Effective In Vivo Topical Delivery of siRNA and Gene Silencing in Intact Corneal Epithelium Using a Modified Cell-Penetrating Peptide

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INTRODUCTION

The eye, and in particular the ocular surface, is one of the most accessible sites for local drug treatment, allowing direct application without the need for systemic administration. This, taken together with the fact that the area to be treated is small and the success of any treatment is easily monitored, makes topical drug delivery an attractive option for ophthalmology. In addition, an immune-privileged status of the cornea is sought, large hydrophilic oligonucleotides represent a unique and effective option for ophthalmology. However, despite its unique attributes, drug delivery through and to the cornea, which represents one of the main components of the ocular surface, has proved to be challenging.1,4 The main obstacles to achieving a therapeutic dose, by the diffusion of a drug through the eye surface, are the protective mechanisms and underlying ocular anatomy.1,2 The cornea is a tear film-covered, 500-μm-deep tissue composed of three layers, from anterior to posterior, the epithelium, stroma, and endothelium, separated by Bowman’s layer and Descemet’s membrane, respectively. A drug may be eliminated from the ocular surface by various mechanisms including lacrimation and tear turnover, drug metabolism, and preferential conjunctival absorption.4,6 The corneal epithelium is a non-keratinized, stratified squamous epithelium, approximately five to six cell layers thick, joined together by tight junctions. This, together with the tear film, make the cornea a difficult barrier to overcome, and when the desired target is other parts of the eye, such as the retina, bypass of the cornea by direct injection of the drug (e.g., intravitreal injection) is often the preferred pathway to delivery in the clinic.5,7

Although methods such as intrastromal injection7 and iontophoresis8,9 have been shown to be successful for delivery to the cornea, the development of a user-friendly, non-invasive method such as self-administered eye drops has proved challenging.10-12 Drugs and delivery agents need to be able to overcome the tear barrier and remain associated with the corneal epithelium for the time necessary to allow cellular internalization. Subsequently, once internalized into the target cell, the drug cargo must be released to function within the cell with optimal bioavailability.

Among the different drugs for which delivery to the front of the eye is sought, large hydrophilic oligonucleotides represent a unique and...
effective approach for selective gene therapy treatment of a wide spectrum of corneal diseases.13–15 In particular, small interfering RNA (siRNA)-induced gene silencing has been shown to hold great potential for the treatment of different ocular pathologies, reaching phase II and III clinical trials for glaucoma and dry eye,2,16 and more recently for corneal pathologies13,15 such as corneal dystrophies (CDs). CDs represent a spectrum of eye diseases associated with one or more different layers of the cornea, affecting its shape and transparency, and in some cases, leading to a partial or complete loss of vision.17 Corneal transplantation is the only intervention that can be used currently in the case of a damaged cornea. CDs are frequently caused by missense mutations or small in-frame insertions or deletions,18 and therefore stable gene editing and transient gene silencing are promising tools for a gene therapy approach.

To investigate this further, our group previously developed promising siRNA molecules for a personalized therapeutic approach for CDs.19–23 siRNAs that can be used for a transient, reversible, and dosage-variable treatment were developed that were highly specific with single-nucleotide discrimination at the mutation site.26,27

Due to the short half-life of siRNA molecules, a daily reversible and dosage-variable treatment regimen by non-invasive topical delivery is necessary. However, effective delivery remains a challenge, and presently no published research reports significant siRNA delivery to an intact corneal epithelium.11,28

In addition to overcoming pre-corneal tear film turnover and the other protective mechanisms described, it is necessary to promote cellular uptake of siRNA through the cellular membranes of the epithelial cells and increase corneal bioavailability. Shielding the negatively charged siRNA with positively charged delivery molecules represents a promising option.29 A positively charged formulation can, in the first instance, interact with the negatively charged ocular components, such as the epithelial cell membranes and the external mucus, in order to increase the persistence of the drug on the eye surface,30 and then mediate cellular uptake.

Cationic polymers have been extensively used for drug delivery, in particular of nucleic acids. Examples of these polymers include polyethyleneimine (PEI), polyamidoamine dendrimers (pAMAMs), and chitosan, which are routinely used for cell transfection in vitro and in vivo.31 Different cationic polymers may be used to deliver oligonucleotides to the eye surface,32 and among these, cationic cell-penetrating peptides (CPPs) are very versatile and promising.33 Their positive charges can be exploited both to generate anionic interaction with the negatively charged siRNA and to drive ocular penetration, and when compared with other nanoparticles, CPPs have the advantage of forming nanoparticles by simply mixing the peptide with siRNA in aqueous solution. CPPs have been used extensively to deliver various macromolecules,34 including siRNA,35,36 to cells both in vitro and in vivo. They can be easily modified by the addition of chemical blocks to address different delivery hurdles; for example, lipid moieties (such as palmitoyl- and cholesteryl-) may be added in order to increase the hydrophobicity of CPPs, and thus favor the destabilization of the endosomal membrane. CPPs can also be used together with other molecules involved in delivery, offering a wide range of potential combinations. Moreover, in contrast with other cationic polymers, CPPs are well-defined chemical entities, allowing better control of the CPPs/siRNA molar ratio.

CPPs, such as POD37,38 and PEP-1,39 have demonstrated the ability to penetrate the corneal tissues when applied topically to the eye. Herein, we present the development of a modified POD for corneal delivery of siRNA that overcomes poor endosomal escape (when siRNA remains trapped in endosomes and is trafficked into lysosomes, where it is degraded).34,35,40 In this study, chloroquine (Chlq)41 was first applied together with the polyplexes (i.e., nucleotide-peptide complexes) to elicit siRNA release from the endosomes, in order to confirm the endosomal entrapment and to determine whether a lysogenic compound was able to enhance siRNA release in vitro and in vivo. Subsequently, the combination that showed the best delivery properties when tested in vitro and in vivo was selected and covalently modified with a Chlq analog. A corneal epithelium cell line was used as an in vitro model, whereas in vivo experiments were performed on a novel murine model expressing, under the regulation of the corneal-specific Krt12 promoter, the luciferase gene and Meesmann epithelial CD mutations.

This covalently modified peptide, once topically applied on the eye surface, proved capable of delivering bioavailable siRNA into corneal epithelial cells, allowed effective release of the siRNA from the endosomes, and achieved significant knockdown of gene expression.

