Trace element-incorporating octacalcium phosphate porous beads via polypeptide-assisted nanocystal self-assembly for potential applications in osteogenesis

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ABSTRACT

The promising future of calcium phosphates (CaP) as a group of biomedical materials with a wide range of functions, might ultimately depend on tuning their composition and microstructure. However, the disorderly growth and aggregation of CaP nanocrystals limit their practical application. This paper reports a strategy for designing polypeptide(trace elements (TE), dual mediating the self-assembly of octacalcium phosphate (OCP) nanocrystals, with multilayered porous cross section and TE dilute doping. Intriguing advantages such as bead morphology, mesoporous structure, tunable diameter (20–1000 µm) and TE contents, biodegradability and bioactivity are obtained. The microcomputerized-tomography reconstruction reveals an interconnective macroporous architecture and a void volume of over 49.02% for the nearly close-packed bead scaffolds. The specific surface area and average mesopore size are 89.73 m² g⁻¹ and 2.75 nm for the 180 µm diameter bead group, and those of 500 µm diameter beads are 130.17 m² g⁻¹ and 3.69 nm, respectively. It is demonstrated that the bead production mechanism is a multistep process including liquid-like precursor formation, nanocrystal nucleation and aggregation, aggregate combination and bead growth. Such a multilayer structure of TE–OCP porous beads would have adequate physical strength to maintain their shape, in contrast to the physical weakness of pure OCP hollow shell. The beads exhibit good biocompatibility and degradability and encourage bone mineralization in the early stage in vivo. This study demonstrates the feasibility of developing highly porous calcium phosphate giant beads via biomimetic self-assembly for direct application in reconstructive surgery and other widespread applications such as tissue engineering and drug delivery.

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1. Introduction

The use of dilute dopants or impurities to control the behavior of materials is at the heart of many technologies. Doping is widely critical for semiconductors [1], superconductors [2], new energy materials [3] and biomaterials [4–6], which would otherwise fall far below their performance requirements. The biogenic apatite is non-stoichiometric and contains relatively high levels of magnesium, sodium and carbon (in the form of carbonate groups, CO₂⁻), and lower levels of trace elements (TE) [7]. These foreign TE ions at critical levels are considered to play pivotal roles on the process of biomineralization as well as other diverse effects on nanocrystal size, dissolubility and bioactivity of synthetic hydroxyapatite (HA) [8]. As a family of attractive materials with great chemical similarity to biological apatite, calcium phosphates (CaP) have been widely investigated in particle, ceramic, cement or film form in skeletal repair, drug delivery, cell scaffolds and interference coatings. Some investigations have demonstrated that the CaP ceramics could be endowed with ectopic osteoinductivity by changing the surface texture and microstructure [9–11], whereas a major drawback of the sparingly soluble CaP biomedical device is its low surface reactivity in orthotopic bone regeneration [12,13]. Systematic efforts to mediate stoichiometric CaP structure by impurity dilute doping can be dated back to the 1990s, when a variety of foreign TE ions were incorporated into CaP using sintering or solution-phase reactions [14,15]. The selection of TE as dopants was motivated by their pharmacologically beneficial and biologically properties in activating cell response, nuclei acid syn-
thesis and tissue development [16,17]. Currently, great endeavors are being devoted to investigating the impact of TE dilute doping on the biological performance of CaP bulk devices, or gaining better control and understanding of the TE doping process itself [18–22]. Both activities are essential prerequisites for the improvement of diverse properties, including biological reactivity, bulk biodegradability, and textural and structural features. A system designed as TE-doped CaP biomaterials would ideally be bioresorbable three-dimensional (3D) networks with tunable interconnected macropores for the migration of cells and vascular ingrowth; specifically, practical applications also require mesoporous structure (i.e. high surface area) for incorporation of therapeutic agents. Evidently, opportunities may emerge for porous spheres, which might not only pack 3D porous network to fill various types of vacant sites, but also provide 100% connectivity allowing for mass transfer [23,24]. The sphere-like bulk structure and macroporous ceramics could be conveniently prepared using sintering reactions, but the high-thermal process results in appreciable crystallinity or grain growth and distortion of the solid/pore structure [25–27]. Therefore, artificial building of a delicate 3D mesoporous—macroporous architecture via TE mediating CaP nanocrystal growth by a bottom-up approach is a valuable pursuit, but with many challenges.

