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Research article

Remineralization of dentine tubules induced by phosphate-terminated PAMAM dendrimers

Yajie Wen [a,b,1], Jichao Wang [c,1], Jun Luo [d], Jiaojiao Yang [a,*]

[a] State Key Laboratory of Oral Diseases, National Clinical Research Center for Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu, 610041, China
[b] School of Environment and Resource, Southwest University of Science and Technology, Mianyang, 621010, China
[c] Department of Infection, The People's Hospital of Qiannan, Qiannan, 558000, China
[d] College of Polymer Science and Engineering, Sichuan University, Chengdu, 610064, China

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ABSTRACT

Various sealants have been developed to treat the exposure of dentinal tubules (DTs) and further dental hypersensitivity. Herein, the phosphate-terminated fourth generation polyamidoamine dendrimers (PAMAM-PO_3H_2) was successfully synthesized. Six duplicates of demineralized dentin and type I collagen matrix were incubated in artificial saliva solution with or without PAMAM-PO_3H_2 treatment at 37 °C for 2 weeks, respectively. The artificial saliva solution was replaced every day. These regenerated crystals on the surface of dentin and collagen matrix were confirmed to be hydroxyapatite (HAp). The approach thus demonstrates that PAMAM-PO_3H_2 can offer an efficient therapy for seal DTs in clinical dentistry.

1. Introduction

Demineralization of dentin leads exposure of dentinal tubules (DTs) and further dental hypersensitivity (DH), which is characterized by dental pain due to acidic stimuli, bacterial infection and so on [1]. Traditional strategies to seal DTs include occluding resins, dentin adhesives and so on. However, these methods have been proved to cause various clinical risks, such as short-time stability [2]. Indeed, the exposure of DTs usually occurs when the intrinsic equilibrium between the remineralization and demineralization of dentin break, which is caused by oral acid-producing bacteria or acidic diet [3]. Therefore, regulating the equilibrium to remineralization is a valuable choice to seal DTs.

Dentin is composed of hydroxyapatite (HAp), water and organic matrix, in which non-collagenous proteins (NCPs), can play the role of templates to control the nucleation and growth of HA within the collagen fibrils matrix [4]. However, NCPs have not enough ability to induce remineralization in the mature dentin [5]. In that cause, it is desirable to develop effective templates to realize the remineralization of demineralized dentin and DTs closure [6, 7].

Representing a kind of polymers with mono-dispersed polymeric molecular structures, dendrimers have been recognized as 'artificial proteins' due to their biomimetic properties and widely applied in biomedical field, such as drug and gene delivery. Dendrimers have a spherical shape and regular branched structure with high amount of surface groups [8]. Polyamidoamine (PAMAM) dendrimers have been deeply investigated for different biomedical applications [9]. In previous studies, PAMAM dendrimers with different surface chemical groups have been proved to self-assemble similar to amelogenin [10], anchor on the surface of enamel [11], immobilized within the collagen matrix of dentin [12, 13] and induce in situ remineralization [11, 12, 13]. Further, a type of phosphate-terminated PAMAM dendrimers were synthesized and obtained a satisfy ability to induce remineralization of enamel [14] and dentin [15] due to the similarity between of the phosphate-terminated PAMAM dendrimers and proteins in tooth (i.e. amelogenin and dentin phosphophoryn). However, the effect of phosphate-terminated PAMAM dendrimers to seal DTs and induce biominerals formation in collagen matrix in DTs have not been inspected, which is very important to the clinic application of phosphate-terminated PAMAM dendrimers.

The ability of phosphate-terminated the fourth generation PAMAM dendrimers (PAMAM-PO_3H_2) to induce dentin remineralization to seal DTs are discussed in this study, in which the demineralized dentin and recombined collagen matrix were applied.

* Corresponding author.
E-mail address: jiaojiao.yang@scu.edu.cn (J. Yang).
[1] These authors contributed equally to this work.

