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**iRGD and TAT co-modified PEG-PLGA nanoparticles for topical ocular delivery of panax notoginseng
saponins: A novel therapeutic strategy for diabetic retinopathy**

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Abstract

Diabetic retinopathy (DR), a progressive microvascular-neurodegenerative disorder of the retina, is the leading cause of irreversible blindness in adults, for which there is no definitive treatment. *Panax notoginseng* saponins (PNS) and their bioactive components ginsenoside Rg1 (GRg1) and ginsenoside Rb1 (GRb1) have tremendous therapeutic potential in alleviating retinal damage via eye drops. However, the efficacy of conventional eye drops is severely constrained by the biological barriers of the eye, such as poor corneal penetration and inadequate posterior segment delivery. To overcome these challenges, this study designed a co-modified nanoparticles (NPs) delivery system by conjugating PEG-PLGA NPs with iRGD (an integrin-targeting peptide) and TAT (a cell-penetrating peptide). Three types of optimized formulations were developed: (1) 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs, (2) 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs and (3) 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs. Comprehensive characterization revealed that these NPs exhibited uniform spherical morphology, nanoscale particle size, high encapsulation efficiency, superior drug loading capacity, and significant sustained release profiles. *In vivo* and *in vitro* experiments demonstrated that the 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs exhibited excellent ocular biocompatibility, and they could significantly enhance the corneal penetration, elevate aqueous humor drug concentrations, increase the amount of ocular tissue distribution, thus markedly improve the therapeutic efficacy in DR models compared to free APIs. In conclusion, the iRGD and TAT co-modified PEG-PLGA NPs delivery system is a topical ocular delivery system with high bioavailability, which provides a novel, safe, and effective therapeutic strategy for the treatment of DR.

Keywords *Panax notoginseng* saponins, Ginsenoside Rg1, Ginsenoside Rb1, Diabetic retinopathy, Ocular delivery system, PEG-PLGA nanoparticles, iRGD peptide, TAT peptide.

Introduction

Diabetic retinopathy (DR), a progressive microvascular-neurodegenerative disorder of the retina, represents the most prevalent and serious ocular complication of diabetes mellitus (DM) and remains the leading cause of adult blindness worldwide [1, 2]. According to the degree of fundus lesions, DR can be classified into two stages: non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR) [3]. The progression of DR begins with NPDR, characterized by microvascular damage which, if uncontrolled, progresses to vision-threatening PDR marked by pathological neovascularization and frequently culminates in retinal detachment and irreversible vision loss. Current therapeutic approaches for DR include intravitreal injection of vascular endothelial growth factor (VEGF) antagonist, laser photocoagulation, and vitrectomy [4], which are partially effective but have a high risk of complications, such as fibrovascular constriction, persistent elevation of intraocular pressure (IOP), postoperative visual field defects and even retinal detachment [5-7]. These limitations underscore the critical need for developing safer and more effective pharmacological interventions capable of halting DR progression while minimizing adverse effects.

Traditional Chinese medicines (TCMs) demonstrate unique advantages in the treatment of DR, especially those with blood-activating and stasis-resolving properties, such as *Panax notoginseng* (Burk.) F. H. Chen [8], *Salvia miltiorrhiza* Bge. [9], *Ligusticum chuanxiong* Hort. [10], and *Angelica sinensis* (Oliv.) Diels [11], making them particularly suitable for DR patients presenting with blood stasis syndrome. Among those, panax notoginseng saponins (PNS), extracted from the main root or rhizome of *Panax notoginseng* and containing over 30 bioactive components including notoginsenoside R1, (NGR1), ginsenoside Rg1 (GRg1), ginsenoside Re (GRe), ginsenoside Rb1 (GRb1), ginsenoside Rd (GRd), exhibit remarkable efficacy in activating blood circulation, removing blood stasis, and promoting the circulation of veins and collaterals [12, 13]. It has been demonstrated that oral PNS could reduce retinal inflammation through the nuclear factor-kappa B (NF- κ B) signaling pathway inhibition, with GRg1

and GRb1 identified as the primary bioactive components [14]. However, PNS faces significant pharmacokinetic challenges, including gastric degradation, poor membrane permeability, and hepatic first-pass effects, resulting in critically low oral bioavailability [15, 16]. The oral bioavailability of PNS has been reported to be only 0.78% for GRb1 [17] and approximately 1.9% ~ 10% for GRg1 [18]. These absorption limitations severely restrict PNS's clinical potential despite its demonstrated therapeutic effects.

Ocular administration enables more efficient drug delivery to ocular tissues, resulting in higher efficacy. Among various ocular delivery methods, eye drops represent the most common non-invasive approach, offering distinct advantages including excellent patient compliance, cost-effectiveness, and ease of use [19]. Our group investigated the feasibility of using PNS eye drops for the treatment of DR, and the results demonstrated that PNS eye drops had a significant therapeutic effect on NPDR. Notably, at a dose of 40 mg/kg, the eye drops achieved comparable efficacy to oral administration (160 mg/kg), suggesting that PNS eye drops may be more advantageous than oral delivery for NPDR treatment [20]. However, PNS faces penetration challenges due to its large molecular mass and poor membrane permeability, which makes it susceptible to ocular barriers such as the tear film, corneal barrier, conjunctival barrier, blood-aqueous barrier, and blood-retinal barrier (BRB) [21]. These barriers hinder efficient drug delivery to the posterior segment of the eye, which is critical for DR treatment. Therefore, designing an advanced topical drug delivery system capable of overcoming these barriers and effectively delivering the drug to the posterior eye segment is essential for improving DR therapy.

Polymeric nanoparticles (1 ~ 1000 nm in size) can effectively deliver drugs to the posterior segment of the eye through passive or active transport [22]. Drug-loaded polymer nanoparticles have multiple pharmacological advantages, including prolonged precorneal retention time, facilitated corneal penetration, and minimized systemic side effects [23, 24]. As an advanced ocular drug delivery system, polymeric nanoparticles also exhibit superior biocompatibility, controlled drug release, high bioavailability, as well as tissue-specific targeting [25, 26]. Among

various biodegradable polymers, poly (ethylene glycol)-poly (lactic-co-glycolic acid) (PEG-PLGA) has emerged as the most extensively investigated and clinically validated material for nanoparticle fabrication [27]. The amphiphilic nature of PEG-PLGA allows for the effective encapsulation of both hydrophilic and hydrophobic drugs, which makes it promising for pharmaceutical applications [28].

Studies have demonstrated that surface-modified PEG-PLGA nanoparticles have higher drug efficacy. Cell-penetrating peptides (CPPs) are a class of short peptides consisting of 5 ~ 30 acids with unique transmembrane transport capabilities. These short peptides can efficiently mediate the crossing of a variety of therapeutic substances (including small molecule compounds, protein drugs, nucleic acid fragments, and nanoparticles) across complex biological barriers such as the corneal barrier and the blood-retinal barrier (BRB) through covalent coupling or non-covalent binding [29]. Among them, the transcriptional activator (TAT) derived from the human immunodeficiency virus HIV-1 is the most representative CPP, which consists of 11 amino acids (Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg [YGRKKKKRRQRRR]). The TAT has strong cationic properties that can be enhanced by the charge interactions to non-selectively enhance the cell membrane permeation efficiency of drugs [30]. Additionally, iRGD is a target-penetrating cyclic peptide consisting of 9 amino acids (Cys-Arg-Gly-Asp-Lys/Arg-Gly-Pro-Asp/Glu-Cys [CRGDK/RGPD/EC]). Its unique three-dimensional spatial structure enables it to specifically recognize and bind to integrin $\alpha_v\beta_3$ receptors expressed in corneal epithelial cells, retinal pigment epithelial cells, and retinal vascular endothelial cells [31]. Studies have shown that the target penetration process of iRGD involves a multi-step cascade reaction: firstly, high-affinity binding to integrin $\alpha_v\beta_3$ occurs via the C-terminal RGD motif; subsequently, specific cleavage occurs under the action of proteases, such as histone proteases, exposing the CendR motif (R/KXXR/K); and finally, the motif binds to neuropilin protein-1 (NRP-1) to activate cellular endocytosis (including multiple pathways such as lattice protein-mediated endocytosis and fiducial protein-dependent endocytosis) [32]. This target penetration property enables iRGD to significantly enhance the

efficiency of drug transport across the corneal and retinal barrier, which is of great value in the treatment of fundus diseases. Therefore, based on the non-selective transmembrane transport advantage of TAT peptide and the integrin target penetration specificity of iRGD peptide, it is expected to synergistically enhance the ocular drug delivery performance of PEG-PLGA nanoparticles.

In this study, three types of iRGD and TAT co-modified PEG-PLGA NP_s were designed by using PNS and its bioactive components, GRg1 and GRb1, as model drugs, with PEG-PLGA, iRGD-PEG-PLGA, and TAT-PEG-PLGA as carriers, respectively. These formulations were systematically evaluated through characterization, *in vitro* release, ocular irritation, corneal permeability, aqueous humor pharmacokinetics, ocular tissue distribution, and pharmacodynamics studies to assess their potential for enhancing ocular drug delivery.

Materials and Methods

Drugs and reagents

PNS (lot: 20220401, containing 10.40% NGR1, 40.07% GRg1, 5.25% GRe, 27.25% GRb1, and 2.02% GRd) was gifted by Zhongheng Group Co., Ltd. (Guangxi, China). GRg1 (lot: J08IB205578) and GRb1 (lot: J19IB217937) were purchased from Yuanye Biotechnology Co., Ltd. (Shanghai, China). iRGD-SH (molecular weight [MW] 1050 Da), TAT-SH (MW 1662.98 Da), maleimide-poly (ethylene glycol)₃₄₀₀-poly (lactic-co-glycolic acid)₁₅₀₀₀ (Mal-PEG₃₄₀₀-PLGA₁₅₀₀₀, MW 18600 Da, lactic acid [LA]: glycolic acid [GA] 50:50, w/w), maleimide-poly (ethylene glycol)₂₀₀₀-poly (lactic-co-glycolic acid)₁₅₀₀₀ (Mal-PEG₂₀₀₀-PLGA₁₅₀₀₀, MW 17200 Da, LA: GA 50:50, w/w) and methoxy-poly (ethylene glycol)₂₀₀₀-poly (lactic-co-glycolic acid)₁₅₀₀₀ (mPEG₂₀₀₀-PLGA₁₅₀₀₀, MW 17000 Da, LA: GA 50:50, w/w) were bought from Meixing Technology Co., Ltd. (Guangdong, China). Span 80, Tween 80, poloxamer 188 (P188), Evan's blue dye, and streptozotocin (STZ) were acquired from Meilun Biological Co., Ltd. (Dalian, China). Tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), interleukin-1 β (IL-1 β), and VEGF kits were acquired from Biotech Well Technology Co., Ltd. (Shanghai, China). Human corneal epithelial cells

(HCE-T) and Dulbecco's Modified Eagle's Medium/Ham's F12 (DMEM/F12) were obtained from Zhong Qiao Xin Zhou Biotechnology Co., Ltd. (Shanghai, China). Fetal bovine serum (FBS) was obtained from Thermo Fisher Scientific (Waltham, USA).

