

Mucoadhesive polymeric microcarriers delivering diquafosol tetrasodium for dry eye therapy

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Research Article

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Abstract

Dry eye disease (DED) remains a significant challenge in effective drug delivery due to rapid precorneal clearance and suboptimal concentration control, leading to frequent dosing and poor patient compliance. Herein, we developed mucoadhesive phenylboronic acid-modified alginate microcarriers using a microfluidic electrospray strategy for sustained diquafosol tetrasodium (DQS) release and prolonged ocular surface retention. The designed phenylboronic acid-modified alginate polymer enabled both mucoadhesion via dynamic boronate ester bonds to the corneal surface and controllable drug release through interactions with the cis-diol groups in DQS. In a hyperosmolarity-induced human corneal epithelial cell model, the microcarriers demonstrated excellent biocompatibility and effectively protected human corneal

epithelial cells from hyperosmotic stress by reducing intracellular ROS accumulation and suppressing inflammatory responses. Furthermore, in a benzalkonium chloride-induced murine DED model, the microcarrier-based eye drops effectively restored corneal epithelial integrity, improved tear production, and alleviated histological signs of ocular surface damage. These results indicated that our drug-loaded, mucoadhesive microcarrier system represents a promising and potent platform for advanced DED therapy, offering the potential for improved clinical outcomes.

Keywords: dry eye disease; adhesive; microfluidics; microcarrier; drug delivery

Introduction

Dry eye disease (DED) is a prevalent and multifactorial ocular surface disorder that substantially impairs visual function and quality of life, accounting for over 30% of visits to ophthalmology clinics. The pathology of DED involves a vicious cycle of tear film instability, hyperosmolarity, inflammation, and ocular surface damage [1]. Current therapeutic strategies are diverse, ranging from artificial tears and anti-inflammatory medications to mucin secretagogues and punctal plugs, with topical eye drops being the most common administration route [2]. Among them, diquafosol tetrasodium (DQS), a potent agonist of the P2Y purinergic receptor 2 (P2Y₂), a G-protein-coupled receptor [3, 4], has been established as an effective treatment for DED. DQS exerts direct protective effects on corneal epithelial cells by enhancing epithelial barrier integrity, promoting epithelial repair, and attenuating hyperosmotic stress-induced injury. It also promotes fluid secretion and stimulates mucin secretion from conjunctival goblet cells, thereby acting on multiple components of the ocular surface to restore and stabilize the tear film [5-9]. However, like many topical ophthalmic drugs, DQS delivered via conventional eye drops suffers from poor ocular bioavailability [10]. Upon instillation, a majority of the drug is rapidly eliminated from the ocular surface due to blinking and nasolacrimal drainage, resulting in an initial peak concentration followed by a rapid decline below the therapeutic threshold. This pharmacokinetic profile limits efficacy, necessitates frequent dosing, with

conventional 3% DQS generally administered six times daily^[11-13], and often results in suboptimal clinical outcomes and poor patient compliance. Consequently, there is a critical and unmet need for advanced drug delivery systems capable of providing controlled, sustained drug release and prolonged ocular residence time to improve therapeutic efficacy for DED.

Here, we prepared novel adhesive polymeric hydrogel microcarriers via microfluidic technologies for precise and controlled ocular drug delivery, as illustrated in **Figure 1**. Microfluidic electrospray technique provides a robust platform for the continuous and stable generation of highly monodisperse microcarriers, which can be readily solidified into uniform microcarriers^[14, 15]. This high uniformity is crucial for ensuring consistent drug loading and predictable, reproducible release profiles, overcoming the polydispersity issues common to conventional bulk emulsion methods^[16-19]. Concurrently, mucoadhesive polymers have been extensively explored to prolong the residence time of therapeutic formulations on the ocular surface, thereby enhancing drug bioavailability and efficacy. Among various strategies, chemical modification of polymers with phenylboronic acid (PBA) has emerged as a particularly effective approach. The PBA moieties can form reversible, dynamic covalent bonds with the cis-diol groups abundantly present in sialic acid residues of mucins on the corneal and conjunctival epithelium, enabling robust and prolonged mucoadhesion^[20]. Furthermore, this versatile boronate chemistry can be leveraged to tether cis-diol-containing drug molecules simultaneously, such as the ribose rings in DQS^[21, 22]. The dynamic nature of the boronate ester bond provides a potential mechanism for sustained drug release. In addition, PBA/boronate moieties are susceptible to oxidation by H₂O₂ through oxidative deboronation, suggesting the potential for oxidation-sensitive bond cleavage under oxidative conditions. Despite its potential, an integrated approach combining microfluidic electrospray with a dual-functional polymer to deliver DQS for DED therapy remains unexplored.

In this study, we fabricated the PBA-based microcarriers containing DQS using an electrospray microfluidic strategy to achieve sustained drug delivery and enhanced ocular retention for DED treatment. For this purpose, a PBA-modified alginate

(PBA-Alg) polymer was synthesized, which serves as the core component, providing both mucoadhesive property and a matrix for drug tethering. Drug-loaded microcarriers (PBA-Alg@DQS) were subsequently fabricated, and their morphology, size distribution, drug release profile, and mucoadhesive properties were systematically characterized and evaluated. The therapeutic potential and underlying mechanisms of the microcarriers were further investigated in vitro using a well-established hyperosmotic-stressed human corneal epithelial cell model, with emphasis on assessing the mitigation of cell death, inflammation, and oxidative stress. Finally, the in vivo efficacy of the PBA-Alg@DQS microcarrier eye drops was comprehensively validated in a benzalkonium chloride (BAC)-induced DED mouse model. Key clinical signs, including corneal integrity via fluorescein staining, tear secretion volume, and the histological health of the ocular surface, were meticulously assessed. Collectively, this work demonstrates a powerful new platform for DED treatment with significant potential for clinical application.

Results

Design and Fabrication of PBA-Alg@DQS Microcarriers

As illustrated in **Figure 1**, we created mucoadhesive microcarrier eye drops for prolonged medication release in dry eye disease. Our approach is based on a dual-function polymer backbone that is made by grafting sodium alginate with phenylboronic acid. The PBA components establish dynamic covalent bonds with cis-diol groups, facilitating concurrent drug attachment with DQS and strong mucoadhesion to corneal mucins. To guarantee homogeneity, PBA-Alg@DQS microcarriers were created via a microfluidic electrospray method. They were then solidified in a calcium chloride crosslinking solution. By adhering to the ocular surface for extended retention, releasing DQS continuously to restore tear film stability through mucin and water secretion, and reducing the underlying inflammation and oxidative stress, the resultant microcarriers are ultimately designed to break the vicious cycle of DED.

distribution histogram, indicating that the diameter increases as the voltage decreases from 12 kV to 9 kV. This negative correlation between the diameter and the applied voltage is summarized in the curve shown in **Figure 2G**. The flow rate also affects the particle size (**Figure S2**). Based on these results, we chose 12 kV and 50 $\mu\text{L}/\text{min}$ as the optimal conditions to produce MCs with an average diameter of approximately 12.5 μm and a narrow size dispersity. (**Figure 2D**), which is suitable for minimizing eye irritation. Microcarriers with diameters in this range are expected to minimize ocular irritation, potentially enhancing comfort and compliance. These specific MCs were used for all subsequent experiments. Zeta potential measurements showed that both Alg@DQS MCs and PBA-Alg@DQS MCs exhibited negative surface charges (**Figure S3**). The negative surface charge is consistent with the presence of ionized carboxyl groups in the alginate backbone. The rheological properties of the microcarriers were also evaluated (**Figure 2H**). Viscosity decreased with an increase in shear rate. This property is advantageous for topical eye drops, indicating it would provide low foreign body sensation and improved comfort during the high shear rates associated with blinking ^[23]. The drug release behavior was subsequently evaluated in simulated tear fluid under both normal and oxidative conditions. DQS concentration was quantified using the established UV–Vis standard curve (**Figure S4 and S5**). Under normal conditions, PBA-Alg@DQS MCs exhibited a slower and more gradual release profile than Alg@DQS MCs, consistent with the reversible interaction between PBA and the cis-diol groups of DQS. Under H_2O_2 -containing conditions, DQS release from PBA-Alg@DQS MCs was accelerated compared with that under normal conditions, whereas the corresponding change in Alg@DQS MCs was comparatively limited (**Figure 2I**). These results support the oxidation-sensitive release behavior of the PBA-containing microcarriers and are consistent with H_2O_2 -mediated oxidative deboronation of the PBA/boronate moieties.

