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Preparation and optimization of anionic liposomes for delivery of small peptides and cDNA to human corneal epithelial cells

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Abstract

Drug delivery to corneal epithelial cells is challenging due to the intrinsic mechanisms that protect the eye. Here we report a novel liposomal formulation to encapsulate and deliver a short sequence peptide into human corneal epithelial cells (hTCEpi). Using a mixture of Phosphatidylcholine/ Caproylamine/Dioleoylphosphatidylethanolamine (PC/CAP/DOPE), we encapsulated a fluorescent peptide, resulting in anionic liposomes with an average size of 138.8 ± 34 nm and a charge of -18.2 ± 1.3 mV. After 2 h incubation with the peptide-encapsulated liposomes, 66% of corneal epithelial (hTCEpi) cells internalized the FITC-labelled peptide, demonstrating the ability of this formulation to effectively deliver peptide to hTCEpi cells. Additionally, lipoplexes (liposomes complexed with plasmid DNA) were also able to transfect hTCEpi cells, albeit at a modest level (8% of the cells). Here, we describe this novel anionic liposomal formulation intended to enhance the delivery of small cargo molecules *in situ*.

Keywords

peptide; plasmid DNA; ophthalmic delivery; liposomes

Introduction

Advances in our understanding of the biology and pathology of the eye have prompted new strategies for enhancing and prolonging vision. However, the delivery of many promising small molecules and peptides can encounter challenges when transitioning from *in vitro* development to cell-based and *in vivo* analysis. This is particularly the case for drug delivery to the anterior of the eye, which is uniquely designed to exclude small particles, bacteria and viruses. Due to its accessibility to the external environment, eye drops are a logical modality for drug administration. However, the corneal epithelium has poor bioavailability due to the

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Declaration of interest

The authors report no declarations of interest.

intrinsic protective mechanisms of the eye such as blinking, tear drainage, and poor corneal permeability (Akhtar, 2013, Ye et al., 2013, Gaudana et al., 2010). Penetration through the epithelial layers is limited by the corneal epithelial intercellular pore size (estimated to be between 20 and 30 Å) and drug lipophilicity (Toropainen, 2007). These drug delivery limitations result in the need for more frequent drug administration and often cause variations in the drug level achieved (McCalden and Levy, 1990). It is estimated that between 50 and 80% of topically delivered drugs that are administered without a carrier, are lost before they reach the target tissues (Thassu and Chader, 2012, Gaudana et al., 2010). This can result in non-linear drug release with high concentrations of drug released immediately after administration, often causing systemic absorption and undesirable collateral effects (McCalden and Levy, 1990). Therefore, as the number of ocular drugs increases, there is a need to optimize topical bioavailability for enhanced and more consistent drug delivery to help preserve ocular health.

Liposomes have emerged as a leading candidate for topical ocular drug delivery systems. Developed by Bangham in the 1960s (Barauskas et al., 2005), liposomes have shown promise in enhancing the ocular bioavailability of drugs (Kaur et al., 2004, Law et al., 2000, Patel et al., 2010, McCalden and Levy, 1990). Due to the hydrophilic core and hydrophobic bilayer that comprise these lipid vesicles, liposomes are suitable and versatile carriers of both hydrophobic and hydrophilic molecules (Ça da et al., 2014). While their size can be tailored to encapsulate large amounts of cargo in a confined small volume, the liposome surfaces can be modified with small recognition peptides to enhance their interaction with targeted cell surface proteins (Vivès et al., 2008). Moreover, several liposomal formulations have been approved by the FDA (Chang and Yeh, 2012), making these translatable vehicles for future *in vivo* applications.

Liposome formulations can be broadly divided into cationic and anionic subtypes. Of the two, cationic liposomes have been the best studied. Cationic liposomes interact with negatively charged glycoproteins (*i.e.* mucins) on the cell surface of corneal epithelial cells, thereby increasing the affinity and residence time with the target cells, resulting in increased drug delivery (Daull et al., 2012, Calvo et al., 1997, Yamaguchi et al., 2009). However, there are reports that the administration of cationic liposomes results in higher cytotoxicity levels, both *in vitro* (Zhong et al., 2009) and *in vivo* (Balazs and Godbey, 2010). Furthermore, cationic liposomes can be inactivated in the presence of serum (Audouy et al., 2000), are unstable upon storage (Balazs and Godbey, 2010), and present problems for clinical use.