Synthesis and characterization of the conjugated polymers

iRGD-conjugated PEG₃₄₀₀-PLGA₁₅₀₀₀ (iRGD-PEG₃₄₀₀-PLGA₁₅₀₀₀) was synthesized using a one-step method using iRGD-SH and Mal-PEG₃₄₀₀-PLGA₁₅₀₀₀ [33]. Briefly, Mal-PEG₃₄₀₀-PLGA₁₅₀₀₀ was dissolved in acetone, and then the organic solvent was evaporated to make a homogeneous dispersion on the flask wall. The iRGD-SH was dissolved using phosphate-buffered saline (PBS, pH 7.4) and reacted with Mal-PEG₃₄₀₀-PLGA₁₅₀₀₀ at a molar ratio of 1:1 for 48 h at room temperature. The reaction solution was centrifuged at 12,000 rpm for 10 min at 4 °C, then the precipitate was washed three times with PBS to remove the free iRGD-SH and Mal-PEG₃₄₀₀-PLGA₁₅₀₀₀. The collected synthesis products were freeze-dried for 48 h and stored at -20 °C. Synthesis of TAT-conjugated PEG₂₀₀₀-PLGA₁₅₀₀₀ (TAT-PEG₂₀₀₀-PLGA₁₅₀₀₀) followed the same method. The final products were identified using the nuclear magnetic resonance hydrogen (¹H-NMR) spectroscopy method as follows: iRGD-SH and TAT-SH were dissolved in 0.6 mL of D₂O, while Mal-PEG₃₄₀₀-PLGA₁₅₀₀₀, iRGD-PEG₃₄₀₀-PLGA₁₅₀₀₀, Mal-PEG₂₀₀₀-PLGA₁₅₀₀₀, and TAT-PEG₂₀₀₀-PLGA₁₅₀₀₀ were dissolved in 0.6 mL of CCl₃D, respectively. They were subsequently detected via NMR spectroscopy (Ascend™ 600M; Bruker Company, Germany).

Preparation of iRGD-TAT co-modified PEG-PLGA NPs eye drops

5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops were prepared by the double emulsion solvent evaporation method as follows: 15 mg of PNS was dissolved in 1 mL of purified water to form the internal aqueous phase, and 13.5 mg of PEG-PLGA, 0.75 mg of iRGD-PEG-PLGA, and 0.75 mg of TAT-PEG-PLGA was dissolved in 2 mL of dichloromethane containing 0.25% Span 80 to form the organic phase. The internal aqueous phase was

added to the organic phase, and the probe was sonicated for 2 min to form an emulsion. 5 mL of the external aqueous phase containing 3.5% Tween 80 was added to the emulsion, the probe was sonicated for 1 min, and all of it was transferred to the remaining 5 mL of the external aqueous phase. The organic solvent was removed by magnetic stirring (IKA®RT 10, IKA, Germany) at 45 °C for 30 min to obtain 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NP_s suspension. The nanoparticle suspension was added to the ultrafiltration centrifuge filters (50 mL, 30 KDa, Amicon Ultra, Millipore, Germany), centrifuged, and washed three times with purified water to remove the free drug. The collected precipitate was added to an appropriate amount of purified water to formulate a suspension of PNS at a concentration of 50 mg/mL. After adjusting the osmotic pressure with an appropriate amount of sodium chloride (NaCl) to make it isotonic with tear fluid (300 mOsmol/kg), 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops were obtained.

10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NP_s eye drops were prepared by the nanoprecipitation method as follows: 50 mg of GRg1, 42.5 mg of PEG-PLGA, 5 mg of iRGD-PEG-PLGA, and 2.5 mg of TAT-PEG-PLGA were dissolved in 1 mL of acetone-acetonitrile (9:1, v/v) to form an organic phase. The organic phase was added dropwise into 10 mL of aqueous phase containing 1% P188 using a syringe. The organic solvent was removed by magnetic stirring at 1,000 rpm for 3 h to obtain the 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NP_s suspension. The nanoparticle suspension was added to the ultrafiltration centrifuge tube, centrifuged, and washed three times with purified water to remove the free drug. The collected precipitate was added to an appropriate amount of purified water to formulate a suspension of GRg1 at a concentration of 20 mg/mL. After adjusting the osmotic pressure with an appropriate amount of NaCl, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NP_s eye drops were obtained.

10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NP_s eye drops were prepared also by the nanoprecipitation method as follows: 30 mg of GRb1, 25.5 mg of PEG-PLGA, 3 mg of iRGD-PEG-PLGA, and

1.5 mg of TAT-PEG-PLGA were dissolved in 1 mL of methanol-acetonitrile (1:1, v/v) to form an organic phase. The organic phase was added dropwise into 10 mL of aqueous phase containing 1% P188 using a syringe. The organic solvent was removed by magnetic stirring at 1,000 rpm for 3 h to obtain the 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NP_s suspension. The nanoparticle suspension was added to the ultrafiltration centrifuge tube, centrifuged, and washed three times with purified water to remove the free drug. The collected precipitate was added to an appropriate amount of purified water to formulate a suspension of GRb1 at a concentration of 12.5 mg/mL. After adjusting the osmotic pressure with an appropriate amount of NaCl, 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NP_s eye drops were obtained.

Characterization of iRGD and TAT co-modified PEG-PLGA NP_s eye drops

The microstructure of 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NP_s eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NP_s eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NP_s eye drops were observed using transmission electron microscopy (TEM) (JEM-1400 plus, JEOL Ltd, Japan), and the steps are as follows: one drop of NP_s eye drops was taken and evenly applied to the copper mesh, followed by the addition of one drop of 2% phosphotungstic acid (pH 7.0) for staining. After staining, the excess liquid at the edge of the liquid was absorbed by filter paper, dried naturally, and observed.

The particle size, polydispersity index (PDI), and zeta potential of 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NP_s eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NP_s eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NP_s eye drops were determined by Mastersizer Nano ZS (Malvern Instruments, Malvern, UK).

The encapsulation efficiency (EE) and drug loading (DL) of the 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NP_s, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NP_s, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NP_s were determined as follows: 500 μL of the NP_s suspension was accurately added into

the ultrafiltration centrifuge filters (500 μ L, 30 KDa, Amicon Ultra, Millipore, Germany) and centrifuged at 3,500 rpm for 10 min (Centrifuge 5424R, Eppendorf, Germany) to separate the free drug. Quantification of free drug content in the filtrate (W_{free}) was performed using HPLC, with reference to the drug quality evaluation standards established in the 2020 Chinese Pharmacopoeia [34]. The formula for calculating EE (1) and DL (2) is as follows:

$$EE(\%) = \frac{W_{total} - W_{free}}{W_{total}} \times 100 \quad (1)$$

$$DL(\%) = \frac{W_{total} - W_{free}}{W_{nanoparticles}} \times 100 \quad (2)$$

***In vitro* release study**

The dialysis bag method was used to study the drug release behavior [35]. Briefly, 5 mL of PNS eye drops, 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NP_s eye drops (containing 250 mg of PNS), GRg1 eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NP_s eye drops (containing 100 mg of GRg1), GRb1 eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NP_s eye drops (containing 62.5 mg of GRb1) were respectively loaded into dialysis bags and then placed into 100 mL of PBS (pH 7.2) at 37 ± 1 °C and 100 rpm. 1 mL of the samples was taken at 0.5, 1, 2, 4, 6, 8, 12, and 24 h, respectively, and the samples were replenished with an equal volume of release medium. The samples were centrifuged at 15,000 rpm for 10 min, and the amount of drugs in the supernatant was determined by HPLC [34]. The experiment was carried out in triplicate, and the *in vitro* release of the drug was calculated according to the formula (3).

$$Q = \frac{C_n V_2 + (C_{n-1} + \dots + C_2 + C_1) V_1}{M} \times 100\% \quad (3)$$

Where Q is the degree of *in vitro* release of the drug, C_n is the concentration of the drug measured at the n th sampling point, V_2 is the sum of the volumes of the release medium and the liquid in the dialysis bag, V_1 is the sampling volume, and M is the total amount of drug.

Eye irritation study

12 healthy rabbits without eye diseases were randomly divided into 4 groups. During the experiment, the lower eyelids of the rabbits were gently lifted to form a ring around the conjunctival sac, and then 200 μ L of saline, 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops were single dose administered into the conjunctival sacs in both eyes. After administration, the upper and lower eyelids were gently closed, and the eyes were closed for 8 ~ 10 s. The cornea, iris, and conjunctiva tissues of the rabbits were observed at 1, 2, 4, 24, 48, and 72 h, and the degree of irritation was scored and judged according to the scoring criteria of the Draize experiment [36]. At the end of the observation, the animals were anesthetized and executed, the eyeballs were removed, fixed with 4% paraformaldehyde solution, and stained with hematoxylin-eosin (H&E) to observe the pathological morphology of the cornea, iris, and conjunctiva of the rabbits.

Another 12 healthy rabbits were also randomly divided into 4 groups, and then 200 μ L of saline, 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops were administered into the conjunctival sacs in both eyes twice a day at an interval of 1 h for 1 month. After the last administration, the degree of irritation was scored, and the pathological morphology of the cornea, iris, and conjunctiva was observed by the same method.

Corneal cell permeability study

Corneal permeability studies were performed using an immortalized HCE-T cell model. HCE-T cells were cultured with DMEM/F12 containing 10 % FBS (v/v), and 0.5 mL of HCE-T cell suspensions (cell number of 1×10^5) were added to supply chamber of polyester membrane Transwell®-Clear coated with rat tail collagen (1.12 cm² surface area with a pore size of 0.4 μ m, Corning Co., Ltd., Shanghai, China), while 1.5 mL culture medium were added to receiving chamber. The HCE-T cells were maintained at 37 °C under 95%O₂/5%CO₂, and the culture medium was

changed every other day. After 2 ~ 3 weeks, trans-epithelial electrical resistance (TEER) was measured using a Millicell ERS meter (Millipore Corporation, Bedford, MA, USA), and the HCE-T cell layers with TEER values of 200 $\Omega \cdot \text{cm}^2$ were used for transport studies. The culture medium in the supply and receiving chambers was aspirated, and Hanks' Balanced Salt Solution (HBSS) preheated to 37 °C was added and equilibrated in the incubator for 20 min. The HBSS was aspirated, and 0.5 mL of PNS eye drops, 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops (containing 500 μg of PNS), GRg1 eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops (containing 200 μg of GRg1), GRb1 eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops (containing 125 μg of GRb1) were added to the supply chambers, while 1.5 mL HBSS was added to the receiving chamber. 200 μL of samples were collected from the receiving chamber at 30, 60, 90, and 120 min, and then an equal volume of HBSS preheated to 37 °C was immediately added to the receiving chamber to maintain a constant volume. The experiment was repeated 6 times, and the drug content in the samples was analyzed by UPLC-MS/MS as described in reference [14], and the apparent permeability coefficient (P_{app}) of the drug was calculated according to the formula (4).

$$P_{app} = \frac{dC}{dt} \times \frac{1}{A \times C_0} \quad (4)$$

Where P_{app} is the apparent permeability coefficient, dC/dt denotes the amount of drug obtained by the receiving cell per unit time, A denotes the membrane surface area of the Transwell, and C_0 denotes the starting concentration of the drug.

