

# Copolymerized Polymers Based on Cyclodextrins and Cationic Groups Enhance Therapeutic Effect of Rebamipide in the N-Acetylcysteine-Treated Dry Eye Model

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**Purpose:** We aimed to prepare a  $\beta$ -cyclodextrin ( $\beta$ -CD) polymer using radical polymerization with co-monomers, 6-deoxy-6-(2-methacryloyloxyethylsuccinamide)- $\beta$ -cyclodextrin (CD-MSAm) and N,N,N-trimethyl-N-(2-hydroxy-3-metacryloyloxypropyl)-ammonium chloride (QA) to design cyclodextrins suitable for use in ophthalmology. In addition, we evaluated their solubility and inclusion properties with rebamipide (REB), a poorly soluble drug, and investigated the usefulness of the  $\beta$ -CD polymer and REB (REB@CDQA) combination in treating dry eye.

**Methods:** The  $\beta$ -CD polymer (CD-MSAm-co-QA, CDQA) based on CD-MSAm/QA was prepared via radical polymerization, and the usefulness of REB@CDQA in treating dry eye was evaluated using a rabbit treated with N-acetylcysteine (dry eye model).

**Results:** The solubility of the CDQA powder was higher than that of the  $\beta$ -CD powder, and 80 nm colloids were observed in the CDQA solution. No corneal toxicity was observed in human corneal epithelial cells or rat corneas treated with 0.2% CDQA solution. The levels of REB dissolved in the CDQA solution were higher than those of the  $\beta$ -CD solution. Moreover, the application of the CDQA solution enhanced REB retention in the cornea and attenuated the transcorneal penetration of REB. In addition, instillation of REB@CDQA enhanced the volume of the lacrimal fluid and normalized the reduced mucin levels in the dry eye model. The extent of tear film breakup was attenuated by REB@CDQA instillation.

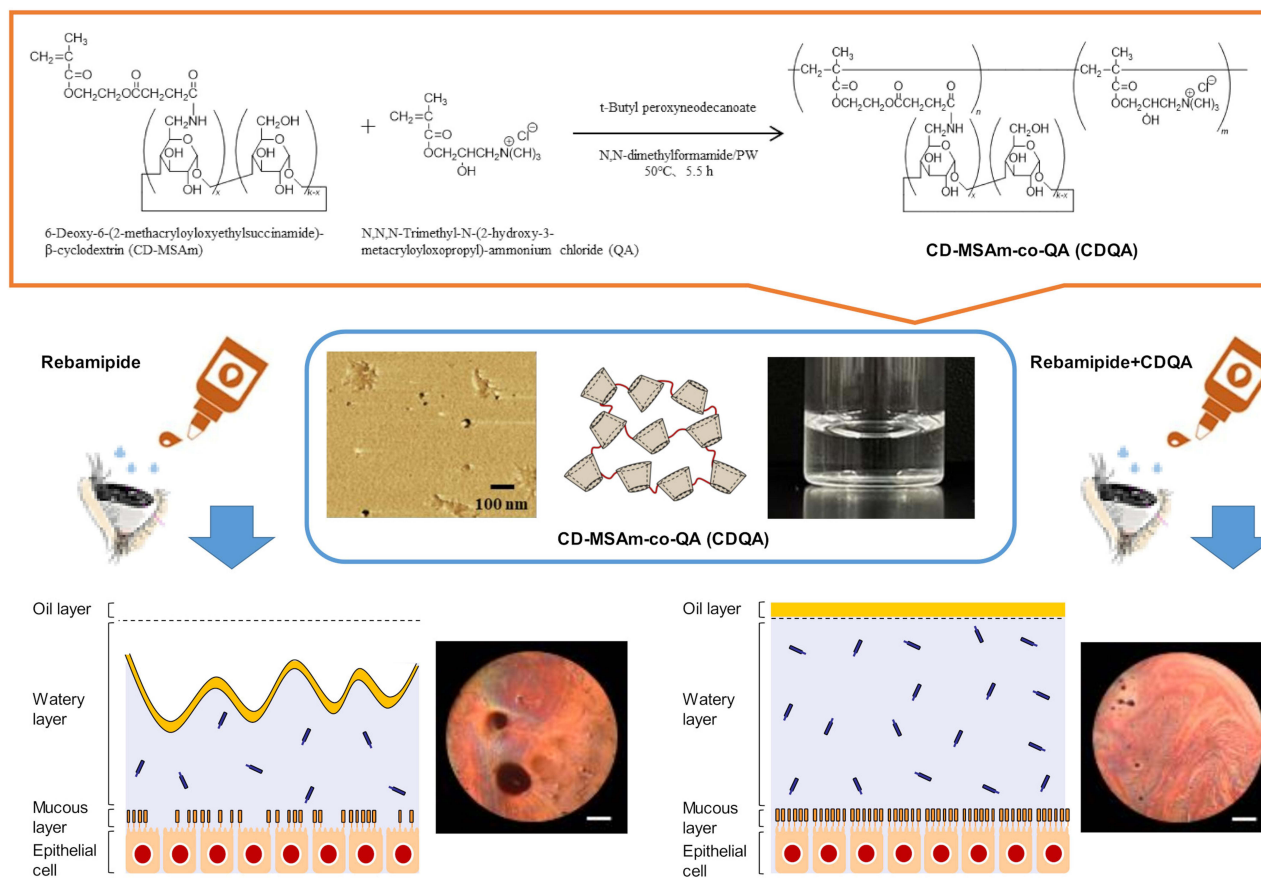
**Conclusion:** The CDQA solution enhanced the solubility of REB, and the combination of CDQA and REB enhanced the drug content in the corneal tissue. Moreover, the therapeutic effect on dry eye was higher than that of REB suspensions without CDQA.

**Keywords:**  $\beta$ -cyclodextrin, polymer, cationic group, rebamipide, dry eye

## Introduction

Tear film instability is caused by a disturbance in ocular surface mucin levels, resulting in dry eye disease. Although vision may not be affected adversely, dry eye disease causes various problems, including photophobia, dryness, and tearing, leading to a measurable adverse impact on daily life.<sup>1,2</sup> If left untreated, dry eye disease does not heal naturally, and its symptoms persist. However, treatments for improving the signs and symptoms may not cure the condition completely.<sup>1,2</sup> The putative pathogenetic mechanisms of dry eye disease include the formation of a hyperosmolar tear film and inflammation of the lacrimal gland, resulting in impaired stabilization of the aqueous layer due to a reduction in mucin levels on the ocular surface.<sup>3</sup> Therefore, enhancing tear rear-film stability via improvement of mucin levels is important for managing dry eye disease. Clinically, mucin secretagogues, corticosteroids, anti-inflammatory medications, autologous serum eye drops, artificial tear products, and punctal plugs are used for treating dry eye disease.

Graphical Abstract



Rebamipide (REB) is a quinolinone derivative with mucin secretagog activity, and the biological effects include prevention of inflammation, wound healing, and cytoprotection in various tissues.<sup>4-7</sup> With respect to dry eye disease, REB increases mucin production in corneal epithelial and conjunctival goblet cells,<sup>8-10</sup> and promotes the healing of conjunctival and corneal injuries by enhancing the secretion of both secreted-type and membrane-associated mucins.<sup>11</sup> Thus, REB possesses multiple pharmacological properties, such as anti-inflammatory activity,<sup>12</sup> and the ability to promote mucin secretion, increase the number of goblet cells,<sup>10,11</sup> and restore the barrier function of the corneal epithelium.<sup>4,5</sup> However, it is used as a suspension due to its poor solubility, and efforts to improve drug retention and solubility in corneal tissue are presently required.

