Corneal Penetrating Elastin-Like Polypeptide Carriers

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Abstract

Purpose: Elastin-like polypeptide (ELP) is a bioengineered protein widely applied as a drug carrier due to its biocompatibility and amenability to modification with cell-penetrating peptides (CPPs) and therapeutic agents. The purpose of this study was to determine whether topically applied ELP or CPP-fused ELPs penetrate the corneal barrier.

Methods: *In vitro* binding and cytotoxicity to human corneal epithelial (HCE) cells were determined for ELP or CPP-ELPs. Corneal binding, clearance, and penetration were assessed in a rabbit model following topical application of the fluorescently labeled proteins by quantitative fluorescence imaging and histology.

Results: ELP bound to HCE cells *in vitro*, and binding/uptake was enhanced 2- to 3-fold by the addition of CPPs. When applied topically to rabbit eyes, ELP accumulated in the cornea at levels 7.4-fold higher than did an equivalent dose of immunoglobulin G. Both ELP and a CPP-ELP penetrated the corneal epithelium and were detectable in the stroma. Addition of CPPs to ELP, however, did not significantly enhance corneal uptake or penetration *in vivo* relative to ELP alone. The polypeptides cleared from the cornea over a period of 20–30 min after application, after which cornea levels reached a steady state of 15–30 µg/mL for up to 3 h.

Conclusions: The ELP drug carrier can penetrate the corneal epithelium and accumulate in the stroma. Given its amenability for fusion to multiple types of therapeutic agents, ELP has the potential to serve as a drug carrier for topical ocular applications.

Introduction

CORNEAL DRUG DELIVERY is hampered by physical barriers and rapid removal of applied therapeutics by the tear film and blinking. The corneal epithelium, a layer 5–7 cells thick¹ connected by tight junctions,² forms an efficient barrier to transport of large or charged therapeutic agents. In addition, dilution by the tear film and physical removal by blinking result in very short residence times for therapeutics applied topically (often as short as 15–30 s, reviewed in Gaudana et al.³). Because of its noninvasiveness and ease of application resulting in high patient compliance, topical drug administration is the desired route for corneal drug delivery. However, the low bioavailability of therapeutics due to these physical barriers often limits the use of this desired route. For example, monoclonal antibodies, which are often used for anterior diseases such as corneal neovascularization and posterior conditions such as diabetic retinopathy or age-related macular degeneration, have very poor penetration when applied topically.⁴ This necessitates the use of more invasive delivery routes, including subconjunctival administration for anterior drug targets, in-

travitreal injection for posterior targets, or very frequent dosing.⁵

The goal of this study was to assess the corneal penetration and corneal residence time of an experimental biopolymer drug carrier. Elastin-like polypeptide (ELP) is a synthetic protein made of repeated units of a 5 amino acid motif, and it has been applied as a drug carrier in several preclinical disease models.^{6–11} ELP is advantageous as a drug delivery vector because it is nonimmunogenic, is easily purified by taking advantage of its unique property of thermally triggered aggregation,^{12–14} and is amenable to fusion with small molecule, peptide, or protein-based therapeutic agents (reviewed in Raucher et al.¹⁵). ELP is produced recombinantly, and its coding sequence is easily modified for fusion of targeting or cell-penetrating peptides (CPPs) and with therapeutic proteins, peptides, or drug conjugation sites using basic molecular biology protocols.^{16–18} Also, ELP's size and thermal aggregation properties are easily altered by changing the number of repeats in the biopolymer or by altering the amino acid composition within the repeated sequence.¹⁹ ELPs have been used extensively for delivery of small molecules and peptides.^{15,20}

