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Original article

Redispersible and stable amorphous calcium phosphate nanoparticles functionalized by an organic bisphosphate

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ABSTRACT

Although much effort has been focused on the preparation of stable amorphous calcium phosphate (ACP) nanoparticles in aqueous solution, the redispersibility and long-term stability of ACP nanoparticles in aqueous solution remains an unresolved problem. In this work, stable colloidal ACPs were prepared by using an organic bisphosphonate (BP) as a sterically hindered agent in aqueous solution. The harvested calcium phosphate nanoparticles were characterized by inductively coupled plasma atomic emission spectrometry (ICP-AES), Fourier transform infrared (FTIR), X-ray diffraction (XRD), dynamic light scattering (DLS) and transmission electron microscopy (TEM). ICP-AES, FTIR and XRD results suggested the particles were ACP. DLS and TEM results indicated that the size of the ACP nanoparticles were in the range of 60 nm with a spherical morphology. The resulting calcium phosphate nanoparticles retained its amorphous nature in aqueous solution for at least 6 months at room temperature due to the stabilizing effect of the organic bisphosphonate. Moreover, the surface of the ACP nanoparticles adsorbed with the organic bisphosphate used showed good redispersibility and high colloid stability both in organic and aqueous solutions.

1. Introduction

Amorphous calcium phosphates (ACPs), a kind of calcium phosphate ceramics, are present in the early stage of bone biomineralization and eventually convert to stable crystalline hydroxyapatites (HAs) [1–3]. ACP has demonstrated better osteoconductivity [4] and crack resistance than hydroxyapatite (HA) [5], and is more biodegradable than tricalcium phosphate (TCP) [6]. Another key feature of the ACP nanoparticles is its intrinsic porosity that is useful for loading proteins, genes, siRNA and drugs [7–11].

From a thermodynamic point of view, amorphous materials are at best metastable [12]. ACP precipitates should be collected and freeze-dried shortly after the preparation (the sooner, the better), because in aqueous or non-aqueous media ACP is spontaneously converted to other crystalline calcium orthophosphates, mainly calcium deficient hydroxyapatite (CDHA) [13]. It is widely accepted that the dissolution of ACP and the nucleation and growth of HA from solution are responsible for the phase transformation processes. Organic solvents or organic additives were widely used to stabilize ACP because the complexes of calcium with organic agents can form during the synthesis. This favors the ACP formation, which is attributed to the coordinated complexing agents remaining in the structure of ACP. However, amorphous calcium phosphate (ACP) synthesized with cyclodextrins (CDs) [14], polyethylene glycol (PEG) [15], terephthaloyl chloride [16] or ethylene glycol (EG) [17] as organic additives at or below room temperature in aqueous solution could remain stable in aqueous solution for several days.

We have synthesized a novel surface modifier for HA, *i.e.*, α-methoxy-ω-dihydrogen phosphate-poly (ethylene glycol) (mPEG-OPO_3H_2). Its organic phosphate groups could strongly bond to Ca^{2+} ions of the growing HA particles during their synthesis in water, forming the HA nanoparticles with peripheral methoxy-poly (ethylene glycol) (mPEG) chains [18]. Bisphosphates (BPs) show higher affinity to calcium phosphate than their mono-phosphate counterparts and are widely used for the treatment of orthopedic diseases, such as Paget bone disease, osteoporosis, fibrous dysplasia, myeloma and bone metastases [19–22]. Based on this, we proposed that organic bisphosphates could be used to stabilize ACP for a longer time by being tightly bonded on the surface of ACP and provide the ACP particles with good redispersibility and colloid stability. Therefore, we synthesized a bisphosphate, *i.e.* 3-(*N*,*N*-dimethylamino)-*N*′,*N*′-bis(phosphonomethyl) propylamine (NDBP), a biocompatible aminophosphonic acid [23]. So we prepared ACP by using NDBP as an organic phosphate and surface modifier. The synthetic route of NDBP and the supposed complexation mechanism of NDBP with calcium ions [24] are shown in Scheme 1.

