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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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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 hr. Intrafibrillar mineralization was examined using a 2-D fibrillar collagen model; successful mineralization was confirmed using transmission electron microscopy. To date, this is

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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.

Keywords

amorphous calcium phosphate; amine functionalization; biomineralization; collagen; expanded pore mesoporous silica

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 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 1,800), toluene, calcium chloride (CaCl₂), potassium phosphate dibasic (K₂HPO₄), 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 hr 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 hr. The white powder was retrieved by filtration, rinsed with ethanol and dried at 80 °C for 8 hr.

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 hr.

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

Eighteen mM CaCl₂ and 8.4 mM K₂HPO₄ solutions were prepared separately. The pH of each solution was adjusted to 7.4 using Tris-buffered saline. Polyacrylic acid was added to the CaCl₂ solution to achieve a final PA concentration of 500 µg/mL. Equal volumes of PA-containing CaCl₂ and K₂HPO₄ solutions were mixed together, with the supersaturated calcium phosphate solution stabilized by PA in the form of PA-ACP.

Two hundred mg of AF-eMSN was suspended in 50 mL of PA-ACP containing solution and shaken for 1 day to promote infiltration of the PA-ACP complex into the mesopores within AF-eMSN. The white precipitate was retrieved, rinsed with deionized water, filtered, and stored as wet specimens at -20 °C until use.

2.5. Characterization of nanoparticles

Ultrastructural features of eMSN, AF-eMSN, and PA-ACP@AF-eMSN was examined by transmission electron microscopy (TEM) using a JEM-1230 microscope (JEOL, Tokyo, Japan). Elemental mapping was obtained by a field-emission scanning TEM (Tecnai G2, Hillsboro, USA) with energy dispersive x-ray spectroscopy (EDS). Small-angle and wide-angle X-ray diffraction (XRD) patterns of the nanoparticles were analyzed with a X-ray diffractometer (X'Pert PRO, PANalytical B.V., The Netherlands). Fourier transform-infrared spectra (FT-IR) was obtained with a Nicolet 6700 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Thermogravimetric analysis (TGA) was performed by thermogravimetric analyzer (NETZSCH STA 449 F3, Selb, Germany) using nitrogen atmosphere. X-ray photoelectron spectroscopy (XPS) was performed using a Kratos Axis Ultra spectrometer (DLD-600W, Kratos, Japan) with a monochromatic Al KR X-ray source. ¹³C and ²⁹Si solid-state nuclear magnetic resonance (NMR) spectroscopy was performed with a 270 MHz spectrometer (JEOL, Tokyo, Japan) equipped with a 7 mm Magic Angle Spinning (MAS) probe at a frequency of 91.369 MHz for ¹³C and 53.762 MHz for ²⁹Si in the cross polarization (CP) mode. Zeta potential measurements were performed

with a Zetasizer Nano ZS system (Malvern Instruments Ltd., Malvern UK). Nitrogen adsorption/desorption measurements were performed using ASAP 2020 analyzer (Micromeritics Instrument Corp., Norcross, GA, USA).

2.6. In vitro release of ACP

To avoid interference from Ca and P ions present from simulated body fluid (SBF), HEPES buffer was used to evaluate the actual amount of Ca and P released from PA-ACP@AF-eMSN. Specifically, 100 mg of PA-ACP@AF-eMSN was immersed in 10 mL HEPES (10 mM, pH 7.4). The concentration of Ca and P released was measured at 0.5, 1, 3, 6, 12, 24, 48, 72, 96, and 120 hr. At each designated time interval, the mixture was centrifuged at 4000 rpm for 4 min. Then, a 1 mL aliquot of the supernatant was removed and replaced with fresh solution. The aliquots were analyzed for Ca and P content using inductively coupled plasma-atomic emission spectroscopy (ICP-AES, Perkin Elmer, MA, USA).

