Colloids and Surfaces B: Biointerfaces 136 (2015) 168–174

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Fine-tuning in mineral cross-linking of biopolymer nanoparticle for incorporation and release of cargo

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ARTICLE INFO

Article history:
Received 5 June 2015
Received in revised form 13 August 2015
Accepted 5 September 2015
Available online 9 September 2015

Keywords:
DNA
Nanoparticle
Alternative dialysis
Calcium phosphate
Mineralization
Cross-linking
Release control

ABSTRACT

We developed a mineral cross-linking strategy to prepare a biopolymer-based nanoparticle using calcium phosphate (CaP) as a cross-linker. Nanoparticles were first formed by mixing deoxyribonucleic acid (DNA) with cationic surfactants, and were cross-linked by CaP precipitation. After removal of the surfactants, we carried out the alternative dialysis of nanoparticles against $CaCl_2$ aqueous solution and phosphate buffered solution for further mineral cross-linking. XRD and FT-IR studies revealed that the resultant nanoparticles were produced by mineral cross-linkages of hydroxyapatite (HAp) and the crystal amount and properties such as morphology and crystallinity could be well-controlled by the reaction conditions. Chemical dyes could be incorporated into nanoparticles via their affinities with crystal faces of HAp and DNA. Their release was tunable by crystal amount and properties of mineral cross-linkages. Also, the release could be triggered by mineral dissolution in response to pH. Such a mineral cross-linking will open up a potential way to provide a nanoparticle with versatile functions such as cleavable cross-linking, binding affinity for cargos, and pH-responsive release.

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1. Introduction

There have been growing interests in development of gel materials since they are capable of absorbing and retaining substances within their matrix and often exhibit their unique properties such as volume changes in response to external stimuli. Among them, the gel particles are often produced by precipitation and assembling of polymers through the interaction with oppositely-charged polymers, surfactants and ions and the association of polymer chains. These assembled structures are often fragile against dilution so that cross-linking process is prerequisite to maintain their colloidal stability without dissolution. In biological systems, we can find an analogue of cross-linked structures, which is in the form of milk proteins known as casein micelles. Several casein micelles are assembled into submicron-sized particles and cross-linked by precipitation of calcium phosphate. Kappa casein has phosphoserine residues in the sequences, which act as binding sites for calcium ions and thereby leading to precipitation of calcium phosphate (CaP) [1]. Calcium phosphate is also known as a main constituent of hard tissues in vertebrates, such as bones and teeth, and is the most abundant inorganic material in living organisms. In terms of materials design, pH-dependent dissolution of CaP is also advantageous to enable pH-responsive release of cargos, which is useful in drug delivery system. There have been numerous reports on tuning of crystal properties of nano-sized CaP in the presence of polymers for dissolution of CaP and release of cargos [2,3]. In those cases, polymers were added as an assistance for CaP mineralization. In addition, there are some reports that mineralization was carried out in preformed polymer particles to render them colloidal robustness and pH-responsiveness [4–8]. For instance, Akiyoshi et al. reported preparation of hybrid nanoparticles via utilizing a nanogel as a template for mineralization [9–12]. Han et al. produced mineralized nanoparticles of PEGylated hyaluronic acid, which possessed a pH-dependent drug releasability [13]. In both cases, the polymer nanoparticles were chemically cross-linked prior to mineralization and were used as a template for mineralization.

Herein, inspired from cross-linking structures of casein micelles, we intended to produce hybrid nanoparticles via cross-linking of biopolymer by means of mineralization of CaP (mineral cross-linking). By tuning in the amount and crystal properties of CaP, we also aimed to provide nanoparticles with functionalities designed for incorporation and release of cargos. Among various crystal structures of CaP, we especially focused on hydroxyapatite (HAp) because it possesses both positively and negatively-charged crystal faces and is often utilized as a chromatography column for separation of charged biopolymers such as proteins and DNA [14].

