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RESEARCH ARTICLE

Eye drop delivery of nano-polymeric micelle formulated genes with cornea-specific promoters

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Abstract

Background This study evaluates the eye drop delivery of genes with cornea-specific promoters, i.e., keratin 12 (K12) and keratocan (Kera3.2) promoters, by non-ionic poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) polymeric micelles (PM) to mouse and rabbit eyes, and investigates the underlying mechanisms.

Methods Three PM-formulated plasmids (pCMV-*Lac* Z, pK12-*Lac* Z and pKera3.2-*Lac* Z) containing the *Lac* Z gene for β-galactosidase (β-Gal) whose expression was driven by the promoter of either the cytomegalovirus early gene, the keratin 12 gene or the keratocan gene, were characterized by critical micelle concentration (CMC), dynamic light scattering (DLS), and atomic force microscopy (AFM). Transgene expression in ocular tissue after gene delivery was analyzed by 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) color staining, 1,2-dioxetane β-Gal enzymatic activity measurement, and real-time polymerase chain reaction (PCR) analysis. The delivery mechanisms of plasmid-PM on mouse and rabbit corneas were evaluated by EDTA and RGD (arginine-glycine-aspartic acid) peptide.

Results The sizes of the three plasmid-PM complexes were around 150–200 nm with unimodal distribution. Enhanced stability was found for three plasmid-PM formulations after DNase I treatment. After six doses of eye drop delivery of pK12-*Lac* Z-PM three times a day, β-Gal activity was significantly increased in both mouse and rabbit corneas. Stroma-specific *Lac* Z expression was only found in pKera3.2-*Lac* Z-PM-treated animals with pretreatment by 5 mM EDTA, an opener of junctions. *Lac* Z gene expression in both pK12-*Lac* Z-PM and pKera3.2-*Lac* Z-PM delivery groups was decreased by RGD peptide pretreatment.

Conclusions Cornea epithelium- and stroma-specific gene expression could be achieved using cornea-specific promoters of keratin 12 and keratocan genes, and the gene was delivered with PM formulation through non-invasive, eye drop in mice and rabbits. The transfection mechanism of plasmid-PM may involve endocytosis and particle size dependent paracellular transport.

Keywords specific gene expression; eye drops; polymeric micelles; particle size

Introduction

The delivery of genes by eye drop holds promise for the treatment of a wide range of corneal epithelial defects (e.g. corneal neovascularization, dystrophies, neurotrophic keratopathy, recurrent erosion, and dry

eye syndrome, etc.) and stromal disorders (corneal scar, corneal haze, excessive deposition of extracellular matrix, angiogenesis, and inflammation, etc.). It offers a non-invasive, simple delivery strategy with large corneal surface absorption area and has numerous advantages over more invasive delivery approaches. Without the removal of epithelium, or invasive injection, most viral vectors fail to deliver DNA into corneal cells. For example, Behrens *et al*. used eye drop delivery of a retroviral vector containing a dominant-negative form of the anti-keratocyte proliferative cyclin G1 gene to prevent the activation of keratocytes only after phototherapeutic keratectomy (PTK), and subsequently block haze formation [1]. Although viral vectors are the most commonly used in experimental and clinical gene therapy studies, they evoke important safety issues, particularly inflammatory and immune responses to viral proteins [2–4].

Gene delivery to the corneal epithelium is greatly facilitated because of relatively rapid cell division and the anatomical fact that corneal epithelial and stromal layers are on the top surface of the cornea [5]. Unfortunately, the cornea is also protected from noxious substances in the environment by rapidly secreted tears to flush its surface as well as an impermeable epithelium with tight junctions between cells [2]. Thus, attempts to improve the bioavailability of a topically delivered gene in ocular tissues should extend the contact time between the delivered gene and ocular tissue, as well as develop an optimally designed vesicle to achieve efficient and successful cell- or tissue-specific gene therapy with low toxicity.

In general, synthesized polymers are thought to have the potential for repeated administration and large-scale production. Among polymeric carriers, a non-ionic PEO-PPO-PEO (poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide)) triblock copolymer has been shown to be non-immunogenic, have relatively low toxicity, be resistant to protein/serum absorption, is stable in an enzymatic environment [6,7], and has been approved by the Food and Drug Administration for use in food, ophthalmic pharmaceuticals [8–10], and a wide range of consumer products [11]. We have previously reported [12] that in topical administration of PEO-PPO-PEO polymer micelles (PM) with pCMV-*Lac* Z plasmid DNA via eye drop, β-galactosidase (β-Gal) expression could be detected in various ocular tissues including the anterior chamber, cornea, iris, choroid sclera, conjunctiva, retinal pigmented epithelium, and the vitreous body. Likewise, this plasmid-PM formulation can efficiently deliver and express the transgene in liver and brain by oral administration [13].

For clinical applications, cell- or tissue-specific promoters should meet the purpose of targeted gene expression due to specific promoters that can drive genes to express exactly in target tissue. In previous studies [14–17], using direct plasmid injection, the 3.2-kb 5′-flanking region of the keratocan gene (Kera3.2) was able to drive the expression of a *Lac* Z or EGFP gene in corneal stroma. In addition, the corneal epithelium-specific K12 promoter was able to drive *Lac* Z gene expression after particle-mediated gene transfer (Gene-Gun). However, intrastromal injection and gene-gun delivery are invasive and may cause ocular surface damage. Thus, the aim of this study was to evaluate a non-invasive devise for *in vivo* gene delivery. We examined the non-ionic ABA-type PEO-PPO-PEO copolymer-mediated transfer of a gene whose expression is driven by a corneal tissue-specific promoter (the promoter of keratin 12 or keratocan genes) to mouse and rabbit eyes. The underlying mechanism of plasmid-PM transfer to the cornea was further evaluated by pretreatment with EDTA to enhance the paracellular pathway or with RGD (arginine-glycine-aspartic acid) peptide to influence endocytosis. All together, our results indicate that PM-mediated delivery of genes whose expression is driven by tissue-specific promoters was significantly increased and the trans-gene expression was restricted to the cornea. We found that the particle size of the plasmid-PM complex may influence the complex permeating through the paracellular pathway, and the preliminary results suggest that the transfection mechanism may involve endocytosis. Our results suggest that this novel gene delivery strategy may be useful to improve some corneal dystrophies and dysfunctions by cell-specific rescue of the defective gene [18].

Materials and methods

Materials

PEO-PPO-PEO copolymer with an average molecular weight of 8400 was obtained from the BASF Corporation (Ludwigshafen, Germany). EDTA and RGD peptide were obtained from Sigma Chemical (St. Louis, MO, USA). PEG (polyethylene glycol) was obtained from American Polymer Standards Corporation (Mentor, OH, USA). All other chemicals were analytical reagent grade and used without further purification.