RESULTS

Evaluation of In Vitro siRNA Delivery Using Modified Versions of Peptide for Ocular Delivery (POD)

To improve the delivery and bioavailability of siRNA by the POD peptide, novel chemical modifications were introduced (Table 2). To minimize the number of possible candidates to be tested in vivo with the mouse corneal reporter model, the different modified versions of POD were first tested to determine their delivery activity in vitro in a corneal epithelial cell model.43

Initially, POD was modified with either a palmitoyl group, a cholesterol group, or poly(lactic-co-glycolic acid) (PLGA)-PEG and tested for its ability to deliver siRNA in corneal epithelial cells. POD was functionalized with a palmitoyl- or a cholesterol- group because these modifications, increasing the ability of a peptide to fuse with the plasma membrane, were previously shown to enhance the performance of other siRNA-delivering peptides.34,45 PLGA-PEG-POD was selected for this study because PEG-POD was reported to efficiently deliver nucleotides in vivo, whereas the addition of PLGA was shown to enhance the capability of PEG-POD to penetrate corneal tissues.37,46

Evaluation of POD-siRNA Complexes

A molar ratio of 35:1 PODs/siRNA was used to determine the POD-siRNA complex formation in a gel retardation assay. This molar
ratio was chosen because molar ratios between 30:1 and 50:1 have been demonstrated previously to result in the maximum incorporation of siRNA into complexes for other CPPs. Moreover, a molar ratio of 25:1 was shown to achieve efficient knockdown of GFP expression in transiently transfected cells, whereas Vasoncelos et al. determined the cell viability in cells treated with the PLGA-PEG-POD to be higher than 60% at a concentration of 2.5 mg/mL (herein we used 1.4 mg/mL PLGA-PEG-POD). The size and ζ-potential of each POD-siRNA complex was therefore determined. The analysis showed that the particles from each formulation in PBS had mean diameters and mean charges (Table 1) that fall within the described parameters and are thus suitable for cell delivery. The analysis was also performed in water to assess whether a buffered pH may have an effect on the properties of the complexes (data not shown). However, only a minimal reduction of the charge and dimension was observed when prepared in PBS, which was used from this point to prepare the other formulations described in this study.

Evaluation of siRNA Cellular Delivery

A human epithelial corneal epithelial cell line (HCE-S) was used for initial in vitro screening in order to evaluate cellular transfection and not escape from the wells; this POD/siRNA ratio was used for all further experiments.

PODs ζ-Potential and Size

CPP-siRNA complexes that have the highest rate of endocytic uptake and tissue diffusion are generally smaller than 200 nm and have a positive ζ-potential (generally lower than +40 mV) in aqueous solution. The size and ζ-potential of each POD-siRNA complex was therefore determined. The analysis showed that the particles from each formulation in PBS had mean diameters and mean charges (Table 1) that fall within the described parameters and are thus suitable for cell delivery. The analysis was also performed in water to assess whether a buffered pH may have an effect on the properties of the complexes (data not shown). However, only a minimal reduction of the charge and dimension was observed when prepared in PBS, which was used from this point to prepare the other formulations described in this study.

Evaluation of siRNA Cellular Delivery

A human epithelial corneal epithelial cell line (HCE-S) was used for initial in vitro screening in order to evaluate cellular transfection and
toxicity properties of the POD-siRNA complexes and to reduce the number of animals needed for the subsequent in vivo analysis. Although corneal epithelial cell lines have molecular features that differ from the original epithelium and might respond differently to the treatment if grown in different culture conditions, they represent a valid cellular model of the cornea to initially investigate cellular transfection and toxicity. Delivery of a green fluorescently labeled nontargeting siRNA (siGLO) into HCE-S cells by each of the different PODs was tested. The majority of HCE-S cells, transfected with all three different POD-siRNA formulations, showed punctuate cytoplasmic fluorescence (Figure 1B) with a perinuclear concentration (nuclei stained blue, DAPI), in agreement with previous observations in corneal epithelial cells of a rabbit cornea treated with the commercially available cationic lipid transfection agent RNAiMAX (Figure 1B). The perinuclear distribution pattern of the POD-delivered siGLO is detectable in cells treated with non-complexed siGLO (Figure 1B). No intracellular fluorescent dots, larger than those observed using the commercially available cationic lipid transfection agent RNAiMAX (Figure 1B). The perinuclear distribution pattern of the POD-delivered siGLO is characterized by fluorescent dots, larger than those observed using the commercially available cationic lipid transfection agent RNAiMAX (Figure 1B). The perinuclear distribution pattern of the POD-delivered siGLO suggests that the nanoparticles are internalized along an endocytic pathway.27,54,55

### Evaluation of POD Cellular Toxicity

siRNAs used herein were previously reported not to elicit any toxicity or immunological response in HCE-S cells. The cellular toxicity of POD-siRNA complexes was assessed by measuring cell viability using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in HCE-S cells treated for 24 h with PODs at different concentrations (Figure 1C). Under these conditions, cells treated with Chol- and Palm-POD showed, when compared with the untreated control, an ~87% and 75% cell viability with 17.5 and 35 μM POD that fell to ~55% at 70 μM. PLGA-PEG-POD was instead showing ~100% viability. Similar results were obtained for the POD-siRNA comparison, showing that the presence of Chol-, Palm-, and PLGA-PEG groups did not elicit cellular toxicity.

### Evaluation of Gene Knockdown

The 35:1 PODs/siRNA ratio, determined by a gel retardation assay to be sufficient for complexing all of the siRNA, was then used to assess knockdown of luciferase reporter gene expression in HCE-S cells using a dual-luciferase assay, as previously described.22,26 No significant knockdown was observed when cells were transfected with luciferase targeting siRNA (siLuc) complexed with any of the four different PODs (Figure 1D, black bars) compared with PODs complexed with non-targeting siRNA (NSC4) (Figure 1D, gray bars), whereas knockdown was achieved in the positive control using RNAiMAX (p < 0.001). The results obtained are consistent with those previously observed in studies with other CPPs where, even in the case of strong cell association, no significant gene expression knockdown was measured.47

### Evaluation of Endosomal Release

Because all of the PODs tested were able to deliver siRNA in vitro but failed to knock down luciferase expression, we hypothesized that the siRNA-peptide complexes were entrapped in endosomes, a well-known cellular barrier that prevents the cytosolic release of siRNAs.56-58 To test this hypothesis, we repeated assessment of delivery and knockdown, treating the cells with the POD-siRNA formulations together with Chlq,41 reported to increase endosomal escape. This should result in a release of siRNA to the cytoplasm, where it can target mRNA and be observed as reduction in luciferase reporter expression.