In contrast, anionic liposomes offer a number of attributes, relative to the challenges associated with cationic formulations. Anionic liposomes demonstrate greater stability in solution (Zhigaltsev et al., 2002, Phillips and Heydari, 1996), lead to lower aggregation when compared with neutrally charged liposomes, and have increased endocytosis when compared with cationic (Bajoria et al., 1997) and neutral liposomes. Furthermore, two FDA-approved ophthalmic drugs utilize negatively charged, non-liposomal delivery modalities – Restasis (Allergan, for the treatment of chronic dry eye) and Durezol (Alcon, for ocular inflammation) (Lallemant et al., 2012, de la Fuente et al., 2010). These drugs lend support to the utilization of negatively charged liposome carriers to enhance ocular drug delivery. Based on these findings, we hypothesized that anionic liposomes would provide a viable

means for delivering peptides and other small molecules to the corneal epithelium, while helping to overcome the limitations of drug delivery.

In the present study, we developed a novel platform for delivering small molecules to corneal epithelial cells using anionic liposomes. The Phosphatidylcholine/Caproylamine/Dioleoylphosphatidylethanolamine (PC/CAP/DOPE) liposomes were synthesized to encapsulate small peptides, with the long-term goal of using these liposomes for drug delivery into the corneal epithelium. Caproylamine is a lipid with a free amino group on the lipid head, providing the versatility to attach biomolecules that are critical for drug delivery or target tissue specificity. The structure of the liposome vesicles additionally provides a protective shield to the molecules of interest (Figure 1) against the degradation, biotransformation, or elimination, that “free” molecules may undergo (Allen and Cullis, 2004). Additionally, this formulation is predicted to reduce the cytotoxicity of drug molecules (Pleyer et al., 1995), a major limitation when topically administering drugs to the eye (Furrer et al., 2002, Pellinen et al., 2012, Kovoov et al., 2004). This liposomal formulation is both easy to prepare and reproducible for generating carriers that have consistent dimensions, charge, and a high peptide delivery efficiency. In addition, the anionic liposomes can be used for plasmid DNA transfection of monolayers of corneal epithelial cells. Together, these liposomes introduce a promising new delivery alternative to the current treatment modality of eye drops, while overcoming several barriers of topical drug delivery to the corneal epithelium.

Materials

Phosphatidylcholine (PC), Caproylamine (CAP), Dioleoylphosphatidylethanolamine (DOPE) and a Mini-Extruder set were purchased from Avanti Polar Lipids Inc. (Alabaster, Alabama). Chloroform, digitonin and NaCl were acquired from Sigma (St. Louis, MO). FITC-labelled short sequence peptide (Sequence: IRNTNEpYTEGPTV – molecular weight: 1493.5 Da; pI: 4.53) was custom synthesized by Biomatik USA, LLC (Wilmington, DE). pRk7-histone 2B-RFP plasmid DNA was used as previously described (Hu et al., 2009).

hTCEpi cells were acquired from Geron Corp. (Menlo Park, CA) (Robertson et al., 2005). Cells were maintained in defined keratinocyte serum free media (K-SFM) supplemented with growth serum and Penicillin/Streptomycin (100 U/ml of penicillin and 100 µg/ml of streptomycin). TrypLE™ Express Enzyme was used to passage cells. All tissue culture supplies were purchased from Gibco (Life Technologies, Grand Island, NY). Cells were maintained in a Thermo Scientific Heracell 150i CO₂ incubator (Waltham, MA) at 37°C and 5% CO₂.

Methods

Liposome synthesis

Liposomes were synthesized using PC, CAP, and DOPE at a molar ratio of 4:2:1. The lipid mixture was dissolved in 3 ml of chloroform through brief stirring resulting in a 10 mM (7.75 mg/ml) PC solution. The suspension was then allowed to form a thin film using a rotary evaporator (Heidolph Collegiate Rotary, Elk Grove Village, IL) at 60°C and 250 rpm

for approximately 10 minutes until the solvent was completely evaporated. The lipid film was then hydrated and incubated with the molecules of interest. Empty liposomes were prepared by sonicating the thin film with 3 mls of 0.9 % NaCl, at 60°C for 3 h.

