



Deliver protein across bio-barriers via hexa-histidine metal assemblies for therapy: a case in corneal neovascularization model

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ABSTRACT

Because of their high specificity and low side effects, protein drugs possess a substantial global market. However, the low bioavailability of protein is still a major obstacle to their expanded applications, which is expected to be answered with proper protein formulations. Taking corneal neovascularization (CNV) as an example, we demonstrated a co-assembled system of hexa-histidine and Ava (Avastin) with metal ions (HmA@Ava) could cross the cornea, the most important bio-barrier during the treatment of most diseases of the anterior segment in clinics. We found that the nanosized HmA@Ava efficiently encapsulated Ava with impressive loading capacity without destroying the bioactivity of Ava and assisted Ava penetration through the corneal barriers to effectively inhibit CNV development in an alkali burn rat model with sustained and pH-dependent Ava release. Our results suggested that the co-assembled strategy of protein and HmA is a proper formulation to protein drugs, with promising penetration ability to deliver protein across bio-barriers, which could open a path for topical administration of protein drugs for treatment of various ocular diseases and hold enormous potential for delivery of therapeutic proteins not only for ocular diseases but also for other diseases that require protein treatment.

1. Introduction

Compared with small molecular drugs, which dominate the current pharmaceutical market, protein drugs have high specificity and few side-effects [1]. As such, protein-based therapeutics have experienced rapid development in past decades, with sales in the global market of nearly \$250 billion in 2020, which was a more than 40-fold increase in the past 30 years. It is predicted that the development of the market is still in its infancy, and sales will double in the next 5 years. To date, protein drugs have been extensively explored as promising candidates to treat various incurable and chronic diseases, including cancer and diabetes [2]. The structural complexity of proteins offers not only the advantages of potency and high specificity but also the disadvantages of limited ability to cross biological barriers, such as mucosal membranes, skin, and cellular membranes, which give enough time to enzymatic degradation and immune clearance, ultimately resulting in very low bioavailability [3–5]. Low bioavailability gives rise to frequent administration to achieve the

desired therapeutic effect, which leads to poor patient compliance and enormous economic burden [6,7]. Obviously, efficient delivery of protein across bio-barriers to specific sites is an urgent problem to be solved to extend its further applications.

There are diverse bio-barriers, depending on the administration routes and where the protein needs to be delivered, e.g. oral administration vs. harsh acidic and enzymatic environments of the gastrointestinal tract and intestinal epithelium [6], skin administration vs. low-permeable stratum corneum [5], pulmonary and nasal administration vs. tight junctions of the epithelium layer through the entire respiratory tract and immune response [8], etc. Even when protein is injected into the body, the primary mode of administration currently faces various tissue barriers and immune clearance [9]. In addition, the pain, inconvenience, and high skills required for injection into some organs are other major concerns. The eye, as one of the most important organs of humans, accounts for more than 80% of the external information received by the human brain, and its unique anatomical and

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physiological structure makes it hard to directly administer through injection and poses some bio-barriers [10]. Compared with systematic administration, eye drops are the most popular administration method to treat most diseases of the anterior segment in clinics because of their convenience and good patient compliance. Although the concentration of active ingredients in eye drops reached the standard, the overall bioavailability of the drug was still less than 5% [11], even for most small molecular drugs, because of the presence of various bio-barriers in the anterior segment, including the conjunctival barrier, tear film barrier, and corneal barrier [12]. Therefore, the key challenge in the treatment of anterior segment diseases is how drugs cross these biological barriers into the eyes. Among these bio-barriers, the cornea is considered the primary barrier and can be divided into five layers: the epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium [13]. The tight junctions between corneal epithelial cells, with space less than 10 Å (1 nm), restrict the molecules larger than this size from entering [14], which means most of the protein drugs are impossible to cross the cornea.

Corneal neovascularization (CNV) often occurs in a wide variety of corneal pathologies, such as chemical burns, trauma, infectious keratitis, contact lens-related hypoxia, inflammatory disorders, corneal graft rejection, limbal stem cell deficiency, allergy, congenital diseases, and autoimmune diseases [15–17]. CNV-related diseases affect millions of people's daily lives, with an estimated 1.4 million people developing CNV per year, 12% of whom suffer subsequent loss of vision [18]. In CNV, the genesis of abnormalities in the cornea and new blood vessels erupt from pre-existing corneal vasculature in pathological corneas [19]. Avastin (Ava) with a molecular weight (Mw) of 149 kDa, composed of two 214-residue light chains and two 453-residue heavy chains, was engineered to target VEGF-A (vascular endothelial growth factor A) and block angiogenesis [20,21]. Ava has been widely applied in treating neovascularization-related diseases, including cancer and age-related macular degeneration, since it has been approved by the United States Food and Drug Administration in 2004 for clinical use [22,23]. Recently, Ava and its analogous antibodies have been widely used in ophthalmology because of their safety and effectiveness in inhibiting fundus neovascular diseases (diabetic retinopathy, exudative age-related macular disease, neovascular glaucoma) in the clinic [24], which has attracted extensive interest in treating CNV [25]. However, because of the abovementioned bio-barriers of the eye, the bioavailability of biologicals after subconjunctival delivery is less than 10% [26].

Proper formulation or modification of proteins is well accepted to enhance their bioavailability and avoid enzymatic degradation, but it is highly challenged by the sensitivity of the protein quaternary structure to environmental changes (pH, heat, salt, etc.), which could lead to the loss of bioactivity or denaturation of proteins during the formulation process [27]. Although various systems (such as hydrogels, micro-particles/nanoparticles, micelles, etc.) have been explored to formulate protein drugs and successfully deliver proteins through various bio-barriers [28–30], very few have been applied in corneal-related disease treatment [31,32]. Here, we developed a co-assembled system of hexa-histidine and Ava with metal ions (HmA@Ava) under very mild conditions, into which Ava was encapsulated. The physicochemical properties of HmA@Ava and concerns about protein delivery, including loading efficiency and capacity during the co-assembly process, bioactivity, and release profile of encapsulated protein, were thoroughly investigated. The ability of HmA@Ava to across various bio-barriers of eyes received special attention in the alkali burn-induced CNV rat model. Our findings suggested that HmA can be used as a formulation of proteins to nanoparticles with promising penetration ability to deliver proteins across bio-barriers, which could open a path for topical administration of protein drugs for the treatment of various ocular diseases and hold enormous potential for delivery of therapeutic proteins not only for ocular diseases but also for other diseases that require protein treatment.

2. Experimental section

2.1. Materials

Zinc nitrate hexa-hydrate ($\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, $\geq 99\%$), polyvinylpyrrolidone (PVP, Mw~58k), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 40% formaldehyde, acetic acid, and 80% ethanal were purchased from Aladdin (China). Hexa-histidine (His_6) was purchased from Kare Bay Bio-chem, Inc. and used without further purification. Anti-CD31 antibody (ab119339), anti-VEGF-A antibody (ab1316), and goat anti-mouse IgG H&L (Alexa Fluor 594) (ab150116) were purchased from Abcam. FITC (fluorescein isothiocyanate) labeled goat anti-human IgG (H + L) and DAPI were purchased from Beyotime (China). Ava was provided by Roche. Cell Counting Kit-8 (CCK-8) reagent was purchased from Dojindo (Japan). Cell lines were provided by ATCC. Mammalian cell culture media, fetal bovine serum (FBS), optimal cutting temperature (OCT) compound, 4% paraformaldehyde, and ProLong Gold Antifade kits were purchased from Thermo Scientific. F-Ava was Ava labeled with FITC by a previously reported method [33].

2.2. Synthesis and characterization of HmA@Ava particles

2.2.1. Synthesis of HmA@Ava particles

The synthesis of HmA@Ava was conducted by following the drug encapsulation procedure for HmA [34]. In brief, 10 μL of $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (30 mg/mL) was added dropwise to a 200 μL solution containing Ava, His_6 (0.84 mg), and polyvinyl pyrrolidone (1 mg) and buffered with 50 mM HEPES at a pH of 8.0. The entire encapsulating procedure was conducted at 4 °C under sonication. When the HmA@Ava particles were generated, they were collected by centrifugation (10,000 rpm, 10 min) and washed three times with deionized H_2O . The particles were stored at 4 °C and sonicated 1 min before characterization and cellular testing.