2.7. Cytotoxicity evaluations

The PA-ACP@AF-eMSN delivery system has to possess low cytotoxicity before it may be used for future dental remineralization procedures. Accordingly, human dental pulp stem cells derived from adult teeth (hDPSCs; DP003F, All Cells, LLC, Alameda, CA, USA) were used to evaluate the cytotoxicity of PA-ACP@AF-eMSN using the MTT assay. The hDPSCs were seeded in 96-well plates at a density of 5,000 cells per well and cultured in α -modified Eagle medium (Gibco, BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco), 100 units/mL penicillin and 100 mg/mL streptomycin (Sigma-Aldrich) in 5% CO₂ at 37 °C for 24 hr to achieve 80-85% confluency. The cells were exposed to a series of increasingly concentrated PA-ACP@AF-eMSN (0, 10, 20, 40, 80, 160, 320, 640 μ g/mL). After 24 hr of incubation, 10 μ L of MTT solution (5 mg/mL) was added to each well and incubated for 4 hr. Dimethyl sulfoxide-NH₄OH was then added to the culture medium to dissolve the formazan produced from MTT by intracellular mitochondrial dehydrogenases. The optical density of the formazan salt was measured with a plate reader (Synergy H1, Biotek Instruments Inc., Winooski, USA) at 490 nm. Background absorbance of media was subtracted from the measured absorbance. The experiment was conducted in sextuplicate. The data was statistically analyzed using one-factor ANOVA and Holm-Sidak pairwise comparison procedures (versus control without nanoparticles) at $\alpha = 0.05$ after validation of the normality and equal variance assumptions of the data set.

2.8. Collagen reconstitution

A 2-D collagen model was used for TEM examination. Reconstituted fibrillar collagen was prepared. 2 mg/mL of collagen/acetic acid solution was prepared by dissolving bovine skin-derived type I collagen lyophilized powder in 17.5 mM acetic acid. Carbon-formvar-coated Ni TEM grids (Electron Microscopy Sciences, Hatfield, PA, USA) were placed over a droplet of the solution. Self-assembly of collagen fibrils was achieved by increasing the pH of the collagen solution with 1% ammonia vapor. The reconstituted collagen fibrils were cross-linked with 0.3 M EDC for 4 hr to increase their stability.

2.9. Collagen remineralization

Twenty mg of PA-ACP@AF-eMSN was suspended in 1 mL of HEPES. Reconstituted type I collagen-coated TEM grids were floated on top of the solution. The assembly was incubated in a 100% humid chamber at 37 °C. Grids were retrieved after 15 min, 1, 2 and 4 days of collagen mineralization for TEM examination at 110 kV. Selected area electron diffraction (SAED) was performed to identify the crystallinity of the intrafibrillar CaP minerals.

3. Results and Discussion

Successful preparation of eMSN was achieved through a pore expansion strategy that involves the use of TMB, a pore expansion agent, to enlarge the CTAB-templated mesopores [30]. The TEM images (Figs.1a and 1b) showed that eMSN was well-dispersed and was about 200 nm in diameter. An image of an ultrathin section of epoxy resin-embedded eMSN prepared with an ultramicrotome (Fig.1c) showed that most of the mesopores were arranged radially from the center to the surface of nanoparticle. Unlike non-expanded MSN that displayed short-range order of their internal pore architecture that is manifested as several definitive Bragg diffraction peaks in small-angle XRD [31], no typical diffraction peak was identified in the small-angle XRD pattern of eMSN (Fig.1d). This indicates that the original hexagonal pore order in MCM-41 type MSN was lost following TMB-directed pore expansion. The broad band identified from wide-angle XRD at $22^{\circ} 2\theta$ (Fig.1e) was indicative of non-crystalline scattering of amorphous solids [32]. Nitrogen sorption isotherms of all undoped and doped eMSN exhibited typical type IV isotherm and a hysteresis loop, attributed to the “slit” between intraparticle partitions (Fig.1f) [32]. This result correlated well with the TEM images and XRD spectra. The Brunauer-Emmett-Teller (BET) specific surface area, Barrett-Joyner-Halenda (BJH) pore volume, and pore width were $349.14 \text{ m}^2/\text{g}$, $1.18 \text{ cm}^3/\text{g}$ and 14.8 nm (Fig.1g), respectively, suggesting that eMSN has potential to be ACP carriers.

