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Delivery of siRNA to the Eye: Protocol for a feasibility study to assess novel delivery system for topical delivery of siRNA therapeutics to the ocular surface

Running head: Feasibility study of novel ocular siRNA carrier

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Abstract

Drug delivery to the eye remains a real challenge due to the presence of ocular anatomical barriers and physiological protective mechanisms. The lack of effective siRNA delivery mechanism has hampered the real potential of RNAi therapy, but recent literature suggests that nanocarrier systems show great promise in enhancing siRNA bioavailability and reducing the need for repeated intraocular injections. A diverse range of materials are under exploration worldwide, including natural and synthetic polymers, liposomes, peptides, and dendrimeric nanomaterials. This chapter describes a simple workflow for feasibility assessment of a proposed ocular surface siRNA delivery system. Gel retardation assay is used for investigation of optimal siRNA to carrier loading ratio. Fluorescent siRNA allows for initial *in vitro* testing of cellular uptake to corneal epithelial cells and investigation of *in vivo* siRNA delivery into mouse cornea by live animal imaging and fluorescence microscopy.

Key Words

siRNA, drug delivery, ocular delivery, cornea, eye, topical, corneal epithelial cells, gel retardation, fluorescence, IVIS

1. Introduction

The eye is a particularly attractive target for developing innovative therapies due to its small size and external accessibility for local drug treatment ***(1)***. Moreover, it is quite isolated from the rest of the body, limiting the access of the drug administered into this organ to systemic circulation and minimalizing the risk of possible off-target effects *(2)*. However, most drugs exhibit difficulties for overcoming the eye-associated barriers, and a delivery of large hydrophilic charged siRNA molecules is particularly challenging. While eye tissues are accessible by injection, it is a very delicate administration route and a non-invasive topical

administration is considered preferable, especially for siRNA therapeutics with short *in vivo* half-life which requires repeated treatment regimen to achieve a continuous ocular presence of intact nucleic acid ***(3, 4)***. However, the ocular surface is a complex biological barrier that has proven challenging for topical drug delivery due to the combined effect of short contact time, the presence of tear film and the barrier properties of the cornea ***(5)***.

Designing a drug delivery system for topical ocular administration effective in siRNA delivery to the eye is a major challenge for scientists developing RNAi therapies. Broad delivery strategies and a wide range of materials are under exploration worldwide, including natural and synthetic polymers, liposomes, peptides, and dendrimeric nanomaterials ***(6)***. Drug-loading capacity, release rate, and ocular retention time of drug delivery systems would have a great impact on the bioavailability of siRNA at the target site ***(7)***. Herein we describe an easy approach to test the potential of a new carrier system being engineered for topical delivery of siRNA to the cornea. The methodology described could be also adapted for evaluation of siRNA delivery system developed for other anterior or posterior eye tissues. Commonly used gel retardation assay, also known as gel shift assay, has been adapted to investigate siRNA entrapment by a delivery system and optimise the siRNA loading ratio. The electrophoretic separation of complexes formed with various increasing weight ratios of carrier to siRNA allows to quickly determine if the carrier is capable of binding to a given RNA duplex and identify the optimal loading conditions for the most effective siRNA entrapment ***(8, 9, 11)***.

An efficient internalization of siRNA is a matter of great importance for topical ophthalmic RNAi drug delivery, therefore we present a protocol for testing cellular uptake using commercial siGLO marker. This fluorescently tagged siRNA enables visual assessment of uptake into cornea cells *in vitro* ***(9–11)*** as well as *in vivo* imaging ***(9, 11)***. Topical delivery of labelled siRNA into mouse cornea can be assessed by live animal imaging using IVIS

Spectrum and further evaluated in eye sections with fluorescence microscopy. Two variants of siGLO transfection indicator are available, however due to distinct spectral properties of green and red fluorophores, performance may differ based on the delivery and detection methods employed. As per manufacturer's recommendations, siGLO Green (FAM-labelled) represents the ideal choice for studying cellular uptake by microscopy or flow cytometry following *in vitro* transfection, whereas siGLO Red (tagged with DY-547), which exhibits a stronger signal at lower concentrations, would be a better selection for *in vivo* imaging.