2.2.2. Encapsulation efficiency and loading capacity

The Ava encapsulation efficiency (EE%) and loading capacity (LCwt%) were calculated as per previously reported equations [34].

$$EE (\%) = \frac{\text{Vol}_0 \cdot A_0 - \text{Vol}_s \cdot A_s}{\text{Vol}_0 \cdot A_0} \cdot 100\% \quad (a)$$

$$LC (\text{wt}\%) = \frac{W_{\text{added drug}} \cdot EE (\%)}{W_{\text{added polyhistidine}} \cdot \text{Yield} (\%)} \cdot 100\% \quad (b)$$

A_0 and A_s represent the UV-vis absorbance of the original and supernatant solutions of Ava, respectively; Vol_0 and Vol_s are the volume of the original and supernatant solutions, respectively, and W_{Abs} and W_{His_6} are the weight of the added Ava and added His_6 , respectively. The absorbance peaks at 280 nm for Ava were used as the quantitative position, and three repeats were conducted.

2.2.3. Characterization of HmA@Ava particles

Dynamic lighting scattering (DLS) was used to determine the size and zeta potential of the HmA@Ava particles using a Zetasizer Nano ZS instrument (Malvern, UK). Fourier transform infrared spectrometry (FTIR, Bruker TENSOR II) and UV-vis (Perkin-Elmer, Lambda 25, USA) were used to study the encapsulation of Ava into HmA. A field emission scanning electron microscope (FESEM, SU8010 HITACHI) and transmission electron microscope (TEM, Talos F200S FEI) were used to observe the morphology of HmA@Ava particles.

2.3. In vitro test

2.3.1. In vitro release test

The *in vitro* release of Ava from HmA@Ava particles in Bis-Tris buffer solutions (pH = 4.5, 5.8, 6.5, 7.2) was conducted as per a previous

method [34,35]. Briefly, 1 mL of HmA@Ava (containing 5 mg of Ava) was added to a 5 mL dialysis tube (molecular weight cutoff [MWCO]: 300 kDa). Tubes were then incubated in centrifuge tubes filled with 49 mL of Bis-Tris buffer solutions (pH = 4.5, 5.8, 6.5, 7.2) under 200 rpm stirring at 4 °C. One milliliter of the test solution was withdrawn at regular time intervals, followed by the addition of the same volume of fresh medium, followed by quantitative UV-vis analysis. The cumulative release (%) at each time point was obtained following the same calculation method as we reported previously [34].

2.3.2. *In vitro* bioactivity preservation test

The bioactivity preservation of Ava in HmA was detected after the release test. Samples including free Ava, HmA@Ava, and Ava released from HmA@Ava were evaluated with circular dichroism (CD) spectroscopy (Applied Photo-physics Ltd.), separately. In the test, HmA@Ava particles were centrifuged at 12,000 rpm for 12 min, followed by resuspension in 100 mM HEPES (pH = 6) for 1 h. The suspension was dialyzed to remove free His₆ and Zn²⁺ using a dialysis tube (MWCO of 8 kDa) at 4 °C for 24 h. Two hundred microliters of the dialyzed solution (Ava concentration of ~0.01 mg/mL) or free Ava solution (0.01 mg/mL) was examined via CD spectroscopy and analyzed using CDNN software.

2.3.3. *In vitro* cytotoxicity test

Human lens epithelial cells (HLECs) were cultured in DMEM (Dulbecco's Modified Eagle Medium). ARPE-19 (retinal pigmented epithelium) cells were cultured in DMEM:F12. Human corneal epithelial cells (HCECs) were cultured in DMEM with an additional dose of 5 µg/ml insulin. Ten percent FBS, 100 U/mL penicillin, and 100 U/mL streptomycin were added to all the media.

All cells were cultured at 37 °C in a 5% CO₂ atmosphere. Cytotoxicity tests were performed using a CCK-8 assay. The standard procedure was as follows: First, cells were seeded in 96-well plates at a density of 8000 cells per well. After incubation for 24 h, the culture medium in plates was removed, and the cells were washed with PBS (phosphate buffer solution) buffer once. Then, medium containing suspended particles at various concentrations was added. Cells without treatment were used as the control. After being further incubated for a certain time, the plates were washed with PBS buffer three times to remove the treatments. Then, 100 µL of fresh 1640 medium supplemented with 10% CCK-8 solution was added to each testing well, and the cells were incubated for another 2 h. Finally, the plates were read at 450 nm with a microplate reader (Varioskan LUX Multimode Thermo, USA). Five repeats were conducted for each point.

2.3.4. *In vitro* cellular penetration test

HCECs were seeded on a 24-well plate at a density of 50,000 cells per well. After incubation for 24 h, the culture medium in plates was removed, and the cells were washed with PBS buffer once. Then, 500 µL of HmA@Ava (HmA: 50 µg mL⁻¹; Ava: 10 µg mL⁻¹) particles containing medium was added to each well. The free Ava (Ava: 10 µg mL⁻¹) containing medium was used as the control. After 6 h of incubation, the medium was removed, and the cells were washed with PBS buffer three times. Then, the cells were fixed with 4% paraformaldehyde for 20 min and submersed in 1% Triton and 5% goat serum solutions for 1 h. FITC-labeled goat anti-human IgG (H + L) (1:500) was used as the secondary antibody for intracellular Ava staining. Nuclei were stained with DAPI. Subsequently, the samples were mounted with a ProLong Gold Antifade kit and imaged via laser scanning confocal microscopy (LSCM, A1, Nikon).

2.4. *In vivo* test

2.4.1. Animals

Male Sprague-Dawley rats with weight of 180~220 g and male albino rabbits with weight of 2.5~3 kg were used for the study. All animals were in good condition. In all experimental animals, the right eye was

selected as the experimental eye and the left eye as the normal control eye. Before the experiment, the animals were examined by the slit lamp microscope, and the lesions of the anterior segment of both eyes were excluded. The animal experiments were performed in accordance with the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision and were approved by the Animal Experimental Committee of Wenzhou Medical University (Zhejiang province, China).

2.4.2. *In vivo* biocompatibility of HmA

The ocular surface biocompatibility of HmA was studied on male albino rabbits and evaluated by the Draize test [36]. HmA solutions (3.12 mg/mL, 50 µL) were topically administered into the conjunctival sac of the right eyes of rabbits. NaCl solution (0.9%, 50 µL) was administered to the left eyes as the control. The ocular irritation reactions of the rabbits were observed and imaged by a slit lamp at 1 h, 6 h, and 24 h. At 6 h, the corneas were stained with fluorescence sodium to evaluate the integrity of the corneal epithelium. The histology of the cornea was examined via hematoxylin and eosin (H&E) staining at 24 h after instillation.

2.4.3. Alkali burn-induced rat CNV and Ava treatment

Here, alkali burn-induced rat CNV was used to evaluate Ava treatment. Compared with the suture model, the alkali burn model is better to simulate the pathological process of human corneal alkali burn disease and does not cause corneal perforation, which brings the difficulties to following immunofluorescence and determination of neovascularization [37]. In addition, the alkali burn model has low cost and easy operation [37]. Alkali burn-induced CNV is the most widely used model in the study of CNV [38–40].

The alkali burn-induced rat CNV model was established on male Sprague-Dawley rats as previously described [41]. In brief, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (30 mg/kg) for general anesthesia. Proparacaine hydrochloride (0.5%, Alcon, USA) was topically administered for topical anesthesia. Considering the alkali burn model of cornea neovascularization has a highly variable response, the alkali-burned conditions (volume and concentration of sodium hydrate, the immersing time into sodium hydrate solution, and the time to burn) and process (cleaning after burn to remove excess sodium hydrate) were strictly controlled. After the anesthetization takes effect, the pipette gun sucks 4 µL of 1 M sodium hydrate solution and drops it on a circular filter paper with a diameter of 3 mm. After the alkali solution completely saturates the filter paper (timing 1.5 min), wipe off the excess liquid in the conjunctival sac, quickly place the filter paper in the center of the rat cornea (timing 40 s), quickly remove the filter paper, and continuously rinse the conjunctival sac with 30 ml of pre-cooled (4 °C) saline to avoid further burn. Wiping off the excess liquid in the conjunctival sac, 20 µL of 0.9% NaCl solution (NS group), HmA solution (HmA group) (HmA: 3.12 mg/mL), Ava solution (free Ava group) (Ava: 5 mg/mL), or HmA@Ava group (HmA: 3.12 mg/mL; Ava: 5 mg/mL) were immediately administered, three times a day for 7 days. To further avoid the variation of produced results, about 100 male Sprague-Dawley rats were averaged and randomly divided into abovementioned 4 groups (25 rats in each group). More than 6 rats were randomly picked out at designed time points (days 3, 7, and 14 after administration) for assessment of CNV, histology test, or immunostaining test, and the results were reported from the average of these duplications.

