Surface Engineering of FLT4-Targeted Nanocarriers Enhances Cell-Softening Glaucoma Therapy

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ABSTRACT: Primary open-angle glaucoma is associated with elevated intraocular pressure (IOP) that damages the optic nerve and leads to gradual vision loss. Several agents that reduce the stiffness of pressure-regulating Schlemm’s canal (SC) endothelial cells, in the conventional outflow pathway of the eye, lower IOP in glaucoma patients and are approved for clinical use. However, poor drug penetration and uncontrolled biodistribution limit their efficacy and produce local adverse effects. Compared to other ocular endothelia, FLT4/VEGFR3 is expressed at elevated levels by SC endothelial cells and can be exploited for targeted drug delivery. Here, we validate FLT4 receptors as clinically relevant targets on SC cells from glaucomatous human donors and engineer polymeric self-assembled nanocarriers displaying lipid-anchored targeting ligands that optimally engage this receptor. Targeting constructs were synthesized as lipid-PEGx-peptide, differing in the number of PEG spacer units (x), and were embedded in micelles. We present a novel proteolysis assay for quantifying ligand accessibility that we employ to design and optimize our FLT4-targeting strategy for glaucoma nanotherapy. Peptide accessibility to proteases correlated with receptor-mediated targeting enhancements. Increasing the accessibility of FLT4-binding peptides enhanced nanocarrier uptake by SC cells while simultaneously decreasing the uptake by off-target vascular endothelial cells. Using a paired longitudinal IOP study in vivo, we show that this enhanced targeting of SC cells translates to IOP reductions that are sustained for a significantly longer time as compared to controls. Confocal microscopy of murine anterior segment tissue confirmed nanocarrier localization to SC within 1 h after intracameral administration. This work demonstrates that steric effects between surface-displayed ligands and PEG coronas significantly impact the targeting performance of synthetic nanocarriers across multiple biological scales. Minimizing the obstruction of modular targeting ligands by PEG measurably improved the efficacy of glaucoma nanotherapy and is an important consideration for engineering PEGylated nanocarriers for targeted drug delivery.

KEYWORDS: VEGFR3, FLT4, drug delivery, targeting ligand, rational design, IOP, nanoparticle

INTRODUCTION

Glaucoma is the leading cause of irreversible blindness, lacks a cure, and is currently estimated to affect 76 million people worldwide.1 The prevalence of glaucoma continues to rise, with the number of individuals living with the disease projected to increase to ~112 million people by 2040.2 The hallmark of glaucoma is elevated intraocular pressure (IOP ≥ 21 mm Hg),2 which progressively damages retinal ganglion cells.3 Multiple lines of evidence demonstrate that mechanical dysfunction in pressure-regulating tissues contributes to this increase in IOP.4–13 In the eye, IOP is determined by the balance between the rate of aqueous humor production by the ciliary body and the rate of aqueous humor outflow through tissues of the conventional14–17 and uveoscleral18–21 outflow pathways. Ocular hypertension results from increased resistance to aqueous humor outflow through the conventional outflow pathway22 that is positioned at the iridocorneal angle of the anterior segment and consists of the trabecular meshwork (TM), Schlemm’s canal (SC), collector channels, intrascleral venous plexus, and the aqueous veins. Decreased porosity4–8 of the SC endothelium contributes to the increase in outflow resistance in glaucoma, and these porosity changes arise from an increase in SC stiffness that impairs pore formation.12 Most recently, it was demonstrated that the source of outflow resistance is localized to a region within 1 μm of the inner wall of the SC endothelial surface.17

Currently, first and second line glaucoma therapeutics decrease IOP by either (i) decreasing aqueous humor inflow or (ii) driving a greater proportion of the aqueous humor outflow through the unconventional outflow pathway.23 However, these treatments do not address the source of the
increased outflow resistance that contributes to ocular hypertension. Cell-softening agents, such as actin depolymerizers and Rho kinase inhibitors, improve aqueous humor outflow by directly addressing dysfunction in the conventional outflow pathway. Cell-softening agents are approved for clinical use in the United States (Netarsudil) and abroad (Ripasudil) but suffer from side effects, including conjunctival hyperemia, conjunctival hemorrhage, cornea verticillata, eye pruritus, reduced visual acuity, and blurred vision. Targeted drug delivery vehicles may address these issues by directing cell-softening agents to their site of action while minimizing adverse events arising from off-target effects.

To target the delivery of cell-softening agents to SC cells, we previously developed a lipid-anchored targeting peptide that binds to FLT4/VEGFR3 receptors. FLT4 receptors are enriched on the SC endothelium in animal models. Most recently, FLT4 receptors have been detected in cultured SC cells derived from healthy human donors and in human donor eyes by single-cell RNA sequencing. Despite this early work, multiple uncertainties remain regarding the use of an FLT4-targeting strategy. One uncertainty pertains to clinical utility and whether FLT4 receptors are expressed on the SC surface in the eyes of glaucoma patients to permit the targeted delivery of cell-softening drugs. Second, to maximize the efficacy and minimize the side effects of these agents, it is important that the engineered vehicles optimally deliver drug payloads to SC cells instead of off-target endothelial cell types, in particular the corneal endothelium. Regarding our initial SC-targeting approach, steric considerations involving the PEG chains of the drug delivery vehicle chassis and their potential for obstructing receptor access to the peptide ligand motivated us to rationally design alternative ligand structures to improve performance. Suboptimal receptor binding is a general problem with targeted drug delivery systems, and it is often left unresolved due to a lack of methods for optimizing ligand interactions at the nanocarrier surface. While useful insights into the role of ligand length on organ-level nanocarrier accumulation are available in the literature, empirical relationships between ligand steric effects at nanocarrier surfaces and their consequences on multiscale targeting performance and the impact on clinically relevant outcomes remain unestablished. Here, we address these issues by developing a novel assay to measure peptide accessibility to proteolysis, which enabled us to quantify the availability of ligands for binding events. We were particularly interested in determining whether lipid-anchored targeting constructs can be optimized to maximize binding to FLT4 receptors and whether differences in receptor engagement produced measurable differences in drug delivery vehicle performance in a clinically relevant setting.