2. Materials

2.1. Gel retardation assay

1. Tested carrier system, suspended in nuclease-free water or buffer to form stable solution
2. Chosen siRNA oligonucleotide, diluted with nuclease-free water to 5µM concentration
3. Agarose gel: agarose, 10X TBE (Tris-Borate-EDTA) buffer, GelRed® Nucleic Acid Stain 10,000X, distilled water
4. Gel imager (a standard UV transilluminator or gel documentation system)

2.2. Human Corneal Epithelium (HCE) cell line maintenance

1. HCE cells
2. DMEM low glucose with GlutaMAX™ supplement
3. Fetal Bovine Serum
4. Trypsin/EDTA (0.25%)
5. Phosphate Buffered Saline

2.3. *In vitro* cellular uptake of fluorescent siRNA to corneal epithelial cells

1. Tested carrier system, suspended in nuclease-free water or buffer to form stable solution
2. Fluorescent siRNA marker: siGLO Green Transfection Indicator (Dharmacon)
3. HCE cells
4. DMEM low glucose with GlutaMAX™ supplement
5. Fetal Bovine Serum
6. Trypsin/EDTA (0.25%)
7. Phosphate Buffered Saline
8. OptiMEM
9. 24-well plate
10. 13mm coverslips and microscope slides
11. 4% Paraformaldehyde solution in PBS
12. DAPI mounting medium
13. Zeiss Axio Scope A1 fluorescence microscope equipped with DAPI and FITC filter

2.4. *In vivo* siRNA uptake into mouse cornea

1. Tested carrier system, suspended in nuclease-free water or buffer to form stable solution
2. Fluorescent siRNA marker: siGLO Red Transfection Indicator (Dharmacon)
3. Wild type adult mice
4. IVIS Spectrum system with LivingImage software

5. Isoflurane
6. Phosphate buffered saline
7. 4% Paraformaldehyde
8. O.C.T. embedding medium
9. Dry ice
10. Isopentane
11. Cryostat
12. Microscope slides and coverslips
13. DAPI mounting medium
14. Zeiss Axio Scope A1 fluorescence microscope equipped with DAPI and Cy5 filter

3. Methods

3.1. Gel retardation assay

1. To prepare 1% agarose gel, dissolve 0.5 g of agarose in 50 ml 0.5X TBE by warming the solution in the microwave. Allow the dissolved gel solution to cool and add 5 µl GelRed® Nucleic Acid Stain. Pour the gel in appropriate tray with well combs inserted and allow it to solidify (see *Note 1*).
2. Prepare tested samples of siRNA-carrier formulation at increasing loading ratio (see *Note 2*) in a total volume of 8 µl and an equal final siRNA concentration of 1.25 µM, as described in Table 1. Incubate samples at room temperature for 30 minutes or according to previously optimized conditions (see *Note 3*).

Place here Table 1.

3. Add 2 µl of 5X loading dye to each sample and mix by pipetting.

4. Place the agarose gel in a tank filled with 0.5X TBE buffer, remove the comb and load the samples to the gel wells (10 µl per lane).
5. Run electrophoresis at constant 100 mA for about 40 minutes (at that point siRNA band should be at about ¾ of a 5 cm gel).
6. Visualize the gel under the UV gel imager. An example of gel imaging output is shown in Figure 1.

3.2. Human Corneal Epithelium (HCE) cell line maintenance

1. HCE cells are cultured in a standard 1X Dulbecco's modified eagle medium (DMEM) supplemented with GlutaMAX™ and 10% heat-inactivated Fetal Bovine Serum (FBS) in an incubator at 37 °C with 5% CO_2. Sub-confluent cultures (70-80%) are split at 1:2 to 1:4 ratio as described below.
2. Remove and discard the growth medium.
3. Gently rinse the cell layer with 3mL phosphate-buffered saline. Aspire and discard the wash solution.
4. Add the pre-warmed 1X Trypsin/EDTA dissociation reagent and incubate cells for 5-10 minutes at 37˚C. Control the cells under the microscope for detachment.
5. When ≥ 90% of the cells have detached, add pre-warmed complete growth medium to inactivate trypsin (volume at least equal to the volume used for the dissociation reagent).
6. Transfer cell suspension to a conical tube and centrifuge at 1000 x g for 5 minutes.
7. Aspirate the supernatant and resuspend cells in 10 mL pre-warmed complete growth medium.
8. Take a sample for counting and determine the total number of cells using a

hemocytometer or cell counter.

9. Dilute cell suspension to the appropriate seeding density, and move 1.5 x 10^6 cells into a new $75cm^2$ flask.
10. Place the cells in a 37°C incubator at 5% CO_2. Monitor cell growth daily.