In combination with Chlq, POD-siRNA complexes were observed in a more diffuse cytoplasmic pattern (Figure 2A) when compared with the same complexes without Chlq (Figure 1B), and achieved a significant knockdown of luciferase expression (Figure 2B). Based on these in vitro analyses, Chol- and Palm-POD were selected for the subsequent in vivo experiments. In further experiments, these two PODs were tested to identify which demonstrated the best in vivo delivery and might thus be covalently modified with an endosomal disruptor. Direct derivatization of a POD is sought to maximize endosomal release while minimizing corneal toxicity. PLGA-PEG-POD was excluded from this in vivo comparison because its chemical and structural features made it unsuitable for any further chemical derivatization.

### Corneal Delivery and Knockdown of Luciferase Expression by Intrastromal Injection of Accell-siRNA

Previous in vivo studies have demonstrated that direct intradermal injection of siRNA can: (1) specifically silence co-injected target alleles in murine epidermis,27,59,60 (2) silence expression of epidermal reporter transgene,61 and (3) show efficacy in a phase Ia clinical trial.62 "Pressure-fecion" intrastromal injection of plasmid into the corneal stroma has been shown to result in GFP expression in all layers of the cornea.63,64 To determine whether the siLuc siRNA was able to knock down luciferase expression in the cornea of the Krt12-/luc2 mice, we first sought to demonstrate that intrastromal injection can deliver siRNA to all layers of the murine cornea. Live animal imaging was performed following intrastromal injection of Cy3-labeled Accell siRNA, a nuclease-resistant siRNA with “self-delivery” properties.27 Strong fluorescent signals were observed in the mouse eye for up to 72 h following injection; however, the signal was most intense at 6 h post-injection (Figure 3A). Fluorescence microscopy showed that Cy3-labeled Accell siRNA localized to the corneal epithelium and stroma after the initial injection, with pronounced expression in all layers of the cornea.63,64 To determine whether the siLuc siRNA was able to knock down luciferase expression in the cornea of the Krt12-/luc2 mice, we first sought to demonstrate that intrastromal injection can deliver siRNA to all layers of the murine cornea. Live animal imaging was performed following intrastromal injection of Cy3-labeled Accell siRNA, a nuclease-resistant siRNA with “self-delivery” properties.27 Strong fluorescent signals were observed in the mouse eye for up to 72 h following injection; however, the signal was most intense at 6 h post-injection (Figure 3A). Fluorescence microscopy showed that Cy3-labeled Accell siRNA localized to the corneal epithelium and stroma after the initial injection, with pronounced expression in all layers of the cornea.63,64 To determine whether the siLuc siRNA was able to knock down luciferase expression in the cornea of the Krt12-/luc2 mice, we first sought to demonstrate that intrastromal injection can deliver siRNA to all layers of the murine cornea. Live animal imaging was performed following intrastromal injection of Cy3-labeled Accell siRNA, a nuclease-resistant siRNA with “self-delivery” properties.27 Strong fluorescent signals were observed in the mouse eye for up to 72 h following injection; however, the signal was most intense at 6 h post-injection (Figure 3A). Fluorescence microscopy showed that Cy3-labeled Accell siRNA localized to the corneal epithelium and stroma after the initial injection, with pronounced expression in all layers of the cornea.
12 h. These findings confirmed that intrastromal injection results in siRNA delivery to the corneal epithelium and suggest that the retention times should be sufficient to study siRNA gene silencing in living Krt12+/luc2 mice.

In order to assess the ability of the described siRNAs to knock down the expression of the luciferase gene in vivo, intrastromal injections of Accell-modified siRNA in mice expressing luciferase in the cornea were performed.

Before in vivo treatment experiments began, corneal luciferase activity in Krt12+/luc2 mice was quantified every 24 h for 3 days to confirm a consistent right-to-left ratio. Accell control (Accell-NSC4) or luc2 siRNAs (Accell-siLuc) were delivered by intrastromal injection (n = 3 mice/group), and corneal epithelial luciferase expression was evaluated daily by live animal imaging over 7 days (Figure 3C). Accell-siLuc inhibited luciferase expression in vivo, with >50% repression achieved 72 h post-injection. Maximal inhibition (64%) was observed at day 5, and silencing persisted at day 6 (Figure 3D); data were statistically significant (p < 0.05) for both time points. Importantly, intrasomal injection of non-targeting Accell-NSC4 had no significant effect (Figure 3D).

In parallel experiments, no significant knockdown of expression was observed when luciferase expression was measured following topical application of Accell siRNA (data not shown). Therefore, to investigate whether siRNA-POD polyplexes can mediate knockdown of corneal gene expression following topical application, we chose to use the siLuc siRNA that we had proved to knock down luciferase expression in vivo by intrastromal injection. However, we combined PODs with native and not Accell-modified siRNA because it is not known whether this modification interferes in the peptide-siRNA interaction and CPP-mediated delivery.

In Vivo Evaluation of Topical Delivery of POD-siRNA Complexes to the Cornea Using a Fluorescent siRNA

Following the demonstration that successful delivery and gene knockdown in vitro were facilitated by the addition of Chlq, the two modified versions of the POD (Palm-POD and Chol-POD) were assessed for in vivo delivery of siRNA. PODs were first complexed with red fluorescent siGLO at the same molar ratios used for in vitro delivery and applied topically to the eye surface of wild-type mice, and fluorescence was monitored for up to 24 h. All of the eyes treated with siGLO in combination with a POD showed fluorescence up to 24 h, whereas the siGLO-only-treated eyes did not show any visible fluorescence (Figure 4A). At 3 and 6 h after application, fluorescence signals from siGLO-Palm-POD and of siGLO-Chol-POD were significantly higher than the siGLO-only control. siGLO-Palm-POD fluorescence was between three and four times more intense than the one measured for Chol-POD (Figure 4B).

Twenty-four hours after the application of POD-siRNA, the eyes were collected and distribution of the siRNA throughout the cornea observed. Red fluorescence was detected in all of the treated sections.
throughout the corneal layers, particularly the corneal epithelium, whereas no fluorescence above background was observed in the siGLO-only control (Figure 4C).

Because PODs alone are not sufficient to deliver siRNA into the cytoplasm, with the siRNA-POD complexes probably retained into the endosomes, and Chlq has been proved to elicit endosomal escape in vitro, but is known to result in in vivo toxicity, we decided to covalently modify POD with an analog of Chlq that shows low toxicity. We also chose to use Palm-POD rather than Chol-POD because it showed better delivery of siGLO in vivo (Figure 4B).