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For intracellular targets, ELPs are often modified with CPPs,^{21,22} short and usually positively charged peptides that mediate uptake across the plasma membrane (reviewed in refs.^{23–26}). These peptides are highly efficient for facilitating delivery into cells in culture, but there are fewer studies testing their ability to mediate uptake across the corneal barrier. In a comparison of the Tat, penetratin, low molecular weight protamine, and poly-arginine CPPs, Liu et al. found that all CPPs tested were more permeable across excised corneas than was a control peptide, with penetratin being the most efficient.²⁷ When applied *in vivo* in rats, penetratin accumulated strongly in the corneal epithelium and endothelium and was diffusely localized in the stroma. Tat was shown to deliver acidic fibroblast growth factor into the retina and protect from ischemia–reperfusion injury after topical administration in a rat model.²⁸ A CPP based on nucleolin-binding peptide was found to enter the superficial corneal epithelial cells, but did not penetrate to the corneal stroma after topical application.²⁹ Johnson et al. described a novel peptide for ocular delivery (POD) composed of 4 repeats of an ARKKAAGA motif.³⁰ When applied topically in a mouse model, a fluorescently-labeled POD bound to the cornea, sclera, and dura of the optic nerve. Within the cornea, the POD was localized within the corneal epithelium at early time points and in 50% of the animals was weakly detectable deeper in the cornea 24 h after administration. POD was used to deliver green fluorescent protein (GFP) to the mouse cornea after topical application, where it entered the corneal epithelium, but was not detected in the corneal stroma.³¹ Furthermore, a POD-fused polyethylene glycol nanoparticle was able to bind plasmid DNA and achieve transgene expression in the optic nerve head following subretinal injection.³² These data, along with the studies on conventional CPPs, suggest a potential role for the use of CPPs to enhance uptake or corneal penetration of drugs following topical ocular application.

In the current study, we utilized an ELP carrier modified with a single cysteine residue for cargo (or fluorophore) attachment. Also, similar ELPs fused to the CPPs SynB1³³ or Tat³⁴ were produced. The ability of these CPPs to mediate uptake into human corneal epithelial (HCE) cells was assessed in cell culture. Using a rabbit model, we tested the total corneal uptake, corneal penetration, and corneal residence time after topical administration of each agent. These data indicate that ELP does penetrate the corneal barrier and can deliver a cargo fluorophore into the corneal stroma. The addition of CPPs to ELP, while effective *in vitro*, did not significantly enhance the total corneal levels or corneal penetration. Overall, these data suggest that ELP is a promising drug delivery platform for corneal drug delivery by topical administration, and that addition of CPPs may not be necessary or beneficial.

Methods

Generation and purification of polypeptides

ELP and CPP-ELP expression vectors were generated as previously described.³⁵ Proteins were expressed recombinantly in *Escherichia coli* and purified using inverse transition cycling as in George et al.¹⁴ and Bidwell and Raucher.³⁵

Fluorescent labeling

ELP and CPP-ELPs were labeled on a unique cysteine residue with Alexa Fluor 633 C₅-maleimide or tetramethylrhodamine-5-

maleimide (Life Technologies) as described previously.³⁵ Immunoglobulin G (IgG; Sigma) was reconstituted in 0.1 M sodium bicarbonate buffer (pH 8.3) at a final concentration of 92.5 μ M, and 5/6-carboxy-tetramethylrhodamine succinimidyl ester (Life Technologies) was added to a 10-fold molar excess and incubated at 4°C overnight. Excess unreacted label was removed using an Amicon spin filter with a 3,000 Da molecular weight cutoff. Labeling efficiency was determined spectrophotometrically as described in Bidwell and Raucher.³⁵

Cell culture

Immortalized HCE cells were cultured in the KGM[®]-2 SingleQuots media from Lonza. The cells were maintained at 37°C in a humidified incubator at 5% CO₂.