2.4.4. Assessment of CNV

All images were pictured by the same angle (from front and side of the eyeball) and same magnification (16X) when the CNV was well focused. The area of CNV was calculated using the following equation:

$$S = \frac{C}{12} \times \pi \times [r^2 - (r - L)^2] \quad (c)$$

where S is the area of CNV, C is the number of limbus hours of CNV, L is the length of CNV (from limbus to the border of the vessel), and r is the radius of the cornea [42]. As per the previous report [43], the neovascularization area was calculated from the slit lamp images of front eyeball, in which the corneal was divided by four quadrants. In each quadrant, S_{quadrant} was calculated by Eq. (c), and S was the sum of S_{quadrant} in four quadrants. C , L , and r were measured with assistance of image J , and the average values of more than 10 times of the random test were used for calculation. The distance from the center of the corneal to the edge at the junction of corneoscleral (can be clearly seen) was used as r . To obtain C , the cornea was circled and clock calibrated, and C was sum hours at the clock calibration. L was the longest neovascularization length in each quadrant.

2.4.5. Precorneal retention time evaluation

The precorneal retention time was evaluated in alkali burn-induced rats and monitored by a multimode optical living imaging system (PerkinElmer IVIS Lumina XRMS Series III American). One drop of free F-Ava, F-HmA, and F-HmA@Ava (20 μL) was instilled into the alkali-burned eyes (left), which were imaged after 0 h, 0.5 h, 1 h, 2 h, and 3 h of instillation. Before imaging, 10% chloral hydrate (0.3 ml/100 g) was injected intraperitoneally for anesthesia, and the head region was imaged using an imaging system equipped with filter sets (excitation/emission, 495/520 nm). F-HmA and F-HmA@Ava were synthesized using the same protocol as HmA and HmA@Ava, but 10% His₆ was replaced with F-His₆.

2.4.6. Corneal distribution of Ava

The rats were anesthetized and sacrificed at 0.5 h, 6 h, and 24 h after corneal injury and Ava treatments. The eyeballs were harvested within 30 min and fixed in 4% paraformaldehyde at 4 °C for 6 h. Then, the eyeballs were cut open, and the crystalline lens was removed. The eyeballs were rapidly embedded in the OCT compound and cut into 5 μm sections. The samples were pre-treated with 1% Triton and 5% goat serum solutions. FITC-labeled goat anti-human IgG (H + L) (1:500) was used as the secondary antibody for Ava staining. Cell nuclei were stained with DAPI. Subsequently, the samples were mounted with a ProLong Gold Antifade kit and imaged via LSCM.

2.4.7. Histology test

The histology test was conducted as previously described [44]. Briefly, the rats were sacrificed at 7 days and 14 days after corneal injury and Ava treatments. Eyeballs were harvested within 30 min and fixed in fixative solution (40% formaldehyde/acetic acid/80% ethanol = 1: 1: 8; v/v/v) at 4 °C overnight. Before embedding, the crystalline lens was removed from the eyeballs. Then, the samples were dehydrated, embedded in paraffin, and cut into 5 μm sections. H&E staining was performed as previously described [44]. The samples were observed and photographed via ortho-microscopy (Nikon, Japan).

2.4.8. Immunostaining test

An immunostaining test was performed to evaluate the presentation and distribution of VEGF-A and CD31. As mentioned previously, the rats were sacrificed at 7 days and 14 days after corneal injury and Ava treatments. Eyeballs were fixed, embedded in the OCT compound, and cut into 5 μm sections. The samples were pre-treated with 1% Triton and 5% goat serum solutions. Anti-CD31 antibody (1:500) and anti-VEGF-A antibody (1:500) were used as the primary antibodies for CD31 and VEGF-A targeting. Goat anti-mouse IgG H&L (Alexa Fluor 594) (1:500) was used as the secondary antibody. After immunostaining, the samples were mounted with a ProLong Gold Antifade kit and imaged via LSCM.

2.5. Statistical analysis

Student's t -test was used to assess the significance of the difference in means between two groups. $p < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS software (IBM SPSS statistics 22.0). The error bars were standard error, which indicates how different the population mean is likely to be from a sample mean.

3. Results and discussion

3.1. Synthesis of HmA@Ava particles

As schematically demonstrated in Fig. 1a, following a previously reported method, HmA@Ava was produced in the presence of Ava in His₆ solution during the formation of HmA by adding zinc ions into His₆ solution at pH ~ 8 [34]. As shown in Fig. 1b, the His₆ solution containing

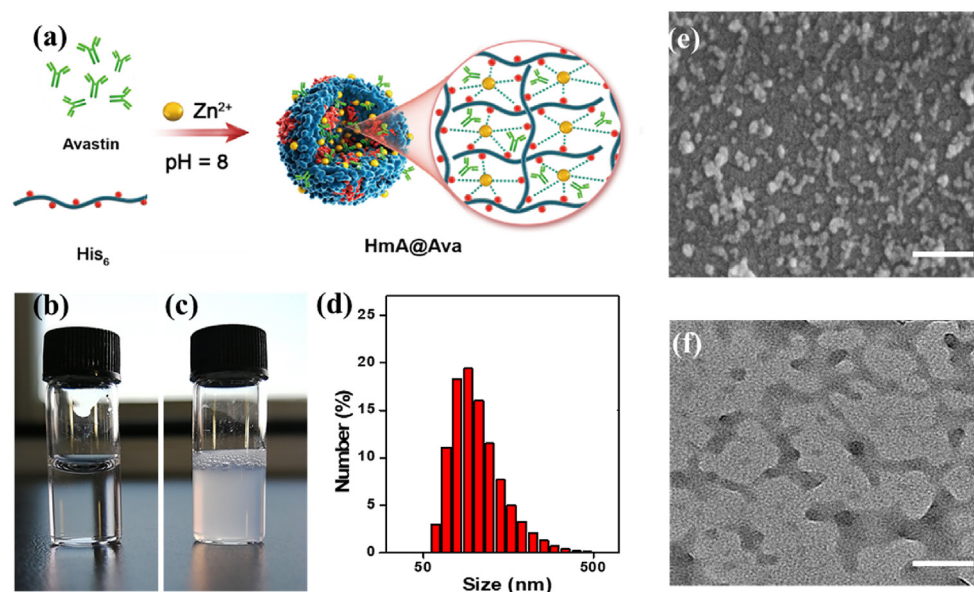


Fig. 1. (a) Schematic showing the formation of HmA@Ava; formation images of HmA@Ava particles before (b) and after (c) the addition of zinc nitrate solution; (d) size distribution of HmA@Ava particles; (e) SEM and (f) TEM images of HmA@Ava particles. The scale bars in (e) and (f) were 200 nm and 50 nm, respectively.

Ava was colorless before the addition of zinc ions but changed to light blue after the addition of zinc ions, which indicated the formation of HmA@Ava particles (Fig. 1c). Because the size and zeta potential of particles are critical characteristics of nanomedicine, a DLS analysis was carried out. In the DLS test, HmA@Ava particles displayed an average size of 169.7 nm (Fig. 1d) and a narrow polymer dispersity index (PDI) of 0.147, whereas the HmA particles were much larger (with an average size of ~60 nm) [34] but had a similar PDI of ~0.15. The increased size of HmA@Ava was probably caused by the loaded Ava. The small PDI values indicated that HmA@Ava has a narrow size distribution. The zeta potential of the HmA@Ava particles was +20 mV, higher than that of HmA (+10 mV) [34]. Notably, these properties (size, size distribution, and zeta potential) of HmA@Ava were maintained even after several weeks, which permitted subsequent delivery of Ava into cells and *in vivo* treatments. Under the SEM (Fig. 1e) and TEM (Fig. 1f), no spherical aggregated particles with irregular shapes were clearly observed.

3.2. Encapsulation and release

The successful loading of Ava into HmA was supported by the UV–vis spectra of HmA@Ava, HmA, Ava, and His₆ (Fig. 1e). In the tested range, no absorbance peak was observed for His₆, but a strong absorbance peak at 280 nm attributed to a typical protein absorbance was observed for free Ava solution. For the generated HmA, the baseline increased because of the light scattering of nanoparticles but did not show an absorbance peak at 280 nm. For HmA@Ava, the baseline also increased, and the absorbance peak of Ava at 280 nm was clearly observed.

