Reducible PEG-POD/DNA Nanoparticles for Gene Transfer In Vitro and In Vivo: Application in a Mouse Model of Age-Related Macular Degeneration

Bhanu Chandar Dasari,1 Siobhan M. Cashman,1 and Rajendra Kumar-Singh1

1Department of Developmental, Molecular, and Chemical Biology, Program in Genetics, Sackler School of Graduate Biomedical Sciences, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111, USA

Non-viral gene delivery systems are being developed to address limitations of viral gene delivery. Many of these non-viral systems are modeled on the properties of viruses including cell surface binding, endocytosis, endosomal escape, and nuclear targeting. Most non-viral gene transfer systems exhibit little correlation between in vitro and in vivo efficiency, hampering a systematic approach to their development. Previously, we have described a 3.5 kDa peptide (peptide for ocular delivery [POD]) that targets cell surface sialic acid. When functionalized with polyethylene glycol (PEG) via a sulphydryl group on the N-terminal cysteine of POD, PEG-POD could compact plasmid DNA, forming 120- to 180-nm homogeneous nanoparticles. PEG-POD enabled modest gene transfer and rescue of retinal degeneration in vivo. Systematic investigation of different stages of gene transfer by PEG-POD nanoparticles was hampered by their inability to deliver genes in vitro. Herein, we describe functionalization of POD with PEG using a reducible orthopyridyl disulfide bond. These reducible nanoparticles enabled gene transfer in vitro while retaining their in vivo gene transfer properties. These reducible PEG-POD nanoparticles were utilized to deliver human FLT1 to the retina in vivo, achieving a 50% reduction in choroidal neovascularization in a murine model of age-related macular degeneration.

INTRODUCTION

During the previous decade, there has been significant progress in the field of gene therapy.1–11 Much of this has been fueled by the successful application of adeno-associated virus (AAV) as a gene transfer vector.12 Despite this progress, critical challenges to the application of AAV in gene therapy persist, namely a significant limited transgene capacity,13 immunotoxicity,14 and hepatotoxicity,15 as well as the cost of production and standardization of these novel biologics.10 To address the limitations of AAV and other viral vectors, a surge of interest in the development of non-viral gene transfer technologies has led to the identification of an expansive repertoire of polymers, peptides, and lipids with gene transfer capability.16 However, while these formulations lead to efficient gene transfer to cells in vitro, they have not generally been found to deliver transgenes efficiently in vivo, particularly to post-mitotic cells such as neurons in the brain or the retina.12,13

Similar to viral vectors, non-viral gene transfer vectors need to efficiently package DNA, dock at the cell surface, become endocytosed, escape the endosome, and enter the nucleus to enable transgene expression.14 A single non-viral agent that efficiently replicates all of these steps has thus far not been identified, and it is likely that a successful non-viral vector will require multiple components to overcome different aspects of the delivery process, from cell targeting to nuclear entry.15 Non-viral DNA delivery vectors will, therefore, need to be systematically assembled rather than identified. The process of non-viral vector development has been hampered by the lack of correlation of in vitro transfection data with that observed in vivo.12,15 and in-depth analysis of the interaction of non-viral vectors with each step of the delivery pathway, from cell surface to nucleus, is technically challenging in vivo.16 Bridging the gap between in vitro and in vivo gene transfer efficiency is, therefore, critical to the development of non-viral gene delivery systems. Previously, we have described a novel peptide known as peptide for ocular delivery (POD) that can efficiently compact DNA and enable gene transfer to cells in culture.17 These POD/DNA nanoparticles, however, failed to deliver DNA to post-mitotic cells in vivo.18 We determined that the addition of polyethylene glycol (PEG) to POD prior to complexing with DNA led to the formation of homogeneous nanoparticles of approximately 120–150 nm in size, not substantially larger than an adenovirus (~100 nm).19 We found that these PEGylated POD/DNA nanoparticles could now enable delivery of DNA to retinal cells of mice and attenuate the progression of light-induced apoptosis and ensuing retinal degeneration in a murine model of retinitis pigmentosa.18,19 Relative to viral vectors, however, the levels and duration of transgene expression are limited, needing further development before PEG-POD/DNA nanoparticles could be considered to have clinical potential. Continued development of PEG-POD/DNA nanoparticles, however, has been hampered by their inability to transfet cells in vitro.

Received 14 February 2017; accepted 8 June 2017;
http://dx.doi.org/10.1016/j.omtn.2017.06.004.
Correspondence: Rajendra Kumar-Singh, Department of Developmental, Molecular, and Chemical Biology, Program in Genetics, Sackler School of Graduate Biomedical Sciences, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111, USA.
E-mail: rajendra.kumar-singh@tufts.edu

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
In the current study, we describe the development of a reducible PEGylated POD/DNA nanoparticle that enables gene transfer in vitro and in vivo. This novel nanoparticle is subject to disruption of the PEG-POD bond by the extracellular environment. The reducible PEG-POD/DNA nanoparticle (PEG-SS-POD/DNA) has the same structure and DNA binding capacity as the previously described non-reducible PEGylated nanoparticle but exhibits efficient gene transfer in vitro without adversely affecting its transfection efficiency of the murine retina in vivo. The reducible PEG-SS-POD/DNA nanoparticles were used to deliver a transgene expressing soluble FLT1, an isoform of vascular endothelial growth factor (VEGF) receptor 1, to cells in culture and to murine retina, and were shown to significantly reduce growth of neovascular lesions in a murine model of choroidal neovascularization (CNV). These reducible PEG-SS-POD/DNA nanoparticles provide a critical step toward understanding the obstacles to intracellular trafficking using in vitro data that can be applied to post-mitotic cells in vivo.

RESULTS

Conjugation of PEG to POD Peptide with a Reducible Disulfide Bond

Previously we attached a 10 kDa PEG to the free sulfhydryl group on the N-terminal cysteine residue of POD using the maleimide group of methoxy-PEG-maleimide (mPEG-MALM). This PEG-POD conjugation, involving a thioether bond, is hereafter referred to as PEG-S-POD. To determine whether conjugation of PEG with POD using a reducible disulfide bond restores transfection of cells by PEG-POD/DNA nanoparticles in vitro, we attached PEG to POD using an orthopyridyl disulfide functionalized 10 kDa PEG (methoxy-PEG-orthopyridyl disulfide [mPEG-OPSS]). This PEG-POD we hereby refer to as PEG-SS-POD. Following purification on a size exclusion column, conjugation of PEG with POD was confirmed by PAGE (Figure 1A). Previously we have shown that the 3.5 kDa POD migrates at a position corresponding to ~17.5 kDa, likely because of formation of a pentamer. Consistent with addition of a 10 kDa PEG to the pentamer, PEG-SS-POD migrates at a position on the gel corresponding to ~27.5 kDa (Figure 1A). A band of reduced intensity migrating at ~37.5 kDa is also observed for PEG-SS-POD (Figure 1A), likely caused by the attachment of an additional PEG to the POD pentamer. When POD is conjugated with mPEG-OPSS, the majority of the PEG-SS-POD complex also migrates at ~27.5 kDa, with some of the PEG-SS-POD also observed migrating at ~37.5 kDa (Figure 1A).