Flow cytometry

HCE cells were seeded at a density of 3×10^5 cells/well in 6-well tissue culture plates and incubated at 37°C in humidified incubator with 5% CO₂ overnight. The cells were washed and treated with fluorescein-labeled proteins (ELP, SynB1-ELP, and Tat-ELP) at final concentration of 10 μ M and incubated at 37°C in a humidified incubator with 5% CO₂ overnight. At the end of the incubation, the cells were washed with DPBS twice and 500 μ L of nonenzymatic cell dissociation buffer (Mediatech, Inc.) was added in each well followed by addition of 1 mL of Dulbecco's phosphate buffered saline (DPBS). The cell suspension was removed to fresh polystyrene tubes and centrifuged at 400 g. The cell pellets were resuspended in 400 μ L of DPBS and analyzed by flow cytometry (BD Gallios analyzer). The mean fluorescence intensity of 10,000 cells per sample was measured. The fluorescence intensity was corrected for differences in labeling efficiency among the individual proteins, and the data represent the mean $\pm$ standard error of the means (SEM) of 5 independent experiments, each performed in duplicate.

Cell proliferation

HCE cells were seeded at 10,000 cells/well in 96-well plates and incubated at 37°C in humidified incubator with 5% CO₂ overnight.

The cells were treated with proteins (ELP, SynB1-ELP, and Tat-ELP) in 100 μ L volume in complete media at final concentrations of 5, 10, 20, and 40 μ M and incubated for an additional 72 h. Viable cells were detected using the MTS cell proliferation assay (Promega). Data represent the mean $\pm$ SEM of 5 independent experiments, each performed in quadruplicate.

In vivo corneal penetration

All animal studies were approved by the University of Mississippi Medical Center's Animal Care and Use Committee and carried out according to the guidelines specified in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research. New Zealand White rabbits (female, 1–2 kg; Charles River) were used for corneal uptake studies. Twenty-five microliters of a 3% solution of each rhodamine-labeled or Alexa Fluor 633-labeled protein was applied topically to the left eye, and saline control was applied to the right eye in conscious

rabbits using 1 of 2 treatment schedules. The standard treatment schedule involved 3 applications of protein, each 2 h apart, followed by tissue harvest 2 h after the final application. A more frequent treatment regimen was also tested in which protein was applied every 15 min for the first hour, then every 30 min for 5 additional hours, and then eyes were harvested 1 h after the final treatment. At tissue harvest, the rabbits were anesthetized with isoflurane, a blood sample was drawn from an ear vein, the animals were euthanized by isoflurane overdose, and eyes were removed for *ex vivo* analysis and histology.

Ex vivo imaging and quantitative fluorescence analysis

Whole eyes were imaged using an IVIS Spectrum (Caliper; Perkin Elmer) in fluorescence mode with 535 nm excitation and 580 nm emission for detection of rhodamine or 605 nm excitation and 660 nm emission for detection of Alexa Fluor 633, and autoexposure. Fluorescence intensity of the cornea was determined by drawing a region-of-interest (ROI) around each cornea (using the photographic image) and measurement of mean fluorescence radiant efficiency in each ROI with Living Image Software (Caliper). Autofluorescence, determined by measurement of saline-treated eyes, was subtracted, and the data were averaged for all animals in each group. An *n* of 5 rabbits per treatment group was used. To correct for any differences in labeling efficiency among the different proteins, a standard curve of each protein was diluted into a black 96-well plate and imaged with the same imaging parameters as were used for the tissue imaging. The mean corneal fluorescence radiant efficiency was fit to the standard curve for each protein to determine corneal levels.

For detection of systemic protein, direct fluorescence measurements of plasma samples were made using a fluorescence plate reader and a Nanoquant™ plate (Tecan) using 610 nm excitation, 660 nm emission to detect the Alexa Fluor 633 labeled protein, and a gain setting of 170.

Histology and fluorescence microscopy

Eyes were flash frozen in liquid nitrogen, embedded in a freezing medium, and cut into 20 μ m sections in the sagittal plane using a cryomicrotome. Slides were scanned using a fluorescence slide scanner with a 543 nm laser and equal laser power and photomultiplier tube voltage were used for all scans. After scanning, sections were fixed with 4% paraformaldehyde for 10 min, stained with 4',6-diamidino-2-phenylindole (DAPI, 2.5 μ g/mL) for 5 min, and mounted with the Antifade mounting medium. Images were collected using an epifluorescence microscope (Nikon) and MetaMorph software (Molecular Devices). Equal exposures were used for all protein images.