Aqueous humor pharmacokinetic study

36 healthy rabbits without eye diseases were randomly divided into 6 groups. During the experiment, the lower eyelids of the rabbits were gently lifted to form a ring around the conjunctival sac, and then 200 μL of PNS eye drops, 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, GRg1 eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, GRb1 eye drops, and 10% iRGD-5% TAT co-modified

253 GRb1@PEG-PLGA NPs eye drops were administered into the conjunctival sacs in both eyes. After administration,
254 the upper and lower eyelids were gently closed and the eyes were closed for 8 ~ 10 s, and approximately 50 μ L of
255 aqueous humor was withdrawn from the corneal limbus with a syringe to Eppendorf tubes at 0.083, 0.25, 0.5, 0.75,
256 1, 1.5, 2, 4, 6, 8, 12, and 24 h. The aqueous humor samples were processed using the following method: 10 μ L of
257 sample was mixed with 90 μ L of saline, 300 μ L of methanol, and 100 μ L of internal standard solution (saikosaponin
258 A, 20 ng/mL). After vortexing for 3 min and centrifugation at 15,000 rpm for 10 min, 400 μ L of supernatant was
259 dried under nitrogen and reconstituted in 100 μ L of 70% methanol, and then the supernatant was analyzed using
260 UPLC-MS/MS according to the method described in reference [14].

261 **Ocular tissue distribution study**

262 108 healthy rats without eye disease were randomly divided into 3-time point groups of 36 for each. The 36 rats
263 at each time point were further randomly divided into 6 groups of 6 for each. During the experiments, the lower
264 eyelids of rats were gently lifted to form a ring around the conjunctival sac, and then 20 μ L of PNS eye drops, 5%
265 RGD-5 % TAT co-modified PNS@PEG-PLGA NPs eye drops, GRg1 eye drops, 10% iRGD-5 % TAT co-modified
266 GRg1@PEG-PLGA NPs eye drops, GRb1 eye drops, and 10% iRGD-5 % TAT co-modified GRb1@PEG-PLGA
267 NPs eye drops were administered into the conjunctival sacs in both eyes. After administration, the upper and lower
268 eyelids were gently closed, and the eyes were closed for 8 ~ 10 s. At 0.5, 2, and 4 h, the rats were anesthetized and
269 euthanized. The eyeballs were promptly enucleated, and the aqueous humor was collected. The cornea, sclera,
270 retina, and vitreous humor were dissected and transferred into pre-labeled Eppendorf tubes. After precise weighing,
271 the solid tissues were homogenized in saline (1:40, g/mL), followed by centrifugation at 15,000 rpm for 10 min.
272 For analysis, 100 μ L of the supernatant (or 10 μ L of aqueous humor mixed with 90 μ L of saline) was combined
273 with 300 μ L of methanol and 100 μ L of internal standard solution (saikosaponin A, 20 ng/mL). The mixture was
274 vortexed for 3 min and centrifuged (15,000 rpm, 10 min). Subsequently, 400 μ L of the supernatant was evaporated

under nitrogen and reconstituted in 100 μ L of 70% methanol. After centrifugation (15,000 rpm, 10 min), the drug content in the supernatant was quantified via UPLC-MS/MS, as previously described [14].

NPDR efficacy study

112 healthy rats without eye disease were intraperitoneal injections with STZ (65 mg/kg) dissolved in sodium citrate buffer solution (pH 4.5). After 7 days, rats with fasting blood glucose (FBG) ≥ 16.67 mmol/L were classified as diabetic rats, and then these diabetic rats were randomly divided into a model group and six administration groups of 16 for each. The other 16 rats were injected intraperitoneally with blank sodium citrate buffer solution as the control group. After 2 months, the rats in the administration groups were respectively treated with PNS eye drops, 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops (40 mg/kg), GRg1 eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA eye drops (16 mg/kg), GRb1 eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops (10 mg/kg) in both eyes at 20 μ L each time, 6 times a day, at an interval of 1 h each time, and were administered continuously for 1 month. Saline of equal frequency and equal amount was administered to both eyes of rats in the control and model groups, respectively. Throughout the experimental period, the body weight and FBG of the rats were monitored. Upon treatment completion, four rats per group were subjected to retinal acellular capillary analysis, H&E staining, and immunofluorescence staining. Six rats per group underwent Evan's blue dye leakage assay, while four additional rats per group were used for retinal leukocyte adhesion assessment. The remaining animals were euthanized under anesthesia, with serum and retinal tissues collected and stored at -80 $^{\circ}$ C. The methods of detection of relevant indices were referred to in the reference [14].

Data analysis

Data analyses were carried out using GraphPad Prism 8 statistical software and were expressed as mean $\pm$ standard

deviation (SD). The significance of differences between groups was assessed via one-way ANOVA, followed by Bonferroni post-hoc tests, with statistical significance defined as $P < 0.05$.

Results and discussion

Characterization of the conjugated polymers

iRGD and TAT co-modified PEG-PLGA NPs were prepared by conjugating the drugs with PEG-PLGA, iRGD-conjugated PEG-PLGA, and TAT-conjugated PEG-PLGA. In this study, iRGD-PEG₃₄₀₀-PLGA₁₅₀₀₀ was synthesized through the addition reaction between iRGD-SH's sulfhydryl groups and maleimide groups in Mal-PEG₃₄₀₀-PLGA₁₅₀₀₀, while TAT-PEG₂₀₀₀-PLGA₁₅₀₀₀ was similarly prepared using TAT-SH and Mal-PEG₂₀₀₀-PLGA₁₅₀₀₀ [32]. The ¹H-NMR of iRGD-SH, Mal-PEG₃₄₀₀-PLGA₁₅₀₀₀, iRGD-PEG₃₄₀₀-PLGA₁₅₀₀₀, TAT-SH, Mal-PEG₂₀₀₀-PLGA₁₅₀₀₀, and TAT-PEG₂₀₀₀-PLGA₁₅₀₀₀ is shown in Fig. 1. Mal-PEG₃₄₀₀-PLGA₁₅₀₀₀ and Mal-PEG₂₀₀₀-PLGA₁₅₀₀₀ showed a distinctive single peak at δ (ppm) ≈ 6.7 corresponding to maleimide double bonds. Notably, this peak disappeared in both iRGD-PEG₃₄₀₀-PLGA₁₅₀₀₀ (abbreviation iRGD-PEG-PLGA) and TAT-PEG₂₀₀₀-PLGA₁₅₀₀₀ (abbreviation TAT-PEG-PLGA), confirming a near-complete reaction of iRGD-SH and TAT-SH with Mal-PEG-PLGA and successful synthesis of the target conjugates.

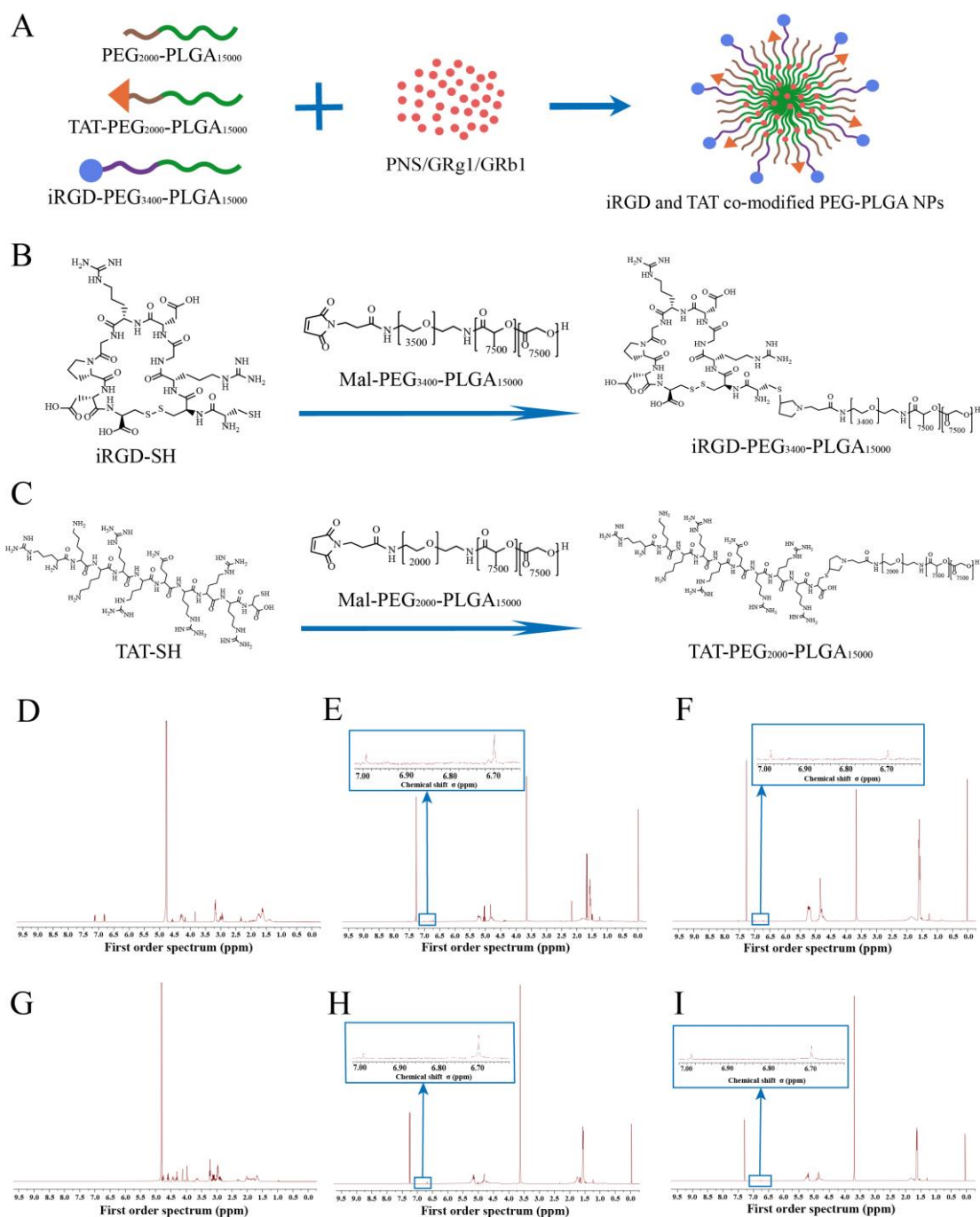


Fig. 1 Schematic of the synthesis and characterization of the conjugated polymers. (A) Schematic of the preparation of iRGD and TAT co-modified PEG-PLGA NPs. (B) Schematic of the synthesis of iRGD-PEG₃₄₀₀-PLGA₁₅₀₀₀. (C) Schematic of the synthesis of TAT-PEG₂₀₀₀-PLGA₁₅₀₀₀. (D) Nuclear magnetic resonance hydrogen spectroscopy of iRGD-SH. (E) Nuclear magnetic resonance hydrogen spectroscopy of Mal-PEG₃₄₀₀-PLGA₁₅₀₀₀. (F) Nuclear magnetic resonance hydrogen spectroscopy of iRGD-PEG₃₄₀₀-PLGA₁₅₀₀₀. (G) Nuclear magnetic

resonance hydrogen spectroscopy of TAT-SH. (H) Nuclear magnetic resonance hydrogen spectroscopy of Mal-PEG₂₀₀₀-PLGA₁₅₀₀₀. (I) Nuclear magnetic resonance hydrogen spectroscopy of TAT-PEG₂₀₀₀-PLGA₁₅₀₀₀.