Peptide encapsulation and liposome characterization

The lipid film was resuspended in a 0.9 % NaCl solution (250 µl) using a water bath sonicator (Branson CPX5800H, Danbury, CT) at 60°C for 4 h. The FITC-labelled peptide (327.5 µg/ml) was added to the liposomal suspension to encapsulate it into the lipid vesicles. Peptide encapsulation was performed through ten repeated freeze-thaw cycles (Torchilin, 2003). Each freeze and thaw cycle consisted of ten minutes incubation in liquid nitrogen, ten minutes on ice, and ten minutes at room temperature. The liposomes encapsulating the peptide were extruded through a 200 nm pore size membrane [Nucleopore Track-Etch Membranes, Whatman (Piscataway, NJ)], using an Avanti® Mini-Extruder set. Dialysis against 0.9 % NaCl was performed using a 10 kDa molecular weight cut-off cassette (Thermo Scientific, Waltham, MA) to remove the “free” peptide present in solution. The dialysis was conducted at 4°C for 12 h with a buffer change every 4 h. The average size and charge of empty liposomes and peptide-encapsulated liposomes were determined using dynamic light scattering analysis (DLS) with a Zetasizer Nano Series DLS system (Malvern Instruments, Westborough, MA). Optical spectral evaluation was also conducted using a spectrophotometer (BioTek Synergy HT, BioTek, Winooski, VT) to compare the blank liposome spectrum with the peptide-encapsulated liposome spectrum. Liposomes were stored at 4°C for less than a week, with no detectable loss of transfection efficiency.

Cell culture and incubation with liposomes encapsulating FITC-short peptide

Liposomes encapsulating the FITC-short peptide were incubated with hTCEpi cells. hTCEpi cells were plated on glass coverslips and allowed to grow until 80% confluent, followed by 2 h of serum starvation. Serum-starved cells were then incubated with the liposomal suspension (1 ml/well; previously diluted 1:20 using serum free medium) for 2 h. As a negative control, intact cells received the FITC-short peptide (50 ng/ml) in the absence of liposomes. As a positive control for peptide delivery, cells were permeabilized using 0.15 mg/ml of digitonin (in PBS) for 5 min at 4 °C, followed by incubation with the FITC-labelled peptide (50 ng/ml). Following incubation with the liposomal suspension, the cells were washed 3× with PBS supplemented with 0.5 mM CaCl₂ and 0.5 mM MgCl₂ (PBS⁺⁺). Cells were then fixed with a 4% formaldehyde solution for 20 min, followed by three washes with PBS⁺⁺. Coverslips were mounted on microscope slides using Prolong antifade reagent containing DAPI (Invitrogen Life Technologies).

Image acquisition using fluorescence microscopy

After mounting the coverslips on the microscope slides, the slides were visualized under the Nikon Eclipse TE-2000U fluorescence microscope (Melville, NY) equipped with a 60X objective. Images were collected using Nikon NIS Elements software and exported to Adobe Photoshop for figure preparation.

Peptide encapsulation and release from liposomes

To measure the encapsulation efficiency of FITC-labelled peptide in the liposomes, immediately after the synthesis of the peptide containing liposomes, the peptide was extracted with acetonitrile and water co-solvent (9:1 water:acetonitrile, v/v) (Lutsiak et al., 2002). Briefly, a 2 μ L liposome solution was diluted 1:10 in PBS, followed by a 1:15 dilution in 9:1 water:acetonitrile. The sample was vortexed for 1 min and centrifuged for 15 min at 13,000 rpm. The fluorescence intensity of a portion of the supernatant was measured using a BioTek multiplate reader ($\lambda_{\text{ex}} = 495\text{nm}$, $\lambda_{\text{em}} = 519\text{nm}$) and quantified using a FITC-peptide standard curve.