Recently, there has been growing attention to morphogenesis of octacalcium phosphate (OCP) in material chemistry. Fibrous spherulitic OCP with macroscale dimension could be synthesized using a bottom-up approach, although they grew slowly in agar gels [28]. Bigi et al. found that hollow OCP spherical shells could also be conveniently prepared by polyelectrolyte-mediated crystallization [29,30]. The low concentrations of fluoride ion may provoke OCP nanocrystal cluster formation [31]. OCP offers a fascinating alternative as a transient intermediate to the biogenic apatite for bone regeneration, owing to its attractive biodegradability and osteoinductivity with potential in medicine, as well as their biological applications [31–33]. To address the drawbacks of existing CaP biomaterials, a layer-by-layer assembly method for encapsulating TE into Silica@OCP core–shell nanospheres was reported [34]. However, this technique is not suitable for producing biomaterial in large quantities because of the labor-intensive procedures. The present authors’ research group recently studied the oriented growth of OCP nanobelts under the collaborative action of amphiphilic surfactant vesicle/lamellar templates [35]. They also developed a polypeptide/surfactant compromised dual-mediation approach to self-assemble OCP nanocrystals into a variety of infrequent hierarchical architectures [36]. In light of the improvement in the mechanism of OCP self-assembly, it was hypothesized that polyanion and inorganic hetero-ions might play a part in defining activity in OCP nanostructures. In that case, the convenient control of OCP microstructure, morphology and dimension during porous aggregate manufacture potentially offer avenues for the design of advanced biomaterials.

Here, it is reported how the TEs-mediated OCP (TEs–OCP) beads were generated with multilayer architecture. An array of TE ions (strontium, zinc, silicon, magnesium) were doped into an OCP structure by polysparatic acid (PAsp)-assisted wet-chemical reactions. These foreign ions were chosen because they play pivotal roles in biology, nutrition and medicine, as well as their biologically active effect in health [8,16]. Intriguing advantages such as mesoporous microstructure, bead morphology, tunable size, TE contents and bioactivity were obtained.

2. Materials and methods

2.1. Chemicals and materials

High-purity-grade inorganic reagents (Ca(CH₃COO)₂, Na₂HPO₄, NaH₂PO₄, SrCl₂, MgCl₂, ZnCl₂, Na₂SiO₃) were purchased from BBI, Canada. Tris(hydroxymethyl)aminomethane (Tris; Bio-Rad) and PAsp (Mₙ = 5.5 kDa, 30 wt.% in water; Taihe Co.) were used without further purification. Reagent-grade sodium cacodylate, formaldehyde, ethanol, HCl and ethylenediaminetetraacetic acid (EDTA) were received from Sinopharm Chemical Co., Shanghai. Ultrapure water (18.0 MΩ cm⁻¹) was used in the experiments. Concentrated Ca(CH₃COO)₂ (500 mM) and Na₂HPO₄/NaH₂PO₄ solution (500 mM, with respect to phosphate) were prepared.

2.2. Synthesis of OCP

Representative synthetic conditions are listed in Table 1. In a typical procedure for preparation of OCP as reported by Bigi et al. [29,30], a dilute solution (600 ml) of Ca(CH₃COO)₂ was added dropwise (≤2 drops per second) to a stoichiometric amount of pH-adjusted Na-phosphate solution (2400 ml; pH 5.0) containing Na₂HPO₄/NaH₂PO₄ and PAsp (2.5 μM). The molar ratio of Ca²⁺/(HPO₄²⁻ + H₂PO₄⁻) was 4:3 in the total solution. The reaction in the 3000 ml flask with mechanical stirring (450 rpm) was undertaken in a 60 °C water bath while recording the pH value (Hanna Instruments pH211). The suspensions were stirred for 2 h (ageing time), and the products were filtered, washed three times with ultrapure water and dried in vacuum. To understand the structure and size evolution, more OCP were prepared by changing only the stirring speeds and ageing time, while the other conditions remained the same.

2.3. Synthesis of TE–OCP

In a procedure similar to that mentioned above, a mixed solution (600 ml) of Ca(CH₃COO)₂ (500 mM) and SrCl₂ (6.0 mM, variable in different experiments) was added dropwise to a stoichiometric amount of pH-adjusted Na-phosphate solution (2400 ml) in the presence of PAsp (2.5 μM). The molar ratio of Ca₂⁺ + Sr²⁺/(HPO₄²⁻ + H₂PO₄⁻) was 4:3 in the total solution. The reaction was undertaken at 60 °C with mechanical stirring (500 rpm). After ageing for 2–16 h, the TE–OCP beads were washed three times with dilute HCl solution (pH = ~4.2) and ultrapure water and dried in vacuum. To understand the morphology stability, other individual TE (i.e. zinc, magnesium, silicon) were added to the aqueous medium, and TE–OCP were synthesized, changing only the TE species and concentrations.