Cyclodextrins (CD) are cyclic oligosaccharides D-glucopyranose molecules with hydrophilic surface and hydrophobic cavity. CDs can accommodate guest molecules via the formation of inclusion complexes because of the presence of a lipophilic domain.<sup>13</sup> Three types of CDs were classified based on the number of D-glucopyranose units they contain: α-CD (six units), β-CD (seven units), and γ-CD (eight units). The principal pharmaceutical application of these CDs are aimed to increase the bioavailability, dissolution rate, and solubility of drugs with poor aqueous solubility.<sup>14</sup> In general, the torus opening for α-CD, with six glucopyranose units, is too small for many drugs, while γ-CD, with eight glucopyranose units, was weakly conducive for inclusion complexation. The geometry of β-CD is suitable for inclusion complex formation with many drugs of varying sizes, because of which it has been used extensively. In contrast, the application of CDs is limited by the low solubilization of β-CD, which has been overcome by chemical modification of β-CDs with various functional groups. 2-Hydroxypropyl-β-CD (HPβCD), the most widely used modified β-CD, has excellent inclusion properties for many

compounds, is less toxic, safe, and an effective drug carrier.<sup>15–17</sup> Moreover, methyl- $\beta$ -CD (Me $\beta$ CD) is water-soluble, less toxic, biodegradable, and inexpensive,<sup>18,19</sup> and can be used widely in drug delivery systems to increase the bioavailability, stabilization, and solubilization of drugs.<sup>18–20</sup> Sulfobutylether- $\beta$ -CD (SBE $\beta$ CD) has also been extensively used for the same reasons.<sup>21</sup> Thus, enhancement of  $\beta$ -CD solubilization and its ability to include poorly soluble drugs is important for its pharmaceutical application. Additionally, the corneal surface carries a negative charge, which causes drugs with a positive charge to adhere more readily to it.

Hence, in this study, we aimed to prepare a  $\beta$ -CD polymer (D-MSAm-co-QA, CDQA) with cationic charge using radical polymerization with co-monomers, 6-deoxy-6-(2-methacryloyloxyethylsuccinamide)- $\beta$ -cyclodextrin (CD-MSAm) and N, N,N-trimethyl-N-(2-hydroxy-3-methacryloyloxypropyl)-ammonium chloride (QA), and evaluate its solubility and ability to form inclusion complex with REB. In addition, we investigated whether a CDQA solution containing REB (REB@CDQA) was useful for dry eye treatment by monitoring the ocular surface of rabbit. No CDQA has ever been created, and this is also the first time that REB@CDQA has been shown to enhance the therapeutic effect of REB in a dry eye model. Thus, these findings on unknown drugs, design, development, and therapy in the field of ophthalmology are highly novel and will be useful for developing drugs for dry eye disease.

## Material and Methods

### Animals

The experiments using the animals were performed according to the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) and Kindai University, and the protocol was approved by the Kindai University (approval number: KAPS-2021-003, KAPS-2021-004, April 1, 2021). Male 7-week-old Wistar rats (weight,  $2.87 \pm 3.31$  g; N = 30) were obtained from Kiwa Laboratory Animals Co., Ltd. (Wakayama, Japan), and male adult rabbits (weight,  $2.52 \pm 0.69$  kg; N = 47) were purchased from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan). These animals were housed under standard conditions (7:00 am–7:00 pm, fluorescent light, 25°C) and allowed free access to a commercial diet [CE-2 (rat) and CR-3 (rabbit); Clea Japan Inc., Tokyo, Japan] and water.

### Chemicals

Conventional REB powder,  $\beta$ -CD, Me $\beta$ CD, and methyl p-hydroxybenzoate were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). CD-MSAm and QA (BLEMMER QA) were supplied by the NOF CORPORATION (Tokyo, Japan). HP $\beta$ CD was obtained from Nihon Shokuhin Kako Co., Ltd. (Tokyo, Japan). SBE $\beta$ CD was provided by Funakoshi Co., Ltd. (Tokyo, Japan). Dulbecco's modified Eagle's medium/Ham's F12, penicillin, streptomycin, and fetal bovine serum were obtained from Gibco (Tokyo, Japan). N, N-dimethylformamide and cell count reagent SF were purchased from Nacalai Tesque, Inc. (Kyoto, Japan), and the tear mucin assay enzyme-linked immunosorbent assay (ELISA) kit was obtained from Cosmo Bio Co., Ltd. (Tokyo, Japan). The protein assay kit and pentobarbital were purchased from Bio-Rad (Hercules, CA, USA) and Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan), respectively. All chemicals used were of the highest purity.

### Synthesis of CDQA (CD-MSAm-Co-QA)

The synthesis of CDQA was performed by modifying the protocol in previous patents (JP 6561728 B2). CD-MSAm (4.65 g, 3.45 mmol) and QA (0.70 g, 1.48 mmol) were dissolved in a mixture of purified water (PW; 5.19 g) and N, N-dimethylformamide (13.51 g). Nitrogen gas was bubbled into the solution for 30 min to remove oxygen and heated to 50°C with stirring. t-Butyl peroxyneodecanoate (30.6 mg, 0.125 mmol) was added and stirred at 50°C for 6 h. The solution was cooled to room temperature and purified using a Spectra/Por MWCO 3500 dialysis membrane for 48 h. The reagents were freeze-dried using the FREEZE DRYER FDU-2100 (TOKYO RIKAKIKAI CO., LTD., Tokyo, Japan) for 48 h, and stored until further use. In this study, the structures of the obtained reagents were identified by JNM-ECS400 (JEOL Ltd., Tokyo Japan) (Figure S1). Other polymerization technique, it was difficult to quantitatively introduce the cationic structural units. Therefore, we applied this present method to synthesis of CDQA. In this study, the polymer formation was confirmed by using the NMR spectra. Figure S2 shows the <sup>1</sup>H NMR spectrum of CD-MSAm (A), QA (B), and CDQA (C). Briefly, we confirmed that polymerization had proceeded, because peak a from the starting material

disappeared in the CDQA spectrum. <sup>1</sup>H NMR spectrum of CD-MSAm showed characteristic peaks b and c derived from the constituent monomers, indicating that we had obtained CDQA ([Figure S2](#)).

## Preparation of Formulations Containing REB

We synthesized CD, QA and CDQA, followed by lyophilization to obtain them in powdered form. Using this powder, formulations containing REB were prepared. Twenty milligrams of CD, QA, and CDQA powder were dissolved in PW (10 mL), and stirred at 22°C for 30 min, and prepared solutions were used as 0.2% CD, QA, and CDQA solutions, respectively. REB@PW, REB@CD, REB@QA, and REB@CDQA which contain 2% REB, were prepared by suspending 100 mg of REB (2%) in 5 mL of 0.2% PW, CD, QA, and CDQA solutions, respectively. At this stage, a 1 h stirring was conducted. The prepared formulation was stored in a dark place at 20°C until use. In this study, the concentrations of CD and QA were determined according to the concentrations of the corresponding substances in the CDQA solution.