Characterization of iRGD and TAT co-modified PEG-PLGA NPs eye drops

PEG-PLGA nanoparticles are commonly prepared by nanoprecipitation, solvent diffusion, and double emulsion solvent evaporation methods [37-39]. Due to the poor water solubility of GRg1 (0.21 mg/mL) and GRb1 (0.58 mg/mL), the nanoprecipitation method was used to prepare 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs in this study. Notably, the saponin structure contains both hydrophilic and hydrophobic groups, enabling spontaneous formation of inner hydrophobic and outer hydrophilic micelles in aqueous solution [40]. This amphiphilic property, combined with the presence of NGR1, GRg1, GRe, GRb1, and GRd, significantly enhances PNS water solubility (>1,000 mg/mL). Consequently, 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs were prepared using the double emulsion solvent evaporation method.

The prepared 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs exhibited spherical morphology with uniform particle size distribution and no apparent aggregation (Fig. 2A). As shown in Table 1, the 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs measured 305.26 ± 16.98 nm in diameter, while the 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs and the 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs showed slightly smaller particle sizes with 276.06 ± 8.20 nm and 263.60 ± 14.20 nm, respectively. The particle size of the nanoparticles further affects the amount of iRGD and TAT modification. Surface modification of PNS@PEG-PLGA NPs with 10% iRGD and 5% TAT resulted in particle size of more than 400 nm, which is not favorable for cellular uptake (Fig. 2B). Therefore, we determined 5% iRGD-5% TAT to be the optimal modification ratio for PNS@PEG-PLGA NPs, while the PNS@PEG-PLGA NPs and PNS@PEG-PLGA NPs were

10% iRGD-5% TAT.

Due to the complex composition of PNS, the EE of PNS was calculated as the sum of EE values for its major components (NGR1, GRg1, GRe, GRb1, and GRd). In 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs, the individual EE values were $47.44 \pm 3.16\%$ (NGR1), $54.28 \pm 3.27\%$ (GRg1), $56.02 \pm 2.98\%$ (GRe), 100% (GRb1), and 100% (GRd), yielding a total PNS EE of $72.30 \pm 1.25\%$. Comparatively, the EE was $50.79 \pm 0.47\%$ for 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs and $97.60 \pm 0.75\%$ for 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs (Table 1). Among the internal components of PNS, GRb1, and GRd (protopanaxadiol/PPD-type saponins) had higher EE values than NGR1, GRg1, and GRe (protopanaxatriol/PPT-type saponins), which can be explained by their structural differences [41]. Compared with PPT-type saponins, PPD-type saponins are more lipid soluble, easier to bind to PEG-PLGA, and have a higher EE [42]. In addition, the study also found that the EE of GRg1 and GRb1 in 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs was higher than that of GRg1 in 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs and GRb1 in 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs. This phenomenon may be attributed to the fact that GRg1 and GRb1 in PNS bind not only to the excipient PEG-PLGA but also to the internal components, thus reducing the amount of the drug in the free state. Furthermore, in terms of the 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs, the EE of the GRg1 only reached a maximum of $49.99 \pm 0.57\%$, while GRb1 reached $97.50 \pm 0.75\%$. The reason for this phenomenon may be related to the solubility of GRg1 and GRb1. GRb1 is more lipid soluble than GRg1 and binds more readily to excipients, resulting in a higher EE.

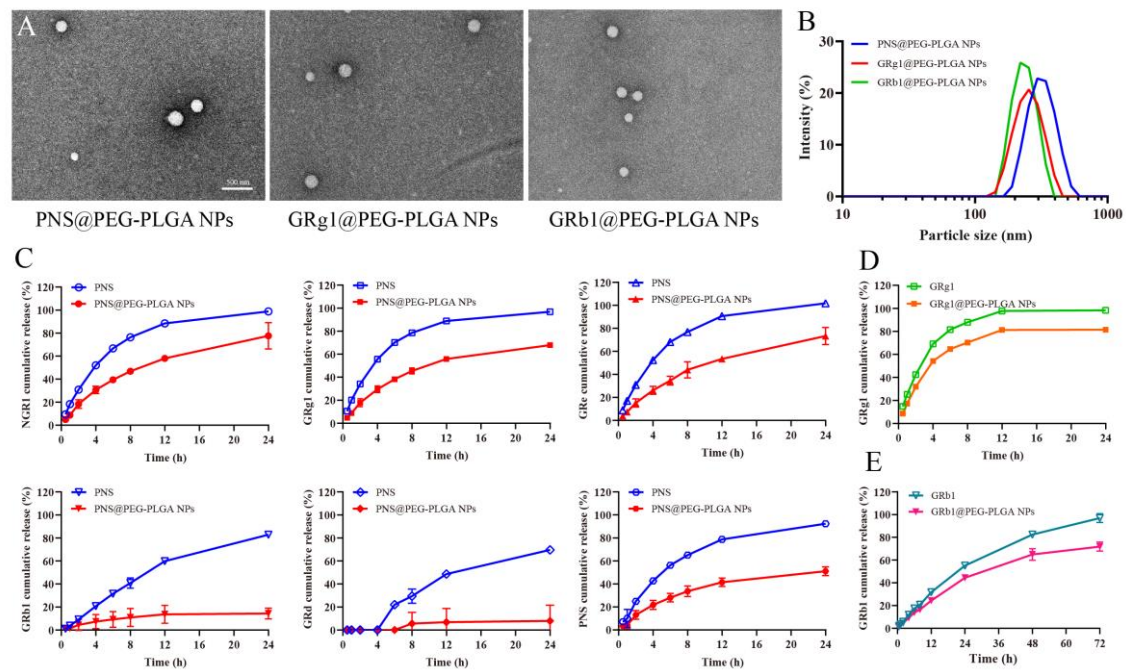


Fig. 2 Characterization and *in vitro* release of iRGD and TAT co-modified PEG-PLGA NPs eye drops. (A) Transmission electron microscope images. (B) Particle size distribution diagram. (C) *In vitro* release profiles of NGR1, GRg1, GRe, GRb1, GRd, and PNS in PNS eye drops and 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops ($n = 3$). (D) *In vitro* release profiles of GRg1 eye drops and 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops ($n = 3$). (E) *In vitro* release profiles of GRb1 eye drops and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops ($n = 3$).

365 **Table 1** Related parameters of iRGD and TAT co-modified PEG-PLGA NP_s eye drops (*n* = 3).

Parameters	A						B	C
	NGR1	GRg1	GR _e	GRb1	GR _d	PNS		
Particle size (nm)			305.26 ± 16.98				276.06 ± 8.20	263.60 ± 14.20
Polydispersity index			0.310 ± 0.024				0.234 ± 0.014	0.203 ± 0.025
Zeta potential (mV)			-10.8 ± 1.2				-11.5 ± 1.3	-10.9 ± 1.3
Encapsulation efficiency (%)	47.44 ± 3.16	54.28 ± 3.27	56.02 ± 2.98	100 ± 0.00	100 ± 0.00	72.30 ± 1.25	50.79 ± 0.47	97.60 ± 0.75
Drug loading (%)	2.91 ± 0.04	13.35 ± 0.22	1.73 ± 0.09	14.56 ± 0.00	1.09 ± 0.00	33.36 ± 0.28	33.73 ± 0.18	49.38 ± 0.19

366 Notes: A: 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NP_s eye drops; B: 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NP_s eye drops; C: 10% iRGD-5% TAT
367 co-modified GRb1@PEG-PLGA NP_s eye drops.

368

***In vitro* release study**

Controlled drug release from ocular delivery systems is critical for therapeutic efficacy [43]. The cumulative release degree-time profiles of PNS eye drops, 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NP_s eye drops, GRg1 eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NP_s eye drops, GRb1 eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NP_s eye drops are shown in Fig. 2C-E. The cumulative release degrees of NGR1, GRg1, GRe, GRb1, GRd, and PNS in PNS eye drops at 24 h were $98.88 \pm 2.20\%$, $96.89 \pm 1.85\%$, $101.74 \pm 1.67\%$, $82.87 \pm 0.90\%$, $69.67 \pm 2.71\%$, and $92.35 \pm 1.58\%$, respectively, whereas the cumulative release degrees of NGR1, GRg1, GRe, GRb1, GRd, and PNS in 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NP_s eye drops at 24 h was $77.64 \pm 11.41\%$, $67.87 \pm 1.29\%$, $73.40 \pm 7.41\%$, $14.40 \pm 4.77\%$, $7.98 \pm 13.82\%$, and $51.05 \pm 3.77\%$, respectively (Fig. 2C). The GRg1 eye drops were almost completely released at 24 h with a cumulative release degree of $98.40 \pm 2.65\%$, whereas the cumulative release of 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NP_s eye drops at 24 h was $81.44 \pm 1.03\%$ (Fig. 2D). The GRb1 eye drops was almost completely released at 72 h with a cumulative release degree of $96.96 \pm 3.95\%$, whereas the cumulative release degree of 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NP_s eye drops at 72 h was $72.01 \pm 3.94\%$ (Fig. 2E). 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NP_s eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NP_s eye drops and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NP_s eye drops had a slowing effect on the release degree of active pharmaceutical ingredient (API) eye drops, demonstrating that the backbone structure of PEG-PLGA could prevent drug diffusion.

In 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NP_s eye drops, the release degree of NGR1, GRg1, and GRe was faster than GRb1 and GRd, and the different release degree may be related to the structure of the drug [42]. Compared to the PPT-type saponins NGR1, GRg1, and GRe, the PPD-type saponins GRb1 and GRd are more lipophilic, bind more readily to the excipient PEG-PLGA, and are more difficult to release from the

backbone material, resulting in a slower release degree, which is consistent with the conclusion of the EE of PNS. Furthermore, compared with the release degrees of GRg1 in 10% iRGD-5% TAT GRg1@PEG-PLGA NPs eye drops and GRb1 in 10% iRGD-5% TAT GRb1@PEG-PLGA NPs eye drops, the release degrees of GRg1 and GRb1 were slower in 5% iRGD-5% TAT PNS@PEG-PLGA NPs eye drops. This may be the result of internal components in the PNS binding to each other and impeding drug diffusion. In addition, it was also found that the release degree of 10% iRGD-5% TAT GRb1@PEG-PLGA NPs eye drops was slower than that of 10% iRGD-5% TAT GRg1@PEG-PLGA NPs eye drops, which could be explained by the higher lipid solubility of GRb1 and its tighter binding to the excipients [44], which is also in agreement with the findings of the EE of GRg1 and GRb1.

Eye irritation study

Ideal eye drops need to have not only good therapeutic efficacy but also a high level of safety and not cause damage to the organism. Reversible or irreversible inflammation of the cornea, iris, conjunctiva, and surrounding tissues caused by direct exposure of the eye to the drug is termed an ocular irritation response. This irritant response leads to significant tear spillage, which reduces the concentration and permeability of the drug in the eye [45, 46]. In clinical practice, irritation of eye drops reduces patient compliance and compromises drug efficacy. Therefore, safe eye drops must have low ocular irritation.