2.4. Synthesis of TES–OCP (one-stage addition of TE ions)

Solutions with 25 mM of MgCl₂, ZnCl₂ and SrCl₂ were mixed with Ca(CH₃COO)₂ (500 mM) under stirring in an appropriate proportion to give Sr:Ca, Mg:Ca and Zn:Ca molar ratios of 0.9:100 (0.9%), respectively. A solution of Na₂SiO₃ (25 mM) was also mixed with sodium phosphate solution to give a Si:P molar ratio of 0.9:100 (0.9%). The molar ratio of Ca²⁺ + Sr²⁺ + Zn²⁺/(HPO₄²⁻ + H₂PO₄⁻) was 4:3 in the total solution. The synthesis of TES–OCP was similar to the procedure described above. To understand the changes in dopant capacity and particle beads, more TES–OCP beads were prepared, changing only the foreign ion concentrations, stirring speeds and ageing times, while the other conditions remained the same.

2.5. Synthesis of TES–OCP (two-stage addition of TE ions)

As above, half the solutions with 25 mM of MgCl₂, ZnCl₂ and SrCl₂ were mixed with Ca(CH₃COO)₂ (500 mM) under stirring in an appropriate proportion to give Sr:Ca, Mg:Ca and Zn:Ca molar ratios of 0.45:100 (0.45%), respectively. A solution of Na₂SiO₃ (25 mM) was also mixed with sodium phosphate solution (500 mM with respect to phosphate) in an appropriate proportion
to give a Si:P molar ratio of 0.45:100 (0.45%). After the burst nucleation occurred, the other halves of the solutions with 25 mM of MgCl₂, ZnCl₂, SrCl₂ and Na₂SiO₃ were also added to the suspension solution. Finally, the beads were washed three times with dilute HCl solution, ultrapure water and absolute ethanol and dried in vacuum.

2.6. ICP analysis

The as-dried beads (20 mg) were dissolved into HCl (10 vol.%) with three replicates prior to chemical analysis. The ion concentrations were determined using inductively coupled plasma (ICP; Thermo).

2.7. Structure and phase analysis

The phase of the beads was identified by X-ray diffraction (XRD; Rigaku) using Cu Kα radiation at a scanning rate of 3°/min and Fourier transform infrared spectroscopy (Nicolet). The surface and cross-sectional morphology of the beads were observed using scanning electron microscopy (SEM; Hitachi S4800). In order to evaluate the lattice constant, the Rietveld refinement analysis of the samples (denoted by OCP, Si–OCP, Zn–OCP, Sr–OCP, Mg–OCP, TEs–OCP, respectively) was conducted with the assistance of the MAUD computer program [35,37].

2.8. Porosity estimation and measurement

Porosity measurements were performed by imaging the bead-packed cylinder-like scaffold (Ø8 × 10 mm) with three types of bead diameter distribution (45 ± 15, 180 ± 30 and 500 ± 50 μm) using X-ray micro-computerized tomography (μCT, Scanco Medical). Using 3D μCT reconstruction and a segmentation value of 75, porosities were estimated via the quantitative 3D evaluation program included with the μCT software package (n = 3). The porosity was confirmed using mercury porosimetry. Brunauer–Emmett–Teller (BET) surface areas and Barrett–Joyner–Halenda (BJH) pore-size distribution were measured with an accurate surface area and porosimetry system (Micromertitics ASAP2010).

2.9. TE release measurement

The TE release was investigated for the TEs–OCP180 beads (bead size, 180 ± 30 μm; Si/P, 0.34%; Zn/Ca, 2.16%; Sr/Ca, 0.47%; Mg/Ca, 0.25 mol.%) and TEs–OCP500 beads (bead size, 500 ± 50 μm; Si/P, 0.31%; Zn/Ca, 2.04%; Sr/Ca, 0.41%; Mg/Ca, 0.19 mol.%) by incubating in Tris buffer at physiological temperature to simulate the weak alkaline environment in vivo. The Si/P, Zn/Ca, Sr/Ca and Mg/Ca molar ratios were 0.31%, 2.04%, 0.41% and 0.19% in the TEs–OCP. The TEs–OCP beads (100 mg) were incubated in 25 ml Tris buffer (0.05 M) with an initial pH 7.25 in a 37 °C water bath. After soaking for different time intervals (1, 2, 4, 8, 24,