## High-Performance Liquid Chromatography (HPLC) of REB

REB concentration was measured using the HPLC LC-20AT system (Shimadzu Corp. Kyoto, Japan). Methyl p-hydroxybenzoate was used as the internal standard, and 50 mm phosphate buffer/acetonitrile (75/25 v/v) was used as the mobile phase at the flow rate of 0.25 mL/min. The samples were diluted with N,N-dimethylformamide, and 100 µL of each sample was added to a sample cup. Subsequently, 50 µL methyl p-hydroxybenzoate was added. After that, 10 µL sample was injected using a SIL-20AC auto-injector, and REB was separated at 35°C using an Inertsil<sup>®</sup> ODS-3 column in a CTO-20AC chromatography chamber (GL Science Co., Inc., Tokyo, Japan) and its concentration was determined at 287 nm. The R-value was 0.9994 in the calibration curve, and the detection sensitivity was 70.8 ng/mL.

## Solubility of β-CD and CDQA

Excess β-CD- and CDQA powders were added to 5 mL PW, and the dissolved β-CD and CDQA were obtained after centrifugation at 17,900 × g for 15 min, 4°C. The concentrations were measured using the method described above.

## Image and Viscosity of β-CD, QA, and CDQA Solutions

The CDQA solution, containing 0.2% CDQA, and the β-CD and QA solutions, prepared according to the concentration of the corresponding substance in the CDQA solution, were used. Atomic force microscopy (AFM) images were obtained using SPM-9700 (Shimadzu Corp) and created by combining the phase and height images. The viscosities were measured using SV-1A at 10–50°C (A & D Company Limited, Tokyo, Japan).

## Corneal Toxicity of β-CD, QA and CDQA Solutions

The human corneal epithelial cells (HCE-T cells, RCB1384) used in this study were prepared by Araki-Sasaki et al<sup>22</sup> and the HCE-T cell was donated from RIKEN BRC CELL BANK (Ibaraki, Japan). The HCE-T cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 with heat-inactivated fetal bovine serum (5%), penicillin (1,000 IU/mL), and streptomycin (0.1 mg/mL). In total, 1 × 10<sup>4</sup> HCE-T cells were seeded in 96-well microplates (Iwaki, Chiba, Japan) and incubated for 24 h. Subsequently, the cells were treated with 100 µL β-CD, QA, and CDQA solutions and incubated for 120 s. This was followed by the addition of the cell count reagent SF, and the absorbance (Abs) at 450 nm was measured according to the manufacturer's protocol. Cell viability (%) was represented as the Abs ratio of the treatment group and the non-treatment group.<sup>23</sup> In biological systems, after instillation, the drug is diluted by tear fluid and rapidly decreases in concentration due to drainage through the lacrimal punctum, typically within 300 s. However, in this study, the drug was applied at a concentration equivalent to that immediately following administration, resulting in the cells being exposed to a high concentration for 120 s. Based on this context and previous study,<sup>23</sup> we determined the maximum exposure time to 120 s in this study. Thus, the cell toxicity evaluation in this study was conducted under conditions more severe than those encountered in vivo.

For in vivo evaluation, twenty-eight male 7-week-old Wistar rats were divided into four groups, and each rat was repetitively instilled with PW, β-CD, QA, and CDQA solutions for five days (third a day, 10:00, 14:00, and 18:00); on the third day of instillation, the 18:00 instillation was omitted, and the experiments were performed at 18:00. The rats were

euthanized by injecting a lethal dose (200 mg/kg) of pentobarbital, and the corneas were removed and fixed at room temperature for 24 h using tissue-quick 10% formalin solution (Super Fix, Kurabo Industries, Osaka, Japan). The fixed tissues were prepared in frozen blocks using the OCT compound and liquid nitrogen, and 3  $\mu$ m-thick serial sections were prepared using the Leica cryostat, CM3050S (Leica Biosystems Nussloch GmbH, Nussloch, Germany). Hematoxylin and eosin (H & E) staining was performed for morphological analysis. The specimens were observed using Shimadzu BA210E (Shimadzu), and the central area of the cornea was photographed.<sup>24</sup>

## Solubility of REB in Various Types of CD Solution

The excess REB (5 mg) was added to 5 mL PW,  $\beta$ -CD, QA, and CDQA solutions and solutions containing 0.2% HP $\beta$ CD, Me $\beta$ CD, and SBE $\beta$ CD. Subsequently, the suspensions were incubated for 24 h, and the dissolved REB was collected via centrifugation (17,900  $\times$  g, 15 min, 4°C). REB concentration was measured using HPLC, as described above.

## Particle Size of REB in Formulations Used in This Study

The particle size of formulations was measured with the NANOSIGHT LM10 (Malvern, Worcestershire, UK) utilizing nano tracking analysis. The conditions for measuring were as follows: wavelength, 405 nm; measurement time, 60s; viscosity, 1.27 mPa·s.

## Zeta Potential and Osmolality of Formulations Used in This Study

The zeta potential was measured using a micro-electrophoresis zeta potential analyzer (Sanyo Trading Co., Ltd., Tokyo, Japan). Diluted each formulation 20 times with water and measured under the following conditions: electrophoresis distance, 120  $\mu$ m; strength of electric field, 10 V/cm; sample temperature, 26°C. The osmolality was measured by using Micro osmometer at 26°C (Biomedical science, Tokyo, Japan).

## REB Release from CDQA

MF<sup>TM</sup> membrane filters (220 nm pore size) were set on a Franz diffusion cell attached a plate with a 5 mm diameter hole.<sup>25</sup> The reservoir chamber (12.2 mL) of the Franz diffusion cell was filled with 10 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) consisting of 1 mm K<sub>2</sub>HPO<sub>4</sub>, 5.5 mm glucose, 136.2 mm NaCl, 1.7 mm CaCl<sub>2</sub>, and 5.3 mm KCl, and 10  $\mu$ L of REB@PW and REB@CDQA were instilled to donor chambers (0.196 cm<sup>2</sup>, diameter 5 mm). The experiments for transcorneal penetration were performed at 37°C for 1 h, and 100  $\mu$ L aliquots were withdrawn from the reservoir chamber. REB concentrations in the samples were measured using HPLC, as described above.

## Behavior of REB in Cornea Treated with Formulations Used in This Study

The twelve male 7-week-old Wistar rats were euthanized by injecting a lethal dose (200 mg/kg) of pentobarbital. Thereafter, twenty-four corneas were removed and set on a Franz diffusion cell attached a plate with a 5 mm diameter hole.<sup>25</sup> The reservoir chamber (12.2 mL) of the Franz diffusion cell was filled with 10 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) consisting of 1 mm K<sub>2</sub>HPO<sub>4</sub>, 5.5 mm glucose, 136.2 mm NaCl, 1.7 mm CaCl<sub>2</sub>, and 5.3 mm KCl, and 10  $\mu$ L of REB@PW (control), REB@CD, REB@QA, and REB@CDQA were instilled to donor chambers (0.196 cm<sup>2</sup>, diameter 5 mm). The experiments for transcorneal penetration were performed at 37°C for 6 h, and 100  $\mu$ L aliquots were withdrawn from the reservoir chamber at 0.5, 1, 2, 4, and 6 h. REB concentrations in the samples were measured using HPLC, as described above. Moreover, the cornea after 6 h treatment was collected, homogenized in 100  $\mu$ L N,N-dimethylformamide to extract REB, and centrifuged at 17,900  $\times$  g, 4°C for 15 min. The supernatants were used as samples. As described above, corneal REB levels were also measured using HPLC and expressed as mg/mg protein. Protein levels were measured by Bradford method using a Bio-Rad Protein assay kit (Bio-Rad laboratory, CA, USA) according to the manufacturer's protocol. Additionally, apparent permeability of REB (*P*<sub>app</sub>) at 1 h after the start of the experiment were calculated following equation

$$P_{app}(cm/h) = (dQ/dt)/(C_0 \times S)$$

Where,  $dQ/dt$ ,  $C_0$ , and  $S$  were permeation rate ( $\mu\text{g/s}$ ), initial concentration of the drug ( $\mu\text{g/L}$ ), and surface area ( $\text{cm}^2$ ).

