Epigallocatechin-3-gallate/mineralization precursors co-delivery hollow mesoporous nanosystem for synergistic manipulation of dentin exposure

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ABSTRACT

As a global public health focus, oral health plays a vital role in facilitating overall health. Defected teeth characterized by exposure of dentin generally increase the risk of aggravating oral diseases. The exposed dental tubules provide channels for irritants and bacterial invasion, leading to dentin hypersensitivity and even pulp inflammation. Cariogenic bacterial adhesion and biofilm formation on dentin are responsible for tooth demineralization and caries. It remains a clinical challenge to achieve the integration of tubule occlusion, collagen mineralization, and antibiofilm functions for managing exposed dentin. To address this issue, an epigallocatechin-3-gallate (EGCG) and poly(allylamine)-stabilized amorphous calcium phosphate (PAH-ACP) co-delivery hollow mesoporous silica (HMS) nanosystem (E/PA@HMS) was herein developed. The application of E/PA@HMS effectively occluded the dentinal tubules with acid- and abrasion-resistant stability and inhibited the biofilm formation of Streptococcus mutans. Intrafibrillar mineralization of collagen fibrils and remineralization of demineralized dentin were induced by E/PA@HMS. The odontogenic differentiation and mineralization of dental pulp cells with high biocompatibility were also promoted. Animal experiments showed that E/PA@HMS durably sealed the tubules and inhibited biofilm growth up to 14 days. Thus, the development of the E/PA@HMS nanosystem provides promising benefits for protecting exposed dentin through the coordinated manipulation of dentin caries and hypersensitivity.

1. Introduction

In terms of physical, psychological, and biological points of view, oral health in nature can be considered indispensable to overall health throughout the life course [1]. The presence of natural teeth lays a foundation for oral health and contributes to their function. As the main component of teeth, dentin (surrounded by outside enamel) plays an essential role in protecting inside pulp tissue and has a complex tubular structure [2]. Clinically, the exposure of dentin caused by wedge-shaped defects, sleep bruxism, caries (or acid erosion), and trauma generally leads to recurrent discomfort or pain and has become one of the most common dental complaints expressed by patients [3]. This is mainly related to the fact that the opened tubules provide available pathways for irritants (from mechanical, chemical, cold, and thermal aspects) or bacterial invasion and are likely to induce hypersensitivity and pulp infection [4]. Therefore, the appropriate management and protection of the exposed dentin remain an arduous challenge for clinicians.

Based on the hydrodynamic hypothesis, the conventional strategy for alleviating relevant clinical symptoms depends on the occlusion of dentinal tubules to decrease fluid movement and impulse transduction [5]. For this reason, various approaches, for instance, treatment by oxalates, adhesives, bioglass, and lasers, have been introduced over the past decades [6,7]. However, most of the previous studies concentrate on the effects of tubule occlusion, and the accompanying potential
threats involved in exposed dentin have not been adequately addressed. First, the instability of tubule occlusion limits its long-lasting application, and the physicochemical property of dentin can be impacted by the continuous demineralization of collagen fibrils [8]. Second, compared to enamel, dentin has a lower mineralization degree, which is more susceptible to cariogenic bacterial attack [9]. The acid production with a decreased pH microenvironment by bacteria metabolism inside the biofilm often brings about dental caries [10]. Besides, the penetration of irritants or bacteria along the tubules also increases the risk of pulp inflammation [11]. Thus, desirable strategies to manage and prevent diseases after dentin exposure demand a considerable emphasis on the achievement of tubule occlusion, collagen mineralization, and biofilm control.

According to the biomimetic concept, applying mineral compounds that approximate natural dentin in main composition is rational for tubule occlusion [12]. Previous research has confirmed that amorphous calcium phosphate (ACP) is capable of obstructing the tubules [13,14] and is involved in the biomineralization of teeth and bone as precursors [15]. ACP can be stabilized in a moldable, nanoscale state based on the polymer-induced liquid precursor phase (PILP) with the participation of matrix protein polyanionic analogs [16]. Inspired by the function of long-chain polyamines on stabilization of orthosilicic acid in the course of silica-contained organisms biosilicification, a poly(allylamine) hydrochloride-stabilized ACP (PAH-ACP) precursor developed by Niu et al. has been proven to induce intrafibrillar mineralization of collagen fibrils [17]. This finding implies that PAH-ACP may be appropriate for tubule occlusion and remineralization of dentin matrix as a promising candidate. However, it is difficult to maintain sufficient content of polymer-stabilized ACP phases on dentin while applying a dispersion of these nanoparticles that are yielded by liquid–liquid phase separation from supersaturated calcium and phosphate-contained solutions. Accordingly, the development of an efficient delivery system both for storage and release of ACP precursors and durable tubule occlusion is highly needed [18].

Since cariogenic bacterial adherence and biofilm formation on dentin considerably contribute to the sustained dissolution and loss of minerals [19], the antibiofilm capability to combat caries is also urgently anticipated. Plenty of measures have been employed in combating the formation of bacterial biofilms in recent years, such as chlorhexidine, silver diamine fluoride, triclosan, and quaternary ammonium silane [20–22]. Compared to these functional agents, epigallocatechin-3-gallate (EGCG), a natural active component of green tea, exhibits safer biocompatibility with high biological activity in anti-inflammatory, antioxidant, and antimicrobial applications. The adhesion and biofilm growth of Streptococcus mutants (S. mutans) on tooth surfaces can be suppressed by EGCG via inhibition of specific virulence factors and glucosyltransferase–related gene expression [23,24]. EGCG also causes cell membrane damage in bacteria and inhibits the enzymes involved in fatty acid biosynthesis, limiting the production of toxic bacterial metabolites [25]. Previous literature has demonstrated that EGCG can promote the osteogenic differentiation of bone mesenchymal stem cells and inhibit the inflammatory response of dental pulp cells [26,27]. These favorable characteristics endow EGCG with promising benefits for inhibiting biofilm formation and protecting dental pulp as a secure antimicrobial. However, retaining EGCG at a high concentration appears to be difficult to achieve in the complex oral environment.

To maximize the advantages of PAH-ACP and EGCG in managing exposed dentin, co-delivery of these two active components is necessary. Over the past decades, mesoporous silica nanoparticles featured by large surface area and pore volume, ordered framework, and chemical/thermal stability have been extensively used as enzyme, protein, gene, and drug delivery nanocarriers [28,29]. Due to its favorable mechanical and acid-resistant performance, mesoporous silica has been shown to be effective in tubule sealing by our previous studies [30,31]. Hollow mesoporous silica (HMS) attracts intense attention on account of its effective in tubule occlusion and remineralization effects, as well as inhibit the biofilm formation of cariogenic bacteria on dentin. E/PA@HMS also has the ability to sustainably release calcium, phosphorus, and EGCG. As such, E/PA@HMS acts as a biomimetic functional barrier to protect exposed dentin and pulp tissue and is promising in collaborative manipulation of dentin caries and hypersensitivity.