Table 1

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</table>

a The mixture solution containing Mg²⁺, Sr²⁺ and Zn²⁺ ions was first added to the aqueous sodium phosphate (containing PAsp and silicate ions), and then the Ca(CH₃COO)₂ solution was added dropwise.

b The concentration preconditions of the TE were designed as: M/Ca = 0, 0.9%, 1.2%, 1.5, 1.8% (where M: Mg, Sr or Zn); Si/P = 0, 0.9%, 1.2%, 1.5, 1.8%, in the original aqueous solution.

c The average sizes were measured when the beads had aged for 2 h in the aqueous media.

d Gelatinous nanocrystal aggregates.
36, 48, 72, 96, 120 h), 2.0 ml of supernatant was centrifuged for ICP analysis, and an aliquot amount of fresh buffer (2 ml) was added to the Tris buffers to maintain the medium volume constant.

2.10. In-vivo testing I (subcutaneous implantation)

Sprague–Dawley (SD) rats 12 weeks old (~280 ± 10 g) were used in this study. Incisions were made bilaterally in the back region under general anesthesia. The surgical protocol was in accordance with the Zhejiang University Animal Care Committee guidelines for the care and use of laboratory animals. Subcutaneous implantation of TEs–OCP500 and OCPx (x denotes bead diameter, \( x = 45 \), 180 and 500 \( \mu \)m; ageing time, 1.5 h) in rats for periods of 2–6 weeks were carried out. Four pockets for sterilized pure OCP (three-group: OCP45 with diameter 45 ± 15 \( \mu \)m, OCP180 with diameter 180 ± 30 \( \mu \)m, and OCP500 with diameter 500 ± 50 \( \mu \)m) and TEs–OCP500 (one-group: 500 ± 50 \( \mu \)m) were made in the subcutaneous tissue on either side of the spinal cord ~5 cm from the base of the neck in each rat (Scheme 1A). Wounds were closed with absorbable surgical sutures (Huawei Medical Products Co. Ltd). At 2, 4 and 6 weeks (\( n = 4 \)) post-surgery, the beads and surrounding tissue were harvested, recorded by digital camera (Nikon). For the histological study, the specimens were dehydrated in grade alcohols, partly decalcified in 10% EDTA in 0.1 M sodium cacodylate buffer (pH 7.0), post-fixed for 6 h in formaldehyde and then embedded in paraffin. Sections (7 \( \mu \)m) were cut with a microtome and stained with hematoxylin and eosin (HE; Sigma–Aldrich) for histological evaluation was carried out as described above.

2.11. In vivo testing II (femur defect implantation)

Incisions were made bilaterally in the femur region of SD rats (\( n = 24 \)) under general anesthesia. Using the sterile surgical technique, the OCP500 and TEs–OCP500 beads were implanted in the anterior cortex of both femurs. Under continuous saline irrigation, unicortical holes (2.5 \( \times \) 4 mm) were drilled in a standard manner in the anterior cortex of the femur, using a dental drill bit of conical design. The sterilized beads were inserted into the holes with a press-fit technique. Then the subcutaneous tissue and skin were closed in layers. Rats were sacrificed at 6 weeks, and the femur specimens with the implants were harvested, cleared of all soft tissue for \( \mu \)CT reconstruction with an isotropic resolution of 20 \( \mu \)m in all three spatial dimensions. After scanning, the \( \mu \)CT images were segmented using a nominal threshold value of 225, and the 3D cross-section analyses were performed automatically. Then the histological evaluation was carried out as described above.