Because both PA-ACP (-33.84 mV) and eMSN (-9.52 mV) are negatively-charged (Fig. 3F), it is difficult to achieve high loading capacity only by capillary action. Electrostatic attraction between the adsorbate and adsorbent is the major driving force for loading drugs, proteins and genes in MSNs [33]. Hence, eMSN was amine-functionalized to create positively-charged surfaces for loading PA-ACP. Through a post-grafting method with APTES, primary amine groups were introduced on the surfaces of the eMSN [34]. After amine functionalization, the BET specific surface area was reduced from 394.14 to $323.80 \text{ m}^2/\text{g}$, while the pore volume decreased from 1.18 to $0.61 \text{ cm}^3/\text{g}$. Their pore size was reduced from 14.8 to 8.1 nm (Fig.1g), which is still larger than the mean pore size ($< 4 \text{ nm}$) of conventional non-amine-functionalized MCM-41 MSN with hexagonal pore architecture [31]. This renders the AF-eMSN more favorable for loading biomineralization precursors. The AF-eMSN was ultrastructurally non-distinguishable from the eMSN (not shown).

Covalent bonding of aminopropyl groups to eMSN was confirmed by FTIR, TGA, XPS and NMR. As shown in FTIR spectra (Fig.2a), absorbance peaks of eMSN were observed at 804 cm^{-1} (Si-O-Si bending vibration), 960 cm^{-1} (Si-OH bending vibration) and 1070 cm^{-1} (Si-O stretching vibration). For AF-eMSN, primary amine vibrations and NH_3^+ deformation were observed at 1570 , 1490 and 1630 cm^{-1} , suggesting that there was successful graft of

functional amine groups ($-\text{CH}_2-\text{CH}_2-\text{CH}_2\text{NH}_2$) [34,35]. Thermogravimetric analysis (Fig. 2b) and differential thermal gravimetry (DTG; Fig. 2c) were used to evaluate the thermal stability of nanoparticles and to estimate the amount of amine groups attached to the eMSN. For eMSN, a total weight loss of 7.05% was observed due to the loss of physisorbed water (69.7 °C) and chemisorbed water (117.4 °C). The AF-eMSN showed an overall weight loss of 21.75 % occurring in two stages: water loss was similar to eMSN in the first stage (7.68%), while decomposition of aminopropyl groups (349.1 °C) and dehydroxylation of silanol groups (567.5 °C) occurred in the second stage (14.07%) [36,37]. X-ray photoelectron spectroscopy of AF-eMSN showed a strong C1s peak and an intensive N1s peak in the wide-scan spectrum that were associated with the incorporation of APTES (Fig. 3a). From the C1s narrow-scan spectrum (Fig. 3b), the peaks at 284.6, 285.5 and 286.2 eV could be assigned to C-H, C-C, C-N in the $-(\text{CH}_2)_3-\text{NH}_2$ chain, while the peak at 284.3 eV was attributed to C-Si in the $\text{Si}-(\text{CH}_2)_3-\text{NH}_2$ functionality. From the N1s narrow-scan spectrum (Fig. 3c), the peaks at 399.9 eV and 399.2 eV were respectively assigned to the $-\text{NH}_2$ and C-N bonds from APTES. ^{29}Si CP-MAS NMR spectrum of AF-eMSN exhibited T2 (−58 ppm) and T3 (−67 ppm) peaks that were confirmative of covalent grafting of the aminopropyl functionality to the silica matrix via three siloxane ($\text{Si}-\text{O}-\text{Si}$) bridging bonds (Fig. 3d). The dominant Q4 peak (−112 ppm) suggested that there was further condensation of neighboring $\text{Si}-\text{OH}$ groups present on the silica walls [38,39]. For the ^{13}C NMR spectra (Fig. 3e), covalent bonding of the aminosilane to the silica surface was validated by the appearance of three peaks with chemical shifts C^1 (10.658 ppm), C^2 (26.368 ppm) and C^3 (43.867 ppm), representing the carbon atoms in $\text{Si}-\text{O}-\text{Si}-\text{C}^1-\text{C}^2-\text{C}^3-\text{NH}_2$ [40].