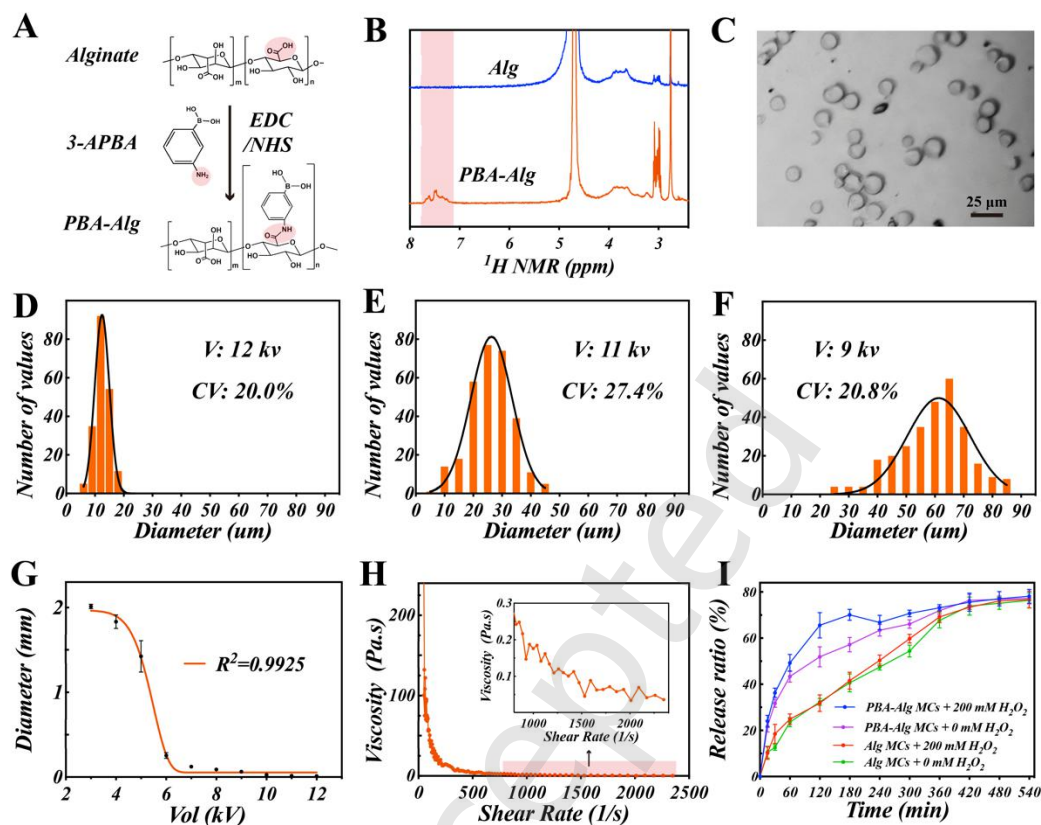


Figure 2. Synthesis and characterization of PBA-Alg microcarriers. (A) Diagram representation of the chemical synthesis process for PBA-Alg. 3-APBA: 3-Aminophenylboronic acid. (B) ^1H NMR spectra of alginate and PBA-Alg. (C) Representative bright-field microscopy image of the optimized microcarriers. Scale = 25 μm . (D-F) Particle size distribution histograms of microcarriers produced under varying applied voltages: (D) 12 kV, (E) 11 kV, and (F) 9 kV. (G) The correlation between applied voltage and microcarrier diameter. (H) Rheological characterization of the PBA-Alg@DQS microcarriers, illustrating viscosity as a function of shear rate. (I) In vitro cumulative release profiles of DQS from Alg@DQS and PBA-Alg@DQS microcarriers in simulated tear fluid under normal and H_2O_2 -containing oxidative conditions.

Mucoadhesive and Ocular Retention Properties

To verify the mucoadhesive properties imparted by the PBA moieties, we first conducted an in vitro mucin-binding assay. PBA-Alg@DQS MCs exhibited a

significantly higher mucin-binding efficiency than Alg@DQS MCs (**Figure 3A**). We next evaluated ocular surface retention ex vivo using isolated porcine eyeballs. Yellow dye was incorporated into the formulations for visualization. After rinsing with simulated tear fluid, most of the PBA-Alg@DQS microcarriers remain on the surface of the cornea, while the unmodified microcarriers and free DQS are largely cleared (**Figure 3D**). Quantitative analysis further confirmed the enhanced ocular retention of PBA-Alg@DQS MCs (**Figure 3B**).

These findings have been verified in vivo through a mouse model. The in vivo imaging system shows that compared with the almost cleared free DQS and Alg@DQS microcarriers, the residual time of PBA-Alg@DQS microcarriers on the ocular surface is extended (**Figures 3C, E**). These data show that PBA modification increases the residual surface of the eye, highlighting the potential of the system as an effective long-acting drug delivery platform. These findings demonstrate that PBA modification enhances ocular surface retention, supporting the potential of the microcarrier system for prolonged ocular delivery.

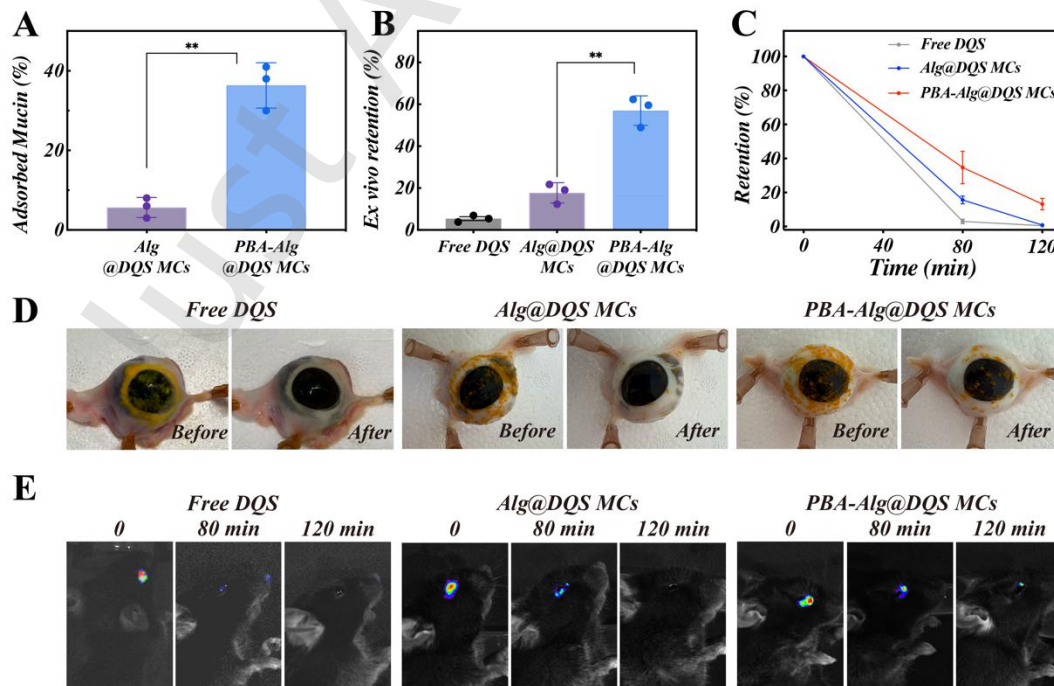


Figure 3. Evaluation of the mucoadhesive and ocular retention properties of PBA-Alg@DQS MCs. (A) Mucin-binding efficiency of Alg@DQS and

PBA-Alg@DQS MCs after incubation with mucin (n=3). (B) Quantitative analysis of ex vivo ocular retention of free DQS, Alg@DQS MCs, and PBA-Alg@DQS MCs on porcine eyeballs after irrigation (n=3). (C) Quantitative analysis of fluorescence intensity over time in mouse eyes, corresponding to the in vivo study (n=3). (D) Representative digital images of porcine eyeballs treated with free DQS, unmodified Alg@DQS MCs and PBA-Alg@DQS MCs, shown before and after a washing period. Yellow dye was used for visualization. (E) Representative in vivo fluorescence images of mouse eyes following administration of free DQS, Alg@DQS MCs, and PBA-Alg@DQS MCs.

In Vitro Therapeutic Effects

First, the biocompatibility of the microcarriers was confirmed in human corneal epithelial cells (HCECs). Live/Dead staining (**Figure S6**) and quantification of percentage of live cells (**Figure 4A**) demonstrated high cell viability in all the groups. Next, we established an in vitro DED model by subjecting HCECs to hyperosmotic stress. A Cell counting kit-8 (CCK8) assay revealed the relative cell survival. While hyperosmotic conditions significantly reduced cell viability, pretreatment with PBA-Alg@DQS microcarriers markedly rescued the cells and restored the viability (**Figure 4B**). To clarify the underlying mechanism, we studied the properties of PBA-Alg@DQS MCs to interrupt the DED pathological cascade in a hyperosmotic stress model. We evaluated its effect on oxidative stress [24, 25]. Intracellular ROS levels were evaluated using the DCFH-DA probe. Hyperosmotic conditions resulted in marked intracellular ROS accumulation, whereas PBA-Alg@DQS treatment significantly reduced the fluorescence signal (**Figure 4D**). This reduction was further confirmed by fluorescence intensity quantification (**Figure 4C**) and flow cytometry analysis (**Figure 4E, F**). These findings demonstrate that PBA-Alg@DQS treatment attenuated intracellular ROS accumulation under hyperosmotic stress. The reduction in intracellular ROS was accompanied by suppression of downstream inflammatory responses, as evidenced by quantitative real-time PCR (RT-qPCR) analysis of several key genes. These included matrix metalloproteinase-9 (MMP-9) [26, 27] and the

inflammatory markers tumor necrosis factor-alpha (TNF- α) [28, 29] and interleukin-1 β (IL-1 β) [30, 31]. PBA-Alg@DQS treatment effectively inhibits the mRNA expression of these markers, indicating that it has a strong anti-inflammatory effect (**Figure 4G**). In general, these in vitro experimental results show that the PBA-Alg@DQS microcarrier system protects corneal cells by concurrently decreasing both oxidative and inflammatory damage from hyperosmotic stress.