## Monitoring the Ocular Surface of Rabbits Instilled with the Formulations Used in This Study

The dry eye model was established by instilling 30  $\mu\text{L}$  of 10% N-acetylcysteine six times per day in the right eyes of 42 rabbits (at 9:00, 11:00, 13:00, 15:00, 17:00, and 19:00) per our previous report.<sup>26</sup> From the next day, the dry eyes were repetitively instilled with PW (N = 5),  $\beta$ -CD (N = 5), QA (N = 5), and CDQA solutions (N = 5), REB@PW (N = 5), REB@CD (N = 5), REB@QA (N = 5), and REB@CDQA (N = 7) at third a day for three days (10:00, 14:00, and 18:00). The volume of the lacrimal fluid and the tear film breakup level changes in the ocular surface were evaluated 4 h (18:00) after the last application (14:00) of the PW,  $\beta$ -CD, QA, and CDQA solutions with or without REB. The volume of lacrimal fluid in rabbits was measured using Schirmer's tear test strips. Changes in the ocular surface were monitored using a dry eye monitor, DR-1 (Kowa Co., Ltd., Aichi, Japan) per our previous study.<sup>26</sup> The tear film breakup area was measured 2 s after the last blink using the Image J software, and the measurement was performed thrice; the mean was used as the value. The tear film breakup levels (%) were expressed as the tear film breakup area in N-acetylcysteine-treated rabbits without instillation for each group.

## Mucin Levels in Rabbits Instilled with the Formulations Used in This Study

The dry eye model described above was used to measure mucin levels. Briefly, rabbits were instilled with 30  $\mu\text{L}$  of 10% N-acetylcysteine six times daily (at 9:00, 11:00, 13:00, 15:00, 17:00, and 19:00). From the next day, the dry eyes were repetitively instilled with  $\beta$ -CD, QA, and CDQA solutions with or without REB at third a day for three days (10:00, 14:00, and 18:00). Mucin content was measured using a tear mucin assay ELISA kit according to the manufacturer's instructions. In brief, lacrimal fluid was collected using Schirmer's tear test strips, which were added to the elution buffer of a tear mucin assay ELISA kit to extract the mucin. Next, the concentration of the extracted mucin was measured using the tear mucin assay ELISA kit and a fluorescence microplate reader (absorption/emission = 336 nm/383 nm).<sup>26</sup> The mucin levels (%) were estimated from the Abs of instilled dry eye model/Abs of non-instilled normal rabbit ( $0.73 \pm 0.10$  mg/mL, N = 5).

## Statistical Analysis

Data are presented as the mean  $\pm$  standard error of the mean (SEM). Statistical differences were analyzed using the JMP software ver. 5.1 (SAS Institute, Cary, NY, USA). One-way repeated-measures analysis of variance (ANOVA), followed by the Tukey-Kramer test, was used for the statistical analysis of multiple group comparisons. Statistical differences between the two groups were analyzed using the unpaired Student's *t*-test. *P*-values < 0.05 were considered statistically significant.

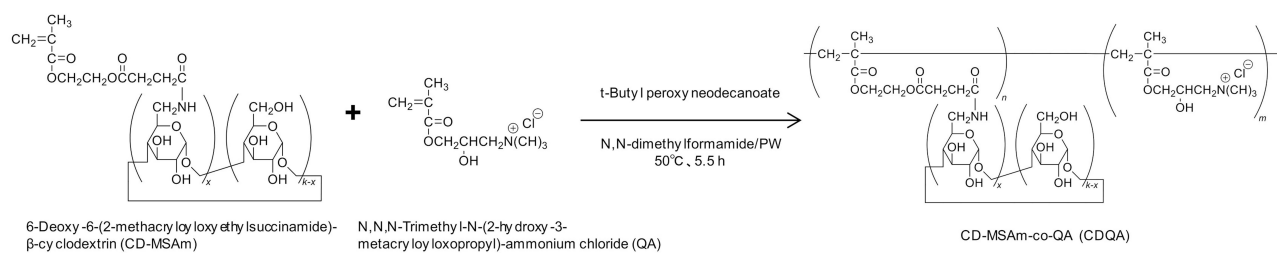
## Results

### Designing of CDQA and Evaluation of Its Characteristics

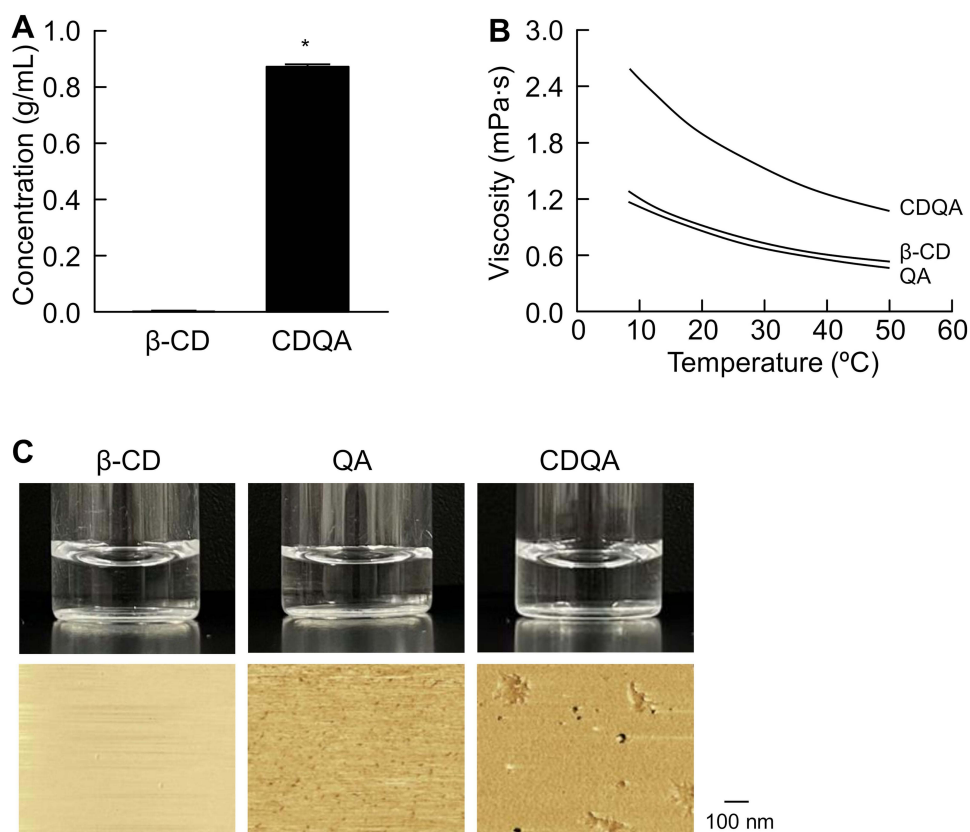
First, we attempted to design a  $\beta$ -CD polymer with cationic charge and successfully prepared CD-MSAm-co-QA (CDQA) via radical polymerization (Figure 1). We also determined the characteristics of CDQA (Figure 2). The solubility of CDQA was higher than that of  $\beta$ -CD, and the saturation solubility of CDQA was 0.86 g/mL at 20°C (Figure 2A). In addition, the viscosity of the CDQA solution was significantly higher than those of the  $\beta$ -CD and QA solutions (Figure 2B). The CD, QA, and CDQA solutions were clear under visual inspection, and differences were not observed (Figure 2C). However, only polymers less than 40–100 nm in size in the CDQA solution were detected in the SPM images.