3. Results and discussion

3.1. Morphology of TE-mediated OCP

To understand the effect of TE ions on both the microstructure and size of the OCP particles, a series of experiments was conducted to determine the parameters, including stirring speed, TE species and concentrations, in driving OCP bead production towards a tunable size (see Table 1). The starting point for this synthetic effort was the protocol developed by Bigi et al. [30], who identified a morphology-controlled growth mechanism based on pure OCP hollow spherical shell on acidic polyelectrolyte mediation. The solutions containing quaternary TE behave similarly to those containing each individual TE. These TE mainly impair bead growth, but not its morphology. These primary investigations indicated that the critical factors in controlling bead size were indeed mechanical stirring, ageing time and TE concentration (typically from sub-micrometer colloidal aggregates to tens and hundreds of micrometer beads). Reducing stirring speed and prolonging ageing time lead to multilayer self-assembly and produce giant beads ~1 mm in diameter. The representative SEM images of OCP are shown in Fig. 1a–d, in which precipitation from TE-free solutions (control experiments) yielded spherical particles. The diameter of the microspheres could be roughly controlled by tuning stirring speed (450–600 rpm). Similar bead morphology was clearly seen for TE–OCP and TEs–OCP. With the addition of zinc, magnesium, strontium or silicon precursors at the same stirring speed of 500 rpm, different bead sizes were produced (Fig. 1i–o). In particular, the OCP was investigated as a function of ageing time (Fig. 1c and e–h). This comparison showed that an 'eggshell' was reserved via core dissolution or collapse over time, whereas the TEs–OCP exhibited a multilayered porous microstructure (Fig. 1m). The SEM images at progressively increasing magnifications revealed that OCP were sponge-like, porous beads by nanocrystals self-assembling over several dimensions (nanoplate-like individuals assembling into macrobeads: Fig. 1n–p). It suggested that TE were insufficient to influence the bead morphology, but sufficient to increase the core stability of TEs–OCP.

3.2. Phase transformation of beads

The TE-mediated beads over ageing time were examined by XRD. The first definable mineral phase appeared later in the burst nucleation period and was a distinct, partly crystalline OCP phase identified a morphology-controlled growth mechanism based on pure OCP hollow spherical shell on acidic polyelectrolyte mediation. The solutions containing quaternary TE behave similarly to those containing each individual TE. These TE mainly impair bead growth, but not its morphology. These primary investigations indicated that the critical factors in controlling bead size were indeed mechanical stirring, ageing time and TE concentration (typically from sub-micrometer colloidal aggregates to tens and hundreds of micrometer beads). Reducing stirring speed and prolonging ageing time lead to multilayer self-assembly and produce giant beads ~1 mm in diameter. The representative SEM images of OCP are shown in Fig. 1a–d, in which precipitation from TE-free solutions (control experiments) yielded spherical particles. The diameter of the microspheres could be roughly controlled by tuning stirring speed (450–600 rpm). Similar bead morphology was clearly seen for TE–OCP and TEs–OCP. With the addition of zinc, magnesium, strontium or silicon precursors at the same stirring speed of 500 rpm, different bead sizes were produced (Fig. 1i–o). In particular, the OCP was investigated as a function of ageing time (Fig. 1c and e–h). This comparison showed that an ‘eggshell’ was reserved via core dissolution or collapse over time, whereas the TEs–OCP exhibited a multilayered porous microstructure (Fig. 1m). The SEM images at progressively increasing magnifications revealed that OCP were sponge-like, porous beads by nanocrystals self-assembling over several dimensions (nanoplate-like individuals assembling into macrobeads: Fig. 1n–p). It suggested that TE were insufficient to influence the bead morphology, but sufficient to increase the core stability of TEs–OCP.
indicating the metastable nature of OCP. Under these conditions, the corresponding pH profile showed a multi-step process of precipitate formation (Fig. 2b). This included an initial period of 50 min during which the pH quickly increased by 0.9 units (stage I). This was followed by a short stable period and fast reduction in pH to a value of 5.7 (stage II and III). After nucleation, the curves were still time dependent (stage IV and V).

3.3. TE contents in OCP beads

The procedures developed for TE doping in an OCP structure were perhaps the most critical aspect of the preparation of bioactivity-enhanced beads, because only the substantially incorporated TE in the OCP structure would maintain sustained release. Thus, two basic approaches were designed to explore this possibility. The first was to grow the beads in situ in the presence of quaternary TE (Mg2+, Sr2+, Zn2+ and SiO4\(^{4-}\) ions) via one-step TE addition before bead nucleation, to clarify the morphological characteristics by TE dilute doping. Attempts to prepare TE–OCP beads by this procedure succeeded, because porous spheres were observed. The products exhibited as spherical beads with multilayered porous microstructure (Fig. 1m). Interestingly, sufficient physical adsorption of zinc occurred, as manifested by ICP analysis of the products before and after dilute HCl washing. However, this physical adsorption was far lower than the doping ratios (data not shown). The result that the TE simultaneously incorporate in the OCP structure via a wet-chemical reaction is encouraging.