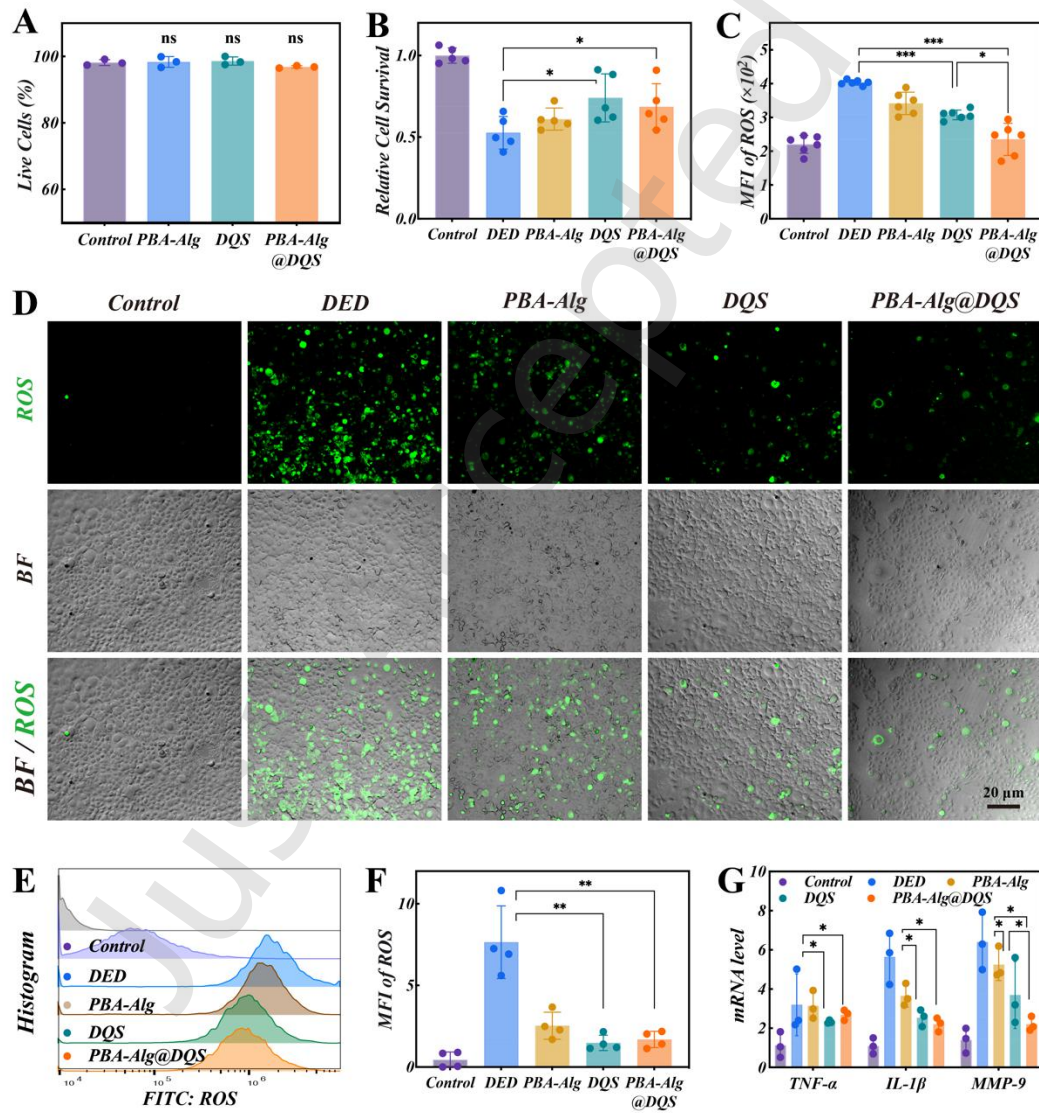


Figure 4. The in vitro therapeutic effect of PBA-Alg@DQS microcarrier on

HCECs under hyperosmolar stress. (A) The biocompatibility of microcarriers was evaluated before the treatment evaluation. Under standard conditions, the percentage of live cells determined by live/dead staining was quantitatively evaluated after 24 hours of co-culture of different groups and HCECs (n=3). In order to simulate dry eye disease in vitro, HCECs were pretreated with different preparations before exposure to hyperosmotic stress. (B) Relative cell survival was assessed using a CCK-8 assay, normalized to the control group (n=5). (C) Quantification of intracellular ROS by measuring average fluorescence intensity (MFI) by fluorescence microscope (n=6). (D) A representative fluorescence microscope image showing the reactive oxygen in the cell. Scale = 20 μ m. (E) The histogram shows the level of intracellular ROS quantitatively analyzed by flow cytometry. The gray peak represents the unstained negative control cell group. (F) Quantitatively analyze the average fluorescence intensity (MFI) of ROS according to the flow cytometry data shown in (E) (n=4). (G) Determine the relative expression of TNF- α , IL-1 β and MMP-9 in HCEC by RT-qPCR (n=3).

Transcriptomic Analysis of Redox-Related Responses

To further investigate the molecular changes associated with the reduction in intracellular ROS, RNA sequencing was performed in HCECs from five experimental groups. The overall transcriptomic profiles and numbers of differentially expressed genes are summarized by PCA and DEG analysis, respectively (Figures S7 and S8), while selected disease- and treatment-responsive genes are shown in Figure S9. GSEA showed significant enrichment of the VAV-mediated RAC1 activation gene set in DED versus CTRL, whereas PBA-Alg@DQS treatment significantly reversed this transcriptional program (Figure 5A, B). Directional enrichment analysis further identified coordinated changes in a NOX/RAC1-associated superoxide-generation program (Figure 5C). Consistent with the pathway-level analysis, VAV3 and NCF2 were markedly increased under hyperosmotic stress and reduced following treatment. These expression changes were further confirmed by RT-qPCR (Figure 5D). Collectively, these findings indicate that the reduction in intracellular ROS was

associated with attenuation of a hyperosmotic stress-induced VAV–RAC1/NOX-related superoxide-generating transcriptional program.

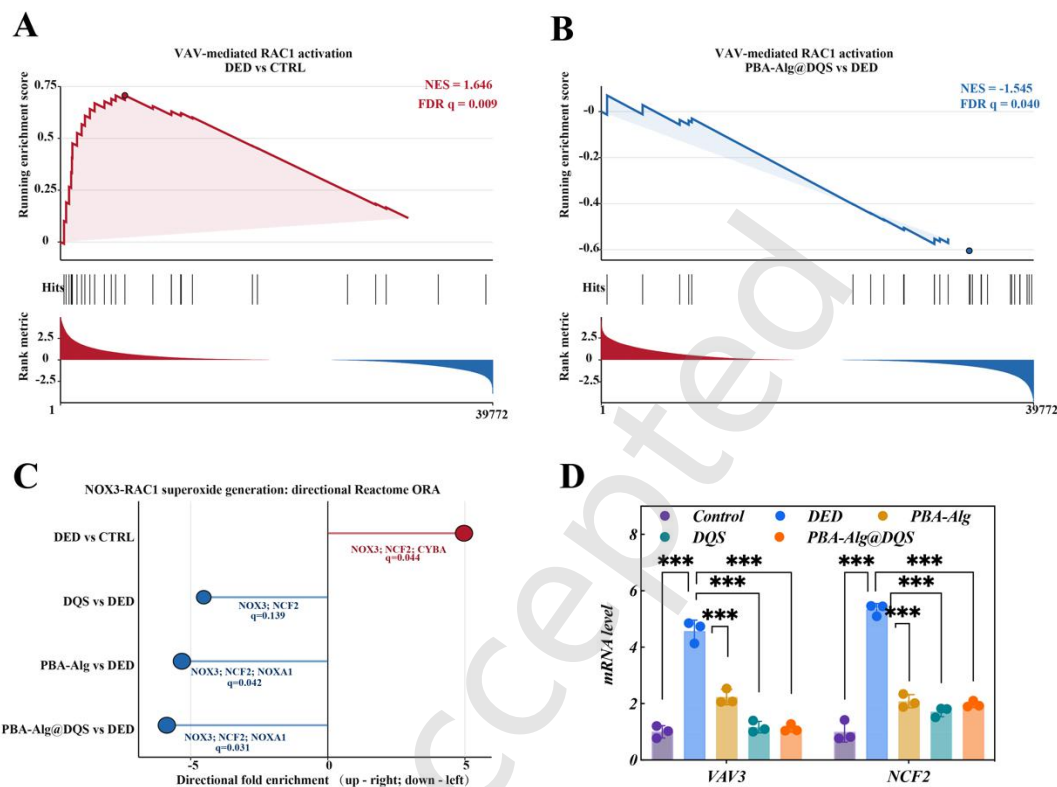


Figure 5. Transcriptomic analysis of redox-related responses in HCECs under hyperosmotic stress. (A) GSEA of the VAV-mediated RAC1 activation gene set in DED versus CTRL. (B) GSEA of the VAV-mediated RAC1 activation gene set in PBA-Alg@DQS versus DED. (C) Directional over-representation analysis of the NOX/RAC1-associated superoxide-generation program. (D) RT-qPCR analysis of VAV3 and NCF2 expression in HCECs (n=3).

Therapeutic Efficacy and Mechanism in a Mouse Model of DED

First, the in vivo biocompatibility of PBA-Alg@DQS microcarriers was verified. During the 14-day administration, the mice receiving the microcarriers showed no apparent signs of systemic toxicity and maintained body weight gain comparable to the control group receiving no treatment (**Figure S10**). Ocular surface TUNEL

staining showed that cell apoptosis was not significantly increased, indicating good local tolerance (**Figure S11**). Following the biocompatibility assessment, the therapeutic efficacy was evaluated in a murine DED model. According to the timeline shown in **Figure 6A**, a mouse model of dry eye disease was established by applying 0.075% BAC. All treatment groups were administered twice daily to enable a direct comparison between free DQS and PBA-Alg@DQS under the same dosing frequency. Key clinical indicators were evaluated. The integrity of the cornea was measured by staining with sodium fluorescein. The PBA-Alg@DQS microcarrier treatment group showed significant corneal surface repair (**Figure 6B**). The significant decrease in corneal staining score confirms this observation (**Figure 6C**). The test results of phenol red filament showed that PBA-Alg@DQS treatment significantly restored tear secretion (**Figure 6D**). These results demonstrate a potent recovery of ocular surface health at a clinical level in PBA-Alg@DQS group. The therapeutic effects of PBA-Alg@DQS microcarriers were examined at the molecular level within corneal tissues. RT-qPCR analysis demonstrated that treatment with PBA-Alg@DQS MCs markedly reduced the expression of MMP-9 and pro-inflammatory cytokines (TNF- α and IL-1 β) (**Figure 6E**).