Animals

The animal protocol was approved by the Laboratory Animal Research Committee of Taipei Medical University. The nude mice (BALB/cAnN.Cg-*Foxn1*^{nu}/CrlNarl, male, 6–8 weeks old) used in the *in vivo* topical eye drop delivery study were maintained under specific pathogen-free conditions and were purchased from the National Laboratory Animal Center (Taipei, Taiwan). Male albino New Zealand rabbits (Animal Center of National Taiwan University, Taipei, Taiwan), weighing 2 to 3 kg, were also used in the *in vivo* eye drop studies.

Plasmid DNA

Three plasmids, containing the *Lac* Z gene for β-galactosidase (β-Gal), were constructed such that *Lac* Z

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expression was driven by the promoter of cytomegalovirus early gene (pCMV-*Lac* Z), the 2.0-kb 5′-flanking region of the keratin 12 gene (pK12-*Lac* Z) [13,14] or the 3.2-kb 5′-flanking region of the keratocan gene (pKera3.2-*Lac* Z) [15]. In addition, *Eco* RI and *Xho* I were used to remove the CMV promoter from pCMV-*Lac* Z, generating a promoterless plasmid, p*Lac* Z. All plasmid DNAs were amplified in the *Escherichia coli* host DH5α strain, and were purified by equilibrium centrifugation in a cesium chloride/ethidium bromide (CsCl-EtBr) gradient [19]. The purity and stability of plasmid preparations were investigated by agarose gel electrophoresis followed by EtBr staining, and the DNA concentration was measured by UV absorption at 260 nm.

Preparation of plasmid-PM complexes

In this study, plasmid-PM complexes were prepared freshly on a weight percentage basis as in previous studies [11,12]. Different amounts of polymer were added to water containing 0.08 μg/μl of plasmid with gentle mixing at room temperature for 2 h.

Characterization of plasmid-PM complexes

The formation of critical micelle concentration (CMC) of PM was confirmed by a pyrene fluorescence probe [20]. As the micelle formed, pyrene was partitioned into the micelle hydrophobic phase, and was determined using the ratio of peak I_1/peak I_3 of pyrene as previously reported [21]. The fluorescence emission spectrum of pyrene in the PM solution was measured from 350 to 460 nm using a fixed excitation wavelength of 339 nm with a constant pyrene concentration of 6×10^{-7} M. The PEO-PPO-PEO copolymer varied from 0.001% (w/v) to 10%. The spectral data were recorded using a Hitachi F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). All fluorescence experiments were carried out at 25 °C. The above CMC of non-ionic polymer was used to form PM for plasmid delivery.

Size distribution and zeta potential of plasmid-PM and size distribution of PEGs

The size and zeta potential of 0.3% (w/v) PM containing 0.08 μg/μl of plasmid DNA were measured by quasi-elastic laser dynamic light scattering (DLS) (Zetasizer 3000, Malvern Instruments, Malvern, UK) with an assumed refractive index ratio of 1.33 and viscosity of 0.88. All measurements were performed at 25 °C at a measurement angle of 90° and results are presented as mean ± standard deviation (SD) [11,12]. In addition, 5% of PEG (Mw: 194, 330, 400, 450, 610, 700, 975, and 1150) was used as reference particle size measurement.

Atomic force microscopy

Atomic force microscopy (AFM) was used to reveal the morphology of complexes of plasmids, PMs, and plasmid-PMs. A volume of 3 μl of plasmid-PM solution was placed on a mica surface with no further treatment. The microscope (SPA-400, SII Seiko NanoTechnology Inc., Tokyo, Japan) was operated in dynamic force mode (DFM). The cantilevers were standard silicon for DFM levers. The scanning rate was 1 Hz and all images were collected within a 1.5×1.5 μm^2 area. Unless otherwise stated, all images shown were subjected only to the normal image processing of leveling.

In vitro stability of plasmid-PM with DNase I treatment

Protection of plasmid by PM against DNase I was carried out with modified methods as described in other reports [22]. Briefly, the reaction mixture contained 150 μl of digestion buffer, 1.5 units of RQ1 RNase-free DNase I (Promega Biotech Co., Ltd., Madison, WI, USA) and 12 μg of plasmid complex with or without PM. The reaction was carried out for 105 min at 37 °C. This mixture was sampled in 10 μl volumes at fixed time intervals and was then stopped by immediate addition of 1 μl of 20 mM EGTA stop solution (Promega Biotech Co., Ltd.). The resulting solutions were loaded onto a 0.8% agarose gel for electrophoresis and then the gel was stained with EtBr. Qualification of band intensities was performed with a Kodak EDAS290 Analysis system (Kodak Scientific Imaging System, New Haven, CT, USA).

Gene eye drop delivery to ocular tissues *in vivo*

For the *in vivo* eye drop delivery studies, the eyes were delivered with 0.08 μg/μl plasmid in 0.3% PM (10 μl per mouse eye, and 50 μl per rabbit eye, six doses of eye drop three times a day) [11]. To evaluate PM gene transfer *in vivo*, each animal treatment group was sacrificed either by cervical dislocation for nude mice or intravenous (i.v.) injection with 5% pentobarbital for rabbits. The excised eyes were immediately removed after 48 h of the first topical administration. For gene transfer enhancement, pretreatment with 5 mM EDTA (10 and 50 μl for mice and rabbits, respectively) was applied in eye drops for 10 min followed by the same dose of delivery of the plasmid-PM complexes. RGD peptide (10 mM) was used to treat animal eyes for 10 min before plasmid-PM administration [23].

Determination of β-Gal activity

β-Gal activity was quantified using the Galacto-Light Plus™ system (Applied Biosystems, Foster City, CA, USA)

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according to the manufacturer's protocol. Briefly, the ocular tissues were excised after six doses of eye drops, washed twice with phosphate-buffered saline (PBS) and then homogenized in lysis buffer. The resulting lysates were frozen at −80 °C. Following thawing and centrifugation at 13 000 rpm for 10 min at 4 °C, 20 μl of the supernatant was analyzed for chemiluminescence with a Veritas™ microplate luminometer (Turner BioSystems, Inc., Sunnyvale, CA, USA) with the addition of diluted Galacton-Plus reaction buffer and incubation at room temperature for 45 min. Total tissue protein was measured using the *DC* protein assay kit (BioRad, Hercules, CA, USA), which was used to normalize β-Gal activity in each sample. Statistical comparisons were made with analysis of variance (ANOVA) tests with Dunnett's multiple comparison tests at a 95% confidence level. All results are presented as mean ± standard error of the mean (SEM).