Using a multidisciplinary approach that included molecular characterization, in vitro targeting studies, and direct evaluation in a murine model, we examined the potential for these optimized nanotherapeutics to target a cell-softening agent to SC in vivo and lower IOP. C57BL/6J mice were used as an animal model in these studies due to their similar conventional outflow pathway anatomy, physiology, and pharmacological responses to humans. This work holds important clinical implications for the use of cell-softening nanotherapies as safe and efficacious tools for managing glaucoma. Furthermore, the rational design principles established herein, as well as the empirical evidence supporting their utility, can be harnessed to meet diverse challenges in drug delivery.

### EXPERIMENTAL SECTION

#### Chemicals

Unless otherwise stated, all chemical reagents were purchased from the Sigma-Aldrich Chemical Company.

#### Solid-Phase Peptide Synthesis

Fmoc-N-amido-dPEG6-acid, Fmoc-N-amido-dPEG24-acid, and Fmoc-N-amido-dPEG48-amido-dPEG24-acid (Quanta Biodesign) were purchased for use in the synthesis of the PG6, PG24, and PG48 peptide constructs, respectively. Standard Fmoc solid-phase peptide synthesis was performed to synthesiz PG6, PG24, and PG48 peptides on a 0.125 mmol scale. Information regarding each synthesized peptide is presented in Table S2. The chemical structure of each peptide is presented in Figure S1.

#### Preparation of PEG-b-PPS Micelle Formulations

The PEG45-b-PPS23 polymer was synthesized using established procedures (Table S1). Briefly, sodium methoxide was used to deprotect PEG thioate. This deprotected PEG thioate is then used to initiate the polymerization of propylene sulfide through an anionic ring-opening polymerization reaction. Micelle (MC) nanocarriers were self-assembled from the PEG45-b-PPS23 polymer via cosolvent evaporation using established protocols. For uptake studies in vitro, MCs were prepared to load Dil hydrophobic dye (Invitrogen), whereas formulations intended for IOP studies in vivo were prepared to load latrunculin A (LatA; Cayman Chemical Company) and Dil. The formed MCs were split into aliquots of equal volume prior to the addition of peptide. PG6, PG24, or PG48 targeting peptides were dissolved in dimethyl sulfoxide and were added to the specified MC aliquots at either a 1 or 5% molar ratio (peptide/polymer). Blank MC controls (lacking peptide) were included in cellular uptake studies. All formulations were prepared under sterile conditions and were filtered using a Sephadex LH-20 column.

#### Determination of Peptide Concentration

The peptide concentration in purified MCs was determined by measuring tryptophan fluorescence ($\lambda_{em} = 270$ nm and $\lambda_{em} = 350$ nm), calibrated against a peptide concentration series, using a SpectraMax M3 microplate reader (Molecular Devices, LLC). The peptide is readily detectable using these parameters (Figure S3). The concentration of peptide was determined using a simple linear regression model that was obtained by fitting the calibration data (see Figure S4 for a representative calibration curve). To account for background fluorescence from the polymeric MCs, the emission from a blank MC control formulation (lacking peptide) was subtracted from the peptide-containing MC formulations. The peptide-containing formulations and control formulations were prepared in the same solvent and contained the PEG-b-PPS polymer at an identical concentration. This procedure isolates the analyte signal (the specified peptide) by removing contributions from the PEG-b-PPS polymer and solvent.

#### Small-Angle X-ray Scattering

Small-angle X-ray scattering (SAXS) was performed using synchrotron radiation at the DuPont-Northwestern-Dow Collaborative Access Team (DND-CAT) beamline at the Advanced Photon Source at the Argonne National Laboratory (Argonne, IL, USA). A 7.5 m sample-to-detector distance, 10 keV ($\lambda = 1.24$ Å) collimated X-rays, and 3 s exposure time were used in all SAXS experiments. The q-range of 0.001 – 0.5 Å$^{-1}$ was used to analyze scattering, and silver behenate diffraction patterns were used for calibration. The momentum transfer vector ($q$) is defined in eq 1, where $\theta$ denotes the scattering angle.

$$q = 4\pi \frac{\sin(\theta)}{\lambda}$$

PRIMUS software (version 3.0.3) was used for data reduction. SasView (version 5.0) software was used for model fitting. A core–shell sphere model (eq 2) was fit to the scattering profiles of blank MCs (prepared without peptide) as well as MCs displaying PG6, PG24, or PG48 peptides at a 1 or 5% molar ratio (peptide/polymer)
TOF MS instrument. BioSight (Table 1) and were cultured using established procedures. Spectrometry (MS) using a Bruker rapid.”

under hot air using a heat gun. Data were acquired using matrix-applied to 384-spot polished stainless-steel plates and were dried.

displaying PG6, PG24, or PG48 at a 5% molar ratio were incubated under these conditions. Reaction aliquots were mixed 1:1 with 50:50 methanol/methanol and was placed at 4000 × g for 10 h. A subsequent blocking step, cells were incubated with an APC-conjugated anti-human VEGF(R3) (FLT4) antibody (BioLegend) for 20 min at 4 °C and were then subjected to two iterative rounds of washing (and centrifugation) per manufacturer’s recommendations. Cells were fixed using paraformaldehyde-cell fixation buffer (BioLegend). Flow cytometry was performed using a BD LSRFortessa flow cytometer. Cytobank software was used to analyze the acquired data.