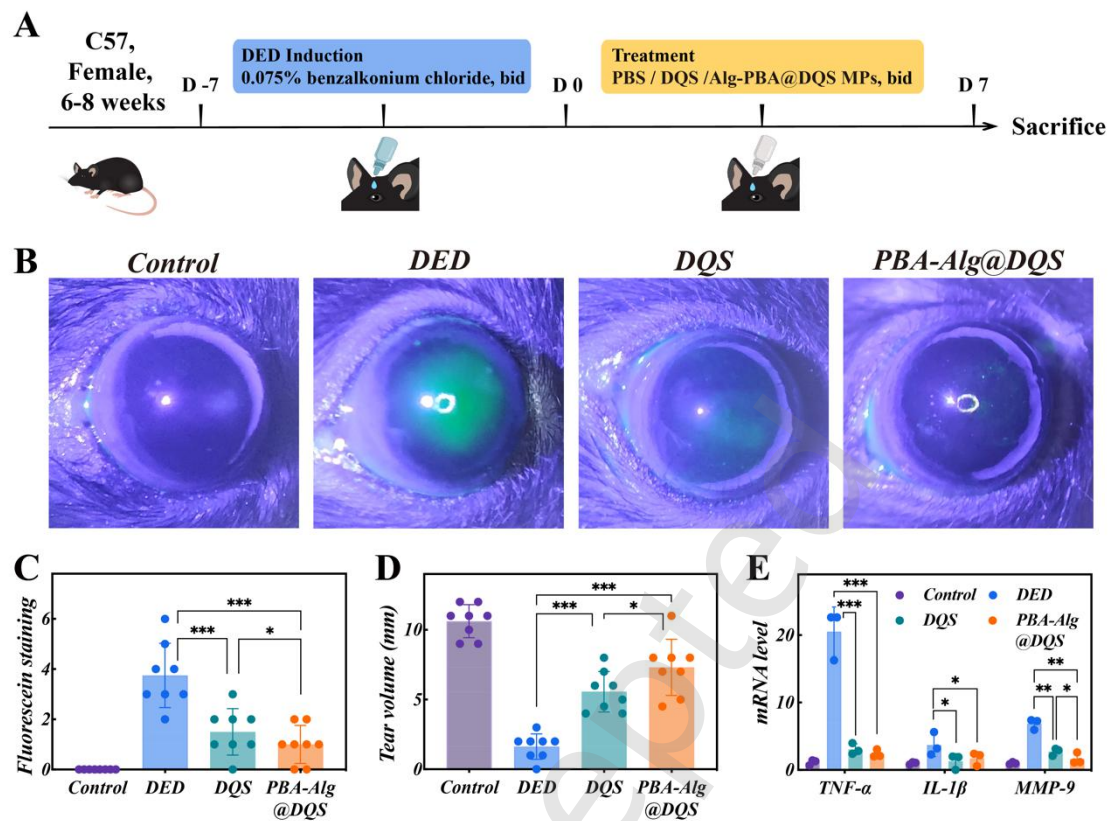


Figure 6. Therapeutic efficacy of PBA-Alg@DQS microcarriers in a BAC-induced mouse model of dry eye disease. (A) Schematic diagram of the experimental timeline for the induction and treatment of DED. (B) Representative images depicting fluorescein sodium staining on the seventh day of treatment. (C) Evaluation scores derived from fluorescein staining of the cornea (n=8). (D) Assessment of tear production using phenol red threads (n=8). (E) RT-qPCR analysis of inflammatory gene expression (TNF- α , IL-1 β , and MMP-9) in corneal samples (n=3).

Additionally, the therapeutic advantages were validated through the analysis of protein levels using immunofluorescence labeling. We focused on TNF- α , the master pro-inflammatory cytokine, and MMP-9, the enzyme that degrades the corneal epithelial barrier, both of which are highly implicated in DED pathogenesis. The levels of the TNF- α (**Figure 7A, C**) and MMP-9 (**Figure 7B, D**) were significantly decreased in the corneas of the PBA-Alg@DQS treated group. The histological examination of the ocular surface via PAS staining was carried out. The dry eye model led to a significant decrease in goblet cells. PBA-Alg@DQS microcarrier

therapy successfully retained this cell group (**Figure 7E, G**). To confirm if these cells regained their function, we examined the expression of MUC5AC, the primary gel-forming mucin. Immunofluorescence staining showed a robust re-establishment of MUC5AC protein levels in the conjunctiva (**Figure 7F, H**), confirming the restoration of the protective mucin layer. This effect is consistent with the pharmacological action of DQS as a P2Y2 receptor agonist, which promotes mucin secretion from goblet cells [32]. The restoration of MUC5AC is essential for reestablishing the protective mucous layer of the tear film, which is fundamental to the treatment of DED. In conclusion, the PBA-Alg@DQS microcarrier eye drops efficiently mitigated the signs and symptoms of DED. Under the twice-daily dosing regimen used in this study, the formulation inhibited inflammation and restored the mucin-secreting function of goblet cells, which collectively contributed to ocular surface healing and tear-film restoration.

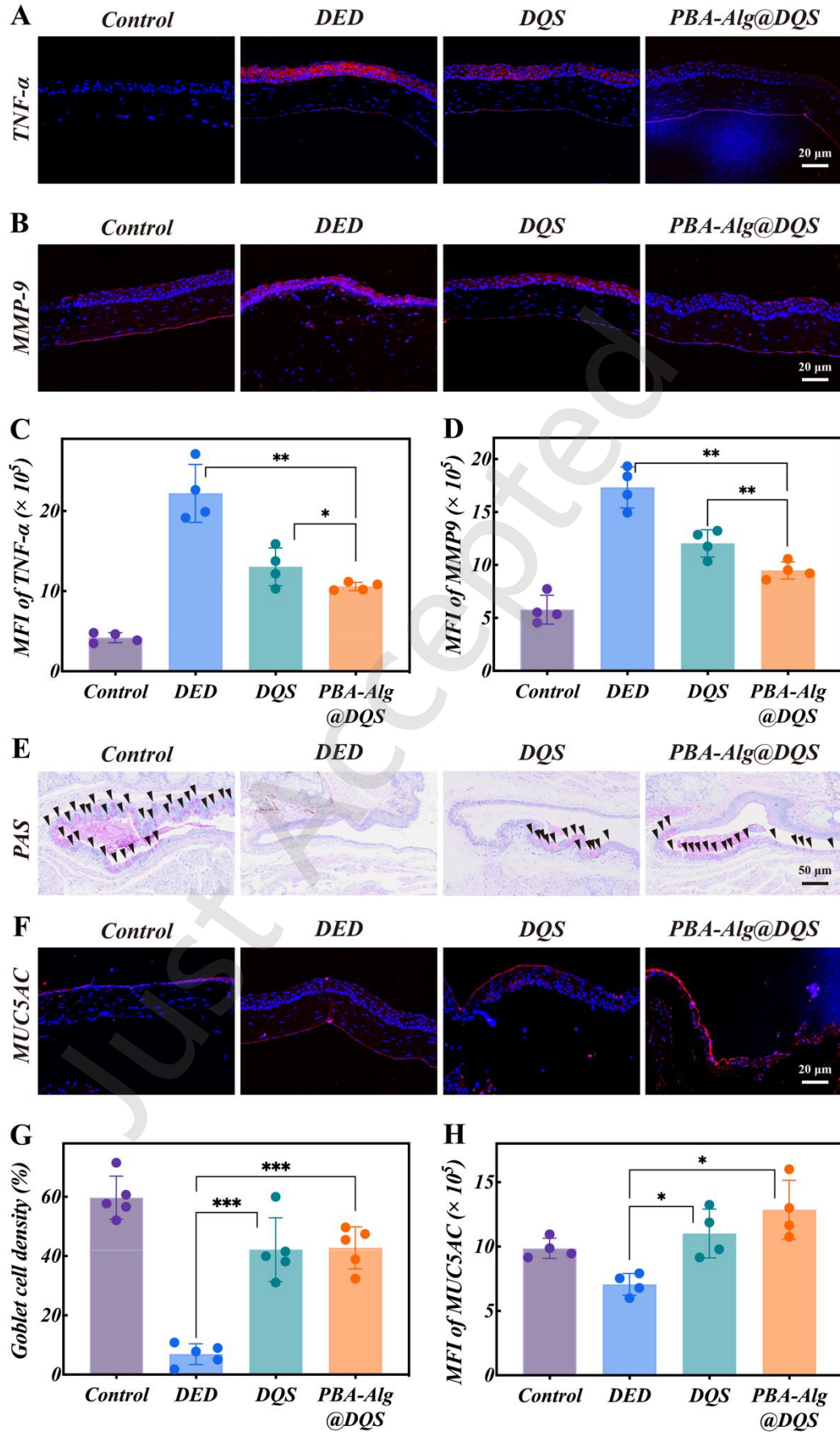


Figure 7. Immunofluorescence analysis of key markers in corneal tissues. Immunofluorescence staining of TNF- α (A) and MMP-9 (B) expression. Blue fluorescence indicates DAPI-stained nuclei. Scale bar: 20 μ m. Quantitative analysis of the fluorescence intensity for TNF- α (C) and MMP-9 (D) (n=4). (E) PAS staining of conjunctival tissue showing conjunctiva goblet cells. Scale bar, 50 μ m. (F) Immunofluorescence staining of MUC5AC expression. Blue fluorescence indicates DAPI-stained nuclei. Scale bar: 20 μ m. (G) Quantitative analysis of the percentage of conjunctiva goblet cells areas (n=5). (H) Quantitative analysis of the fluorescence intensity for MUC5AC (n=4).

Discussion

In this study, we developed a phenylboronic acid-modified alginate microcarrier platform (PBA-Alg@DQS) fabricated by microfluidic electrospray for ocular delivery of diquafosol tetrasodium in DED therapy. By integrating dynamic covalent chemistry into uniformly sized hydrogel microcarriers, the system exhibited sustained DQS release in simulated tear fluid and enhanced ocular surface retention. In vitro, the microcarriers protected human corneal epithelial cells from hyperosmotic injury by attenuating oxidative stress and downstream inflammatory signaling, while in a BAC-induced murine model they restored corneal integrity, tear secretion, and goblet cell density under a twice-daily dosing regimen. Together, these findings demonstrate the therapeutic potential of PBA-Alg@DQS as an ocular drug delivery platform for DED.