Detection of mRNA expression by real-time quantitative polymerase chain reaction (qPCR)

In order to confirm transferred gene expression, excised eyes or corneas of nude mice or corneas of rabbits after six doses of topical administration within 48 h were obtained and total RNA was extracted with TRIZOL® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Real-time qPCR was used to determine the levels of β-Gal mRNA. Total RNA (1.5 μg) was reverse-transcribed with SuperScript™ II reverse transcriptase (Invitrogen Life Technologies) primed with oligo-dT (10 μM). Real-time qPCR was performed using SYBR® Green PCR Master Mix in an ABI PRISM 7300 sequence detection system (Applied Biosystems 7300 System Sequence Detection System (SDS) software version 1.3). The primers for β-Gal [24] (forward: 5′-CTA CAC CAA CGT AAC CTA TCC C-3′ and reverse: 5′-TTC TCC GGC GCG TAA AAA TGC G-3′) and 18S rRNA [25] (forward: 5′-GAG CCG CCT GGA TAC CG-3′ and reverse: 5′-CGC TCT GGT CCG TCT TGC-3′) were used. The conditions for the PCR were as follows: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Plasmid standards were made by preparing 10-fold serial dilutions of plasmid (pCMV-*Lac* Z) from 1×10^6 to 1×10^1 copies. All the cDNA samples were run in duplicate with a plasmid standard curve that contained 1×10^1 to 1×10^6 copies of the *Lac* Z gene. The quantification values were obtained from the threshold cycle (Ct) number at which the increase in signal associated with an exponential growth of PCR products began to be detected using SDS software. At the end of the PCR cycles, amplification specificity was confirmed by a dissociation curve analysis and the products were separated on a 3% agarose gel and stained with EtBr for visual confirmation of the PCR product. The identity of the amplified DNA was further confirmed by direct sequencing of the PCR product. The β-Gal mRNA was normalized and expressed as copies per microgram (μg) of total RNA. All results are presented as mean ± SEM.

Histochemical staining of β-Gal

The localization of β-Gal expression was determined by histochemical analysis using X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) substrate (Invitrogen Life Technologies) following the method of Oshima *et al*. [26]. Excised eyes were washed with PBS twice and fixed with 4% paraformaldehyde at 4 °C for 90 min, and then rinsed with PBS. The ocular tissue was incubated with 10 mM $K_4Fe(CN)_6$, 10 mM $K_3Fe(CN)_6$, 0.01% sodium deoxycholate, 0.02% Nonidet-40 (NP-40), and 2 mM $MgCl_2$ in PBS containing 1 mg/ml of X-Gal substrate, pH 7.4, at 37 °C for 48–72 h. Cryosections (10 μm) of the O.C.T. (Sakura Finetek U.S.A., Torrance, CA, USA)-embedded stained tissues were washed in an acetone/methanol (1 : 1) solution, and then stained with hematoxylin-eosin for histological assessment. β-Gal expression was visible under a microscope (Olympus BX-40, Japan) when a green-blue color appeared.

Results

Characterization of plasmid-PM complexes

The PM formation and the critical micelle concentration (CMC) of PM were confirmed by a fluorescence probe, pyrene. By measuring the ratio of peak I_1/peak I_3 of pyrene, we found that the ratio decreased above 0.1% of polymers (Figure 1). The peak I_1/peak I_3 ratio of the pyrene fluorescence probe has been correlated to the hydrophobicity of the environment of the pyrene probe and indicated that PM were formed above the CMC (0.1%) [11,21]. In this study, 0.3% (w/v) of polymer was used in all formulations to form PM.

The particle size of PM was 36.7 ± 20.8 nm measured by dynamic light scattering (DLS) (Table 1). The particle sizes of three plasmids alone were 69.7 ± 36.1, 70.0 ± 31.0 and 72.9 ± 28.6 nm for pCMV-*Lac* Z, pK12-*Lac* Z and pKera3.2-*Lac* Z, respectively. The particle sizes of three plasmid-PM complexes were all unimodal distributions and the particle size distributions of pK12-*Lac* Z-PM and pKera3.2-*Lac* Z-PM were significantly larger than that of pCMV-*Lac* Z-PM. The surface zeta potentials of pCMV-*Lac* Z, pK12-*Lac* Z and pKera3.2-*Lac* Z were −26.2 ± 8.4, −27.9 ± 13.0 and −24.7 ± 11.0 mV (Table 1), respectively, and it was −5.5 ± 0.7 mV for the non-ionic PM alone. After formulation, the zeta potentials were increased to −8.9 ± 3.8, −12.1 ± 1.2 and −12.1 ± 1.1 mV for pCMV-*Lac* Z-PM, pK12-*Lac* Z-PM and pKera3.2-*Lac* Z-PM, respectively. AFM was also used to visualize the morphology of PM, plasmid and three plasmid-PM complexes (Figure 2). The results showed that these plasmid-PM complexes appear round-shaped

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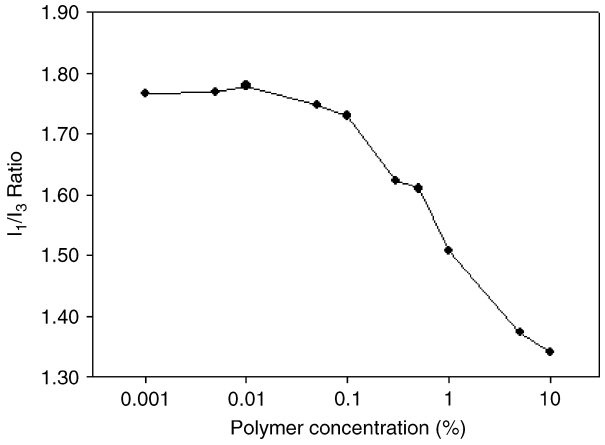

Figure 1. Intensity of the I_1/I_3 ratio pyrene fluorescence spectrum with PEO-PPO-PEO copolymer concentration profile. The ratio of I_1/I_3 decreased at and above 0.1% (w/v) polymer concentration indicating that the critical micelle concentration (CMC) is 0.1%

with a measured particle size similar to that detected by DLS.

The stability of plasmid alone and plasmid-PM was observed by electrophoresis after 2 days at room temperature, 4 °C, or 37 °C and after two cycles of freezing and thawing. The plasmid-PM showed similar a electrophoresis pattern to that of plasmid alone (Figure 3). However, plasmid alone incubated at 37 °C for 2 days was observed to undergo some conformational changes.

In vitro stability of plasmid-PM complexes with DNase I treatment

Naked plasmid DNA administration *in vivo* is rapidly degraded by nucleases [27]. The protection of plasmid with PM was analyzed by DNase I treatment. We found that after incubation of plasmid along with DNase I, all tested plasmids were completely diminished within 3 min (Figure 4). Exposure of three plasmid-PM complexes to DNase I resulted in a delayed DNA degradation during the experimental period; complete DNA degradation occurred after 105-, 90- and 45-min reactions for pCMV-*Lac* Z-PM, pK12-*Lac* Z-PM and pKera3.2-*Lac* Z-PM, respectively. It suggests that PM could protect plasmids from nucleases.

