Published in final edited form as:

ChemMedChem. 2014 January ; 9(1): 189–196. doi:10.1002/cmdc.201300371.

Synthesis and Characterization of Glycol Chitosan DNA Nanoparticles for Retinal Gene Delivery

Dr. Rajendra N. Mitra^[a], Dr. Zongchao Han^[a], Mr. Miles Merwin^[a], Dr. Muhammed Al Taai^[a], Dr. Shannon M. Conley^[a], and Dr. Muna I. Naash

Muna I. Naash: muna-naash@ouhsc.edu

^[a]Department of Cell Biology, University of Oklahoma Health Sciences Center, 940 Stanton L Young Blvd. BMSB781, Oklahoma City OK, 73104 USA, Phone: 405-271-2388, Fax: 405-271-3548

Abstract

Given the number of monogenic ocular diseases and the number of non-monogenic degenerative ocular diseases for which gene therapy has been considered as a treatment, the development of effective therapeutic delivery strategies for DNA is a critical research goal. Here we generate, characterize, and evaluate non-viral nanoparticles composed of glycol chitosan (GCS) and plasmid DNA (pDNA). We show that these particles are stable, do not aggregate in saline, are resistant to DNases, and have a hydrodynamic diameter of ~250 nm. We further show that the plasmid in these NPs maintains its proper conformation and can be released and expressed inside the cell. To determine whether these NPs would be suitable for intraocular use, pDNA carrying the ubiquitously expressed CBA-eGFP expression cassette was compacted and subretinally injected into adult WT albino mice. At post-injection (PI) day 14, we observe substantial GFP expression exclusively in the retinal pigment epithelium (RPE) in eyes treated with GCS NPs but not in uncompact pDNA or vehicle (saline) treated eyes. We observe no signs of gross retinal toxicity and at PI-30 days, there is no difference in electroretinogram function between GCS NP-, pDNA-, or vehicle-treated eyes. These results suggest that with further development GCS NPs may be a useful addition to our available repertoire of genetic therapies for the treatment of RPE-associated diseases.

Keywords

Glycol Chitosan; Nanoparticles; Non-Viral Gene Delivery; Polymers; Retinal Pigment Epithelium

Introduction

Due to the large number of identified disease genes associated with the retina and retinal pigment epithelium (RPE), ocular gene therapy has long been a research focus.^[1] The most basic gene therapy is the direct delivery of plasmid DNA (pDNA), but the efficacy of this approach is limited by the fact that pDNA is inefficiently taken into most cells and nuclei, and is unstable in the cytosol due to degradation by nucleases.^[2] As a result, significant

effort has been directed toward identifying effective, biocompatible agents to facilitate delivery of therapeutic DNAs to the target sites.

Adeno-associated virus (AAV) based vectors have been widely and successfully used for the treatment of degenerative ocular diseases, with research efforts culminating in a series of clinical trials for RPE65-associated Leber's congenital amaurosis (LCA).^[1, 3] While AAVs have proven to be an excellent delivery method for the eye, there remains room for improvement in therapeutic efficacy, and many genes are too large for the limited genetic capacity of AAVs (<5 kbp) such as the retinal disease genes *ABCA4*^[4] and *USH2A*,^[5] and the RPE disease genes *BEST-1*^[6] and *LRAT*.^[7] In addition, AAVs can be costly to produce, driving us to identify safe, biocompatible alternative DNA packaging options.^[8]

Cationic polymers can condense pDNA by strong electrostatic interactions to form different types of nanoparticles (NPs).^[9] Various cationic polymers have been employed so far for non-viral gene delivery^[10] including polylysine,^[11] polyethyleneimines,^[12] chitosan,^[13] and polyamidoamine dendrimers.^[14] Among these, chitosan-based cationic oligomers have been established as a safe DNA condensing agent that can effectively deliver therapeutic agents to the target site in vivo.^[13] However, chitosan is limited by its insolubility in water or any medium with a pH over 6,^[15] making chitosan polymers alone ineffective for DNA compaction. As a result, different types of chemical modifications have been incorporated on chitosan side chains to increase solubility.^[15] One such modification is the conjugation of ethylene glycol branches to the chitosan, which confers water solubility at a neutral pH. This glycol chitosan (GCS) has been adopted as a potential candidate for delivery of therapeutic agents to in vivo systems due to its hydrophilicity, acceptable biodegradability, and low immunogenicity.^[15-16] Importantly, it has been observed that GCS NPs exhibit a significant amount of cellular and tissue internalization in tumor layers in vivo.^[15, 16f] However, GCS has not been explored for ocular non-viral gene delivery.

Here we formulate, characterize, and test GCS NPs for gene delivery to the eye. We demonstrate that these particles are well-tolerated after subretinal injection and drive gene expression preferentially in the RPE. These results suggest that GCS based NPs represent an additional avenue for potential therapeutic delivery of cargos for the treatment of genetic diseases associated with the RPE.