To compare the efficiency of PEG conjugation with POD using mPEG-OPSS instead of mPEG-MALM, we employed Ellman’s reagent/dithionitrobenzoic acid (DTNB) to measure free sulfhydryl groups in each reaction (Figure 1B). Sulfhydryl quantitation revealed no free sulfhydryl groups in either the PEG-SS-POD or PEG-SS-POD reactions, indicating that PEG conjugation occurs with comparable efficiencies using mPEG-MALM or mPEG-OPSS (Figure 1B).
To determine the capacity for reduction of the disulfide bond in the PEG-SS-POD, unconjugated POD, PEG-S-POD, and PEG-SS-POD were incubated with the reducing agent, β-mercaptoethanol, and samples were run on a SDS-polyacrylamide gel (Figure 1C). Of the three samples tested, only PEG-SS-POD exhibited a reduction in the ratio of the 27.5 kDa to 17.5 kDa band, corresponding to the presence of β-mercaptoethanol (Figure 1C). This confirms that the disulfide bond of PEG-SS-POD can be reduced, releasing the POD peptide.

The shape and size of peptide/DNA nanoparticles have been found to depend on whether the peptide is equilibrated with trifluoroacetic acid (TFA) or with ammonium acetate (AmAc) prior to DNA compaction. However, we have not observed a difference in nanoparticle structure when PEG-S-POD was equilibrated in either buffer (data not shown). However, TFA has been found to inhibit reduction of the disulfide bonds. 

To determine whether the use of either TFA or AmAc as a counterion for equilibration of PEG-SS-POD could inhibit reduction of the disulfide bond, we incubated PEG-SS-POD equilibrated in each buffer with β-mercaptoethanol (Figure 1D). Indeed, we observed that the disulfide bond of PEG-SS-POD (~27.5 kDa) was more efficiently reduced, releasing unconjugated POD pentamer (~17.5 kDa), when suspended in AmAc buffer than when suspended in TFA (Figure 1D). Quantitation of the intensity of the ~27.5 kDa band corresponding to PEG-SS-POD equilibrated in either TFA or AmAc in the presence or absence of β-mercaptoethanol confirmed the higher efficiency of reduction of the disulfide bond in AmAc (p < 0.05; Figure 1E).

PEG-SS-POD Does Not Alter the Efficiency or Size of Compacted DNA Nanoparticles

Plasmid DNA encoding luciferase, pCAGLuc, was compacted using PEG-SS-POD in a nitrogen:phosphate (N:P) ratio of 8:1. Plasmid DNA compaction by PEG-SS-POD was confirmed by an inability of the compacted plasmid to migrate in an agarose gel (Figure 2A), because of neutralization of negatively charged DNA by positively charged POD peptide. Incubation of the PEG-SS-POD compacted pCAGLuc plasmid in trypsin prior to gel analysis released the charged POD peptide. Pre-incubation of the nanoparticles with trypsin (T) to digest the POD releases the pCAGLuc plasmid for migration in the gel. (B) Analysis of nanoparticle size and zeta potential by dynamic light scatter shows that PEG-S-POD/pCAGLuc and PEG-SS-POD/pCAGLuc nanoparticles are similar in size (nm, mean ± SD) and colloidial stability (mV, mean ± SD). (C) Quantitation of luciferase activity in extracts of ARPE-19 cells at 24 hr following incubation with either uncompacted pCAGLuc, PEG-S-POD/pCAGLuc nanoparticles, or PEG-SS-POD/pCAGLuc nanoparticles shows a significant (p = 0.002) 2,800-fold increase in luciferase activity in cells treated with PEG-SS-POD/pCAGLuc nanoparticles relative to that of cells treated with PEG-S-POD/pCAGLuc nanoparticles. (D) Quantitation of luciferase activity in extracts of ARPE-19 cells following incubation with PEG-SS-POD/pCAGLuc nanoparticles containing either linearized plasmid or circular plasmid shows an 85-fold (p = 0.002) lower transfection efficiency when PEG-SS-POD is used to deliver linear plasmid than when used to deliver circular plasmid. (E) LDH assay of ARPE-19 cells incubated with PEG-SS-POD/pCAGLuc nanoparticles indicates low levels of cytotoxicity when nanoparticles contain either linearized (Lin) or circular (Circ) pCAGLuc plasmid. Data are presented as the percentage of dead cells relative to cells treated with the detergent, Triton X-100, which causes 100% cell lysis. Data are presented as mean ± SE, except where otherwise stated. N, naked (uncompacted) DNA; RLU, relative luciferase unit. **p ≤ 0.01.
**PEG-SS-POD Nanoparticles Mediate Greater In Vitro Gene Transfer Than PEG-S-POD Particles**

To determine whether use of the disulfide bond for conjugation of PEG to POD allowed for more efficient cellular uptake and expression of plasmid DNA than conjugation of PEG with POD using a thioether bond, we incubated ARPE-19 cells in 200 ng of uncompacted pCAGLuc, 200 ng of plasmid in PEG-SS-POD/pCAGLuc nanoparticles, or 200 ng of plasmid in PEG-S-POD/pCAGLuc nanoparticles. Twenty-four hours following incubation, the ARPE-19 cells were harvested for quantification of luciferase activity (Figure 2C). As was previously observed, PEG-S-POD/pCAGLuc-incubated cells exhibited no significant difference in luciferase activity relative to those cells incubated with uncompacted pCAGLuc (Figure 2C). However, cells incubated with PEG-SS-POD/pCAGLuc exhibited an ~2,800-fold increase in luciferase activity relative to PEG-S-POD/pCAGLuc-treated cells (p = 0.002; Figure 2C).

Plasmid DNA can exist as one of three different topological forms: supercoiled, relaxed circular, and linear. It has been found that these different forms transfect mammalian cells with different levels of efficiency. In order to determine whether transfection by PEG-SS-PEG/pCAGLuc nanoparticles is influenced by DNA topology, both non-linearized (circular) and linearized pCAGLuc plasmid was compacted using PEG-SS-POD, and the resulting nanoparticles were used to transfect ARPE-19 cells. At 24 hr following transfection, cells were harvested for quantification of luciferase activity (Figure 2D). Luciferase activity in cells incubated in PEG-SS-POD/pCAGLuc (linear plasmid) was observed to be 85-fold (p = 0.002) lower relative to cells incubated in PEG-SS-POD/pCAGLuc (circular plasmid) (Figure 2D).