Quantiﬁcation of Loaded Drug Concentration. Aliquots of puriﬁed LatA-loaded MC formulations were frozen at −80 °C and were lyophilized overnight. The resulting powder was resuspended in methanol and was placed at −20 °C for 1 h. Samples were centrifuged at 4000g for 5 min to sediment the polymer. After this extraction procedure, the supernatant (containing drug) was collected for analysis. The concentration of LatA was determined using high-performance liquid chromatography (HPLC) calibrated against a concentration series of LatA prepared in methanol (Figure S6). The absorbance of 235 nm light was measured. Data were acquired from three replicates. HPLC was performed using a C18 XDB-Eclipse column (Agilent) and a static methanol/water (95:5) mobile phase.

Quantitative Protease Protection Assay for Examining the Biochemical Accessibility of Targeting Peptides. PEG-P-PSP MC nanocarriers displaying PG6, PG24, or PG48 at a 5% molar ratio were incubated with trypsin gold protease (Promega) at 37 °C, 80 rpm for 10 h. A peptide concentration of 40 nM and an enzyme concentration of 800 nM (1:20 ratio) were used in these experiments. Reaction aliquots were quenched in 2% formic acid at the speciﬁed timepoints. Quenched reaction aliquots were mixed 1:1 with 50:50 methanol/acetoni trile with 0.1% triluoroacetic acid and a saturating quantity of the ε-cyano-4-hydroxycinnamic acid (Sigma) matrix. Samples were applied to 384-spot polished stainless-steel plates and were dried under hot air using a heat gun. Data were acquired using matrix-assisted laser desorption ionization time-of-ﬁght (TOF) mass spectrometry (MS) using a Bruker RapidI X MALDI Tis sueTyper TOF MS instrument.

Primary Cell Culture. Normal and glaucomatous SC endothelial cells were isolated from postmortem human eyes obtained from Biosight (Table 1) and were cultured using established procedures.8,9 Human umbilical vein endothelial cells (HUVECs) were pooled donors were purchased from Lonza, Ltd. The passage number of all primary cells used in these studies was less than six. SC cells were cultured in low-glucose Dulbecco’s modiﬁed Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum (FBS) and 1X penicillin–streptomycin–glutamine (Gibco). HUVECs were cultured in endothelial cell growth basal medium-2 (Lonza) supplemented with FBS and an EGM-2 BulletKit (Lonza) optimized for HUVEC culture. All cells were cultured at 37 °C, 5% CO2 in T25 or T75 flasks.

Quantitative of FLT4/VEGF(R3) Expression by Flow Cytometry. SC cells, glaucomatous SC (SCg) cells, or HUVECs (n = 3) were seeded in 24-well plates at a density of 100,000 cells per well. Cells were cultured in their appropriate media types (described elsewhere in the Experimental Section) and were allowed to adhere overnight at 37 °C, 5% CO2. On the following day, media were aspirated, cells were washed with phosphate-buffered saline (PBS), and single-cell suspensions were prepared following established procedures.6,44 Cell pellets were resuspended in the cell staining buffer with zombie aqua cell viability stain (BioLegend), incubated at 4 °C for 15 min, and washed with the cell staining buffer. After a subsequent blocking step, cells were incubated with an APC-conjugated anti-human VEGF(R3) (FLT4) antibody (BioLegend) for 20 min at 4 °C and were then subjected to two iterative rounds of washing (and centrifugation) per manufacturer’s recommendations. Cells were ﬁxed using paraformaldehyde-cell ﬁxation buffer (BioLegend). Flow cytometry was performed using a BD LSRFortessa flow cytometer. Cytobank software was used to analyze the acquired data.

Live-Cell Imaging by High-Throughput Wideﬁeld Fluorescence Microscopy. SC cells, SCg cells, or HUVECs (n = 3) were seeded in glass-bottom 96-well plates (Greiner) at a seeding density of 25,000 cells/well and were placed at 37 °C, 5% CO2, overnight. Cells were washed, blocked, and then placed in media containing an APC-conjugated anti-human FLT4/VEGF(R3) antibody (BioLegend) for 30 min at 37 °C. Afterward, the media were removed by aspiration and cells were gently washed twice using fresh media. After the final wash step, cells were treated with media supplemented with cell-permeant NucBlue (Hoechst 33342) counterstain to visualize cell nuclei. High-throughput widefield microscopy was performed using an ImageXpress High-Content Imager (Molecular Devices). Images of live cells were acquired at 40× magniﬁcation in brightﬁeld, DAPI, and Cy5 channels.

Nanocarrier Uptake Studies. SC cells or HUVECs were seeded at a density of 100,000 cells/well in 48-well plates and were allowed to adhere overnight at 37 °C, 5% CO2. Cells were treated with the speciﬁed Dil-loaded MC formulations (0.5 mg/mL polymer) for 2 h at 37 °C, 5% CO2. All experiments included untreated cells and a PBS-treated group, as well as three biological replicates per treatment group (n = 3). MC uptake was quantiﬁed by ﬂow cytometry using a BD LSRFortessa ﬂow cytometer, and the acquired data were analyzed using Cytobank software. The median ﬂuorescence intensity (MFI) above the PBS-treated background was calculated to remove cellular autoﬂuorescence contributions to the measured values.

Longitudinal Evaluation of IOP In Vivo. The mice were anesthetized with ketamine (60 mg/kg) and xylazine (6 mg/kg). IOP was measured using rebound tonometry (TonoLab; Icare) immediately upon the cessation of movement (i.e., in light sleep). Each recorded IOP was the average of six measurements, giving a total of 36 rebounds from the same eye per recorded IOP value. IOP was measured three times prior to nanocarrier treatment and 24, 30, 48, 72, and 96 h following nanoparticle injection. A total of nine mice were used for the 0–48 h timepoints. The IOP of four and three mice were measured at 72 h and 96 h timepoints, respectively.