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http://dx.doi.org/10.1016/j.colsurfb.2015.09.011
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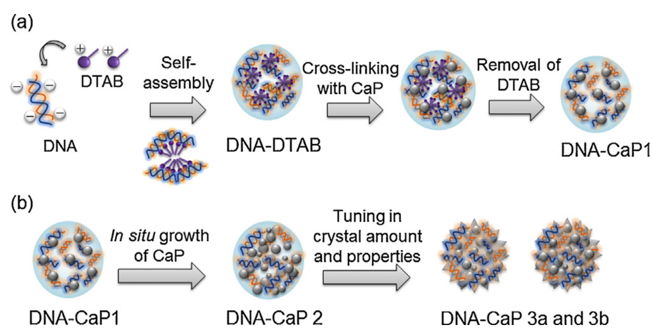

Scheme 1. Schematic illustration for preparation of DNA gel nanoparticles cross-linked by multi-step mineralization of calcium phosphate (CaP). (a) DNA was assembled into nanoparticles with the aid of cationic surfactant, DTAB, and mineralization was carried out to produce mineral cross-linkages inside nanoparticles. (b) By the use of the resultant nanoparticles as a template, multi-step mineralization was conducted to induce *in situ* CaP growth and control its crystal amount and properties.

Therefore, we expected that mineralization of HAp inside the nanoparticles would make it possible to generate binding sites suitable for incorporating various charged substances. Furthermore, we also expected that dissolution of HAp and release of cargos could be controlled by tuning in the amount and properties of crystals deposited inside the nanoparticles.

It is well known that phosphate groups act as suitable sites for nucleation and growth of CaP [15–17]. We previously reported that HAp mineralization was facilitated on a DNA-coated surface [18]. Therefore, in this study, DNA was selected as a biopolymer for construction of nanoparticles and subsequent mineral cross-linking via *in situ* deposition of HAp. We intended to control the solubility of mineral cross-linkages by tuning crystal properties of HAp through the multi-step mineralization as shown in Scheme 1.

Scheme 1a illustrates the assembly of DNA into nanoparticles with the aid of a cationic surfactant and CaP mineralization inside the nanoparticles. The surfactant was then removed via dialysis to prepare mineralized nanoparticles (DNA-CaP1) for mineral cross-linking. By utilizing DNA-CaP1 as a template, further growth of CaP was carried out by multi-step mineralization to give rise to DNA nanoparticles (DNA-CaP2, DNA-CaP 3a and DNA-CaP 3b) as shown in Scheme 1b. The hydrodynamic diameter and the zeta potential of the obtained nanoparticles were measured and their morphologies were observed by transmission electron microscopy (TEM). Crystal structures and crystallinity of CaP were interpreted by utilizing infrared spectroscopy (IR) and X-ray diffraction analysis (XRD). Chemical dyes were incorporated by the affinities with DNA and HAp crystal faces of nanoparticles. We also investigated the dye release from mineralized DNA nanoparticles to reveal how the release could be influenced by the mineral cross-linking.

2. Experimental

2.1. Materials

DNA derived from salmon spermary, dodecyltrimethylammonium bromide (DTAB), calcium chloride ($CaCl_2$) dehydrate, disodium hydrogenphosphate 12-water, sodium dihydrogenphosphate dehydrate, 8-anilino-1-naphthalenesulfonic acid (ANS) and acridine orange (AO) were all purchased from Wako Pure Chemical Industries, Ltd, Japan. Albumin from bovine serum (BSA) and Arsenazo III were purchased from Sigma-Aldrich, USA. Rhodamine 6G (Rh6G) and erythrosine (ER) were purchased from Junsei Chemical Co., Japan. Water used in all experiments was prepared in a water purification system and had a resistivity higher than 18.2 M Ωcm.

2.2. Self-assembly of DNA into gel nanoparticles

Nanometer-sized DNA particles were prepared as follows. At first, DNA and DTAB were dissolved in HEPES buffer (10 mM, pH 7.4) and milli-Q water, respectively. 0.17 mL of the DTAB solution (0.03–0.17 mM) was added drop-wise into 5 mL of the DNA solution (0.26 mg/mL) with gentle stirring. Thereafter, the sample was stirred for 30 min followed by incubation without stirring for another 30 min to make DNA assembled into DNA nanoparticles (DNA-DTAB). Unless otherwise stated, preparation of DNA-DTAB was carried out at 25 °C. For *in situ* precipitation of CaP, 0.7 mL of $CaCl_2$ aqueous solution (1 mM, pH 7.4) was added to the suspension of DNA-DTAB. After 30 min-incubation, 0.7 mL of Na_2HPO_4/NaH_2PO_4 phosphate buffered solution (5 mM, pH 7.4) was added, too. The resultant suspension was incubated for 1 d at 25 °C to induce sufficient mineral cross-linking. Then, in order to remove DTAB and obtain mineralized nanoparticles (DNA-CaP1), the suspension of nanoparticles was dialyzed against several types of solutions including HEPES buffer, phosphate buffer or $CaCl_2$ aqueous solution (5 mM, pH 8.0) for 18 h.

2.3. Multi-step crosslinking with calcium phosphate

In situ precipitation of CaP was repeated to tune mineral cross-linking of gel nanoparticles with the assistance of the alternative dialysis as follows. After removal of DTAB, the suspension of DNA-CaP1 in $CaCl_2$ aqueous solution (5 mM, pH 8.0) was dialyzed with a Slide-A-lyzer dialysis cassette (Thermo Fisher Scientific Incorporation, USA) against phosphate buffer (10 mM, pH 8.0) for 3 h at 25 °C to produce mineralized nanoparticles (DNA-CaP2). For further mineralization, the suspension of DNA-CaP2 in phosphate buffer (10 mM, pH 8.0) underwent centrifugal separation at 15,000 r.p.m for 5 min and the supernatant solution was removed. Then, 0.25 mL of $CaCl_2$ aqueous solution (2 mM, pH 7.4) was added to the nanoparticles. Phosphate buffer (10 mM, pH 7.4) was added after 30 min-incubation. The resultant suspension was further incubated for 24 h at 25 °C to obtain mineralized nanoparticles (DNA-CaP3a). In order to promote crystallization, mineralization was performed by utilizing $CaCl_2$ aqueous solution (1 mM, pH 9.0) and phosphate buffer (5 mM, pH 9.0), and then the incubation was carried out for 24 h at 37 °C to prepare mineralized nanoparticles (DNA-CaP3b).

2.4. Characterization of nanoparticles

To determine the content of DNA in nanoparticles, DNA remaining in the supernatant solution was collected via centrifugal separation at 70,000 r.p.m. for 45 min. The amount of DNA was estimated from its absorbance at 260 nm using the spectrophotometer. The hydrodynamic diameters of nanoparticles were evaluated by dynamic light scattering using a photon correlator (PAR-3, Otsuka Electronics, Japan). The electrophoretic mobility of nanoparticles was measured using a zeta-potential analyzer (ZC-2000, Microtec Co., Ltd., Japan). The mobility (u) was converted into zeta-potential (ζ) utilizing Smouluchowski relation $\boldsymbol{\zeta} = u\eta/\epsilon$, where η and ϵ are the viscosity and the permittivity of the solution, respectively. For morphological observations, the sample was prepared by depositing a nanoparticle suspension upon a carbon-coated copper grid, followed by air-drying, and then it was observed with a field emission transmission electron microscope (FE-TEM, FEI company, USA).

2.5. Characterization of calcium phosphate in mineralized nanoparticles

Elemental analysis of mineralized nanoparticles was performed by energy-dispersive X-ray spectrometry (EDX). The content of Ca in mineralized nanoparticles was evaluated by using calcium ion-

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