The efficiency with which a reagent transfects cells is generally directly correlated with the amount of cellular toxicity elicited. To determine whether this is also the case for PEG-SS-POD/pCAGLuc nanoparticles, we collected media from ARPE-19 cells transfected with either PEG-SS-POD/pCAGLuc (circular plasmid) nanoparticles or PEG-SS-POD/pCAGLuc (linear plasmid) nanoparticles at 24 hr following incubation in nanoparticles and measured for activity of lactate dehydrogenase (LDH). The amount of toxicity of nanoparticle-treated cells is presented (Figure 2E) as a percentage relative to that of cells treated with the detergent, Triton X-100, in which the LDH activity was considered to represent 100% toxicity. The percentage toxicity observed in both PEG-SS-POD/pCAGLuc (linear plasmid) nanoparticle-incubated cells and cells incubated with PEG-SS-POD/pCAGLuc (circular plasmid) nanoparticles was not observed to be significantly above that of untransfected cells not exposed to nanoparticles (Figure 2E), indicating that the increased transfection efficiency of circular DNA by PEG-SS-POD is not a result of increased toxicity.

**Increased Transfection by PEG-SS-POD/DNA Nanoparticles Is the Result of Increased Uptake by Cells In Vitro**

Although PEG prevents large aggregate formation in nanoparticle formulations, it is speculated that it can reduce the efficiency with which the attached moiety (in this case, POD) binds its receptor on the cell surface. It has previously been found that the cell culture media provides a reducing environment for disruption of disulfide bonds. To determine whether this could allow for improved transfection efficiency by PEG-SS-POD/pCAGLuc nanoparticles in ARPE-19 cells relative to PEG-S-POD/pCAGLuc nanoparticles because of increased uptake of the PEG-SS-POD/pCAGLuc nanoparticles by the cell, pCAGLuc was labeled with a fluorophore (Cy5) and compacted using either PEG-SS-POD or PEG-S-POD. The resulting nanoparticles were tested for compaction and DNA concentration (data not shown), and added to ARPE-19 cells using equivalent amounts of labeled pCAGLuc. Cells exposed to either PEG-SS-POD/pCAGLucCy5 nanoparticles or PEG-S-POD/pCAGLucCy5 nanoparticles were incubated with trypsin at two different time points postexposure, and the cells were resuspended for analysis of Cy5 fluorescence by flow cytometry (Figure 3). At 2 hr (data not shown) and 24 hr (Figure 3) postexposure, cells incubated with uncompacted Cy5-labeled luciferase plasmid indicated very few Cy5-positive cells (1.38%). Of those cells incubated with Cy5-labeled luciferase plasmid delivered using Lipofectamine, 34.5% were positive for Cy5 fluorescence (Figure 3). At 2 hr and 24 hr postexposure, cells treated with PEG-S-POD/pCAGLucCy5 nanoparticles had 0.08% and 2.1% of cells, respectively, positive for Cy5 signal (Figure 3). Among the cells incubated with PEG-SS-POD/pCAGLucCy5 nanoparticles, however, 23.8% of cells exhibited Cy5 fluorescence at 2 hr postexposure (Figure 3). At 24 hr postexposure, the number of ARPE-19 cells treated with PEG-SS-POD/pCAGLucCy5 nanoparticles that indicated Cy5 fluorescence was observed to be 67.4% of the cell population (Figure 3). These data are consistent with the luciferase expression data presented above (Figure 2C) and consistent with the hypothesis that PEG-SS-POD/pCAGLuc nanoparticles are more efficient than PEG-S-POD/pCAGLuc nanoparticles at binding the cell surface for uptake.

**The Disulfide Bond in PEG-SS-POD Nanoparticles Is Reduced in the Extracellular Environment**

The increased transfection efficiency of PEG-SS-POD/pCAGLuc nanoparticles in vitro relative to that of PEG-S-POD/pCAGLuc nanoparticles is consistent with reduction of the disulfide bond in the extracellular environment, release of the PEG moiety, and increased accessibility of the POD for attachment to the cell surface. To determine whether extracellular release of PEG occurs, we quantified the amount of both PEG and POD remaining in the cell medium at 4 hr following incubation of ARPE-19 cells with either PEG-SS-POD/pCAGLuc nanoparticles or PEG-S-POD/pCAGLuc nanoparticles. The medium collected after 4 hr of incubation of cells with nanoparticles was analyzed by SDS-PAGE (Figure 4). To detect POD peptide, we stained the gel with Coomassie blue (Figure 4A). A positive control was included on the gel, consisting of the PEG-SS-POD/pCAGLuc nanoparticles that had been pre-incubated with the reactive sulfhydryl donor, cysteine (Figure 4A). As anticipated, PEG-SS-POD/pCAGLuc nanoparticles pre-incubated with cysteine exhibited a band migrating at ~17.5 kDa, consistent with dePEGylated POD peptide. Neither of the unconjugated PEG reagents, mPEG-OPSS nor mPEG-MALM, stained positive with Coomassie blue.
blue, and there was no band detected in media from control (untrans- 
fected) cells (Figure 4A). The media harvested from cells incubated 
with PEG-S-POD/pCAGLuc nanoparticles for 4 hr exhibited a 
band migrating at \( \sim 27.5 \text{ kDa} \), consistent with a PEGylated POD com-
plex (Figure 4A). However, in the media harvested from cells incu-
bated with PEG-SS-POD/pCAGLuc nanoparticles for 4 hr, there 
was no band observed consistent with either PEGylated (27.5 kDa) 
or dePEGylated (17.5 kDa) POD. These data indicate ef-
cient release 
of PEG from PEG-SS-POD/pCAGLuc nanoparticles in the extracel-
ular environment and efficient uptake of the dePEGylated POD 
nanoparticles (Figure 4A).

In order to detect PEG, we performed a second SDS-PAGE and 
stained with 0.1 N iodine (Figure 4B). As above, unconjugated PEG 
reagents, mPEG-MALM and mPEG-OPSS, were used as controls; 
both reagents migrated between 15 and 20 kDa, with the slight differ-
ence in migration patterns likely because of the OPSS and MALM side 
chains. PEG-SS-POD/pCAGLuc nanoparticles pre-incubated with 
cysteine were also analyzed on the iodine-stained gel; in this lane, a 
band was observed at \( \sim 17 \text{ kDa} \), similar in size to that observed for 
mPEG-OPSS (Figure 4B). Media collected after 4 hr from cells incu-
bated with PEG-S-POD/pCAGLuc nanoparticles exhibited two bands 
on the gel: one band migrating at \( \sim 17 \text{ kDa} \), consistent with free PEG, 
and a second band at \( \sim 25 \text{ kDa} \), consistent with PEGylated POD (Fig-
ure 4B). That some of the PEG conjugated via a thioether bond is 
released from the PEG-S-POD/pCAGLuc nanoparticles is not unexpected; 
considering the increased transfection efficiency of ARPE-19 cells by PEG-
SS-POD/pCAGLuc nanoparticles. As expected, no bands were detected in media collected from control 
(untransfected) cells.