Using New Zealand white rabbits as test animals, the eye irritation of 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops was investigated in the combination of Draize assay and H&E staining. According to the Draize eye irritation evaluation criteria, the composite score of the cornea, iris, and conjunctiva between 0 ~ 3 is considered non-irritating, between 4 ~ 8 is considered mildly irritating, between 9 ~ 12 is considered moderately irritating, and between 13 ~ 16 is considered belligerently irritating [47]. As shown in Table 2, after single-dose administration, the saline group, 5% iRGD-5% TAT co-

modified PNS@PEG-PLGA NPs eye drops group, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops group, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops group had a composite score of 0 at each observation time point, indicating that there was non-irritating. The microstructure of the cornea, iris, and conjunctiva was further evaluated by H&E staining, and the results were shown in Fig. 3A. The cornea tissues of rabbits in each group were structurally intact and demarcated, with well-stratified corneal epithelial cells and regular arrangement of stromal fibers; the iris blood vessels were not abnormal or congested; the conjunctival blood vessels were loosely arranged, and the conjunctiva was free of congestion, edema, and secretion adherence. No morphological abnormalities were observed in the cornea, iris, and conjunctiva after a single dose administration. Compared with the saline group, there was no significant difference in the eye irritation scores and pathological histological structure of rabbits in drug groups, indicating that 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops did not have eye irritation after single-dose administration.

In the multiple doses administration scores, the saline group, 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops group, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops group, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops group were scored below 3 at each observation time point, which belonged to the range of non-irritating (Table 2). The results of H&E staining of eye tissues in Fig. 3B also showed that no abnormalities were observed in the cornea, iris, and conjunctiva of rabbits in all groups. Compared with the saline group, there were no significant differences in the eye irritation scores and pathological histological structures of the rabbits in the drug groups, indicating that 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, and 10%

iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops did not have eye irritation after multiple doses of administration.

The results of single dose and multiple dose administration of eye irritation showed that 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops did not show eye irritation, which is suitable for ocular administration.

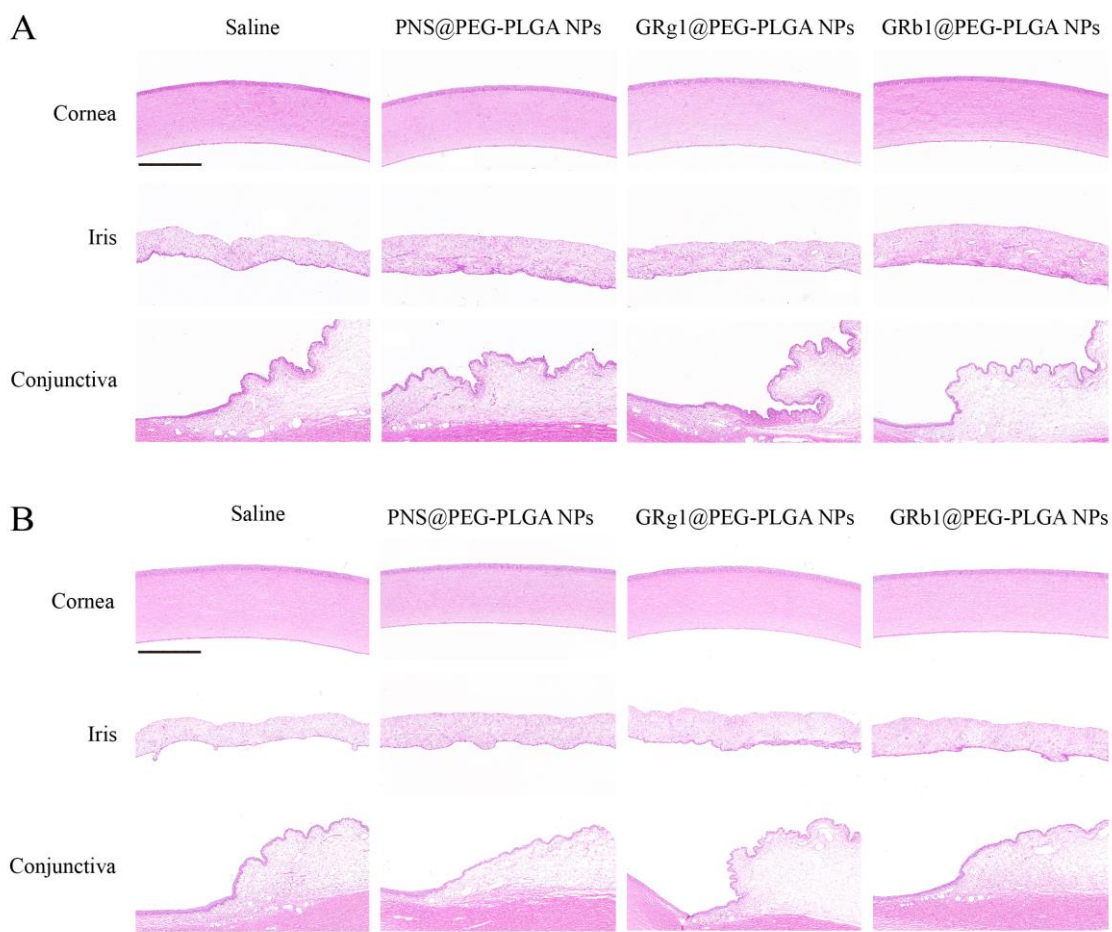


Fig. 3 Hematoxylin-eosin staining of the cornea, iris, and conjunctiva after a single dose and multiple dose administration of iRGD and TAT co-modified PEG-PLGA NPs eye drops. (A) Single dose administration of iRGD and TAT co-modified PEG-PLGA NPs eye drops (scale bar: 500 μm). (B) Multiple doses administration of iRGD and TAT co-modified PEG-PLGA NPs eye drops (scale bar: 500 μm).

445 **Table 2** Eye irritation scores after single dose and multiple dose administration of iRGD and TAT co-modified PEG-PLGA NP_s eye drops (*n* = 3).

Time (h)	Single dose				Multiple doses			
	A	B	C	D	A	B	C	D
1	0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.50 ± 0.55	0.33 ± 0.52	0.33 ± 0.52
2	0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.33 ± 0.52	0.33 ± 0.52	0.17 ± 0.41
4	0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.17 ± 0.41	0.17 ± 0.41	0.00 ± 0.00
24	0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
48	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
72	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

446 Notes: A: Saline; B: 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NP_s eye drops; C: 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NP_s eye drops; D: 10% iRGD-
447 5% TAT co-modified GRb1@PEG-PLGA NP_s eye drops.

Corneal cell permeability study

The efficacy of drugs in the eye is largely dependent on the amount of drug absorption by ocular tissues, among which corneal epithelial cells are the most important barrier limiting drug absorption [48, 49]. Corneal cell permeability assay refers to culturing a monolayer corneal cell model in Transwell plates to assess drug uptake and transport on both sides of the cell membrane [50]. It can provide a powerful system for studying drug uptake, transport, and other properties [51], and data obtained using the HCE-T cell can serve as a useful model for predicting drug behavior in the eye [52].

The corneal permeability of PNS eye drops, 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, GRg1 eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, GRb1 eye drops and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops was investigated on HCE-T cells, and the result are shown in Fig. 4. The P_{app} of NGR1, GRg1, GRe, GRb1, GRd, and PNS in 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops was 3.35, 2.66, 1.90, 3.48, 2.76 and 3.31 times higher than that in PNS eye drops, respectively (Fig. 4A). The P_{app} of 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops was 2.33 times higher than that in GRg1 eye drops (Fig. 4B). The P_{app} of 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops was 2.38 times higher than that in GRb1 eye drops (Fig. 4C). The P_{app} of 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops were significantly higher than those of the API eye drops, suggesting that iRGD and TAT co-modified PEG-PLGA NPs could effectively penetrate the corneal barrier, thus significantly improving drug permeability. The increased permeability may be attributed to the iRGD can be hydrolyzed after binding to integrin $\alpha_v\beta_3$ on the surface of corneal epithelial cells, and the hydrolyzed peptide can bind to NRP-1, which activates the endocytosis pathway [30]. In addition, TAT can also non-specifically open the cellular junctions, which further improves the ability of

nanoparticles to penetrate the corneal barrier. It has been reported that the expression level of integrin $\alpha_v\beta_3$ was lower in the tissue of the anterior segment of the eye, and the permeation function of iRGD and TAT co-modified PEG-PLGA NPs was mainly dependent on TAT. The integrin $\alpha_v\beta_3$ was highly expressed on the surface of retinal endothelial cells, and the uptake of iRGD and TAT co-modified PEG-PLGA NPs in the posterior segment of the eye was mainly dependent on iRGD [53].

It was found that GRd had the highest P_{app} in 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, followed by GRb1, GRg1, GRe, and NGR1. The reason for the different permeability of the different components may be related to their properties. The lipid solubility of the five components, GRd > GRb1 > GRg1 $\approx$ GRe > NGR1, was demonstrated by the fact that in the C18 reversed-phase chromatographic column, the peak of NGR1 appeared first, followed by GRg1 $\approx$ GRe, GRb1 appeared fourth, and GRd appeared last [14]. The strong lipid solubility favored the penetration of biofilm, thus, GRd had the highest P_{app} , and NGR1 had the lowest P_{app} . In addition, the P_{app} of GRg1 in 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops and GRb1 in 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops were stronger than those of GRg1 and GRb1 in 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops. The stronger corneal permeability could be attributed to the smaller particle size and the higher iRGD modification of nanoparticles, which is more favorable for cellular translocation. Meanwhile, due to the higher lipid solubility of GRb1 than GRg1, the P_{app} of 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops was slightly higher than that of 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops for the same particle size and iRGD and TAT modifications.