3.3. *In vitro* cellular uptake of fluorescent siRNA to corneal epithelial cells

1. Place coverslips in wells of 24-well plate. Sterilize under UV for 30 minutes.
2. Seed $1x10^5$ cells per well in 500 µl of DMEM GlutaMAX™ enriched with 10% FBS and grow the cells 24 hours at standard culture conditions (section 3.2).
3. Mix siGLO with tested formulation according to Table 2 to prepare samples of siRNA-carrier at optimized loading ratio (section 3.1) and incubate as required. Use Opti-MEM medium as diluent.

   Place here Table 2.
4. As a positive control, complex siGLO with Lipofectamine RNAiMAX transfection reagent according to Table 3, following manufacturer's protocol.

   Place here Table 3.
5. As a negative control, prepare naked siRNA sample by diluting siGLO with Opti-MEM medium according to Table 4.

   Place here Table 4.
6. Remove and discard 50 µl of growth medium from each well.
7. Add 50 µl of above prepared siGLO solutions (step 3 – tested sample, 4 – positive control and 5 – negative control) to separate wells (see *Note 4*).
8. Incubate the cells for 24 hours in an incubator at 37 °C with 5% CO_2 (see *Note 5*).
9. After incubation, remove and discard medium from each well.

10. Wash the cells with Phosphate Buffered Saline (PBS) three times by adding 500 µl of PBS to each well and removing by aspiration.
11. Add 300 µl of 4% paraformaldehyde (PFA) to each well and incubate 10 minutes at room temperature.
12. Remove and discard PFA solution.
13. Collect coverslips using forceps and mount them separately on a drop of mounting medium with DAPI placed on the microscope slide. Make sure that a coverslip is placed reversing side, so that cells are in contact with dye solution.
14. Incubate the slides for 10 minutes in the dark.
15. Visualize the slides under fluorescence microscope. Measure green siGLO signal using FITC filter and compare with the image of cell nuclei acquired with DAPI filter. An example of imaging output is shown in Figure 2.

3.4. *In vivo* siRNA uptake into mouse cornea

1. Initialize the IVIS.
2. Anaesthetise the mouse using 1.5–2% isoflurane in ~1.5 L/min flow of oxygen (setting 2.5 on isoflurane control valve).
3. Place the mouse inside the IVIS Spectrum imager chamber.
4. Acquire image sequence according to the established protocol to measure fluorescence signal (see *Note 6*).
5. Return the mouse safely to the home cage and control while it recovers consciousness.
6. Repeat fluorescence measurement with IVIS (step 1-5 above) on a daily frequency for 3 days prior to treatment using all experimental mice. Calculate ocular fluorescence baseline as an average signal intensity for right eye and left eye in separate.

7. On the treatment day, mix siGLO with tested formulation to prepare eye drop samples of 25 µM siRNA-carrier at optimized conditions (see *Note 7*). Also, dilute siGLO with the vehicle buffer to prepare control sample (25 µM naked siRNA).
8. Anaesthetize the mouse and apply a 4 µl drop of siGLO-carrier on one eye of the animal and a 4 µl drop of naked siGLO control on the other eye of the animal (see *Note 8*). Allow sample to absorb for 1 minute.
9. Return the mouse safely to the home cage and control while it recovers consciousness.
10. Perform fluorescence measurement with IVIS (according to step 1-5 above) at least 3 hours after the treatment (see *Note 9*).
11. Compare post-treatment ocular fluorescence intensity with baseline measurements (from step 6) in separate for treated (i.e. siGLO-carrier) and control (i.e. naked siGLO) eye.
12. After taking *in vivo* measurements, the fluorescence signal can be further examined in eye sections as explained below.
13. Euthanize the mice using carbon dioxide and gently enucleate the ocular globe from the sacrificed animal. Wash the enucleated eyes with phosphate buffered saline to remove blood if required.
14. Collect the tissues into labelled tubes filled with 4% paraformaldehyde and fix for 2 hours at room temperature.
15. Transfer the collected tissues into tubes filled with phosphate buffered saline to wash.
16. Place the tissue for a few minutes in OCT in a labelled small weigh boat (to acclimate the tissue).
17. Transfer the tissue into fresh OCT in a labeled plastic cryomold (with just enough OCT to cover the tissue) and orientate the eye with the cornea facing a side position allowing proper sectioning.