Table 1. Particle sizes and zeta potentials of polymeric micelles (PM), plasmid alone and plasmid-PM[a]

	PM alone	Plasmid alone	Plasmid-PM
Particle size (nm)			
pCMV-*Lac* Z (7.2 kb)	36.7 ± 20.8	69.7 ± 36.1	142.9 ± 8.7[b]
pK12-*Lac* Z (8.6 kb)		70.0 ± 31.0	182.1 ± 6.1
pKera3.2-*Lac* Z (10.9 kb)		72.9 ± 28.6	187.3 ± 9.1
Zeta potential (mV)			
pCMV-*Lac* Z	-5.5 ± 0.7	-26.2 ± 8.4	-8.9 ± 3.8
pK12-*Lac* Z		-27.9 ± 13.0	-12.1 ± 1.2
pKera3.2-*Lac* Z		-24.7 ± 11.0	-12.1 ± 1.1

[a]Results are expressed as the mean ± standard deviation (n = 6).
[b]Denotes a significant difference ($P < 0.01$) compared with pK12-*Lac* Z-PM and pKera3.2-*Lac* Z-PM.

Determination of *in vivo* β-Gal activity and real-time qPCR analysis

After six doses of plasmid-PM eye drop, the β-Gal activity was evaluated in mouse eyes. For mice receiving pCMV-*Lac* Z alone, p*Lac* Z (the promoter-less plasmid)-PM complex, no significant increase in *Lac* Z gene expression could be detected in any tissue (Table 2). For the mice administered with pCMV-*Lac* Z-PM and pK12-*Lac* Z-PM, a significant increase in β-Gal activity was detected compared with that detected in the mice receiving

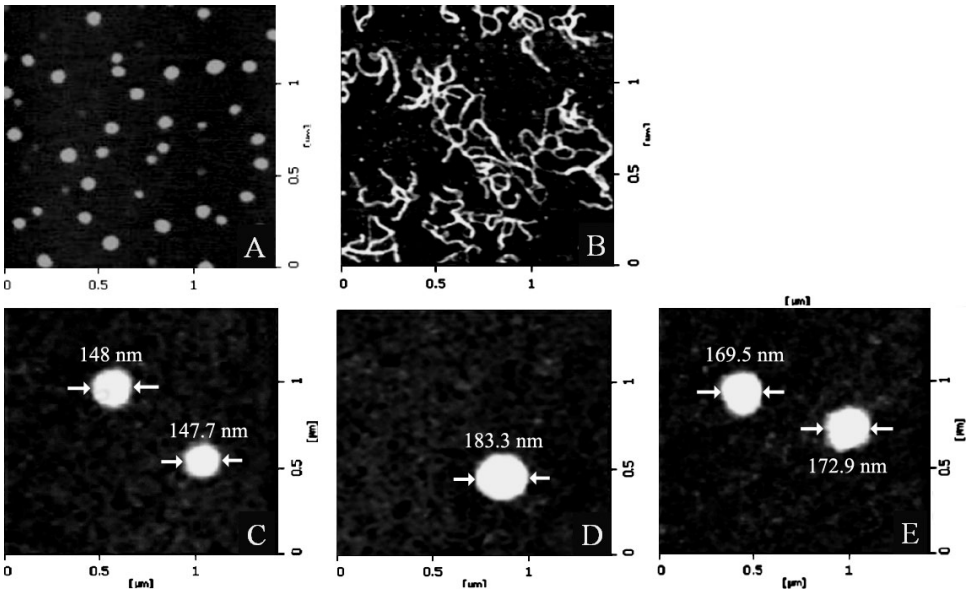

Figure 2. AFM images of PM, plasmid, and plasmid-PM (3 μl of 0.08 μg/μl plasmid with 0.3% PM solution on a mica surface): (A) 0.3% PM; (B) pCMV-*Lac* Z alone; (C) pCMV-*Lac* Z-PM; (D) pK12-*Lac* Z-PM; and (E) pKera3.2-*Lac* Z-PM

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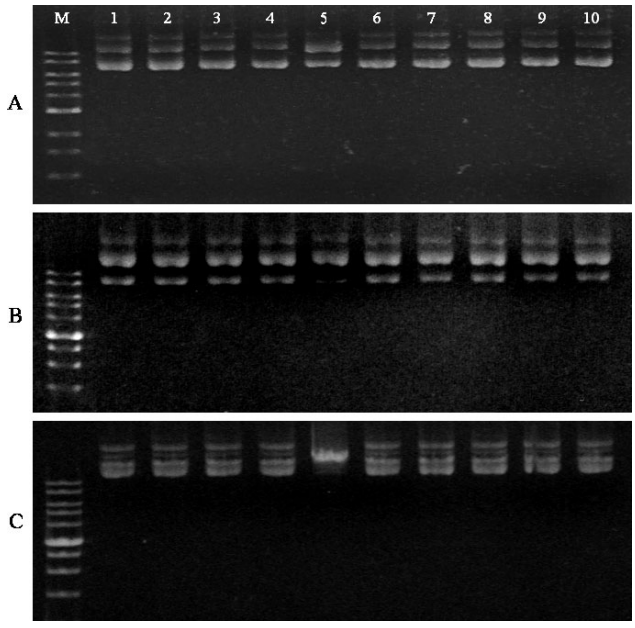

Figure 3. Temperature effect on the stability of three plasmid-PM complexes: (A) pCMV-*Lac* Z; (B) pK12-*Lac* Z; and (C) pKera3.2-*Lac* Z. Lanes 1–5: plasmid alone; lanes 6–10: plasmid-PM; lanes 1 and 6: incubated at room temperature for 2 h; lanes 2 and 7: two cycles of freeze/thaw; lanes 3 and 8: incubated at room temperature for 2 days; lanes 4 and 9: stored at 4 °C for 2 days; lanes 5 and 10: incubated at 37 °C for 2 days. Markers (M) were 1 kb DNA ladders