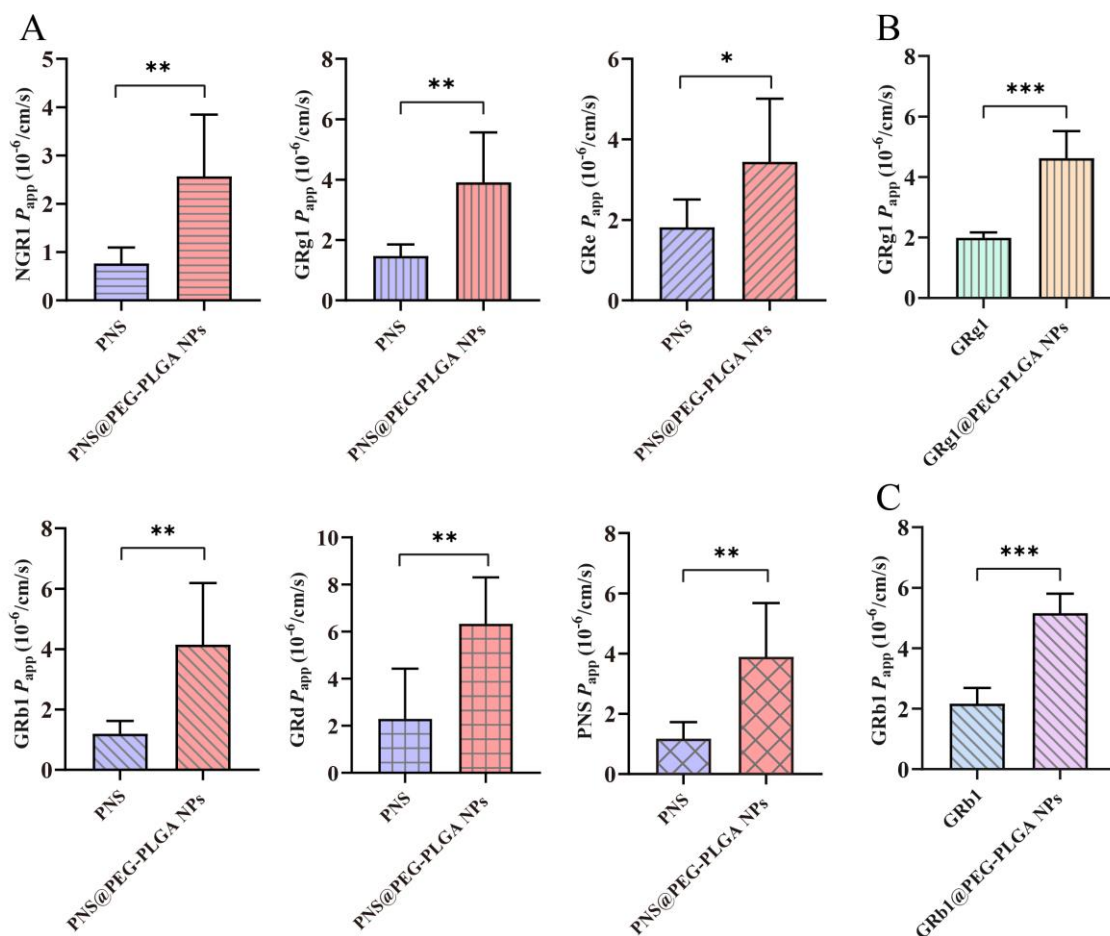


Fig. 4 Apparent permeability coefficient of iRGD and TAT co-modified PEG-PLGA NP_s eye drops in human corneal epithelial cells. (A) PNS eye drops and 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NP_s eye drops ($n = 6$). (B) GRg1 eye drops and 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NP_s eye drops ($n = 6$). (C) GRb1 eye drops and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NP_s eye drops ($n = 6$). Compared with the corresponding API eye drops: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Aqueous humor pharmacokinetic study

Drugs are absorbed through the cornea into the aqueous humor and then distributed to the sclera, ciliary body, retina, and other tissues to play a role. Aqueous humor pharmacokinetics is the application of kinetic principles and mathematical models, quantitatively describing the dynamic law of the absorption, distribution, metabolism, and excretion of drugs through the local administration into the aqueous humor [54]. It is indispensable for the

rational application of ophthalmic drugs, optimization of therapeutic dosage, as well as the design and drug evaluation of novel ophthalmic drugs [55].

To study the dynamic changes of drugs in the eye, aqueous humor pharmacokinetic experiments were carried out. The mean drug concentration-time curves in the rabbit aqueous humor are shown in Fig. 5, and the pharmacokinetic parameters of drugs in the aqueous humor calculated by DAS software are shown in Tables S1-3. After administration of 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops to the rabbit, the mean concentration-time curves of NGR1, GRg1, GRe, GRb1, GRd, and PNS in the aqueous humor were higher than those of PNS eye drops (Fig. 5A). The $C_{\max}$ and $AUC_{(0-\infty)}$ of NGR1, GRg1, GRe, GRb1, GRd, and PNS were significantly increased, and the bioavailability was 3.62, 2.44, 3.06, 3.46, 5.94, and 3.96 times than that of PNS eye drops, respectively (Table S1). After administration of 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops to rabbit, the mean concentration-time curves of GRg1 in the aqueous humor was higher than that of GRg1 eye drops (Fig. 5B). The $C_{\max}$ and $AUC_{(0-\infty)}$ of GRg1 were significantly increased, and the bioavailability was 6.74 times higher than that of GRg1 eye drops (Table S2). After administration of 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops to rabbit, the mean concentration-time curves of GRb1 in the aqueous humor was higher than that of GRb1 eye drops (Fig. 5C). The $C_{\max}$ and $AUC_{(0-\infty)}$ of GRb1 were significantly increased, and the bioavailability was 4.15 times than that of GRb1 eye drops (Table S3). 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops had significantly higher bioavailability than API eye drops, suggesting that iRGD and TAT co-modified PEG-PLGA NPs increased the concentration of PNS, GRg1, and GRb1 in the aqueous humor. The reason for this phenomenon may be related to their ability to increase the permeability of PNS, GRg1, and GRb1 in the cornea.

In addition, the experimental results also showed that the concentration of GRg1 and GRb1 in the aqueous

humor was higher than that of NGR1, GRe, and GRd in 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, which may be related to their contents and properties [14]. In 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs, GRg1 and GRb1 had the higher DL (13.35% and 14.56%), whereas NGR1, GRe, and GRd had the lower DL (2.91%, 1.73%, and 1.09%, respectively). Despite the differences in the P_{app} of the five components, their content in 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs played a decisive role in their content in the aqueous humor. Therefore, a higher concentration of GRg1 and GRb1 was observed in the aqueous humor than in NGR1, GRe, and GRd. Furthermore, the C_{max} and $AUC_{(0-\infty)}$ of GRg1 in 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops and GRb1 in 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops were higher than those of GRg1 and GRb1 in 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops. This phenomenon may be related to the smaller particle size, higher iRGD modification, and greater corneal permeability of 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops than 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops. In addition, 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops showed smaller C_{max} and larger $AUC_{(0-\infty)}$ in the aqueous humor compared to 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, which may be a result of the combination of dose and $t_{1/2}$ [56, 57]. Due to the different contents of GRg1 (40.07%) and GRb1 (27.25%) in PNS (200 μ L, 50 mg/mL), 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops were administered in a volume of 200 μ L at a concentration of 20 mg/mL, whereas 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops were administered in a volume of 200 μ L at a concentration of 12.5 mg/mL in this study. Although the P_{app} of 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops was slightly higher than that of 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, the higher GRg1 administration dose mainly resulted in a higher C_{max} in a short time. In addition, the stronger lipid solubility of GRb1 compared to GRg1 makes it less likely to be cleared

544 from the aqueous humor, resulting in larger $AUC_{(0-\infty)}$, and longer MRT and $t_{1/2}$ of 10% iRGD-5% TAT co-modified
545 GRb1@PEG-PLGA NPs eye drops than 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops.
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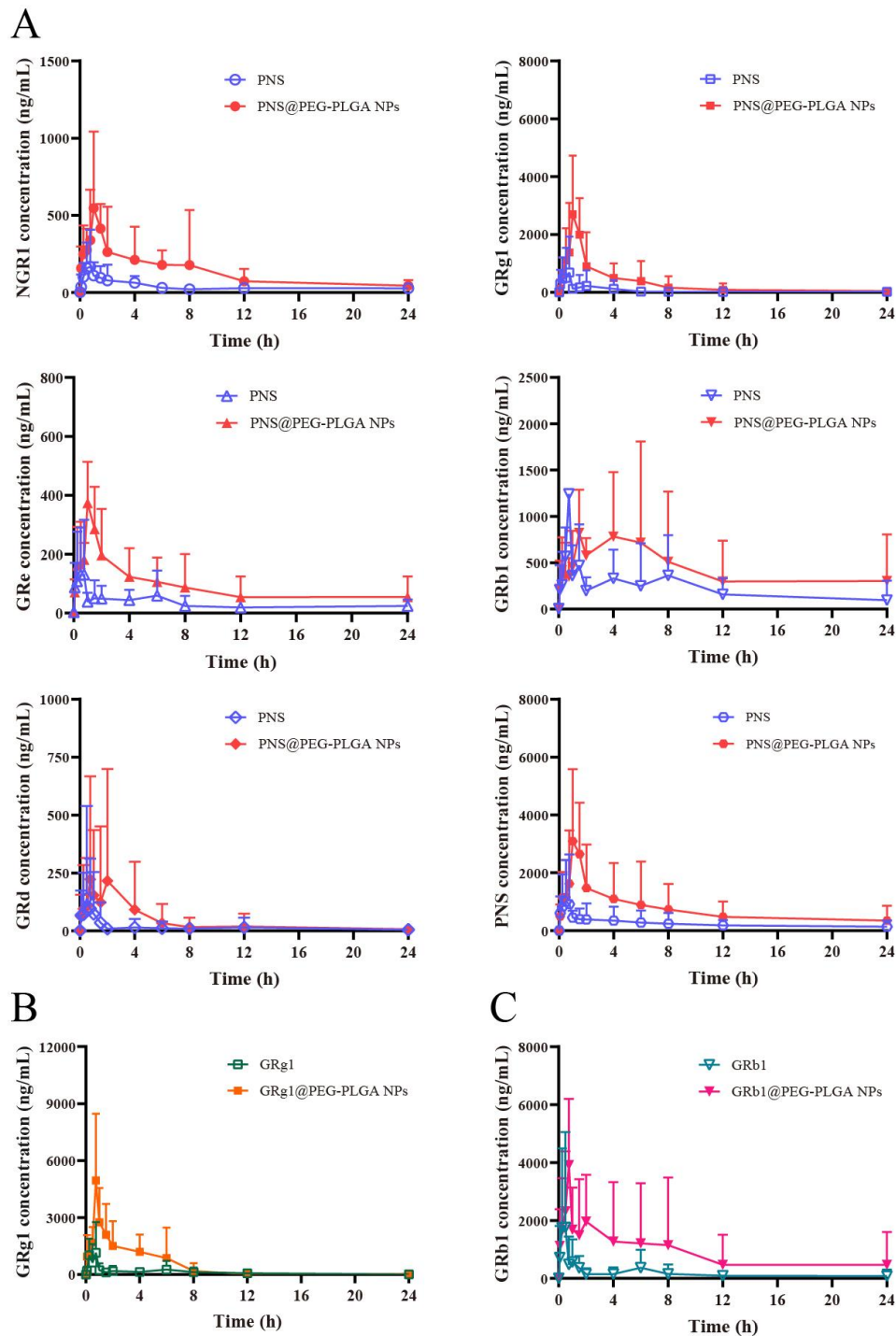


Fig. 5 Mean drug concentration-time curves of iRGD and TAT co-modified PEG-PLGA NPs eye drops in the rabbit aqueous humor. (A) PNS eye drops and 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops ($n = 6$). (B) GRg1 eye drops and 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops ($n = 6$). (C) GRb1 eye drops and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops ($n = 6$).

Ocular tissue distribution study

When eye drops are used to treat diseases of the posterior segment of the eye, the drug not only needs to overcome the ocular barrier to enter the eye but also needs to have a high concentration in the tissue sites to give full play to its efficacy. The tissue distribution combined with the UPLC-MS/MS method to determine the drug content in the cornea, aqueous humor, sclera, retina, and vitreous tissues can give an idea of the distribution of the drug in various tissues of the eye.

The distribution amounts of PNS eye drops, 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, GRg1 eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, GRb1 eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops in the cornea, aqueous humor, sclera, retina and vitreous humor of the eye at 0.5, 2 and 4 h are shown in Fig. 6. The distribution amounts of NGR1, GRg1, GRe, GRb1, GRd and PNS in 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops in the cornea, aqueous humor, sclera, retina and vitreous humor were significantly higher than those of PNS eye drops (Fig. 6A). The distribution amounts of GRg1 in 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops in the cornea, aqueous humor, sclera, retina and vitreous humor was significantly higher than that of GRg1 eye drops (Fig. 6B). The distribution amounts of GRb1 in 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops in cornea, aqueous humor, sclera, retina and vitreous humor was significantly higher than that of GRb1 eye drops (Fig. 6C). The ocular tissue distribution results showed that iRGD and TAT co-modified PEG-PLGA NPs eye drops increased the distribution amounts of PNS, GRg1 and GRb1 eye drops in ocular tissues, which may be attributed to their ability to facilitate drug penetration into the cornea and improve the ocular bioavailability of drugs.