18. Freeze prepared cryomold samples in slush of dry ice and isopentane in a metal container placed in a foam cooler and surrounded with crushed dry ice. Do not fully submerge the samples.
19. Transfer frozen samples to a covered foam cooler filled with dry ice while continuing on to other samples. Wrap all samples in labelled foil and store at -80°C until further processing.
20. Remove the frozen block from the freezer and allow it to equilibrate to temperature in the cryostat chamber for approximately 30 minutes.
21. Perform a series of sections 5-10μm thick using cryostat and collect sections on microscopy slides.
22. Air dry the slides at room temperature for 1 hour.
23. Add a drop of mounting medium with DAPI to cover each tissue section and place a coverslip on top.
24. Incubate the slides for 10 minutes in the dark.
25. Visualize the slides under fluorescence microscope. Measure red siGLO signal using Cy5 filter and compare with the image of cell nuclei acquired with DAPI filter. An example of imaging output is shown in Figure 3.

4. Notes

1. Use microwave on medium power for approximately two minutes but do not let the solution overflow when boiled. Heat with occasional stirring until solution is clear. Make sure the agarose is completely dissolved (no visible clumps in the solution). Remove the flask from the microwave with a glove and let it cool under tap water until you can comfortably pick it up without protection.
2. Loading ratio of siRNA to carrier can be expressed in multiple way, such as molar

ratio (mol/mol), weight ratio (μg/μg), or commonly used N/P ratio, described as the ratio of positively-charged amine (N = nitrogen) groups in a carrier system to negatively-charged nucleic acid phosphate (P) groups. Thus, it depends on the investigator how to load siRNA onto carrier system. For a simple demonstration of sample preparation procedure at fixed siRNA concentration and at variable loading ratio, the increasing volume of carrier solution per volume unit of siRNA (v/v ratio) is used in the attached table.

3. Various factors may have an effect on the process of siRNA complexation with the carrier system, such as type of siRNA-carrier interaction, loading ratio, temperature, physicochemical properties of the carrier, buffer composition, pH, salt concentration, etc. The procedure of siRNA loading requires optimization for each carrier system. Gel retardation assay described here is a method that may help to optimize loading ratio for a cationic carrier.
4. Described procedure would result in 50nM siGLO transfection using a single carrier/siRNA ratio of choice (based on previously performed gel retardation assay). However, it may be necessary to re-optimize siRNA delivery conditions for a certain carrier system in order to maximize transfection efficiency while minimizing cytotoxicity. It is a good practice to perform a screening study using triplicate wells containing siGLO at different concentrations (12.5, 25, 50, or 100 nM) and variable amount of carrier formulation per well.
5. The incubation time can be adjusted and the influence of the incubation time on cellular uptake of siRNA complexed with a certain carrier can be investigated.
6. Signal acquisition protocol should be optimised for specific application. For siGLO Red detection epi-fluorescence measurement with 535nm excitation filter and 580nm emission filter at auto-exposure settings should be most suitable, although we

recommend contacting IVIS Spectrum support team for protocol establishment.

7. Prior to advancing to *in vivo* studies, the formulation should be optimised for ophthalmic use. Most eye drops are sterile, isotonic, aqueous solutions that may contain excipients, which, for example, regulate osmotic pressure, the pH, and viscosity of the preparation. Moreover, the concentration of formulation should be optimised to enable sufficient siRNA dose to be applied within a limited volume of eye drop. Due to a low capacity of conjunctival sac, the volume between 2 to 5 µl should be used for topical installation to mouse eye.
8. Following the implementation of the 3Rs, we recommend using a split body control experiment and compare the formulated siGLO in one eye (tested carrier) with a negative control of naked siGLO (vehicle) in the other eye of the same animal. The amount of siGLO administered shall be constant within the experiment, although the dose may require preliminary optimisation.
9. Due to the use of isoflurane for brief anaesthesia throughout both topical administration and fluorescence measurement it is recommended to keep at least three-hour break between procedures.