Results

Characterization of NPs

NP compaction with GCS (Fig. 1A) and pDNA occurs via strong electrostatic interactions. For all studies here, we used the pscCBA-GFP (pDNA) vector in which GFP reporter gene expression is driven by the well-characterized chicken β -actin (CBA) heterologous promoter.^[17] As part of our initial study, we first optimized the ratio of GCS:DNA. A constant amount of pDNA (10 μ g) was mixed with varying amounts of GCS (as described in the methods). As the amount of GCS used for compaction increases, the mobility of the pDNA is gradually retarded (Fig. 1B, labels indicate GCS:DNA, μ g: μ g). We found that a ratio of 25:1 (μ g GCS: μ g DNA) was sufficient to entirely compact the DNA, (observe that

the DNA band does not leave the well), and this ratio was therefore used for all subsequent experiments.

To assess whether the NPs were prone to aggregate in physiological buffer (saline), we conducted turbidity analysis. The turbidity parameter was determined by measuring the UV absorbance of NPs from 330-410 nm, and calculating the slope of log (abs) vs. log (wavelength),^[19] with slopes ranging from -3.5 to -5.0 indicating minimal or no aggregation.^[19-20] The GCS NPs generated a slope of -3.9 (Fig. 1C), indicating that they did not exhibit aggregation in saline. We next assessed the zeta potential of the NPs, an important index for average surface charge of the NPs in solution.^[13a, 21] The NPs have a positive surface potential of 24.17 ± 0.74 mV (Fig. 1D), compared to the negative potential of naked pDNA (-25.90 ± 0.32 mV). All the DLS and zeta potential measurements were carried out in saline.

Finally, to assess NP size and shape we conducted dynamic light scattering (DLS) and TEM experiments. Analyzing the DLS data we found that the GCS-pDNA NP possessed significantly ($p < 0.01$) lower effective diameter (253.3 ± 3.18 nm) than the naked pDNA (499 ± 52.54 , Fig. 1E), in good agreement with other GCS based delivery systems.^[16c] TEM (Fig. 1F) showed that the GCS NPs are ellipsoidal in shape. The particle size as measured by TEM was lower (10-15 nm in diameter) than the effective hydrodynamic diameter determined from the DLS (Fig. 1E). This is a common occurrence,^[22] and likely arises due to a combination of factors. These include the hydration state of particles measured by DLS vs. the vacuum dried state for TEM as well as the ionic strength of the solution in which the particles were measured by DLS. Our DLS measurements were conducted in 150mM saline, a condition which has been shown to increase DLS-based size measurements by 2-3 fold.^[22b]

Combined, these results suggest that GCS NPs meet generally accepted quality control standards^[16c, 23] thus prompting us to proceed with in vitro and in vivo testing.

Integrity of pDNA in the GCS NPs

Our next step was to confirm that the pDNA inside the NPs was intact and maintained a proper conformation. Because the NPs must spend some time in the nuclease rich cytosol, DNase resistance is a key feature of effective delivery vehicles.^[2, 18] To assess the ability of GCS NPs to protect genetic cargo from nucleases, NPs or uncompact pDNA were incubated with DNase I and then chitosanase (which hydrolyzes beta-1,4-linkages and releases the DNA from the NP) (Fig. 1C). We observed no significant degradation of nanocompacted DNA (Fig. 1C) while naked pDNA was completely digested, demonstrating that the GCS NPs were nuclease resistant. Importantly, when the NPs were incubated with chitosanase after DNase treatment, we observed that intact plasmid was released (Fig. 2A). The open coil and super coiled DNA conformations were preserved even after release from the NP network; significant as supercoiled DNA is a prerequisite for gene expression.^[24] We next asked whether the secondary structure of the pDNA was preserved in the NPs. CD spectra of pDNA (Fig. 2B) show a positive and negative band at 273 and 246 nm respectively, consistent with the B-form of pDNA.^[25] This pattern is preserved in the

compacted GCS NPs which show similar spectral peaks at 283 nm (positive) and 246 nm (negative, Fig. 2B). As expected, GCS alone exhibits no specific CD spectra.

There are no good tissue culture models which mimic the intraocular environment, so extensive tissue culture testing is not relevant. However, as a final test prior to animal experiments, we wanted to confirm that the plasmid DNA could be released from the NPs and drive gene expression. We have previously observed that NP formulations which transfect retinal cells well (such as CK30 NPs [26]) do not readily transfect tissue culture cells, and we observed a similar phenomenon with the GCS particles (not shown). Therefore to test the integrity of the expression cassette we combined the NPs with a commercial transfection reagent, lipofectamine 2000. Since lipofectamine is a cationic lipid reagent, we first tested whether it could package the positively charged GCS NPs. We confirmed that both pDNA and GCS NPs are packaged by the lipofectamine: pDNA and GCS NPs then used the lipofectamine-packaged pDNA or NPs to transfect HEK cells. We observed efficient GFP expression in both cases (Fig. 2C) indicating that the expression cassette is intact and can be released from the GCS inside the cell and drive gene expression.

GFP expression after subretinal injection

We next assessed the ability of GCS NPs to drive ocular gene expression. GCS NPs ($0.2\mu\text{g}\mu\text{l}^{-1}$), pDNA ($0.2\mu\text{g}\mu\text{l}^{-1}$), or saline (vehicle) were subretinally injected ($1\mu\text{l}$) into wild-type albino mice at post-natal day (P) 30. To assess GFP expression *in vivo*, fundus images were captured at post-injection (PI)-14 days using GFP filters. As shown in Fig. 3A, we observed GFP expression in NP-injected but not pDNA-injected or saline-injected eyes at PI-14 days. This expression was primarily localized to the region of injection (superior temporal quadrant). Brightfield imaging shows no gross changes in fundus phenotype were observed as a result of NP treatment (Fig. 3B).