In order to determine whether inhibition of thiols in the extracellular 
environment would adversely affect the efficiency of transfection of 
cells by PEG-SS-POD/pCAG-Luc nanoparticles, we added cell-
permeable DTNB to the media of ARPE-19 cells and incubated 
them for 30 min prior to addition of PEG-SS-POD/pCAGLuc nano-
particles. At 24 hr posttreatment with nanoparticles, the ARPE-19 
cells were harvested for quanti-
fication of luciferase activity (Fig-
ure 4C). ARPE-19 cells cultured in media exposed to a 1 mM concen-
tration of DTNB prior to treatment with nanoparticles did not reveal 
any alteration in luciferase activity relative to cells cultured in media 
that were not exposed to DTNB prior to addition of PEG-SS-POD/pCAGLuc nano-
particles. At 24 hr posttreatment with nanoparticles, the ARPE-19 
cells were harvested for quantification of luciferase activity (Fig-
ure 4C). ARPE-19 cells cultured in media exposed to a 1 mM concen-
tration of DTNB prior to treatment with nanoparticles did not reveal 
any alteration in luciferase activity relative to cells cultured in media 
that were not exposed to DTNB prior to addition of nanoparticles 
(Figure 4C). However, ARPE-19 cells cultured in media pre-exposed 
to 3 mM DTNB had a significant 85% (p < 0.05) reduction in lucif-
erase activity following incubation with PEG-SS-POD/pCAGLuc nano-
particles relative to cells grown in media not exposed to 
DTNB; this strongly indicates an extracellular reduction of the disul-
fitide bond of PEG-SS-POD/pCAGLuc nanoparticles by thiols in the 
media as a key mechanism of the increased transfection efficiency 
of ARPE-19 cells by PEG-SS-POD/pCAGLuc nanoparticles.

**PEG-SS-POD/DNA Nanoparticles Can Deliver Plasmid DNA to 
the Murine Retina In Vivo**

Previously we have shown delivery of plasmid to retinal pigment 
epithelium (RPE) cells in vivo by PEG-S-POD/DNA nanoparticles 
following subretinal injection. In order to determine whether
Figure 4. PEG Is Released from PEG-SS-POD in the Extracellular Environment

(A) SDS-PAGE analysis of media harvested from cells 4 hr after incubation with PEG-SS-POD/pCAGLuc or PEG-S-POD/pCAGLuc nanoparticles. Coomassie blue staining of gel detects PEGylated and free POD peptide. Unconjugated PEG does not stain, as evidenced by loading of PEG-MALM and PEG-OPSS. PEG-SS-POD/pCAGLuc nanoparticles pre-incubated with cysteine (Cys) were used as a positive control for POD peptide release from conjugate following reduction of disulfide bond; this band is observed to migrate as expected for POD pentamer. Media from cells treated with PEG-S-POD/pCAGLuc nanoparticles show two bands, one migrating at ~17 kDa and the other band migrating at ~25 kDa, consistent with free PEG. The absence of a band which migrates at ~17 kDa, consistent with free PEG. The absence of a band which migrates at ~17 kDa, consistent with free PEG. The absence of a band migrating at 25 kDa is consistent with the observations of the Coomassie-stained gel above. (B) SDS-PAGE analysis of the same samples described in (A); this gel, however, was stained with iodine solution for detection of PEG. The free PEG reagents, PEG-OPSS and PEG-MALM, migrate at ~17 kDa. PEG-SS-POD/pCAGLuc nanoparticles pre-incubated with cysteine (Cys) show a similar 17 kDa band, indicating reduction of the disulfide bond and release of PEG from the nanoparticle. Media from cells treated with PEG-S-POD/pCAGLuc nanoparticles show two bands, one migrating at ~17 kDa and the other band migrating at ~25 kDa, indicating the presence of both free PEG and PEG-S-POD. Media from cells treated with PEG-SS-POD/pCAGLuc nanoparticles shows only one band, which migrates at ~17 kDa, consistent with free PEG. The absence of a band migrating at 25 kDa is consistent with the observations of the Coomassie-stained gel above. (C) Quantitation of luciferase activity in extracts of ARPE-19 cells incubated in the presence of 0, 1, and 3 mM cell-impermeable DTNB prior to transfection with PEG-SS-POD/pCAGLuc nanoparticles. A significant 85% reduction (*p < 0.05) in transfection efficiency was observed when cells were pre-incubated with 3 mM DTNB prior to addition of nanoparticles. Data are presented as mean ± SE.

Our data also suggest that either reduction of the disulfide bond to release PEG from the PEG-SS-POD/pCAGLuc nanoparticles does not occur as efficiently in the in vivo extracellular environment as in the in vitro environment, or that PEG “shielding” of the nanoparticle is not the major limiting factor to transfection in vivo. Once the initial barrier to cell entry imposed by the plasma membrane is overcome, the nanoparticle is faced with a new challenge in encountering the nuclear membrane. A critical difference between ARPE-19 cells in vitro and RPE cells of the mouse eye is that the ARPE-19 cells are actively dividing with concomitant breakdown of the nuclear membrane, likely allowing access of the plasmid DNA and/or nanoparticle to the nucleus. RPE cells of the adult murine eye are largely mitotically quiescent, providing no such easy access to the nucleus. Injury to the retina, however, has been shown to induce RPE cell proliferation through the process of epithelial-to-mesenchymal transition (EMT); one such injury is laser-induced photocoagulation of murine RPE.

In order to determine whether transfection of RPE in vivo by PEG-SS-POD/pCAGLuc nanoparticles could be enhanced by induction of RPE cell division by laser-induced photocoagulation, we injected C57BL/6J mice in the subretinal space with pCAGLuc nanoparticles pre-incubated with cysteine (Cys). Following quantification, eyes injected with PEG-SS-POD/pCAGLuc nanoparticles following laser treatment exhibited a 239% increase (p = 0.008) in luciferase activity relative to eyes injected with uncompacted plasmid. These data indicate that conjugation of PEG to the nanoparticle via a reducible linker does not adversely affect gene transfer in vivo.

