Colloids and Surfaces B: Biointerfaces 162 (2018) 135–145

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Full Length Article

Facile one-pot fabrication of calcium phosphate-based composite nanoparticles as delivery and MRI contrast agents for macrophages

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ARTICLE INFO

Article history:
Received 18 July 2017
Received in revised form 20 October 2017
Accepted 14 November 2017
Available online 15 November 2017

Keywords:
Calcium phosphate
Iron oxide nanoparticle
Composite nanoparticle
Magnetic resonance imaging (MRI)
Macrophage

ABSTRACT

We developed a facile one-pot fabrication process for magnetic iron oxide–calcium phosphate (IO–CaP) composite nanoparticles *via* coprecipitation in labile supersaturated CaP solutions containing IO nanocrystals. All the source solutions used were clinically approved for injection, including water and magnetic IO nanocrystals (ferucarbotran, used as a negative magnetic resonance imaging (MRI) contrast agent). This ensured that the resulting nanoparticles were pathogen- and endotoxin-free. The dispersants used were clinically approved heparin sodium (heparin) or adenosine triphosphate disodium hydrate (ATP), which were added to the IO-containing labile supersaturated CaP solutions. Both heparin and ATP coprecipitated with CaP and ferucarbotran to form heparin- and ATP-modified IO–CaP nanoparticles, respectively, with a hydrodynamic diameter of a few hundred nanometers. Both the resulting nanoparticles exhibited relatively large negative zeta potentials, caused by the negatively charged functional groups in heparin and ATP, which improved the particle dispersibility when compared to non-modified IO–CaP nanoparticles. The heparin-modified IO–CaP nanoparticles were effectively ingested by murine macrophages (RAW264.7) without showing significant cytotoxicity but barely ingested by non-phagocytotic human umbilical vein endothelial cells, indicating the potential of these nanoparticles for targeted delivery to macrophages. The heparin-modified IO–CaP nanoparticles exhibited a negative contrast enhancing ability for MRI. Our results show that IO–CaP nanoparticles have potential as delivery and MRI contrast agents for macrophages.

1. Introduction

Calcium phosphates (CaPs) are mineral components found in humans, and can be easily synthesized from common and inexpensive inorganic sources. Certain types of CaPs exhibit good biocompatibility, high affinity for various biomolecules, and degradability under acidic conditions. Nano-sized (1–1000 nm) CaP particles can be injected *via* intravenous administration, wherein they travel through the blood stream. They are taken up by cells with minimal toxicity, and then dissolve into serum ions (calcium and phosphate ions). These characteristics of CaPs make them suitable as delivery carriers of various diagnostic and therapeutic agents [1–4]. It has been demonstrated by *in vivo* studies that CaP nanoparticles can deliver diagnostic and therapeutic agents without noticeable adverse effects for the detection and treatment of various diseases including cancer [5–7].

Macrophage cells are associated with many inflammatory diseases, including atherosclerosis, arthritis, cancer, diabetes, and neurological diseases [8,9]. The safe and efficient delivery of imaging agents to macrophage cells is of importance for early diagnosis and accurate evaluation of these diseases. In general, nanoparticles with a size of few hundred nanometers are useful for targeted delivery to macrophages because macrophages with phagocytic capacity more easily take up such nanoparticles compared to non-

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https://doi.org/10.1016/j.colsurfb.2017.11.034
0927-7765/

phagocytic cells [10]. Thus, we aimed to fabricate CaP nanoparticles with a size of a few hundred nanometers containing imaging agent for targeted delivery to macrophages. We employed magnetic iron oxide (IO) nanocrystals, a negative magnetic resonance imaging (MRI) contrast agent, as the imaging agent, which shortens the transverse (T_2) relaxation time and decreases the signal intensity of T_2 weighted image [11].

Previously, composite nanoparticles comprising CaP and magnetic IO have been prepared from chemical reagents *via* several methods, including a coprecipitation process [12–14], a mechanochemical milling process [15], and a pulsed laser irradiation process in coprecipitation solutions [16,17]. Among these processes, the coprecipitation process is most useful since CaP-based nanoparticles are spontaneously formed in supersaturated CaP solutions under mild conditions (neutral pH, normal pressure, and relatively low temperature) without the need for specific instruments. However, a relatively long processing time (>10 h) and/or multiple complex processes are required to obtain composite nanoparticles. In addition, IO nanocrystals that coprecipitate within the CaP matrix can cause side effects in the body when isolated from the CaP nanoparticles.

In this study, we used a clinically approved T_2 negative MRI contrast agent as the magnetic IO source and achieved, for the first time, a facile (30 min) one-pot fabrication for magnetic IO–CaP composite nanoparticles *via* coprecipitation in supersaturated CaP solutions containing IO and a specific dispersant. The supersaturated solutions were prepared only from injection solutions (even water and dispersant were of injectable quality) to secure the safety level (pathogen-free, endotoxin-free, and so on) of the products for future preclinical and clinical applications. For the magnetic IO source, we chose ferucarbotran: maghemite (γ-Fe_2O_3) nanoparticles coated with carboxydextran (Fig. S1a), which has been clinically used as a T_2 negative MRI contrast agent for the liver [18]. It was expected that ferucarbotran, which has been approved for intravenous administration, would enter the metabolic pathway for iron after being isolated from the CaP nanoparticles. For CaP sources, we used several infusion fluids based on previous reports by Sogo et al. [19]. For the particle dispersants, we selected heparin sodium (heparin, Fig. S1b) and adenosine triphosphate disodium hydrate (ATP, Fig. S1c)—negatively charged pharmaceutical agents approved for injection. The use of these dispersants was inspired by our previous finding that negatively charged DNA molecules carrying phosphate groups coprecipitate with CaP in supersaturated CaP solutions to form well-dispersed and size-regulated DNA–CaP composite nanoparticles because of their surface charge repulsion [20,21]. We hypothesized that heparin with sulfo and carboxyl groups and ATP with phosphate groups (Fig. S1, yellow parts) would interact with CaP similar to DNA molecules and coprecipitate with CaP and ferucarbotran in supersaturated CaP solutions, affording well-dispersed and size-regulated IO–CaP nanoparticles.