Infrared spectroscopy showed the features of Ava, HmA@Ava, and HmA (Fig. 2b). The orange curve indicates the characteristic peaks of Ava, including three strong peaks in the regions of 1656 cm⁻¹ and 1545 cm⁻¹ representing amide I and amide II, respectively, and we can also see the –CH stretch at 2936 cm⁻¹ [45,46]. Interestingly, the amide II region in HmA moving to a short wave is due to the coordination between

the imidazole group in His₆ and zinc ions, which was also mentioned in our previous research [34]. Moreover, the amide II region of HmA is not obvious compared with the protein and the particles containing the protein because HmA is only a peptide of hexa-histidine with fewer amide bonds. Obviously, the characteristic peaks of Ava can be seen in HmA@Ava, which indicates that our drugs are well contained in the carrier.

These data suggested that Ava was successfully loaded into the HmA@Ava particles. Here, PVP (polyvinyl pyrrolidone) acted as a dispersant, as previously reported [47]. Without PVP, the sizes of generated HmA@Ava or HmA were relatively uncontrollable, and a large amount of precipitation was easily formed. As a control, without His₆, PVP and/or Ava did not form any particles or aggregates with zinc ions under the same conditions and the same procedure, suggesting that the presence of His₆ was the only force that generated HmA@Ava or HmA.

EE and *LC* are the two main parameters indicating the loading ability of a vehicle. The *EE* and *LC* of Ava for HmA particles at different drug concentrations were investigated and are shown in Fig. 2a. When the concentration of Ava was 1 mg/mL, the *EE* and *LC* were approximately 94% and 30%, respectively. When the concentration rose to 10 mg/mL, the *EE* and *LC* were approximately 50% and 160%, respectively. In other cases, Inés Luis de Redín et al. [48] reported Ava-loaded HSA nanoparticles displaying a high payload of approximately 13%, and annexin A5-associated phospholipid vesicles reported by Benjamin M. Davis et al. [49] reached a 25% Ava encapsulation efficiency. As far as we know based on reports on Ava encapsulation efficiency, HmA demonstrated the highest *LC* of Ava with a comparatively high encapsulation efficiency. As a control, the generated HmA particles were dispersed into Ava solution (1 mg/mL). After they were repelleted, the *EE* and *LC* were calculated to be approximately 3% and 0.9%, respectively, much lower than the values obtained from the *in situ* encapsulation. These results strongly support that Ava was primarily encapsulated inside the HmA during the formation of HmA@Ava and partially absorbed onto the surface. In HmA@Ava,

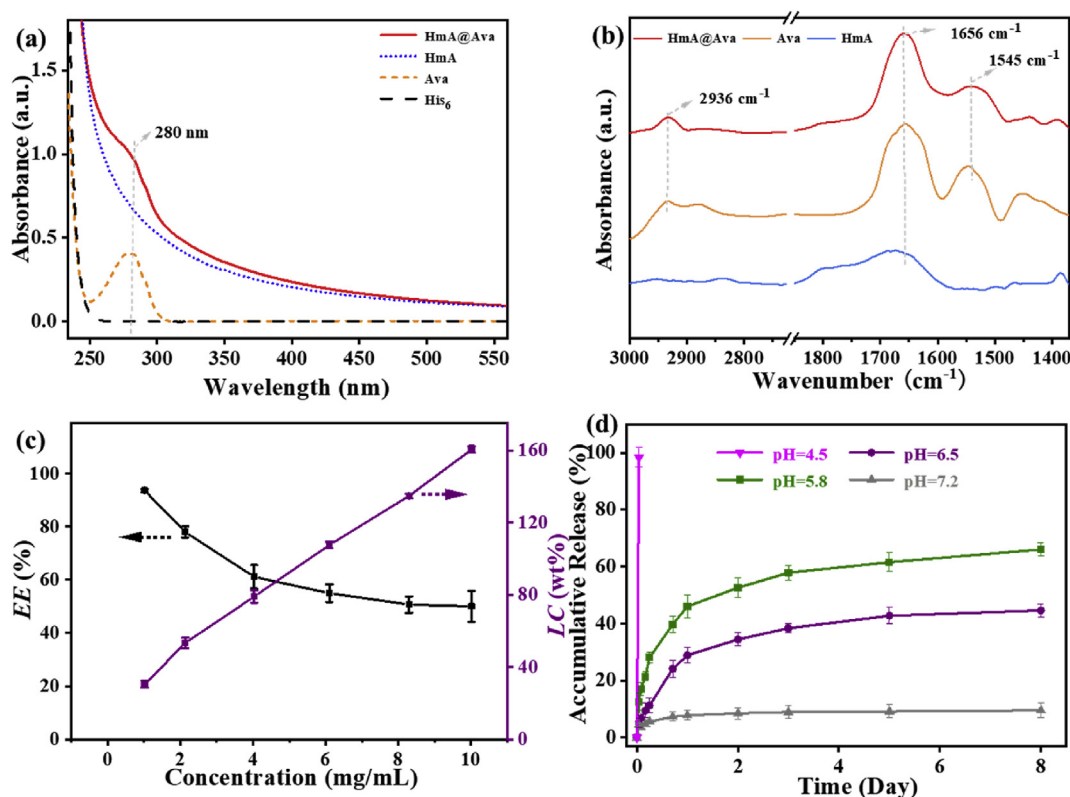


Fig. 2. (a) UV–vis absorbance of HmA@Ava, HmA, Ava, and His₆ solutions; (b) FTIR spectra of Ava, HmA, and HmA@Ava; (c) *EE* and *LC*wt% of Ava for HmA particles at different drug concentrations; (d) *in vitro* release profiles of Ava (5 mg) from HmA@Ava particles at various pH values in Bis-Tris solutions.

His6 and zinc formed a coordinative network in which Ava was trapped, as schematically demonstrated in Fig. 1a. The reasons for the encapsulation of Ava into HmA@Ava with a high EE and LC are multiple and complicated. The coordinative side groups in Ava (such as carboxyl groups from glutamine and aspartic acid, imidazole groups from histidine, and phenol groups from tyrosine) likely take part in the encapsulation during the formation of HmA@Ava. In addition, electrostatic interactions between slightly positively charged His6 and/or zinc ions and negatively charged Ava also contributed. Moreover, other weak interactions, such as hydrogen bonding and hydrophobic-hydrophobic interactions between His6 and Ava, might also play a role in trapping Ava into HmA@Ava. Such a significant improvement in Ava encapsulation efficiency provided a strong advantage in the delivery of Ava. Moreover, for all generated HmA@Ava particles at various Ava concentrations, all the HmA@Ava particles exhibited a nanoscale (<200 nm) size, a small PDI (<0.2), and a positive charge (~20 mV). As reported, nanoscale and positively charged particles are beneficial for effective endocytosis [50]. Therefore, these results indicate effective endocytosis of HmA@Ava particles by corneal cells.

The release profile of Ava from HmA@Ava particles at different pH conditions was conducted in Bis-Tris buffers (pH = 4.5, 5.8, 6.5, or 7.2). As presented in Fig. 2b, Ava showed a burst release (100%) at pH = 4.5, and the particles disappeared at once, which indicated rapid decomposition of HmA@Ava particles at pH = 4.5. At pH = 7.2, Ava showed a continuous and very slow release (10% release of Ava after 5 days). In addition, the lower the pH value, the faster and greater the release of Ava, which demonstrated pH-responsive and sustained release of Ava from the HmA@Ava particles. These results were similar to the previously reported release profile of drugs (fluorescence sodium or dextran-40k) from HmA@drug particles [34]. Slow and sustained release of Ava could provide a long-term effective drug concentration in the cornea. No big difference in released behaviors was observed at 4 °C and 37 °C.

3.3. Bioactivity preservation

Bioactivity preservation is a critical issue for delivering protein drugs because it is related to the therapeutic outcome. Proteins have quaternary structures, which are very fragile and may become deactivated because of their sensitivity to environmental changes (pH, temperature, solvent, salt, etc.) during the particle generation and delivery process. Denaturation of proteins normally accompanies changes in secondary conformation, which can be detected by CD spectroscopy. Therefore, the CD spectra of Ava during the generation of HmA@Ava and the Ava delivery process were recorded. As displayed in Fig. 3a, for the original free Ava (black line), the negative peak with the center at approximately 220 nm was attributed to the α -helix, and the positive peak with the center at approximately 198 nm was assigned to the β -sheet. After forming HmA@Ava, the spectrum curve of Ava in HmA@Ava (red line) was very

similar to that of the original Ava, suggesting that the generation process does not transform the secondary conformation. The curves of Ava showed consistency even though HmA@Ava was incubated in PBS solution at 37 °C for over 24 h (green line) and then released (blue line) under acidic conditions. With the assistance of the affiliated software, the composition of the secondary structure of Ava was analyzed in detail, and the results are shown in Fig. 3b. This explicitly illustrated that the secondary structure did not obviously change, indicating that the function of Ava was well maintained during the processes of generating HmA@Ava, under the tested conditions and during the release of Ava from HmA@Ava.

