Electronic Supplementary Material (ESI) for Nanoscale Advances.
This journal is © The Royal Society of Chemistry 2023

Supporting Information

Fusogenic liposome-coated nanoparticles for rapid internalization into donor corneal endothelial tissue to enable prophylaxis before transplantation

*Thanuja M. Y.[$], Suraksha S. Tellakula[$], Samarth V. Suryavanshi, Keerthana G. S., Chandan Vasudev S., Sudhir H. Ranganath**

Experimental Section

Materials

Poly-L-lactic-co-glycolic acid (PLGA; Resomer® RG 502H; 7-17 kDa, #719897), Pluronic® F-127 (#P2443), Poly-L-lysine (150-300 kDa, #P4707), DiO (3,3′-Dioctadecyloxacarbocyanine perchlorate, #D4292), DAPI (4'6-diamidino-2-phenylindole, #D9542), Trypan Blue (#T8154), cholesterol (from sheep wool; in powder form), chloroform (with 100-200 ppm amylenes as a stabilizer), acetone (analytical grade), Attachment factor (#123-100), and antibiotic–antimycotic (A5955) were procured from Sigma Aldrich (Bengaluru, India). DOTAP (Dioleol-3-trimethylammonium propane), DOPE (Dioleylphosphatidylethanolamine), and Soya-PC (Soya Phosphatidylcholine) were purchased from Avanti Polar Lipids (USA) and Lipoid (Germany). Dulbecco's Modified Eagle's Medium (#10313021), fetal bovine serum (#10082147), and Trypsin EDTA (0.25 %; #59428C) were procured from Gibco (Bengaluru, India). Texas Red®-X Phalloidin (T7471) was procured from ThermoFisher (Bengaluru, India). Lactate dehydrogenase (LDH) assay kit (#CCK058) and Triton X-100 (#MB031) were procured from HiMedia Laboratories (India) and Cornisol® from Aurolab (India).

Preparation of DiO-loaded PLL-coated PLGA nanoparticles

To characterize nanoparticle internalization in the cells, we prepared DiO-loaded nanoparticles (DNPs). DiO is a fluorescent dye (Ex λ_{max} = 483 nm; green emission at Em λ_{max} = 501 nm). Nanoprecipitation method was employed to prepare DiO-loaded PLGA nanoparticles (DNPs). Briefly, the organic phase consisting 50 mg PLGA (lactic acid to glycolic acid ratio of 50:50; carboxylic end terminated) and 1 % (w/w) DiO (with respect to PLGA, was weighed using an analytical microbalance (Shimadzu, Model AUW220D) with a four-digit accuracy) and dissolved in 5 mL acetone. The surfactant Pluronic F127 (1% w/w) was dissolved in 10 mL deionized water, which acts as the aqueous phase. The organic phase was added dropwise into the aqueous phase using a syringe with 23 G needle with constant stirring at 2000 rpm

to allow formation of nanoemulsion. The mixture was then stirred for another 5 h to allow evaporation of acetone. Acetone removal allowed the nanoparticles to precipitate, and the nanoparticles were collected by centrifuging at 26,000 rpm at 4 °C for 30 min. After removing the supernatant, the nanoparticles' pellet was washed thrice with deionized water to remove the surfactant. The obtained nanoparticles were lyophilized for 48 h and stored at 4° C before further use.

To enhance internalization, we also altered the surface charge of the nanoparticles by coating the nanoparticle surface with poly L-lysine (PLL) as described earlier.[1] This was achieved by incubating the nanoparticles (5 mg) with 1 mL of aqueous PLL solution (0.01% (w/v)) at 37 °C for 2 h to allow surface adsorption of PLL. PLL-DNPs thus obtained were centrifuged at 26,000 rpm at 4 °C for 30 min and the nanoparticles were washed three times with deionized water to remove unbound PLL. The obtained PLL-DNPs were lyophilized for 48 h and stored at 4° C before use. The nanoparticles were assessed for shape and morphology using TEM. Average size of nanoparticles, distribution of size (Polydispersity index, PDI), and surface charge (zeta potential) were determined by dynamic light scattering (DLS) analysis using Zeta Sizer (ZS Nano90, Malvern, UK).

Preparation of DiO-loaded fusogenic liposome-coated PLGA nanoparticles

DiO-loaded PLGA nanoparticles (DNPs) were first prepared by the nanoprecipitation method as describe above. Later, 25 mg of DOTAP and 25 mg of DOPE or Soya-PC were dissolved in 5 mL of chloroform in a round bottomed flask (25 mL). Chloroform was evaporated using a vacuum rotary evaporator for 10 min at 45 °C at a vacuum of about 55 Pa. This led to the formation of a thin lipid film on the wall of the round bottomed flask, which was subsequently hydrated with 6.25 mL of 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid) buffer containing 50 mg of DiO-loaded PLGA NPs for 2 h at 45 °C. The hydration led to spontaneous self-assembly of the lipids into bi-layered liposomes. The resulting suspension