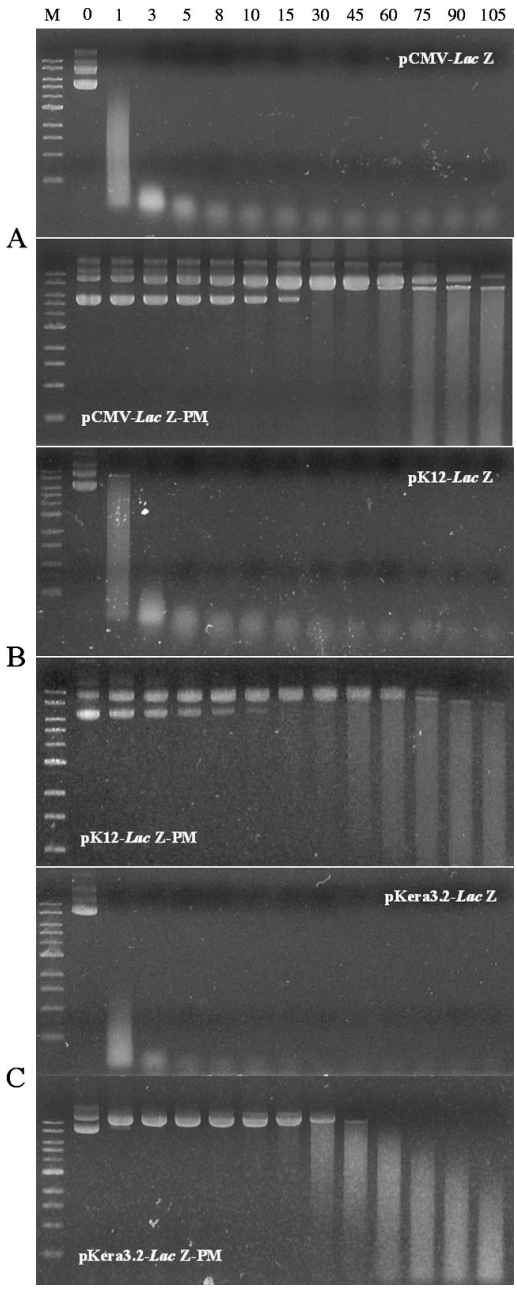

Figure 4. Time course (min) of *in vitro* stability with DNase I treatment of plasmid-PM: (A) pCMV-*Lac* Z; (B) pK12-*Lac* Z; and (C) pKera3.2-*Lac* Z. Markers (M) were 1-kb DNA ladders

pCMV-*Lac Z* plasmid alone, or p*Lac* Z-PM complex from 3.09 ± 0.09 to 3.90 ± 0.14 and 3.58 ± 0.19 mU/mg protein in mice whole eyes, respectively (Table 2). There was no significant β-Gal activity found in mice receiving pKera3.2-*Lac* Z-PM. When tissues were pretreated with 5 mM EDTA, known to open tight junctions and increase paracellular transport [28], the β-Gal activity was significantly increased in the group receiving pKera3.2-*Lac* Z-PM delivery (3.73 ± 0.25 mU/mg protein). No further improvement for pK12-*Lac* Z-PM was observed in conjunction with EDTA pretreatment. On the other hand, after pretreatment with RGD peptide, β-Gal activities were influenced in whole eyes of mice that received pK12-*Lac* Z-PM and pKera3.2-*Lac* Z-PM in conjunction with EDTA pretreatment (Table 2).

To confirm tissue-specific β-Gal expression after six doses of plasmid-PM delivery, β-Gal activity was analyzed on corneas dissected from whole eyes. The β-Gal activity detected in the cornea and the other parts of the eye post-pCMV-*Lac* Z-PM delivery all increased significantly (Table 2). After pK12-*Lac* Z-PM, pK12-*Lac* Z-PM delivery in conjunction with EDTA pretreatment or pKera3.2-*Lac* Z-PM delivery in conjunction with EDTA pretreatment, β-Gal activities in corneal tissue all significantly increased, with no difference in β-Gal activity in other ocular surface tissues, such as conjunctiva and sclera, when compared to the control group. The pretreatment with EDTA, known to open junctions, did not statistically significantly further enhance the β-Gal activity for pK12-*Lac* Z-PM delivery. In addition, pretreatment with RGD peptide for 10 min decreased corneal β-Gal activity when mice received pK12-*Lac* Z-PM (Table 2). A combination of pretreatment with EDTA and RGD peptide inhibited β-Gal activity in corneas compared to corneas treated with pKera3.2-*Lac* Z-PM in conjunction with EDTA pretreatment. In rabbits receiving pK12-*Lac* Z-PM, pK12-*Lac* Z-PM delivery in conjunction with EDTA pretreatment or pKera3.2-*Lac* Z-PM in conjunction with EDTA pretreatment, corneal β-Gal activity exhibited an increased level similar to that detected in mice, and no significant difference in the others (Table 2). RGD peptide pretreatment also decreased rabbit corneal β-Gal activity.

In order to quantify β-Gal mRNA levels, a standard curve for *Lac* Z DNA was obtained using SYBR® Green PCR

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Table 2. β-Galactosidase activity on mouse and rabbit eyes after eye drop delivery of plasmid-PM[a]

Treatment	*β-Gal activity* (mU/mg protein)				
	Mice			*Rabbits*	
	Whole eye (n = 16)	*Cornea* (n = 10)	*Others* (n = 10)	*Cornea* (n = 5)	*Iris* (n = 5)
p*Lac* Z-PM	3.10 ± 0.08	3.12 ± 0.12	2.52 ± 0.08	3.21 ± 0.55	5.58 ± 0.77
pCMV-*Lac* Z	3.09 ± 0.09	3.19 ± 0.19	2.46 ± 0.07	3.26 ± 0.47	5.86 ± 0.81
pCMV-*Lac* Z-PM	3.90 ± 0.14^{b}	4.52 ± 0.29^{b}	2.85 ± 0.08^{b}	5.49 ± 0.41^{b}	6.92 ± 0.63^{b}
pK12-*Lac* Z-PM	3.58 ± 0.19^{b}	3.89 ± 0.12^{b}	2.57 ± 0.06	4.86 ± 0.63^{b}	5.68 ± 0.93
EDTA-pK12-*Lac* Z-PM	3.49 ± 0.10^{b}	3.91 ± 0.21^{b}	2.45 ± 0.11	4.75 ± 0.71^{b}	5.45 ± 0.67
RGD-pK12-*Lac* Z-PM	3.25 ± 0.12^{c}	3.24 ± 0.15^{c}	2.26 ± 0.09	3.74 ± 0.54^{c}	5.73 ± 0.87
pKera3.2-*Lac* Z-PM	3.09 ± 0.11	3.45 ± 0.12	2.55 ± 0.09	3.84 ± 0.41	5.45 ± 0.67
EDTA-pKera3.2-*Lac* Z-PM	3.73 ± 0.15^{bd}	3.90 ± 0.13^{bd}	2.57 ± 0.08	4.54 ± 0.49^{bd}	5.62 ± 0.62
EDTA-RGD-pKera3.2-*Lac* Z-PM	3.29 ± 0.17^{e}	3.45 ± 0.14^{e}	2.28 ± 0.10	3.91 ± 0.42^{e}	5.98 ± 0.74

[a]BALB/cAnN.Cg-*Foxn1nu*/CrlNarl nude mice and albino New Zealand rabbits were eye dropped with six doses (0.08 μg/μl of plasmid in 0.3% PM, 10 μl per mouse eye, and 50 μl per rabbit eye) of formulations for 2 days. Whole eye, cornea and other parts of eyes from mice, cornea and iris from rabbits were collected at 48 h after the first dosage delivered. The numbers in parentheses are numbers of specimens examined in each treatment group. β-Gal activity (mU/mg protein) is expressed as mean ± SEM.
[b]Significant increase ($P < 0.05$) compared with the same ocular tissue from pCMV-*Lac* Z and p*Lac* Z-PM treatment.
[c]Significant decrease ($P < 0.05$) compared with the same ocular tissue from pK12-*Lac* Z-PM and EDTA-pK12-*Lac* Z-PM treatment.
[d]Significant increase ($P < 0.05$) compared with the same ocular tissue from pKera3.2-*Lac* Z-PM treatment.
[e]Significant decrease ($P < 0.05$) compared with the same ocular tissue from EDTA-pKera3.2-*Lac* Z-PM treatment