To monitor the release of peptide from the liposomes, 600 μ L [of the 7.5 mM (theoretical) liposome stock] was diluted 1:10 in PBS (pH 7.4). The liposome solution was dialyzed using a 12–14,000 Da MWCO membrane in 1000 mL 1X PBS with constant stirring at room temperature ($\sim 22^\circ\text{C}$). Fifty microliter liposome aliquots were directly taken from the dialysis membrane at 0.5, 2, 4, 8, 24, 48, 72 h and 1 wk time points. The amount of FITC-labelled peptide released from the liposome was determined as described above using fluorescence intensity. All samples were measured in triplicate unless otherwise noted.

FACS Analysis

Following liposome incubation for 0.5, 2, or 16 h, cells were washed three times with PBS to remove the associated liposomes, trypsinized, and resuspended in PBS. The cells were evaluated for the presence of FITC using a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR). Non-transfected cells were used to determine the basal fluorescence intensity; basal fluorescence was subtracted from the liposome transfected cells.

Lipoplex formation

Liposomes complexed with cDNA (lipoplexes) were synthesized to evaluate gene transfection efficiency in hTCEpi cells. The thin lipid film with the same lipid composition was formed as described previously. Since anionic liposomes have a negative net charge it is important to use cations (CaCl_2) to mediate the electrostatic interaction between the negatively charged DNA and liposomes (Patil et al., 2004). The dried thin film was hydrated with 1 mL of 2X HEPES [4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine- N' -(2-ethanesulfonic acid)] buffered saline pH 7.4. The solution was briefly vortexed leading to a white opaque solution. To disrupt any visible aggregates, the suspension was subjected to extensive sonication (≈ 4 h) at 60°C in a water bath until the preparation was free of aggregates visible to the naked eye (determined empirically). The liposomes were then extruded using the same methods described earlier. Dynamic light scattering (DLS) was performed in order to determine the size distribution of the particles. Plasmid DNA complexation was done by mixing the plasmid DNA with Ca^{2+} . Different Ca^{2+} concentrations (1.1 mg/mL to 14.7 mg/mL) and volumes were mixed with the diluted DNA to achieve the final Ca^{2+} concentrations. After mixing the DNA with the Ca^{2+} , the mixture was allowed to rest for 10 min at room temperature. The amount of liposomes was also varied (ranging between 1 and 35 μ L/mL). All of the buffers, liposomes and Ca^{2+} solutions were sterile filtered prior to incubation with the cells. Complexation of the DNA

with the liposomes was done through drop-wise mixing of the DNA (600 ng) with the liposome suspension under gentle vortexing for approximately 1 min. Lipoplexes were allowed to form for 30 min at room temperature with vortexing of the vial every 10 min (Patil et al., 2004).

hTCEpi cells were plated at 100,000 cells/well on glass coverslips (Size: 12RD, thickness: 1, Propper Manufacturing Company, Long Island City, NY) in 24-well plates. The cells grew for 24 h, were washed twice with PBS, and were subsequently serum-starved for 2 h prior to incubation with the lipoplexes. Each well contained 1 ml of K-SFM, and lipoplexes (60 μ l/well, 600 ng of DNA/well) were added dropwise to the cells. The lipoplexes were distributed evenly across the coverslips to maximize distribution of the complexes and the cells were incubated for 2 h at 37°C. Following incubation, the cells were washed twice with cell medium supplemented with serum and replaced every 24 h. After 72 h, cells were fixed in 4% formaldehyde for 20 min, washed in PBS/0.5 mM CaCl_2 /0.5 mM MgCl_2 (3 $\times$ for 5 min each) and mounted on microscope slides using Prolong antifade reagent with DAPI (Invitrogen, Grand Island, NY).

For experiments that examined the co-localization of the FITC-labelled peptide with Early Endosome Autoantigen 1 (EEA1), after fixation, cells were permeabilized with 0.1% saponin, and probed with a mouse monoclonal EEA1 antibody (Cell Signalling, Danvers, MA), followed by a secondary Alexa 468-conjugated goat anti-mouse secondary antibody as previously described (Dinneen and Ceresa, 2004). Coverslips were mounted on microscope slides using Prolong reagent containing DAPI (Invitrogen). Cells were then visualized and imaged using a Nikon Eclipse TE-2000U microscope (Melville, NY) with a 60X objective.