Table 1. Donated Human SC Endothelial Cell Strains Used in This Study

<table>
<thead>
<tr>
<th>cell strain</th>
<th>disease state</th>
<th>age</th>
<th>gender*</th>
<th>race*</th>
<th>dex test†</th>
<th>cell source</th>
<th>bank</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC 78</td>
<td>normal</td>
<td>77</td>
<td>male</td>
<td>white</td>
<td>negative</td>
<td>whole eye</td>
<td>Kansas Eye Bank</td>
</tr>
<tr>
<td>SC 84</td>
<td>normal</td>
<td>67</td>
<td>NA</td>
<td>NA</td>
<td>negative</td>
<td>whole eye</td>
<td>NC Eye Bank</td>
</tr>
<tr>
<td>SC 57g</td>
<td>glaucoma</td>
<td>78</td>
<td>male</td>
<td>NA</td>
<td>negative</td>
<td>whole eye</td>
<td>Life Legacy</td>
</tr>
<tr>
<td>SC 90g</td>
<td>glaucoma</td>
<td>71</td>
<td>female</td>
<td>white</td>
<td>negative</td>
<td>whole eye</td>
<td>NC Eye Bank</td>
</tr>
</tbody>
</table>

“NA = Information not available. †Dex (dexamethasone) test. A negative Dex test indicates a lack of myocilin induction upon treatment with dexamethasone, which is a response that is expected of SC cells and is used to distinguish isolated SC cells from the major possible contaminant, TM cells (a positive Dex test is expected for TM cells).
Intracameral Injection of Nanocarrier Formulations. For IOP studies, three-month-old female C57 mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). A drop of 0.5% proparacaine, a topical anesthetic, was applied to both eyes. Two pulled microglass needles filled with nanocarrier formulation (labeled "A" or "B") and connected to a pump were alternatively inserted into both mouse anterior chambers. A volume of 2 μL of nanocarrier formulation "A" or "B" containing 18 μM LatA was infused into the anterior chamber of contralateral mouse eyes at a rate of 0.67 μL/min. Thus, this procedure delivered 15.2 ng of LatA per eye. After infusion, the needles were withdrawn, and topical erythromycin antibiotic ointment was applied to both eyes. All the mice were maintained on a warm water-circulating blanket until they had recovered from the anesthesia, and the animals were subsequently returned to the animal housing rack. Unmasking of treatment identity occurred after all IOP measurements were recorded.

Confocal Microscopy. Mouse eyes were dissected into eight radial wedges. Each wedge was immersed in Vectashield mounting media containing DAPI (Vector Laboratories) in a glass-bottom dish and was imaged along one of the two sagittal planes using a Zeiss LSM 700 confocal microscope (Carl Zeiss). The un.injected contralateral eyes were used as the negative control. Single images and tile images were acquired using a 20× objective to capture either SC and its surrounding structures or the entire sagittal plane using the ZEN2010 operating software (Carl Zeiss).

Statistical Analysis. Statistical analyses were performed using Prism software (version 9.0.0; GraphPad Prism Software, LLC). Details of each statistical analysis are provided in the corresponding figure legends.

■ RESULTS

Target Validation: Glaucamatos Schlemm’s Canal Endothelial Cells Highly Express FLT4/VEGFR3 Receptors. The SC endothelium is somewhat unique in that it expresses both vascular and lymphatic characteristics. FLT4 receptors are lymphatic markers expressed by SC cells. Developing strategies to promote nanocarrier interactions with FLT4 receptors offers a potential strategy for increasing their uptake by SC endothelial cells relative to blood vascular cells of the iris and ciliary body. While past studies detected FLT4 receptors on the surface of SC cells derived from normal...
human patients, it is unclear whether glaucoma alters the expression of these receptors. To determine whether FLT4 is a viable cell surface marker for targeting SC cells in patients with glaucoma, FLT4 expression was examined in two normal and two glaucomatous SC cell strains from human donors (Figure 1a and Table 1).

SC cells expressed FLT4 at significantly greater levels than HUVECs, a representative model of vascular endothelial cells, in both the normal and glaucomatous cell strains examined (Figure 1b,c). The quantification of FLT4 expression by flow cytometry (Figure 1b) was consistent with the FLT4 expression levels observed by widefield fluorescence microscopy (Figure 1c). While low FLT4 expression was detected on HUVECs by flow cytometry, this lower expression level is below the limit of detection of the less-sensitive fluorescence microscopy technique. The relatively high FLT4 expression by SC cells in the conventional outflow tissues, and its maintenance during glaucoma (Figure 1), suggests that FLT4 is available for targeting the delivery of IOP-reducing agents directly to SC cells in glaucoma patients.

**Figure 2.** PEG-b-PPS MCs displaying lipid-anchored FLT4-/VEGFR3-binding peptides that differ in the length of their PEG spacers. (a,b) Illustration of PEG-b-PPS MCs (a) and cryo-TEM of blank MCs (i.e., without peptide) (b). (c) MC characterization by SAXS. (d) Illustration of the designed FLT4-binding peptide constructs and LC−MS performed on the synthesized peptide products. (e) Deconvoluted mass spectra of purified peptides. Peaks are 2151.1, 3046.7, and 4071.4 Da for PG6, PG24, and PG48, respectively. (f) Cryo-TEM of MCs displaying the specified targeting peptides at a 5% molar ratio (peptide/polymer). (g-i) Characterization of MCs displaying FLT4-binding peptides at a 5% molar ratio. The magnification is 10,000×, and the scale bar is 100 nm for all cryo-TEM micrographs. In all cases, SAXS was performed using synchrotron radiation at the Argonne National Laboratory and a core-shell model was fit to the data. \( \chi^2 < 1.0 \) was obtained for all model fits (a good fit is indicated by \( \chi^2 < 1.0 \)).
The terminus of the PEG hydrophilic block (Figure 2a) humor proteins, which are present at a relatively low concentration, was therefore used to minimize the adsorption of aqueous carriers at a 1 or 5% molar ratio (peptide/polymer) and were prepared without peptide (Figure S2). FTIR spectra of purified peptide samples demonstrated that the methoxy surface chemistry was therefore used to minimize the adsorption of aqueous humor proteins, which are present at a relatively low concentration and are compositionally similar to plasma, since adsorbed protein layers would otherwise shield targeting ligands and hinder binding interactions. We hypothesized that the FLT4-binding peptides incorporating longer PEG spacers would more easily bind to the FLT4-binding receptors of the SC cells as the longer spacer would minimize obstruction by the 45-unit MC PEG corona. To test this hypothesis, peptide constructs were designed with 6- (PG6), 24- (PG24), or 48- (PG48) unit PEG spacers (Figures 2d and S1). Peptides were synthesized using standard Fmoc solid-phase peptide synthesis, and their size similarity across different formulations, is consistent with the expected MC morphology. These results further suggest that the nanocarrier morphology is not perturbed by the palmitoleic lipid anchor of the peptide.