**Evaluation of In Vitro and In Vivo siRNA Delivery Using a Palmitoylated Version of POD Functionalized with Chlq**

An analog of Chlq, trifluoromethylquinoline (QN), was selected to covalently functionalize Palm-POD because a peptide previously developed for siRNA delivery was similarly derivatized and showed successful in vivo delivery with low toxicity (Figure 5A).

**Evaluation of PODs-siRNA Complexes**

To assess whether the functionalization of Palm-POD with QN altered the ability of the POD to bind siRNA and to determine the optimum ratio of QN-Palm-POD/siRNA for POD-siRNA complex formation, QN-Palm-POD was titrated at different molar ratios with siRNA. Although partial complexation of siRNA was observed at 35:1 and 70:1 (with slightly more siRNA bound to the POD at a 70:1 molar ratio), a 140:1 molar ratio achieved complete siRNA complexation (Figure 5B). This result suggests that the presence of the covalently attached QN molecule reduces the ability of the peptide to interact with siRNA by approximately 75% because a 35:1 molar ratio of Palm-POD/siRNA was sufficient to achieve complete complexation (Figure 1A).

**QN-Palm-POD ζ-Potential and Size**

Biophysical analysis of the QN-Palm-POD-siRNA complexes (140:1 ratio) showed that they had a mean diameter (±SD) of 107.1 ± 3.2 nm and a mean charge (±SD) of +14.9 ± 4.2 mV in PBS (Table 2). Thus, the presence of the QN groups does not have an effect on the charge of complexes formed in PBS (14 mV), and although the dimensions of the QN-Palm-POD complexes are reduced compared with Palm-POD in PBS (142 to 107 nm), these values remain in the range suitable for cellular delivery (as described earlier in PODs ζ-Potential and Size).
Evaluation of QN-Palm-POD Cellular Toxicity

Cellular toxicity of QN-Palm-POD-siRNA in HCE-S cells treated with POD concentrations of 9, 17.5, and 35 μM (140:1 molar ratio) was measured with an MTT assay (Figure 5C). Cell viability was reduced to ~80% in cells treated with 9, 17.5, and 35 μM QN-Palm-POD when compared with untreated cells. Thus, to achieve complete complexation of siRNA (140:1 molar ratio) while minimizing cellular toxicity, the concentrations used for the transfection experiments of QN-Palm-POD and siGLO/siLuc were reduced to 17.5 μM and 125 nM, respectively.

Evaluation of Knockdown of Reporter Gene Expression

Knockdown of luciferase reporter gene expression was assessed in HCE-S using QN-Palm-POD:siLuc complexes. Seventy-two hours after transfection, a 50% knockdown of luciferase expression (p < 0.05) was observed. Although addition of 30 μM Chlq to the QN-Palm-POD:siLuc complex-transfected cells increased knockdown of luciferase gene expression to 62% (p < 0.01), this was not significantly higher than with the QN-Palm-POD:siLuc complex alone (Figure 5E).

In Vivo Evaluation of QN-Palm-POD-siRNA Complex Delivery to the Cornea

To assess the ability of the QN-functionalized POD to deliver siRNA in vivo, we measured fluorescence as described above using a 140:1 molar ratio (700 μM QN-Palm-POD and 5 μM siGLO), following topical application of siGLO-QN-Palm-POD complexes. Although the fluorescence intensity was lower than that observed for the non-functionalized Palm-POD, fluorescence in the cornea treated with QN-Palm-POD-siRNA persisted for up to 24 h (Figure 6A) and is significantly higher than in corneas treated with the siGLO alone at all the time points (p < 0.001 at 3 h, p < 0.05 at 6 and 24 h) (Figure 6B). Sections of the treated corneas show uptake of siGLO in all of the corneal layers (epithelium, stroma, and endothelium) (Figure 6C), whereas siGLO was not observed in the posterior segment (data not shown).
In Vivo Evaluation of QN-Palm-POD-siRNA Nanoparticle Knockdown of Luciferase Expression

The ability of QN-Palm-POD to deliver siRNA to the cornea and achieve knockdown of corneal epithelial gene expression in vivo was assessed using siLuc knockdown of luciferase expression in a Krt12+/luc2 mouse model. In a split body control experiment, mice (n = 5) were treated with QN-Palm-POD complexed with siLuc (right eye) and NSC4 (left eye) (140:1 molar ratio) in parallel daily for 4 days. NSC4 was shown earlier not to decrease luciferase signal when injected in the stroma, suggesting that any observed effect on luciferase gene expression is not due to non-specific or toxic effects.

Although no significant knockdown of expression was observed during the 4 days of treatment, a significant knockdown was detected in the 3 days following the termination of the treatment with QN-Palm-POD-siRNA, reaching a maximum of 30% (p < 0.001) at day 9 (day 3 after withdrawal of treatment) (Figures 6D and 6E), whereas luciferase gene expression returned to pretreatment level 4 days after treatment.

DISCUSSION

The results presented here are the first example of successful topical delivery of a mixed siRNA-delivery agent to the cornea. siRNAs have been successfully and extensively used to treat different diseases, but their application for the treatment of corneal pathologies has proved to be difficult, despite the external accessibility of this organ. To date, administration of oligonucleotides by intrastromal injection is the preferred route, although it is not suitable for a...
prolonged and repeated treatment regimen. Commercially available transfection agents, including Lipofectamine 2000, Entranster—in vivo, PEI, and PEO-PPO-PEO polymers, are unable to deliver Cy3-siRNA to mouse cornea in vivo.

For successful polynucleotide delivery, a vehicle should fulfill three requirements: (1) delivery to the desired tissue, (2) release of cargo into the cytoplasm, and (3) low toxicity. Cell-penetrating peptides have been proved to satisfy all of these requirements, delivering siRNA and peptides to cells, with an increasing number of peptides used for this purpose. Some CPPs have been used in vivo to deliver siRNA, targeting tumors, the brain-blood barrier, and other tissues, but none was topically applied, either to the skin or to the ocular surface. A CPP with proven ability to overcome the corneal barrier, with a PEG moiety, showed improved functionalizing able to deliver a luciferase expression vector to retinal cells in vivo and more efficiently than other CPPs such as HIV-Tat and CK30. Further modification of POD by the addition of one moiety of PLGA, previously proved to be medically compatible and with recognized delivery features, resulting in PLGA-PEG-POD, improved the in vivo bioavailability of POD.