Herein, we fabricated magnetic IO–CaP nanoparticles using injection solution-derived IO-containing supersaturated CaP solutions, with and without dispersant (heparin or ATP). The resulting nanoparticles were analyzed for their physicochemical properties, dispersion stability, cellular uptake, and cytotoxicity. In the cellular uptake and cytotoxicity analyses, we used murine RAW264.7 macrophages and non-phagocytotic human umbilical vein endothelial cells (HUVECs) to demonstrate the potential of these nanoparticles in macrophage-targeted delivery. The selected nanoparticles were further analyzed for their MRI contrast enhancing ability. To the best of our knowledge, this is the first report to investigate the MRI contrast enhancing ability of the magnetic IO–CaP nanoparticles, although there are some studies which have reported that the magnetic IO–CaP nanoparticles have the potential as heating elements in hyperthermia [15,22,23] and as transfer agents in magnetofection [24–26].

2. Materials and methods

2.1. Preparation of nanoparticles from injection solutions

We prepared three types of IO-containing labile supersaturated CaP solutions—without dispersant, with heparin, and with ATP—and obtained three products—**CaP-Fer**, **CaP-Fer-Hep**, and **CaP-Fer-ATP**, respectively. The injection solutions and the volumes used to prepare 30 mL of the supersaturated solution are shown in Table 1. The volume compositions of these supersaturated solutions were determined from our previous study on the fabrication of DNA–CaP composite nanoparticles (ferucarbotran and/or heparin or ATP were added instead of DNA to the solution X1.0 [20]).

All operations were performed under aseptic conditions. First, we prepared four source solutions: Fe, Ca, and P-containing solutions, and an alkalinizer. The Fe-containing solution (Fe: 5 mM) was prepared by mixing ferucarbotran (Resovist® Inj., KYOWA CritiCare Co., Ltd., Japan) with either injection water (Water for Injection, Fuso Pharmaceutical Industries, Ltd., Japan) for **CaP-Fer**, heparin (Heparin Na LOCK 100Units/mL SYRINGE OTSUKA 5 mL, Otsuka Pharmaceutical Co., Ltd., Japan) for **CaP-Fer-Hep**, or ATP (ATP Injection 20 mg, Koa Isei Co., Ltd., Japan) for **CaP-Fer-ATP**. The Ca-containing solution (Ca^{2+}: 4.78 mM), P-containing solution ($H_2PO_4^-$ and HPO_4^{2-}: 20.0 mM), and alkalinizer (HCO_3^-: 167 mM) were each prepared by mixing two injection solutions, as follows: (1) Ca-containing solution: Ringer's Solution OTSUKA (Otsuka Pharmaceutical Co., Ltd.) and Calcium Chloride Corrective Injection 1 mEq/mL (Otsuka Pharmaceutical Co., Ltd.), (2) P-containing solution: Klinisalz® (KYOWA CritiCare Co., Ltd.) and Dibasic Potassium Phosphate Injection 20mEq Kit (Terumo Co., Japan), and (3) alkalinizer: MEILON® Injection 7% (Otsuka Pharmaceutical Co., Ltd.) and injection water [20,27]. These source solutions were prepared in sterile centrifuge tubes (capacity: 50 mL) that were placed in a dry bath with a setting temperature of 18 °C. The Ca-containing solution, the P-containing solution, and the alkalinizer were added sequentially to the Fe-containing solution in the dry bath. The final solution (30 mL) was immediately mixed by shaking the tube a few times. The nominal concentrations of heparin, ATP, Fe, Ca, and P in the resulting IO-containing labile supersaturated CaP solutions are listed in Table S1. The prepared three solutions had constant concentrations of Fe (0.25 mM), Ca (3.68 mM), and P (1.83 mM) and differed only in dispersant (heparin or ATP, or none of them). The solutions were tightly sealed in the tubes and then placed in an incubator with a setting temperature of 37 °C to allow coprecipitation. After coprecipitation for 30 min, the products were collected *via* centrifugation (6,000 rpm (3,700*g*), 5 min) and washed twice with injection water. The resulting products are hereafter referred to as the 'final products'.

2.2. Characterization of the products

The morphology, chemical composition, and crystalline structure of the final products were examined *via* scanning electron microscopy (SEM; S-4800, Hitachi High-technologies Corporation, Japan), energy dispersive X-ray spectroscopy (EDX; EMAX x-act, HORIBA, Ltd., Japan), X-ray photoelectron spectroscopy (XPS; PHI 5000 VersaProbe, ULVAC-PHI, Inc., Japan) with AlKα X-rays, and thin-film X-ray diffractometry (XRD; M18X, MAC Science, Japan and Ultima IV, Rigaku Corporation, Japan) with CuKα X-rays. The micro and crystalline structures of the selected product (**CaP-Fer-Hep**) were further investigated *via* transmission electron microscopy and transmission electron diffraction (TEM and