Table 2. Physicochemical Characterization of PEG-b-PPS MCs Displaying FLT4-Targeting Peptide Constructs

<table>
<thead>
<tr>
<th>formulation</th>
<th>$D_{total}$ (nm)</th>
<th>PDI</th>
<th>$D_{total}$ (nm)</th>
<th>$R_{core}$ (nm)</th>
<th>shell thickness (nm)</th>
<th>$\chi^2$</th>
<th>zeta potential (mV)$^\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL MC</td>
<td>21.9</td>
<td>0.06</td>
<td>21.0</td>
<td>4.5</td>
<td>6.0</td>
<td>0.011</td>
<td>$-1.2 \pm 0.2$</td>
</tr>
<tr>
<td>MC + PG6 (1%)</td>
<td>21.8</td>
<td>0.05</td>
<td>21.2</td>
<td>6.3</td>
<td>4.3</td>
<td>0.007</td>
<td>$1.7 \pm 0.4$</td>
</tr>
<tr>
<td>MC + PG6 (5%)</td>
<td>21.5</td>
<td>0.06</td>
<td>20.9</td>
<td>6.1</td>
<td>4.3</td>
<td>0.008</td>
<td>$2.5 \pm 0.1$</td>
</tr>
<tr>
<td>MC + PG24 (1%)</td>
<td>20.4</td>
<td>0.07</td>
<td>21.2</td>
<td>6.1</td>
<td>4.5</td>
<td>0.006</td>
<td>$0.6 \pm 0.2$</td>
</tr>
<tr>
<td>MC + PG24 (5%)</td>
<td>21.6</td>
<td>0.08</td>
<td>21.4</td>
<td>6.1</td>
<td>4.6</td>
<td>0.014</td>
<td>$1.3 \pm 0.7$</td>
</tr>
<tr>
<td>MC + PG48 (1%)</td>
<td>20.7</td>
<td>0.07</td>
<td>23.0</td>
<td>6.3</td>
<td>5.2</td>
<td>0.019</td>
<td>$0.4 \pm 0.2$</td>
</tr>
<tr>
<td>MC + PG48 (5%)</td>
<td>22.4</td>
<td>0.05</td>
<td>24.2</td>
<td>6.1</td>
<td>6.0</td>
<td>0.012</td>
<td>$2.1 \pm 0.7$</td>
</tr>
</tbody>
</table>

$^\dagger$Number-average hydrodynamic diameter ($D_{total}$) and PDI measured by DLS. $^\beta$SAXS performed using synchrotron radiation at the Argonne National Laboratory. The fit values for the total diameter ($D_{total}$), thickness of the shell, and radius of the core ($R_{core}$) are displayed together with the chi squared ($\chi^2$) value for the final model fit. See the Experimental Section for details. $^\ddagger$Mean zeta potential ± s.d. (n = 3) measured by ELS.

While the incorporation of peptide did not disrupt the spherical morphology expected for PEG-b-PPS MCs, as demonstrated by morphological analysis using cryo-transmission electron microscopy (cryo-TEM; Figure 2f; see the Supporting Information for cryo-TEM procedures) and SAXS performed using synchrotron radiation (Figure 2g–i and Table 2). In cryo-TEM micrographs, PEG-b-PPS MCs appear as small dark circles in two dimensions. These visible structures are the high-resolution structural details. SAXS is not perturbed by the palmitoleic lipid anchor of the peptide.

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Evaluation of Peptide Biochemical Accessibility and Targeting SC Cells In Vitro. We hypothesized that targeting peptides designed with longer PEG spacers would minimize ligand obstruction by the MC PEG corona to biomolecules in the surrounding environment and that these accessibility enhancements would improve peptide binding with FLT4 receptors (Figure 1). To examine differences in peptide accessibility, we developed an MS-based proteolysis kinetic assay. This assay uses trypsin protease (23.3 kDa) as a biochemical probe (~5.8 nm diameter), which cleaves arginine (WHWLPNLHYAS) in the peptide sequence to induce mass shifts that are detectable in quenched reaction aliquots (Figure 3a). A crude proteolysis rate is determined by monitoring the ratio of intact versus cleaved peptide signal intensities with time. Comparing differences in proteolysis kinetics provides a means to examine how accessible peptide constructs are to biomolecules in their environment when displayed on nanocarriers.

When presented on MCs in the PG48 form, the FLT4-binding peptide substrate was cleaved to completion almost immediately, whereas buried PG6 and PG24 constructs were cleaved at much slower rates (Figure 3b). This result indicates that protease access to the peptide component of the PG6 and PG24 constructs is obstructed by the 45-unit PEG corona of the MC nanocarriers to an extent that depends on the depth it is buried. The ligand obstruction observed for PG6 and PG24 constructs further suggests the potential for suboptimal engagement with FLT4/VEGFR3 receptors on the surface of the SC cells.