In the present study, we compared PLGA-PEG-POD with the native POD and two other PODs modified with either a palmitoyl- or a cholesterol- group (Palm-POD and Chol-POD), in an attempt to improve the performance of siRNA delivery, and more efficiently than other CPPs such as HIV-Tat and CK30. Further modification of POD by the addition of one moiety of PLGA, previously proved to be medically compatible and with recognized delivery features, resulting in PLGA-PEG-POD, improved the in vivo bioavailability of POD.

In vitro, all of the PODs tested were able to achieve cellular delivery of siRNA with a low toxicity, which did not exceed that previously reported for the cationic lipid transfection agent, Lipofectamine, and CPPs at high concentrations. However, bioavailability was not achieved, and none of the formulations achieved gene silencing of a luciferase reporter gene in in vitro-transfected HCE-S cells, consistent with other CPPs, where cellular delivery of siRNA was not matched with gene silencing.

We attributed the lack of bioavailability and gene silencing in cells treated with POD-siRNA complexes to endosomal entrapment, assuming whichever internalization pathway is utilized by CPP, it is fundamental to develop a method that permits the siRNA to escape from the endosomes. Herein, we demonstrated Chlq, a known endosomal disruptor (coupled to and derivatized with the POD peptide), to elicit siRNA endosomal release and gene silencing, in agreement with previous reports. However, the structural and chemical features of PLGA-PEG-POD make it unsuitable for further covalent modification with endosomal disruptors and because the latter are essential to improve the release and decrease corneal toxicity, PLGA-PEG-POD was excluded from the in vivo study. Both Palm- and Chol-POD achieved a significantly higher knockdown than the POD, and they were thus selected for the subsequent in vivo analysis.

Topical drug delivery to the eye surface has proved difficult because of several anatomical barriers. The tear film in particular reduces the contact time of an applied drug to the eye surface. Absorption through the conjunctival pathway is responsible for the removal of more than 75% of any administrated drug on the ocular surface. Topical delivery of POD to the cornea is promising: a fluorescently tagged POD was visible in mouse corneas 45 min after application and persisted, with a decreased intensity, for 24 h afterward, penetrating into the different corneal layers. Similarly, a fluorescently labeled PLGA-PEG-POD was visible in rabbit corneas 2 h after topical application. siRNA alone cannot penetrate in vivo into the murine cornea unless injected under pressure into the stroma. In comparison, topical delivery of siRNA combined with Chol- and Palm-POD effectively penetrated into all corneal layers and demonstrated some gene silencing of the target gene. The persistence of the fluorescent siRNA for up to 48 h after topical application suggests that these PODs have the capacity to interact with the ocular surface, thus increasing the effective time of exposure and the amount of complex that can be internalized, which is in contrast with results previously described for modified, single-filament fluorescent siRNA, which completely cleared from the cornea in about 3 h.

We demonstrate here that to achieve knockdown of gene expression, an endosomal disruptor, like Chlq, is necessary to release the siRNA into the cytoplasm. However, because Chlq has been reported to display strong systemic and corneal toxicity, we sought a way to use it in vivo that would minimize these harmful side effects. An analog of Chlq, QN, linked to the peptide PepFect6, was previously shown to deliver siRNA and miRNA and achieve knockdown, both in vivo and in vitro without significantly enhancing cytokine levels in serum and cellular toxicity in kidney, lung, liver, and spleen. We modified Palm-POD by the covalent addition of two moieties of QN. The ability of the Palm-POD to complex siRNA was reduced by QN-functionalization, probably because of the presence of the bulky QN moiety; to compensate for this, subsequent in vitro experiments were all conducted with a 140:1 molar ratio and 125 nM siRNA. Enhanced endosomal release was confirmed through bioavailability and significant knockdown of luciferase expression 72 h after transfection, which was not significantly increased when the cells were pre-treated with Chlq.

| Table 2. POD Structures and Molecular Weights |
|-----------------------------------------------|
| Name     | Structure                  | MW (g/mol) |
| POD      | CGGG[ABKAAKA]₅             | 3,592.4    |
| Palm-POD | CH₃(CH₂)₃₇-CO-POD          | 3,829.9    |
| Chol-POD | C₂₀H₄₀O-CO-POD             | 4,041.1    |
| QN-Palm-POD | C₆₀H₃₆F₄₀N₂₀-O₂-CO-POD   | 4,873.0    |
| PLGA-PEG-POD | [C₆H₁₀O₂]₅[C₇H₈O₃]₂[C₆H₈O]₁ | 29,500–43,500 |

In vitro transfected HCE-S cells, consistent with other CPPs, where cellular delivery of siRNA was not matched with gene silencing.
The functionalized formulation was also able to deliver siRNA \textit{in vivo}, performing as well as unmodified PALM-POD, with the siGLO fluorescent signal persistent in the cornea for up to 24 h. This is despite the fact that, as a consequence of the reduced complexation capacity of QN-Palm-POD, the amount of siGLO applied was reduced. Furthermore, the luciferase reporter gene expression knockdown observed (up to 30%) in the corneal epithelium was greater than previously achieved despite a reduced amount of siRNA, and the effect was prolonged for 3 days after treatment and had no observable toxic or inflammatory effect on the cornea \textit{in vivo}. Enhancing gene silencing within the cornea, using non-invasive eye drop delivery, to a therapeutic level remains a challenge, and we have not matched the 60% knockdown previously reported in mice skin using Accell-siRNA.\textsuperscript{61} We can match this level of corneal gene silencing using intrastromal injection of Accell siRNA (64%) as described within, but this is not suitable for repeated and long-term siRNA therapeutic application in the ophthalmology clinic. To our knowledge, the modest 30% gene silencing result we achieved is the first report of a decreased protein expression in corneal epithelium after siRNA-mediated knockdown persisting up to 72 h after eye drop treatment. Taketani et al.\textsuperscript{84} observed knockdown in mouse cornea only at the mRNA level and only for 24 h after the treatment, whereas mRNA expression returned to the untreated level by 48 h.

Moreover, in agreement with reports that PepFect6-siRNA does not elicit an inflammatory response \textit{in vivo}\textsuperscript{86,86} and does not alter the lipid bilayer,\textsuperscript{87} no toxic effect of QN-Palm-POD-siRNA was observed in mouse corneas \textit{in vivo}, suggesting that the reduced amount of this Chlq analog is not toxic to the corneal epithelium.

In summary, we designed a novel version of a cell-penetrating POD capable of complexing the siRNA, delivering it into the corneal layers, and releasing functional siRNA into the cytoplasm, which ultimately results in targeted gene expression reduction.