After covalent coupling of aminopropyl groups, the zeta potential of eMSN increased from −9.52 mV to +25.77 mV (Fig. 3F). Reversal of the zeta-potential of AF-eMSN facilitates doping of PA-ACP (−33.84 mV) via electrostatic attraction. The surface area, pore volume and pore size of PA-ACP@AF-eMSN were further reduced to 152.46 m^2/g , 0.30 cm^3/g and 3.5 nm (Fig. 1g), respectively. Because it was not possible to identify doped amorphous mineral precursors within the PA-ACP@AF-eMSN in TEM images of both unsectioned 3-D nanoparticles (Fig. 4a) and epoxy resin-embedded sections (Fig. 4b), STEM-EDX mapping was used to identify the elemental composition of PA-ACP@AF-eMSN in epoxy resin-embedded ultrathin sections (Fig. 4c). The strong carbon signal could be ascribed to the grafted amine functional group and adsorption of PA in the mesopores. Presence of Ca and P signals is indicative of adsorption of ACP precursors within the mesopores. The elemental mapping results were supported by the presence of three new peaks (1718, 1558, and 1400 cm^{-1}) in the FTIR spectrum of PA-ACP@AF-eMSN (Fig. 2a). These peaks are attributed to the $-\text{COOH}$ carbonyl, $-\text{COO}-$ asymmetric and symmetric stretches of PA, respectively [41].

The release kinetics of mineralization precursors from PA-ACP@AF-eMSN (Fig. 4d) was characterized by an initial period of rapid Ca and P release that gradually slowed down and reached a plateau after 120 hr. The Ca/P ratio of released mineral phases was almost constant at 1.5 (Fig. 4e), which is the typical Ca/P ratio of ACP at neutral pH [42]. Release of PA-ACP from the AF-eMSN is most likely attributed to the competitive displacement of physisorbed PA-ACP by the HEPES zwitterion [43,44]. Because neutral amino acids exist as zwitterions at physiologic pH, AF-eMSN represents a feasible carrier for *in vivo* delivery of the intermediate precursors of biomineralization. Polyacrylic acid was employed as a

biomimetic analog for stabilizing amorphous calcium phosphate in the present study because it possesses rich polycarboxyl groups, and has been used as a biomimetic analog to mimic the function of matrix proteins involved in biomineralization in previous studies using solution-based biomineralization systems [45]. According to the size exclusion theory of intrafibrillar collagen infiltration [46], molecules larger than 40 kDa cannot diffuse into the water compartments within collagen fibrils. Conversely, molecules small than 6 kDa can enter freely into these water compartments. The molecule weight and size of polyacid-stabilized ACP prenucleation clusters was about 2000 and 0.87 ± 0.20 nm, respectively [11]. Hence, PA-ACP was not only small enough to be adsorbed into the mesopores of eMSN by electrostatic attraction, but also satisfied the size limits of collagen biomineralization.

Relative mitochondrial dehydrogenase activities of hDPSCs exposed to different concentrations of PA-ACP@AF-eMSN (0-640 $\mu\text{g/mL}$) are shown in Fig 4f. Significant difference was identified among the various groups ($P = 0.003$). There were no significant differences in all pairwise comparisons except for the comparison between 0 $\mu\text{g/mL}$ (control) and 640 $\mu\text{g/mL}$ ($P = 0.001$). The results indicate that PA-ACP@AF-eMSN did not induce more than 20% cell death even at the highest concentration (640 $\mu\text{g/mL}$). Thus, PA-ACP@AF-eMSN is highly biocompatible and suitable for *in vivo* applications.