In an attempt to determine the cell type transfected by PEG-SS-POD/DNA nanoparticles in laser-treated mice, a lacZ-expressing plasmid
(pCAGLacZ) was compacted using PEG-SS-POD and injected into the subretinal space of mice immediately following laser treatment. At 48 hr post-laser treatment, injected eyes were harvested for in situ enzyme assay for lacZ activity in the whole eyecup, followed by cryosectioning (Figure 5C). In PEG-SS-POD/pCAGLacZ-injected eyes, occasional lacZ-positive cells were observed only within the region of laser damage (Figure 5C1, arrow). The scarcity of lacZ-positive cells observed was not consistent with the increase in luciferase activity observed in laser-treated eyes relative to that of untreated mice, ***p < 0.01. (C) Retinal cryosections of eyes injected with either PEG-SS-POD/pCAGLacZ nanoparticles or uncompacted pCAGLacZ plasmid following laser treatment. (C1) Retinal section of PEG-SS-POD/pCAGLacZ-injected eye shows lacZ-positive cell (arrow) in the choroid. Closed arrowheads mark the single RPE cell layer, whereas open arrowheads denote the area of disruption of the RPE cell layer. Dotted line denotes border between RPE and choroid. (C2) Higher magnification of lacZ-positive cell (arrow) shows more clearly that it occurs within the choroid and not below the boundary with the RPE (denoted by the dotted line). (C3) Further magnification confirms the absence of pigment in the lacZ-positive cell, excluding the likelihood of transfection of a choroidal melanocyte. (C4) Retinal section of eye injected with uncompacted pCAGLacZ plasmid shows the absence of lacZ-positive cells in the choroid or in the RPE. Closed arrowheads mark the single RPE cell layer, whereas open arrowheads denote the area of disruption of the RPE cell layer. n = 5 eyes for each treatment. Data are presented as mean ± SE.

**PEG-SS-POD-Mediated Delivery of Plasmid Expressing Soluble FLT1 Ameliorates Laser-Induced CNV**

In addition to causing RPE cell proliferation, laser photocoagulation of the murine RPE induces proliferation of endothelial cells, resulting in CNV through increased expression of VEGF. Laser-induced CNV is the most commonly utilized animal model of the “wet” form of age-related macular degeneration, the most common cause of blindness among people over the age of 65 years. In order to determine whether the transfection efficiency of PEG-SS-POD/DNA nanoparticles following laser photocoagulation is therapeutically relevant, we compacted a plasmid expressing human soluble FLT1 (hFLT1) using PEG-SS-POD and injected it into the subretinal space of C57BL/6 mice immediately following laser treatment, and the effect on growth of CNV was quantified. The plasmid employed, pCAGhFLT1, was generated to express hFLT1 from a chicken b-actin promoter/CMV enhancer. Prior to compaction, expression of hFLT1 from pCAGhFLT1 was confirmed by western blot analysis of media harvested from ARPE-19 cells at 24 hr postinfection with either pCAGhFLT1 or a control plasmid, pCAGLuc (Figure 6A). Media from pCAGhFLT1 transfected cells showed a specific band at ~120 kDa (Figure 6A), consistent with previous analyses of glycosylated soluble FLT1. Media from untransfected cells and cells transfected with pCAGLuc exhibited no detectable bands. Cells transfected with pUNO1-hFLT1(s7) were used as a positive control (Figure 6A) for expression of hFLT1. Compaction of pCAGhFLT1 with
PEG-SS-POD was confirmed by an agarose gel retardation analysis (Figure 6B). Pre-incubation of the nanoparticles with trypsin prior to gel electrophoresis released the pCAGhFLT1 for migration in the gel (Figure 6B). Following confirmation of the integrity of the plasmid and nanoparticles, C57BL/6 mice were administered laser treatment and immediately injected in the subretinal space with either PEG-SS-POD/pCAGhFLT1 or PEG-SS-POD/pCAGLuc nanoparticles. At 2 days after laser followed by injection of the nanoparticles, the posterior eyecups of some of the mice were harvested for analysis of hFLT1 expression (Figure 6C). qRT-PCR of extracted RNA showed a significant (p = 0.003) level of expression of hFLT1 mRNA, as measured by the number of copies of hFLT1 mRNA, in PEG-SS-POD/pCAGhFLT1-injected eyes after laser relative to control injected eyes (Figure 6C). Seven days following injection, eyes of the remaining mice were harvested and the size of CNV growth quantified by staining of RPE/choroid with the endothelial-specific lectin, Griffonia Simplicifolia lectin I (GSL I; Figures 6D and 6E). Following quantification of the CNV area, eyes injected with PEG-SS-POD/pCAGhFLT1 nanoparticles exhibited an ~50% reduction in CNV area relative to eyes injected with PEG-SS-POD/pCAGLuc nanoparticles (p = 0.007).

DISCUSSION

Previously, we have found that PEGylation of POD/DNA nanoparticles was necessary for delivery of the nanoparticle to RPE cells in vivo.18 Conjugation of PEG to the nanoparticle, however, abolished its capacity for transfection of cells in vitro.18 Herein, we discovered that use of a reducible PEG-POD/DNA nanoparticle (PEG-SS-POD), in which PEG is attached to the nanoparticle via a disulfide linker, allowed for a very significant increase in transfection of ARPE-19 cells in vitro relative to a non-reducible PEGylated POD/DNA nanoparticle (PEG-S-POD), without adversely affecting gene transfer efficiency to the murine eye in vivo. This reducible PEG-SS-POD/DNA nanoparticle should provide a valuable tool to study intracellular trafficking of the relevant POD/DNA complex in cells in vitro.

Importantly, our data demonstrate that the increased transfection efficiency of PEG-SS-POD/DNA relative to that of PEG-S-POD/DNA is unlikely due to alterations in nanoparticle structure—i.e., size of the particle or DNA-condensing capacity of the PEG-SS-POD—or to an increase in cellular toxicity of the PEG-SS-POD/DNA nanoparticle. The increased in vitro transfection by the PEG-SS-POD/DNA relative to PEG-S-POD/DNA nanoparticles was found to be coincident with increased cellular uptake of the reducible nanoparticle by ARPE-19 cells. A reduction of ARPE-19 cell transfection by PEG-SS-POD/DNA nanoparticles was found to be coincident with increased cellular toxicity of the PEG-SS-POD/DNA nanoparticle. The increased in vitro transfection by the PEG-SS-POD/DNA relative to PEG-S-POD/DNA nanoparticles was found to be coincident with increased cellular uptake of the reducible nanoparticle by ARPE-19 cells. A reduction of ARPE-19 cell transfection by PEG-SS-POD/DNA nanoparticles was found to be coincident with increased cellular toxicity of the PEG-SS-POD/DNA nanoparticle. The increased in vitro transfection by the PEG-SS-POD/DNA relative to PEG-S-POD/DNA nanoparticles was found to be coincident with increased cellular uptake of the reducible nanoparticle by ARPE-19 cells.