Scheme 1. Schematic illustration of EGCG and PAH-ACP co-delivery hollow mesoporous silica (E/PA@HMS) nanosystem for synergistic management of exposed dentin. (a) Diagram of the synthetic process of E/PA@HMS. (b) The exposure of dentinal tubules is susceptible to external stimuli and cariogenic bacterial attack, thereby leading to dentin hypersensitivity, caries, and pulp inflammation. (c) The application of E/PA@HMS can durably occlude the dentinal tubules with acid-/abrasion-resistant stability and remineralization effects, as well as inhibit the biofilm formation of cariogenic bacteria on dentin. E/PA@HMS also acts as a biomimetic functional barrier to protect exposed dentin and pulp tissue and is promising in collaborative manipulation of dentin caries and hypersensitivity.
2. Materials and methods

2.1. Chemicals

All materials were used as received. Sodium hydroxide (NaOH), ammonia solution (28%), sodium carbonate (Na₂CO₃), calcium chloride dihydrate (CaCl₂·2H₂O), disodium hydrogen phosphate (Na₂HPO₄), citric acid, acetic acid, ethylene diamine tetraacetic acid (EDTA), absolute ethanol, glutaraldehyde, crystal violet, Coomassie blue, and phosphate buffer saline (PBS) were purchased from Sinopharm Chemical Reagent (China). Poly(allylamine) hydrochloride (PAH; Mw: 17,500), tetraethaethylorthosilicate (TEOS), cetyltrimethylammonium bromide (CTAB; Mw: 364.45), dimethylsulfoxide (DMSO), Tris-buffered saline (TBS), epigallocatechin-3-gallate (EGCC), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), cetylpyridinium chloride, agar, sucrose, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), hexamethyldisilazane (HMDS), paraformaldehyde, dexamethasone, β-phosphoglycerol, Vitamin-C, polyformaldehyde, penicillin/streptomycin, and Triton X-100 were obtained from MilliporeSigma (USA). Fetal bovine serum (FBS) and α-modified essential medium (α-MEM) were supplied by Gibco (Australia) and HyClone (USA), respectively.

2.2. Synthesis of Hollow Mesoporous Silica (HMS) Nanoparticles

The synthesis of HMS was implemented based on a previously reported approach under minor modification [34]. Briefly, 2.8 mL of 0.5 M NaOH, 0.23 g of CTAB, and 100 mL of deionized water were mixed and stored at 80 °C for 15 min. TEOS (0.75 mL) was dissolved into 5 mL of ethanol and then added dropwise to the mixture under stirring for 2 h. The mixture was centrifuged and triple-washed with deionized water and ethanol. After oven-drying overnight at 60 °C, a white powder (SiO₂ nanospheres) was obtained. A self-templating method was used to transform SiO₂ nanospheres into HMS [35,36]. Fifty mg of the white powder was dispersed into 25 mL of deionized water containing 15 mL of ethanol, 75 mg of CTAB, and 0.275 mL of ammonia. This solution was stirred at room temperature for 30 min. TEOS (0.6 mL) was then added to the solution under constant stirring for 6 h. After centrifugation, the coating of SiO₂ nanospheres was accomplished, and a white precipitate was achieved. The precipitate was then dispersed in 20 mL of deionized water, followed by adding 210 mg of Na₂CO₃ and stirring at 60 °C for 22 h for etching to produce a hollow cavity. After centrifugation and triple-washing with deionized water and ethanol, the precipitate was oven-dried at 60 °C overnight and calcined at 550 °C for 6 h to generate the powdery product (HMS).

2.3. Fabrication of EGCC/PAH-ACP-Loaded HMS (E/PA@HMS)

Firstly, 50 mL of CaCl₂·2H₂O (9 mM) solution in TBS (pH 7.4) was prepared, and 50 mg of PAH powder was added to the CaCl₂ solution under stirring for 15 min. After that, 50 mL of Na₂HPO₄·12H₂O (4.2 mM) solution prepared in TBS was mixed with the above mixture to prepare the PAH-ACP mineralization precursors, which had a concentration of 500 μg/mL for PAH. Subsequently, HMS (100 mg) was dispersed in 25 mL of PAH-ACP-containing solution (pH 7.2) under stirring for 24 h at room temperature and shaken for another 24 h to facilitate the infiltration of PAH-ACP into the interior of HMS. The precipitate collected by centrifugation was washed with deionized water and stored at −20 °C until use. The precipitate was centrifuged, washed, filtered, and stored at −20 °C until use.

2.4. Characterization

Transmission electron microscopy (TEM; Talos F200S, Thermo Scientific, USA) was employed to determine the morphology and ultrastructure of HMS, PA@HMS, and E/PA@HMS. Scanning TEM equipped with energy dispersive spectroscopy (EDS) was used to examine the mapping patterns of elements. The crystallinity was identified by selected area electron diffraction (SAED). X-ray diffraction (XRD) analysis with small-angle and wide-angle patterns was performed using a PANalytical Empyrean diffractometer (the Netherlands). A Nicoleti7000 spectrophotometer (Thermo Scientific, USA) with Fourier transform-infrared spectroscopy (FTIR) was conducted to detect typical functional groups. The pore size distribution and surface area calculated by the Brunauer–Emmett–Teller and Barrett–Joyner–Halenda (BET/BJH) methods were measured using an ASAP2020 analyzer (Micro meritics Corp., USA) with nitrogen adsorption-desorption. A Zetasizer Nano ZSP system (Malvern Instruments, UK) was used to monitor the size distribution and zeta potential. The loading efficiency was inspected using a STA449F3 thermogravimetric analyzer (NETZSC, Germany) under an N₂ environment at a heating rate of 10 °C/min by thermogravimetric analysis (TGA) and derivative thermogravimetry (DTG).

2.5. Release measurement of Ca, P, and EGCG

The TBS was used to measure the in vitro release of Ca, P, and EGCG from E/PA@HMS to eliminate the interference from Ca and P existing in artificial saliva, simulated body fluid, or PBS. Specifically, E/PA@HMS (100 mg) was immersed in 10 mL of TBS at pH 7.4. The amounts of Ca and P released were detected at 0.5, 1, 3, 5, 7, 14, 21, and 28 days, respectively. This mixture at each time interval was centrifuged at 4000 rpm for 5 min. One mL aliquot of supernatant was immediately collected and supplemented with fresh TBS at an equal volume [37]. These collected aliquots were analyzed by a Spectord200 UV–vis spectrophotometer (Analytik Jena, Germany) at a 325 nm wavelength to determine the content of released EGCC. Meanwhile, the aliquots were analyzed by Intrepid II XSP inductively coupled plasma-atomic emission spectrometry (ICP-AES, Thermo Fisher, USA) to measure the contents of released Ca and P.

2.6. Intrafibrillar Mineralization of Reconstituted Collagen

The effects of PAH-ACP release from the E/PA@HMS delivery nanosystem on intrafibrillar mineralization were inspected using a single-layer reconstituted collagen model [18]. Briefly, bovine skin-derived type I collagen lyophilized powder (MilliporeSigma, USA) was dissolved in acetic acid to prepare a collagen/acetic acid solution at a concentration of 2 mg/mL, followed by immersing 200-mesh Ni TEM grids in the solution. The pH was adjusted to 8.0 with 1% ammonia vapor to achieve the self-assembly of collagen fibrils. After EDC (0.3 M) cross-linking for 4 h, rinsing with deionized water, and air-drying for 30 min, the reconstituted collagen fibrils were deposited on Ni TEM grids.

E/PA@HMS (50 mg) was suspended in 1.5 mL of TBS at pH 7.4. The reconstituted collagen fibrils-covered TEM grids were floated on top of the suspension. The assembly was cultivated at 37 °C inside a 100% humidity control chamber. TBS without E/PA@HMS was set as control. The grids were retrieved after 1, 3, and 5 days for TEM (HT7700, Hitachi, Japan) observation of collagen mineralization. The crystallinity of intrabifibrillar calcium/phosphorus minerals was identified by SAED.