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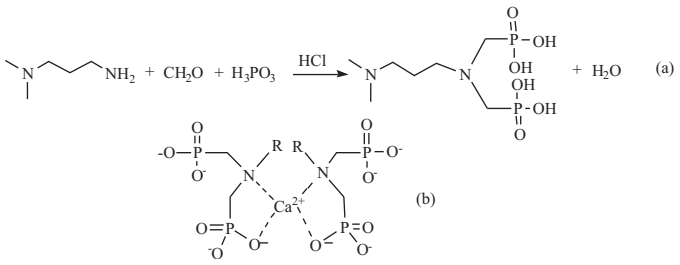

Scheme 1. (a) The synthetic route of NDBP; (b) complexation of calcium ion by BPs.

2. Experimental

2.1. Materials

Aqueous ammonia (25% by mass fraction), concentrated hydrochloric acid and formaldehyde solution (30–40% by mass fraction) and $Ca(NO_3)_2{\cdot}4H_2O$, $(NH_4)_2HPO_4$ and H_3PO_3 were all of analytical grade and purchased from Chengdu Kelong Chemical Reagent Company, Chengdu, Sichuan, China. 3-Dimethylamino-propylamine ($C_5H_{14}N_2$) was purchased from Adamas Reagent Co., Ltd., Shanghai, China.

2.2. Synthesis of 3-(N,N-dimethylamino)-N′,N′-bis(phosphonomethyl) propylamine (NDBP)

NDBP was prepared using the Mannich reaction [25]. Briefly, 3-dimethylaminopropylamine (1.02 g, 0.01 mol), H_3PO_3 (1.64 g, 0.02 mol) and a stirring bar were added to a three-necked round-bottom flask, and deionized water (28 mL) was added. The flask was placed in an ice-salt-bath and concentrated hydrochloric acid (5.4 mL) was slowly added to the flask drop wise. After heating to 90 °C, formaldehyde solution (5.4 mL) was added drop wise, and the reaction mixture was kept at reflux temperature for 3 hours. The solvent was removed using a rotary evaporator and the product was washed with anhydrous ethanol and stored in a dry environment. The product was characterized by NMR and FTIR. ^{1}H NMR in D_2O gave the following resonances: δ 2.15 (m, 2H, C–CH_2–C), 2.79–3.12 (s, 6H, –CH_3), 3.22–3.26 (d, 4H, N–CH_2–P), 3.41–3.45 (m, 4H, N–CH_2–C); ^{13}C NMR (D_2O): δ 54.09(d, N–CH_2–P), 53.14 (–CH_2–N), 51.78 (N–CH_2–), 42.81 (–CH_3), 19.13(C–CH_2–C); FTIR (cm^{-1}): 3435.48 (ν_{P-OH}), 1473.83 ($\nu_{P=O}$), 923.32 (ν_{P-O}).

2.3. Synthesis of amorphous calcium phosphates (ACPs)

ACP nanoparticles were synthesized with NDBP as a surface modifier by using the similar synthetic procedures that we have reported recently [18]. The phosphate solution was prepared by dissolving 0.0609 g of NDBP (organic phosphate 0.41 mmol) and 0.0238 g of $(NH_4)_2HPO_4$, (inorganic phosphate, 0.18 mmol) in 16 mL of water. The calcium solution was prepared by dissolving 0.236 g of $Ca(NO_3)_2{\cdot}4H_2O$ (1 mmol) in 14 mL of water. The total Ca/P molar ratio was 1.67. The pH values of both solutions were adjusted to 10.5 by aqueous ammonia. For the preparation of ACP, the calcium solution was added drop wise into the phosphate solution at room temperature in 15 min, followed by ultrasonic irradiation for 30 min, during which the pH value of the mixture was kept at 10.5. Thereafter, the mixture was aged at 85 °C for 5 h. The resulting light blue hydrocolloid was subjected to 30 min ultrasonic treatment and then stayed overnight before purification.