3.4. In vitro experiments

To fully verify the cytotoxic effect of HmA *in vitro*, we selected three commonly used ocular cell lines as representatives. The cell lines represent cells located in the front (HCEC), middle (HLEC), and back (RPE) of the eyeball. The viabilities of HCECs, HLECs, and ARPE cells treated with HmA for 24 h are shown in Fig. 4a, b and c. All cell types demonstrated good viabilities, ranging from 94.5% to 126%, which indicated low cytotoxicity of HmA in ocular cells.

The main mechanism of CNV formation is increased expression of VEGF-A induced by various factors. Studies have shown that corneal epithelial cells and infiltrating neutrophils can express a large amount of VEGF-A under inflammatory conditions [51]. Therefore, the cell penetration ability of Ava can significantly promote VEGF-A inhibition. To evaluate the cell penetration ability of HmA@Ava, *in vitro* cellular endocytosis was first tested. HCECs were first treated with HmA@Ava particles or free Ava for 6 h. Then, immunostaining of Ava (green) in HCECs was conducted to demonstrate the intracellular Ava. As shown in Fig. 4d, the free Ava group did not show any green fluorescence, whereas the HmA@Ava group demonstrated strong fluorescence inside the HCECs, which indicated more potent cellular endocytosis of HmA@Ava than of free Ava itself. In addition, the pH-responsive release of HmA promoted endosomal escape when HmA@Ava particles were endocytosed by the epithelium or inflammatory cells at the site of injury and facilitated VEGF inhibition.

3.5. In vivo experiments

Before administration to animals, a modified Draize test was performed to evaluate the ocular surface cytotoxicity of HmA [52]. No apparent abnormalities, such as conjunctival congestion and edema, were observed in the anterior segment by slit lamp observation at 24 h after instillation (Fig. 5a). Damage to the corneal epithelium was evaluated by the fluorescein sodium staining test, which is used clinically to assess the integrity of the corneal epithelium [53]. Fluorescein sodium staining of rabbit corneas at 6 h after instillation of HmA particles

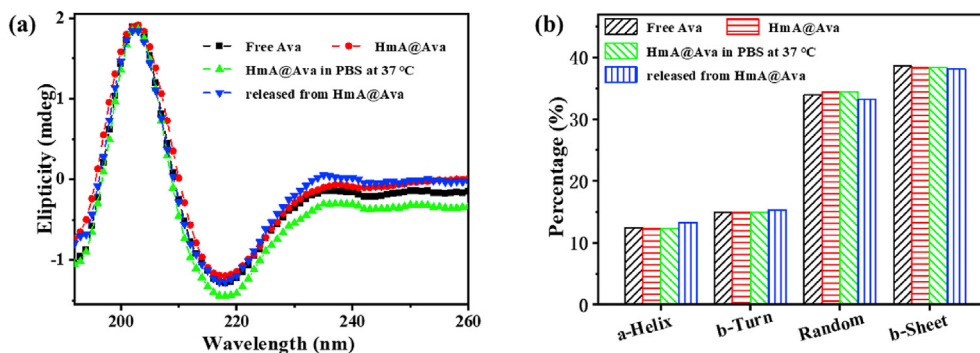


Fig. 3. (a) CD spectra and (b) detailed composition of the secondary structure of Ava in different states; (b) was derived from (a) with the assistance of the affiliated software.

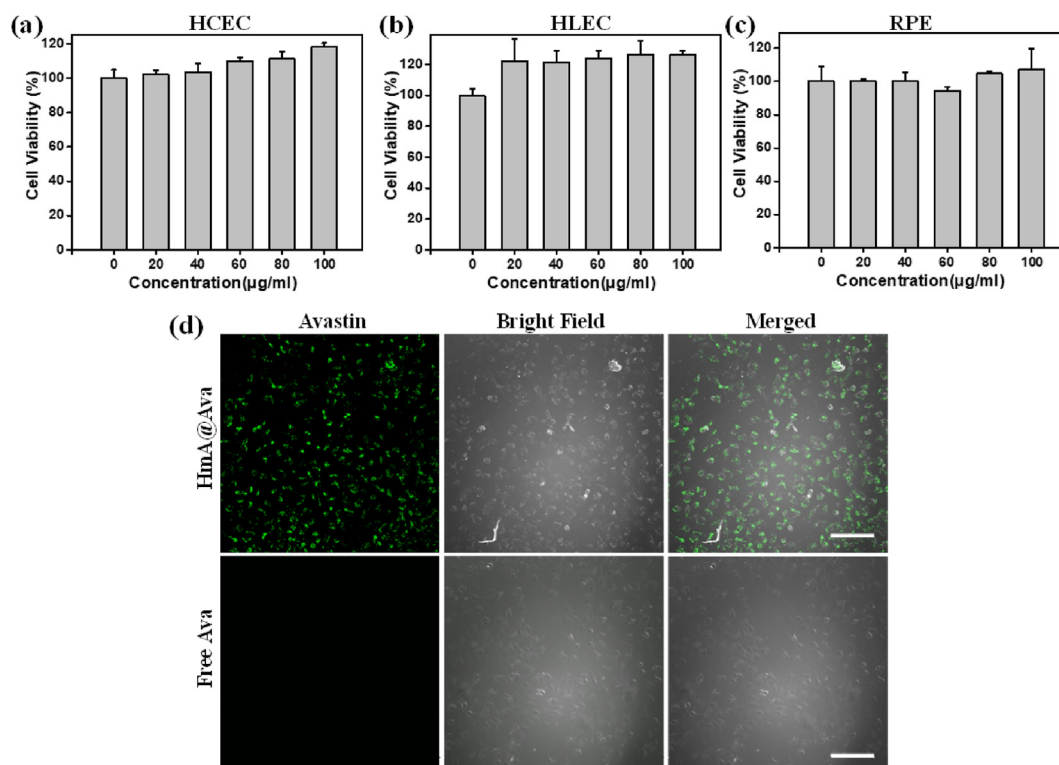


Fig. 4. Viabilities of (a) HCECs, (b) HLECs, and (c) RPE cells after treatment with different concentrations of HmA particles for 24 h; (d) Ava fluorescence staining images of HCECs after treatment with HmA@Ava or free Ava for 6 h; Ava (green). The scale bar is 200 µm.

showed no corneal epithelial defects. Moreover, the H&E-stained histological sections demonstrated a normal morphology and architecture at 24 h after instillation. All results indicated low cytotoxicity of HmA.

The precorneal retention time of drug formulations is one of the most important parameters to the bioavailability of the delivered drugs in eye drops. A longer retention time normally indicates higher bioavailability and better therapeutic outcomes. Fluorescence imaging technology is a well-established method to evaluate precorneal retention *in vivo* and was used to observe the attachment of F-Ava, F-HmA, and Ava@F-HmA onto corneas. From the fluorescent images at different time intervals demonstrated in Fig. 5b, the fluorescent intensities of F-Ava and F-HmA declined much faster than that of Ava@F-HmA at the same tested time. The plot of fluorescent percentage against tested time (Fig. 5c) further supported the quantified fluorescent intensities in Fig. 5b. In detail, it could be easily seen that the fluorescence intensities of F-Ava decreased sharply; at 0.5 h, the fluorescence intensity directly dropped by approximately 80% for F-Ava. Then, the fluorescence intensity continued to decrease and remained at only approximately 10% after 3 h. In contrast, the fluorescence intensity of Ava@F-HmA continued to decrease at the tested time, but the decrease rate was much slower than that of free F-Ava. The fluorescence intensity remained nearly 60% even after 3 h. These results suggested that the formulated Ava by HmA had a much longer attachment time onto precorneal than free F-Ava did, which might bring Ava deeper penetration into the cornea, and better therapeutic outcomes could be expected.