In vivo corneal clearance

Adult Dutch-belted rabbits (female, 2–3 kg) were used for *in vivo* imaging because the dark fur around their eyes reduced autofluorescence. The rabbits were sedated with acepromazine (3 mg i.m.) and then anesthetized with isoflurane. Each rabbit was immediately transferred to the Xenogen IVIS fluorescent imaging system, where anesthesia was continuously maintained with 1.25–2% isoflurane. With

the animal lying on its right side, the lids of the left eye were retracted with a 1- $\frac{3}{4}$ " Barraquer eye speculum (Integra). Before application of the protein, autofluorescence was recorded by imaging the eye with the 535 nm excitation and 580 nm emission filters. Rhodamine-labeled proteins (ELP and SynB1-ELP) were applied directly on the left eye with a total volume of 25 μ L and a final concentration of 3%. Fluorescence imaging was measured at times 0, 1, 5, 20, 15, 20, 25, 30, 60, 120, and 180 min after application of the protein to the left eye. The Barraquer eye speculum was removed between time points for imaging, to allow the eye to remain moist and viable. Image analysis was performed with Living Image 4.3.1 software (Caliper Life Sciences). Mean fluorescence intensity was calculated inside a ROI encompassing only the cornea and reported in units of radiant efficiency. A standard curve of known concentrations of each labeled protein in a 96-well plate was imaged with identical imaging parameters, and the mean corneal fluorescence intensity at each time point was fit to the standard curve. The experiment was performed thrice for each protein. The same rabbit was used for all 3 trials (1 rabbit used per protein), and at least 48 h was allowed between each trial to ensure no fluorescent protein remained. The data represent the mean $\pm$ SEM of 3 independent experiments for each protein. Clearance data were fit with a single exponential decay using GraphPad Prism.

Statistical analysis

Differences between protein uptake in HCE cells were compared with a 1-way analysis of variance (ANOVA) and a *post-hoc* Bonferroni multiple comparison. Cell viability was analyzed using a 2-way ANOVA for factors of protein treatment and concentration, and a Bonferroni multiple comparison was used to assess statistical significance of group differences. Corneal protein levels were compared using a 1-way ANOVA with a *post hoc* Bonferroni multiple comparison or a Student's *t*-test as appropriate. A *P*-value of <0.05 was considered statistically significant for all studies. All statistics were performed using GraphPad Prism.

Results

Polypeptide uptake and toxicity in HCE cells

CPPs have been used to deliver many types of cargo into multiple cell types. The efficiency of plasma membrane transport varies for various CPPs, and it is dependent on the cargo and the cell type.³⁶ Previous studies have used CPPs to deliver ELP and ELP carrying many types of cargo to cancer cell lines.^{21,22} To determine whether CPPs could mediate uptake of ELP in primary HCE cells, HCE cells were exposed to fluorescently labeled proteins, and their cellular levels were determined 24 h after exposure by flow cytometry. As shown in Fig. 1A, the unmodified ELP was detectable in HCE cells at levels much higher than autofluorescence. When ELP was modified with the SynB1 or Tat CPPs, the cellular levels were increased 2.3- or 3-fold, respectively (*P*=0.029, 1-way ANOVA). We also determined whether the ELP or CPP-ELPs had any effect on HCE cell proliferation. Previous studies have demonstrated cytotoxicity from some CPP-ELPs,^{22,37} and the level of toxicity is highly CPP and cell line dependent. When HCE cells were exposed to varying concentrations of ELP or of

FIG. 1. Protein uptake and toxicity in corneal epithelial cells. **(A)** human corneal epithelial (HCE) cells were exposed to 10 μ M fluorescently labeled elastin-like polypeptide (ELP), SynB1-ELP, or Tat-ELP. After a 24-h continuous exposure, protein levels were determined by flow cytometry. **(B)** HCE cells were exposed to the indicated concentration of ELP, SynB1-ELP, or Tat-ELP for 72 h. Cell survival was assessed using the MTS cell viability assay. Bars, standard error of the means (SEM) * $P < 0.05$ relative to ELP control, 1-way analysis of variance (ANOVA) **(A)** or 2-way ANOVA for factors of protein treatment and concentration **(B)** with *post hoc* Bonferroni multiple comparisons.