The colloid was centrifuged at 15,000 rpm, followed by water washing and re-dispersing repeatedly until the conductivity of the re-dispersed colloid was near that of water, indicating that water soluble impurities were totally removed. Most centrifuged precipitate was freeze-dried for 24 h to obtain powders. The residue was added to water or methanol and then subjected to 15 min of ultrasonication irradiation to form a stable colloid. The controlled sample was prepared using the same synthesis as mentioned above but without any surface modifiers and designated as BLK.

In order to investigate the long term stability of the ACP particles in water, the re-dispersed ACP aqueous colloids were stored at room temperature for 2, 4 and 6 months, respectively, before being centrifuged and freeze-dried. The samples were designated as ACP-2m, ACP-4m and ACP-6m, respectively. The freeze-dried powders of ACP-2m, ACP-4m and ACP-6m were subjected for the XRD study.

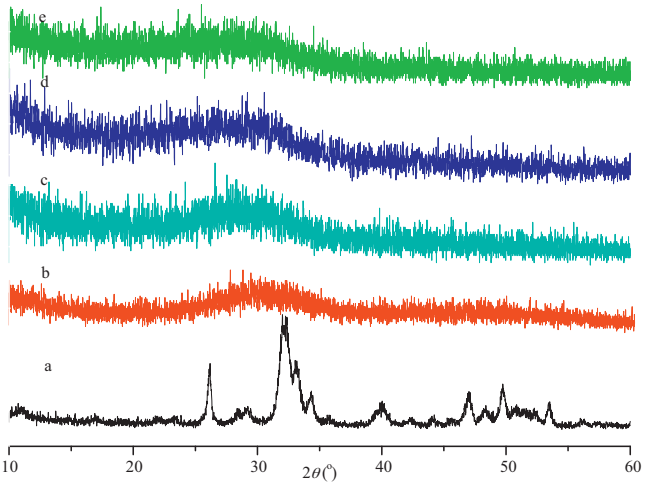

Fig. 1. XRD patterns of ACP dried powder (a, BLK; b, ACP-0m; c, ACP-2m; d, ACP-4m; e, ACP-6m).

2.4. Characterization

The X-ray diffraction (XRD) patterns of the ACP and BLK powders were recorded on an X'Pert Pro MPD diffractometer (PANalytical BV, Netherlands) using a Cu Kα radiation operated at 40 (kV) and 35 (mA). Fourier transform infrared (FTIR, KBr pellets) was carried out on a Nicolet IR200 spectrometer (Thermo Electron, USA), in the wavenumber range 400–4000 cm^{-1} with a resolution of 4 cm^{-1}. The Ca/P ratio of the ACP was measured by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES)

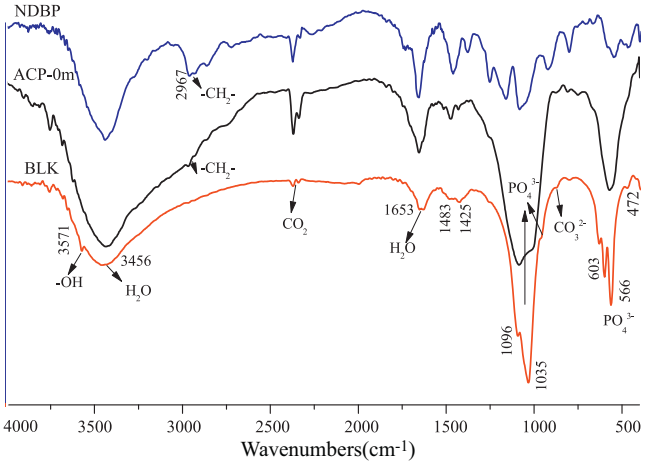

Fig. 2. FTIR spectra of ACP-0m, BLK and NDBP.