An alkali-burned rat model of CNV was used to evaluate the anti-CNV efficacy of HmA@Ava *in vivo*. This modeling method is one of the simplest and most frequently used methods in CNV animal model preparation [54]. The experimental method was performed as described previously. As per previous observations, the limbus vascular network grows rapidly on the cornea and extends deeply into the cornea 3 days after alkali burn. The vessels reach a growth peak within 7 days and basically stabilize at 14 days [55]. In this article, the rats were divided into 4 groups, namely, a control group, HmA group, free Ava group, and

HmA@Ava group. Eye drops were administered immediately after the alkali burn. The corneal images and quantitative CNV results for rats after alkali burn and drug administration for different times are presented in Fig. 6a and b, respectively. Three days after treatment, CNV began growing from the corneal limbus, with obvious corneal opacity and edema in all groups. After 7 days, the CNV continued to grow in each group, and the edema subsided. The area of CNV in the control group and HmA group demonstrated fast expansion (nearly 80%) but was less in the free Ava group (62.5%) and lowest in the HmA@Ava group (23.6%). Both the free Ava group and HmA@Ava group were significantly different from the control group ($p < 0.01$). The free Ava and HmA@Ava solutions both inhibited the growth of CNV, but HmA@Ava performed much better. This may be attributed to the better penetration ability of HmA@Ava. After 14 days, the CNV was basically stabilized in each group, and the edema had receded. There was no significant difference between the control group (96.2%), HmA group (88.5%), and free Ava group (88.0%). Notably, the HmA@Ava group showed the best and most persistent CNV inhibition efficacy among all the groups (27.7%). The fast CNV growth tendency in the free Ava group may be due to continued secretion of VEGF from corneal epithelial cells and inflammatory cells after Ava administration was stopped [56]. The persistent CNV inhibition efficacy in the HmA@Ava group may be attributed to the corneal penetration ability of HmA@Ava and the sustained release of Ava from HmA after drug withdrawal. Moreover, Ava in the HmA@Ava group (Ava: 5 mg/mL for 7 consecutive days) demonstrated a better inhibition effect than Ava hydrogel (Ava: 12.5 mg/mL for 14 consecutive days) and a CNV inhibition effect comparable with that of Dexp-Ava combination hydrogel (Dexp [dexamethasone]: 5 mg/mL and Ava: 12.5 mg/mL for 14 consecutive days) reported by Huang et al. [31]. This may be attributed to the better corneal penetration ability of HmA than hydrogel. Although the hydrogel can extend precorneal retention, as a macromolecule, Ava alone can rarely penetrate the cornea.

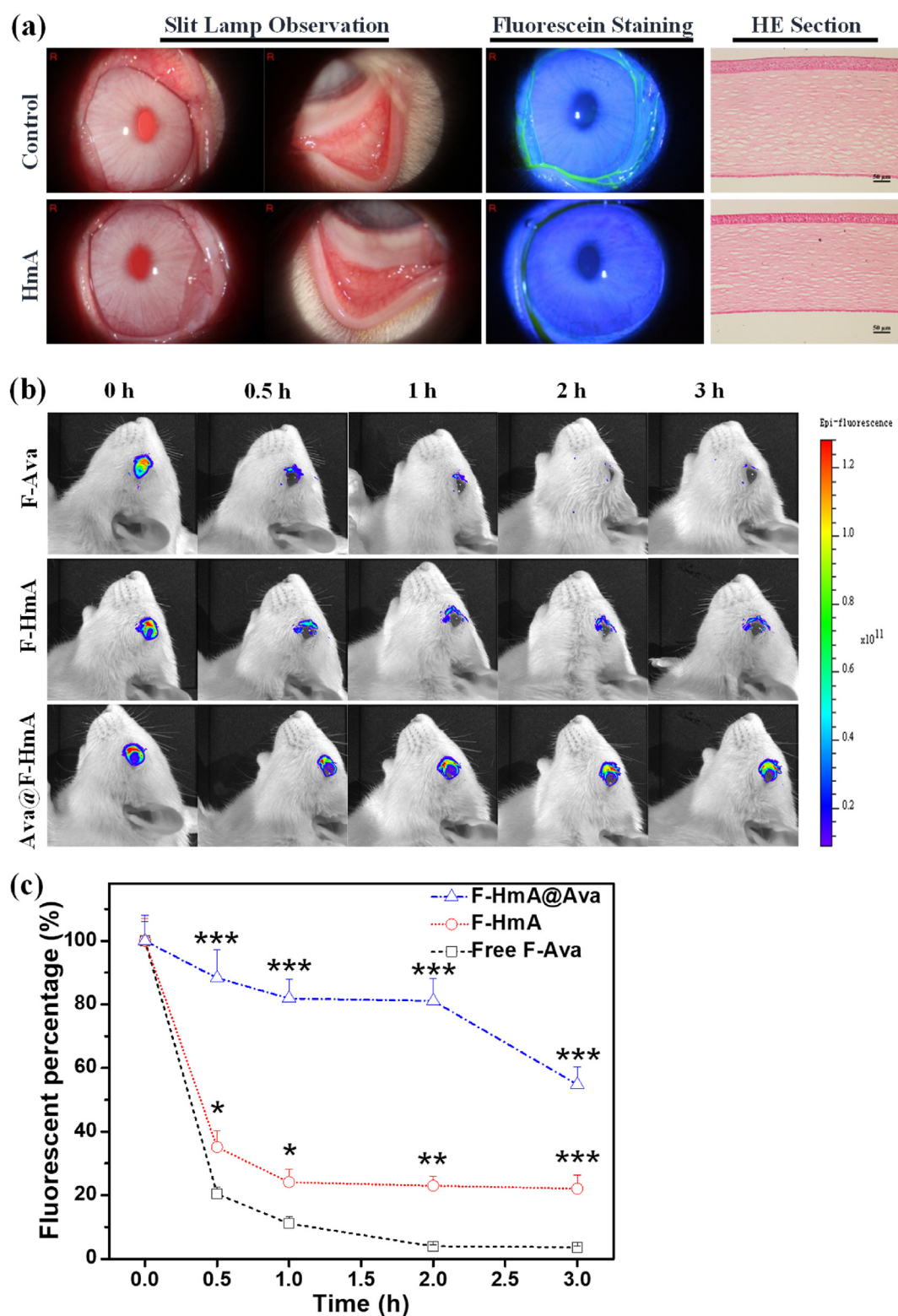


Fig. 5. (a) Clinical signs and histopathological staining of the ocular anterior segment after a single instillation of 50 μ L NS or HmA particle solutions in rabbit eyes; slit lamp observation of the ocular anterior segment 24 h after the instillation; fluorescein staining of the ocular surface 6 h after the instillation; H&E staining images of corneal histopathological sections 24 h after the instillation. The scale bar in the H&E section of (a) is 50 μ m. (b) *In vivo* fluorescence imaging of free F-Ava, F-HmA, and Ava@F-HmA at different time intervals; (c) The quantified fluorescent intensities from (a), the plot of fluorescent percentage against tested time. *Indicates the differences between the control group and the experimental group: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. *In vivo* fluorescence imaging of NP eye drops.