To further assess this expression, GFP was examined in retinal cross sections. To avoid confusion due to outer segment autofluorescence,^[26] immunofluorescence was carried out with GFP antibodies (red). GFP expression was clearly observed in the RPE layer (Fig. 4A) in GCS NP injected eyes while controls (pDNA and saline) exhibited no signs of GFP expression. This RPE specific expression occurred in all our experiments, Fig. 4B.

We next assessed native GFP fluorescence in RPE whole mounts as indicated previously.^[27] After removing the neural retina, eyecups were mounted RPE side up and imaged. GFP positive cells were mostly distributed near the injection site (superior temporal quadrant) at PI-14 days (Fig. 4C). Consistent with what we saw on the cross sections, GFP fluorescence is detected throughout the cytosol in the binucleated RPE cells (Fig. 4C, lower panels). No significant fluorescence was observed in flatmounts from pDNA or saline-injected eyes. These results clearly show that GCS NPs can drive gene expression in the RPE.

Lack of toxicity following NP injection

Although we did not observe any signs of gross retinal degeneration in retinal sections or fundus images at PI-14 days, we wanted to confirm that the GCS NPs do not impair retinal function. We therefore conducted full-field ERGs on saline, pDNA and NP-injected animals at PI-30 days. PI-30 was selected because we have previously observed that this amount of

time is sufficient for full functional recovery from the retinal detachment which is a consequence of the subretinal injection procedure.^[27] Representative scotopic (Fig. 5A) and photopic (Fig. 5B) traces are shown, with quantification of mean ($\pm$ SEM) maximum amplitudes presented in Fig. 5C. There were no significant differences in scotopic or photopic ERG amplitudes between the NP group and pDNA or saline. Because we observed gene expression in the RPE, we also wanted to assess a marker of general RPE health. We therefore measured mRNA levels for a key RPE protein, RPE65, and found there were no significant differences between any groups and compared to wild type (WT) (Fig. 5D) at PI-14 days. Combined, these results suggest that GCS NPs are well-tolerated in the eye and do not induce acute retinal or RPE toxicity.

Discussion

Here we conduct a systematic physicochemical characterization of NPs formulated with GCS, a novel compaction agent for ocular gene delivery. We selected the GCS biopolymer not only because it is biocompatible but also because its biological function in vivo in other tissue^[15-16] suggested it could be useful in the eye. We showed that these particles are stable, homogeneous, carry a slight positive charge, and can efficiently transfect the RPE in vivo. They have a hydrodynamic diameter of $\sim$ 250 nm, consistent with other reported values for similar particles^[16c, 23a]. While in-depth toxicity studies will be conducted in future, we observe no histological or functional signs of gross retinal toxicity or degeneration following subretinal delivery of GCS NPs. This is particularly important as unmodified chitosan in the eye has been shown to induce some toxic effects^[28] (although other modified chitosans, such as deoxycholic acid-substituted chitosan were shown not to be cytotoxic^[29]).

Successful non-viral gene therapy requires a delivery agent that is 1) readily internalized into cells, 2) protected from degradation in the cytosol during delivery to the nucleus, and 3) capable of being expressed once in the nucleus. We showed that our GCS NPs met all these criteria. Here we did not undertake any specific characterization of the uptake of GCS NPs in the eye; however our in vivo expression data indicate that the particles are taken up exclusively by RPE cells. GCS particles have been used in the lung, and it has been observed that the positive surface charge of GCS NPs facilitates electrostatic interactions with the anionic surface of epithelial cells, promoting uptake and possibly sustained release.^[23a] Similarly, chitosan NPs formulated to have positive surface charge had significantly better cellular uptake than similarly compacted chitosan particles with neutral or negative surface charge.^[30] However, it is not clear to what extent charge plays a role in uptake of GCS NPs into RPE cells, as we have previously shown good gene expression (with uptake as a prerequisite) after delivery of charge neutral polylysine NPs and some negatively charged pDNA.^[31] RPE cells are highly phagocytic and will take up a variety of materials from the subretinal space.

However, effective gene expression requires more than just cellular uptake. Indeed one of the historical limitations of non-viral gene delivery has been degradation in the lysosomes or degradation by cytosolic DNases. Although the intracellular transport pathways for GCS particles are not known, positively charged chitosan particles have been shown to be effectively released from lysosomes.^[30] In addition, we here showed that the GCS NPs are

resistant to degradation by DNases at levels which exceed those commonly found inside the cell,^[32] while pDNA was (as expected) rapidly degraded. The lack of gene expression from pDNA in the current study indicates that the GCS plays a protective role for the pDNA inside the cell. Importantly our CD spectra/chitosanase release experiments, in vitro transfection studies, and most importantly in vivo gene expression data, confirm that the plasmid can be released intact from the particles and mediate efficient gene expression.

In addition to these biological criteria, an ideal therapeutic formulation also has some other features. These include stability in physiological conditions, ease of production, low cost, and persistent expression (to avoid repeat dosing). Here we show expression for up to two weeks post-injection and our future studies may address the duration of this expression. In addition, GCS NPs are already known to have some of these other benefits. They are easy to produce and low cost,^[30] and we show that they are stable and do not aggregate in physiological saline (likely due to their positive surface charge).