### Biosafety of the CDQA Solution in the Cornea

Understanding ocular toxicity is important when using CDQA solution as an additive in ophthalmic treatments. Therefore, we evaluated corneal toxicity in HCE-T cells and rats. Cell viabilities in the presence of the CD, QA, and CDQA solutions were similar, and it was approximately 90% for the HCE-T cells (Figure 3A). Therefore, we

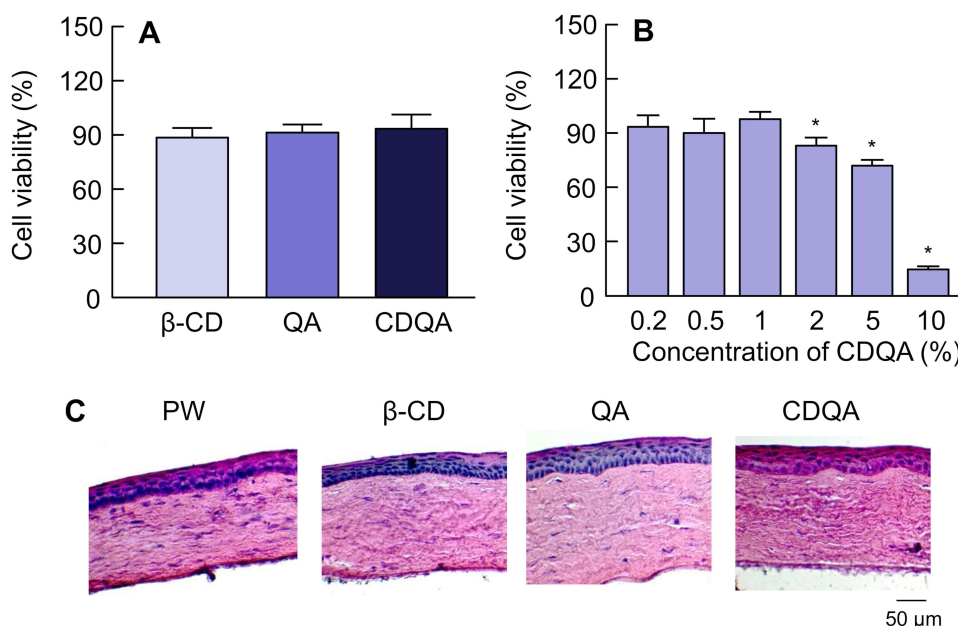


**Figure 1** Structural formula for CDQA used in this study. The  $\beta$ -CD polymer with cationic charge (CD-MSAm-co-QA, CDQA) was prepared via radical polymerization.



**Figure 2** Changes in the solubilities and viscosities of CD, QA, and CDQA. The concentration of CDQA in the CDQA solution was set at 0.2%, and the concentrations of  $\beta$ -CD and QA in the  $\beta$ -CD and QA solutions were determined according to the concentration of the corresponding substances in the CDQA solution. **(A)** Solubilities of  $\beta$ -CD and CDQA powder in PW. **(B)** Viscosity of CD, QA, and CDQA solutions at 10–50°C. **(C)** Digital and SPM images of  $\beta$ -CD, QA, and CDQA solutions. The bar in the image (Figure 2C) indicates 100 nm.  $N = 8$  \* $P < 0.05$  vs  $\beta$ -CD (Student's *t*-test). The solubility of CDQA powder was higher than that of the  $\beta$ -CD powder, and aggregation (approximately 80 nm) was observed in the CDQA solution. Moreover, the viscosity of the CDQA solution was higher than that of  $\beta$ -CD and QA solutions.

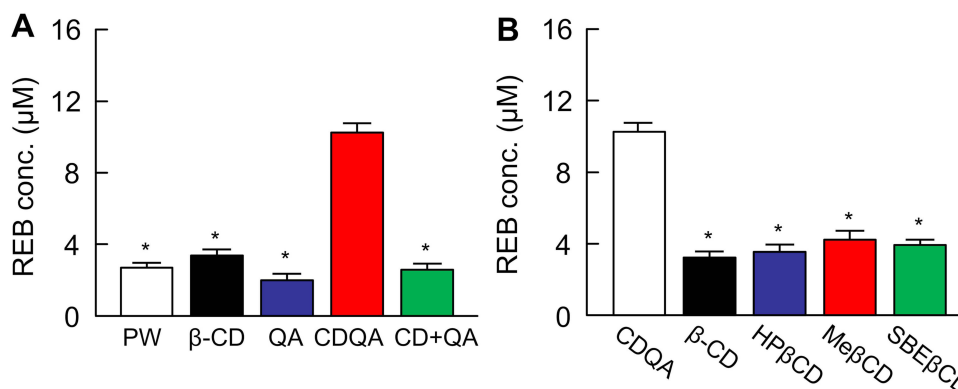
investigated the relationship between CDQA concentration and corneal toxicity (Figure 3B). The viability of HCE-T cells treated with 0.2–1% CDQA solutions exceeded 90%, and a reduction in cell viability was observed at concentrations >2%. The viabilities of HCE-T cells treated with 2, 5, and 10% CDQA solutions were 83.3, 72.1, and 14.5%, respectively. Therefore, we investigated whether repeated instillations of CDQA solution (0.2%) induced corneal damage in rats (Figure 3C). Tissue injury was not observed in rat cornea repetitively instilled with CD, QA, or CDQA solutions, and the images of rat corneal tissue after the instillation of CD, QA, and CDQA solutions were similar to those obtained after the instillation of PW.



**Figure 3** Corneal toxicity in the HCE-T cells and rats treated with the CDQA solution. The concentration of CDQA in the CDQA solution was set at 0.2–10% and the concentrations of  $\beta$ -CD and QA in the  $\beta$ -CD and QA solutions were determined according to the concentration of the corresponding substance in the CDQA solution. **(A)** Changes in the viability of HCE-T cells treated with CD, QA, and CDQA solutions (0.2%). **(B)** Changes in the viability of HCE-T cells treated with 0.2–10% CDQA solution. **(C)** H & E staining of the rat cornea repetitively instilled with CD, QA, and CDQA solutions (0.2%). The CD, QA, and CDQA solutions were instilled thrice a day for 5 days. N = 7. \* $P < 0.05$  vs non-treated group for each category (Tukey-Kramer test). Significant difference was not observed between 0.2% CDQA solution- and PW-treated group, although cell viability decreased after treatment with 2%, 5%, and 10% CDQA solutions. No stimulation was observed in the rat cornea repetitively instilled with CD, QA, and CDQA solutions (0.2%).