SynB1-ELP, no effect was observed on cell proliferation or survival (Fig. 1B). However, when exposed to Tat-ELP, a concentration-dependent reduction in cell number was observed. These results are consistent with previous studies in which Tat-ELP was found to be cytotoxic.^{22,37} These data indicate that ELP and SynB1-ELP enter corneal epithelial cells *in vitro* and are nontoxic.

Corneal uptake and penetration of ELPs

To determine whether ELP and CPP-ELPs accumulate in the cornea *in vivo*, a rabbit model was used for topical administration of the proteins. ELP, SynB1-ELP, and Tat-ELP were fluorescently labeled and administered topically at

times 0, 2, and 4 h. Also, given the use of monoclonal antibodies for ocular neovascularization disorders and the known limitations of corneal penetration for topical antibody use, we compared ELP and CPP-ELP corneal accumulation to that of IgG (as a surrogate for a therapeutic monoclonal antibody). Two hours after the final topical application, rabbits were sacrificed and eyes were removed for *ex vivo* imaging. Standard curves of each agent were also imaged, and fluorescence data were fit to these standard curves to correct for any differences in labeling levels among the proteins. As shown in Fig. 2A, all proteins were detectable in the eye above autofluorescence levels following this administration protocol. More protein was apparent in the sclera than in the cornea. Corneal IgG levels were

FIG. 2. Ocular accumulation of ELPs relative to immunoglobulin G (IgG). **(A)** A 3% solution of fluorescently labeled ELP, SynB1-ELP, Tat-ELP, or a nonspecific IgG was applied topically to rabbit eyes thrice over 6 h. Two hours after the final administration, the eyes were removed and examined by *ex vivo* quantitative fluorescence to determine polypeptide levels. **(B)** Corneal levels of each protein were determined from images in **(A)** by measuring fluorescence intensity and fitting to a standard curve of the labeled protein. Bars, SEM * $P < 0.05$, 1-way ANOVA with *post hoc* Bonferroni multiple comparisons. Color images available online at www.liebertpub.com/jop

FIG. 3. Corneal penetration of ELP or SynB1-ELP. Rabbit eyes were harvested and rapidly frozen after exposure to 3 topical applications of 3% solutions of labeled proteins over a period of 6 h. Eyes were cut into sagittal sections using a cryomicrotome, and sections were stained with 4',6-diamidino-2-phenylindole (DAPI) to mark cell nuclei. Images were collected with a fluorescence microscope with a 20× magnification objective. All images were collected with identical exposure times. Color images available online at www.liebertpub.com/jop

very low (Fig. 2B). In contrast, ELP levels in the cornea were 7.4-fold higher than IgG, despite being administered at the same dose. SynB1-ELP levels were also significantly increased relative to IgG, but the addition of the SynB1 or Tat CPPs did not enhance ELP corneal accumulation over the unmodified ELP.

In addition to measuring total corneal uptake by *ex vivo* imaging, we also assessed the ability of ELP and SynB1-ELP to penetrate the corneal epithelium and enter the corneal stroma. Rabbits were treated with fluorescently labeled ELP or SynB1-ELP applied topically for 3 applications, each 2 h apart, as described above. Two hours after the final application, the rabbits were sacrificed, and the eyes were rapidly frozen and sectioned. DAPI was used to stain cell nuclei and clearly indicate the epithelial layer. Both ELP and SynB1-ELP were detectable at very high levels in the corneal epithelium (Fig. 3). Also, significant amounts of both proteins traversed the epithelial layer and were detectable in the corneal stroma. Although the epithelial layer was strongly autofluorescent with this filter pair (top panel in Fig. 3), levels of ELP and SynB1-ELP (pseudocolored green in Fig. 3 for image clarity) were increased in both the epithelium and the stroma relative to autofluorescence controls (quantified in Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/jop). Consistent with the total protein levels seen in the previous experiment, no increase in epithelial or stromal levels was apparent in the SynB1-ELP-treated eyes relative to the unmodified ELP-treated eyes (Supplementary Fig. S1).