Our results clearly show that packaging pDNA in GCS yields much more efficient gene expression than pDNA alone. The plasmid used in this study, psc-CBA-GFP, has previously been shown not to drive efficient gene expression when delivered without compaction to the eye^[17c] even at high concentration. Similarly, when the RPE-specific vector pEPI-VMD2-GFP (VMD2 is the RPE-specific vitelliform macular dystrophy 2 promoter) was delivered without compaction, only low levels of gene expression were observed.^[27] In contrast, when a highly engineered plasmid containing a scaffold/matrix attachment region (S/MAR) was used to deliver the VMD2-eGFP reporter cassette, gene expression from both nanocompacted and uncompacted DNA was high and persistent.^[31] These observations indicate that the vector itself can play a role in efficiency of expression and that vector engineering strategies combined with nanocompaction strategies may yield optimal results.

One of the limitations of GCS as a compacting agent is the hydrogelation property of the polymer. This physical property has two negative outcomes; first, it makes concentrating GCS NPs very difficult, and second, the viscosity and stickiness of the particles prevents widely distributed gene expression. We have previously delivered polylysine NPs at a concentration of $4.3 \mu\text{g } \mu\text{l}^{-1}$ DNA, while here we delivered particles at a concentration of $0.2 \mu\text{g } \mu\text{l}^{-1}$. Positively, we detect gene expression, even at this low concentration, but given the limited injection volume for subretinal delivery the ability to further concentrate particles is beneficial. In addition, wide distribution of expression throughout the subretinal space will be critical for maximum therapeutic efficacy. It is likely that judicious chemical modifications of the GCS may improve these parameters and will be a focus of future work.

One interesting outcome of these studies is the RPE-specific nature of the gene expression we observe. It is not clear why RPE cells appear to be targeted, although neurons (including retinal neurons) are difficult to transfect.^[33] Given the urgent need for treatments for RPE based disease such as Leber's Congenital Amaurosis (LCA), this specificity is beneficial. Further rational chemical modifications of the GCS and use of cell specific promoters (e.g. VMD2) might further improve the efficiency of RPE gene transfer.

Conclusions

In this study, we have synthesized and systematically characterized GCS NPs. The introduction of the glycol moiety in GCS led to improved solubility of the NP formulation compared to chitosan. The pDNA was stably incorporated inside the NPs and retained its native conformation and functional integrity thereby facilitating gene expression in the RPE after subretinal delivery. The RPE is a critical therapeutic target and the lack of gross retinal toxicity coupled with positive gene expression profiles suggest that, with further characterization, GCS NPs may be a useful strategy for targeting RPE-associated disease. As the demand for ocular gene therapy continues to grow along with the need for alternatives/additions to the available complement of viral therapeutics, development of efficient gene delivery strategies based on biocompatible polymers is critical.

Experimental Section

Materials

Glycol chitosan (GCS, MW 250 kDa, degree of deacetylation 82.7%) was purchased from Sigma (St. Louis, MO, USA). The acetate groups are found on the amine groups of the chitosan ring. The pscCBA-GFP plasmid DNA (5.7 kbp) was kindly provided by Dr. Arun Srivastava, Department of Medicine at the University of Florida. This vector contains the 544 bp chicken β -actin (CBA) promoter that has been widely used for gene therapy,^[17] and the GFP reporter gene. All centrifuge tubes, tips, water, filtering membranes, saline and other related reagents were purchased as endotoxin free. HEK-293 cells were purchased from American Type Culture Collection (ATCC, USA).

GCS NP formulation

Glycol chitosan (GCS) was dissolved in endotoxin free (Lonza, LAL reagent water) water at a concentration of 25mg ml⁻¹ (5ml total volume) and warmed at 55°C in a water bath for 2 hours. Concentrated acetic acid (0.013ml) was added and the mixture was warmed for another 2 hours at 55°C. The clear solution was passed through 0.2 μ m endotoxin free PURADISC™ 25 PES membranes (Whatman Inc., USA) to create the GCS stock solution. 10 μ l of this GCS stock was mixed well with 1.5 μ l of 5_M saline (Sigma, endotoxin tested & sterilized). Separately, 10 μ l of pscCBA-GFP plasmid DNA (pDNA, 1.0 mg ml⁻¹) was added to 28.5 μ l of saline (0.9% w/v) in a separate tube and mixed vigorously. The pDNA and GCS solutions were warmed at 55°C for 10-15 minutes and then the pDNA solution was added immediately to the GCS solution under constant vortexing at maximum speed for 1 min and then incubated for 1 hour at room temperature. Formulation was confirmed by electrophoresis on a 1% agarose gel with 1X tris-acetate (TAE) running buffer at 120V for 30 min. The concentration of pDNA in the compacted NPs was measured by using Eppendorf BioPhotometer plus UV/Vis Photometer (Eppendorf Inc., USA).