## Changes in the Solubility and Corneal Penetration of REB Suspensions After Adding CDQA

It was known that the addition of  $\beta$ -CD enhances the solubility of sparingly soluble drugs by forming inclusion compounds, although, the REB was poorly soluble in the  $\beta$ -CD solution (Figure 4) and that the solubilities of REB@CD and REB@QA did not differ significantly. In addition, the solubility of REB in a solution containing both  $\beta$ -CD and QA ( $\beta$ -CD + QA) was similar to that of REB@PW. In contrast, the CDQA solution enhanced the solubility of REB, and the concentration of REB was 10.27  $\mu$ M (Figure 4A). Moreover, the enhanced solubility of REB in the CDQA

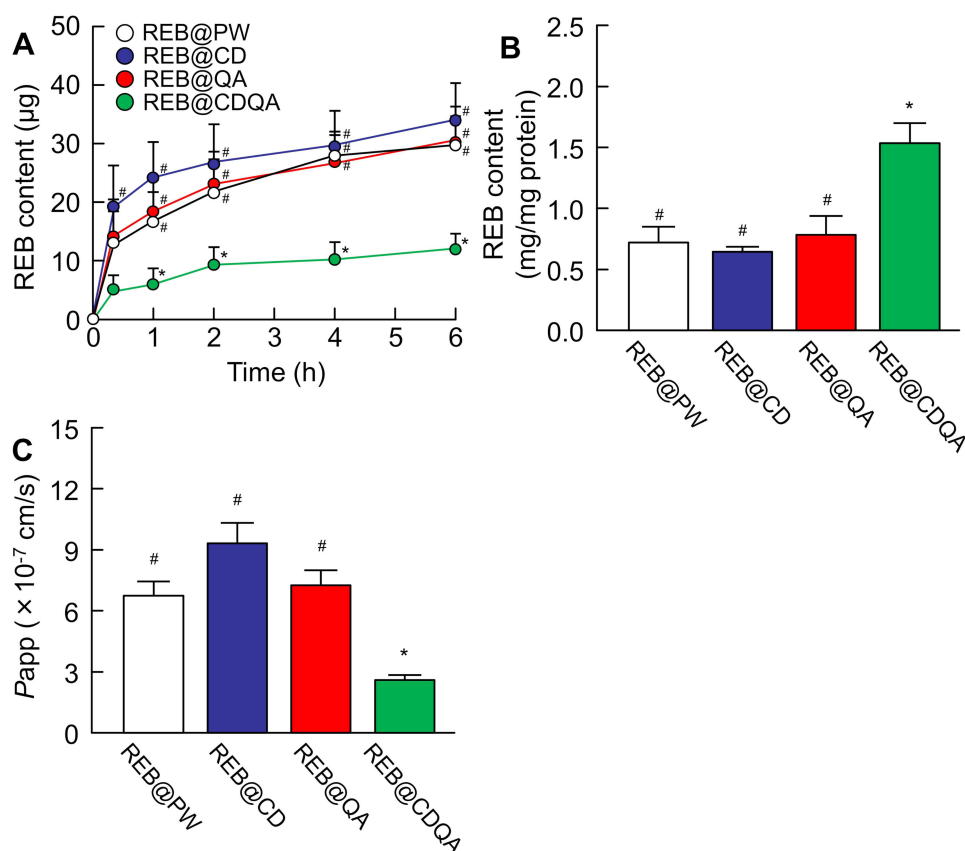


**Figure 4** Effect of CDQA solution on the solubility of REB. The concentration of CDQA in the CDQA solution was set at 0.2%, and the concentrations of  $\beta$ -CD and QA in the  $\beta$ -CD and QA solutions were determined according to the concentration of the corresponding substances in the CDQA solution. **(A)** Changes in the dissolved REB in the PW, CD, QA, and CDQA solutions.  $\beta$ -CD + QA shows the dissolved REB levels in a solution containing both  $\beta$ -CD and QA. **(B)** The dissolved REB in the CDQA solution and various traditional CD solutions ( $\beta$ -CD, HP $\beta$ CD, Me $\beta$ CD, and SBE $\beta$ CD). N = 6. \* $P < 0.05$  vs CDQA for each category (Tukey-Kramer test). The levels of dissolved REB in the CDQA solution were higher than those of  $\beta$ -CD and QA solutions. In addition, the solubility of REB in the CDQA solution was also significantly higher than those of various traditional CD solutions.

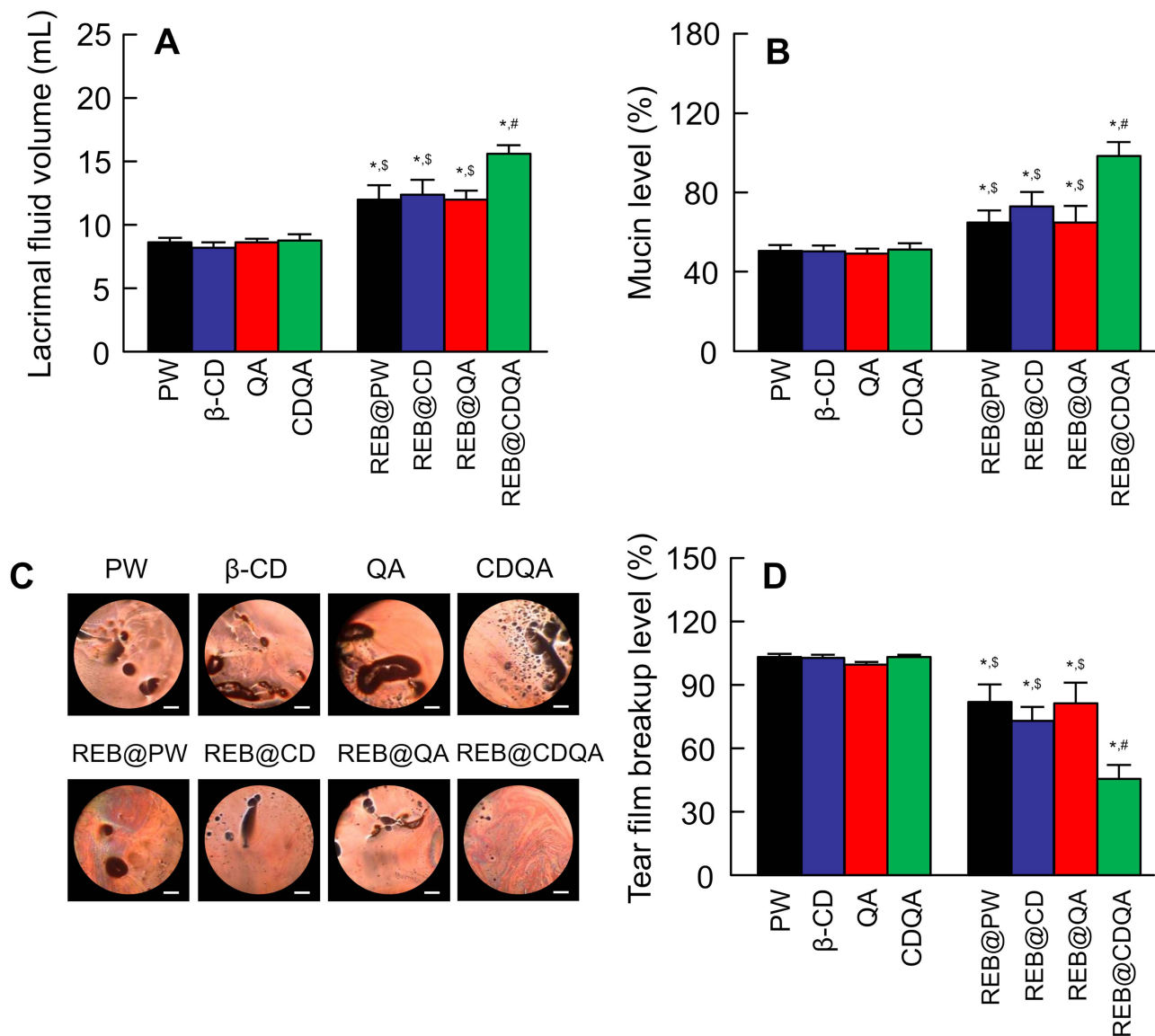
solution was significantly higher than those containing corresponding concentrations of traditional CDs ( $\beta$ -CD, HP $\beta$ CD, Me $\beta$ CD, and SBE $\beta$ CD) (Figure 4B). Next, we evaluated whether the application of the CDQA solution affected the corneal penetration behavior of REB (Figure 5). The corneal penetration behavior of REB did not change with the application of REB@QA, and the corneal penetration levels of REB tended to increase with REB@CD concentration (Figure 5A). The REB content in the cornea was similar for REB@CD and REB@QA (Figure 5B). In contrast, the corneal penetration levels of REB were attenuated in REB@CDQA (Figure 5A); however, the REB contents in corneas treated with REB@CDQA were higher than those treated with REB@PW, REB@CD, and REB@QA (Figure 5B). In this study, the  $P_{app}$  was also calculated. The  $P_{app}$  values exhibited a similar trend to the corneal permeation behavior of the REB, decreasing with the addition of CDQA, with the value being 0.38 times that of REB@PW (Figure 5C). Furthermore, the characteristics of EB@CDQA were measured in this study. The pH, osmolality, mean particle size and zeta potential of REB@CDQA were 7.4, 285 mOsm, 106 nm - 971 nm, 42.1 mV, respectively. The zeta potential of REB (-76.5 mV) was shifted to a positive charge from negative charge by the addition of CDQA (51.0 mV).