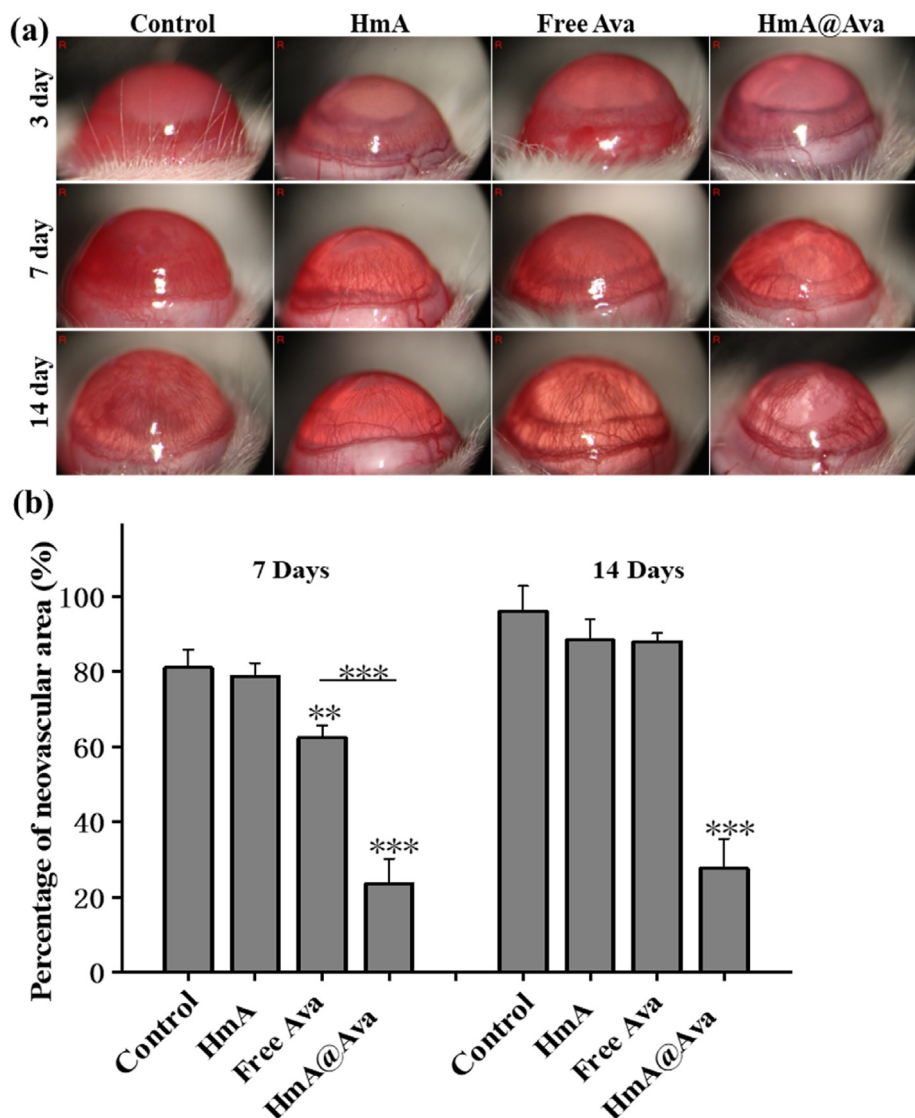


Fig. 6. (a) Representative cornea images (16X) of each group (control group, HmA group, free Ava group, and HmA@Ava group) at different time points (3 days, 7 days, and 14 days) after corneal alkali burn in rats; (b) The area of blood vessel growth 7 days and 14 days after alkali burn; * indicates the differences between the control group and the experimental group: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3.6. Penetration and immunological analysis

Furthermore, the *in vivo* corneal distribution of Ava was investigated to further evaluate the penetration ability of HmA@Ava. The rats were divided into four groups: a control group, HmA group, free Ava group, and HmA@Ava group. The corneas were instilled with normal saline solution (control group), HmA particle solution (HmA group), free Ava solution (free Ava group), or HmA@Ava particle solution (HmA@Ava group) immediately after the alkali burn. Immunostaining of Ava in corneas was conducted at 0.5 h, 6 h, and 24 h after formulation instillation to demonstrate the corneal distribution of Ava. The Ava immunostaining results are presented in Fig. 7. As expected, the control group and HmA group did not show any green fluorescence at any time point. The free Ava group and HmA@Ava group both demonstrated green fluorescence. In detail, Ava was already distributed in the superficial cornea at 0.5 h and penetrated through the corneal stroma at 6 h in both the free Ava group and HmA@Ava group. This verified penetration of Ava into the corneal tissue after free Ava and HmA@Ava instillation. However, only in the HmA@Ava group did Ava arrive at the corneal endothelial layer at 24 h, which showed much brighter green fluorescence than that in the free Ava group throughout the experiment. In

addition, compared with the free Ava group, the HmA@Ava group was able to adhere to much more Ava onto the superficial cornea and corneal stroma before 24 h. These results indicated that HmA@Ava improved the corneal penetration ability of Ava.

To further evaluate the CNV inhibition ability of HmA@Ava, histological analyses were conducted. In terms of inflammation, there are few cells in the normal corneal stromal layer. H&E staining (Fig. 8a) showed that in the control, HmA, and free Ava groups, there were more inflammatory cell infiltrations at 7 days and 14 days, whereas at the same time in the HmA@Ava group, the infiltration of inflammatory cells was very less, even hardly found. The HmA@Ava group exhibited the best therapeutic effect and had recovered to a normal cornea level. From the view of corneal structure, the corneal edema and tissue of the control group and HmA group were very disordered at 7 days and 14 days, whereas they are complete and the edema was basically retracted in the free Ava group and the HmA@Ava group. About new blood vessels, it can be seen from Figs. 6(a) and 8(a) that the new blood vessels in the control group are worst, and they are also present in the stromal layer (the position indicated by the black arrow) in the HmA group and the free Ava group. However, no new blood vessels at all were observed in the HmA@Ava group. Interestingly, in Fig. 8a, the epithelial in the control, HmA, and

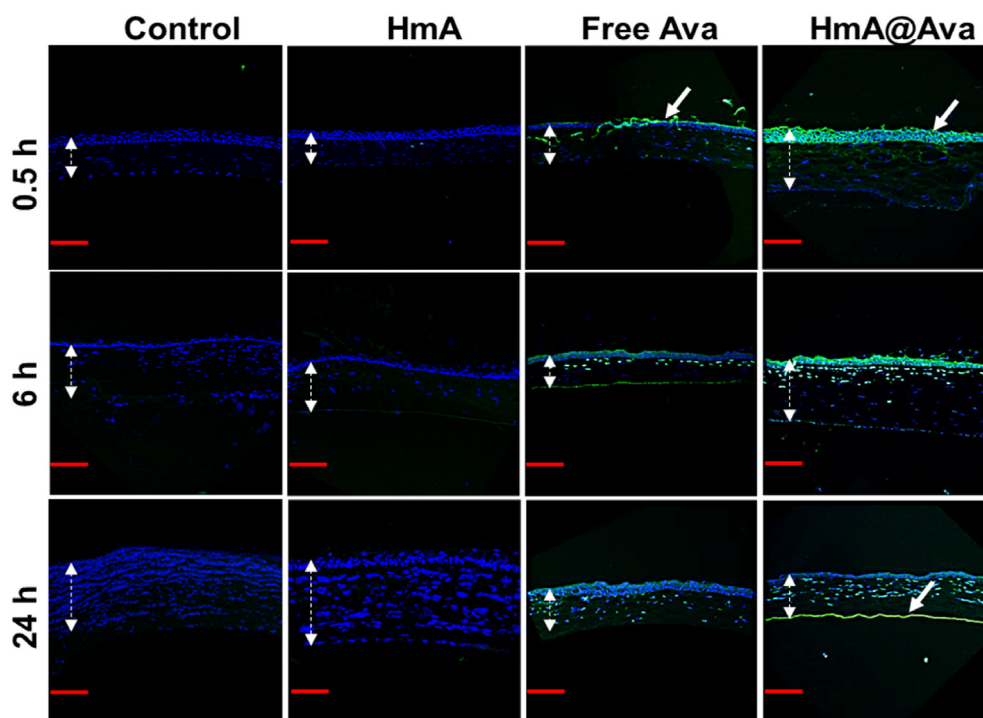


Fig. 7. Fluorescence staining images showing the corneal Ava distribution at 0.5 h, 6 h, and 24 h after induction of rat corneal alkali burns in the control group, HmA group, free Ava group, and HmA@Ava group; DAPI (blue); Ava (green). The scale bar is 20 μm .

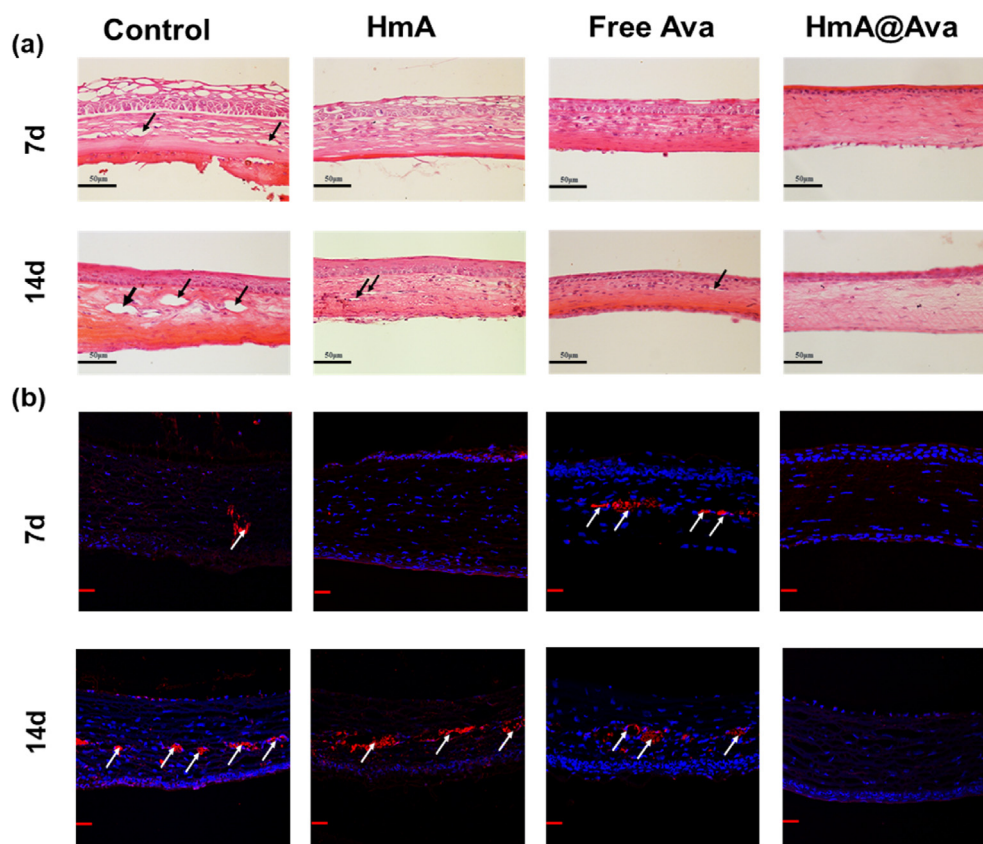


Fig. 8. (a) H&E staining and (b) CD31 fluorescence staining images of rat corneal pathological slices at 7 days and 14 days after alkali burn in each group (control group, HmA group, free Ava group, and HmA@Ava group). The black and white arrows refer to the location of new blood vessels. The scale bars in Fig. a and b are 50 μm and 20 μm , respectively. In Fig. b, DAPI is (blue), and CD31 is (red).