The therapeutic performance of the system can be attributed to the rational design of the PBA-Alg backbone, which integrates several complementary functions within a single material. First, reversible boronate ester interactions between PBA and cis-diol-containing components of ocular mucins contribute to mucoadhesion, consistent with the enhanced mucin binding, ex vivo retention, and prolonged in vivo ocular residence observed for PBA-Alg@DQS MCs. Second, the same reversible boronate chemistry can interact with cis-diol groups in DQS, providing a drug-affinity

mechanism that suppresses burst release and prolongs drug release relative to Alg@DQS MCs. Third, PBA/boronate moieties are chemically susceptible to H₂O₂-mediated oxidative deboronation. Under oxidative conditions, this reaction may disrupt PBA–cis-diol interactions and could thereby facilitate drug release while consuming H₂O₂ during the deboronation process. Although the H₂O₂-dependent release experiment supports oxidation-sensitive drug release, direct oxidant consumption was not quantitatively measured in the present study. Therefore, these effects should be regarded as mechanistically plausible rather than experimentally established. Collectively, these properties couple ocular adhesion, drug retention, sustained release, and oxidation-sensitive chemistry within the same delivery platform.

Compared with previously reported strategies for prolonging ocular drug residence—such as viscosity enhancers, contact lens-based reservoirs, in situ-forming gels, and nanoparticle suspensions—the present platform offers several distinguishing advantages. Microfluidic electrospray affords precise control over particle dimensions and a narrow size dispersity, which is difficult to achieve with bulk emulsification or conventional spray techniques and which directly translates into more reproducible drug loading and release profiles. The optimized diameter of approximately 12.5 μm together with the shear-thinning rheology of the formulation is expected to balance ocular retention against patient comfort, since the suspension flows readily during blinking but reverts to a more viscous state at rest. Mechanistically, the dual-functional PBA-Alg design also distinguishes our system from earlier mucoadhesive carriers based on thiolation, catechol chemistry, or cationic polymers, which generally provide adhesion without intrinsic drug-tethering or oxidation-sensitive functionality.

Beyond the delivery aspect, the in vitro and in vivo data collectively suggest that the therapeutic benefit arises from intervention at multiple components of the DED pathological cycle. DQS released from the carrier acts pharmacologically on P2Y₂ receptors, contributing to tear and mucin secretion, as reflected by the recovery of tear

volume, goblet cell density, and MUC5AC expression in treated mice. In parallel, PBA-Alg@DQS treatment markedly reduced intracellular ROS accumulation under hyperosmotic stress. Transcriptomic analysis provided additional mechanistic context for this redox effect. Several redox-defense transcripts showed treatment-associated restoration, while PBA-Alg@DQS was also associated with reversal of a hyperosmotic stress-induced VAV–RAC1/NOX-related superoxide-generating transcriptional program. VAV proteins regulate RAC-family GTPases, whereas RAC participates in NADPH oxidase activation together with regulatory components such as NCF2/p67phox, thereby providing a plausible transcriptional link to cellular superoxide production.

In addition to these redox-related effects, PBA-Alg@DQS treatment suppressed inflammatory responses, as demonstrated by reduced expression of MMP-9, TNF- α , and IL-1 β at the transcriptional level and decreased TNF- α and MMP-9 protein levels *in vivo*. These effects, together with restoration of tear and mucin secretion, indicate that the formulation acts on multiple interconnected components of the DED pathological cycle. The integrated PBA-Alg@DQS system therefore combines the pharmacological and cellular protective effects of its components with the carrier-specific advantages of mucoadhesion and controlled drug release. This multi-level action may contribute to the improved therapeutic performance of PBA-Alg@DQS compared with free DQS under the matched dosing conditions used in this study.

Several limitations should be acknowledged. The therapeutic evaluation was conducted in a BAC-induced acute DED model over a 7-day window, which captures epithelial damage and inflammation but does not fully recapitulate the chronic, immune-mediated features of human DED such as Sjögren-associated disease or meibomian gland dysfunction; longer-term studies in additional models, including evaporative and aqueous-deficient phenotypes, will be needed to define the breadth of indication. Detailed pharmacokinetic profiling of ocular DQS concentrations, further characterization of oxidation-dependent release kinetics across physiologically

relevant oxidative conditions, and large-animal safety and tolerability studies are warranted before clinical translation. Direct quantification of H₂O₂ consumption and structural characterization of PBA oxidative deboronation would also be valuable for establishing the contribution of this mechanism to the observed reduction in oxidative stress. In addition, the transcriptomic findings identify pathway-level associations but do not establish direct changes in RAC1 activation or NADPH oxidase enzymatic activity, which will require dedicated protein- and activity-level validation in future studies. Finally, scalable and GMP-compatible manufacturing of microfluidic electrospray products, together with formulation stability during storage, remains an engineering challenge that will need to be addressed in subsequent development.

In summary, the PBA-Alg@DQS microcarrier platform integrates microfluidic electrospray fabrication with dual-functional boronate chemistry to provide mucoadhesion, sustained in vitro DQS release, and prolonged ocular surface retention within a single ocular formulation. The therapeutic efficacy observed under twice-daily administration suggests that this platform has the potential to reduce dosing burden, although direct pharmacokinetic comparison and comparison with the conventional six-times-daily DQS regimen will be required to confirm this advantage. More broadly, this work highlights the potential of dynamic covalent chemistry for improving ocular drug delivery.

Materials and Methods

Materials

Analytical chemicals (alginate, NHS, EDC purchased from Sigma; 3-APBA, DQS purchased from Aladdin) are used directly. All aqueous solutions are prepared with deionized water. Gibco provides the required cell culture medium and additives (DMEM/F-12, FBS, P/S, trypsin-EDTA). CCK-8 kits, live/dead cell staining kits and ROS test kits are all purchased from Beyotime Biotechnology.

Synthesis of PBA-Alg Hydrogel

The synthesis involved the conjugation of PBA to alginate by EDC/NHS chemistry ^[33]. An alginate solution (1 g in 0.1 M MES) was titrated to pH 5.5, thereafter including EDC (700 mg), NHS (100 mg), and 3-aminophenylboronic acid (300 mg) in succession. Following a 24-hour reaction period, the resultant polymer was purified by dialysis with deionized water for 72 hours (MWCO: 6–8 kDa).

Microfluidic Electrospray Device and Optimization of microcarriers

The experimental apparatus consisted of a syringe and a glass capillary linked via a microtube. The capillary was tapered by heating it over a flame, and the tip was polished with sandpaper to achieve the specified diameter. Pregel solutions were prepared by mixing DQS with 1% PBA-Alg solution, with the DQS feed concentration adjusted to 3% (w/v). This concentration has been validated in numerous clinical studies and is widely used in clinical practice^[10]. For the control Alg@DQS MCs, unmodified sodium alginate (1%, w/v) was used instead of PBA-Alg, while the DQS concentration (3%, w/v) and all other fabrication and crosslinking conditions were kept identical. This solution was then processed through a microfluidic electrospray device. PBA-Alg@DQS solution was electrosprayed into 2% calcium chloride solution ^[34] under variable factors including voltage (3–12 kV) and flow rate (50–275 $\mu\text{L}/\text{min}$). To identify the ideal fabrication conditions, particle morphology was examined under light microscopy and the size distribution was examined using ImageJ software.

Zeta Potential Measurement

The zeta potentials of Alg@DQS MCs and PBA-Alg@DQS MCs were measured using a Zetasizer (Malvern Panalytical, UK) ^[35]. The microcarriers were dispersed in KCl solution prior to measurement. Each sample was measured independently three times.

In Vitro Drug Release.

The in vitro release of DQS from Alg@DQS MCs and PBA-Alg@DQS MCs was evaluated in simulated tear fluid under normal and oxidative conditions. Microcarriers loaded in dialysis bags were immersed in 1 mL of simulated tear fluid either (Jiacheng Technology) without H₂O₂ or supplemented with 200 μ M H₂O₂ and incubated at 37°C with shaking at 100 rpm. At predetermined time points, 100 μ L of the release medium was collected and replaced with an equal volume of fresh corresponding medium. The concentration of DQS in the release medium was determined by UV–Vis spectrophotometry (General 0401) using the external standard method.

In Vitro Mucin Binding Assay

The mucin binding capacity of the microcarriers was quantified using a spectrophotometric method [36, 37]. Briefly, 10 mg of either PBA-Alg@DQS MCs or unmodified Alg@DQS MCs were suspended in porcine gastric mucin solution. The mixture was kept at 37°C for two hours with gentle shaking. The samples were then spun down at 10,000 rpm for 15 minutes. The concentration of non-adsorbed mucin was determined via supernatant absorbance at 255 nm. By measuring drops in mucin concentration in the supernatant relative to the starting concentration, the mucin binding efficiency was determined. Every experiment was carried out three times.

Ex Vivo Ocular Surface Adhesion Test

Fresh porcine eyeballs obtained from a local slaughterhouse were used to evaluate the mucoadhesive properties of the PBA-Alg@DQS MCs by measuring their retention on the ocular surface under physiological settings. Three formulations, including free DQS, Alg@DQS MCs, and PBA-Alg@DQS MCs, were evaluated. Yellow dye was added to each formulation for easy visibility. After the ocular surface was gently rinsed with PBS to remove debris, 20 μ L of each formulation was administered onto the corneal surface. Images were acquired immediately after administration and after irrigation with 3 mL of simulated tear fluid over 30 s to mimic tear clearance [38, 39]. ImageJ software was used to examine the covering area on the ocular surface. We calculated retention rates based on the percentage of the

area remaining after washing to the initial area ^[40]. The results were compared to assess the superior mucoadhesion properties of the bio-inspired PBA-Alg@DQS MCs.