Particle characterization

To assess the nuclease resistance of GCS/DNA NPs, particles or uncompacted pDNA (1 μ g) were incubated with 2 U of DNase I (Life Technologies) and DNase buffer for 20 min at room temperature followed by agarose electrophoresis. For chitosanase digestion,^[34] 1 μ g

GCS/DNA NP solution was incubated with 0.1 U of chitosanase at pH 5.5 for 24 hours at room temperature, followed by agarose electrophoresis. Turbidity was determined by measuring the UV absorbance of NPs from 330-410 nm in UV-Vis spectrophotometer (Shimadzu Inc., USA). Triplicate values were collected from three separately prepared NPs. The turbidity was determined from the slope of the log (abs) vs. log (wavelength). NP size and surface charge were assessed by dynamic light scattering (DLS) and zeta potential (ZetaPALS, Brookhaven Inc., USA) using helium neon (HeNe) laser system that was operated at 659 nm and 25 °C, as described in.^[19] The naked pDNA and NP solutions were prepared in 2.0 ml saline with 50 µg ml⁻¹ of DNA. The scattered light by these solutions was determined at an angle of 90°. NP shape was determined by using TEM (JEOL 100CS) at 80 kV. Samples were prepared as described in earlier literature.^[35] Briefly, 5 µl of NP solution with a concentration of 0.2 µg µl⁻¹ was placed on 400 mesh carbon coated copper grid (Ted Pella, Inc., USA), excess water was removed using kimwipes held at the bottom of the grid, washed with water and air-dried. Before taking TEM images, the sample on the grid was stained with 2% uranyl acetate solution. The grid was then washed, cleaned, and imaged with TEM instrument.

The circular dichroism (CD) studies were carried out using JASCO J-715 (JASCO, Japan) following the earlier method.^[36] 300 µl of naked pDNA and NP solutions at 0.2 µg µl⁻¹ were assessed using a 0.1 cm path length quartz cuvette and the CD spectra were collected from 325-220 nm at 20 °C with a speed of 200 nm min⁻¹, resolution of 1.0 nm, and five accumulations. The spectra were corrected by subtracting the saline background (0.9% w/v).

In vitro transfection

HEK293 cells were grown in DMEM (Life Technologies) supplemented with 10% FBS (Atlanta Biologicals) and 1% of Antibiotic-Antimycotic (Life Technologies, 100X). Cells were seeded in 6-well plates on cover slips at a density of 5×10⁵ cells/well for 24 hours prior to transfection. On the next day media was replaced with 500 µl of serum/antibiotic-free DMEM. Transfection was carried out using 5 µl lipofectamine 2000 (Life Technologies) and 1 µg of pDNA or NPs according to the manufacturer's instructions. After 6 hours, transfection media was replaced with fresh DMEM supplemented with 10% FBS and 1% antibiotics. After 48 hours, cells were washed with 1X sterile PBS and mounted on slides with Prolong[®] Gold mounting media with DAPI (Life Technologies, USA). Native GFP fluorescence was imaged using a Zeiss Axio microscope (Carl Zeiss, Germany) with GFP filter and images were taken at 20X.

Animals and subretinal injections

Albino mice were bred in-house and maintained in the breeding colony under cyclic light (12L: 12D) conditions throughout the study. All animal studies were approved by the University of Oklahoma Health Sciences Institutional Animal Care and Use Committee and conformed to the guidelines of the Association of Research in Vision and Ophthalmology (ARVO, Rockville, MD). Trans-scleral subretinal injections were done as described previously.^[27] 1 µl of saline (vehicle), pDNA (0.2 µg µl⁻¹), or NPs (0.2 µg µl⁻¹) was subretinally injected into the superior temporal region.

Immunofluorescence

Whole eyes were enucleated at post injection (PI)-14 days as previously described,^[17a, 26-27, 37] and fixed with 4% paraformaldehyde in phosphate-buffered saline (1X PBS), for 2 hours at room temperature. Eyes were dissected, cryoprotected, and retinal cryosections (10 μ m) were collected as described earlier.^[17a, 26-27, 37] After washing with 1X PBS and blocking (5% BSA/PBS, 1.0% Triton X-100, 2% donkey serum) for 1 hour, sections were incubated overnight at 4°C with rabbit-anti GFP polyclonal antibody (1:1000 Life Technologies). Subsequently, slides were washed with 1X PBS (4x, 10 minutes), incubated with secondary antibody (1:2000 donkey-anti-rabbit Alexa-Fluor 555, Life Technologies) for 1 hour at room temperature, and washed again prior to being mounted with Prolong[®] Gold mounting media with DAPI (Life Technologies). RPE flatmounts were prepared as described previously,^[27] after fixation and removal of the cornea, lens, and neural retina. Flatmounts were mounted on glass slides using Prolong[®] Gold mounting media with DAPI (Life Technologies) after making radial incisions to flatten the tissue. Slides and flatmounts were imaged using a BX-62 spinning disk confocal microscope (Olympus, Japan) equipped with Slidebook[®] version 4.2.

Electroretinography

Full-field scotopic and photopic electroretinograms (ERG) were performed as described previously.^[17a, 26-27, 37] Briefly, mice were dark-adapted overnight, then anesthetized and eyes were dilated with 1% Cyclogyl[®] (PSI, Inc., Tulsa OK) and Gonak (hypromellose 2.5%, PSI, Inc., Tulsa OK) was applied to the eye. Scotopic recordings were collected in response to a single flash at 1.89 log cd s m⁻² in a ganzfeld (Model GS-2000; LKC Technologies, Gaithersburg, MD). After 5 minutes light adaptation at 30 cd m⁻² intensity for 5 minutes photopic recordings were averaged from a series of 25 flashes at 1.89 log cd s m⁻².