A 2-D fibrillar collagen model was used to examine whether ACP precursors released from PA-ACP@AF-eMSN still have the ability to mineralize collagen fibrils. After the reconstituted collagen fibrils were immersed in the mineralization assembly for 15 min, PA-ACP@AF-eMSN could be identified in the vicinity of unmineralized collagen fibrils (Fig. 5a). The TEM image of one-day specimen (Fig.5b) showed released PA-ACP precursors attached to the surface of collagen fibrils as well as their conversion into needle-shaped crystallites within the fibrils. Although collagen fibrils were not completely mineralized along their entire lengths after two days (Fig.5c), electron-dense crystallites could be clearly identified along the longitudinal axis of those regions of the fibrils that had been mineralized (Fig.5d). After four days, most of the collagen fibrils were heavily mineralized (Fig.5e), with conversion of extrafibrillar PA-ACP (see Fig.5d) into extrafibrillar needle-shaped crystallite clusters (Fig.5f). Selected area electron diffraction of the intrafibrillar minerals (inset in Fig. 5f) confirmed that they were apatite crystallites.

In conclusion, biomineralization with the use of the PA-ACP@AF-eMSN delivery system and collagen fibril as the mineralization template may be interpreted as follows: negatively-charged PA-ACP precursors are continuously released from AF-eMSN in the presence of competitive displacement by zwitterions derived from HEPES. These precursors bind to regions of net positive charges such as the a-bands of collagen fibrils [47] and infiltrate the fibrils by mechanisms which may include capillary action [48] or electrostatic interaction [47,49]. Once infiltrated into the collagen, the liquid-like amorphous mineral phase diffused through the spaces between the collagen molecules and solidified [50]. The amorphous calcium phosphate was subsequently transformed into apatite crystallites along longitudinal axis of the collagen fibril [50]. Similar to the preservation of genetic materials from external environmental degradation when those materials are stored within the mesopores of eMSN [24], storage of highly hydrated PA-ACP within those mesopores may at least be partially accountable for retention of these biomineralization precursors in their fluidic state when the

doped AF-eMSN is stored under hydrated conditions. This probably prevents solidification of the biomineralization precursors within the mesopores.

The PA-ACP@AF-eMSN employed in the present study have several distinct advantages over the non-expanded MSN carrier that was presented in the authors' previous work [31]. Firstly, large differences in surface area, pore volume and pore size were observed among the eMSN, AF-eMSN, and PA-ACP@AF-eMSN. These differences suggest that expanded-pore MSN with irregular pore structure (14.8 nm) are more favorable to be amine-functionalized and loaded with intermediate precursors of calcium phosphate biomineralization than conventional small-pore MCM-41 type MSN with hexagonal pore architecture (2-4 nm). In the latter, only minor differences in surface area, pore volume and pore size were observed among the MSN, AF-MSN and PA-ACP@AF-MSN. Secondly, the amount of Ca and P released from PA-ACP@AF-eMSN in the present study was almost twenty times higher than that of previous work. The data infers that the present delivery system functions better as a carrier for biomineralization precursors. Thirdly, the more uniform size, monodispersity and biocompatibility of PA-ACP@AF-eMSN greatly expands the application profile of the previous delivery system.

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 (Fig.6). The present translational strategy represents an important step in the application of contemporary biomineralization concepts, especially for *in-situ* remineralization of bone and teeth. For example, the translational strategy of biomineralization presented here has potential clinical applications in the remineralization of localized osteoporotic bone defects [51]. Another perceived *in vivo* application of the delivery strategy is the incorporation of PA-ACP@AF-MSN paste as nanofillers in hydrophilic dental adhesive resins that are used to bond tooth-colored fillings to decayed teeth. Lowering of the environmental pH by acidogenic bacterial biofilms causes dissolution of apatite from dentin, while host-derived matrix metalloproteinases and cysteine cathepsins present in dentin degrade the dentin collagen matrix [52]. Release of biomineralization precursors from PA-ACP@AF-eMSN incorporated in adhesives employed for bonding tooth fillings to dentin may arrest dentinal caries by backfilling the acid-demineralized collagen matrix with apatite crystallites and preventing collagenolytic enzymes from irreversibly denaturing the collagen fibrils [53]. Related studies are in order.

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Figure 1.