Animals

Female C57BL/6J mice aged 6 to 8 weeks were used, which were purchased from Jiangsu Huachuang Sino PharmaTech. These animals were kept at a facility at the Medical School of Nanjing University. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Affiliated Drum Tower Hospital, Medical School of Nanjing University (Protocol No. 2024AE01096). The studies were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and complied with the ARRIVE guidelines and the NIH Guide for the Care and Use of Laboratory Animals.

In Vivo Retention Study

For in vivo ocular retention studies, three formulations were evaluated: free DQS, Alg@DQS MCs, and PBA-Alg@DQS MCs. Sodium fluorescein was added to each formulation as a fluorescent tracer for ocular surface tracking, with the tracer concentration kept identical among all groups. For in vivo imaging, female C57BL/6J mice (8 weeks old) were anaesthetized with isoflurane. To replicate natural tear dynamics, manual blinking was used. We administered it into the conjunctival sac and measured fluorescence retention using an AniView 100 system at 0, 80, and 120 minutes post-instillation^[41]. Background fluorescence was subtracted from all measurements to ensure accurate quantification of retention on the ocular surface. The fluorescence intensity was quantified in the ocular region, and retention percentages were calculated relative to the initial fluorescence signal.

Cell Culture

Log-phase HCECs (CRL-11135) were propagated in DMEM/F12 containing 10% FBS and 1% antibiotics under standard conditions (37°C, 5% CO₂).

Biocompatibility Assessment

HCECs were co-cultured with PBA-Alg MCs in 96-well plates and 6-well plates, with normal culture media serving as control. Calcein AM/PI staining was used to discriminate between live and dead cells (30 minutes at 37°C) [42], and visualized under a fluorescence microscope (MICA, LEICA). Cell viability was calculated as the percentage of live (green) cells relative to the total number of cells.

In Vitro Dry Eye Model Establishment and Therapeutic Evaluation

HCECs were selected to model the corneal epithelial component of DED, particularly hyperosmolarity-induced epithelial injury, oxidative stress, and inflammation. HCECs were seeded in plates at appropriate densities and allowed to attach overnight. To replicate dry eye conditions, a hyperosmotic stress media (450 mOsm/L) containing NaCl was utilized as previously reported with slight modifications^[7]. After 12 hours of exposure to hyperosmotic stress medium, cells were treated with PBA-Alg MCs, free DQS or PBA-Alg@DQS MCs for 12 hours - with the final DQS concentration adjusted to 120 μ M in the DQS-containing groups. The cell viability was evaluated using the CCK-8 test at 450 nm (Tecan) [43].

RT-qPCR

RNA isolated using TRIzol (Invitrogen) was transcribed to cDNA by a kit (Vazyme). The qPCR was performed on a Q5 machine using SYBR Green. Expression levels were measured with β -actin used for the internal control (Primer: Table S1).

RNA Sequencing and Bioinformatics Analysis

HCECs from five groups—CTRL, DED, PBA-Alg, DQS, and PBA-Alg@DQS—were analyzed, with three samples per group. Hyperosmotic treatment consisted of 12 h of exposure to NaCl-supplemented medium (450 mOsm/L), followed by 12 h of treatment under continued hyperosmotic conditions. The nominal DQS-equivalent concentration was 120 μ M in both DQS-containing

groups. Total RNA was extracted using TRIzol (Thermo Fisher Scientific, 15596018). Poly(A) RNA was enriched with oligo(dT) beads, fragmented, and used to construct strand-specific cDNA libraries. Libraries were sequenced on an Illumina NovaSeq X Plus platform with 150-bp paired-end reads by LC-Bio Technology Co., Ltd.. Reads were processed using fastp (v0.24.0) and aligned to the human reference genome from Ensembl release 115 using HISAT2 (v2.2.1). Transcript assembly and abundance estimation were performed using StringTie (v2.1.6). Gene-level counts were analyzed by the sequencing provider using DESeq2 (v1.22.2). P values were adjusted using the Benjamini–Hochberg procedure within each comparison. Provider-reported adjusted P values and $\log_2$ fold changes were used to identify differentially expressed genes (DEGs), defined by $q < 0.05$ and $|\log_2 FC| \geq 1$. Upregulated and downregulated DEGs were counted separately for DED versus CTRL and each treatment versus DED. Principal component analysis was performed in R using the gene-expression matrix. All 15 samples were included, and the first two principal components were displayed with their explained variance. Gene set enrichment analysis (GSEA, v4.1.0) was performed using Reactome gene sets and the provider’s expression matrix. Genes were ranked by Signal2Noise without prior DEG selection. Gene sets represented in the ranked list were analyzed using weighted enrichment and gene-set permutations. Normalized enrichment scores (NES) and GSEA-derived FDR q values were reported, with FDR $q < 0.05$ considered significant. Positive and negative NES values indicated enrichment toward higher expression in the first- and second-named groups, respectively. The VAV-mediated RAC1 activation gene set (R-HSA-3928628) was selected for presentation after examination of the enrichment results. Curves were reconstructed from the provider’s ranked lists and gene-set definitions, retaining the original NES and FDR values. For the directional Reactome over-representation analysis (ORA), a secondary filtering and enrichment analysis was applied to the existing DESeq2 output. Genes with counts ≥ 10 in at least 3 of the 15 samples were retained. Within this filtered set, Benjamini–Hochberg correction was reapplied to the nominal P values reported for each contrast. Protein-coding genes with recalculated q

< 0.05 and $|\log_2 FC| \geq 1$ were separated into upregulated and downregulated sets and analyzed independently. One-tailed hypergeometric tests were performed using the testable protein-coding genes with Reactome annotations as the analysis background. Reactions containing 5–1,000 annotated genes and at least two overlapping DEGs were tested, and P values were adjusted using the Benjamini–Hochberg method separately for each contrast and direction. Fold enrichment was calculated as $(k/N)/(K/M)$, where k and N denote the numbers of overlapping and input genes, respectively, and K and M denote the reaction size and annotated background size, respectively. The Reactome reaction “NOX3 complex:RAC1: GTP generates superoxide from oxygen” (R-HSA-5668731) was selected for presentation because of its direct relevance to the intracellular ROS phenotype. For the selected-gene heatmap, gene counts were normalized to counts per million using total gene count and transformed as $\log_2 (CPM + 0.5)$. Values were standardized for each gene across all 15 samples by subtracting the mean and dividing by the sample standard deviation. Fourteen genes were selected based on biological relevance and observed disease- and treatment-associated expression patterns. Genes were arranged by functional category and samples by group, without hierarchical clustering. Row z-scores represent within-gene relative expression and do not constitute a separate significance test.

Intracellular ROS Measurement

Intracellular ROS generation was employed by the DCFH-DA probe following the ROS Assay Kit ^[44]. The resulting fluorescence was visualized and recorded using a Nikon fluorescence microscope, and quantitative analysis of ROS levels was performed by measuring average fluorescence intensity using flow cytometry (BD FACSCalibur). Data analysis was conducted using FlowJo software version 10.4.

Evaluation of In Vivo Biocompatibility

PBA-Alg@DQS MCs (5 μ L per eye, twice daily) were given to 6 healthy female C57BL/6J mice (8 weeks old) for 14 days to evaluate the *in vivo* bio compatibility of the MCs. Under the same circumstances, a control group was not given any treatment. Throughout the trial, the body weight of mice was recorded at regular intervals to track systemic toxicity. The mice were sacrificed after the treatment was finished, and their eyes were removed for examination. Following the instructions, the enucleated eyeballs were sectioned and stained using TUNEL test to check for possible apoptosis on the ocular surface. A fluorescent microscope was used to image the resultant sections.

BAC-Induced DED Model and Treatment

A Dry Eye Disease mice model was established by administering 0.075% BAC twice daily for a duration of 7 days, as previously stated [30]. Following this, the mice were randomly allocated into three groups receiving treatment with either PBS (DED group), free DQS solution (3%, w/v; DQS group), or PBA-Alg@DQS MCs prepared using a 3% (w/v) DQS feed concentration (PBA-Alg@DQS group). A fourth group of healthy, non-induced mice receiving PBS served as the healthy control (Control group). All treatments (5 μ L) were administered twice daily for 7 days. Then corneal fluorescein staining, photographs of eyes, tear volume test, histopathological examination, RT-qPCR and immunofluorescence staining test were taken.

Evaluation of Clinical Signs

Tear volume test was assessed by phenol red thread (Tianjin Jingming). The length of the red segment of the thread was measured in millimeters after 3 minutes. Sodium fluorescein test paper (Tianjin Jingming) was used topically to examine the fluorescein staining of cornea ^[45], producing a score ranging from 0 to 15 under a previously established grading system ^[46] by the slit-lamp biomicroscope under cobalt blue light.

Histological Analysis

Enucleated eyeballs were embedded in paraffin, sectioned, and fixed in ocular fixation solution (Servicebio) for histological examination. To visualize goblet cells, sections were stained with the PAS kit and examined under an optical microscope (VS200) [47].

Immunofluorescence Staining

Sections of ocular tissue were deparaffinized before being exposed to sodium citrate buffer and then washed by PBS washing. The sections were incubated in 5% BSA for an hour at 37°C. After that they were permeabilized using 0.2% Triton X-100. Primary antibodies, rabbit anti-TNF- α (1:200, Proteintech), mouse anti-MMP-9 (1:200, Servicebio), and rabbit anti-MUC5AC (1:200, Proteintech), were used and incubated at 4°C for one entire night. After washing, slices were incubated in secondary antibodies that were correspondingly Alexa Fluor-conjugated (1:1000, Invitrogen). DAPI was used as a counterstain for the nuclei. Using a Nikon fluorescent microscope, pictures were taken.

Statistical Analysis

The data were reported as mean $\pm$ SD. Unpaired t-tests were performed to assess statistically significant differences between two groups, and one-way ANOVA was used to analyze multiple groups (Prism 10). A P-value of <0.05 was judged statistically significant. $P < 0.05$ is denoted by *, $P < 0.01$ by **, $P < 0.001$ by ***, while ns denotes no significance.