Fundus imaging

The eyes were dilated and anesthetized as described earlier.^[27] A drop of Gonak was placed on each eye for optimal refraction. Eyes were examined using the Micron III (Phoenix Research Laboratories, Pleasanton, CA) fundus imaging system using either white light or a GFP filter (excitation at ~482 nm and emission at ~536 nm).

Statistical analyses

The P-values of DLS and zeta studies (n=3) were reported from two-tailed, unpaired Student's t-tests and ERG analysis data are presented as means $\pm$ SEM. ERG results were analyzed by one way ANOVA with Bonferroni's post-hoc test where P<0.05 was considered a statistically significant difference.

Acknowledgments

The authors would like to thank Dr. Karla Rodgers (University of Oklahoma Health Sciences Center) for her technical assistance with CD experiments. This work was supported by the National Eye Institute (EY018656-MIN, EY22778-MIN), the Foundation Fighting Blindness (MIN), the Oklahoma Center for the Advancement of Science and Technology (SMC, ZH, and MIN), and Fight for Sight (RNM).

References

1. Bainbridge JW, Tan MH, Ali RR. *Gene Ther.* 2006; 13(16):1191–1197. [PubMed: 16838031]
2. Sasaki A, Kinjo M. *J Control Release.* 2010; 143(1):104–111. [PubMed: 20035810]
3. a) Bainbridge JW, Smith AJ, Barker SS, Robbie S, Henderson R, Balaggan K, Viswanathan A, Holder GE, Stockman A, Tyler N, Petersen-Jones S, Bhattacharya SS, Thrasher AJ, Fitzke FW, Carter BJ, Rubin GS, Moore AT, Ali RR. *N Engl J Med.* 2008; 358(21):2231–2239. [PubMed: 18441371] b) Cideciyan AV, Aleman TS, Boye SL, Schwartz SB, Kaushal S, Roman AJ, Pang JJ, Sumaroka A, Windsor EA, Wilson JM, Flotte TR, Fishman GA, Heon E, Stone EM, Byrne BJ, Jacobson SG, Hauswirth WW. *Proc Natl Acad Sci U S A.* 2008; 105(39):15112–15117. [PubMed: 18809924] c) Maguire AM, Simonelli F, Pierce EA, Pugh EN Jr, Mingozzi F, Bennicelli J, Banfi S, Marshall KA, Testa F, Surace EM, Rossi S, Lyubarsky A, Arruda VR, Konkle B, Stone E, Sun J, Jacobs J, Dell'Osso L, Hertle R, Ma JX, Redmond TM, Zhu X, Hauck B, Zelenia O, Shindler KS, Maguire MG, Wright JF, Volpe NJ, McDonnell JW, Auricchio A, High KA, Bennett J. *N Engl J Med.* 2008; 358(21):2240–2248. [PubMed: 18441370]
4. Allikmets R. *Nat Genet.* 1997; 17(1):122. [PubMed: 9288113]
5. Liu X, Bulgakov OV, Darrow KN, Pawlyk B, Adamian M, Liberman MC, Li T. *Proc Natl Acad Sci U S A.* 2007; 104(11):4413–4418. [PubMed: 17360538]
6. Bitner H, Mizrahi-Meissonnier L, Griefner G, Erdinest I, Sharon D, Banin E. *Invest Ophthalmol Vis Sci.* 2011; 52(8):5332–5338. [PubMed: 21467170]
7. den Hollander AI, Roepman R, Koenekoop RK, Cremers FP. *Prog Retin Eye Res.* 2008; 27(4):391–419. [PubMed: 18632300]
8. Wu Z, Yang H, Colosi P. *Mol Ther.* 2010; 18(1):80–86. [PubMed: 19904234]
9. Mastrobattista E, Hennink WE. *Nature materials.* 2012; 11(1):10–12.
10. Kim TH, Park IK, Nah JW, Choi YJ, Cho CS. *Biomaterials.* 2004; 25(17):3783–3792. [PubMed: 15020154]
11. Boylan NJ, Kim AJ, Suk JS, Adstamongkonkul P, Simons BW, Lai SK, Cooper MJ, Hanes J. *Biomaterials.* 2012; 33(7):2361–2371. [PubMed: 22182747]
12. Cheng W, Yang C, Hedrick JL, Williams DF, Yang YY, Ashton-Rickardt PG. *Biomaterials.* 2013; 34(14):3697–3705. [PubMed: 23422590]
13. a) de la Fuente M, Ravina M, Paolicelli P, Sanchez A, Seijo B, Alonso MJ. *Advanced drug delivery reviews.* 2010; 62(1):100–117. [PubMed: 19958805] b) Puras G, Zarate J, Diaz-Tahoces A, Aviles-Trigueros M, Fernandez E, Pedraz JL. *Eur J Pharm Sci.* 2013; 48(1-2):323–331. [PubMed: 23201002]
14. Wang H, Shi HB, Yin SK. *Exp Ther Med.* 2011; 2(5):777–781. [PubMed: 22977574]
15. Kim JH, Kim YS, Park K, Lee S, Nam HY, Min KH, Jo HG, Park JH, Choi K, Jeong SY, Park RW, Kim IS, Kim K, Kwon IC. *Journal of controlled release : official journal of the Controlled Release Society.* 2008; 127(1):41–49. [PubMed: 18234388]
16. a) Kim JH, Kim YS, Park K, Kang E, Lee S, Nam HY, Kim K, Park JH, Chi DY, Park RW, Kim IS, Choi K, Chan Kwon I. *Biomaterials.* 2008; 29(12):1920–1930. [PubMed: 18289669] b) Lee SJ, Huh MS, Lee SY, Min S, Lee S, Koo H, Chu JU, Lee KE, Jeon H, Choi Y, Choi K, Byun Y, Jeong SY, Park K, Kim K, Kwon IC. *Angew Chem Int Ed Engl.* 2012; 51(29):7203–7207. [PubMed: 22696263] c) Hyung Park J, Kwon S, Lee M, Chung H, Kim JH, Kim YS, Park RW, Kim IS, Bong Seo S, Kwon IC, Young Jeong S. *Biomaterials.* 2006; 27(1):119–126. [PubMed: 16023198] d) Lee SJ, Koo H, Lee DE, Min S, Lee S, Chen X, Choi Y, Leary JF, Park K, Jeong SY, Kwon IC, Kim K, Choi K. *Biomaterials.* 2011; 32(16):4021–4029. [PubMed: 21376388] e) Key J, Cooper C, Kim AY, Dhawan D, Knapp DW, Kim K, Park JH, Choi K, Kwon IC, Park K, Leary JF. *Journal of controlled release : official journal of the Controlled Release Society.* 2012; 163(2):249–255. [PubMed: 22902594] f) Na JH, Lee SY, Lee S, Koo H, Min KH, Jeong SY, Yuk SH, Kim K, Kwon IC. *Journal of controlled release : official journal of the Controlled Release Society.* 2012; 163(1):2–9. [PubMed: 22846988]
17. a) Cai X, Nash Z, Conley SM, Fliesler SJ, Cooper MJ, Naash MI. *PloS one.* 2009; 4(4):e5290. [PubMed: 19390689] Han Z, Berendzen K, Zhong L, Surolia I, Chouthai N, Zhao W, Maina N, Srivastava A, Stacpoole PW. *Mol Genet Metab.* 2008; 93(4):381–387. [PubMed: 18206410] c)