These data are consistent with a study investigating the effect of a reducible PEG conjugation on DNA delivery using polylysine, CK30.19 That study found a 10-fold increase in transfection efficiency of the reducible nanoparticle was very likely due exclusively to the release of PEG by thiols in the extracellular rather than intracellular

![Figure 6. Subretinal Delivery of PEG-SS-POD/pCAGhFLT1 Nanoparticles Reduces CNV](image)

(A) Western blot analysis of hFLT1 in media of untransfected cells or cells transfected with either pCAGhFLT1, pCAGLuc, or pUNO-hFLT1 (α(7)) shows the presence of a unique band migrating at ~120 kDa, consistent with glycosylated hFLT1, in media of cells transfected with hFLT1 encoding plasmids, but not in media of controls. (B) Agarose gel retardation analysis of the compaction of pCAGhFLT1 by PEG-SS-POD. Uncompacted pCAGhFLT1 loading as a control. PEG-SS-POD/pCAGhFLT1 nanoparticles show retardation in the gel, consistent with efficient compaction. Pre-incubation of the nanoparticles with trypsin (T) releases the pCAGhFLT1 plasmid for migration in the gel. (C) qRT-PCR of FLT1 expression in murine eyecups at 2 days after laser and injection of PEG-SS-POD/pCAGhFLT1 nanoparticles shows a significant presence of FLT1 mRNA. Copy number of mRNA was determined using a standard curve of predetermined copy number of pCAGhFLT1. (D) Quantitation of the area of CNV in eyecups of mice at 7 days following injection of either PEG-SS-POD/pCAGhFLT1 nanoparticles or control (PEG-SS-POD/pCAGLuc) nanoparticles shows a significant ~50% reduction (p = 0.007) in the size of CNV in mice injected with PEG-SS-POD/pCAGhFLT1 nanoparticles relative to CNV size of control injected mice. n = 70 spots for naked plasmid injection; n = 68 spots for PEG-SS-POD/pCAGEN-hFLT1 injections. (E) Representative micrographs of CNV lesions stained with FITC-GSL I from eyecups of mice injected with either PEG-SS-POD/pCAGhFLT1 nanoparticles or PEG-SS-POD/pCAGLuc nanoparticles. Data are presented as mean ± SE. **p ≤ 0.01.
environment and subsequent aggregation of nanoparticles on the cell surface. To our knowledge, such nanoparticles have not yet been tested in vivo.

In our study, the reducible linkage in PEG-SS-POD did not impact transfection by PEG-SS-POD/DNA nanoparticles of murine RPE in vivo. While this is possibly due to differences in redox potential between the in vivo and in vitro extracellular environments, it has been found that cells grown in vitro condition the media such that the redox state is similar to that of the extracellular matrix in vivo. It is, therefore, more likely that the difference in performance of the reducible nanoparticles in vitro versus in vivo is due to the mitotic status of the RPE cells. To date, nanoparticles conjugated with PEG via a disulfide linkage have only been delivered to mitotic cells in vivo. When liposomes conjugated with the reducible linker were delivered intravenously to a murine model of B cell lymphoma, the “reducible” liposomes had an improved therapeutic efficacy relative to “non-reducible” liposomes, despite a reduced stability in the plasma, likely because of PEG release by blood components such as cysteine. In vivo gene transfer to a murine model of glioblastoma using a reducible PEGylated polymer was also increased following intravenous injection, albeit a “modest” increase, relative to that of the non-reducible PEGylated polymer; the modest increase is likely also due to increased clearance of the reducible polymer in the plasma.

In the case of subretinal injection, however, nanoparticles are delivered adjacent to the RPE and, therefore, stability of nanoparticles in plasma is not a significant concern. That the PEG-S-POD/DNA and PEG-SS-POD/DNA nanoparticles exhibit equivalent competency in gene transfer to the murine eye following subretinal injection suggests that release of PEG is not a critical factor for subretinal delivery to RPE cells. Changing the proliferative status of cells in the choroid and RPE in vivo by laser photocoagulation resulted in a significant increase in transfection by PEG-SS-POD/DNA nanoparticles, suggesting that the nuclear membrane is a more significant barrier than the plasma membrane to DNA delivery in vivo. This increase in transfection efficiency by the reducible nanoparticles is therapeutically relevant and was sufficient to reduce the growth of neovascular lesions in the laser-induced CNV, a murine model for the wet form of AMD.

Laser photocoagulation induces its effects through absorption of energy by melanin in the choroidal melanocytes and RPE cells; the melanocytes and RPE cells are damaged by the heat that ensues, inducing a burst of cellular proliferation among surrounding RPE cells, as well as vascular endothelial cells in the choroid and macrophages, and microglia in the retina and choroid. Following subretinal injection of PEG-SS-POD/pCAGLuc nanoparticles in lasered mice, a significant increase in luciferase-positive cells was observed relative to non-lasered mice. It is possible that, similar to other studies investigating reducible nanoparticles in vivo, extracellular release of PEG from the PEG-SS-POD/DNA nanoparticles in the laser-damaged choroid increases susceptibility of the dePEGylated particles to uptake by infiltrating macrophages and/or microglia. The absence of pigment in the lacZ-positive cells, as well as their location in the choroid, of lasered mice injected with PEG-SS-POD/DNA nanoparticles is consistent with uptake of the nanoparticles by either macrophages or microglia. The possibility of transfection of endothelial cells by these particles was excluded by the absence of staining of lacZ-positive cells with GSL I (data not shown).

Considering the efficiency with which PEG-SS-POD/DNA nanoparticles reduce the size of CNV lesions in laser-treated mice, however, we consider it likely that the number of cells observed positive for lacZ activity in PEG-SS-POD/pCAGlacZ-injected eyes is an underestimate. Previous studies indicate that subretinal injection of an AAV-8 vector expressing soluble FLT1 prior to laser treatment of mice resulted in an ~38% reduction in the size of CNV lesions.

Considering the efficiency of PEG and photoreceptor transduction by AAV-8 shown in the study, relative to the efficiency of ocular transfection of PEG-SS-POD/DNA nanoparticles as indicated by lacZ staining, the difference in the percentage of reduction in CNV size in our study (~50%) versus the AAV-8 study is more likely explained by a lack of sensitivity of the detection of lacZ-positive cells in laser-treated eyes pre-injected with PEG-SS-POD/pCAGlacZ nanoparticles.

Laser photocoagulation of C57Bl6/J mice results in a process of CNV mediated by increased levels of VEGF and is a much exploited murine model of neovascular AMD. Soluble FLT1 is a naturally occurring isomer of VEGF receptor 1 and has been found to reduce the progression of CNV in the murine and non-human primate models of laser-induced CNV. The efficiency with which PEG-SS-POD/DNA nanoparticles expressing a growth-factor-inhibiting protein are observed to inhibit aberrant cellular proliferation in the murine eye suggests that the PEG-POD/DNA nanoparticles may warrant further investigation in proliferative retinopathies, such as proliferative vitreo-retinopathy, proliferative diabetic retinopathy, and neovascular AMD.