The second approach explored was another synthetic route in which the TE ions were added in solution by stages. Half of the foreign divalent metal ions were mixed originally with the Ca\(^{2+}\) solution, and Na\(_2\)SiO\(_4\) was added directly to the Na-phosphate solution, and the other half was added dropwise at the end of the induction period, when the burst nucleation occurred. The resulting beads displayed a considerable size and porous microstructure, indicating reducing synergistic inhibition on bead growth (Fig. 1n–p). This two-stage approach was also briefly tested for TE contents in OCP compared with that in the one-step approach. It can be seen from Fig. 3 that the two-stage TE addition significantly increased the doping ratio by 10–20% in OCP beads. For instance, the 1.5% composition of TE in original solution (relative to Ca and P, respectively) doped the highest levels of TE (i.e. 2.31% of Zn, 0.43% of Sr, 0.33% of Si and 0.19% of Mg). However, a significant level of Mg\(^{2+}\) ions remained in the mother solutions. This experimental result accorded very well with Matsunaga’s first-principle prediction that zinc could more favorably substitute for the specific calcium site of OCP compared with magnesium [38]. With respect to silicate anions the effective doping ratio was over 20% of the total dopant existing in the solution (i.e. 0.34% of Si/P ratio in the solid phase vs 1.5% in the solution phase). This is classically considered to be the reason for size or heterovalent competition between SiO\(_4^{4-}\) and PO\(_4^{3-}\) ions [14].

3.4. Lattice parameters of TE- and TEs-doped OCP beads

Deeper insight into the effects of TEs on OCP structure was revealed by calculating the lattice parameters, as summarized in Table 2. The lattice parameters of Mg-doped OCP samples were approximately the same as those of pure OCP, mainly because of the small amount of dopant (~0.2%). However, considerable Zn-doping (~2.31%) or a definitely limited Si-doping rate (~0.34%) both led to a slight decrease in cell constants. In contrast, the cell dimensions increased in the Sr-containing OCP, and those containing multi-doping foreign ions exhibited a modest increase with respect to those of OCP. The ionic radius of different cations is usually used to explain how zinc, magnesium and strontium may affect the cell parameters of OCP. The first-principle calculation shows that the substitution of foreign metal ion in OCP with the smaller ions (e.g. Mg\(^{2+}\), Zn\(^{2+}\)) or larger ions (e.g. Sr\(^{2+}\)) would give rise to lattice contraction or expansion of the surrounding atoms [38,39]. These variances are consistent with the experimental results reported elsewhere [20–22]. For silicate anion substitution, this lattice strain can be understood in terms of interdiffusion of Si substituting for P in the PO\(_4\) lattice and in terms of a deviation of the oxygen concentration from stoichiometry. The doping of the Si ion arising from the corresponding adjustment of the Si valency can supply additional contributions to lattice strain.
3.5. TE release analysis

The feasibility of TE release in the early stage was investigated in the Tris buffer at 37 °C for TEs–OCP beads with different sizes (Fig. 4a and b). Strontium maintained sustained release in the whole stage. The magnesium concentrations increased steadily with time, although its effective doping ratio was very low (<15%) in comparison with zinc ions. Interestingly, the silicon and zinc concentrations increased significantly with the rapid release of calcium and phosphate ions in the first hour, but then decreased to a steady concentration after 2 h. This implied that part of the zinc and silicate ions returned into the hydrated layer of OCP nanocrystals. Casey et al. found that the silicate anions preferentially reconstructed to form a network in the near-surface amorphous region of the mineral and were present in solution before being incorporated into a growing secondary phase[40]. Zinc is a hydrolysable metal of higher polarity, which is arguably a more common TE in human metabolism and possesses the ability to attach electrostatically to the negatively charged surface at pH 5–8.3[41]. Actually, it can be seen that the beads dissolved readily from the slow increase in cumulative release rate of calcium and phosphorus (Fig. S1, Supplementary data), accompanying the change in its surface structure. The low-magnification SEM images of TEs–OCP show the smooth surface of the beads before soaking (Fig. 4c and e). Meanwhile, relatively uniform morphology but a very rough surface can be clearly observed from the high-magnification SEM images after soaking for 7 days (Fig. 4d and f). The randomly distributed dissolution-corroded cracks were further observed clearly in the bead surface after soaking in the buffers. This suggests that the TEs–OCP beads would be biodegradable in vivo.

3.6. Microstructure of TEs–OCP beads

Fig. 5a is a digital picture of as-prepared TEs–OCP, which are spherical and from tens of micrometers to millimeters in diameter. In the present porous scaffold-like frameworks stacked with beads of nearly uniform size, the overall 3D architecture and void volume were validated using μCT reconstruction (Fig. 5b–e). This scaffold-like framework ensures adequate pore volume for nutrient transfer and tissue regeneration, and also allows flexibility in choosing an optimal overall pore size due to controllable bead sizes. For the 45-, 180- and 500-μm bead groups, the measured percentage pore volumes (38–50%) were higher than the theoretical values (24–26%) of the close-packed microsphere system (Table 3). However, mercury porosimetry reported far higher values (68–85%) than those from μCT analysis, mainly because the μCT software cannot discriminate void space <10 μm within the individual beads.