Results

Liposome synthesis, peptide encapsulation and characterization

Preparation of the PC/CAP/DOPE liposomes was straightforward and highly reproducible. The average hydrodynamic size of the peptide-encapsulated liposomes was 138.8 ± 34 nm (Figure 2A) and displays the relatively narrow size distribution for a representative preparation. The sizes of empty liposomes and lipoplexes were 169 nm and 159 nm, respectively, and showed no statistically significant differences in diameter relative to peptide-encapsulated liposomes. The average zeta potential for PC/CAP/DOPE liposomes was -18.2 ± 1.3 mV.

Since the objective of these studies is to develop a new strategy for delivering small molecules and/or peptides to the corneal epithelium, we tested the ability of these liposomes to encapsulate a 13 amino acid, fluorescently-tagged peptide (described in Material and Methods), that was originally designed to enhance growth factor receptor-mediated signalling (Kumar et al., 2012). However, the peptide's instability in the cytosol precluded that function. Here, the encapsulation of the peptide in the liposomes was achieved and verified through spectral evaluation and fluorescence microscopy. First, the short sequence peptide had a fluorescent FITC-tag attached to the N-terminus that was detected within the optical absorption spectrum collected for the sample (Figure 2B); empty liposomes did not exhibit an absorption peak in the region of 480–490 nm.

Next, the encapsulation efficiency (EE) of peptide within the liposome was quantified using fluorescence spectroscopy after disruption of the lipid vesicles with acetonitrile (Lutsiak et al., 2002). We found that the liposomes encapsulated 149.7 µg/ml (total added = 327.5 µg/ml) FITC-labelled peptide, resulting in an EE of 45.7%. In addition, the rate of peptide release from the liposomes was evaluated; the liposomes were allowed to release the encapsulated peptide into the surrounding PBS eluent, and the amount of remaining peptide present in the liposomes was measured. We determined that 50% of the peptide was released from the liposomes within ~2.5 h and 100% of the peptide was released after 48 h (Figure 3).

Cell culture and incubation with liposomes encapsulating FITC-short peptide

The peptide-encapsulated PC/CAP/DOPE liposomes were evaluated for their ability to deliver the peptide to corneal epithelial cells (Figure 4). The liposomes were incubated with immortalized human corneal epithelial (hTCEpi) cells in serum-free media. Delivery of the peptide to the hTCEpi cells was determined by monitoring for the presence of the FITC-tag, either by fluorescence microscopy (Figure 4) or FACS (Figure 5). After 2 h incubation with the peptide-encapsulated PC/CAP/DOPE liposomes, approximately 69% of the cells were positive for FITC (Figure 4C). Cells were incubated with the FITC-labelled peptide alone as a negative control (Figure 4A; 0% of cells were positive for fluorescence) and the peptide with digitonin as a positive control (91% positive cells) (Figure 4B). Cells were sorted by FACS (Figure 5) as a secondary method of determining FITC-peptide containing cells. A clear, time-dependent increase in the fluorescence intensity was observed after treatment with liposomes containing FITC-peptides. The mean fluorescence intensity increased to 7.4-fold after 16 h of liposome incubation.

Next, we wanted to determine how the liposomes deliver peptide to the cells. Two hours after liposomes incubation, there is a near uniform distribution of fluorescence in the cytosol (Figure 4C). This suggested to us that an equilibrium in peptide internalization had been reached and shorter time points should be examined. To determine if the liposomes were entering the cell via the endocytic pathway, we examined the distribution of the FITC-peptide 30 min after liposome incubation. We fixed the cells and processed them for indirect immunofluorescence using an early endosome specific antibody [Early Endosome Autoantigen 1, or EEA1 (Mu et al., 1995) (Figure 4E)] to determine if the FITC-labelled peptide was delivered directly to the cytosol or entered the cell via vesicular trafficking (*i.e.* endocytosis or phagocytosis). At this early time point, there are distinct peptide-containing vesicles (green) that are separate from the EEA1-positive vesicles (red). These data suggest that the FITC-labelled peptide is being delivered via a non-endocytic mechanism and via membrane fusion.