In summary, we have generated a reducible PEG-SS-POD/DNA nanoparticle that efficiently transfects cells in vitro without negatively impacting transfection of cells in vivo. We have found that the increase in in vitro transfection is coincident with extracellular release of PEG and increased uptake of the nanoparticle. The reducible PEGylation permits maintenance of the benefits of PEG, i.e., reduced aggregation, increased solubility, and in vivo transfection, while restoring transfection by the nanoparticles in vitro, thereby making in vitro study of intracellular trafficking of the relevant nanoparticle accessible. We consider the PEG-SS-POD/DNA nanoparticle to provide a valuable tool in development of this technology for gene therapy for ocular diseases.

MATERIALS AND METHODS

Reagents

POD peptide [CGGG(ARKKAAKA)₄] was synthesized and purified by high-performance liquid chromatography at Tufts University.
Peptide Synthesis Core Facility (Tufts University). Methoxy-PEG-orthopyridyl disulfide (mPEG-OPSS; molecular weight [MW], 10,000) and mPEG-MALM (MW 10,000) were purchased from Laysan Bio. Ellman’s reagent [5, 5’-dithio-bis-(2-nitrobenzoic acid); DTNB] was purchased from Sigma. Plasmid labeling kit was purchased from Mirus Bio. Cell culture reagents were purchased Life Technologies. Micro Bio-Spin P6 chromatography columns, Any kD TGX protein gels, and Quick Start Bradford Protein Assay were purchased from Bio-Rad. Luciferase Assay System was purchased from Promega. Barium chloride was purchased from Thermo Fisher Scientific. The LDH kit was purchased from ScienCell Research Laboratories.

Cloning
The plasmids pCAGLuc and pCAGLacZ were described previously.\textsuperscript{18} The plasmid pCAGhFLT1 was constructed by Cyagen Biosciences. The hFLT1(s7) encoding cDNA from pUNO1-hFLT1(s7) (Invivo- gen) was cloned into pCAGEN.\textsuperscript{45}

Conjugation of PEG with POD and Plasmid Compaction
PEGylation of POD peptide and DNA compaction was performed as previously described,\textsuperscript{15} with some modifications. In brief, POD peptide, mPEG-MALM, and mPEG-OPSS were dissolved in 0.1 mM sodium phosphate buffer containing 5 mM EDTA. PEGylation of POD via a thioether linkage (i.e., PEG-S-POD) was made by mixing 1:1 molar ratio of POD:mPEG-MALM. PEGylation of POD via a disulfide linkage (i.e., PEG-S-S-POD) was prepared using a 2:1 molar ratio of mPEG-OPSS:POD. The reaction mixture was incubated overnight at room temperature on a shaker. PEGylated POD was then purified using Micro Bio-Spin P6 chromatography columns equilibrated with either 50 mM AmAc or 0.1% TFA. The PEG-S-POD and PEG-S-S-POD were quantified by SDS-PAGE analysis alongside POD standards of known concentration using Any kD Mini-PROTEAN TGX protein gels. DNA was compacted by adding dropwise to either PEG-S-POD or PEG-S-S-POD in a final concentration of 0.1% dextrose using BioMax 10K centrifugal filters (Millipore) and stored in 0.1 M Tris (pH 8)/C14 C water bath in the dark for 3 hr.

Quantitation of Sulfhydryl Groups
Sulfhydryl groups were quantified using Ellman’s reagent according to the manufacturer’s instructions (Sigma). In brief, a DTNB stock solution (50 mM sodium acetate/2 mM DTNB) and a 1 M Tris (pH 8) solution were prepared. 1 mL of POD samples was diluted with 99 mL of DTNB working reagent (1:2.168 of DTNB stock/Tris solution:H2O), and absorbance was read at 412 nm on a microplate reader (Molecular Devices) following a 5 min incubation. The concentration of thiols in the solution was determined by comparison with a series of cysteine standards.

Labeling of Plasmid DNA
For analysis of uptake of nanoparticles by ARPE-19 cells (American Type Culture Collection), plasmid DNA was labeled with the fluorophore Cy5, using a labeling kit from Mirus Bio according to the manufacturer’s instructions. The ratio of Cy5 to plasmid used was 1:1 (v/v). The reaction mixture was incubated in a 50°C water bath in the dark for 3 hr.

Cell Culture, In Vitro Transfection, Luciferase Assay, and DTNB Inhibition
ARPE-19 cells were grown in DMEM supplemented with 10% fetal bovine serum. Cells were seeded in 96-well plates at an initial density of 4 × 10^4 cells/well at 1 day prior to transfection. At the time of transfection, the medium in each well was replaced with 100 μL of fresh Opti-MEM containing nanoparticles (to deliver 200 ng plasmid/well). Following a 24 hr incubation with nanoparticles at 37°C, the cells were harvested for luciferase assay. Luciferase activity was assessed using a luciferase assay kit according to manufacturer’s protocol and a GloMax 20/20 Luminometer over a 10 s integration (Promega). Luciferase assay was normalized to total protein concentration and expressed as relative luciferase units (RLUs) per milligram protein. Protein concentration was measured using a Quick Start Bradford Protein Assay. For DTNB inhibition experiments, the cells were incubated for 30 min with DTNB dissolved in serum-free media at the indicated concentrations. The DTNB-containing media were aspirated and replaced with fresh serum-free media containing nanoparticles in the presence of the indicated concentration of DTNB for a further 1 hr 45 min. After 24 hr incubation, luciferase assay was performed on cell lysates. Experiments were performed three times independently.

Detection of PEG in Cell Culture Media
ARPE-19 cells were incubated with PEG-S-POD and PEG-S-S-POD nanoparticles as described above. Following incubation at 37°C for the indicated times, the media were harvested and centrifuged at 10,000 rpm for 2 min at 4°C. The centrifuged media were analyzed by SDS-PAGE. Following electrophoresis, the gel was soaked in 5% barium chloride solution for 15 min, rinsed with distilled water for 30 min, and stained with 0.1 N iodine solution for 15 min. The positive control for dePEGylation was generated by incubation of PEGSS-POD nanoparticles with 4 mM cysteine for 15 min.

LDH Assay
ARPE-19 cells were incubated with nanoparticles as described above. Following a 24 hr incubation, media were collected and LDH activity was measured using a kit provided by ScienCell Research Laboratories according to the recommended protocol.

Subretinal Injection and Laser Photocoagulation of Mice
All experiments involving animals were in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Tufts University. C57BL/6J mice between 6 and 8 weeks were purchased from Jackson Laboratories and
maintained in a 12 hr dark/12 hr light cycle in accordance with federal, state, and local regulations. Subretinal injections of nanoparticles containing 1 μg of plasmid DNA in a 2 μL volume were performed with a 32G needle (Becton Dickinson) and a 5 μL glass syringe (Hamilton) by a transscleral transchoroidal approach. Two days after injection, animals were sacrificed by CO2 inhalation followed by cervical dislocation and eyes processed for luciferase assay. The eyes were harvested and the anterior chamber was removed. The posterior eyecup was homogenized using a VWR PowerMax AHS 200 homogenizer (VWR). Laser photocoagulation was performed as previously described.46 In brief, mice were sedated with an intraperitoneal injection of ketamine (0.1 g/kg)/xylazine (0.01 g/kg), and pupils were dilated with 2.5% phenylephrine HCl (Bausch & Lomb) and 1% tropicamide (Bausch & Lomb). To minimize corneal injury, we applied 2.5% hypermellose (Goniovisc). Four laser spots were made per eye using an argon laser (532 nm, IRIS Medical Light Solutions, IRIDEM; IRIDEX) set to a spot size of 75 μm in diameter, 150 mW, and 100 ms pulse time.