Master Mix with a series of *Lac* Z plasmid DNA dilutions (Figure 5). Since the Ct value increases in a linear fashion with decreasing *Lac* Z plasmid DNA copy number and all of the amplification curves were located within this linear amplification range, the amount of *Lac* Z transcript could be measured based on this standard curve. After six doses of pCMV-*Lac* Z-PM, pK12-*Lac* Z-PM or pKera3.2-*Lac* Z-PM in conjunction with EDTA pretreatment, β-Gal mRNA/μg total RNA was detected at 1924.14 ± 949.60, 282.57 ± 40.24 and 279.02 ± 66.43 copies in whole mouse eyes and 2324.00 ± 388.44, 580.13 ± 96.41 and 558.01 ± 93.16 copies/μg total RNA in mouse corneas via real-time PCR, respectively (Figure 5). β-Gal mRNA expression in rabbit corneas was detected at 3421.14 ± 2712.40, 864.46 ± 742.05 and 796.33 ± 652.78 copies/μg total RNA for pCMV-*Lac* Z-PM, pK12-*Lac* Z-PM and pKera3.2-*Lac* Z-PM with EDTA pretreatment, respectively. There was no β-Gal mRNA detected after p*Lac* Z-PM, pCMV-*Lac* Z, pK12-*Lac* Z or pKera3.2-*Lac* Z delivery and without reverse transcription of RNA from tissues after pCMV-*Lac* Z-PM, pK12-*Lac* Z-PM and pKera3.2-*Lac* Z-PM with EDTA pretreatment.

Histochemical staining of β-Gal

Mouse eyes were excised at 48 h after eye drop administration and subjected to X-Gal staining to localize β-Gal expression. β-Gal expression was distributed in the entire cornea in pCMV-*Lac* Z-PM-treated mice (Figure 6D). β-Gal expression was restricted to the corneal epithelium of the pK12-*Lac* Z-PM-treated mice (Figures 6E–6H). Stromally restricted β-Gal expression (indicated by blue-green staining) (Figures 6I–6L) was observed in pKera3.2-*Lac* Z-PM mice in conjunction with EDTA pretreatment, with the bulk of the expression located in the anterior stroma (Figures 6J and 6L). It should also be noted that occasional β-Gal expression was observed at the central or posterior stroma in these mice (Figure 6K). No X-Gal-positive expression was found in control eyes (Figures 6A–6C).

Particle sizes of PEGs

EDTA has been shown to open the tight junctions in the paracellular pathway and could increase corneal permeation of PEG 450 to PEG 700 [29]. To further correlate the relevance between particle size and permeation of PEGs with different molecular weights via the paracellular (tight junction) route, we used DLS to measure the particle sizes of different molecular weight PEGs. The particle sizes of PEG 194, 330, 400, 450, 610, 700, 975, and 1150 were determined to be 118.2 ± 24.2, 120.9 ± 8.6, 99.4 ± 8.6, 151.0 ± 19.3, 142.5 ± 21.4, 206.8 ± 24.9, 237.4 ± 30.9 and 341.8 ± 44.5 nm, respectively (Figure 7). These results indicate that EDTA pretreatment allows larger-sized particles (PEG 450 to PEG 700: about 150–200 nm) to pass into the tissue via the paracellular pathway.

Discussion

Recently, Yang *et al*. [30] have reported that polymeric micelles (PM) enhance reporter gene expression in a mouse strain- and promoter-type-dependent manner. Their data showed that there was PM-enhanced reporter gene expression in immunocompetent C57Bl/6 and Balb/c mice, but not in athymic Balb/c nu/nu mice. In addition, this PM-enhanced reporter gene expression driven by the CMV promoter was through the NF-κB- and p53-mediated signaling pathways activated by inflammatory responses, but no cytotoxicity was observed of this PM at which gene expression was increased [31]. We therefore thought that it might be appropriate

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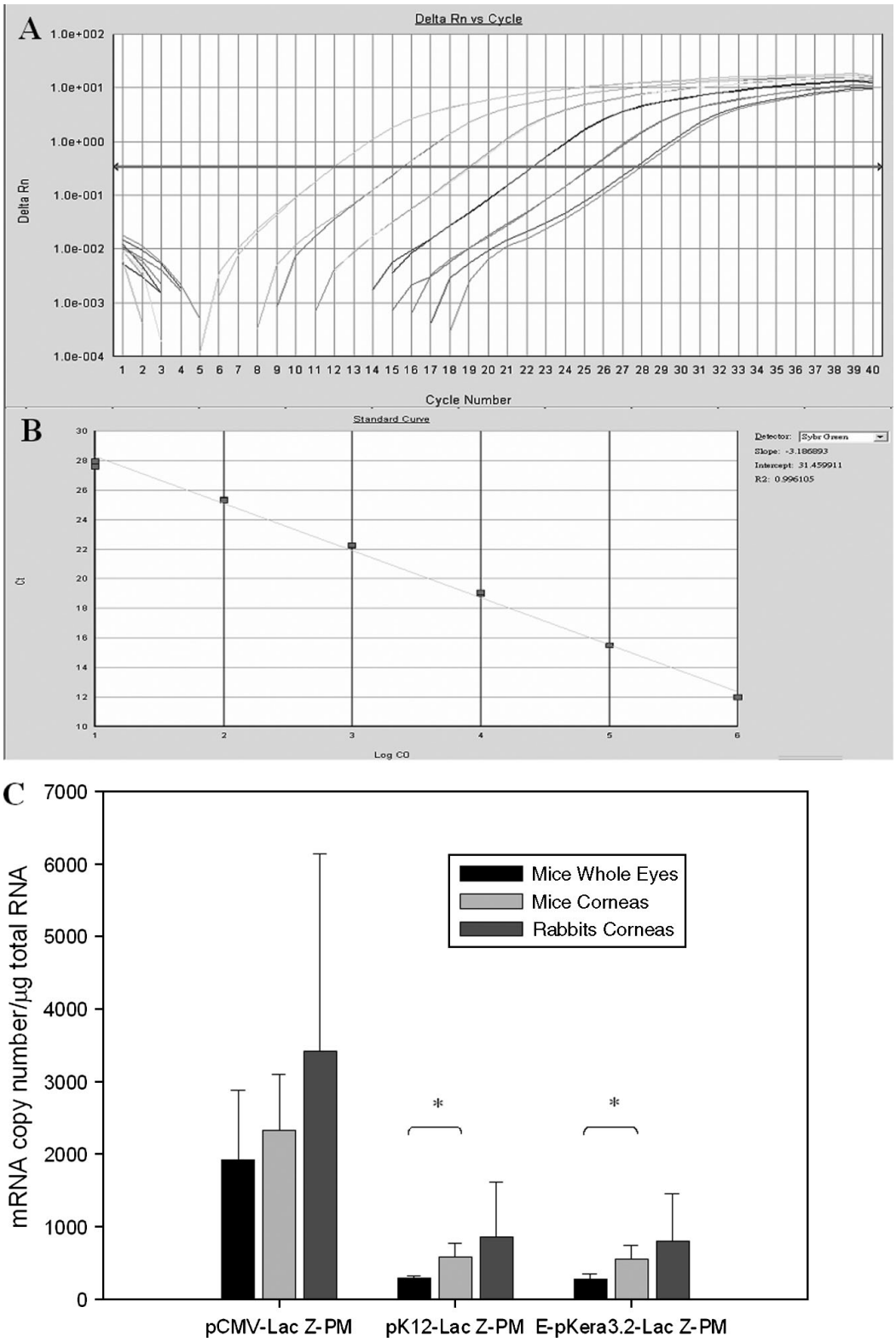