containing multilamellar fusogenic liposome-coated DNPs was sonicated for 150 min to produce unilamellar liposome coating on DNPs. Next, this suspension was extruded sequentially through polycarbonate membranes of pore sizes 5, 1.2, and 0.45 μm held in an Avanti Lipid Mini-extruder (Alabaster, AL, USA), one membrane at a time at the phase transition temperature of the lipid (45 °C). After 20 passes through the extruder with each membrane, we obtained a suspension of uniformly sized unilamellar fusogenic liposome-coated DNPs (FL-DNPs). The resulting FL-DNPs were stored at 4° C before further use. They were assessed for shape and morphology by TEM. Average nanoparticle size,size distribution (Polydispersity Index, PDI), and surface charge (zeta potential) were determined by dynamic light scattering (DLS) analysis using Zeta Sizer as described earlier.

Isolation of cornea endothelium for *ex vivo* studies

Porcine eye globes (6-10-month old pigs) were obtained in sterile phosphate buffer saline from a local slaughter house within 1 h after sacrifice. Laminar air flow chamber was cleaned with 70% ethanol and UV sterilized. Eye globes were transferred to the laminar air flow chamber and rinsed in 10% povidone iodine in phosphate buffer saline for 3-5 min and transferred to fresh phosphate buffer saline. Extra ocular muscles and conjunctival tissue present around the eye globes were removed. Eye globes were washed 2x with sterile PBS for 3-5 min and placed in a sterile tissue paper and wrapped around the cornea. The wrapped eye globe was held in the hand with maintaining adequate pressure. The remaining conjunctiva present around the cornea was removed using iris scissors. The incision around the cornea was made just below the 3-4 mm limbus region and this incision was extended to 360°. Cornea was separated from eye globe and iris was removed using teethed forceps. It was washed 2x with PBS without touching the corneal endothelial surface and the cornea was placed in the eye cup with endothelium facing upward. Eye cups were placed in a sterile petri-dish and filled with sterile PBS for further use.

Culture of porcine corneal endothelial cells for *in vitro* studies

Culture plates were coated with the extracellular matrix mixture, which contains attachment factor and incubated for 30 min at 37 °C. Fresh porcine eyes were obtained from the slaughterhouse in a phosphate buffer saline (PBS) under strict sterile conditions. Eye globes were washed with PBS containing 10% povidone-iodine. Then, the cornea was isolated and placed in a Petri dish with the endothelial layer facing up as described earlier. The endothelial layer was stained with Trypan blue for better visualization, and endothelial explants (with Descemet's membrane) were peeled off as described previously.[2] The explants were cultured on Petri dishes coated with the attachment factor in a medium containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% fetal bovine serum and 1% antibiotic–antimycotic solution in a humidified incubator at 37 °C with 5% CO_2. Corneal endothelial cells migrated from the explants onto the tissue culture plates for 4 - 6 days, leading to confluence and were employed for further experiments.

Internalization of nanoparticles in corneal endothelial cells *in vitro*

Corneal endothelial cells (first passage) were cultured on 12 mm coverslips coated with a mixture of attachment factor and grown until confluence (80-90%). DNPs, PLL-DNPs or FL-DNPs were suspended at a 0.4 mg/mL concentration in a culture medium containing DMEM, 1% FBS, and 1% antibiotic-antimycotic mixture and subjected to sonication (40 kHz) for 30 min at 37 $^{\circ}$C. Sonication was performed to remove nanoparticle aggregation. The suspension of nanoparticles was added on the coverslip present in a Petri dish and placed in a humidified incubator at 37 °C with 5% CO_2 for internalization for 3 or 6 h. After this, the cells were washed with PBS in order to remove free or non-internalized nanoparticles. Then, the cells were fixed with 3.7% PFA (paraformaldehyde) at room temperature for 20 min. After washing with PBS, the cells were permeabilized with PBS containing 0.1% Triton-X 100 for 2 min and incubated with Texas Red®-X phalloidin (1:500) at room temperature for 60 min followed by incubating the cells with 1 µg/mL DAPI for 10 min to stain the nucleus. Then, the cells were mounted on

glass slides with antifade reagent and was visualized by Zeiss LSM 880 confocal microscope with a 63x oil dipping objective. Internalization of nanoparticles was quantified by measuring green fluorescence (DiO dye) intensity in the cells by Zen 2.1 SP3, Zen Black software (Zeiss, Bangalore, India).