Acknowledgements

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Electronic Supplementary Materials

Mucoadhesive polymeric microcarriers delivering diquafosol tetrasodium for dry eye therapy

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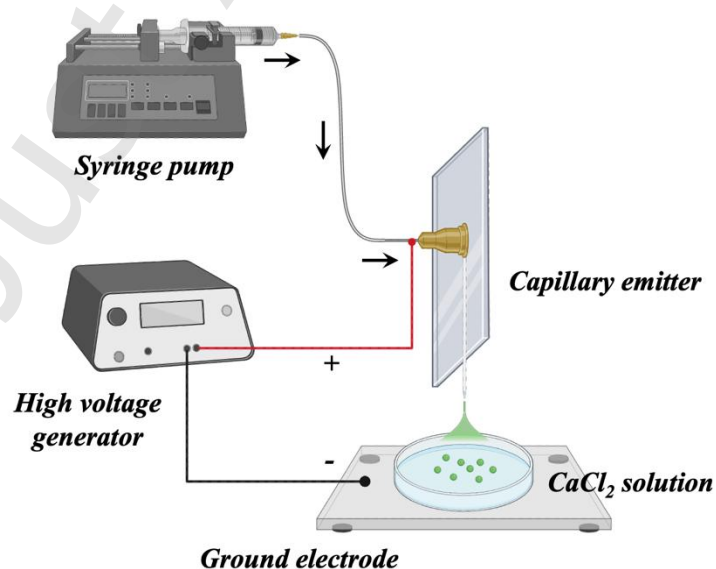


Figure S1. Schematic diagram of the electrospraying apparatus used for microcarrier fabrication.

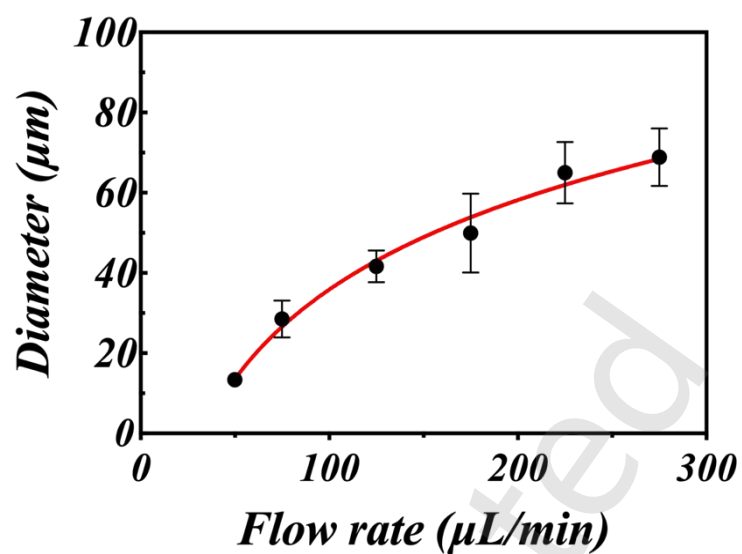


Figure S2. The influences of flow rate of pre-gel on microcarrier diameter were detected and summarized (n=3).

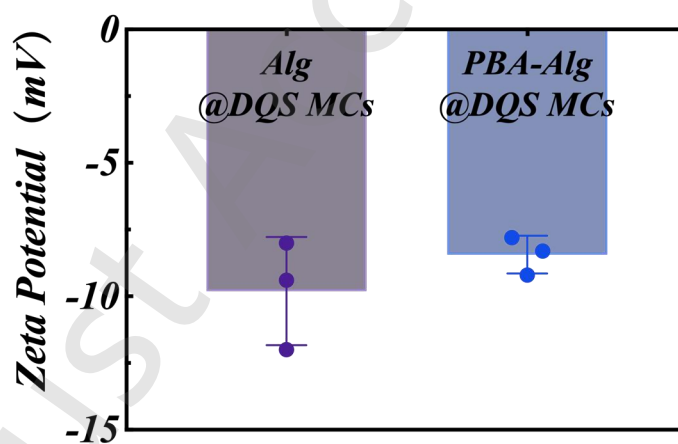


Figure S3. Zeta potentials of Alg@DQS and PBA-Alg@DQS microcarriers (n=3).

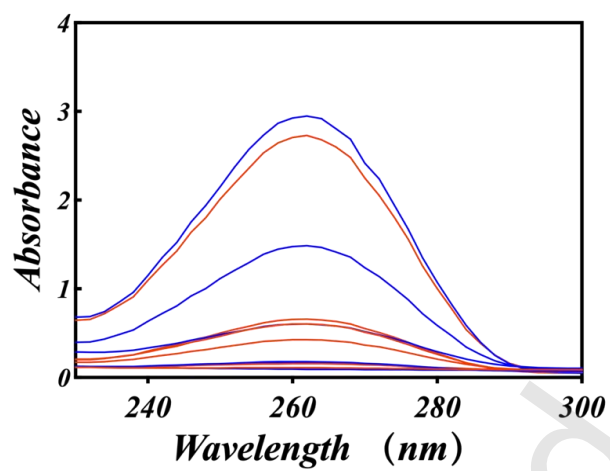


Figure S4. UV-Vis absorption spectra of DQS.

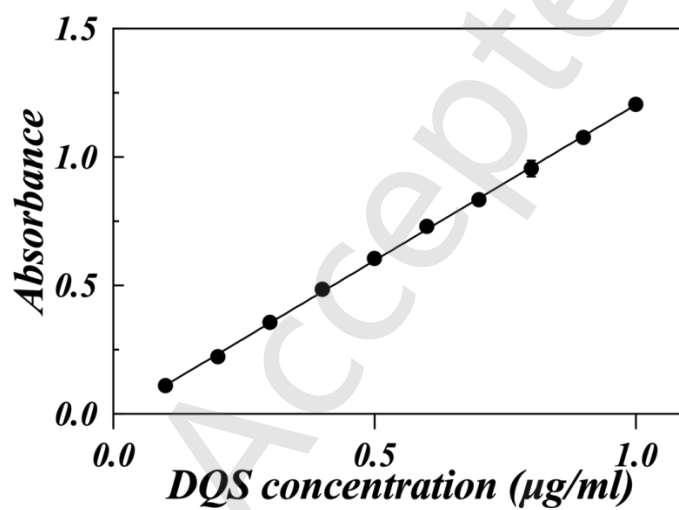


Figure S5. Standard Curve for DQS quantification.

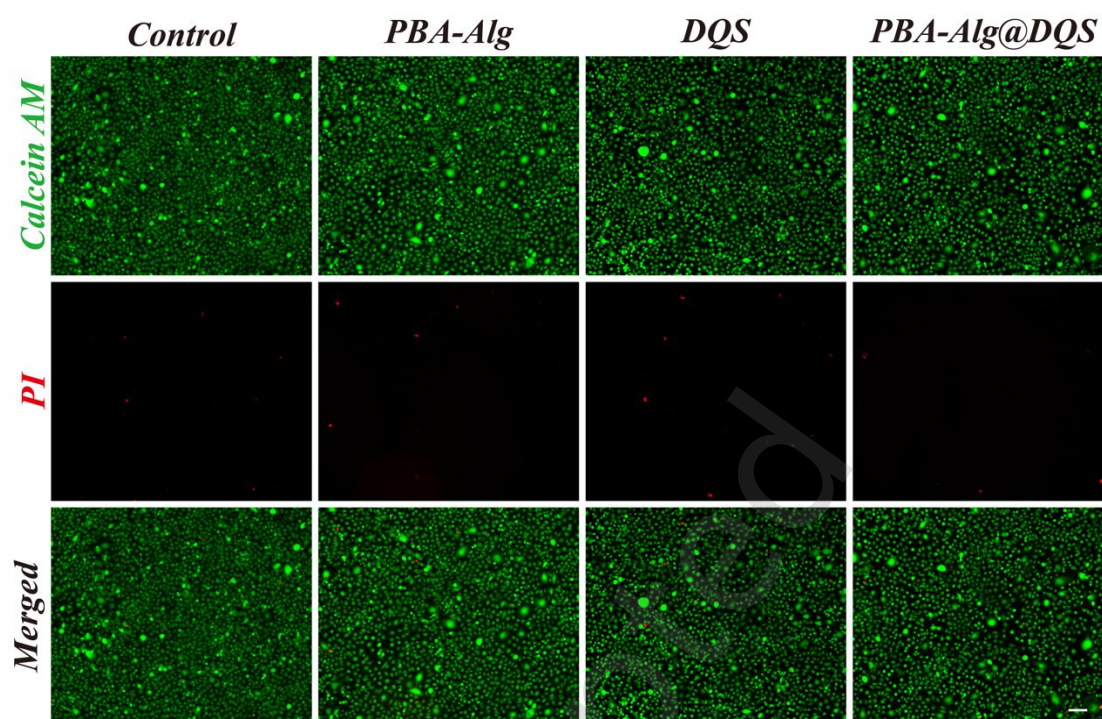


Figure S6. Biocompatibility Assessment of Microcarriers with HCECs. Cells were stained with Calcein AM (green, live cells) and Propidium Iodide (PI; red, dead cells).