In addition, the experimental results also revealed that the ocular tissue distribution amounts of GRg1 and GRb1 in 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops were higher than those of NGR1, GRe,

574 and GRd. The reason for the different ocular tissue distribution amounts of the different components may be
575 dependent on their concentration in the aqueous humor, with higher drug concentration in the aqueous humor
576 determining the higher tissue distribution amounts. Furthermore, GRg1 in 10% iRGD-5% TAT co-modified
577 GRg1@PEG-PLGA NPs eye drops and GRb1 in 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye
578 drops had higher tissue distribution amounts than those of GRg1 and GRb1 in 5% iRGD-5% TAT co-modified
579 PNS@PEG-PLGA NPs eye drops. The higher corneal permeability and aqueous humor concentration of 10%
580 iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops and 10% iRGD-5% TAT co-modified
581 GRb1@PEG-PLGA NPs eye drops compared to 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops
582 may account for the higher ocular tissue distribution amounts of GRg1 and GRb1. It was found that the distribution
583 amounts of 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops in ocular tissues were higher than
584 those of 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops, which may be related to the higher
585 administration dose of GRg1 than that of GRb1. The amount of drug distribution in the tissues after a single
586 administration over a short time depended mainly on the concentration of the drug in the aqueous humor.

NPDR efficacy study

Effect on retinal damage

The aim of developing iRGD and TAT co-modified PEG-PLGA NPs was to improve the permeability of the drug in the cornea and increase the amount of drug distribution in the ocular tissues to enhance the therapeutic efficacy of the drug. To study the efficacy of iRGD and TAT co-modified PEG-PLGA NPs on DR, we established a rat NPDR model and measured the relevant test indexes. Changes in the body weight and FBG of the rats were recorded during the experiment. As shown in Fig. 7A, the body weight of rats in the control group gradually increased, while that of rats in the DM group increased slowly and then leveled off. However, PNS eye drops, 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, GRg1 eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, GRb1 eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops did not affect DM rats. In addition, the FBG of control rats was normal and stable, and that of DM rats was significantly higher than that of control rats, whereas PNS eye drops, 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, GRg1 eye drops, 10% iRGD-5% TAT co-modified GRg1 NPs eye drops, GRb1 eye drops, and 10% iRGD-5% TAT co-modified GRb1 eye drops also did not affect FBG in DM rats (Fig. 7B).

To assess the effects on the microstructure of the retina, retinal tissues were stained with H&E. As shown in Fig. 7C, the retinal structure of the control group was clear and intact, and the cells in each layer were tightly arranged with the ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), and outer nuclear layer (ONL), in descending order, whereas the retinal structure of the rats in the DM group was intact, but the number of cells was reduced and loosely arranged. However, the reduction of the number of retinal cells was significantly improved in DM rats treated with PNS eye drops, 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, GRg1 eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, GRb1 eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA

NPs eye drops. In addition, the reduction in cell number further led to a reduction in cell layer thickness. As shown in Fig. 7D, the retinal ONL thickness was significantly reduced in rats in the DM group compared with rats in the control group, while the retinal ONL thickness was significantly increased in DM rats treated with PNS eye drops, 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, GRg1 eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, GRb1 eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops.

The effects on the retinal vasculature involved both vascular morphology and function [14]. As shown in Fig. 7E-F, the number of acellular capillaries in the retinal vasculature of DM rats was significantly increased compared with that of the control group, suggesting that the retinal morphology was severely disrupted. However, DM rats treated with PNS eye drops, 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, GRg1 eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, GRb1 eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops, the number of acellular capillaries in the retinal vasculature was significantly reduced, and the retinal vascular morphology was restored.

Evan's blue dye leakage test was used to detect the damaging effect of BRB. As shown in Fig. 7G, Evan's blue dye leakage was significantly increased in the retinas of rats in the DM group compared with the control group, which indicated an increase in retinal vascular permeability after the STZ model. PNS eye drops, 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, GRg1 eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, GRb1 eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops significantly reduced retinal Evans' blue dye leakage, suggesting that all of the above eye drops significantly reduced retinal vascular permeability in DM rats.

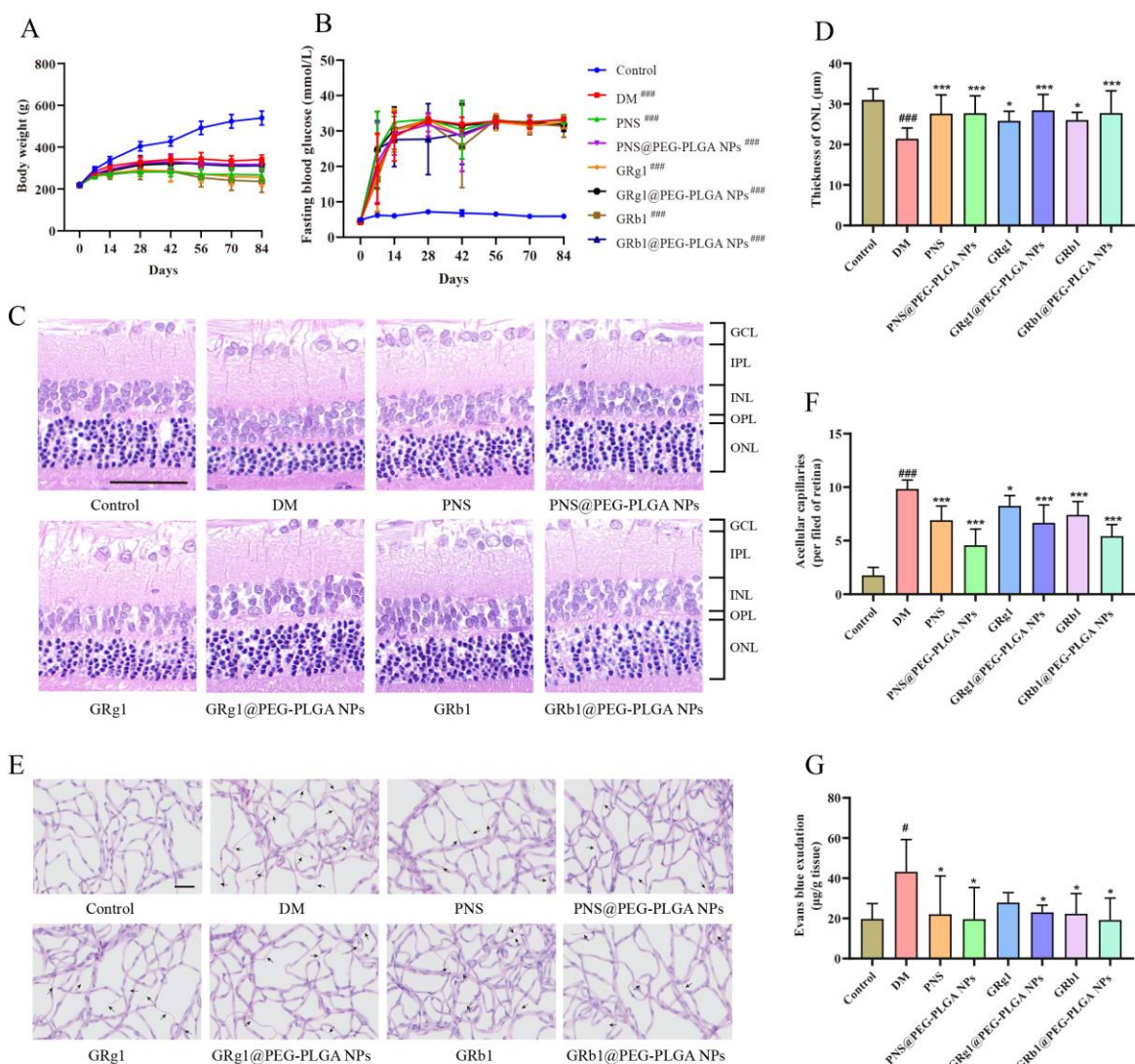


Fig. 7 The effect of iRGD and TAT co-modified PEG-PLGA NPs eye drops on retinal damage. **(A)** Body weight ($n = 16$). **(B)** Fasting blood glucose ($n = 16$). **(C)** H&E staining of retinas (scale bar: 50 μm). **(D)** Quantitative analysis of the retinal ONL thickness ($n = 4$). **(E)** H&E staining of retinal capillaries after trypsin digestion (scale bar: 50 μm). Black arrows indicate acellular capillaries. **(F)** Quantitative analysis of the number of acellular capillaries per field in retinas ($n = 4$). **(G)** Evan's blue dye leakage assay ($n = 6$). Data are expressed as mean $\pm$ SD. # $P < 0.05$, ### $P < 0.001$ compared with control; * $P < 0.05$, *** $P < 0.001$ compared with DM.

The results of the above indexes indicated that iRGD and TAT co-modified PEG-PLGA NPs significantly improved the retinal damage in NPDR rats compared with PNS, GRg1, and GRb1, and the improvement effect of

5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops was better than that of 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, and the improvement effect of 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops was better than that of 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops.

Effect on retinal inflammation

Emerging evidence suggested that inflammation is a key factor in the onset and progression of DR. Retinal microglia, as resident immune cells, actively participate in inflammatory processes, and their activation has been implicated in exacerbating neuronal damage and vascular dysfunction [58]. Immunofluorescence analysis of ionized calcium-binding adapter molecule-1 (Iba-1), a well-established microglial marker [59], revealed significant upregulation in the ganglion cell layer (GCL), outer plexiform layer (OPL), and inner plexiform layer (IPL) of diabetic rats (Fig. 8A). These findings indicate that microglia are widely activated in the diabetic retina following STZ administration. PNS eye drops, 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, GRg1 eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, GRb1 eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops treated DM rat retinas showed a significant decrease in the number of Iba-1, indicating that microglial cell activation was significantly inhibited.

Furthermore, inflammatory processes may promote leukocyte adhesion to retinal vascular endothelium, exacerbating vascular leakage and contributing to DR pathogenesis. In this study, FITC-ConA was used to label the adherent leukocytes in the retinal vessels, and the results are displayed in Fig. 8B-C. Compared with the normal group, the number of adherent leukocytes in the retinal blood vessels of DM rats was significantly increased, whereas PNS eye drops, 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, GRg1 eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, GRb1 eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops reversed the increase in the number of adherent leukocytes.

Inflammatory factors can accelerate retinal tissue damage [60]. To assess retinal inflammatory status, the serum levels of TNF- α , IL-6, and IL-1 β were measured. As illustrated in Fig. 8D, the expression levels of TNF- α , IL-6, IL-1 β , and VEGF in the serum of DM rats were significantly elevated. However, PNS eye drops, 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, GRg1 eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, GRb1 eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops effectively reduced the expression of TNF- α , IL-6, IL-1 β , and VEGF in serum.

The results of the above indexes indicated that iRGD and TAT co-modified PEG-PLGA NPs significantly inhibited retinal inflammation in NPDR rats compared with PNS, GRg1, and GRb1, and the degree of inhibition of retinal inflammation was 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops in descending order.