Lipoplex formation and lipofection

Last, the PC/CAP/DOPE liposomes were tested for DNA delivery to corneal epithelial cells. Corneal epithelial cells are notoriously resistant to the uptake of genetic material, with standard transfection protocols (*i.e.* calcium phosphate, Lipofectamine, Fugene) resulting in less than 0.1% cells being transfected. Liposomes and DNA were complexed ("lipoplexes") and upon sonication, produced a suspension that was aggregate-free and had an opaque,

white appearance. Lipoplexes were prepared and delivered to cells as described in Materials and Methods. The plasmid DNA that was used encodes Histone 2B-Red Fluorescent Protein (H2B-RFP) chimeric protein; it co-localizes with the chromosomes in the nucleus and is easily identifiable with fluorescence microscopy (Figure 6). Using fluorescence microscopy, we observed that 8% of cells were transfected.

Discussion

Cationic liposomes have been used in many applications, primarily due to their net charge that enables rapid cellular association and uptake. While cationic and neutrally charged liposomes have been used in many applications, there are several drawbacks associated with the delivery of cationic liposomes. Administration of cationic formulations may lead to cytotoxicity both *in vitro* and *in vivo*, they can be inactivated in the presence of serum, and demonstrate poor storage stability. Moreover, cationic liposomes when injected systemically tend to interact with the circulating blood cells leading to the formation of aggregates; however, this is not seen in anionic (Yeo, 2013) or neutral liposomes (Krasneci et al., 2003). Anionic liposomes have slower kinetics of clearance and reduce phagocytosis by macrophages (Figueiredo et al., 2014), and due to their charge, they extravasate when delivering cargo to tumor vasculature (Krasneci et al., 2003). In addition, they release their cargo faster than neutral liposomes (Nie et al., 2012), which can be of interest for our application - delivery to the corneal epithelium - since release time is critical.

The mechanisms that promote the interaction of the anionic liposomes with cells are unclear (Nie et al., 2012, Zhaorigetu et al., 2014), however it has been demonstrated that liposome uptake occurs when the cell-to-liposome charge ratio reaches a certain threshold (Miller et al., 1998). Liposome uptake consists of two steps: binding and entry in the cells. Lee et al. identified higher interaction of anionic liposomes with cells when compared with neutral and cationic liposomes (Lee et al., 1993, Lee et al., 1992). However, the amount of binding will be dependent on the cell type and charge of the particles (Sahay et al., 2010, Dan, 2002). We have identified an anionic liposome formulation that permits small molecule and cDNA delivery in corneal epithelial cells.

Using a 4:2:1 ratio of PC/CAP/DOPE, we have developed an anionic liposome preparation with a hydrodynamic size within the range for optimal peptide delivery (Chen et al., 2013, Allon et al., 2012). While larger particles can encapsulate more cargo, liposome uptake is highly dependent on and inversely correlated with particle size. In the case of our liposomes, we achieve high encapsulation efficiency (46%) of our FITC-labelled peptide into 138.8 nm particles. The freeze-thaw protocol used to load the peptide changes the molecular spacing and structure between the lipid molecules and allows for the penetration of the peptide molecules to the core of the liposomes (Colletier et al., 2002). Here, we successfully use this methodology as an alternative to the conventional encapsulation methods that require the use of high temperatures to melt the lipid molecules, which may also adversely affect the stability and functionality of the peptide or other small molecules (Zhao and Lu, 2009, Colletier et al., 2002). The negative charge has sufficient association with the cell membrane to permit fusion with the cell. In addition, we have tailored the release of peptide cargo within 48 h, to provide both rapid and enhanced delivery into the cell (vs. free peptide), and

a potential means to extend the typically low (on the order of hours) half-life of free peptide and DNA molecules. Our data indicate that this anionic liposome preparation can be used to minimize the issues associated with cationic liposomes (cytotoxicity and instability).

Further, our studies indicate that our liposomes deliver the peptide via liposome:membrane fusion rather than endocytosis. Early time points show the presence of cell-associated fluorescently-labelled vesicles that are distinct from the early endosomal marker, Early Endosome Autoantigen 1 (EEA1) (Figure 4E), indicating the cargo is not entering the cell through the endocytic pathway. Furthermore, relative to free peptide, we achieve significantly improved uptake of the peptide-encapsulated liposomes in corneal epithelial cells, as measured by FACS and fluorescence microscopy. This indicates their promising potential as a biologic (here, peptide) delivery vehicle to substantially improve cell uptake and remain in the cell for longer durations than free peptide alone.