Principal Components Analysis

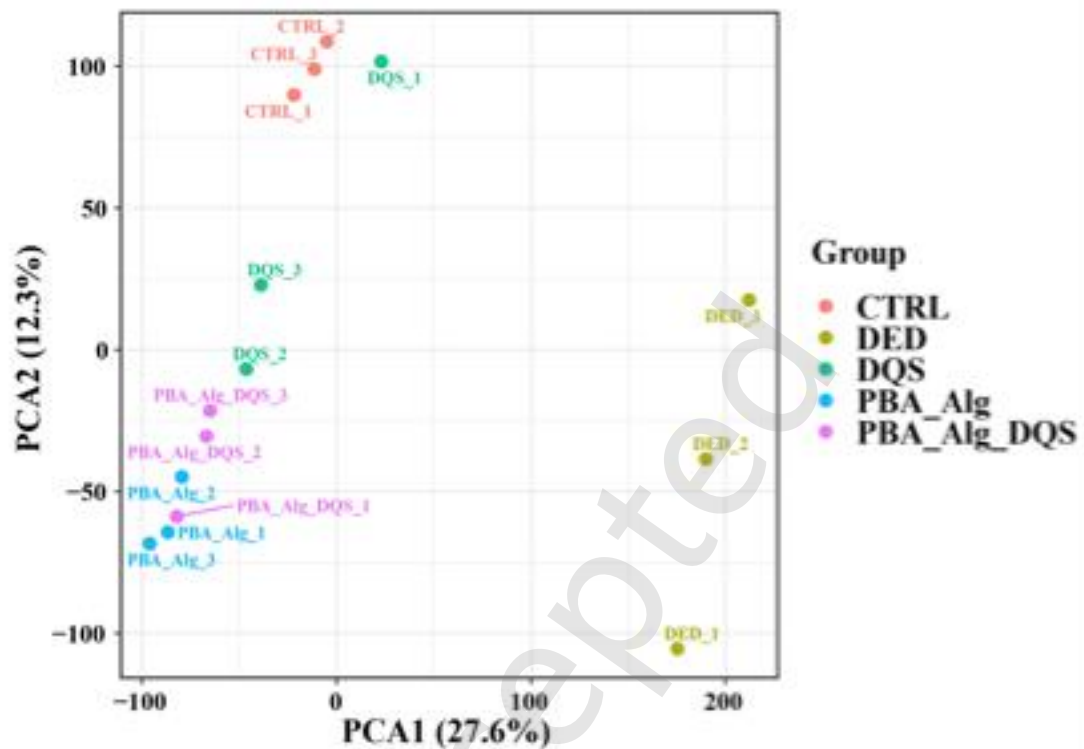


Figure S7. Principal component analysis of RNA-seq profiles from HCECs. Gene-level transcriptomic profiles are shown for the CTRL, DED, DQS, PBA-Alg, and PBA-Alg@DQS groups (n=3 per group). Each point represents one RNA-seq sample; axis labels indicate the proportions of variance explained by PC1 and PC2.

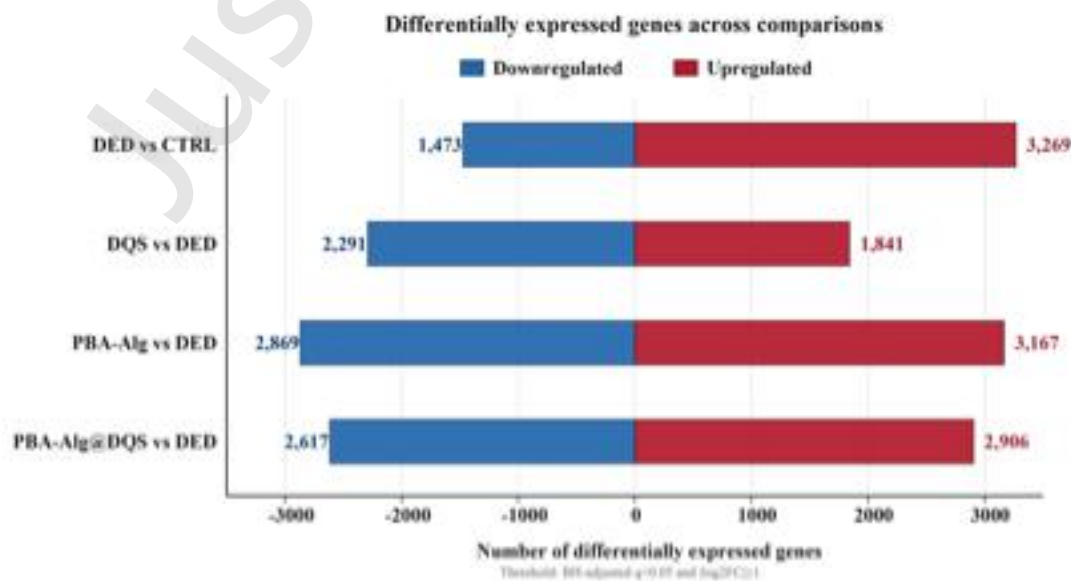


Figure S8. Numbers of differentially expressed genes in the RNA-seq analysis. Upregulated and downregulated DEGs are shown for DED versus CTRL, DQS versus DED, PBA-Alg versus DED, and PBA-Alg@DQS versus DED. DEGs were defined as Benjamini–Hochberg-adjusted $q < 0.05$ and $|\log_2 FC| \geq 1$.



Figure S9. Heatmap of selected disease- and treatment-responsive genes in HCECs. Expression profiles of 14 genes related to ROS generation, redox defense, hyperosmotic stress, and epithelial homeostasis are shown across the CTRL, DED, DQS, PBA-Alg, and PBA-Alg@DQS groups (n=3 per group). Values are row z-scores calculated from $\log_2 (CPM + 0.5)$ -transformed expression values; columns represent individual samples.

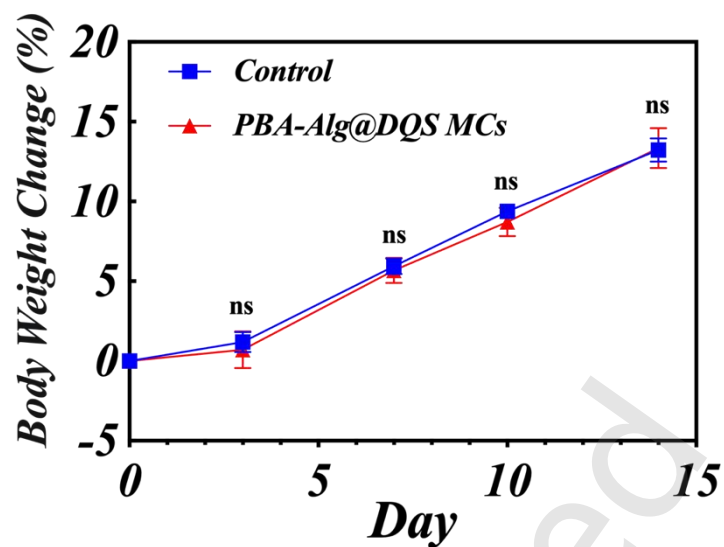


Figure S10. Percentage of body weight change (n=6).

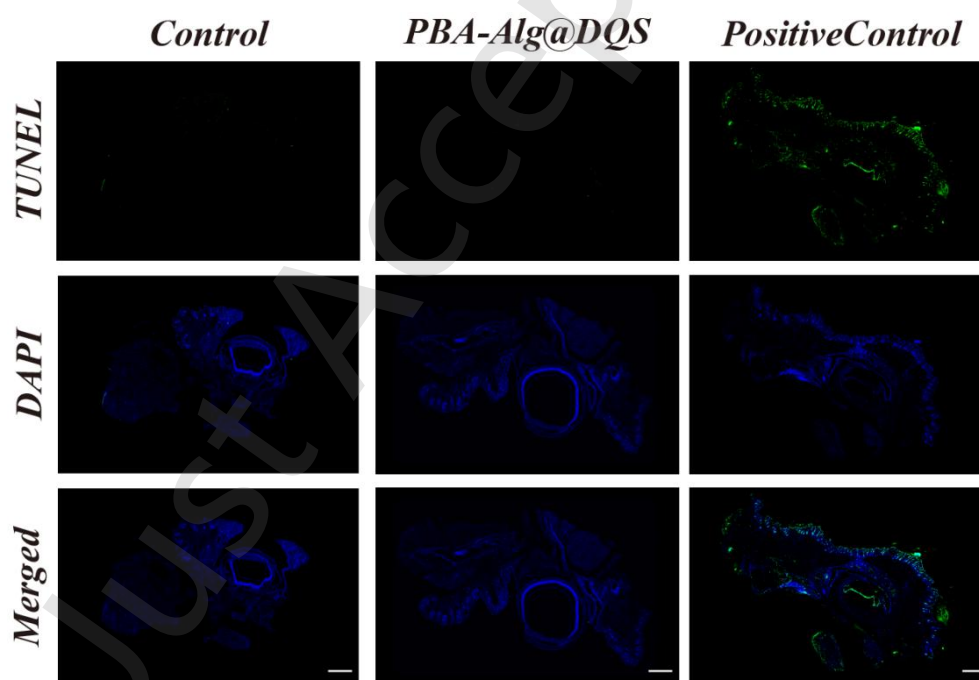


Figure S11. TUNEL staining of eyeballs. The scale bar is 500 μ m.

Table S1. List of individual gene primers.

Gene	Forward	Reverse
hTNF- α	CCTCCCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGA G
hIL-1 β	CGAATCTCCGACCACCACTA	AGGGAAAGAAGGTGCTCAGG

hMMP-9	TGTACCGCTATGGTTACACTCG	GGCAGGGACAGTTGCTTCT
hβ-Actin	GGCACCCAGCACAAATGAAG	CCGATCCACACGGAGTACTTG
hVAV3	AGAGAAACGGACCAATGGACT	GGTGGTGTTCAGAAATAGTTC
		C
hNCF2	CCAGAAGCATTAACCGAGACA	CCTCGAAGCTGAATCAAGGC
	A	
mTNF-α	AGGCACTCCCCCAAAGATG	CTTGGTGGTTTGCTACGACG
mIL-1β	TGTGAAATGCCACCTTTTGA	GGTCAAAGGTTTGGAAGCAG
mMMP-9	CTGGACAGCCAGACACTAAAG	CTCGCGGCAAGTCTTCAGAG
mβ-Actin	TCATCACTATTGGCAACGAGC	AACAGTCCGCCTAGAAGCAC
n		