5. References

1. Moore CBT, Christie KA, Marshall J, et al (2018) Personalised genome editing - The future for corneal dystrophies. Prog Retin Eye Res 65:147–165
2. Jiang DJ, Xu CL, and Tsang SH (2018) Revolution in Gene Medicine Therapy and Genome Surgery. Genes (Basel) 9:575
3. Gaudana R, Ananthula HK, Parenky A, et al (2010) Ocular Drug Delivery. AAPS J 12:348–360
4. Fattal E and Bochot A (2006) Ocular delivery of nucleic acids: antisense

oligonucleotides, aptamers and siRNA. Adv Drug Deliv Rev 58:1203–1223

5. Reimondez-Troitiño S, Csaba N, Alonso MJ, et al (2015) Nanotherapies for the treatment of ocular diseases. Eur J Pharm Biopharm 95:279–293
6. Thakur A, Fitzpatrick S, Zaman A, et al (2012) Strategies for ocular siRNA delivery: Potential and limitations of non-viral nanocarriers. J Biol Eng 6:7
7. Subrizi A, Amo EM del, Korzhikov-Vlakh V, et al (2019) Design principles of ocular drug delivery systems: importance of drug payload, release rate, and material properties. Drug Discov Today 24:1446–1457
8. Chen J, Guo Z, Lin L, et al (2017) Combination therapy of pDNA and siRNA by versatile carriers composed of poly(<scp>l</scp> -serine) modified polyethylenimines. Mater Chem Front 1:937–946
9. Schiroli D, Gómara MJ, Maurizi E, et al (2019) Effective In Vivo Topical Delivery of siRNA and Gene Silencing in Intact Corneal Epithelium Using a Modified Cell-Penetrating Peptide. Mol Ther - Nucleic Acids 17:891–906
10. Dim N, Perepelyuk M, Gomes O, et al (2015) Novel targeted siRNA-loaded hybrid nanoparticles: preparation, characterization and in vitro evaluation. J Nanobiotechnology 13:61
11. Baran-Rachwalska P, Torabi-Pour N, Sutera FM, et al (2020) Topical siRNA delivery to the cornea and anterior eye by hybrid silicon-lipid nanoparticles. J Control Release 326:192–202

Tables

Table 1. Guidance for preparation of siRNA-carrier formulation samples at increasing loading ratio for gel retardation assay.

Sample ID		Loading ratio (*v/v*) carrier to siRNA	Mixing ratio (volume) [µL]		
			siRNA 5 µM	carrier	water
1	Control	0 : 2	2 µl	-	6 µl
2	Sample $L_{0.5}$	1 : 2	2 µl	1 µl	5 µl
3	Sample L_1	2 : 2	2 µl	2 µl	4 µl
4	Sample L_2	4 : 2	2 µl	4 µl	2 µl
5	Sample L_3	6 : 2	2 µl	6 µl	-

Table 2. Example of reagent volumes (defined for one reaction) for preparation of fluorescent siGLO-carrier formulation for *in vitro* cellular uptake assay.

Component	**Volume (single reaction)**
siGLO Green Transfection Indicator, 5µM	5 µl
Tested carrier formulation	10 µl
Opti-MEM medium	35 µl

Table 3. The reagent volumes (defined for one reaction) for preparation of positive control sample for *in vitro* cellular uptake assay.

Component	**Volume (single reaction)**
siGLO Green Transfection Indicator, 5µM	5 µl
Lipofectamine RNAiMAX Reagent	1.5 µl
Opti-MEM medium	43.5 µl

Table 4. The reagent volumes (defined for one reaction) for preparation of negative control sample for *in vitro* cellular uptake assay.

Component	**Volume (single reaction)**
siGLO Green Transfection Indicator, 5µM	5 µl
Opti-MEM medium	45 µl

Figure Captions

Figure 1. Gel retardation assay for optimization of carrier to siRNA ratio. The electrophoretic separation of a carrier-siRNA mixture on agarose gel reflects the fraction of free and complexed siRNA. The unbound siRNA migrates through the gel and shows a band corresponding to free siRNA control (line 1), while the siRNA bound by a carrier system remains immobilized within the wells (line 2-5). The intensity of unbound siRNA band decreases and the intensity of bound siRNA increases with the increasing carrier to siRNA ratio.

Figure 2. Assessment of cellular uptake of siRNA to corneal epithelium cells *in vitro*. Fluorescent green siGLO (25 pmol) was transfected into HCE cells with Lipofectamine RNAiMAX (1.5 µl per 500 µl of growth medium). After 24 hours, cells were washed with PBS, fixed with PFA and stained with DAPI nuclear dye (blue).

Figure 3. Assessment of siRNA delivery to cornea cells *in vivo*. Formulation containing red fluorescent siGLO and naked siGLO control was applied topically onto mouse eye in a split body experiment. Live animal imaging was performed 3 hours after administration using IVIS Spectrum and penetration of siGLO to the cornea was assessed in eye cross sections.