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Self-setting particle-stabilized emulsion for hard-tissue engineering

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ABSTRACT

Injectable self-setting materials have recently attracted interest for use in minimally invasive medical treatments and tissue engineering. In particular, calcium phosphate cements (CPCs) offer certain specific advantages for the treatment of bone defects. Although the inner structures of set CPCs are important for the apposition and remodeling of new bone, there are still limitations to the design of cements with a well-controlled inner structure.

In the present study, we explored self-setting CPCs that generate interconnected macroporous matrices using solid-particle-stabilized emulsion templates. α-Tricalcium phosphate (α-TCP) and poly(ethylene phosphate) sodium salt-coated poly(D,L-lactide-*co*-glycolide) (PLGA) microparticles were mixed with castor oil and water to form an oil-in-water (o/w) emulsion. The α-TCP and PLGA microparticles functioned as an effective particulate emulsifier by adsorption at the oil–water interface. The resulting emulsion spontaneously set in a humidified atmosphere at ambient temperature. The setting behaviors of different emulsions were characterized through X-ray diffraction analysis and compressive-strength measurements. The PLGA microparticles did not hinder the rate of hardening of the emulsions, and they improved the compressive strengths of the set cements. The PLGA particles incorporated within the set cements were hydrolytically degraded, and the degradation of the PLGA particles resulted in the formation of an interconnected pore structure in the set cement. Finally, mouse osteoblastic (MC3T3-E1) cells were cultivated on the set CPCs. The adherent MC3T3-E1 cells adopted a spindle shape, and significant cellular invasion into the set CPCs was observed after degradation of the PLGA microparticles. In conclusion, self-setting emulsions stabilized with α-TCP and PLGA microparticles constitute a novel candidate material for bone regeneration.

1. Introduction

The aging of the population is an issue of considerable concern worldwide. Bone and joint diseases are the primary causes of chronic pain and disability among elderly people. To improve the quality of life of the elderly, the design of novel biomaterials that can be used for hard-tissue repair is important. Self-setting calcium phosphate cements (CPCs) are currently used to repair bone defects and vertebral fractures because these cements are minimally invasive and have a mineral composition similar to that of hard tissues. Brown and Chow discovered fundamental systems for generating CPCs [1]. These authors used an equimolar mixture of tetracalcium phosphate (TTCP; $Ca(PO_4)_2O$) and dicalcium phosphate anhydrous (DCPA; $CaHPO_4$) or dicalcium phosphate dihydrate (DCPD; $CaHPO_4{\cdot}H_2O$). Several compositions have been proposed for such cements to improve the cements' properties [2,3]. α-Tricalcium phosphate (α-TCP; $Ca_3(PO_4)_2$) is one of the most reliable CPC materials for the production of self-setting single-phase calcium orthophosphate formulations [4,5]. The hydrolysis of α-TCP produces calcium-deficient hydroxyapatite (CDHA) through the following reaction (1):

$$3Ca_3(PO_4)_2 + H_2O \rightarrow Ca_9(HPO_4)(PO_4)_5OH \quad (1)$$

This reaction was first described by Monma and Kanazawa [6]. In α-TCP-based CPCs, progressive dissolution of the α-TCP particles and the simultaneous formation of an entangled network of precipitated CDHA crystals are responsible for the setting and hardening of the cement paste [7]. Several methods have been proposed to improve cellular invasion into cement generated with α-TCP [5,8,9]. Ginebra and co-workers have recently summarized various processing approaches for generating macroporous CPCs

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[10,11]. Although these processes successfully produce interconnected macroporous structures in matrices, they suffer from several disadvantages, such as poor reproducibility, weak strength, and irritation resulting from additives.

In the present work, we propose an alternative self-setting CPC that can form an interconnected macroporous structure using a particle-stabilized emulsion template. The formation of particle-stabilized emulsions in the absence of any molecular surfactants is widely used in both research and technology [12–14]. The adsorption of solid particles with predictable and well-defined structures (self-assembly) at oil–water interfaces is of interest for a variety of applications [15]. The surface wettability of the particles at the oil–water interface determines the stability and types of the emulsions that are formed. Moreover, the oil droplet size of the emulsions strongly depends on the concentration of the particles. An effective method of preparing porous solid matrices from emulsions stabilized with solid particles has recently been described. Binks [16] prepared macroporous silica materials using emulsion templates stabilized with a binary mixture of hydrophilized and hydrophobized silica particles. Under certain specific conditions, matrices that contained an interconnected pore structure were obtained after evaporation of the solvents. Bismarck and co-workers have also succeeded in preparing high-internal-phase emulsions (HIPEs) and their polymer forms (poly-HIPE) using functionalized titania nanoparticles as stabilizers [17].

In the present study, we found that α-TCP microparticles can function as an effective particulate emulsifier by adsorption at the oil–water interface to form a stable oil-in-water (o/w) emulsion. The spontaneous setting of the emulsions in a humidified atmosphere at ambient temperature resulted in the formation of macroporous matrices. The size of the pores in the cement could be controlled by changing the powder/liquid ratio. To generate an interconnected pore structure, biodegradable polymer particles were added to the emulsions as a co-emulsifier. The biodegradable polymers did not exert any adverse effects on the hydrolytic setting reaction of α-TCP. Cellular invasion into the CPCs was also evaluated.