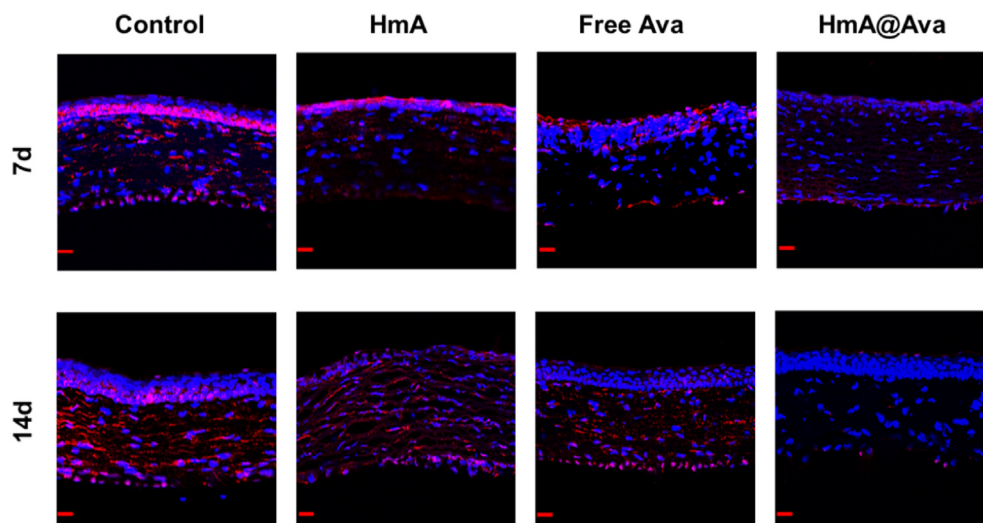


Fig. 9. VEGF-A fluorescence staining images of rat corneal pathological slices at 7 days and 14 days after corneal alkali burn in each group (control group, HmA group, free Ava group, and HmA@Ava group); VEGF-A (red); DAPI (blue). The scale bar is 20 μ m.

free Ava groups was missed on day 7, whereas it is intact in the HmA@Ava group. The missing epithelial in the control, HmA, and free Ava groups is caused by the alkali burn because the 7 days were not long enough to recover the corneal epithelium. The intact corneal epithelium in the HmA@Ava group might be attributed to the therapeutic effect of HmA@Ava (inhibition of CNV, relieved inflammation). It is precisely because of our treatment that the corneal blood vessels caused by alkali burns were reduced, and the inflammation was relieved, and the corneal epithelium can heal more quickly.

Moreover, immunohistochemistry was also conducted to assess CD31 expression in corneal tissue after various treatments at 7 and 14 days; CD31 is a marker of endothelial cells (Fig. 8b). The expression of CD31 was consistent with the clinical observation and H&E staining results. At 14 days, the HmA@Ava group showed the lowest CNV density.

VEGF-A is a key neovascularization regulation factor and can be expressed by many cells [57]. Under inflammation or hypoxia stimulation, the expression of VEGF-A increases and leads to neovascularization [58]. Ava achieves the goal of inhibiting the formation of new blood vessels by binding to VEGF-A and inhibiting its biological activity [59]. Because there is no obvious difference between VEGF-A on day 3 and day 7, only VEGF-A staining images on day 7 and day 14 were given in Fig. 9. On day 7, the expressions of VEGF-A in the cornea of all groups were concentrated in the basal part of the corneal epithelium, which was consistent with a previous report [60]. The control group was similar to the HmA group, showing relatively bright to the Ava group and HmA@Ava group. It is not difficult to see the HmA@Ava group was the weakest and almost invisible, indicating the lowest expression of VEGF-A. These results in Fig. 9 were consistent with abovementioned data in Figs. 6(a) and 8(a). On the 14th day, the expression of VEGF-A in all groups decreased and was likely to distribute in the corneal stromal layer, which was in accordance with a previous report [61]. However, the control group was still the most expressed compared with other groups. For HmA@Ava, the expression remained the least, which further confirmed HmA@Ava had the best therapeutic effect.

Ava inhibited CNV in alkali-burned corneas by blocking VEGF-A and downregulating CD31, which was consistent with the findings of a previous study [62]. However, as a macromolecule, Ava has difficulty penetrating the cornea, which limits its CNV inhibitory effects, especially after healing of the corneal epithelium. However, HmA@Ava suppressed CNV by continually and effectively suppressing VEGF-A. The nanoscale size and positively charged surface of HmA might enhance the corneal

penetration ability, and sustained pH-responsive release may extend the release time of Ava in the cornea. Taken together, HmA is a promising Ava vehicle when used to inhibit CNV.

Zinc is the second most abundant trace metal element in the human body after iron [63], acting as enzyme cofactors to take part in various life activities, such as signal transduction, apoptosis, gene expression, cell proliferation, differentiation, growth, and nucleic acid metabolism in the human body [64]. Compared with other metal ions, zinc ions have a faster metabolic rate and a higher safe concentration range in the human body, and the common dietary deficiencies normally lead to metabolic and organic dysfunctions [63]. As per 'Chinese Residents' Dietary Nutrient Reference Intakes' edition in 2013, the recommended amount of zinc in the dietary nutrient reference intake of adult residents aged 18–49 years is 12.5 mg/day for adult men and 7.5 mg/d for women, and the highest tolerable intake is 40 mg/day. Obviously, the used amount of zinc ions in this work is far below this standard. In the cellular experiments, very low toxicity was found in various ophthalmic cells even for HmA up to 100 μ g/ml (Fig. 4a–c). In vivo (a modified Draize test), no obvious ocular cytotoxicity was produced after administrating HmA, as confirmed by no abnormalities in the anterior segment and normal morphology and structure by H&E staining (Fig. 5a). However, considering many metal ions have long-term cytotoxicity and are highly dangerous to neurocytes, this will bring HmA into very controversial issue when it applies in clinical treatment. Definitely, the long-term cytotoxicity of HmA must be evaluated before any further clinical trial.

4. Conclusions

In summary, we successfully developed a strategy for shepherding Ava through the corneal barrier in the treatment of CNV in an alkali burn rat model. Ava was co-assembled with His₆ and metal to form HmA@Ava with a nanoscale size and positive charge, in which Ava was encapsulated with high *EE* and *LC*. Ava maintained good function during the processes of generating HmA@Ava, after releasing Ava from HmA@Ava, and even after treatment with proteinase. HmA@Ava particles displayed sustained and pH-dependent release of Ava and good biocompatibility with ocular cells and ocular surfaces. In addition, HmA@Ava was able to deliver Ava across the corneal barrier and demonstrated an effective inhibitory effect on CNV development in an alkali burn rat model. The strategy developed here will bring plentiful suggestions for formulating protein nanoparticles with promising penetration ability to deliver protein across bio-

barriers, which might open a path for topical administration of protein drugs for treatment of various ocular diseases and hold enormous potential for delivery of therapeutic proteins not only for ocular diseases but also for other diseases that require protein treatment.

Credit author statement

Hongyan Xu: Writing – original draft; **Bojiao Tang:** Data curation, Formal analysis; **Wenjuan Huang:** Conceptualization; **Shan Luo:** Data curation, Methodology, Software; **Tinghong Zhang:** Methodology, Software; **Jianshu Yuan:** Writing-Reviewing and Editing on revised manuscript; **Qinxiang Zheng:** Investigation, Writing- Reviewing and Editing in original draft; **Xingjie Zan:** Supervision, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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