Lectin Staining

One week following laser photocoagulation and nanoparticle injection, the animals were euthanized with CO2, and the eyes were enucleated. The cornea and lens were removed and the eyecup fixed in 4% paraformaldehyde in 0.1 M phosphate buffer overnight at 4°C. The retina was then removed and the sclera/choroid/RPE complex washed in PBS, blocked in 5% BSA in PBS, and stained with 5 μg/mL fluorescein-conjugated isoelectin (Vectashield) in PBS for 1 hr. Eyecups were flat mounted on glass slides and imaged using an inverted microscope (IX51; Olympus) with relevant filters, a digital camera (Retiga 2000R-FAST; Q-Imaging), and QCapture Pro software (Q-Imaging). CNV area was measured using ImageJ software.

LacZ Detection

PEG-SS-POD/DNA nanoparticles containing 1 μg of plasmid DNA were injected in the subretinal space of 6- to 8-week-old C57BL/6J mice, as described above. After 48 hr, eyes were enucleated, fixed in 0.25% glutaraldehyde for 30 min, and washed three times in PBS for 30 min. The eyes were incubated in X-Gal solution (FisherBiotech, Fisher Scientific) for 16–18 hr and then rinsed in phosphate buffer (pH 7.4) for 45 min. The eyes were fixed for 24 hr in 4% paraformaldehyde (PFA), dehydrated, embedded, and 14 μm sections collected. Bright-field images were taken using an Olympus BX51 upright microscope (Olympus) with Q-capture Pro software.

qRT-PCR

At 2 days after laser and injection of PEG-SS-POD/pCAGhFLT1 and PEG-SS-POD/pCAGluc nanoparticles, eyes were harvested and the cornea, lens, iris, and optic nerve removed. The remaining tissue was homogenized in RLT buffer (QIAGEN) using a VWR AH5200 homogenizer. Total RNA was purified using an RNeasy kit (QIAGEN) according to the manufacturer’s instructions. In brief, RNA was isolated on silica membrane columns using an on-column DNase treatment. qRT-PCR was performed using 100 ng of total RNA and 900 nM human sFLT1 primers (sFLT1 forward, 5′-CATA GTATGCCAAATAAGCAG-3′; sFLT1 reverse, 5′-CGAGTCAAA TAG CGAGCAGAT-3′) with iTaq Universal SYBR Green One-Step kit (Bio-Rad) in a 10 μL reaction. The reaction mix included an iScript Reverse Transcriptase and an antibody-mediated hot-start iTAQ DNA polymerase and was incubated at 50°C for 10 min followed by 1 min at 95°C and 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 60 s on a multicolor real-time PCR detection system (iQ5; Bio-Rad) using optical system software. qRT-PCR of known amounts of pCAGhFLT1 plasmid was performed, and a standard curve of transgene copy number versus threshold cycle generated. The standard curve was used to calculate the number of copies of hFLT1 mRNA in the total RNA isolated from injected eyecups.

Western Blotting

ARPE-19 cells were transfected with pCAGhFLT1 and control plasmids as described above. After 24 hr, the media were harvested and concentrated using a 30k Amicon Ultra centrifugal filter. The media were loaded and electrophoresed on an Any KD gel as described above and transferred onto a membrane. After blocking with Odyssey blocking buffer (LI-COR Biosciences) for 1 hr, the membrane was incubated at room temperature for 1 hr with rabbit monoclonal [Y103] to VEGF receptor 1 (ab32152; Abcam). The membrane was subsequently incubated at room temperature for 1 hr with IRDye 800CW anti-(rabbit IgG) antibody and visualized using an Odyssey IR imaging system (LI-COR Biosciences).

Statistical Analysis

All data analyses were performed using Prism Software 5 (GraphPad Software). In experiments comparing three or more samples, data were analyzed using a one-way analysis of variance with a post hoc Tukey multiple comparison test for significance. All other statistical tests were performed using an unpaired Students t test. All data are presented as the mean ± SE.

AUTHOR CONTRIBUTIONS

Study Design: R.K.-S., B.C.D., and S.M.C.; Experiment Design and Implementation: B.C.D. and S.M.C.; Data Analysis: B.C.D. and S.M.C.; Manuscript Preparation: B.C.D. and S.M.C.; Manuscript Review and Editing: R.K.-S.; Funding Acquisition, R.K.-S.

ACKNOWLEDGMENTS

This study was supported by grants to R.K.-S. from the National Institutes of Health/NEI (EY021805 and EY013837), the Department of Defense/CDMRP (W81XWH-12-1-0374 and W81XWH-16-1- 0650), The Ellison Foundation, and The Paul and Phyllis Fireman Foundation. We wish to thank Dr. Srinivas Sridhar and Dr. Rajiv Kumar (Northeastern University) for providing access to a Zetasizer for these studies.

REFERENCES

1. Bryant, L.M., Christopher, D.M., Giles, A.R., Hinderer, C., Rodriguez, J.L., Smith, J.B., Traxler, E.A., Tycko, J., Wojno, A.P., and Wilson, J.M. (2013). Lessons learned from
43. Ahmed, A., Dunk, C., Ahmad, S., and Khaliq, A. (2000). Regulation of placental vascular endothelial growth factor (VEGF) and placenta growth factor (PIGF) and soluble Flt-1 by oxygen—a review. Placenta 21 (Suppl. A), S16–S24.

44. Lai, C.M., Shen, W.Y., Brankov, M., Lai, Y.K., Barnett, N.L., Lee, S.Y., Yeo, I.Y., Mathur, R., Ho, J.E., Pineda, P., et al. (2005). Long-term evaluation of AAV-mediated sFlt-1 gene therapy for ocular neovascularization in mice and monkeys. Mol. Ther. 12, 659–668.

45. Matsuda, T., and Cepko, C.L. (2004). Electroporation and RNA interference in the rodent retina in vivo and in vitro. Proc. Natl. Acad. Sci. USA 101, 16–22.

46. Cashman, S.M., Ramo, K., and Kumar-Singh, R. (2011). A non membrane-targeted human soluble CD59 attenuates choroidal neovascularization in a model of age related macular degeneration. PLoS ONE 6, e19078.