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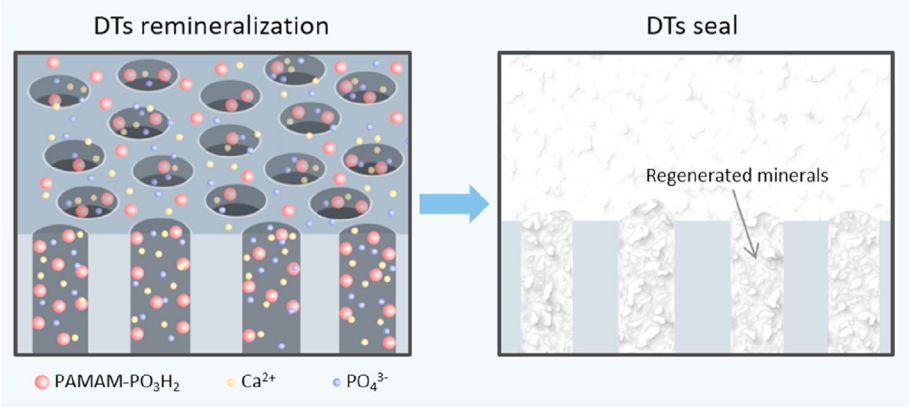

Figure 1. Schematic illustration of DTs seal via remineralization induced by PAMAM-PO_3H_2.

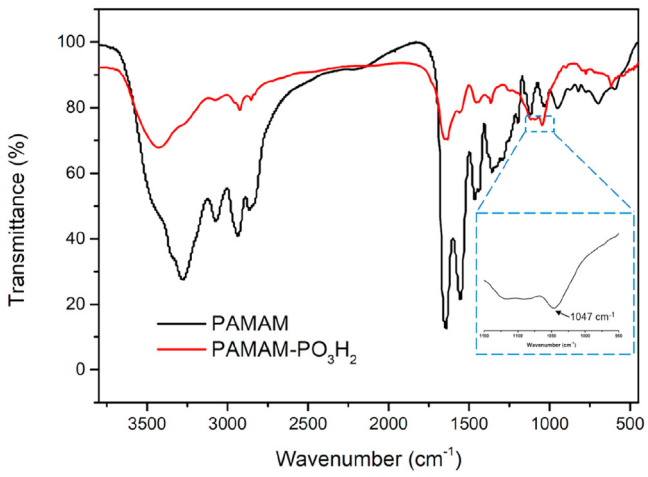

Figure 2. FTIR spectra of PAMAMA and PAMAM-PO_3H_2. The new peak at 1047 cm^{-1} can be attributed to the P–O adsorption peak of phosphate groups.

2. Materials and methods

2.1. Materials

Bromotrimethylsilane (BTMS) and dimethyl hydrogen phosphate (DMP) were obtained from Adams Reagent Company and TCI, respectively. Lyophilized type I collagen powder was obtained from Sigma-Aldrich. All other reagents and solvents were obtained from Tianjin Bodi Chemical Holding Company. Human tooth samples were extracted at West China Hospital of Stomatology, Sichuan University. The ethics approval was obtained from the Research Ethics Committee of West China Hospital of Stomatology, Sichuan University. The informed consent was obtained from all patients for experiments.

2.2. Phosphate-terminated the fourth generation PAMAM dendrimers (PAMAM-PO_3H_2) synthesis

The fourth generation PAMAM dendrimers (denoted as PAMAM) were firstly synthesized following the classical pathway as follows. Briefly, 1,2-ethylenediamine (EA) with excessive methyl acrylate (MA) synthesizes ester-terminated dendrimers that is refereed as 'half-generations'. Then ester-terminated dendrimers with excessive EA produces amine-terminated dendrimers that is refereed as 'full generations'. The above procedure was repeated for 3 times, and then the PAMAM were used for further modification [16].

$PO(OCH_3)_2$-terminatied PAMAM (PAMAM-$PO(OCH_3)_2$) was synthesized by modifying the surface amine groups of PAMAM [17]. The obtained PAMAM (3.200 g, 0.464 mmol) and paraformaldehyde (1.340 g, 44.640 mmol) were added in THF (20 mL) and KOH (1 mol/L) mixture. Then DMP (4.128 g, 37.520 mmol) was dropwise added in the mixture. After vigorous stirring at 70 °C for 1 d, the solution was diluted with lots of deionized water, dialyzed for 24 h and lyophilized to obtain PAMAM-$PO(OCH_3)_2$.