To examine whether these differences in ligand accessibility translate into greater interactions with the SC cells, we performed nanocarrier uptake studies with cultured SC cells from normal human donors (target cell type) and HUVECs (vascular endothelial cell model; off-target cell type) (Figure 3c). Increasing peptide accessibility improved SC targeting in vitro. Compared to all other formulations, MC + PG48 (5%) significantly enhanced MC uptake by human SC cells and decreased uptake by HUVECs in vitro (Figure 3d). The surface-displayed PG48 FLT4-targeting peptide significantly increases MC uptake by human SC cells and decreases uptake by HUVECs in vitro (Figure 3d). The mean ± s.e.m. is displayed (n = 3). (f) SC-targeting specificity defined here as SC/MFI/HUVEC/MFI. For (d–f), statistical significance was determined by ANOVA with post hoc Tukey’s multiple comparison test and a 5% significance level. ****p < 0.0001, ***p < 0.01, **p < 0.05.
corona (Figure 3f) and a 4.5-fold enhancement in SC cell uptake over HUVEC (Figure 3f).

For MCs displaying the PG6 or PG24 constructs, the use of shorter PEG spacers together with the 12 amino acid FLT4-binding peptide obstructs biochemical access to the ligand (Figure 3b) and the extent of this obstruction is a function of ligand display depth within the MC PEG corona. Importantly, differences in ligand biochemical accessibility resulted in significant differences in SC cell-targeting performance (Figure 3d–f). The targeting performance of a nanocarrier measurably decreased with increasing ligand obstruction.

Performance Evaluation of PG48 versus PG6 on Enhancing the Micellar Delivery of IOP-Reducing Agents In Vivo. As our goal was to develop nanocarriers capable of targeting SC cells and delivering payloads that lower IOP, we sought to determine if the optimized nanocarriers displaying the FLT4-binding peptide above the surface would outperform our original, buried peptide construct (PG6) in a more clinically relevant setting. We were particularly interested in evaluating the performance of the original PG6 construct against the more highly targeted PG48 construct in the delivery of the IOP-reducing agent LatA in vivo. LatA is a 16-membered macrolide that acts by blocking the incorporation of actin monomers into actin filaments and thereby depolymerizing actin and lowering cell stiffness. We executed a longitudinal assessment of IOP in C57BL/6J mice receiving LatA MC displaying either PG6 or PG48 in contralateral eyes. Aside from the introduction of the optimized targeting construct, this study incorporated a paired design and greater statistical power than our previous analysis. Animal information and maintenance procedures can be found in the Supporting Information.

The baseline IOP was measured by tonometry prior to treatment. Significant differences were not found in the contralateral IOP measured at the baseline (OS: 19.9 ± 0.1 mmHg and OD: 19.9 ± 0.2 mmHg; Figure S7). Afterward, LatA MC + PG48 and LatA MC + PG6 were administered intracameral to contralateral eyes (i.e., one eye received one formulation type) (Figure 4a). IOP was measured by tonometry at 24, 30, 48, 72, and 96 h after injection (Figure 4a). Compared to baseline IOP values, both formulations led to a significant decrease in IOP at the 24 h timepoint, with an IOP reduction of 7.5 ± 0.9 mmHg in the LatA MC + PG48 treatment group and 7.1 ± 1.4 mmHg in the LatA MC + PG6 group (Figure 4b). Although the decrease in IOP in the PG48 group was greater at 24 h than that in the PG6 group, this difference was not statistically significant.

However, a notable divergence in the efficacy of the two competing formulations was observed after 24 h (Figure 4b). LatA-loaded MCs displaying the PG48 peptide sustained a reduction in IOP through 72 h and did not return to the baseline until day 4 post-treatment (Figure 4b), in contrast to the PG6 treatment group, in which the IOP returned to the baseline by 48 h (Figure 4b). Our paired statistical analysis demonstrated that the IOP of eyes treated with LatA MC + PG48 (18.0 ± 0.7 mmHg) was significantly lower than those treated with LatA MC + PG6 (20.3 ± 0.9 mmHg) at 48 h (Figure 4b). Furthermore, the IOP measured in eyes receiving LatA MC + PG48 was significantly lower than the baseline values through 48 h (i.e., at 24, 30, and 48 h) (Figure 4b). For eyes receiving LatA MC + PG6, significant differences from baseline IOP were only observed through 30 h (Figure 4b).

From these results, we conclude that the targeting performance of MCs displaying the PG48 FLT4-binding peptide is superior to that of MCs displaying the PG6 variant. This improved ability to target cell-softening agent delivery to the SC endothelium enhanced efficacy by achieving a prolonged reduction in IOP that was statistically significant through 48 h after a single injection.

Localization of the Optimal SC-Targeting Nanocarriers in the Murine Conventional Outflow Pathway. We next examined the localization of MCs displaying the PG48 peptide within the murine conventional outflow pathway (Figure 5a). We were particularly interested in the localization of PG48-displaying MCs at an early timepoint that would precede their observed effects in our longitudinal IOP study.
Glaucoma is a progressive disease that, if untreated, slowly but relentlessly destroys ganglion cells and robs vision. The lowering of IOP is the only treatment for glaucoma that is proven to decrease disease progression. Agents such as actin depolymerizers and Rho kinase inhibitors reduce cell stiffness and have been shown to lower the IOP when delivered to tissues of the aqueous humor outflow pathways. However, these agents have significant side effects that are associated with the off-target vasodilation of ocular surface vessels. Using optimized nanocarriers loaded with an actin-depolymerizing agent, LatA, we demonstrate here that we can target SC cells in a highly specific fashion using versatile nanocarriers and that these nanocarriers when loaded with a cell-softening agent significantly lower IOP in murine eyes for an extended period of time.