- Han Z, Conley SM, Makkia R, Guo J, Cooper MJ, Naash MI. *PloS one*. 2012; 7(12):e52189. [PubMed: 23272225] Han Z, Zhong L, Maina N, Hu Z, Li X, Chouthai NS, Bischof D, Weigel-Van Aken KA, Slayton WB, Yoder MC, Srivastava A. *Hum Gene Ther*. 2008; 19(3):267–278. [PubMed: 18303957]
18. Hu WW, Syu WJ, Chen WY, Ruaan RC, Cheng YC, Chien CC, Li C, Chung CA, Tsao CW. *Bioconjug Chem*. 2012; 23(8):1587–1599. [PubMed: 22768969]
 19. Ziady AG, Gedeon CR, Miller T, Quan W, Payne JM, Hyatt SL, Fink TL, Muhammad O, Oette S, Kowalczyk T, Pasumathy MK, Moen RC, Cooper MJ, Davis PB. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2003; 8(6):936–947. [PubMed: 14664796]
 20. Boylan NJ, Suk JS, Lai SK, Jelinek R, Boyle MP, Cooper MJ, Hanes J. *J Control Release*. 2012; 157(1):72–79. [PubMed: 21903145]
 21. Bacalocostantis I, Mane VP, Kang MS, Goodley AS, Muro S, Kofinas P. *Biomacromolecules*. 2012; 13(5):1331–1339. [PubMed: 22515194]
 22. a) Ma PL, Buschmann MD, Winnik FM. *Biomacromolecules*. 2010; 11(3):549–554. [PubMed: 20158194] b) Wu Y, Yang W, Wang C, Hu J, Fu S. *International journal of pharmaceutics*. 2005; 295(1-2):235–245. [PubMed: 15848008] c) Xu Q, Boylan NJ, Suk JS, Wang YY, Nance EA, Yang JC, McDonnell PJ, Cone RA, Duh EJ, Hanes J. *Journal of controlled release : official journal of the Controlled Release Society*. 2013; 167(1):76–84. [PubMed: 23369761]
 23. a) Lee J, Lee C, Kim TH, Lee ES, Shin BS, Chi SC, Park ES, Lee KC, Youn YS. *Journal of controlled release : official journal of the Controlled Release Society*. 2012; 161(3):728–734. [PubMed: 22634071] b) Lee PW, Hsu SH, Tsai JS, Chen FR, Huang PJ, Ke CJ, Liao ZX, Hsiao CW, Lin HJ, Sung HW. *Biomaterials*. 2010; 31(8):2425–2434. [PubMed: 20034662]
 24. Ando S, Putnam D, Pack DW, Langer R. *J Pharm Sci*. 1999; 88(1):126–130. [PubMed: 9874713]
 25. Liu LF, Wang JC. *Proc Natl Acad Sci U S A*. 1987; 84(20):7024–7027. [PubMed: 2823250]
 26. Farjo R, Skaggs J, Quiambao AB, Cooper MJ, Naash MI. *PloS one*. 2006; 1:e38. [PubMed: 17183666]
 27. Koirala A, Makkia RS, Cooper MJ, Naash MI. *Biomaterials*. 2011; 32(35):9483–9493. [PubMed: 21885113]
 28. Prow TW, Bhutto I, Kim SY, Grebe R, Merges C, McLeod DS, Uno K, Mennon M, Rodriguez L, Leong K, Luttly GA. *Nanomedicine*. 2008; 4(4):340–349. [PubMed: 18640079]
 29. Zhou H, Yang L, Li H, Gong H, Cheng L, Zheng H, Zhang LM, Lan Y. *Int J Nanomedicine*. 2012; 7:4649–4660. [PubMed: 22942646]
 30. Yue ZG, Wei W, Lv PP, Yue H, Wang LY, Su ZG, Ma GH. *Biomacromolecules*. 2011; 12(7):2440–2446. [PubMed: 21657799]
 31. Koirala A, Makkia RS, Conley SM, Cooper MJ, Naash MI. *Hum Mol Genet*. 2013; 22(8):1632–1642. [PubMed: 23335596]
 32. Glasspool-Malone J, Malone RW. *Hum Gene Ther*. 1999; 10(10):1703–1713. [PubMed: 10428215]
 33. Dib-Hajj SD, Choi JS, Macala LJ, Tyrrell L, Black JA, Cummins TR, Waxman SG. *Nature Protocols*. 2009; 4(8):1118–1127.
 34. Sun Y, Zhang S, Peng X, Gong Z, Li X, Yuan Z, Li Y, Zhang D, Peng Y. *Mol Biol Rep*. 2012; 39(2):945–952. [PubMed: 21573797]
 35. Mitra RN, Doshi M, Zhang X, Tyus JC, Bengtsson N, Fletcher S, Page BD, Turkson J, Gesquiere AJ, Gunning PT, Walter GA, Santra S. *Biomaterials*. 2012; 33(5):1500–1508. [PubMed: 22078810]
 36. Liu W, Sun S, Cao Z, Zhang X, Yao K, Lu WW, Luk KD. *Biomaterials*. 2005; 26(15):2705–2711. [PubMed: 15585274]
 37. Han Z, Conley SM, Makkia RS, Cooper MJ, Naash MI. *J Clin Invest*. 2012; 122(9):3221–3226. [PubMed: 22886305]