2.7. Preparation of Dentin Specimens

After the achievement of informed consent from donors, non-carious, intact human third molars were gathered in accordance with a protocol approved by the Ethics Committee of Hospital of Stomatolgy, Wuhan University (certificate 2019A11). All molars were thoroughly cleaned, rinsed, and immersed in a 0.5% thymol solution at 4 °C prior to use. A
water-cooled, low-speed Isomet diamond saw (Buehler, USA) was used to segment the molars perpendicular to the long axis beneath the enamel–dentine junction to produce dentin disk specimens, and 600-, 800-, 1200-, 2000-, and 3000-grit silicon carbide sandpapers were employed to sequentially wet-polish the specimens to 1 mm in thickness. Sterile deionized water was used to thoroughly rinse the surface of each specimen.

2.8. Remineralization of demineralized dentin

Fifty mg of E/PA@HMS was dispersed into 10 mL of TBS (pH 7.4) and shaken for 30 min for ACP release to generate the remineralization solution. For determining the remineralization effects, the dentin specimens were etched with 37% phosphoric acid gel for 15 s to demineralize the dentin matrix and thoroughly rinsed with deionized water, followed by immersing in the remineralization solution at 37 °C. After incubation for 1, 7, and 14 days, these dentin specimens were longitudinally sectioned to yield two segments and fixed with glutaraldehyde solution (2.5%) for 24 h. After rinsing with deionized water, gradient dehydration using ethanol solutions was applied. All specimens were subjected to HMDS immersion for 30 min, vacuum-drying for 1 h, g for 30 min, critical-point sputter-coating, and then examined by field-emission scanning electron microscopy (FESEM, JSM-IT800, JEOL, Japan). The EDS-mapping analysis of element distribution and contents were examined by carbon sputter-coating, and then examined by field-emission scanning electron microscopy (FESEM, JSM-IT800, JEOL, Japan). XRD analysis was conducted to investigate the newly formed layer in mineral phase and crystal orientation.

2.9. Evaluation of dentinal tubule occlusion

For evaluation of tubule-sealing effects, the prepared dentin specimens were etched by 0.5 M EDTA solution at pH 7.4 for 4 min to yield an exposed dentin model [38,39]. After rinsing with deionized water, the etched disks were randomly distributed into two groups based on different surface treatment protocols: Group 1 (control), applying deionized water two times using prophy cups for 30 s; Group 2 (E/PA@HMS), applying E/PA@HMS (prepared in Section 2.3) paste (5 mg) two times using prophy cups for 30 s. Subsequently, the specimens were immersed in artificial saliva (prepared by 130 mM KCl, 0.9 mM KH2PO4, 1 mM Na2SO4, 1.5 mM CaCl2, and 20 mM HEPES buffer, pH 7.02) at 37 °C and preserved for 1 or 28 days while replacing the solution every day. After cultivation, the specimens from each group were randomly distributed into two subgroups. One subgroup was challenged by a 6% citric acid solution (pH 1.5) soaking for 60 s to determine acid-resistant stability [40]. Another subgroup was challenged by mechanical brushing with a soft-bristle toothbrush for 3 min to determine abrasion-resistant stability [41].

FESEM was utilized to examine the tubule-occluding capability and acid- and abrasion-resistant stability after the application of E/PA@HMS. Two equal segments were prepared by longitudinally sectioning each disk from the untreated surface to create a groove and then splitting the disk into two halves by exerting pressure on the medial and distal locations. The splitting operation should be carried out such that the treated surface is not influenced. The two segments were observed from the surface and cross-sectional views, respectively. All segments were subjected to desiccation and FESEM examination after gold sputter-coating (JFC-1600, JEOL, Japan).

2.10. Determination of dentin permeability

Dentin permeability was assessed via a modified fluid penetration apparatus as previously reported [38,39]. A water reservoir working under a 20 cm height of water simulating pulpal pressure was linked to a split-chamber unit. Two plexiglass blocks attached with two “O”-type rubber rings were used to tightly compress each specimen. The available area for fluid penetration across the dentin surface was provided at 0.38 cm². The flow rate of fluid was evaluated according to the displacement of a trapped air bubble within a glass tube of 25 μL volume. Values of hydraulic conductance (Lp) were computed by dividing fluid flow rate (μL/min) by water pressure (20 cm H2O) and provided surface area (cm²). Lp data of the specimens monitored at each time point (n = 8 per group), including after etching, treatment, and challenge by acid or brushing, were documented, respectively. The 100% dentin permeability was identified from the Lp value of the specimen after etching. For specimens measured after treatment or challenge by acid or brushing, a percentage (Lp%) of the Lp value of 100% permeability was used to represent the dentin permeability.

2.11. Assessment of dentin microhardness

To assess the microhardness of dentin surfaces after different treatments, three specimens from each group were serially polished by sandpaper under water cooling. After ultrasonic cleaning with deionized water, the Vickers hardness number was determined through a HXD-1000TMC/LCD microhardness testing system (Taiming Inc., China). Five indentations were carried out for each specimen. The load force was applied at 50 g with a dwell time of 10 s.

2.12. Antibiofilm analyses

2.12.1. Preparation of cariogenic bacterial biofilm

*S. mutans* (UA159) was employed for bacterial cultivation and biofilm preparation. The bacterial strain was acquired from Hospital of Stomatology, Wuhan University, and anaerobically incubated in brain heart infusion (BHI) broth (Difco, BD, USA) at 37 °C. The suspension of *S. mutans* (10⁷ CFU/mL) was obtained and supplemented with 1% sucrose to prepare the inoculation medium. As described in Section 2.9, dentin disk specimens were achieved in the same method and treated with deionized water (control group) or E/PA@HMS and stored in artificial saliva at 37 °C for 1 or 28 days. After that, these disks were placed in 24-well plates containing inoculation medium (1 mL for each well) for 24 h of anaerobic cultivation at 37 °C to form biofilm. Subsequently, each biofilm-covered disk was transferred into one well of new 24-well plates after triple-washing with PBS.

2.12.2. Live/dead bacterial staining

The BacLight LIVE/DEAD Bacterial Viability Kits (L13152, Molecular Probes, USA) were used to stain the surface of biofilm-covered disks for 15 min in darkness on the basis of the manufacturer’s instructions. Green and red fluorescence are suggestive of live and dead bacterial cells stained by SYTO-9 and propidium iodide, respectively [42]. Confocal laser scanning microscopy (CLSM; FV1200, Olympus, Japan) was used to observe the fluorescent images of biofilm, and the excitation/emission wavelengths were set at 485/500 nm for SYTO-9 and 594/635 nm for propidium iodide, respectively. By scanning the dentin surfaces from bottom to top at z-stack, two randomly captured areas were recorded with a 2 μm Z-step for each specimen in each group (n = 3). All obtained fluorescent images were analyzed and reconstructed as 3D overlay projections using Imaris 7.4.2 (Bitplane, Switzerland). The biomass distribution of live/dead bacteria in 20 μm of z-stack thickness (10 layers) was calculated. The ratio (%) of biofilm production was expressed as the relative total bacterial biomass of the control group at 1 day.

2.12.3. FESEM morphology of bacterial adhesion

FESEM was employed to observe the morphology of *S. mutans* adhesion on dentin disks. A glutaraldehyde solution (2.5%) was used to fix the specimens at 4 °C. After rinsing with deionized water, gradient dehydration using ethanol solutions (50%, 60%, 70%, 80%, 90%, and 100%) was applied for 15 min each. All specimens were subjected to desiccation and gold sputter-coating, followed by capturing random biofilm images at designated magnifications.
2.12.4. Bacterial metabolic activity

The MTT assay was used to monitor the activities of bacterial biofilm metabolism. One mL of MTT solution (0.5 mg/mL) was added to each well of 24-well plates to immerse the specimens. The plates were anaerobically cultured at 37 °C for 3 h. Then, DMSO (1 mL) was injected after pipetting out MTT solutions from each well. The plates were shaken at room temperature for 15 min protected from light. The specimens (n = 3 per group) were placed into an Elx808 microplate reader (BioTek, USA), and the absorbance at 570 nm was recorded. Triplicate tests were performed for the MTT assay.