The nitrogen sorption isotherm curves of TEs–OCP indicated a nearly linear increase in the amount of adsorbed nitrogen in the low relative pressure range (P/P0 = 0–0.7) (Fig. 6). According to the International Union of Pure and Applied Chemistry [42], it can be classified as a type H3 hysteresis loop deriving from plate-like aggregates with slit-shaped pores. The steep increase in nitrogen sorption at P/P0 = 0.9–1.0 suggested the presence of macropores, which was consistent with the SEM observation in

![Fig. 4. ICP analysis for ion release from (a) TEs–OCP500 and (b) TEs–OCP180 in Tris buffer at 37 °C, and low and high resolution SEM images of the surface morphology and microstructures of (c, d) TEs–OCP500 and (e, f) TE–OCP180 before (c, e) and after (d, f) soaking for 7 days. Bars: 150 μm (c, d); 50 μm (e, f).]
Nevertheless, the majority of the pore volume was contributed by mesopores, that is, most of the pores were in the range 2–50 nm, as shown by the pore size distribution curves (Fig. 6, inset). Calculated from the desorption branch of the isotherm using the BJH method, the average pore size was determined as 2.75–3.69 nm. Moreover, the BET specific surface areas were 130.17 and 89.73 m² g⁻¹ for TEs–OCP180 and TEs–OCP500, respectively (Table 3), which were considerably larger than that of the surfactant-mediated mesoporous CaP nanomaterials [43]. Recently, Ng et al. reported non-ionic surfactant-mediated mesostructured CaP particles with high surface area (>200 m² g⁻¹). Unfortunately, the morphology is still not quite as desired, since the particles have been shown to be disorderly aggregates [44].

### 3.7. Bead growth processes

To evaluate the growth mechanism of TEs–OCP beads in the presence of PAsp and foreign ions, the intermediate products were
examined at different incubation stages by SEM observation (Fig. 7). As illustrated in Fig. 7j, the evolution of morphology and microstructure of the above TEs–OCP aggregates demonstrates a multi-step self-assembly process to form macrobeads. At the initial stage before burst nucleation, PAsp-induced liquid-like precursors (PILP) were produced via the electrostatic interactions between PAsp and metal ions. The long-chain polypeptide with electron-donating side groups (COO⁻/CO⁻) itself is shrunk by electric attraction according to ionic association in a dilute acidic solution. Then the PILP induced colloidal mesocrystal nucleation in a calcium-dose-dependent manner in the metastable aqueous solution. When the reaction was prolonged, the primary mesocrystals tended to oriented aggregation until they dispersed to resemble sphere-like morphology, which became noticeable to the naked eye. Finally, the microspheres grew, accompanying oriented self-assembly of nanocrystals over time, and the primary core as well as the boundary between layers could be clearly recognized from the cross-section view (Fig. S2, Supplementary data). In the absence of foreign ions, the colloidal aggregates formed initially by mesocrystal stack in the solution, and in particular, the polymers could induce the rearrangements to achieve the hollow-core structure in the medium. Bigi et al. demonstrated that the acidic polyelectrolyte could not maintain the core stability without involvement of foreign TE ions [30], which implied that the presence of TE should be an important prerequisite for the multilayered architecture. It is postulated that the foreign ions readily distort the hydrated layer of the primary colloidal nanocrystals, so that the organic molecules provoked a more ordered aggregation that was less susceptible to solution supersaturation with respect to OCP dissolution [45]. Thus, the organic molecules readily bound to the primary amorphous Ca-phosphate nanoparticles, which would consolidate the oriented aggregation of TEs–OCP nanocrystals [46]. Accordingly, it is reasonable to propose that the dual combination of PAsp and TE mediation might control the nucleation of TEs–OCP nanocrystals, and their final assemblies evolve into the multilayered, mesoporous beads. Such multilayer structures of TEs–OCP beads would have adequate physical strength to maintain their shape, in contrast to the physical weakness of hollow OCP eggshell.

