate the osteoblast cell proliferation and osteogenic activity [25,26]. The *in vivo* studies further confirmed that bone regeneration could be enhanced by the Si component [3,11,27]. In addition, our previous studies have shown that the

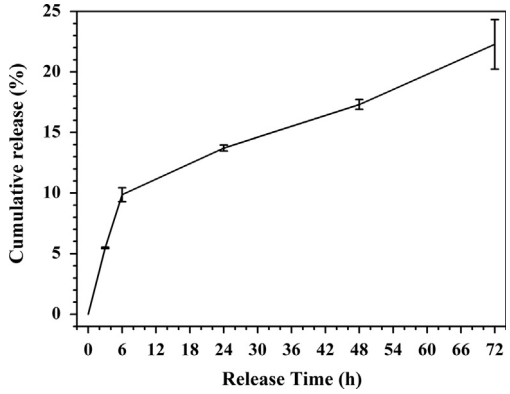

Fig. 6. The cumulative vancomycin release ratio from CS microspheres in PBS.

CS bioceramics possessed excellent bioactivity and bone regeneration ability [10,13–15]. Our recent research works have further shown that the bioactive Si ions released from CS bioceramics provided a preferable extracellular environment for directing BMSCs differentiation toward the osteogenic lineage, enhanced human umbilical vein endothelial cells (HUVECs) proliferation and angiogenesis process even in the absence of extra osteogenic and angiogenic reagents, and significantly promoted early bone formation compared with the traditional calcium phosphate bioceramics [15]. Moreover, the metabolic activity, proliferation and differentiation of human osteoblasts increased by supplement of 0.03 mmol/L silicon in the growth medium [28]. It has been reported that the concentration of Si ions in normal serum is about 2.14×10^{-2} mmol/L [29], which is apparently lower than the values in the present study. It is well known that the amount of Si in bone tissue is much higher than that in blood. Si is present at a high level of ~100 mg/kg in bone and ligaments [30]. Therefore, it can be suggested that a certain high dose of Si may stimulate the proliferation of osteoblasts and their progenitor cells and benefit new bone regeneration [28–30].

3.3. *In vitro study of drug loading and release properties*

The fabricated CS microspheres with micro-porous structures possessed interconnected channels among the micropores, which might have considerable potential applications as drug delivery system. In the present study, the vancomycin model was used to investigate the capability of the CS microspheres as drug carriers. The results showed that the drug loading amount (DLA: $m_{\text{loaded drug}}/m_{\text{carrier}}$, mg/g) reached to 17.19 ± 0.82 mg/g. Fig. 6 shows the cumulative release results of vancomycin from the CS porous microspheres in PBS. It is clear that the vancomycin-loaded CS microspheres had an obvious two-step release behavior with an initial fast release and a subsequent relatively slow release stage. The initial burst release in the first 3 h was around 10 wt% in PBS of the total amount of loaded vancomycin. The subsequent release rate remarkably decreased with time, and the cumulative release crept during 6 and 72 h incubation, reaching a maximum value of 22 wt% in PBS. The slow release rate was

due to the microporous structures and interconnected channels among the microspheres, which decreased the diffusion rate of the loaded drug molecules [2].

4. Conclusions

In this study, the CS microspheres with diameters of 75–100 μm were successfully fabricated by the spray-drying method. The SBF soaking evaluation revealed that the fabricated CS microspheres possessed excellent bioactivity with a fast growth rate of bone-like apatite layer on their surfaces. Compared with the traditional HAp bioceramics, the Si ions released from the CS microspheres could apparently stimulate the proliferation of MC3T3-E1. In addition, the fabricated CS microspheres exhibited sustained drug release property due to their novel microporous structures. The results suggest that the fabricated CS microspheres might be used for applications as injectable bone-regeneration materials and cell/drug-loaded implants.

Acknowledgments

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