2.12.5. CFU counting

CFU counting was used to evaluate the reproduction of bacterial colonies in biofilm after treatments. Three mL of PBS were added to one microtube containing one specimen. The microtubes were vortex-mixed to detach the biofilm coated on the disks for 5 min [43]. After that, serial tenfold dilutions were performed. The BHI agar dishes overspread with each dilution (40 μL) were cultured at 37 °C under anaerobic conditions. The numbers of bacterial colonies from the specimens (n = 3 per group) were counted and calculated after 48 h. Triplicate tests were performed for CFU counting.

2.12.6. Production of lactic acid

Lactic acid production was detected from S. mutans biofilm formed on the dentin disks. Each well containing a respective specimen (n = 3 per group) was added with buffered peptone water (1.5 mL) with 0.2% sucrose. The buffered peptone solution was collected for lactate analysis after 3 h of incubation at 37 °C in 5% CO2 for acid production by biofilms. The enzymatic method was used to determine the concentrations of lactate [44]. The absorbance at 340 nm was recorded. Triplicate tests were performed for each experiment.

2.12.7. pH of biofilm culture medium

The pH variation in the biofilm culture medium of S. mutans was also monitored. Fresh inoculum medium (1 mL) was injected into one well of the new 24-well plates containing biofilm-coated specimens (n = 3 per group). After another 24 h of anaerobic cultivation at 37 °C, an FE20 pH meter (Mettler-Toledo, Switzerland) was used to measure the pH of the culture medium. Triplicate tests were performed for each experiment.

2.13. Biocompatibility evaluation

2.13.1. Cytotoxicity and cell staining

For evaluation of cell proliferation and cytotoxicity, human dental pulp stem cells (HDPSCs) from third molars of healthy adults were extracted after informed consent had been obtained. The α-MEM supplemented with 10% FBS and 1% penicillin/streptomycin was used to culture HDPSCs at 37 °C in a humidified atmosphere of 5% CO2. HDPSCs from the third passage were seeded in 96-well plates (5 × 103 cells/well) for 24 h of cultivation. Subsequently, the cells were cultured with the extract solutions of E/PA@HMS at incremental concentrations of 0, 250, 500, and 1000 μg/mL, followed by 1, 3, 5, and 7 days of incubation. After that, Cell Counting Kit-8 (CCK-8; Dojindo, Japan) solution (10 μL) was added to each well and cultured for 2 h to observe cell proliferation. The absorbance at 450 nm was then recorded. Triplicate tests were performed for each experiment. Relative cell viability (%) of the group only containing culture medium was also used to express the results of each concentration. In addition, crystal violet was used to stain the cells after incubation. The cells were fixed with 4% paraformaldehyde, triple-washed with PBS, and stained with 0.1% crystal violet solution. For Coomassie blue staining, the cells were permeabilized with Triton X-100 (1%), fixed with 4% paraformaldehyde, triple-washed with PBS, and stained with 0.01% Coomassie blue solution. Images of cell morphology and proliferation were recorded using a BX51 inverted phase contrast microscope (Olympus, Japan).

2.13.2. Hemolysis test

Fresh rat blood samples were collected from Sprague-Dawley (SD) rats (8 weeks old) according to the protocol approved by the Ethics Committee of Hospital of Stomatology, Wuhan University. The blood (4 mL) was diluted with 5 mL of sterile PBS and centrifuged to obtain the red blood cells. The cells were washed with PBS until the supernatant was clear and then diluted with PBS. The suspension of E/PA@HMS (1 mL) prepared using sterile PBS was mixed with diluted blood cell suspension (0.2 mL) to achieve the final nanoparticle concentrations of 0.1, 0.25, 0.5, 0.75, 1, 2, 3, 4, and 5 μg/mL. Sterile deionized water and PBS with the same volume were served as positive (+) and negative (−) controls, respectively. After 4 h of incubation at 37 °C, all samples were centrifuged at 1690 g for 5 min. The absorbance of the supernatants at 545 nm was recorded. Triplicate tests were performed for each experiment. The formula for calculating the percentage of hemolysis is listed below: Hemolysis rate (%) = (ODnegative – ODpositive)/(ODpositive – ODnegative) × 100%. A hemolysis rate of >5% suggests the rupture of red blood cells while ≤5% suggests the achievement of safety requirements for clinical use [45].

2.14. In vitro odontogenic differentiation

2.14.1. Alkaline phosphatase (ALP) activity test

The odontogenic induction (OI) medium was prepared by the addition of 100 nM demecosamethylene, 10 mM β-phosphoglycerol, and 50 mg/mL Vitamin-C in α-MEM supplemented with 10% FBS. The odontogenic differentiation of HDPSCs (1 × 105 cells/well in 12-well plate) cultured with the extract solution of E/PA@HMS (1.0 mg/mL) in OI medium was examined after incubation of 7 and 14 days at 37 °C. OI medium without the test material was set as control. For ALP staining, cells were washed with PBS, fixed in 4% glutaraldehyde solution, and stained with a BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, China) after removal of the medium. The inverted microscope was used to record the morphology of the stained cells. For the ALP activity assay, cells were lysed in Triton X-100 (1%) at 4 °C. The ALP activities were determined by an ALP Assay Kit (Beyotime, China). The concentration of total proteins in each sample was measured using a BCA Protein Assay Kit (Biosharp, China). The ALP activity was ultimately normalized to total protein content correspondingly. The absorbance of the mixture at 405 nm was monitored.

2.14.2. Alizarin red staining

Alizarin red staining was performed to determine mineral deposition and calcium nodule formation towards HDPSCs. The cells (1 × 105 cells/well in 12-well plates) were cultivated with the extract solution of E/PA@HMS (1.0 mg/mL) in OI medium at 37 °C. OI medium without the test material was set as control. On the 14th and 21st days, the cells were washed with PBS, fixed in 4% polyformaldehyde solution, and stained with 1% Alizarin red S (Servicebio, China) after removal of the medium. The inverted microscope was used to record the morphology of the stained cells. Ten percent cetylpyridinium chloride (in 10 mM sodium phosphate) was added to destain the samples for analysis of alizarin red staining. The absorbance was detected at 620 nm.

2.14.3. Odontogenic-related genes expression

The expression levels of odontogenic-related genes, including ALP, Runx-related transcription factor 2 (Runx2), osterix (OSX), osteocalcin (OCN), dentin matrix protein-1 (DMP-1), and dentin sialophosphoprotein (DSPP), were evaluated by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). HDPSCs (1 × 105 cells/well in 6-well plates) were seeded in OI medium containing 1.0 mg/mL of the extract solution of E/PA@HMS and cultured for 7 and 14 days at 37 °C. OI medium without the test material was set as control. The total RNA from HDPSCs was isolated using TRIzol reagent (Invitrogen, USA). The concentrations of isolated RNA were determined via a Nanodrop2000 spectrophotometer (ThermoFisher Scientific, USA). The complementary
DNA (cDNA) was synthesized using a PrimeScript RT Reagent Kit (Takara, Japan). qRT-PCR was performed using TB Green Premix Ex Taq II (TaKara, Japan) in a QuantStudio 6 Real-Time PCR System (ThermoFisher Scientific, USA). The relative mRNA expression level of each gene was normalized to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and determined using cycle threshold (Ct) values, followed by analysis with the $2^{-\Delta\Delta Ct}$ method. The forward and reverse primers of the tested genes are summarized in Table S1. Data were expressed as fold regulation of mRNA expression levels, given by $2^{-\Delta\Delta Ct}$.