In an attempt to determine whether corneal levels could be increased with more frequent protein applications and the SynB1 peptide could enhance uptake following a more in-

tensive treatment regimen, rabbits were administered the proteins every 15 min for the first hour and then every 30 min for 5 additional hours. One hour after the final administration, the eyes were removed and the *ex vivo* studies were carried out as described above. As shown in Fig. 4A and quantified in Fig. 4B, more frequent administration did not enhance the ELP levels relative to the 3-dose treatment schedule. Also, the increase in corneal SynB1-ELP levels was not statistically significant relative to the unmodified ELP (Student's *t*-test). Eyes were again frozen and cryo-sectioned and slides were scanned using a fluorescence slide scanner to determine the protein distribution throughout the eye. High levels of ELP and SynB1-ELP were apparent in the corneal epithelium and stroma, and the proteins also accumulated all around the eye, including in the sclera and retina (Fig. 4C). Blood was also sampled from each rabbit before euthanasia to determine whether the proteins reached the systemic circulation. As shown in Supplementary Fig. S2, no ELP or SynB1-ELP was detectable above the autofluorescence background by direct fluorescence measurement in plasma. These data indicate that there is no benefit from adding a cell-penetrating peptide in terms of total protein accumulation. However, the ELP drug carrier does accumulate throughout the eye and penetrate the corneal epithelium, indicating its promise as an ocular drug carrier.

In vivo corneal surface clearance

To determine the clearance rate of topically applied ELP on the cornea, a live rabbit *in vivo* imaging assay was used. Rhodamine-labeled ELP or SynB1-ELP (3%) was applied in a single topical 25 μ L drop, and the eye was imaged at

FIG. 4. Ocular delivery of ELPs following increased application frequency. A 3% solution of fluorescently labeled ELP or SynB1-ELP was applied topically to rabbit eyes every 15 min for 1 h and then every 30 min for 5 additional hours. One hour after the final administration, the eyes were removed and examined by *ex vivo* quantitative fluorescence imaging (**A**) to determine polypeptide levels. (**B**) Corneal fluorescence was quantified relative to a standard curve of each protein. (**C**) Eyes from each treatment group were frozen, sagittally sectioned, and scanned using a fluorescence slide scanner to determine polypeptide distribution throughout the eye. Color images available online at www.liebertpub.com/jop

multiple time points after application. As shown in Fig. 5A, immediately after application, the polypeptides were detectable all over the eye and began to pool around the retracted lids. Over time, the applied proteins continued to wash from the cornea, and the total eye levels decreased as the proteins were eliminated in the tear film. Quantitation of corneal levels (Fig. 5B) revealed that immediately after application, ELP levels in the cornea or overlying tear film were nearly 4-fold higher than SynB1-ELP levels. Both proteins cleared from the cornea and tear film over a period of 15–20 min. Single-exponential decay revealed a corneal half-life of 3.3 min for ELP and 2.6 min for SynB1-ELP (not significantly different). After 20 min, corneal levels stabilized and both proteins were still detectable over autofluorescence. An average level of 25.3 ± 9.3 μg/mL ELP and 18.1 ± 4.8 μg/mL SynB1-ELP remained until at least 3 h after administration (no significant difference between proteins, inset graph, Fig. 5B). It is possible that this remaining fluorescence represents protein that has internalized into the corneal epithelium or penetrated to the stroma and is protected from removal in the tear film.