PAMAM-PO_3H_2 was synthesized from PAMAM-$PO(OCH_3)_2$ following the previous report as follows. In brief, after PAMAM-$PO(OCH_3)_2$ (0.160 g, 0.010 mmol) dissolving in 300 mL anhydrous dimethyl sulfoxide (DMSO), BTMS (0.648 g, 4.236 mmol) was dropwise added and the solution was vigorous stirred at 25 °C for 1 d. The result solution was processed same as the post-treatment of the synthesis of PAMAM-$PO(OCH_3)_2$ to obtain PAMAM-PO_3H_2 product [18].

2.3. Fourier transform infrared (FTIR) spectroscopy

The FTIR spectrum of PAMAM-PO_2H_3 sample was recorded with a spectrometer (IFS 66v/S, Bruker, Germany). Patterns were collected from 3800 to 450 cm^{-1}. A blank scan was scanned for background removal.

2.4. Dentin samples preparation

Fifteen human third molars were collected from people aged from 18 to 40 years in West China Hospital of Stomatology. After cleaned, tooth were cut perpendicular to the long axis of the tooth and the coronal parts were used as dentin disks (5 mm × 5 mm size and 1 mm thickness). The coronal surfaces of these disks were ground flat and polished. Specimens were sealed with acrylic resin except the coronal surfaces. The sample surfaces were painted by acid-resistant varnish and exposed a 3 mm × 3 mm window. All samples were stored in 0.1 thymol solution at 4 °C prior to using.

2.5. DTs exposure

Dentin samples were demineralized in a 0.5 M Ethylene Diamine Tetraacetic Acid (EDTA) solution for 30 min. Then samples were ultrasonically cleaned with deionized water for 10 min. After that, samples were treated with a 4 M guanidine chloride solution for 60 min to remove the NCPs [19]. Finally, samples were ultrasonically cleaned with ethanol for 10 min and stored at 4 °C in phosphate buffer solution (PBS, pH 7.0) before use.

2.6. DTs seal

After rinsed with deionized water and dried, the window of each demineralized dentin sample was covered by 50 μL PAMAM-PO_3H_2 aqueous solution (1 mg/mL). The control groups were covered by 50 μL deionized water. After 1 d, each sample was ultrasonically cleaned in