2.15. Evaluation of in vivo efficacy

In vivo animal studies were conducted under the approval of the Ethics Committee of Hospital of Stomatology, Wuhan University. Male Sprague–Dawley rats (8 weeks old, 220–300 g, 5 rats in each group) were utilized and anesthetized using 1% pelltobarbitalum (0.4 mL/100 g) by intraperitoneal injection. Human dentin specimens with 1 mm thickness were prepared, followed by symmetrically perforating two holes on each specimen to allow the oral fixation in the oral cavity of rats. As the same approach described in Section 2.9, all specimens were wet-polished, etched, and assigned to two treatment groups (i.e. control and E/PA@HMS groups) after rinsing with deionized water. The treated specimens were fixed on the buccal mucosa of the oral cavity of rats using sutures across the two perforated holes. To allow the process of tubule occlusion analogous to that in human mouths, the surface of each specimen was absolutely exposed to native rat’s saliva (which involved varied enzymes and ions). The rats were fed with soft food to avoid possible mechanical damage to the specimens. For in vivo antibiofilm evaluation, the treated specimens were anaerobically cultivated with S. mutans ($10^7$ CFU/mL) for 3 days in vitro to form biofilm in advance and then fixed in rat mouths as the same approach described above. These rats were fed with a cariogenic Keyes 2000 diet and 5% sucrose-contained water to establish a cariogenic environment during incubation. For in vivo efficacy evaluation of E/PA@HMS, all specimens were taken out after 14 days of incubation, rinsed with deionized water, and examined by FESEM.

2.16. Statistical analysis

All data were expressed as means ± standard deviations. One-factor analysis of variance (ANOVA) was performed to evaluate the statistical
significance of variables after equal variances and normality of data were confirmed. Tukey’s test was conducted for post-hoc pair-wise comparisons. If violations appeared in equal variances and normality, a parametric statistical method was selected. SPSS v26.0 (IBM, USA) was utilized for statistical analysis at a \( p < 0.05 \) significance level.

3. Results and discussion

3.1. Characterization of E/PA@HMS

The successful synthesis of HMS was accomplished by transforming the prepared SiO\(_2\) nanospheres into HMS through a self-templating strategy [35,36]. TEM image of HMS in Fig. 1a, indicated that uniform, spherical morphology (approximately 400 nm in diameter) and a core-shell structure with a huge cavity could be visualized, providing it with favorable potential for drug loading. After loading of PAH-ACP and EGCG, the spherical morphology of PA@HMS (Fig. 1b) and E/PA@HMS (Fig. 1c) was well-maintained and their core structure became less clearly defined. The mapping pattern of scanning TEM/EDS was utilized to identify the element distribution since the loaded amorphous mineral precursors within silica nanoparticles were unlikely to be determined by TEM. Compared to HMS (Fig. 1a), the infiltration of ACP precursors into the hollow structure of PA@HMS (Fig. S2), broad bands of non-crystalline scattering around 2\( \theta \) = 22\(^{\circ}\) were suggestive of amorphous solids. FTIR provides evidence for chemical functional groups within as-synthesized materials (Fig. 1e). Typical absorbance peaks at 1631 cm\(^{-1}\) (H–O–H bending vibration), 1096 cm\(^{-1}\) (Si–O–Si stretching vibration), 966 cm\(^{-1}\) (Si–OH bending vibration), and 804 cm\(^{-1}\) and 469 cm\(^{-1}\) (Si–O stretching and bending vibration) were observed for HMS. When compared with HMS, PA@HMS showed additional peaks at 1566 cm\(^{-1}\) (N–H bending vibration) and 1464 cm\(^{-1}\) (CH\(_3\) asymmetric deformation) which were attributed to PAH [18]. As for E/PA@HMS, a series

![Fig. 2. Effects of E/PA@HMS on intrafibrillar mineralization of reconstituted collagen fibrils and remineralization of demineralized dentin matrix. TEM images of collagen fibrils incubated (a–e) without or (f–j) with E/PA@HMS. Non-mineralized collagens were found after 1, 3, and 5 days of incubation without E/PA@HMS. Inset in (a) shows SAED analysis obtained from the location of the asterisk, which reflected no characteristic diffraction ring. For E/PA@HMS, the mineralization precursors released from E/PA@HMS adhered to the collagen surfaces at 1 day (b) and infiltrated into the collagen fibrils resulting in partial mineralization at 3 days (c), complete intrafibrillar mineralization of the collagen fibrils was achieved at 5 days (d). Inset in (b) shows SAED analysis obtained from the location of the asterisk, and arc-shaped (002) and (211) diffraction patterns correspond to apatite deposition along the c-axis of the collagen fibril. FESEM images of remineralization of demineralized dentin after acid etching (e–g, control group) or incubation with E/PA@HMS-containing solution for 7 day (h) and 14 days (i). EDS analysis of (j) elemental distribution and (k) contents of Ca and P in demineralized dentin after incubation with E/PA@HMS for 14 days. (i) XRD analysis of native dentin, acid-etched demineralized dentin, and E/PA@HMS-incubated demineralized dentin after 14 days.]}
of adsorption peaks at 1466 cm⁻¹, 1522 cm⁻¹, and 1636 cm⁻¹ were assigned to C−C stretching vibration of benzene ring from EGCG [46], and vibrations of Si−O− Si and Si−OH were not interfered with. The average particle size of HMS (Fig. 1f) was 408.4 nm in diameter, which corresponded to the TEM results. All three as-synthesized materials were of narrow size distribution. Fig. 1g showed that the average zeta potentials of HMS, PA@HMS, and E/PA@HMS were −15.63 mV, +11.90 mV, and +9.57 mV, respectively. The doping of positively-charged PAH-ACP resulted in the variation of HMS from negative charge to positive charge. For loading drugs or molecules into porous silicon, the major driving force stems from electrostatic interaction between the adsorbent and adsorbate [47]. This phenomenon denotes a probable mechanism for adsorption of precursors and EGCG by the HMS delivery nanosystem.

Results from nitrogen adsorption−desorption revealed that both the undoped and doped HMS manifested type IV isotherms with hysteresis loops (Fig. 1h and S3). The occurrence of such characteristics was ascribed to the presence of a mesoporous framework, which was consistent with TEM and XRD analysis. The specific surface area, pore volume, and pore size of HMS calculated by the BET/BJH method were 384.7 m²/g, 0.451 cm³/g, and 4.69 nm (Table S2), respectively. The specific surface area and pore volume decreased to 101.9 m²/g and 0.156 cm³/g for PA@HMS and further decreased to 28.4 m²/g and 0.059 cm³/g for E/PA@HMS, which is indicative of the infiltration of amorphous mineralization precursors and EGCG molecules into the mesopores of the silica matrix. The amounts of precursors and EGCG encapsulated into HMS and the thermal stability were evaluated by TGA (Fig. 1i) and DTG (Fig. S4). A weight loss of 6.73 wt% for HMS was manifested because of removal of physisorbed water (63.4 °C). A higher weight loss of 15.79 wt% for PA@HMS was observed due to additional decomposition of organic substances of PAH (458.5 °C). The doped amount of PAH was calculated to be 9.06 wt%, illustrating that the adsorbed PAH-ACP amount was higher than this value. For E/PA@HMS, the overall weight loss was 26.81 wt%, which might be attributed to extra thermal degradation of EGCG (287.9 °C) [48]. It is apparent that a significant amount of EGCG molecules (11.02 wt%) was adsorbed inside the HMS. All of the above-mentioned results therefore confirm that the E/PA@HMS was successfully fabricated.