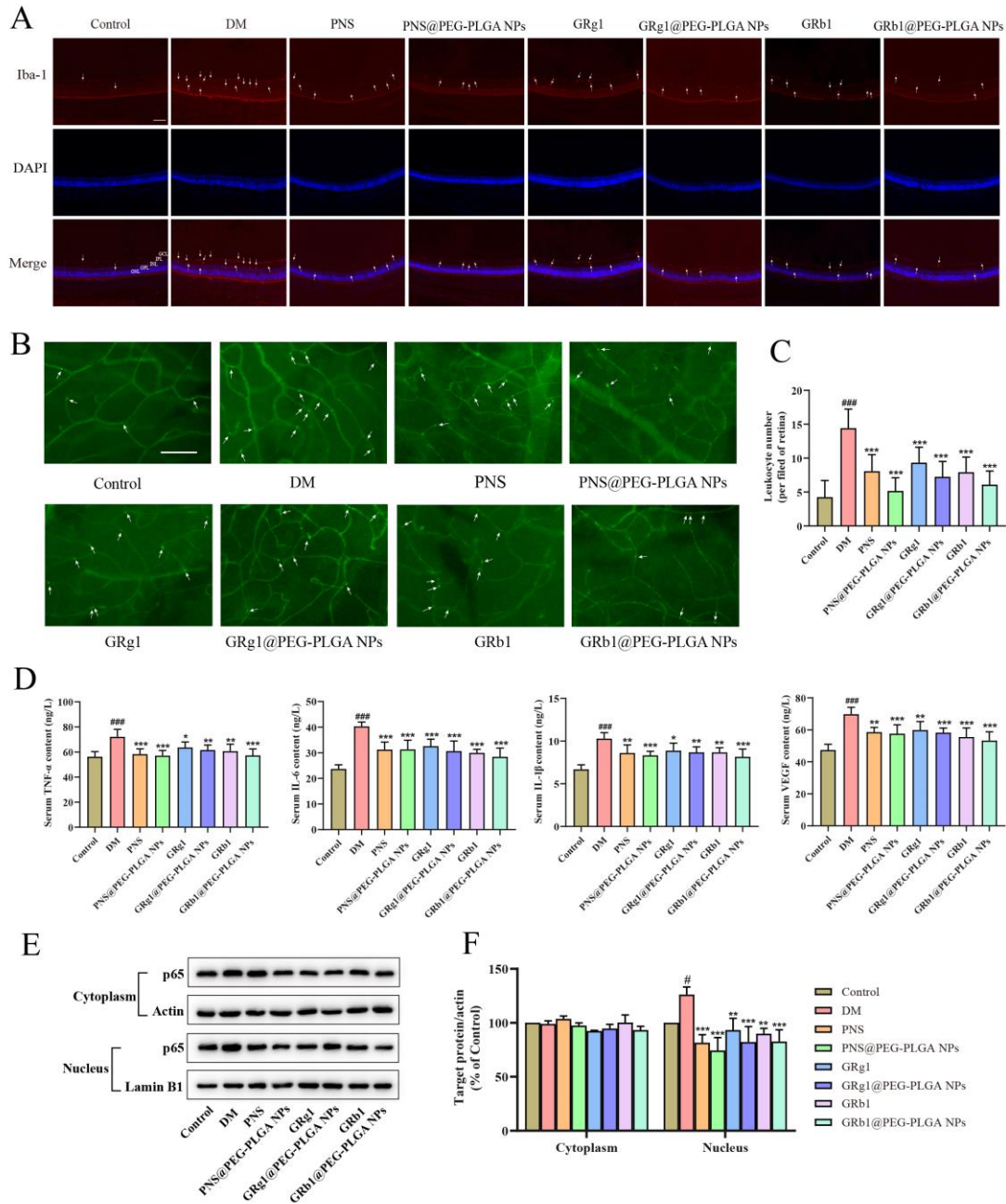


Fig. 8 The effect of iRGD and TAT co-modified PEG-PLGA NP_s eye drops on retinal inflammation and NF-κB signaling pathway. **(A)** Representative images of immunohistochemical staining of Iba-1 in retinal tissues, white arrows indicate retinal microglia (scale bar: 50 μm). **(B)** Representative images of FITC-ConA-labeled vascular endothelial cells and adhered leukocytes in retinas, white arrows indicate adhered leukocytes (scale bar: 100 μm). **(C)** Quantitative analysis of the number of adhered leukocytes in retinal vessels ($n = 4$). **(D)** Serum content of TNF-α, IL-6, IL-1β, and VEGF ($n = 6$). **(E)** Retinal protein expression of nuclear and cytosolic p65 and the results

represent at least three independent experiments. (F) The quantitative densitometric analysis of nuclear and cytosolic p65 and the results are presented as a percentage of control ($n = 3$). Data are expressed as mean $\pm$ SD. $^{\#}P < 0.05$, $^{###}P < 0.001$ compared with control; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with DM.

Effect on NF- κ B signaling pathway

The NF- κ B signaling pathway is a fundamental inflammatory cascade in the pathogenesis of diabetic retinopathy [61]. To elucidate the molecular mechanisms of diabetic retinal inflammation and investigate the potential involvement of the NF- κ B pathway, we quantitatively measured the expression levels of the p65 protein in the retinal cytoplasm and nucleus using Western blot analysis. Our results showed that compared with the control group, p65 protein in the retinas of DM rats exhibited significant nuclear translocation (Fig. 8E-F), which provided direct evidence for the activation of the NF- κ B pathway in diabetic retinas. PNS eye drops, 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, GRg1 eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, GRb1 eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops showed a significant reduction in protein expression of p65 in the retinal nucleus, indicating the activation of NF- κ B signaling pathway was inhibited. In addition, the results also found that 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops significantly inhibited the activation of NF- κ B signaling pathway in NPDR rats compared with PNS, GRg1 and GRb1 eye drops, and the degree of inhibition 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops were stronger than 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops.

Topical administration of 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA

NPs eye drops could reduce retinal inflammation by inhibiting the activation of the NF- κ B signaling pathway, thereby improving NPDR. For the therapeutic efficacy of NPDR, 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops were superior to PNS eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops were superior to GRg1 eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops were superior to GRb1 eye drops, which may be related to the fact that iRGD and TAT co-modified PEG-PLGA NPs could promote the absorption of PNS, GRg1, and GRb1 in the cornea, and increase the concentration of the drug in the aqueous humor and the amounts of distribution in the ocular tissues. Among the different types of eye drops, 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops were superior to 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops, and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops were superior to 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops. Although the dose of 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops (20 mg/mL, 20 μ L per eye, 6 times, 16 mg/kg) was higher than that of 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops (12.5 mg/mL, 20 μ L per eye, 6 times, 10 mg/kg). The 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops were more effective than 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops for the treatment of NPDR. The reason for this phenomenon may be due to the slower intraocular release and longer *MRT* and *t*_{1/2} of 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops compared to 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops, which in turn leads to longer-lasting efficacy after multiple administrations over a long time.

Conclusion

The developed ocular formulations - 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops, 10% iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops and 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops - exhibited optimal physicochemical characteristics including spherical

728 morphology, small particle size, high EE and DL, along with sustained release properties. Both *in vivo* and *in vitro*
729 experiments demonstrated their excellent ocular compatibility (no irritation), enhanced corneal permeability,
730 improved aqueous humor concentration, and favorable tissue distribution. These properties collectively
731 contributed to superior therapeutic outcomes in DR treatment. Notably, formulation performance varied depending
732 on the drug properties: the 5% iRGD-5% TAT co-modified PNS@PEG-PLGA NPs eye drops showed the highest
733 therapeutic efficacy, followed by 10% iRGD-5% TAT co-modified GRb1@PEG-PLGA NPs eye drops, with 10%
734 iRGD-5% TAT co-modified GRg1@PEG-PLGA NPs eye drops demonstrating relatively lower efficacy. These
735 results establish iRGD and TAT co-modified PEG-PLGA nanoparticles as a highly promising topical ocular
736 delivery platform, representing a novel therapeutic strategy for DR that combines enhanced efficacy with excellent
737 safety profiles.

738 **Abbreviations:** API, Active pharmaceutical ingredient; BRB, Blood-retinal barrier; CPPs, Cell-penetrating
 739 peptides; DL, Drug loading; DM, Diabetic mellitus; DR, Diabetic retinopathy; EE, Encapsulation efficiency; FBG,
 740 Fasting blood glucose; FBS, Fetal bovine serum; FITC-ConA, Fluorescein isothiocyanate coupled concanavalin
 741 A; GCL, Ganglion cell layer; GRb1, Ginsenoside Rb1; GRd, Ginsenoside Rd; GRe, Ginsenoside Re; GRg1,
 742 Ginsenoside Rg1; HBSS, Hanks' Balanced Salt Solution; HCE-T, Human corneal epithelial cells; H&E,
 743 Hematoxylin-eosin; Iba-1, Ionized calcium-binding adapter molecule-1; IL-1 β , Interleukin-1 β ; IL-6, Interleukin-
 744 6; INL, Inner nuclear layer; IPL, Inner plexiform layer; NF- κ B, Nuclear factor kappa B; NGR1, Notoginsenoside
 745 R1; ^{1} H-NMR, Nuclear magnetic resonance hydrogen spectroscopy; NPDR, Non-proliferative diabetic retinopathy;
 746 NRP-1, Neurofibrillinase-1; ONL, Outer nuclear layer; OPL, Outer plexiform layer; P_{app} , Apparent permeability
 747 coefficient; PBS, Phosphate-buffered saline; PDI, Polydispersity index; PDR, Proliferative diabetic retinopathy;
 748 PEG-PLGA, Poly (ethylene glycol)-poly (lactic-co-glycolic acid); PNS, Panax notoginseng sponins; PPD,
 749 Protopanaxadiol; PPT, Protopanaxatriol; P188, Poloxamer188; STZ, Streptozotocin; TAT, trans-activator of human
 750 immunodeficiency virus HIV-1; TCMs, Traditional Chinese medicines; TEER, Trans epithelial electrical
 751 resistance; TEM, Transmission electron microscope; TNF- α , Tumor necrosis factor; VEGF, Vascular endothelial
 752 growth factor.

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Author contributions

Yaru Wang: Writing - review & editing, Writing - original draft, Investigation, Formal analysis, Data curation, Visualization. Xin Sun: Investigation, Formal analysis, Data curation. Shusheng Lai: Methodology, Investigation, Formal analysis. Lili Ji: Methodology, Investigation, Formal analysis. Ming Chen: Writing - review & editing, Methodology, Conceptualization. Xiaohui Wei: Writing - review & editing, Methodology, Conceptualization. Changhong Wang: Writing - review & editing, Project administration, Conceptualization, Formal analysis, Funding acquisition. All authors reviewed the manuscript and agreed to the published version of the manuscript.

Data availability

Data will be made available on request.

Declarations

Ethics approval and consent to participate

Animal experiments were conducted under the guidelines for institutional animal care approved and supervised by the Ethics Committee for Animal Experiments of Shanghai University of Traditional Chinese Medicine (Shanghai, China) (Approval No. PZSHUTCM2309070003; PZSHUTCM2401080002).

Consent for publication

Written informed consent was obtained from all participants.

773 **Competing interests**

774 The authors declare that there is no conflict of interest.

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