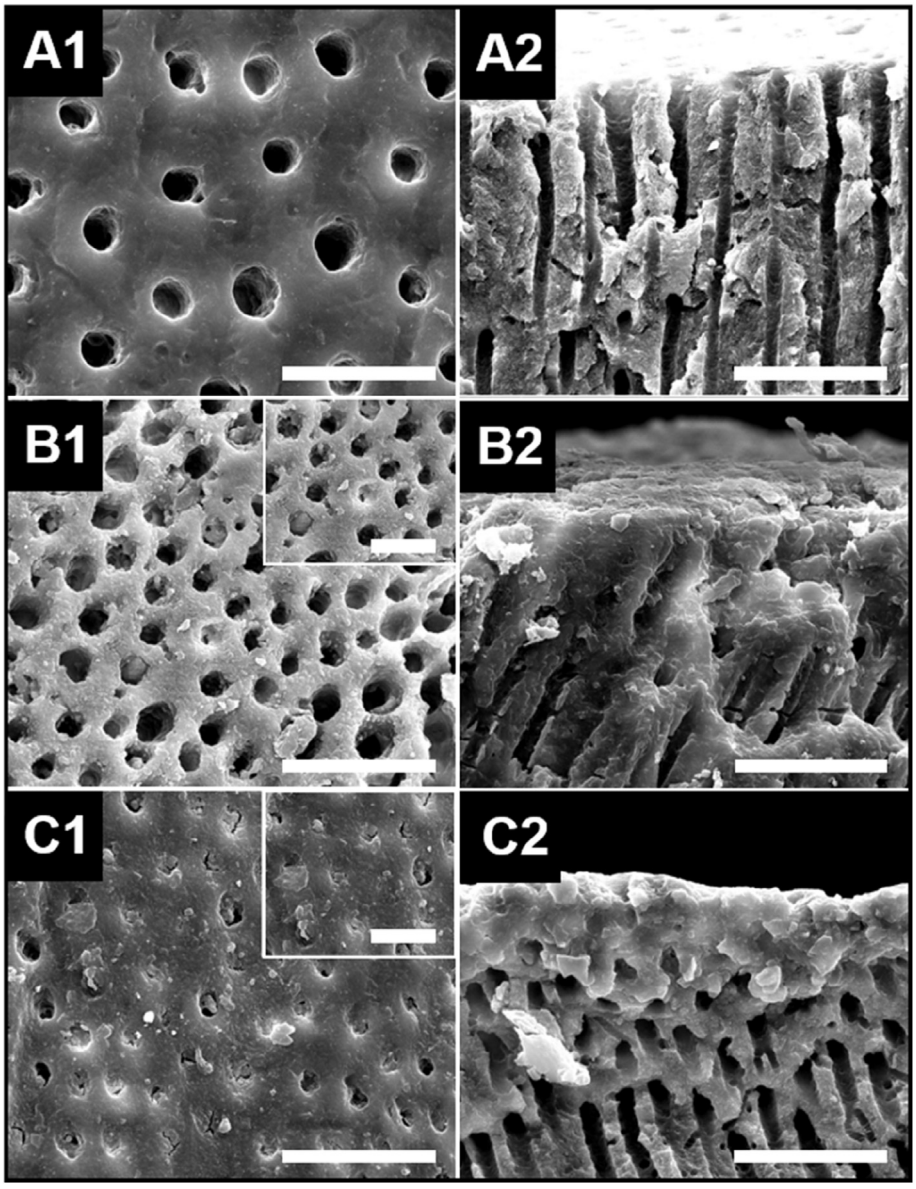

Figure 3. Typical SEM images of demineralized DTs immediately (A1: surface, A2, fracture), without treatment after incubation in artificial saliva at 37 °C 2 weeks (B1: surface, B2, fracture) and with PAMAM-PO_3H_2 treatment after incubation 2 weeks (C1: surface, C2, fracture) (scale bar: 20 μm). The inserts are corresponding enlarged details (scar bars: 10 μm).

deionized water for 5 min to remove free dendrimers. After dried in air, each sample was immersed in 10 mL artificial saliva solution (pH 7.0), which is composed of 1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, 130 mM KCl, 1.0 mM NaN_3 and 20 mM HEPES, at 37 °C. The artificial saliva solution was replaced by fresh solution every day. All samples were removed out after 2 weeks, and then ultrasonically cleaned with deionized water for 5 min for further characterization. Each group had 6 duplicate samples.

2.7. Scanning electron microscopy (SEM)

SEM was carried out with a field emission scanning electron microscope (Merlin, Zeiss, Germany). Each dentin sample was created a slit with around 1 mm depth along the midline. Then the sample was fractured into 2 halves in liquid nitrogen for further occlusal and longitudinal section observation, respectively. After coated with a thin Au layer, samples were analyzed with microscopy at a voltage of 20 kV using the HE-SE2 detector.

2.8. X-ray diffraction (XRD)

The XRD was carried out on a X'Pert PRO MPD, PANalytical (Malvern, United Kingdom) with voltage of 40 kV, current of 40 mA and a zero background holder. Patterns were recorded for angles (2θ) from 10° to 80°, in steps of 0.02° with 0.25 s per step.

2.9. Crosslinking of collagen fibrils

According to the previous report [20], lyophilized type I collagen powder was dissolved in 0.1 M acetic acid at 4 °C to obtain a 0.15 mg/mL collagen stock solution. 50 μL collagen stock solution was dropped on a formvar-and-carbon-coated gold grid. After neutralized by ammonia vapor at 37 °C for 8 h, the grid was stored at 37 °C for 4 d to form gel. Then grids were immersed in 80 μL of 1-ethyl-3-(3-dimethylaminopropy)-carbodiimide, N-hydroxysuccinimide and 2-morpholinoethane sulphonic (EDC-NHS-MES) solution crosslinker at 25 °C for 4 h. The