Effective release of active components from mesoporous silica-based delivery nanocarriers is highly required for subsequent biomedical applications. Thus, in vitro cumulative releasing curves of mineral precursors and drug molecules from E/PA@HMS were detected at pH 7.4. The profiles of Ca and P releasing (Fig. 1j) displayed an initial period of rapid release and gradually slowed down until the end of the 28th day. Moreover, the released minerals tended to maintain a Ca/P ratio of 1.5 (Fig. S5) after 7 days. This obtained ratio was approximately equal to the typical Ca/P ratio of ACP at neutral pH [49]. The release of cumulative EGCG (Fig. 1k) was characterized by an initial quick release during the first 7 days and a relatively slow, sustained release from 7 to 28 days. The sustainable release of minerals and drug molecules as well as the unique combination of a spacious central core and a mesoporous shell endowed E/PA@HMS with great potential to be used as an ideal delivery vehicle.

3.2. Intrafibrillar mineralization of collagen

The ability of ACP precursors released from E/PA@HMS to mineralize collagen fibrils was examined with a single-layer reconstituted collagen model. In Fig. 2a1−a3, TEM images of the collagen fibrils incubated without E/PA@HMS showed no mineralization, and the crystal diffraction ring was not present in SAED (insert in Fig. 2a3). TEM image in Fig. 2b1 showed that the spherical liquid precursors released from E/PA@HMS could be visualized after incubation for 1 day, some of which attached to the collagen surfaces. After incubation for 3 days, Fig. 2b2 revealed that the precursors infiltrated into the collagen fibrils, leading to partial mineralization, and a few needle-shaped crystals were found outside the collagen fibrils. After incubation for 5 days, evidently higher electron densities of the collagen fibrils with complete intrafibrillar mineralization were observed in Fig. 2b3 when compared with those of 1 or 3 days, while partial extrafibrillar mineralization also occurred. SAED analysis (insert in Fig. 2b3) confirmed the deposition of apatite minerals along the c-axis of the collagen fibrils according to the presence of the arc-shaped diffraction pattern at the (002) and (211) planes. These findings indicate that PAH-ACP released from HMS still maintained the capability to infiltrate and mineralize collagen fibrils.

The competitive displacement of zwitterions originated from TBS might contribute to the release of positively charged PAH-ACP precursors from HMS [18]. The probable driving forces underlying the influx of mineralized precursors into collagen could be interpreted from different angles. First, capillary action allows liquid-like amorphous minerals generated by liquid–liquid phase separation to infiltrate into the collagen [50]. ACP diffused via the intrafibrillar water compartments between collagen molecules and solidified, followed by transformation into apatite crystallites along collagen fibrils at the longitudinal axis [51,52]. Furthermore, collagen fibrils might serve as a semipermeable membrane to establish Gibbs–Donnan equilibrium [17], in which the passage of polyelectrolyte nucleation inhibitors (such as PAH) through the semipermeable membrane is restricted, leading to the formation of thermodynamically-stable prernucleation clusters (PNCs) within the collagen intrafibrillar gap zones at the atomic scale, followed by inhibitor-free intrafibrillar apatite assembly [53].

3.3. Remineralization of demineralized dentin

Since intrafibrillar mineralization of reconstituted collagen has been achieved, the remineralization effects of E/PA@HMS on demineralized dentin were further evaluated in vitro. After phosphoric acid etching (control group), both the extrafibrillar and intrafibrillar minerals of dentin were removed while a periodic pattern of collagen fibrils was presented (Fig. 2c1−c4), indicating that minerals within collagen were dissolved. After 7 days of incubation with E/PA@HMS-containing solution, the periodic pattern could not be visualized, and the collagen fibrils were of expanded width and manifested a continuous state, implying that the collagen network was supported by internal minerals (Fig. 2d1−d4). Massive spherical precursor particles adhered to dentin collagen due to sustainable delivery of PAH-ACP from E/PA@HMS, and the contents of Ca and P remarkably increased when compared with those on the 1st day (Fig. S6), denoting a higher degree of mineralization. After 14 days of incubation, demineralized dentin was covered by nanocrystals to generate a homogeneous surface, and the collagen fibrils were adhered by spherical particles with enlarged diameters (Fig. 2e1−e4), indicating a collaborated action of intrafibrillar and extrafibrillar mineralization. There was no substantial change in electron density within the dentinal tubules (Fig. 2f and g, obtained from the location in Fig. S7) when compared with that on the 7th day. A uniform distribution of Ca and P was observed in intratubular and peritubular dentin, and the contents of Ca and P increased slightly (Fig. 2h).

These findings confirm that demineralized dentin can be remineralized by the use of E/PA@HMS from intrafibrillar and then extrafibrillar mineralization, which follows the ‘bottom-up’ mineralization and allows the reconstruction of the hierarchical structure of dentin [54]. Collagen mineralization at an early stage was accordant to that in the single-layer reconstituted collagen model, and the release of PAH-ACP facilitated intrafibrillar mineralization. What’s more, the specific confined environment in demineralized dentin was responsible for the occurrence of extrafibrillar mineralization at a later stage [55]. As such, with the increase of mineralization time, the degree of external mineralization is promoted. In addition, the newly formed crystals on dentin were further measured by XRD analysis. Diffraction peaks in Fig. 2i at 2θ = 25.9° (002), 31.8° (211), and 32.9° (300), respectively, correspond to characteristics of hydroxyapatite (HAp) that appeared in native dentin [56]. These reflections dramatically disappeared or
weakened after acid etching. Importantly, such characteristic diffraction peaks were apparently recovered after incubation with the E/PA@HMS-containing solution. Combined with the EDS results that showed a Ca/P molar ratio of around 1.67 (Fig. 2h), it can be deduced that crystalline HAp was newly formed (which was similar to native dentin), and the remineralization effects were thus induced. Taken together, E/PA@HMS treatment significantly promoted the remineralization of demineralized dentin matrix, thereby offering encouraging benefits in the management of exposed dentin and providing a rational tactic for dental hard tissue mineralization.

3.4. Effectiveness and stability of tubule occlusion

To determine the potential application of E/PA@HMS on exposed dentin, FESEM was used to assess the effectiveness and stability of tubule occlusion. After acid-etched, the control group exhibited patent orifices with a smear-free layer regardless of incubation for 1 or 28 days (Fig. 3a, Fig. 3d). Tubules of dentin specimens treated by E/PA@HMS were absolutely obstructed, and the mineral crystals and silica nanoparticles filled the tubules to more than 13 μm of depth beneath the surface after 1 day (Fig. 3b, Fig. 3h). On the 28th day, fully occluded tubules were well-retained by the filling of minerals and nanoparticles which combined with the tubular wall with no substantial change in depth of infiltration (Fig. 3d, Fig. 3i). When challenged by acid, the control group displayed enlarged, patent tubules and a smooth surface (Fig. 3e, Fig. 3g). As for E/PA@HMS-treated dentin, remaining sealed tubules with a large proportion were observed (Fig. 3f and h). The infiltrated minerals tightly plugged the underlying tubules (12 μm of depth) despite the
superficial orifices being slightly unfolded, and the dentin surface was covered by a membrane-like mineral layer (Fig. 3f). When challenged by brushing, the control group showed open tubules and a rough surface (Fig. 3i). The E/PA@HMS group reflected totally obstructed orifices as well as scattered nanocrystals (Fig. 3j), and the tubules were aggregated by silica spheres and minerals which had a distance of approximately 10 μm from the orifice (Fig. 3l). Dentin permeability was assessed to provide detailed information on the occluding capacity of E/PA@HMS in a simulated pulp-dentin pattern (Fig. 3m). Irrespective of incubation for 1 or 28 days (Figs. S8 and S9 or 3n, 3o), the dentin permeability treated by E/PA@HMS decreased significantly, and its Lp% value was significantly lower than that of the control group. When challenged by acid (Figs. S8 and 3n) or brushing (Figs. S9 and 3o), the dentin permeability of the E/PA@HMS-treated group did not statistically increase, and it showed a significantly lower Lp% value when compared with the control group. The results from dentin permeability measurement support the morphological evidence of FESEM. Furthermore, data from dentin microhardness tests revealed that the values of E/PA@HMS-treated specimens significantly increased compared to those of the acid-etched or control specimens (Fig. 3p). The value measured after 28 days of incubation was 0.601 ± 0.046 GPa, which approached those of native dentin, indicating that the mechanical capacities of the demineralized dentin were recovered by E/PA@HMS treatment.