As the goal was to develop a nanocarrier that selectively targeted SC cells, it was necessary to demonstrate that the target, FLT4, is expressed at high levels not only on normal but also on glaucomatous SC cells (Figure 1). This indicated that promoting nanocarrier engagement with these receptors may provide a clinically useful approach to direct the delivery of cell-softening agents to the SC cells. These data also suggested that the disease state does not hinder the expression of FLT4 by SC cells.

The overarching goal of the present work was to develop an improved nanotechnology platform for targeting the delivery of cell-softening agents to the SC endothelium in order to lower IOP. Previous studies of LatA demonstrated promise in reducing IOP\(^{35,36}\) but did not lead to the development of a clinical product. Our previous biomechanical analyses using atomic force microscopy demonstrated that the untargeted micellar delivery of LatA softens SC cells\(^{in vitro}\),\(^{35}\) thereby confirming the successful lysosomal escape of an actin-depolymerizing agent following cellular internalization and validated PEG-b-PPS nanocarriers as appropriate vehicles for delivering cell-softening agents. In a separate set of studies, we demonstrated that the targeted micellar delivery of LatA (LatA MC + PG6) at a low concentration of 50 nM is sufficient to significantly reduce SC cell stiffness compared to that of the control \(in vitro\),\(^{34}\) and the targeted micellar delivery of 15.5−17 \(\mu\)M LatA using PG6 led to significant reductions in murine IOP for up to 24 h \(in vivo\) following intracameral injection.\(^{34}\)

To improve the efficacy of cell-softening agents delivered by SC-targeting MCs, we rationally designed variants of our modular lipid-anchored FLT4-binding peptide to achieve a greater influx of drug-loaded nanocarriers into the SC cells. We began this design process with the observation that our original

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**Figure 5.** Confocal microscopy of MC + PG48 localization within the murine conventional outflow pathway \(in vivo\). (a) Experimental illustration. DIL dye-loaded MC + PG48 (5%) nanocarriers (20 mg/mL polymer concentration) were administered intracamerally in C57BL/6J mice. After 45 min, eyes were enucleated and fixed for confocal microscopy analysis. (b,c) Images of conventional outflow pathway tissues obtained from the eyes of (b) the negative control group (no injection) and (c) the MC + PG48 treatment group. Images were acquired at 20× magnification. DAPI and Dil signals are overlaid to present the cell nuclei and nanocarrier localization, respectively. Scale bar = 50 \(\mu\)m. Tissue abbreviations: SC: Schlemm’s canal and TM: trabecular meshwork.

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SC-targeting construct,54 denoted in the present work as “PG6”, was likely buried beneath the MC surface where it is less accessible to FLT4 receptors. This design consideration is fairly unique to lipid-anchored targeting peptides embedded in amphiphilic nanocarriers since materials are more commonly prepared for targeting via the direct conjugation of ligand to the surface55 — a strategy that can encounter issues with a lack of modularity, usage of harsh chemical conditions, and control over display density among others.56,57

Understanding the relationship between the biochemical access of a lipid-anchored ligand and targeting performance became a focus of the present work since the use of targeting moieties to direct vehicles to the intended location requires not only (i) successful ligand docking with the cognate receptor, which is enabled by the specific arrangement of functional groups of the ligand with those of the receptor-binding pocket but also (ii) a lack of obstruction that hinders the formation of the ligand—receptor complex. The latter consideration permits successful binding events to occur with a greater frequency. Whereas the first requirement is fulfilled by the WHLPLNLHYAS peptide that was developed by phage display to specifically bind FLT4 receptors, the second requirement was not fully met since the use of the six-unit PEG spacer left the lipid-anchored peptide shrouded within the 45-unit PEG corona of the MCs that self-assemble from the PEG45-b-PPS19 polymer.

These considerations prompted our rational design of optimized targeting peptide constructs that differed in their PEG linker lengths and display depth on MCs (Figure 2). As a prerequisite for understanding the influence of these steric differences on targeting performance, we required an assay that is capable of probing peptide accessibility at the nanoscale. We sought to develop an assay that is high-throughput compatible and avoids the use of receptor proteins, which are notoriously difficult to purify in their native/active form and are expensive when commercially available. We reasoned that the accessibility of nanocarrier-displayed peptide to its cognate receptor would correlate with its susceptibility to proteolysis in vitro. Despite being very different in nature, receptor binding and protease-mediated cleavage both require physical access to the peptide ligand to initiate a successful interaction. It seemed plausible to quantify peptide accessibility by monitoring the occurrence of a peptide-modifying event with time. If the steric differences influenced peptide access, the frequency of a peptide-modifying event should decrease with greater obstruction by the MC PEG corona.

These considerations inspired our development of a novel method that uses trypsin as a biochemical probe. Trypsin acts on each peptide molecule only once since the peptide contains one arginine residue and lysine is absent. Cleavage of the peptide by trypsin produces irreversible mass shifts, and the proteolysis kinetics is monitored to quantify differences in the accessibility of lipid-anchored targeting peptides displayed on amphiphilic nanocarriers (Figure 3a). In this assay, we hypothesized that PG48 is displayed at the MC surface where it is most susceptible to proteolysis, thereby yielding peptide cleavage at a greater rate than the PG24 or PG6 peptides. PG24 and PG6 incorporate shorter PEG linkers (Figure 2), leaving these constructs buried to different extents within the outer PEG corona MCs that self-assemble from PEG45-b-PPS19.

In agreement with our hypothesis, the protease protection kinetic assay developed herein demonstrated that the use of 6-, 24-, or 48-unit PEG spacers achieves significant differences in biomolecular access to the peptide ligand (Figure 3a,b). These molecular-level differences in ligand access translated to differences in targeting specificity in vitro in studies conducted with primary cultures of SC cells obtained from human donors (Figure 3c–f). MCs displaying the most accessible targeting peptide, PG48, achieved the greatest targeting specificity for SC cells in these studies (Figure 3f). This specificity improvement resulted in both an increase in uptake by the SC cells (Figure 3d) and a decrease in uptake by vascular endothelial cells, a model of cells where uptake is not desirable (Figure 3e).