Figure 6. \textit{In Vivo} Topical Application of siRNA with QN-PALM-POD Demonstrated Efficient Delivery and Knockdown in Cornea

(A) \textit{In vivo} comparison of wild-type mice treated with QN-Palm-POD and red siGLO with and without amphotericin B (AmB). The formulation was applied to the right eye, whereas siGLO alone was applied to the left eye (negative control). The images were collected with IVIS Xenogen at 3, 6, and 24 h after treatment. The red and yellow colors represent the intensity of the fluorescence and not the color of the fluorescent signal. (B) Fluorescence measurements of the treated eye at the different conditions reported in (A). The values represent the mean (and the SEs) of two treated eyes in n = 2 mice. (C) Corneal sections of the eyes collected from the treated mice at 24 h reported in (A). The nuclei are stained with DAPI (blue), whereas the red fluorescence represents siGLO. Fluorescence is present in eyes treated with QN-Palm-POD in all of the layers of the cornea. (D) Representative daily images of one of the four mice during the treatment (10 days) with siLuc and QN-Palm-POD in the right eye and with NSC4 and QN-Palm-POD in the left eye. (E) Quantification of luciferase activity for a treatment group (n = 4) expressed as a percentage of the right eye/left eye ratio (R/L ratio %). Significant knockdown of luciferase with siLuc was persistent from day 7 to day 9. The black arrows indicate the days of treatment. The mice were observed for 3 days before the treatment and 4 days after the treatment. *p < 0.05, **p < 0.001. (F) Representative section of the corneas treated with QN-Palm-POD-siRNA and stained with H&E. No abnormality or signs of inflammation were observed in any treated cornea.
The Palm-POD-siRNA complex gave the best knockdown of in vivo gene expression and, when chemically modified by covalent attachment of the Chlq analog, QN, was able to deliver siRNA to the cytoplasm and to knock down gene expression up to 40% in vitro and 30% in vivo. We acknowledge that this knockdown is relatively modest and will require further improvement to reach levels of knockdown sufficient for therapeutic application. This study confirmed that functionalization of a cell-penetrating peptide for siRNA delivery with an endosomal disruptor is an effective approach to target the cornea in vivo. It also represents a valid proof-of-principle that can applied to safer and more effective endosomal disruptors. Different treatment regimens and adjuvants that might increase the persistence of the drug on the eye surface may be tested as well, together with modified siRNAs having an increased resistance to nuclease degradation. The rational design presented in this study, combining an in vitro pre-screening with the in vivo assessment of therapeutic siRNA delivery and function in a corneal bioluminescence reporter mouse, represents a methodology to evaluate the efficacy and topical delivery in the corneal epithelium.

MATERIALS AND METHODS

Synthesis of Peptides and Preparation of Nanoparticles

POD (CGGG[ARKKAAKA])30 was modified in order to obtain a Palm-POD (molecular weight [MW] 3,829.9), a Chol-POD (MW 4,004.1), and a QN-Palm-POD (MW 4,873.0) as described in Figures S1. The lipophilic derivatization was carried out in solid-phase at the N terminus of the POD sequence. A fraction of the peptidyl-resin was treated with 3-fold molar excesses of palmitic acid, N-ε-4-methyltrityl-L-lysine [Fmoc-Lys(Mtt)-OH, 3 equiv] (Novabiochem; Merck Millipore, Darmstad, Germany) amino acid derivative was coupled on solid phase to the N terminus of the POD throughout activation with 2-(1H-7-azabenzo triazole-1-yl)-1,3,3-tetramethyluronium hexafluorophosphate methanaminium (HATU) (3 equiv) (Genscript, Piscataway, NJ, USA) and diisopropyl-lthylamine (DIPEA) (6 equiv) (Fluka-Sigma-Aldrich, St. Louis, MO, USA) in DCM. After removing the Fmoc-protecting group by reaction with piperidine (Fluka-Sigma-Aldrich, St. Louis, MO, USA) in DCM (20% v/v), the palmitic acid was coupled to the free N-ε-4 amino group as described above. Subsequently, the methyltrityl protecting group of the N-ε-amino of the lysine was selectively removed after repeated treatments with 1% TFA in DCM. A Fmoc-Lys(Fmoc)-OH (Novabiochem; Merck Millipore, Darmstadt, Germany) derivative was then incorporated through activation with HATU and DIPEA in DME. Three-fold molar excess of reagents was used. The deprotection of the Fmoc group by repeated treatment with piperidine in DCM (20% v/v) rendered two free amino groups that were afterward treated with succinic anhydride (Fluka-Sigma-Aldrich, St. Louis, MO, USA) (1.5 equiv) and DIPEA (3 equiv) in DME. The efficiency of the reactions was evaluated by the ninhydrin colorimetric test. The synthetic scheme of QN and QN-Palm-POD are described in Figures S2A and S2B.

In order to obtain the final QN-Palm-POD derivative, the QN derivative was first synthesized through reaction of 4-chloro-7-(triﬂuoromethyl)quinoline (Fluka-Sigma-Aldrich, St. Louis, MO, USA) (16.4 mmol) and 2,2’-diamino-N- methylidihcetylamine (TCI, Tokyo, Japan) (194.1 mmol). The product, N-(2-aminoethyl)-N-methyl-N’-[7-(triﬂuoromethyl)-quinolin-4-yl]ethane-1,2-diamine (QN), was characterized by MALDI-TOF (Figure S2C) and proton NMR (NMR-H+) (Figures S2 and 2D). QN (2.5 equiv) was coupled overnight to the succinic acid-modiﬁed peptidyl resin previously activated with 2-(1H-benzo triazole-1-yl)-1,1,3,3-tetramethylammonium tetrafluoroborate (TBTU) (Fluka-Sigma-Aldrich, St. Louis, MO, USA) (3 equiv), HOBt (3 equiv), and DIPEA (6 equiv). Crude peptide was obtained after cleavage and final deprotection of the peptidyl-resin with TFA/water/β-mercaptoethanol/TIS (95:2:2.1).