It can be concluded that E/PA@HMS is effective in occluding the tubules with acid- and abrasion-resistance to reduce dentin permeability and increase dentin microhardness over an extended period, which are beneficial for combating hypersensitivity and demineralization. Importantly, the EGCG and PAH-ACP loaded silica nanoparticles in the form of paste are small enough to penetrate into dentinal tubules to a certain depth. The continuously released Ca and P ions from E/PA@HMS increase the inorganic content and allow their conversion into HAp crystals for mineralization to fully occupy the tubules and integrate with the inner walls [56, 57]. Taking into account the mineralization effects of collagen fibrils and demineralized dentin from ACP precursors, the mechanical properties and stability of mineralized regions can be enhanced and the microhardness recovery can be realized. Moreover, prolonged acid-resistant stability on tubule occlusion was achieved mainly because of the aciduric performance of silica [58]. This character enables the underlying nanoparticles and crystalline minerals to be

**Fig. 4.** Evaluation of antibiofilm efficiency of E/PA@HMS. (a–d) CLSM 3D images of live/dead bacterial staining of *S. mutans* biofilm on dentin treated with or without E/PA@HMS after incubation for 1 or 28 days. Green and red fluorescence are suggestive of live and dead bacteria, respectively. (a1–d1) Corresponding bacterial biomass distribution for each layer (z-stack) of (a–d). (e–h) FESEM images of *S. mutans* adhesion and biofilm formation on dentin treated with or without E/PA@HMS after incubation for 1 or 28 days. Images of (e1–h1) correspond to the high-magnification images of the yellow box region in (e–h). Pointers indicating patent orifices (e1) or obstructed orifices by E/PA@HMS (f1 and h1). (i) MTT metabolic activities, (j) CFU counts, and (l) medium’s pH values of *S. mutans* biofilm formed on dentin treated with or without E/PA@HMS. Different letters denote significant differences (p < 0.05). (k) Representative photographs of *S. mutans* colonies grown on BHI agar plates.
protected from being dissolved despite superficial orifices being slightly unfolded. Dentin erosion and collagen degradation could be prevented due to the inactivation of matrix metalloproteinase activity by EGCG release [59]. Besides, considerable abrasion-resistant stability of tubule occlusion was also obtained since mesoporous silica possesses strong mechanical strength, which has been proposed previously [60]. The strictly as-sealed tubules by E/PA@HMS with available penetrating distance can minimize the likelihood of being removed. In other words, the greater percentage of tubules blocked by E/PA@HMS implies the stronger performance of occluding and acid- and abrasion-resistant stability.

3.5. Antibiofilm function of E/PA@HMS

Cariogenic bacteria adhering to the tooth surface and the subsequent formation of dental biofilms are major contributors to the occurrence and progression of caries [61,62]. Although tubule occlusion approaches could prevent the invasion of external bacteria and stimuli, continuous biofilm formation with acid production and reduced pH atmosphere is likely to compromise the sealing efficacy and induce re-exposure of dentin. The prolonged effectiveness of E/PA@HMS on the adherence and biofilm formation of S. mutans was hence investigated. CLSM images of 3D overlay projections in Fig. 4a–d showed that S. mutans biofilm formed in the E/PA@HMS-treated group was substantially fewer than the control group after 1 and 28 days. The total bacterial biomass exhibited a considerable decrease at the 10th layer in the treated group (Fig. 4d, h) compared to the control group (Fig. 4d, d, h). FESEM images (Fig. 4e–h and e1–h1) manifested that treatment by E/PA@HMS induced far fewer bacteria growth (with obstructed orifices) compared to the control group (with patent orifices). Confocal analysis (Fig. S10), MTT assay (Fig. 4i), and CFU test (Fig. 4j and k) respectively reflected that bacterial biofilm production, metabolic activity, and colony amount in the treated group were significantly fewer than those in the control group. In addition, the treated group represented a much higher pH value (above 6.0, Fig. 4i) and reduced lactic acid generation by biofilm (Fig. S11). The consistent results indicate that E/PA@HMS can persistently inhibit biofilm formation and growth of cariogenic bacteria on dentin.

The mechanism behind this could be attributed to several accounts. On the one hand, the tubule orifices subjected to biofilm challenge were still blocked by E/PA@HMS after incubation, and the released EGCG would perform its function. As a green tea-derived extract, EGCG is of high bio-activity, and previous studies have illuminated the function of EGCG on inhibiting dental biofilms, S. mutans. The acid production process by cariogenic bacteria as well as their virulence factors (for instance, lactate dehydrogenase) can both be suppressed by EGCG [63]. In this regard, the cariogenicity of S. mutans appears to be inhibited. The expression of glucosyltransferase-related genes can also be inactivated via EGCG, which prevents the tooth surface from being adhered to by S. mutans [64]. On the other hand, the calcium and phosphorus released from E/PA@HMS play a significant role in fighting caries. Apart from inhibiting demineralization and facilitating remineralization, the capacity of acid neutralization raised the pH from 4 to >6, signifying the reservation of a safe value approaching neutral rather than a cariogenic value [65]. Fewer acid products would be yielded once the formation and progression of biofilms are inhibited. The control of pH inside local biofilm is highly beneficial to prevent calcium and phosphorus loss to protect dentin. Thus, E/PA@HMS is proved to be effective in co-delivery of PAH-ACP and EGCG and exhibits excellent capability to effectively prevent biofilm formation and obstruct the dentinal tubules. In the future study, multi-species or saliva biofilms will be used to simulate a more realistic oral condition in order to further evaluate the effects of E/PA@HMS treatment on dentin exposure.

3.6. Biocompatibility evaluation

Silicon-based mesoporous nanomaterials possess a high surface area and an ordered framework, which facilitates their extensive applications in disease diagnosis, therapy, and surveillance when compared with conventional drug nanocarriers. Functional nanoparticles are anticipated to fully perform their potential while avoiding adverse impacts on surrounding tissues and cells for biomedical utilization [66]. Previous studies have proved that a negligible effect of mesoporous silica nanoparticles on cell mitosis even upon phagocytosis by vascular endothelial cells was shown when employed as drug vehicles [67,68]. Although silica is acknowledged as having low toxicity, the biocompatibility of E/PA@HMS should be appraised. The CCK-8 assay was conducted to assess the cell proliferation and cytotoxicity of E/PA@HMS with...
different concentrations (0, 250, 500, and 1000 μg/mL) towards HDPSCs cultured for 1, 3, 5, and 7 days. Fig. 5a shows that the OD_{450nm} value of each concentration tested increased with the incubation time. Even though there was a statistical difference between the control group and the 1000 μg/mL group (the highest concentration used), the relative mitochondrial dehydrogenase activities of HDPSCs remained above 90% after 7 days of incubation (Fig. S12). Further observation of HDPSCs stained with crystal violet and coomassie blue is represented in Fig. 5b and c, respectively. Compared to the cells after incubation for 1 day, the cells at each concentration substantially proliferated after incubation for 7 days. The clearly-identified and well-stretched filamentous actins presented in each group stained by Coomassie blue were indicative of cells with healthy status. These results signified that E/PA@HMS induced relatively negligible cytotoxicity to HDPSCs.