Our paired longitudinal IOP studies demonstrated that LatA-loaded MCs displaying the biochemically enhanced PG48 construct led to a 7.5 mmHg reduction in IOP, on average, after 24 h compared to 7.1 mmHg achieved by the treatment with the LatA MC + PG6 formulation (Figure 4b). While this change in IOP was not significantly different between the two groups at 24 h, the greater magnitude decrease observed after treatment with LatA MC + PG48 is consistent with a greater amount of drug reaching SC endothelium in vivo. Strikingly, mice receiving the optimized drug delivery vehicle maintained IOP reductions of 5.0, 1.9, and 1.5 mmHg at 30, 48, and 72 h, respectively, with the return to the baseline observed at 96 h postadministration (Figure 4b). The IOP reductions at 24, 30, and 48 h differed significantly from baseline levels for the PG48 group. In contrast, contralateral eyes receiving LatA MC + PG6 returned to the baseline by 48 h after achieving a significant decrease in IOP at 24 and 30 h (Figure 4b) — an outcome that is consistent with the transient IOP decrease observed in our past reports from two separate trials with this construct.54 Importantly, the IOP reduction in the PG48 group was significantly greater in magnitude than that in the PG6 group at the 48 h timepoint (Figure 4b). Collectively, these results demonstrate the superiority of the PG48 construct for targeting the micellar delivery of a cell-soothing glaucoma therapeutic to SC in the eye. The analysis by confocal microscopy demonstrated that MCs displaying the PG48 FLT4-binding peptide accumulate within SC in vivo (Figure 5c).

Enhancing the biochemical access to the FLT4-binding peptide leads to a greater accumulation of cell-soothing agents within the SC endothelium. Higher drug concentrations at the target site resulted in longer lasting improvements in outflow facility to yield an IOP reduction that is sustained for a longer period of time. We further conclude that MCs displaying the PG48 construct are the highest performing nanocarrier chassis for delivering IOP-lowering cell-soothing agents to SC. PEG-b-PPS MCs efficiently load diverse hydrophobic small molecules54.58 and will accommodate a wide variety of new and currently available therapeutics.59

Looking ahead, the practical challenges of sustaining the therapeutic effect of softening SC cells for a longer period of time remain to be addressed. As the corneal endothelium is permeable only to molecules with molecular weights less than approximately 500 Da,60,61 nanoparticles cannot be delivered to the anterior segment topically. Instead, these agents need to be either (i) injected to the anterior segment or retro-injected into the aqueous veins62 or (ii) released continuously from a long-term depot/device placed in the eye. While injection is plausible for targeted nanocarriers carrying gene therapy to SC, long-term release is required for pharmacological treatments.
such as LatA-carrying MCs as frequent intraocular injections would not be tolerated. Aside from the more conventional delivery of pharmacological payloads, our SC-targeting strategy can be extended to the development of gene delivery technologies. Glaucoma is an attractive target for gene therapy, and modulating the expression of genes that regulate the stiffness of SC endothelial cells may hold the key to producing more permanent enhancements in the outflow facility without stents or surgery. The lipid-anchored FLT4-binding peptides are modular and readily incorporate into alternative PEG-b-PPS morphologies, such as vesicular polymersomes or bicontinuous nanospheres, which are capable of encapsulating the hydrophilic cargoes necessary for genetic intervention. This includes regulatory RNAs for the transient modulation of gene expression or CRISPR/Cas9 components for stable genome editing.

**CONCLUSIONS**

In conclusion, we demonstrate that steric effects between surface-displayed ligands and PEG coronas significantly impact targeting performance across multiple biological scales. Our work holds general implications for the rational design of receptor-targeted nanocarriers and also addresses challenges that are unique to the display of modular ligands anchored to vehicles bearing a common hydrophilic corona (here, PEG2k). We further demonstrate that the assessment of differential proteolysis kinetics provides a powerful tool for quantifying the relative biochemical access of targeting ligand prototypes displayed on nanocarrier surfaces. The biochemical access of a targeting ligand is an engineerable property that can be leveraged to control nanocarrier engagement with receptors on the target cell type over a continuous domain. When applied to the development of glaucoma nanotherapies using our FLT4-targeting approach, improving the biochemical access of peptide ligands increased drug delivery to SC endothelial cells while minimizing off-target interactions with vascular endothelial cells. These performance improvements are achieved at the molecular and cellular levels translated to efficacy enhancements in a paired, longitudinal IOP study in vivo, thereby demonstrating the potential utility of our methods in clinically relevant settings. The technologies developed herein proved to be promising for improving the potency and specificity of cell-softening agents used to manage ocular hypertension. More generally, our rational design principles and methodology can be extended to the development of other targeted nanotherapies to solve diverse challenges in drug delivery.

**ASSOCIATED CONTENT**

1. **Supporting Information**

   The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsami.1c09294.

   Supplementary methods for DLS and ELS; cryo-TEM; FTIR spectroscopy; animals; confocal microscopy of ocular tissues; lipid-anchored FLT4-binding peptide constructs; FTIR spectroscopy spectra of purified MC formulations; fluorescence emission scan of FLT4-binding peptide compared to that of blank PEG-b-PPS MCs; targeting peptide calibration; SAXS of PEG-b-PPS MCs displaying FLT4-binding peptides at a 1% molar ratio; LatA concentration series for calibrating HPLC measurements; baseline IOP comparison between left and right eyes of C57BL/6J mice; PEG-b-PPS MC diblock copolymer; and FLT4-targeting peptide constructs (PDF).

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**Author Contributions**

M.J., E.A.S., W.D.S., G.L., and H.G. contributed to the writing of the manuscript. MPV created all figures and illustrations. All authors have given approval to the final version of the manuscript.

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**Notes**

The authors declare no competing financial interest.

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