The crude peptides were purified by semi-preparative high-pressure liquid chromatography (HPLC; 1260 Infinity; Agilent Technologies, Santa Clara, CA, USA) in an XBridge Prep BEH 130 C18 column (Waters; 5 μm, 10 × 250 mm) at a flow rate of 3 mL/min. The peptides were purified with a linear gradient of 5%–100% B (0.05% [v/v] TFA in acetonitrile) into A (0.05% [v/v] TFA in water) for 20 min. Their identity was confirmed by electrospray ionization mass spectrometry (ES-MS). Thus, purified peptides were characterized by an analytical ultra-performance liquid chromatograph (UPLC; Waters, Milford, MA, USA) coupled to a time-of-flight (LC-TOF) detector, LCT Premier XE (Micromass; Waters, Milford, MA, USA). Samples were analyzed in the UPLC at a flow rate of 0.3 mL/min. The mass spectra were recorded in positive ion mode in the m/z 500–2,500 range. UPLC was performed in an Acuity UPLC BEH C18 reverse-phase column (2.1 × 100 mm, 1.7-μm particle size). Solvent A was 20 mM formic acid in acetonitrile, and solvent B was 20 mM formic acid in water. Elution was performed with linear gradients.
of 5%–100% A into B over 10 min. Figures S3 and S4 show the characterization of the pure peptides by ES-MS and MALDI-TOF.

PLGA-PEG-POD-nanoparticles (NPs) were prepared by covalently binding POD to the pegylated polymer PLGA as described in Figure S5. With this aim, PLGA was preactivated before polyethylene glycosylation (PEGylation) with maleimide-PEG-amine. The obtained PLGA-PEG copolymer was dried under vacuum and stored at 4°C. To conjugate the peptide with the PLGA-PEG-maleimide, POD was dissolved in acetonitrile/DMF and added to the polymer dissolved in chloroform. The mixture was covered tightly and stirred overnight. The product was precipitated with 3 mL of an ice-cold 80/20 mixture of diethyl ether/methanol, centrifuged at 2,600 × g for 10 min, the supernatant discarded, and the product re-dissolved in 1 mL of chloroform. This cycle was repeated twice more and the purified PLGA-PEG-POD dried under vacuum.

1H-NMR was used to assess the grafting of PEG to PLGA and the conjugation with POD. The PLGA-PEG was dissolved in deuterated chloroform and the PLGA-PEG-POD in DMSO-d6. The spectra were recorded at 298K on a Varian Inova 500 MHz spectrometer (Agilent Technologies, Santa Clara, CA, USA). PLGA-PEG-POD NPs were prepared following the solvent displacement technique. In brief, an organic solution of the polymer containing the POD (PLGA-PEG-POD) in acetone was poured, with moderate stirring, into an organic solution of the polymer containing the POD (PLGA-PEG-POD) in acetone. The resulting colloidal suspension was stirred for 5 min, and the acetone was then evaporated and the NP dispersion was concentrated under reduced pressure. The mean particle size, polydispersity index (PI), and ζ-potential were determined by dynamic light scattering (DLS) measurement using a Zetasizer nano ZS (Malvern Instruments, Malvern, UK). Three measurements were collected for each sample, and the values expressed as mean ± SD.

Measurement of Dimension and ζ-Potential
To measure the dimensions and the ζ-potentials of the polyplexes, POD-siRNA formulations were prepared in a final volume of 50 μL by mixing PODs and siRNA in PBS, and incubated for 40 min at 25°C before analysis. For the measurement of particle size, a molar ratio of 140:1 for the QN-Palm-POD and 35:1 for all the other PODs was used. The samples were then diluted to 1 mL in distilled water before the measurement of ζ-potential (the charge of the POD-siRNA polyplexes) using a Nano ZS Zetasizer and DTS software (Malvern Instruments, Malvern, UK). These measurements were collected for each sample, and the values expressed as mean ± SD.

Measurement of Effect of Formulation upon Cell Viability (MTT Assay)
HCE-S cells were plated at a density of 1.5 × 10^4 cells/well in 96-well plates and transfected 24 h later with POD-siRNA polyplexes at 17.5, 35, and 70 μM POD at 35:1 molar ratio in PBS for Chol-, Palm-, and POD, and at 9, 17.5, and 35 μM POD at 140:1 molar ratio for the QN-Palm-POD. For each condition, n = 5 replicates were tested. Twenty-four hours post-transfection, 0.5 mg/mL MTT reagent (Sigma-Aldrich, UK) was added to the media, and the cells were incubated for 2 h at 37°C, under 5% CO2. Absorbance was then measured at 570 and 650 nm in a plate reader (LUMItar OPTIMA; BMG LABTECH, UK). The absorbance at 650 nm, subtracted from that at 570 nm, indicates cell viability; the results obtained were compared with an internal untreated control (maximum cell viability).

In Vitro Fluorescence siRNA Analysis with PODs-siRNA Formulations
Green fluorescent siRNA (siGLO; GE Dharmacon) was complexed with PODs and used to transfect HCE-S cells. Chol-, Palm-, and POD were used at 35:1 molar ratio in PBS with 1 μM green fluorescent siRNA, whereas QN-Palm-POD was used at 17.5 μM with 0.125 μM siGLO, for a final molar ratio of 140:1. HCE-S were seeded on coverslips at 10^5 cells/well in a 24-well plate, 24 h before POD-siRNA transfection, and 24 h after transfection, the coverslips were collected, fixed for 10 min in 4% paraformaldehyde in PBS (Thermo Scientific, USA), and mounted in Ultracruz Mounting media (Santa Cruz Biotechnology). Fluorescence was then assessed with an AxioScope A1 microscope equipped with a 20×/40× N Arachplan lens on an AxioCam MRc camera (Carl Zeiss, Germany).

To study the effect of endosomal disruptors on siRNA release, the experiment was repeated using the formulation at 35:1 molar ratio alone or in combination with 30 μM Chlq (Sigma-Aldrich, UK) added 1 h before transfection.

In Vitro Luciferase Assay with POD-siLuc Formulations
A modified in vitro dual-luciferase assay was performed, as previously reported, in which expression of Firefly luciferase, the siRNA target, is normalized to Renilla luciferase expression as an internal control of cell transfection: HCE-S cells were plated at 6.5 × 10^3 cells/well in a 96-well plate, transfected after 24 h with the luciferase.
reporter plasmids, and then treated 24 h later with the different POD-siRNA formulations (1 μM siRNA 35:1 POD/siRNA in a final volume of 100 μL), using luciferase-specific siRNA (siLuc, 5'-CGACAAGCUGGCGCAGUAUUA-3'), with dTdT overhang at 3' in both strands; Eurogentech, Belgium) and non-specific control siRNA (NSC4, 5'-UAGGACUAACACAUCAUUU-3'), inverted β-galactosidase sequence, with dTdT overhang at 3' in both strands; Eurogentech, Belgium).22,43 Luciferase expression was measured 72 h after POD-siRNA transfection, and the values obtained were expressed as a percentage of the luciferase activity measured with free Chlq (100%). The effect of Chlq upon knockdown of gene expression was investigated by transfecting cells with the POD-siRNA formulation, as above, along with 30 μM Chlq, added 1 h before transfection. As a positive control, cells were transfected with 1 μM siLuc/NSC4 siRNA complexed with RNAiMAX (Thermo Fisher, Invitrogen, UK) as previously described.