## Therapeutic Potential of REB@CDQA for Dry Eye Disease

We also investigated whether REB@CDQA enhanced the usefulness of REB as a therapeutic for dry eye disease using an N-acetylcysteine-treated rabbit model (Figure 6). The lacrimal fluid volume of rabbits treated with N-acetylcysteine was 0.79-fold higher, and the mucin levels were 0.51-fold higher than those of normal rabbits (Figure 6A and B). N-acetylcysteine treatment resulted in strong tear film breakup levels, and the tear film breakup levels were observed to persist three days later in the PW-treated group. Instillation of CD, QA, and CDQA solutions did not improve the lacrimal fluid column, mucin, and tear



**Figure 5** Changes in the corneal penetration of REB in rats treated with REB@PW, REB@CD, REB@QA, and REB@CDQA. The concentration of CDQA in the CDQA solution was set at 0.2%, and the concentrations of  $\beta$ -CD and QA in the  $\beta$ -CD and QA solutions were determined according to the concentration of the corresponding substances in the CDQA solution. The concentration of REB in REB@PW, REB@CD, REB@QA, and REB@CDQA were 2%. (A) Effect of CDQA solution on transcorneal penetration of REB. (B) Effect of CDQA solution on REB retention in the cornea. (C) Effect of CDQA solution on  $P_{app}$  of REB.  $N = 6$ . \* $P < 0.05$  vs REB@PW for each category (Tukey-Kramer test). # $P < 0.05$  vs REB@CDQA for each group (Tukey-Kramer test). Although, the application of the CDQA solution enhanced the REB retention in the cornea, it attenuated the transcorneal penetration and  $P_{app}$  of REB.



**Figure 6** Therapeutic effect of REB@CDQA on the dry eye in N-acetylcysteine-treated rabbit (dry eye model). Rabbits were repetitively instilled with REB@PW, REB@CD, REB@QA, and REB@CDQA at third a day for three days (10:00, 14:00, and 18:00), and the experiments were performed at 18:00. **(A and B)** Effects of REB@CDQA on the lacrimal fluid volume **(A)** and mucin levels **(B)** in the dry eye model. **(C)** Images of the ocular surface in the dry eye model after repetitive instillation of REB@CDQA. The bar indicates 1 mm. The dark spots reflect the tear film breakup. **(D)** Effect of REB@CDQA on tear film breakup levels in the dry eye model. N = 5–7. \**P* < 0.05 vs REB-non-treated group for each group (Tukey-Kramer test). #*P* < 0.05 vs REB@PW for each group (Tukey-Kramer test). §*P* < 0.05 vs REB@CDQA for each group (Tukey-Kramer test). Instillation of REB@CDQA enhanced the volume of lacrimal fluid and normalized the reduced mucin levels in the dry eye model. REB@CDQA instillation also attenuated the levels of tear film breakup.

film breakup levels in N-acetylcysteine-treated rabbits with dry eyes. In contrast, the instillation of REB@CDQA increased both lacrimal fluid volume and mucin levels, which were approximately 1.3- and 1.5-fold higher than those of REB@PW (control), respectively (Figure 6A and B). We also investigated the changes in the levels of tear film breakup in N-acetylcysteine-treated rabbits instilled with REB@CDQA (Figure 6C and D). The tear film breakup levels in the rabbits repetitively instilled with REB@PW (REB suspensions) decreased to 82.1% at three days post-N-acetylcysteine treatment, and the tear film breakup levels were similar to those of REB@CD and REB@QA. Repetitive instillation of REB@CDQA strongly attenuated tear film breakup, and the therapeutic effect of REB@CDQA was significantly higher than that of REB@CD and REB@QA.

## Discussion

Dry eye disease is a common complaint that has recently attracted considerable research interest. Conventional therapy for dry eye disease involves using mucin secretagogues, nonsteroidal anti-inflammatory drugs, and artificial tear products. REB is one of the drugs used conventionally for treating dry eye disease. However, the therapeutic effect and retention time of REB are limited by its low solubilization. In this study, we designed CD-MSAm-co-QA (CDQA) with cationic charge and found that compared with traditional CDs, such as  $\beta$ -CD, HP $\beta$ CD, Me $\beta$ CD, and SBE $\beta$ CD, CDQA improved the solubility of REB. In addition, we found that the instillation of a CDQA solution containing REB (REB@CDQA) increased adherence to the corneal surface and enhanced the volume of lacrimal fluid. Moreover, the instillation of REB@CDQA improved tear film breakup levels in the dry eye model used in this study.

First, we compared the different characteristics (solubility and viscosity) of the  $\beta$ -CD monomer and CDQA, which was prepared using the  $\beta$ -CD polymer (Figure 1). The use of  $\beta$ -CD is limited by its own relatively poor aqueous solubility of 1.85%.<sup>27</sup> The CDQA designed in the study circumvented this problem (Figure 2A), and aggregates <100 nm in size were observed in the CDQA solution (Figure 2C). In addition, the viscosity of the CDQA solution was higher than that of the  $\beta$ -CD and QA solutions (Figure 2B). These results suggested that CDQA formed a polymer in solution, the solubility and viscosity of which differed from those of its monomers.

Next, we measured the toxicity of the CDQA solution because any material that causes eye irritation cannot be used as an additive in ophthalmic formulations. The cytotoxicity of the CDQA solution was similar to those of the  $\beta$ -CD and QA solutions (Figure 3A); in addition, the CDQA solution did not show any cytotoxicity at concentrations below 1% (Figure 3B). Moreover, corneal damage was not observed after repeated instillations of the 0.2% CDQA solution (Figure 3C). Thus, the cell toxicity evaluation in this study is conducted under conditions more severe than those encountered in vivo, although no cellular damage was observed. Furthermore, the results of the tissue section analysis in the in vivo experiments also showed no corneal damage. From these results, we concluded that the 0.2% CDQA solution has low toxicity and may be used for ophthalmic purposes. Compared with REB suspensions (REB@PW), the CDQA solution containing REB (REB@CDQA) improved the symptoms of dry eye disease. In this study, the CDQA solution enhanced the solubility of REB, which was significantly higher than those of traditional CDs ( $\beta$ -CD, HP $\beta$ CD, Me $\beta$ CD, and SBE $\beta$ CD) (Figure 4). In contrast, the enhancement of REB solubility was not observed with  $\beta$ -CD and QA solutions. The polymerization of  $\beta$ -CD (CDQA) may possibly be responsible for the enhanced solubility of REB.