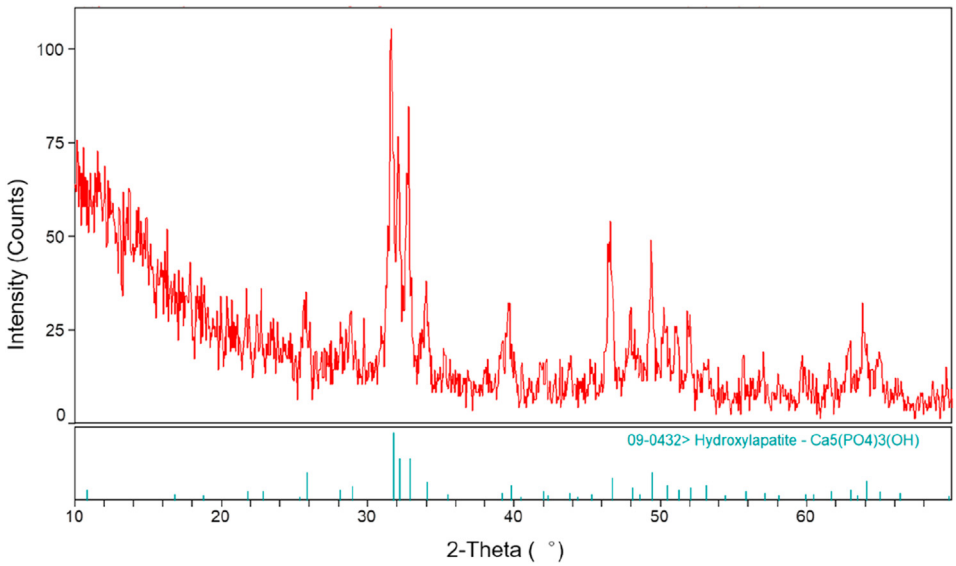

Figure 4. The XRD pattern of PAMAM-PO_3H_2 treated dentin after being incubated in artificial saliva for 2 w and the standard JCPDS card of hydroxyapatite.

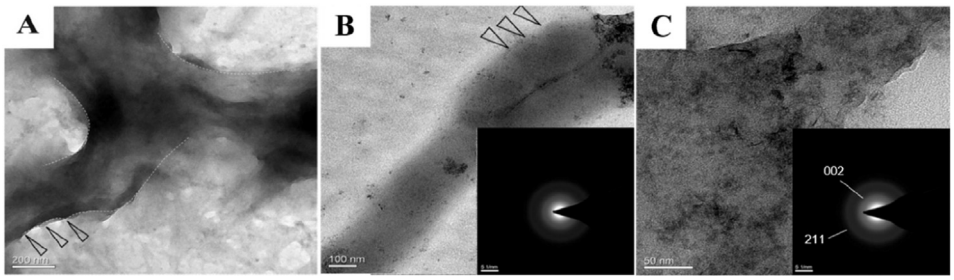

Figure 5. (A) Stained TEM image of reconstituted type I collagen matrix. Unstained TEM images of collagen fibrils treated with PAMAM-PO_3H_2 in artificial saliva at 37 °C for 1 w (B) and 2 w (C). The inserts are electron diffraction spectrum.

EDC-NHS-MES solution includes 0.3 M EDC, 0.06 M NHS and appropriate MES which adjust solution to pH 5.9.

2.10. Collagen mineralization

TEM grids with crosslinking collagen fibrils were immersed in 50 μL PAMAM-PO_3H_2 aqueous solution (1 mg/mL) for 20 min. After that, grids were washed by deionized water. The grids were treated with 1 mL artificial saliva at 37 °C for 1 w and 2 w. Each group had 6 duplicate samples.

2.11. Transmission electron microscopy (TEM) with electron diffraction

TEM with electron diffraction was performed on a FEI Tecnai GF20S-TWIN instrument at 200 kV. The grid with collagen fibrils was stained with 1% uranyl acetate prior to observation, while girds with mineralized collagen fibrils were observed directly.

3. Results and discussion

The effect to seal DTs through mineralization induced by PAMAM-PO_3H_2 and related mechanism are represented in Figure 1.

The PAMAM-PO_3H_2 was successfully synthesized in two steps. In the first step, PAMAM was synthesized following the classical strategy [16]. After that, PAMAM with terminated amide groups was utilized to synthesize PAMAM-PO_3H_2 via a Kabchnic-Fields reaction and hydrolyzation in turn [17, 18]. The successful synthesis of PAMAM and PAMAM-PO_3H_2 was confirmed by FTIR as shown in Figure 2. It can be observed that the peak around 3288 cm^{-1} can be attributed to the amide vibration of PAMAM, while the peak around 2940 cm^{-1} and 1465 cm^{-1} are due to -CH^2- vibration of PAMAM. There is one distinct change in the peak at 1047 cm^{-1} associated with phosphate groups when comparing PAMAM-PO_3H_2 with PAMAM, which is due to the P–O adsorption peak of PAMAM- PO_3H_2.