The experiment was further repeated with QN-Palm-POD at a 140:1 ratio and with QN-Palm-POD in combination with free Chlq.

**Live Animal Imaging**

Animals were used for the following experiments in accordance with the UK Animal Welfare Act; the experiments were approved by the Home Office (Scotland) and the Department of Health, Social Services, and Public Safety (DHSSPS; Northern Ireland). The experiments to assess delivery of fluorescent siRNA (siGLO) to the cornea were performed on wild-type C57BL/6 mice. To assess functionality of the delivered siRNA, we used a transgenic mouse line expressing the luciferase reporter gene luc2l (codon-optimized for mammalian expression) under the control of K12KI.2F: 5'-GCTGTGGAGGCCTCTTTTC-3' in equimolar concentrations, whereas siGLO alone was applied to the left eye of each mouse as a positive control. Measurements were obtained from two untreated mice to determine background fluorescence. Fluorescence was detected as described earlier in Live Animal Imaging. Baseline luciferase reporter gene expression (day 0) was a mean value obtained by measurement of ocular luminescence daily for 3 days before treatment.

**Intrastromal Injection**

Intrastromal injection of Accell siRNA was performed by a trained ophthalmic surgeon as previously described.64 Two microliters of 150 pmol/μL C3y-labeled Accell-modified siRNA was injected intrastromally into the right eyes of WT C57BL/6j mice. To assess the persistence of C3y-labeled siRNA, animals were imaged on the Xenogen IVIS Lumina system at 0, 6, 24, 48, and 72 h post-injection (n = 3). Mice were sacrificed at 0, 6, and 12 h after injection (n = 3); eyes were enucleated and frozen at −80°C. Tissue was fixed in OCT and cryosectioned for fluorescence microscopy. To assess luciferase knockdown, mice were treated in a split body control (untreated versus Accell-siRNA and untreated versus Accell-NSC4) for 7 days after the treatment (n = 3). Luciferase signal was quantified as described earlier in Live Animal Imaging. Baseline luciferase reporter gene expression (day 0) was a mean value obtained by measurement of ocular luminescence daily for 3 days before treatment.

**In Vivo POD-siRNA Studies**

Mice between 12 and 25 weeks old were anesthetized using 1.5%–2% isoflurane in air (Abbott Laboratories, Berkshire, UK) at a flow rate of ~1.5 L/min. Formulations containing 35:1 molar ratio POD/siRNA (Chol- and Palm-), with 18 μM siRNA and 625 μM POD or QN-Palm-POD-siRNA at a 140:1 molar ratio with 5 μM siRNA, in a total volume of 2.5 μL of PBS per eye were prepared, incubated at room temperature for 30 min, and then applied as a drop to the cornea of anesthetized mice, which were maintained with the eye in a horizontal position. After application, the mouse was kept anesthetized for a further 15 min, over which period the droplet was observed to remain on the eye, to allow absorption and maximize uptake. Following treatment, fluorescence and luciferase experiments were performed as described below.

**Assessment of siRNA Uptake by In Vivo Fluorescence Assay**

To assess the uptake of siRNA by the corneal epithelium, in vivo fluorescence assays were performed by treating wild-type mice (n = 2 for each condition). 100 μM red siGLO (D-001630-02; GE Dharmacon) was used in combination with Chol- or Palm-POD. The siRNA-POD polyplexes were applied to the right eye, whereas siGLO alone was applied to the left eye of each mouse as control. Measurements were obtained from two untreated mice to determine background fluorescence. Fluorescence was detected with a Xenogen IVIS with LivingImage 3.2 software (both Perkin Elmer, Cambridge, UK) using DS Red filters (excitation [Ex.] 570 nm, emission [Em.] 620 nm) at 3, 6, and 24 h following application. Fluorescence was quantified after selecting a ROI tightly cropped to the fluorescent regions in the eyes, and the ROI was kept constant in all subsequent measurements. After the final measurement, mice were sacrificed, and the eyes were enucleated, fixed in 4% paraformaldehyde (prepared in PBS, pH 7.4) for 30 min at room temperature, submerged in Poly-Freeze (P0091 SIGMA; Sigma-Aldrich), and immediately frozen at −80°C. Five-micrometer sections were cut with a cryostat (CM 1850; Leica), mounted on 3-aminopropyltriethoxysilane (APES) (Sigma Aldrich, UK)-coated slides, and treated with a mounting medium containing DAPI (DAPI I; Vysis, Molecular Therapy: Nucleic Acids Vol. 17 September 2019 903
USA), to stain the nuclei, and fluorescence was visualized with a fluorescence microscope (as described above).

**Assessment of siRNA-Mediated Gene Expression Knockdown by In Vivo Luciferase Expression Analysis**

*In vivo* luciferase experiments were performed using a split body control by comparing the treatment under a test, in one eye, with a negative control in the other eye of the same animal: the right eye was treated with QN-Palm-POD and NSC4, whereas the left one was treated with QN-Palm-POD and siLuc. QN-Palm-POD:siRNA were at a 140:1 molar ratio with 5 μM siRNA. Experiments and treatment were performed in n = 4 mice. Baseline luciferase reporter gene expression was determined by measurement of ocular luminescence daily for 3 days before treatment. Ocular luminescence was measured before POD-siRNA complexes were applied daily, as described above, for 4 days, and ocular luminescence was then measured daily for a further 4 days after cessation of treatment.

**H&E Staining of the Mouse Cornea**

After the final measurement of luminescence, mice were sacrificed and eyes enucleated, paraformaldehyde fixed, dehydrated through graduated ethanol solutions, and paraffin embedded. Five-micrometer sections were obtained using a microtome (Leica RM 2135), mounted on APES (Sigma Aldrich, UK)-coated slides, dewaxed and paraffin-embedded. D.S., M.J.G., E.M., S.D.A., L.M., K.A.C., D.F.C., and C.M.M. performed the experiments. D.S., E.M., S.D.A., T.M., and I.H. analyzed the data. D.S., E.M., M.A.N., and T.M. wrote the paper.

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