Figure 5. Real-time quantitative PCR analysis of β-Gal mRNA in mouse eyes and rabbit corneas after six doses of topical plasmid-PM eye drops: (A) real-time measurements of PCR products (logarithmic scale) for the plasmid standard curve (serial dilutions from left to right: 10^6 to 10^1 copies); (B) logarithmic amplification plot of real-time measurements of PCR products for a plasmid standard curve (serial dilutions from right to left: 10^6 to 10^1 copies), and the standard curve correlation coefficient (R^2) was: 0.996; (C) real-time quantitative PCR in mice whole eyes, mice corneas and rabbits corneas after pCMV-*Lac* Z-PM, pK12-*Lac* Z-PM and pKera3.2-*Lac* Z-PM delivery with EDTA pretreatment. All of the cDNA samples (n = 5) were duplicated in real-time PCR analysis. β-Gal mRNA level (copy numbers/μg total protein) is expressed as mean ± SEM. The β-Gal mRNA levels of pK12-*Lac* Z-PM and pKera3.2-*Lac* Z-PM with EDTA pretreatment in the cornea were significantly different compared with the same treatment in mouse whole eyes (*$P < 0.05$). No β-Gal mRNA was detected after p*Lac* Z-PM, pCMV-*Lac* Z, pK12-*Lac* Z, pKera3.2-*Lac* Z delivery, without reverse transcription of RNA from tissues after pCMV-*Lac* Z-PM, pK12-*Lac* Z-PM, pKera3.2-*Lac* Z-PM with EDTA pretreatment, and NTC (no template control)

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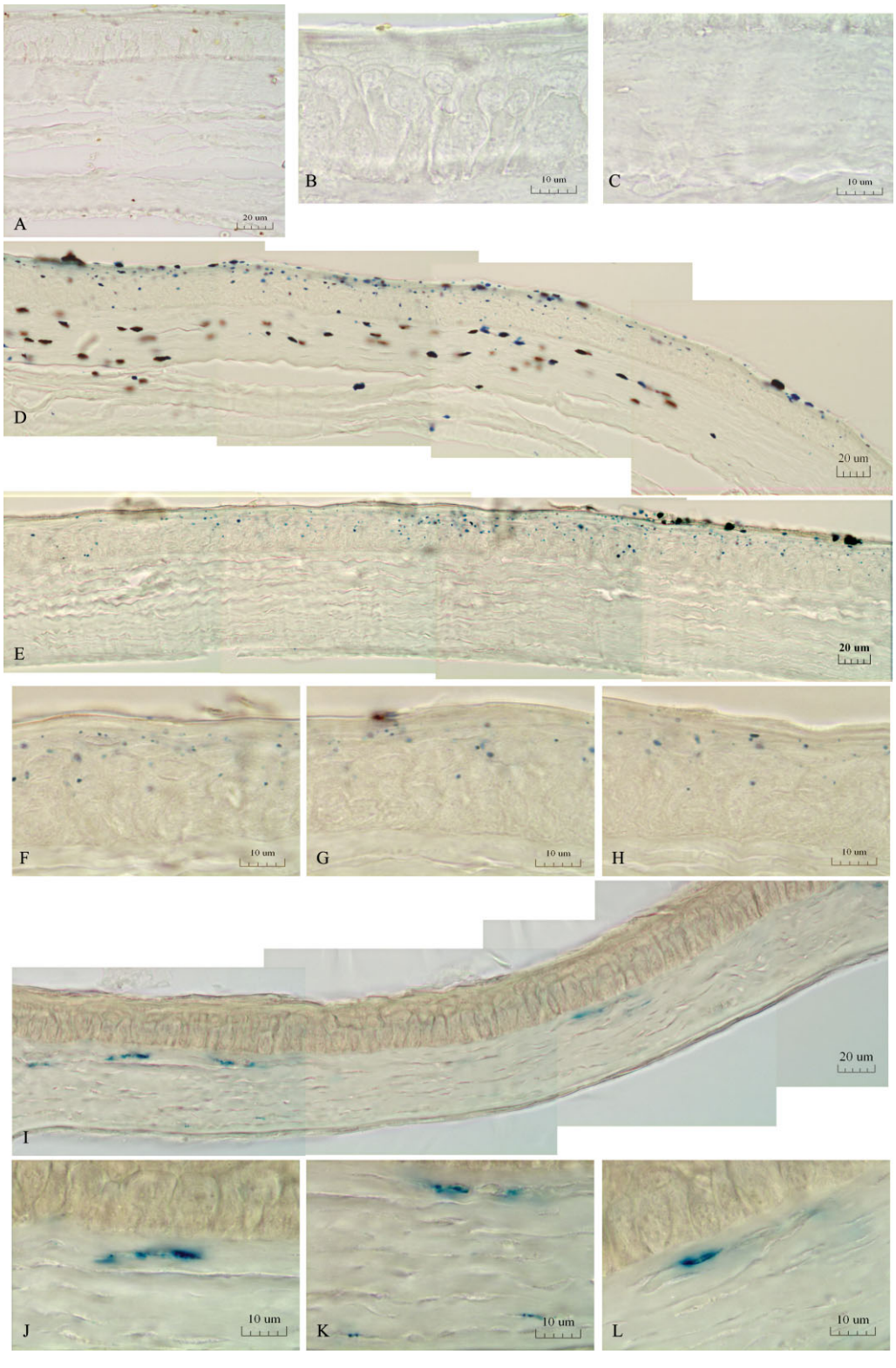

Figure 6. Histochemical analysis (X-Gal staining) of nude mouse eyes 48 h after the start of eye drop delivery of plasmid-PM. A, B and C are control groups (delivered with plasmid alone). (D) Represents gene transfer with pCMV-*Lac* Z-PM and β-Gal expressed in the cornea area. β-Gal expression was visible and restricted to the corneal epithelial and stromal layers after pK12-*Lac* Z-PM (E–H) and pKera3.2-*Lac* Z-PM delivery with EDTA pretreatment (I–L), respectively. Note the blue-green staining corresponding to β-Gal expression. No inflammatory reaction was noted after plasmid-PM delivery

to use the immunodeficient nude mice for the gene delivery experiment to avoid the background signals that were triggered by the NF-κB- or p53-mediated signaling pathway.