Previously, we have proved that PAMAM-PO_3H_2 could stable adhere to minerals and collagen matrix to repair detin [15]. In the present work, PAMAM-PO_3H_2 was utilized to induce remineralization of DTs and seal DTs. Therefore, EDTA-etched bare DTs were first treated with the PAMAM-PO_3H_2 that could form the bonding between Ca^{2+} and chelating groups, i.e. -PO_3H_2 of PAMAM-PO_3H_2. After EDTA etching, the DTs surface in dentine was heavily demineralization and became rough (Figure 3A1), and especially the DTs are thorough exposed (Figure 3A2). After 2 weeks in vitro remineralization, newly formed scattered crystals were covered on the dentin surface and within the DTs when no PAMAM-PO_3H_2 applying, but the exposure of DTs can be observed easily (Figure 3B1, B2). However, when DTs was treated with PAMAM-PO_3H_2, a large amount of minerals fully covered the dentin surface (Figure 3C1). Although the bottom of DTs was empty, which means PAMAM-PO_3H_2 cannot induce the in-depth remineralization of DT, newly minerals achieve to seal DTs totally (Figure 3C). All duplicate samples had similar remineralization results.

The main mineral phase of dentin is HAp, thus newly minerals induced by biomimetic materials is HAp, which can consistent with clinic requirements. The XRD data of remineralization crystals showed peaks at

$2\theta = 25.9°$, 31.8°, 32.2°, 32.8°, 34.1°, 39.8°, 46.7° and 49.5° are corresponding to the characteristic diffraction (002), (211), (112), (300), (202), (221), (222) and (213), respectively, of HAp (Figure 4). Therefore, the remineralization crystals induced by PAMAM-PO_3H_2 are similar to native dentin.

From results above, the presence of newly minerals on the surface and within DTs of dentine shows that PAMAM-PO_3H_2 can successfully seal exposed DTs. However, a large amount of Type I collagen matrix exists in DTs, which can affect the crystal nucleation and growth [20]. Therefore, the mineralization induced by PAMAM-PO_3H_2 in reconstituted type I collagen matrix was verified. Figure 5A shows stained reconstituted type I collagen fibrils, which indicates the diameter of a fibril is about 400 nm. Unstained collagen fibrils treated with PAMAM -PO_3H_2 dendrimer were immersed in artificial saliva for different time periods (1 w and 2 w). Because unstained collagen fibrils cannot be observed in TEM, the visible morphologies in these collagen fibrils are results of newly minerals. As shown in Figure 5B, there are mineral aggregating on the fibrils after being incubated for 1 w. Moreover, the deposited minerals in the fibrils exhibited amorphous feature from the electron diffraction pattern (Figure 5B, insert). After incubation for 2 w, the amount of regenerated minerals grow obviously (Figure 5C). The electron diffraction result (Figure 5C, inset) suggests the crystal nature of the newly minerals. The circle-shaped electron diffraction patterns along the (002) and (211) diffraction plane were corresponding to HAp.

There are several limitations in this study. Even the crystal structure of the newly-formed compound in the surface of demineralized dentin induced by PAMAM-PO_3H_2 has been proved using XRD, other properties of the compound should be characterized, e.g. acid resistance. In the future studies, the in vivo test should also be performed.

4. Conclusion

In summary, we applied synthesized PAMAM-PO_3H_2 to induce remineralization of demineralized DTs. The DTs treated with PAMAM-PO_3H_2 has a improvement to seal exposed DTs, compared with DTs treated without PAMAM-PO_3H_2. The PAMAM-PO_3H_2 successfully induced HAp both in demineralized dentin and reconstituted type I collagen matrix, showing PAMAM-PO_3H_2 could be a potential clinical biomaterials for DTs seal in the future.

Declarations

Author contribution statement

Yajie Wen: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Jichao Wang: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Jun Luo: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Jiaojiao Yang: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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