Fig. 1. Physiochemical characterization of GCS NPs

(A) Structures of chitosan and GCS. (B) Gel retardation analysis of different ratios of GCS:pDNA (X μ g GCS:1 μ g DNA). (C) Nuclease protection assay of the naked pDNA and NPs against DNase I. (D) Turbidity of the NPs in saline (n=3), $r^2 = 0.89$. (E) Zeta potential values of naked pDNA and NPs (n=3), values are significantly different (*** $P < 0.001$). Significance was determined with t-test. (F) Dynamic light scattering (DLS) of naked pDNA and NPs, (n=3), values are significantly different (* $P < 0.05$). ED, effective diameter. (G) TEM image of the NPs, scale bar = 100 nm.

Fig. 2. Functional characterization of GCS NPs

(A) The integrity of pDNA compacted in GCS NPs was determined by chitosanase (Chi) degradation assay. (B) CD spectra of the NPs, naked pDNA and GCS alone. Circles-pDNA, triangles-NPs, dash-GCS alone. (C) In vitro cellular uptake of naked pDNA and NPs after transfection with Lipofectamine 2000. Bright GFP fluorescence was observed from naked pDNA and NPs treated HEK 293 cells after transfection. Scale bar 20 μ m.

Fig. 3. Live fundus images showing GFP expression at PI-14 days

(A) Fundus images were obtained using a GFP filter (excitation at ~482nm and emission at ~536nm). (B) Brightfield images captured at the same time show no gross retinal pathology. To get good quality fundus images, injections were transscleral. Arrow shows GFP expression near the region of injection.

Fig. 4. GFP expression in RPE cells

Eyes were collected at PI-14 days after subretinal injection of 1 μ l of saline or naked pDNA or NPs at a concentration of 0.2 μ g μ l⁻¹. (A-B) Shown representative confocal images from retinal cross sections labeled with antibodies against GFP (red) and overlaid with DAPI (left) or brightfield (right) at 20x (A) or at 40 x (B). GFP expression is restricted to the RPE. (C) Native GFP expression in RPE whole mounts. Nuclei were stained with DAPI. (RPE- retinal pigment epithelium, OS-outer segment, IS- inner segment, ONL- outer nuclear layer, INL- inner nuclear layer). Scale bars 10 μ m.

Fig. 5. Functional Evaluation of treated animals

ERGs were measured at PI-30 from the indicated treatment groups. **(A-B)** Representative scotopic and photopic waveforms. **(C)** Quantification of maximum amplitudes. There is no significant difference between any group. Amplitudes are presented as mean $\pm$ SEM ($n = 6$). AMP, maximum amplitude. **(D)** RPE65 mRNA levels were measured by qRT-PCR. GE, relative gene expression, normalized to actin.— Significance was determined with one-way ANOVA with Bonferroni's post-hoc test.