3.8. In vivo test for biocompatibility

Since the beads prepared in this study were generally larger than 5 μm in particle size, it is of major concern whether the in vivo biocompatibility would be affected by big bead size (x). Subcutaneous implantation of TEs–OCP500 and OCPx (x = 45, 180 or 500 μm) in rats for periods of 2–6 weeks showed no evidence of obvious inflammation response (Fig. 8). At the end of 2 weeks post-implantation, a thin capsule formed in the tissue–beads interface (Fig. 8a–d). After a prolonged time, this in vivo implantation also demonstrated degradation of TEs–OCP500, as evidenced by an increase in the connective tissue-like layer compared with the OCP series (Fig. 8e–h). From histological observation by light microscopy (Fig. 9), some multinucleated giant cells (MNGC) were in contact with the OCP500 and TEs–OCP500 bead residuals at 4 and 6 weeks, whereas there was no such abundance of microvessels surrounding the OCP500 spheres at 6 weeks. These results suggest that the TEs–OCP would be more useful in implant applications where rapid tissue regeneration and concomitant implant resorption are important considerations.

3.9. In vivo test for osteogenic potential at the initial stage

The porous beads were implanted into the rat femur defect model to determine whether bone regeneration could be activated through tissue–material interactions in the early stage. Representative cortical surfaces, 3D μCT construction and histological analysis taken 6 weeks post-implantation are shown in Fig. 10. The OCP500 spheres were exposed in the defect (Fig. 10a) but TEs–OCP500 beads had been submerged by the osteoid-like tissue (Fig. 10e). The 3D microstructure in the TEs–OCP500 group exhibited unmineralized trabecular bone (Fig. 10f). However, in the OCP500 group, there was scarcely new mineralized tissue in the bead scaffold (Fig. 10b). Histological examination of TEs–OCP500 showed more new osteoid-like tissue formation extending homogeneously throughout the bead intervals (Fig. 10g and h). The beads were noted for loose appearance, reflecting their porosity. Many
Fig. 8. Biocompatibility evaluation (digital image) of OCP beads doped with and without TE. (a) The bead aggregates in the open incision demonstrate the adhesion on the surface of muscle, indicating good tissue compatibility. (b–d) TE–OCP shown to assess in vivo compatibility 2 weeks after implantation. (e–g) OCP with different diameters from 45 to 500 µm (control) show the newly formed blood vessel on the encapsulating layer, but TE–OCP 500 µm in diameter (h) were not readily visualized, since the beads were more regularly biodegraded in the thin layer accompanied by new tissue growth after 6 weeks.

Fig. 9. Partially decalcified histological sections stained with HE. Note that numerous MNGC surround the OCP500 and TE–OCP500 implants at 2 and 4 weeks, whereas the number of such phagocytes seemed higher for OCP500 than for TE–OCP500 at 6 weeks. Arrowhead, newly formed vessels; solid arrow, the separated material residuals; hollow arrow, MNGC.
osteoblasts with a cuboidal shape were aligned on the TEs–OCP500 implant, and the relative ratio of osteoid tissue–implant material progressively increased. The remnants of TEs–OCP500 beads were reduced to small islands, which appeared to be completely embedded in the osteoid-like tissue. In the case of OCP500, the limited lamellar bone was adjacent to the material, since the bead was more regularly organized and compact (Fig. 10c and d). These results suggest that the appositional bone growth is faster on TEs–OCP500 than on OCP500 in the early stage after implantation. And thus TEs–OCP500 beads provide preferable cell-scaffolds for bone regeneration in skeletal defects, as seen in the osteoporotic rat femoral marrow space [47].

4. Conclusions

A variety of options were demonstrated for “bottom-up” self-assembling TEs–OCP porous beads with tunable size, microstructure and TE contents to endow nearly optimal microstructure and physicochemical properties. Intentional TE mediating and dilute doping were discussed via unitary TE-mediating OCP (TE–OCP) and quaternary TEs-mediating OCP (TEs–OCP) in aqueous solution. The multilayered porous core of TEs–OCP can be stabilized by different foreign ion dopants with the pure OCP hollow spherical shell synthesized under similar conditions, which is significant for adequate physical strength and shape maintenance. The TEs–OCP beads are expected to be suitable for either direct clinical use in reconstructive surgery or tissue-engineering cell scaffold applications. Moreover, the highly porous beads are potentially convenient for loading biologically active molecules and therapeutic agents to cooperatively enhance the biological performance of the integrated system. It is therefore believed that the combination of these features in TEs–OCP porous beads will offer opportunities for creating a more efficient approach towards bone regenerative medicine.

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Appendix A. Supplementary data

Appendix B. Figures with essential colour discrimination

Certain figures in this article, particularly Figures 1, 2, and 4–10, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.actbio.2011.12.012.

References