Blood compatibility was considered another essential challenge for silicon-based delivery carriers. The interactions between silica and membrane proteins are likely to trigger irreversible damage to erythrocytes from the hemolytic effect [69,70]. Consequently, the hemolysis test of E/PA@HMS was estimated, and the results are shown in Fig. 5d and e. A bright red solution derived from hemoglobin release by red cell rupture was manifested for deionized water (positive control), and a clear, transparent solution denoting no hemolysis occurred was exhibited for PBS (negative control). With the increasing concentrations of E/PA@HMS (0.1, 0.25, 0.5, 0.75, 1, 2, 3, 4, and 5 mg/mL), the hemolysis rate increased correspondingly, implying a concentration-dependent pattern. It is noteworthy that no more than 5% of the hemolysis rate was presented at a concentration of 5 mg/mL, indicating an extremely low hemolytic effect on red blood cells. The findings from cell proliferation and hemolysis assays confirm that E/PA@HMS is highly biocompatible and meets the safety requirements of synthetic biomaterials for in vivo applications.

3.7. In vitro odontogenic differentiation

The formation of mineralized tissues and odontogenic differentiation of stem cells are involved in the regeneration of dental hard tissue [27, 71]. According to the mechanism of biological defense, the irritation of dental pulp cells from the tubules results in odontoblast differentiation and reparative dentin formation based on the pulp-dentin complex [72]. It is therefore expected that the tubule-occluding materials, which had the opportunity to contact HDPSCs, could induce odontogenesis and mineralization to combat pulp inflammation and sensitivity. To validate these effects of E/PA@HMS on HDPSCs, cells were cultivated with 1.0 mg/mL of the extract solution of E/PA@HMS in OI medium. After induction for 7 or 14 days, results of ALP staining images showed that HDPSCs presented normal morphologies in vitro, and the E/PA@HMS group appeared to have more stained areas than the control group (Fig. 6a). The quantitative ALP assay of cell lysates revealed that the
relative ALP activity of E/PA@HMS-treated groups was significantly higher than the control group (Fig. 6c). Alizarin red staining was utilized to determine the extracellular mineral deposition. Fig. 6b and d showed that the calcium nodule formation was dramatically improved in the E/PA@HMS-treated group after 14 and 21 days. The qRT-PCR technique was used to detect the expression of odontogenic-related genes, including ALP, RunX2, OSX, OCN, DMP-1, and DSPP. Among these genes, ALP, RunX2, and OSX are classic biomarkers at the early stages of osteogenic and odontogenic differentiation [73,74]. OCN generally appears at the late stage of osteogenic differentiation [75]. DMP-1 and DSPP are two typical biomarkers that participate in dentin matrix mineralization and influence its initiation and maturation by regulating calcium and phosphate metabolism [76]. The mRNA levels of all these genes in HDPSCs were significantly upregulated after incubation with 1.0 mg/mL of the extract solution of E/PA@HMS for 14 and 21 days (Fig. 6e–j) when compared with the control, indicating a strong and rapid odontogenic inducivity.

Our results reflected that the addition of E/PA@HMS promoted ALP activity and mineral deposition and enhanced the expression of osteo-/odontogenic genes (ALP, RunX2, OSX, OCN, DMP-1, and DSPP) in HDPSCs. Previous studies have certified that the presence of Ca and P ions plays a vital role in hard tissue mineralization [3,77]. Ca ions can bind to plasma membrane or induce stem cell migration to adjust cellular functions through upregulating the gene expression of odontogenesis-related proteins [78]. P ions regulate the function of extracellular matrix proteins during the process of mineralization by influencing the formation of HAp [79]. Furthermore, EGCG has been reported to facilitate mineralized nodule formation and elevate the activity of ALP and the levels of osteo-/odontogenic markers, BSP and Col1, in stem cells from the apical papilla [26,80]. The apoptosis of HDPSCs derived from hypoxia injury and the inflammatory response can both be inhibited by EGCG [27]. Whereas, among different studies, the effective concentrations varied for the effects of odontogenic differentiation, which may be relative to the types of cells used. These findings imply that E/PA@HMS is capable of promoting the odontogenic differentiation and mineralization of HDPSCs, thereby offering essential advantages to protect dental pulp tissue and restore damaged dental hard tissue.

3.8. In vivo efficacy evaluation

In order to investigate the clinical efficiency of E/PA@HMS in managing exposed dentin in a practical oral environment, in vivo animal experiments were designed. Dentin disk specimens treated with or without E/PA@HMS were fixed in the oral cavity of rats (Fig. 7a and b) for tubule-occluding examination. After incubation for 14 days, an environment involving varied enzymes and ions that is approximately that found in humans was provided in the rats’ mouths. In the control group, extremely few dentinal tubules were blocked from FESEM images (Fig. 7e–f). On the contrary, the dentinal tubules of specimens treated by E/PA@HMS were completely occluded (Fig. 7d). Furthermore, the newly formed, mineral-contained layer showed a uniform distribution of Ca and P (Fig. 7g–h) as well as Ca/P ratio of 1.70 (Fig. 7i) by EDS analysis. These results were consistent with those found in experiments in vitro. For in vivo antibiofilm assessment, rats were inoculated with S. mutans to establish a cariogenic environment prior to incubation. After 14 days, the control group exhibited patent tubular orifices and the dentin surface was adhered by a great mass of bacteria (even inside the tubules, Fig. 7g). Notably, E/PA@HMS treatment absolutely plugged the tubules and induced far fewer bacteria grown on the dentin surface (Fig. 7h).

Based on the obtained results, it can be confirmed that the in vivo application of E/PA@HMS achieved effective biofilm inhibition and tubule occlusion with remineralization. It is anticipated that E/PA@HMS will be promising for clinical translation in protecting exposed dentin from synergistic management of hypersensitivity and dental caries. E/PA@HMS could be used as a dentin desensitization agent or immediate sealant to treat dentin hypersensitivity, and/or added to toothpaste or mouthwash to improve the efficacy of traditional oral hygiene.

4. Conclusions

The exposure of dentinal tubules is an essential etiology in the pathogenesis of dentin hypersensitivity and caries. In the present study,
we successfully synthesized a novel EGCG and PAH-ACP co-delivery hollow mesoporous silica (E/PA@HMS) nanosystem for the protection of exposed dentin. The application of E/PA@HMS effectively occluded the dentinal tubules over a prolonged period with acid- and abrasion-resistant stability, and the adhesion and biofilm formation of cariogenic bacteria on the dentin substrate were inhibited. E/PA@HMS can achieve intrafibrillar mineralization of collagen and remineralize the demineralized dentin. Furthermore, E/PA@HMS is highly biocompatible and has the capability to promote odontogenic differentiation and mineralization of dental pulp stem cells. Animal studies showed that E/PA@HMS persistently blocked the tubules and inhibited biofilm growth up to 14 days. Thus, the establishment of the E/PA@HMS delivery nanosystem indicates great potential to serve as a biomimetic protective barrier in dental hard tissue repair and is promising for coordinated management of dentin caries and hypersensitivity.

Conflicts of interest

None.

Ethics approval and consent to participate

All protocols were in accord with the guidelines set by the Ethics Committee of Hospital of Stomatology, Wuhan University (certificate Zhao: 20190210).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2022.11.018.

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