Discussion

These data indicate that the ELP drug carrier is a promising vector for ocular drug delivery. Topically applied ELP accumulated throughout the outer surface of the eye, including in the cornea, sclera, and retina, and in the cornea, the protein penetrated through the epithelial layer and into the stroma. Promisingly, topically applied ELP accumulated in the cornea at levels 7.4-fold higher than an equal dose of

IgG. These results likely reflect the smaller size of ELP relative to full-length monoclonal antibodies as well as its lack of charge. The ELP used in this study has a molecular weight of 61,275.8 g/mol and contains no charged amino acid residues. The protein is made up of 48.6% glycine, 21% valine, 20% proline, and 9.9% alanine (with only a few other amino acids), and it is likely that this lack of any charged amino acids facilitates its penetration through the plasma membranes of the corneal epithelium. Given these favorable features for penetration of ocular barriers, it may be possible to further enhance ELP-mediated ocular delivery by decreasing the size of the biopolymer. Due to the repeated nature of the core ELP sequence and the ease of modifying its DNA coding sequence, changing the size of ELP is easily achieved by increasing or decreasing the number of pentapeptide repeats. We are currently evaluating several ELP sizes to determine if ocular penetration is size dependent and whether we can optimize ELP-mediated delivery by decreasing the ELP size.

In contrast to previous studies with other cargo, the addition of CPPs to ELP did not further enhance its ocular uptake or penetration. This may be due to the already high ocular accumulation of ELP. Previous studies either compared CPP-mediated small molecule delivery to controls of the small molecule alone or used CPPs to enhance the ocular delivery of more hydrophilic cargo such as GFP, fibroblast growth factor, or polyethylene glycol-coated nanoparticles. In these cases, it is logical that the cell-penetrating nature of CPPs is beneficial for increasing the ocular uptake. However, in the case of the much less hydrophilic ELP, which already has good optical penetration properties, the addition

FIG. 5. Clearance of ELPs from the cornea and tear film *in vivo*. **(A)** 25 μ L of a 3% solution of fluorescently labeled ELP or SynB1-ELP was applied topically to the left eye. Immediately following administration of the protein, fluorescence intensity was measured at the indicated times. Mean fluorescence was calculated using a pre-determined region-of-interest, which was restricted to the pupil and iris. **(B)** Corneal levels were calculated by fitting the mean fluorescence intensity to a standard curve of the administered protein. The line represents the fit of a single-exponential decay, and the error bars represent the standard deviation of 3 independent trials per agent. Color images available online at www.liebertpub.com/jop

of CPPs likely cannot further enhance the uptake across ocular membranes. It is also clear that *in vitro* cellular uptake in cultured cells is not a good predictor of *in vivo* penetration as CPPs did enhance ELP uptake into corneal epithelial cells in culture, but had no additional benefit to increase penetration *in vivo*.

These studies lay the groundwork for the use of ELP to deliver topically applied therapeutics for ocular disorders. Due to the simplicity of modifying ELP to add drug-binding domains or to make chimeric fusions with therapeutic peptides and proteins,^{14,15} this system has great potential for noninvasive therapy for ocular disorders. We are currently developing ELP-delivered antiangiogenic agents. Given the good penetration of topically applied ELP in both the cornea and retina, these agents have promise for treatment of corneal neovascularization and retinal neovascular disorders, including macular degeneration. It may also be possible to use ELP to deliver antibacterial, antiviral, or antifungal agents to treat various ocular infections. These represent only a small sample of the potential for this system. Given the ease of modifying ELP for attachment of therapeutics, the convenience of ELP-mediated purification of these therapeutic agents and the good optical biodistribution achieved after topical ELP application, this system has

great potential on which to build novel ocular therapeutic platforms.

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Author Disclosure Statement

G.L.B. is the owner of Leflore Technologies LLC, a private company working to commercialize novel therapeutics for several disease applications. G.L.B. and E.M.G. are authors of patents related to the described research.

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