Several features of the cornea, such as the low permeability of the intact epithelial layer, and the rapid secretion of tears, profoundly reduce the efficiency of gene transfer [2,17]. Experiments using adenovirus or

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herpes simplex viral vectors to directly transfer genes to the corneal epithelium failed to deliver and express transgenes in epithelial layers [32,33]. In addition, topical transfer of the *Lac* Z gene via a herpes simplex viral vector to the corneal epithelium requires prior scarification of the corneal surface [33]. In general, gene transfer with viral vectors fails in the corneal epithelium unless several invasive or destructive approaches are used, such as epithelial wounding, electroporation, or direct injection [33–35]. In this study we transferred the *Lac* Z gene to animal eyes by non-invasive, eye drop delivery of pK12-*Lac* Z-PM. *Lac* Z gene expression was detected and limited to the corneal epithelial layer. Unfortunately, inefficient penetration of epithelial barriers resulted in a lack of stromal gene expression with our pKera3.2-*Lac* Z-PM formulation.

Mohan *et al*., who used an invasive and disruptive lamellar flap technique to transfer genes into rabbit corneal stromal cells through plasmid-cationic lipid complexes, achieved a gene expression of 1.5–2.1 μmol/mg protein/μg plasmid (total dose of 50 μg plasmid) [36]. In this study, EDTA pretreatment for 10 min before pKera3.2-*Lac* Z-PM (total dose 24 μg) delivery achieved a similar level of transferred gene expression (1.0 μmol/mg protein/μg plasmid) in rabbit corneas compared to the results reported by Mohan *et al*. In addition, the β-Gal expression of the cornea receiving pKera3.2-*Lac* Z-PM delivery in conjunction with EDTA pretreatment was restricted to the corneal stroma (Figures 6I–6L), and the distribution pattern of transgene expression was similar to the results of Carlson *et al*. who delivered pKera3.2-EGFP by intrastromal injection [17]. Thus, the efficiency of pKera3.2-*Lac* Z-PM delivery to cornea stromal is enhanced by EDTA, known to open tight junctions, which enhances paracellular transport [12,28]. Hämäläinen *et al*. determined that the paracellular pore diameter of the corneal epithelium was in the nanometer range via PEG oligomer penetration experiments [37]. The corneal permeability of PEG decreased most rapidly at molecular weights above PEG 414. In our previous studies, using a series of PEGs with different molecular weights, we also observed that the molecular weight cutoff of PEG affecting its ability to penetrate cornea was at PEG 450. In addition, we have shown here that EDTA pretreatment can increase corneal permeation of PEG 450 to PEG 700 (Figure 7) [29]. The particle size of PEG 450 to PEG 700 was found to be 150–200 nm via DLS measurements. Thus, exposure of the cornea to EDTA enlarges paracellular pores and enhances the transfer of the larger pKera-3.2-*Lac* Z-PM complex (187.3 ± 9.1 nm), indicating that EDTA pretreatment enhances the transport range of delivered vesicles by opening paracellular tight junctions.

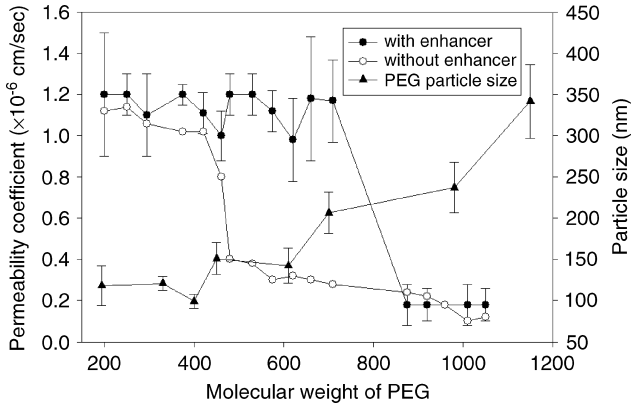

Figure 7. The effect of enhancer on PEG corneal permeation with particle size distribution by DLS. ○: The permeability coefficients of PEG on corneal transport. ●: The corneal permeability coefficients of PEG on EDTA enhancer pretreatment. ▲: The distribution of particle size with PEG molecular weight

It has been demonstrated that PEO-PM was taken up into cells via a receptor-mediated endocytotic mechanism that can be abolished at low temperature [38,39]. Rejman *et al*. demonstrated that internalization of microspheres with a diameter less than 200 nm occurs via clathrin-coated pit-mediated endocytosis [40]. RGD is an endocytotic inhibitor with a critical integrin-binding motif [41,42]. Croyle *et al*. reported that pretreatment of Caco-2 cells with 3.5 mM RGD peptide reduced adenovirus-mediated gene transfer by 80% [43]. Goldman *et al*. also showed that administration of RGD peptide (10 mg/ml), in conjunction with adenovirus-mediated gene transfer to murine respiratory tissues, reduced gene transfer efficiency in both nasal and tracheal epithelia by 65–75% [44]. In this study, when EDTA and RGD peptide were applied for 10 min before pKera3.2-*Lac* Z-PM administration, β-Gal activity decreased 48–70% compared to pKera3.2-*Lac* Z-PM delivery with EDTA pretreatment alone (Table 2). Similar suppressed expression (68–84%) was found with RGD peptide pretreatment in conjunction with pK12-*Lac* Z-PM delivery. On the other hand, pretreatment with EDTA, an opener of junctions, could significantly enhance the β-Gal activity in the pKera3.2-*Lac* Z-PM delivery group, but did not further increase the β-Gal activity after pK12-*Lac* Z-PM delivery to mouse eyes. Thus, the internalization of plasmid-PM in the cornea may be influenced by endocytosis, and enhanced penetration of the stromal layer could be achieved by opening the tight junction.

Taken together, these results provide a promising strategy for corneal gene targeting using nano-PM, a non-viral gene delivery system, and achieve tissue-specific transgene expression with keratin 12 and keratocan promoters. The transfer mechanism of plasmid-PM to the cornea was further enhanced by pretreatment with EDTA to facilitate the paracellular pathway, and with RGD peptide to decrease β-Gal activity. Our studies indicate that plasmid-PM delivery with appropriate pretreatment regimens might be used in conjunction with corneal tissue-specific promoters to alleviate a variety of corneal pathologies.

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