(a, b) Low and high magnification unsectioned TEM images of template-free eMSN examined by direct collection on TEM grids. Bars = 100 nm in (a) and 50 nm in (b); (c) TEM image of a sectioned, epoxy resin-embedded eMSN (bar = 50 nm); (d) Small-angle and (e) wide-angle XRD profiles of eMSN, the broad band at 22° 2θ is indicative of non-crystalline scattering; (f) N_2 adsorption-desorption isotherms and (g) mesopore pore size distribution in eMSN, AF-eMSN and PA-ACP@AF-eMSN.

Figure 2.

(a) FTIR spectra of eMSN, AF-eMSN and PA-ACP@AF-eMSN. (b) TGA and (c) DTG of eMSN and AF-eMSN.

Figure 3.

(a) Wide-scan XPS spectrum of AF-eMSN showing the elemental composition and chemical bonding states. (b) C1s narrow scan XPS spectrum of AF-eMSN. (c) N1s narrow scan XPS spectrum of AF-eMSN. (d) ^{29}Si CP-MAS NMR spectrum of AF-eMSN. The peaks at -58 and -67 ppm are separately assigned to T2 (Si-O-Si symmetric stretching) and T3 (Si-O-Si anti-symmetric stretching). The deconvoluted peaks at -103 and -112 ppm are assigned respectively to Q3 (single silanols) and Q4 (germinal silanols). (e) ^{13}C NMR spectrum of AF-eMSN. (f) Zeta potential of eMSN, AF-eMSN and PA-ACP@AF-eMSN.

Figure 4.

(a) Unsectioned TEM image of PA-ACP@AF-eMSN examined by direct collection on TEM grids (bar = 50 nm). (b) TEM image of sectioned, epoxy resin-embedded PA-ACP@AF-eMSN (bar = 50 nm). (c) STEM-EDX mapping of sectioned PA-ACP@AF-eMSN showing distribution of Si, O, Ca, P, N and C within the nanoparticles (bar = 50 nm). (d) Cumulative Ca and P release profiles of PA-ACP@AF-eMSN in HEPES buffer solution at pH 7.4 for 120 hr. (e) Ca/P ratio of released mineral phase derived from PA-ACP@AF-eMSN. (f) Relative mitochondrial dehydrogenase activities of human dental pulp stem cells exposed to different concentrations (0-640 $\mu\text{g/mL}$) of PA-ACP@AF-eMSN. Data represent means and standard deviations. Columns with the same color are not significantly different ($p > 0.05$).

Figure 5.

TEM of collagen mineralization using the carrier-based delivery system (non-osmicated, unstained, unsectioned specimens) after mineralization was performed for (a) 15 min, (b) 1 day, (c, d) 2 days, and (e, f) 4 days. (a) At 15 min, PA-ACP@AF-eMSN were identified around unmineralized collagen fibrils (bar = 200 nm). (b) At 1 day, released PA-ACP precursors (open arrow heads) attached to the surface of collagen fibrils. Some of these precursors had been converted into needle-shaped crystallites (arrow) within the fibril (bar = 50 nm). (c) At 2 days, partial mineralization occurred, some collagen fibrils were not completely mineralized along their entire lengths (open arrow). Bar = 500 nm. (d) High magnification of (c); electron-dense crystallites (arrow) were identified along the longitudinal axis of those regions of the fibrils that had been mineralized. Released PA-ACP precursors (asterisk) were observed around the collagen (bar = 100 nm). (e) At 4 days, most of the collagen fibrils were heavily mineralized (bar = 200 nm). (f) High magnification of (e) showing dense intrafibrillar apatite crystallites within a collagen fibril (arrow). Extrafibrillar crystallite clusters (pointer) was observed (bar = 100 nm). Inset: selected area electron diffraction of the intrafibrillar minerals taken from the location indicated by the asterisk.

Figure 6.

Schematic illustrating the synthesis of PA-ACP@AF-eMSN and the subsequent intrafibrillar mineralization process. TEOS: tetraethyl orthosilicate; CTAB: cetyltrimethylammonium bromide; TMB: 1,3,5-trimethylbenzene; APTES: (3-aminopropyl) triethoxysilane; PA-ACP: polyacrylic acid-stabilized amorphous calcium phosphate; eMSN: expanded-pore mesoporous silica; AF-eMSN: amine-functionalized eMSN; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid.