Acta Biomaterialia 31 (2016) 378–387

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Full length article

Translation of a solution-based biomineralization concept into a carrier-based delivery system via the use of expanded-pore mesoporous silica

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ARTICLE INFO

Article history:
Received 8 October 2015
Received in revised form 21 November 2015
Accepted 30 November 2015
Available online 2 December 2015

Keywords:
Amorphous calcium phosphate
Amine functionalization
Biomineralization
Collagen
Expanded pore mesoporous silica

ABSTRACT

Mineralization of collagen fibrils using solution-based systems containing biomimetic analogs of matrix proteins to stabilize supersaturated calcium phosphate solutions have been predictably achieved *in vitro*. Solution-based systems have limitations when used for in-situ remineralization of human hypomineralized tissues because periodic replenishment of the mineralizing solution is infeasible. A carrier-based platform designed for delivering mineral precursors would be highly desirable. In the present work, mesoporous silica nanoparticles with expanded pores (eMSN; 14.8 nm) were synthesized. Polyacrylic acid–stabilized amorphous calcium phosphate (PA–ACP) was generated from a supersaturated calcium and phosphate ion-containing solution, and chosen as the model mineralizing phase. After amine functionalization (AF) of the eMSN through a post-grafting method, the positively-charged AF-eMSN enabled loading of PA–ACP by electrostatic interaction. *In-vitro* cytotoxicity testing indicated that PA–ACP@AF-eMSN was highly biocompatible. The release kinetics of mineralization precursors from PA–ACP@AF-eMSN was characterized by an initial period of rapid calcium and phosphate release that reached a plateau after 120 h. Intrafibrillar mineralization was examined using a 2-D fibrillar collagen model; successful mineralization was confirmed using transmission electron microscopy. To date, this is the first endeavor that employs expanded-pore mesoporous silica to deliver polymer-stabilized intermediate precursors of calcium phosphate for intrafibrillar mineralization of collagen. The carrier-based delivery system bridges the gap between contemporary solution-based biomineralization concepts and clinical practice, and is useful for *in-situ* remineralization of bone and teeth.

Statement of significance

Concepts of collagen biomineralization have been reasonably well established in the past few years and intrafibrillar mineralization of collagen fibrils can be predictably achieved with analogs of matrix proteins using solution-based systems. However, solution-based systems have their limitations in clinical applications that require direct application of mineralization precursors *in-situ* because periodic replenishment of the mineralizing solution is impossible. The present work presents for the first time, the use of amine-functionalized mesoporous silica with expanded pores for loading and release of polyacid-stabilized amorphous calcium phosphate mineralization precursors, and for intrafibrillar mineralization of type I collagen fibrils. This strategy represents an important step in the translational application of contemporary biomineralization concepts for *in-situ* remineralization of bone and teeth.

1. Introduction

Over the course of evolution, fibrillar collagen has emerged as a universal template for calcium and silica biomineralization [1,2]. The choice of type I collagen as a template for creating calcium

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http://dx.doi.org/10.1016/j.actbio.2015.11.062
1742-7061/

phosphate-based biocomposites has been rationalized as the end result of evolutionary selection; the idiosyncratic molecular structure of collagen fibrils enables mineralized collagen to function as load-bearing components in living organisms [3]. Because of the elegance and efficacy in which Nature orchestrates these biomineralization schemes, the last decade has witnessed an upsurge in interest in dissecting the science behind collagen biomineralization, and in the art of faithfully replicating those processes via biomimetic approaches. Revolutionary concepts have emerged to elucidate the intriguing phenomenon of replacing the free and loosely-bound water within the intrafibrillar spaces of a collagen fibril with hydrated calcium phosphate precursors [4–8]. Amid these intense pedagogical pursuits, the involvement of amorphous calcium phosphate (ACP) as precursors of biomineralization of bone and teeth has ultimately been confirmed [9]. This highly significant biological advancement was complemented by exciting discoveries in the fields of physical chemistry and chemical physics on formation of organic–inorganic hybrids. Novel models such as the particle-mediated crystallization pathway [10] and polymer-assisted mesocrystal formation [11] are gradually being accepted by the scientific community at large, for interpreting features in collagen biomineralization that are inexplicable by the classical ion-mediated crystallization theory. More recent reports on how calcium and phosphate ions are stabilized as calcium triphosphate prenucleation clusters prior to their coalescence into ACP droplets [12,13] added provocative insight toward understanding collagen biomineralization at the nanoscale. Two-dimensional and 3-D intrafibrillar mineralization of type I collagen matrices can now be predictably achieved in the laboratory using solution-based techniques that incorporate analogs of matrix proteins to stabilize calcium phosphate ions in supersaturated solutions [14–16]. Although some finer aspects of precursor infiltration, such as the fluidic nature of the polymer-stabilized ACP droplets and the mechanism by which these droplets enter the collagen fibril are still under vigorous debate, advancement in the art and science of collagen biomineralization to-date has been nothing short of phenomenal.

For proof-of-concept, a solution-based biomimetic mineralization system is indispensable for examining the fundamental aspects of collagen biomineralization. These solution-based systems may also be used for fabricating mineralized collagen scaffolds for tissue engineering applications [17,18]. Notwithstanding, solution-based systems have their limitations in clinical applications that require direct application of a mineralization system *in-situ*, wherein periodic replenishment of the mineralizing solution is infeasible. Although it may be contended that *in-situ* mineralization may be more easily accomplished using mesenchymal stem cell-seeding or cell-homing strategies [19], there are situations when neither strategy is pragmatic. A notable example is the remineralization of tooth decay, an infectious disease that affects 91% of the United States adult population aged 20–64 [20]. In a typical scenario in which soft decayed dentin is removed, the remaining hypomineralized dentin with intact collagen is sealed from the external environment with a tooth filling which prevents continuous feeding of a solution-based mineralization system. The hypomineralized dentin surface is not in contact with the blood stream for cell-homing, and does not serve as an efficacious scaffold for cell-seeding. Because existing dental filling materials cannot remineralize completely demineralized collagen matrices devoid of seed apatite crystallites [21,22], development of a delivery system for storage of releasable biomimetic ACP precursors would be highly desirable.

Mesoporous silica nanoparticles (MSN) have gained attention as delivery systems for drugs, proteins, enzymes and genetic materials [23] because of their high internal surface area and pore volume, tunable pore sizes [24] and the possibility to functionalize the silanol groups in the internal pores and/or particle surface [25]. Vallet-Regi et al. examined a series of MSN with different pore sizes and found that the rate of drug release declined with decreasing MSN pore size [26,27]. MCM-41 type MSN with small pores (2–4 nm) was found to immobilize or adsorb biomacromolecules mostly on the surface of the particles instead of encapsulating those drugs within the internal pore architecture [28]. The molecular weight of biomimetic analogs that are commonly used to stabilize ACP precursors varied from 1.5 kDa to 27 kDa [29]. Because MSN with small pores in the range of 2–4 nm may not achieve optimal loading capacity, expanded-pore MSN (eMSN) [30] is a better alternative for loading polymer-stabilized ACP. Accordingly, the objective of the present study was to test the hypothesis that the solution-based biomineralization concept may be translated via the use of eMSN into a carrier-based delivery system to release polymer-stabilized ACP precursors for biomineralization of collagen fibrils.

2. Materials and methods

2.1. Reagents

Cetyltrimethylammonium bromide (CTAB), methanol, tetraethyl orthosilicate (TEOS, 99%), ethanol, 1,3,5-trimethylbenzene (TMB), hydrochloric acid (HCl), (3-aminopropyl) triethoxysilane (APTES), polyacrylic acid (PA, MW 1800), toluene, calcium chloride ($CaCl_2$,), potassium phosphate dibasic (K_2HPO_4), Tris-buffered saline, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid solution (HEPES), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), bovine skin-derived type I collagen lyophilized powder and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO, USA) and used without further purification.

2.2. Synthesis of expanded-pore MSN (eMSN)

Expanded-pore MSN (eMSN) was synthesized using a previously-reported technique with modifications [30]. Briefly, 1.97 g of CTAB was dissolved in methanol/water (400 g; 0.4:0.6 w/w), with the pH adjusted to 11 using 1 M NaOH. Three milliliter of TEOS was added to the solution under vigorous stirring. After stirring of the mixture for 8 h and aging overnight, the precipitate was centrifuged and dispersed in ethanol by sonication. Deionized water and TMB were added to the re-suspended MSN (20 mL of 1:1 mixture, v/v) and placed inside a Teflon-lined hydrothermal synthesis autoclave reactor at 140 °C for 4 days to expand the pores within the MSN. The surfactant was removed from the eMSN by refluxing in HCl/ethanol (22 mL of 1:10 mixture, v/v) for 20 h. The white powder was retrieved by filtration, rinsed with ethanol and dried at 80 °C for 8 h.

2.3. Preparation of amine-functionalized eMSN (AF-eMSN)

Five hundred mg of eMSN powder was suspended in 100 mL anhydrous toluene, followed by addition of 2 mL APTES. The mixture was refluxed for 24 h, filtered, rinsed 5 times with ethanol and dried at 80 °C for 12 h.

2.4. Preparation of PA–ACP doped AF-eMSN (PA–ACP@AF-eMSN)

Eighteen mM $CaCl_2$ and 8.4 mM K_2HPO_4 solutions were prepared separately. The pH of each solution was adjusted to 7.4 using Tris-buffered saline. Polyacrylic acid was added to the $CaCl_2$ solution to achieve a final PA concentration of 500 μg/mL. Equal volumes of PA-containing $CaCl_2$ and K_2HPO_4 solutions were mixed together, with the supersaturated calcium phosphate solution stabilized by PA in the form of PA–ACP.