2. Materials and methods

2.1. Materials

Poly(D,L-lactide-*co*-glycolide) (PLGA; supporting Fig. S1) (M_w = 7000–17,000, acid-terminated, L:G = 50:50) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 2-Methoxy-2-oxo-1,3,2-dioxaphospholane (MP) was synthesized following a previously described method [18]. β-Tricalcium phosphate (β-TCP) was purchased from the Taihei Chemical Industrial Co., Ltd. (Osaka, Japan). Other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and were used without further purification.

2.2. Synthesis of poly(ethylene phosphate) sodium salt

MP (20 mmol) was placed into a thoroughly dried 30-mL round-bottom flask equipped with a three-way stopcock. After the mixture was dried under reduced pressure for 2 h, methanol and DBU were added as an initiator and a catalyst, respectively, in an argon gas atmosphere with ice cooling. The reaction was allowed to proceed for 3 h. Poly(2-methoxy-2-oxo-1,3,2-dioxaphospholane) (PMP) was purified through reprecipitation from diethyl ether. The PMP (2.5 g) was dissolved in 50 mL of deionized water (dH_2O) and stirred with two equivalents of trimethyl amine relative to the MP unit. After 24 h, 20 g of cation-exchange resin (Amberlite® IR-120, Merck KGaA, Darmstadt, Germany) was added to the polymer solution, followed by stirring for 1 h. This solution was then filtered to remove the cation-exchange resin. The filtrate was dialyzed overnight using a dialysis membrane (MWCO = 1000) in distilled water to remove formic acid. The dialyzed solution was then freeze-dried. The product, poly(ethylene phosphate) (PHP), was obtained as a viscous colorless liquid. To form a sodium salt of the polymer, the PHP was dissolved in an aqueous solution (20 mg/mL), and the pH of the solution was adjusted to 7.0 via the addition of 0.1 N and 0.01 N sodium hydroxide (NaOH). The solution was dialyzed with distilled water for 1 day. Freeze-drying of the aqueous solution afforded the poly(ethylene phosphate) sodium salt (PHP-Na), as shown in supporting Fig. S1. PHP-Na is a polyelectrolyte and is water soluble.

2.3. Preparation of α-TCP microparticles

Bulk α-TCP was synthesized by annealing β-TCP for 5 h at 1200 °C, followed by quenching to room temperature [19,20]. The α-TCP thus obtained was milled in a planetary ball mill (PM301, Retsch, Hahn, Germany) with stainless balls 2.5 mm in diameter for 1 h at 450 rpm and sieved through a 25-μm sieve.

2.4. Preparation of PLGA microparticles covered with PHP-Na

PLGA particles were prepared using the water-in-oil-in-water (w/o/w) emulsion solvent evaporation method [21]. PLGA (0.2 g) was dissolved in 0.6 g of dichloromethane in a 10-mL glass tube. Then, 60 μL of dH_2O was added, and the mixture was vortex mixed for 90 s. Subsequently, 6 mL of a 0.1% aqueous PHP-Na solution was added, and the mixture was emulsified at 4000 rpm for 5 min (IKA® T25 digital Ultra Turrax®, Staufen, Germany) to form a water-in-oil emulsion. The mixture was then added to 94 mL of a 0.1% aqueous PHP-Na solution and 100 mL of a 2% aqueous isopropanol solution and subjected to rapid stirring for 5 h. The particles were allowed to settle for 15 min, and the resulting clear solution was then decanted. The remaining suspension was centrifuged at 1000 rpm for 5 min to collect the particles, and the collected particles were washed three times with dH_2O. Finally, the particles were lyophilized and stored at −30 °C. Rhodamine-6G-labeled PLGA particles were prepared by simply replacing 60 μL of dH_2O with 60 μL of an aqueous rhodamine-6G solution. The size of the PLGA particles was determined via laser diffraction, and X-ray photoelectron spectroscopy (XPS; ESCA-3400, Shimadzu Co., Japan) measurements were conducted to confirm the presence of PHP-Na on the surfaces of the particles.

2.5. Preparation of particle-stabilized emulsion

α-TCP particles (powder), PLGA particles (powder), castor oil and water were vortex mixed and homogenized using a probe-type ultrasonic homogenizer (Sonifier® S-250A, Branson, USA) for 5 min to form emulsions. The following conditions were employed: α-TCP particles/PLGA particles/castor oil/water (wt%) = 30/0/35/35 (CPC-P0), 30/10/30/30 (CPC-P10), or 30/20/25/25 (CPC-P20). Each emulsion was placed in a 2.5-mL plastic syringe and injected into a silicone rubber ring of 5 mm height and 5 mm diameter. The samples were then allowed to set at 37 °C and 95% humidity.

2.6. Characterization of self-setting particle-stabilized emulsion

In the following investigations, the cements were immersed in 2-propanol to remove the castor oil and were subsequently dried under reduced pressure prior to each measurement.