Internalization of nanoparticles in *ex vivo* porcine cornea

First, the corneal endothelial layer of the excised porcine cornea was placed upward in an eye cup. DNPs, PLL-DNPs or FL-DNPs dispersed in Cornisol medium (cornea preservation medium, Aurolab, India) were added on to the corneal endothelial layer at a concentration of 0.8 mg/mL and incubated in a humidified incubator at 37 °C with 5% CO_2 for 3 or 6 h for internalization. Next, the fixation of the corneal tissue was performed using 3.7% paraformaldehyde at room temperature for 20 min. After washing with PBS, the corneal tissue was permeabilized using PBS containing 0.1% Triton-X 100 for 2 min followed by incubating with Texas Red®-X phalloidin (1:500) at room temperature for 60 min to stain the cell borders and exposure to DAPI at concentration 1 µg/mL for 10 min to stain the nucleus. The stained tissue was mounted on glass slides with antifade reagent and visualized by confocal microscope (Zeiss LSM 880) with a 63x oil dipping objective. Internalization of nanoparticles was determined by measuring the green fluorescence (DiO) intensity in the tissue using Zen 2.1 SP3, Zen Black software (Zeiss, Bangalore, India).

Assessment of cell viability using lactate dehydrogenase (LDH) assay in *ex vivo* porcine corneas internalized with nanoparticles

The entry of the fusogenic liposome-coated nanoparticles involves fusion of the liposomes with the cell membrane before the nanoparticles are released intracellularly[3]. Thus, we investigated if this fusion process and the subsequent nanoparticle internalization would result in any damage to the cell membrane and adversely affect cell viability. To assess this, an established LDH assay was employed[4, 5] to estimate % LDH release, because the intracellular release of

LDH (a stable cytosolic enzyme) correlates to the damage to corneal endothelial cell membrane[6]. First, two types of fusogenic liposome-coated nanoparticles (without DiO loading) i.e., (DOPE + DOTAP)-based fusogenic liposome-coated nanoparticles (FLNP-1) and (Soya PC + DOTAP)-based fusogenic liposome-coated nanoparticles (FLNP-2) were prepared as described earlier in the previous section. 0.8 mg/mL of UV-sterilized FLNPs suspended in Cornisol® medium were added to the endothelial side of *ex vivo* porcine corneas after they were dissected from porcine eyes, to allow uptake by the endothelial cells. Two corneas were used for each experiment and repeated thrice. The corneas were incubated with FLNPs for a period of 3 h at 37 °C with 5% CO_2, after which the medium was replaced with fresh Cornisol® to ensure removal of non-internalized nanoparticles. The nanoparticle-engineered *ex vivo* corneas were incubated for 6, 12, 24, and 48 h at 37 °C with 5% CO_2. Corneas were also treated with 1% Triton X-100 (in PBS) for 15 min as positive control (to ensure membrane damage and LDH release). Untreated corneas were used in the experiment to compare the fold change in LDH release in comparison to nanoparticle-treated corneas.

Next, LDH release was quantified by measuring absorbance of formazan (red color) at 450 nm which was formed when tetrazolium dye (yellow color) was converted into formazan in the presence of LDH, using the LDH assay kit as described next. Briefly, after the nanoparticle-engineered, Triton-treated or untreated corneas were incubated for 3, 6, 12, 24 and 48 h, the corneas were washed with PBS to remove Cornisol® and lysis solution was added to lyse the endothelial cells and release the intracellular LDH. Next, the endothelial layer was scraped to harvest the cells along with released LDH in the obtained solution. The resultant suspension (lysate) was centrifuged at 10,000 rpm for 10 min at 4 °C. The pellet (cell debris) was discarded and the supernatant was mixed with the LDH reagent and incubated for 30 min at 4ºC, after which the stop solution was added to terminate the reaction. The absorbance of formazan was read at 450 nm using UV-Visible spectrophotometer (Shimadzu, Japan). Fold change in the LDH release was calculated using the formula, fold change = (A-B)/(C-B), where A is the

average absorbance value for treated cells, B is the average absorbance value for background medium (PBS without cells) and C is the average absorbance value for untreated cells.

Statistical analysis

Statistical analysis was performed with one-way analysis of variance using Microsoft Excel (version 2016). A value of $p < 0.05$ was considered statistically significant and $p < 0.01$ was considered statistically significant for the cell viability assay, since these experiments involve *ex vivo* tissues, having high degree of heterogeneity. Data are expressed as mean ± standard deviation. "n" represents multiple independent experiments.

References

1. S. H. Ranganath, Z. Tong, O. Levy, K. Martyn, J. M. Karp and M. S. Inamdar, *Stem Cell Reports*, 2016, **6**, 926-939.
2. S. M. Koh, T. Coll, D. Gloria and N. Sprehe, *Cornea*, 2017, **36**, 476-483.
3. B. Kim, H. B. Pang, J. Kang, J. H. Park, E. Ruoslahti and M. J. Sailor, *Nat Commun*, 2018, **9**, 1969.
4. C. Anupama, A. Shettar, S. H. Ranganath and S. P. Srinivas, *J Ocul Pharmacol Ther*, 2023, **39**, 70-79.
5. M. Y. Thanuja, B. S. Suma, D. Dinesh, S. H. Ranganath and S. P. Srinivas, *J Ocul Pharmacol Ther*, 2021, **37**, 399-411.
6. E. A. Lahagu, J. Fachiroh, A. S. Anugrah, W. Gunawan, I. T. Mahayana and Suhardjo, *Int J Ophthalmol*, 2020, **13**, 1148-1151.