We also investigated the changes in the corneal permeability and retention time of REB, as the CDQA solution bears cationic charge and affects the physical properties of REB more than the traditional  $\beta$ -CD solution. Previous studies have reported that the  $\beta$ -CD related drug absorption and was used as a drug delivery system.<sup>18–20,28,29</sup> We showed that the application of  $\beta$ -CD tended to enhance the transcorneal penetration of REB. Unlike the  $\beta$ -CD solution, the CDQA solution increased the REB content in corneal tissue instead of transcorneal penetration of REB (Figure 5). In general, high viscosity increases drug concentration on the ocular surface after instillation; however, this was an in vitro study using Franz diffusion cells, and REB constantly remained on the corneal surface. Moreover, the maximum amount of REB in the reservoir chamber was lower than that in REB@PW (control). Additionally, the REB release rate (%) from REB@CDQA was  $53 \pm 6.9\%$  (N=5). These results suggested that viscosity was not directly related, and the REB in polymer network may be related to the attenuation of the transcorneal penetration of REB in REB@CDQA. Similar to other CDQA characteristics, QA in CDQA bears a cationic charge, and drugs with a cationic charge adhere better to the cornea, which harbors anionic charge. Therefore, the high retention and decreased transcorneal penetration of REB in REB@CDQA was possibly due to the cationic charge of QA in CDQA. Aggregation (~80 nm particles) was also observed in the CDQA solution (Figure 2C), and the solubility of REB in REB@CDQA was higher than that in REB@CD. These factors [CDQA aggregation and formulation of the  $\beta$ -CD-REB inclusion complexes] may also affect the changes in corneal permeability and retention of REB. Further studies are required to clarify the mechanism underlying the enhancement of REB solubility in the CDQA solution.

The therapeutic effects of REB@CDQA in dry eye disease should be evaluated using an animal model for dry eye. N-acetylcysteine is a mucolytic and reducing agent,<sup>30</sup> and instillation causes the breaking of mucoprotein disulfide bonds in the conjunctiva and cornea, resulting in reduced levels of corneal and conjunctival mucin-like substances. In addition,

the instillation of REB enhances glycoconjugate levels on the ocular surface and decreases the enhanced Rose Bengal staining scores of the cornea and conjunctiva in N-acetylcysteine-induced models.<sup>11,31</sup> Moreover, N-acetylcysteine reduces the levels of mucin produced and the ability to retain mucin in the conjunctiva and cornea.<sup>30–33</sup> Thus, the N-acetylcysteine-treated rabbit model has been widely used as an animal model of dry eye, which was also used in this study. Compared to those in the non-treated group, the lacrimal fluid volume, mucin levels, and tear film breakup levels decreased after treatment with N-acetylcysteine, and the instillation of  $\beta$ -CD, QA, and CDQA solutions did not affect these changes (Figure 6). These results showed that the  $\beta$ -CD, QA, and CDQA solution did not affect dry eye treatment. REB is a mucosal protective agent that increases the secretion of both membrane-associated and secreted-type mucins by increasing the levels of endogenous prostaglandins, E2 and I2.<sup>34,35</sup> In addition, REB scavenges oxygen free radicals and performs other anti-inflammatory actions.<sup>34,35</sup> Thus, the therapeutic effects of REB could be attributed to the enhancement of mucin levels in the cornea and conjunctiva. Although REB attenuated these changes in lacrimal fluid volume, mucin levels, and tear film breakup levels, significant differences among REB@PW, REB@CD, and REB@QA were not observed. In contrast, the instillation of REB@CDQA, but not REB@PW (control), REB@CD, and REB@QA, significantly enhanced lacrimal fluid volume and mucin levels, and decreased tear film breakup levels (Figure 6). In this study, we showed that the REB content in the corneas treated with REB@CDQA was higher than those that were treated with REB@PW (REB suspensions without CDQA) (Figure 5B). This strong therapeutic effect of REB may be due to its enhanced solubility and content in the cornea. Further studies are required to evaluate the mechanism underlying the enhancement of REB solubility and content in the cornea in the presence of CDQA. However, the dose-dependent therapeutic effects of REB@CDQA remain to be elucidated, which is a limitation of this study. In the future, we intend to measure the ratio of drug/ $\beta$ -CD inclusion complexes and drug release from CDQA. In addition, we will investigate the affinity between CDQA and the cornea to understand why corneal permeability and retention changed. In addition, it is essential to elucidate the long-term impact of the polymer and its complex with REB, and this will be an important focus of future investigations.

## Conclusion

We prepared CD-MSAm-co-QA, which is  $\beta$ -CD polymer with cationic charge (CDQA), and showed that the instillation of the CDQA solution (0.2%) did not cause corneal toxicity. Moreover, we found that the CDQA solution enhanced the solubility of REB and that it was more effective than traditional CDs ( $\beta$ -CD, HP $\beta$ CD, Me $\beta$ CD, and SBE $\beta$ CD). Additionally, the combination with CDQA (REB@CDQA) enhanced the REB content in the corneal tissue, and its therapeutic effect on dry eye was higher than that of the REB without CDQA (REB@PW). No CDQA has ever been created, and this is also the first time that REB@CDQA has been shown to enhance the therapeutic effect of REB in a dry eye model. Thus, these findings on unknown drugs, design, development, and therapy in the field of ophthalmology are highly novel and will be useful for developing drugs for dry eye disease.

## Abbreviations

Abs, absorbance; AFM, atomic force microscopy; ANOVA, one-way repeated measures analysis of variance; CD, cyclodextrin; CD-MSAm, 6-deoxy-6-(2-methacryloyloxyethylsuccinamide)- $\beta$ -cyclodextrin; CDQA, 6-deoxy-6-(2-methacryloyloxyethylsuccinamide)- $\beta$ -cyclodextrin-co-N,N,N-trimethyl-N-(2-hydroxy-3-metacryloyloxopropyl)-ammonium chloride; H & E, hematoxylin and eosin; HCE-T, human corneal epithelial cell; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HP $\beta$ CD, 2-hydroxypropyl- $\beta$ -cyclodextrin; HPLC, high-performance liquid chromatography; Me $\beta$ CD, methyl- $\beta$ -cyclodextrin; PW, purified water; QA, N,N,N-trimethyl-N-(2-hydroxy-3-metacryloyloxopropyl)-ammonium chloride; REB, rebamipide; REB@CD, cyclodextrin solution containing rebamipide; REB@CDQA, CDQA solution containing rebamipide; REB@PW, rebamipide suspensions; REB@QA, QA solution containing rebamipide; SBE $\beta$ CD, sulfobutylether- $\beta$ -cyclodextrin; SEM, standard error of mean.

## Data Sharing Statement

The data generated in the present study may be requested from the corresponding author.

## Ethics Approval

All animal experiments were performed in accordance with the guidelines of Kindai University, the Japanese Pharmacological Society, and the National Institutes of Health (NIH). The experimental protocols were approved on April 1, 2021, by the Kindai University under project identification codes KAPS-2021-003 and KAPS-2021-004, and the study was conducted in compliance with the ARRIVE and AVMA euthanasia guidelines, 2020.

## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

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