For gene transfection applications, it is well known that corneal epithelial cells are notoriously difficult to transfect. The commercially available transfection agents (Lipofectamine, Fugene, and Nucleofection) often yield little to no cDNA transfection (Shankardas, 2008). Although cationic liposomes are most commonly used for nucleic acid transfection (Patil et al., 2004), the use of anionic liposomes, like these PC/CAP/DOPE liposomes, have been reported to have a lower cytotoxicity (Navarro et al., 2013). Despite the moderate cDNA transfection efficiency, our transfection efficiency is higher than other established methods (Shankardas, 2008), and this methodology introduces a viable approach to express genes in hTCEpi cells. In addition, gene expression can be induced in monolayer and differentiated corneal epithelial cell cultures with minimal disruption.

Here we demonstrate that our novel anionic liposomes can provide controlled delivery of small cargo into corneal epithelial cells, with corresponding enhanced delivery of peptide relative to free peptide alone. Additionally, the biofunctionality of cDNA is maintained and enhanced relative to other corneal cell transfection options, as demonstrated by the expression of H2B-RFP in hTCEpi cells. Based on these promising results, we expect that topical administration of these particles will be useful for future *in vivo* studies. Combined with the ability of liposomes to increase the residence time on the ocular surface to provide efficacy and enhanced drug retention, this delivery modality may help circumvent some of the intrinsic protective mechanisms of the eye responsible for poor drug bioavailability. In addition to corneal epithelial delivery, we anticipate the utility of this delivery platform to encapsulate other molecules of interest for delivery to other tissues. Liposomal delivery systems are currently being tested in a variety of applications [e.g., drug delivery to tumoural sites, cosmetics, antigen delivery (immunology), enzymatic delivery]. Furthermore, liposomes are being formulated for intravenous, subcutaneous, peritumoural and intraperitoneal delivery, in addition to topical delivery. Delivery of small cargo to the corneal epithelium still requires optimization based on the selected target molecule; however, we believe this study can serve as a preliminary work to motivate additional research in this field.

Conclusions

The present study illustrates an easy, novel and reliable method to deliver peptides to human corneal epithelial cells. Anionic liposomes complexed with plasmid DNA resulted in a modest, although beneficial, level of transfection, relative to the scarcity of efficient transfection methods that currently exist. Additionally, peptide delivery was enhanced, relative to the delivery of free peptide alone, demonstrating the feasibility of improved biologic delivery into the cell. With this formulation, we also anticipate the delivery of other compounds including analgesics (diclofenac, ketorolac), antibiotics (bacitracin, erythromycin, ofloxacin) or VEGF inhibitors in future *in vitro* and *in vivo* studies. By increasing the amount of drugs delivered to the corneal epithelium, we postulate that less frequent administrations will be required as well as achieving more accurate dosing and less cytotoxicity. These factors will reduce patient discomfort and recovery time (Fathalla et al., 2015, Monem et al., 2000). Efficacy, stability, *in vivo* fate and safety are future studies of interest for translation of this platform for *in vivo* applications.

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Figure 1. Schematic of liposome formation and peptide encapsulation

The phospholipids are originally suspended in an organic phase. Upon removal of the solvent through rotary evaporation the dried thin film is hydrated with an aqueous buffer and mixed with the peptide of interest. Through repeated freeze and thaw cycles the peptide is then encapsulated in the liposomes. The free peptide is removed through extensive dialysis.

Figure 2. Characterization of liposomes

A) Size distribution by number. The DLS evaluation of this liposomal preparation had an average particle size of 132.7 nm. The chart indicates a relatively homogeneous liposome size distribution. **B)** The optical density for two different liposome preparations (empty and peptide-encapsulated liposomes) was acquired to verify the presence of the fluorescent peptide in the liposomes. The presence of a “peak” in the 488 nm region of the spectrum for the peptide-encapsulated liposomes indicates the presence of the FITC-labelled peptide.

Figure 3. Peptide release from liposomes

Liposomes were analysed for FITC-labelled peptide release by measuring the amount of remaining peptide during dialysis. Data are plotted as the average percentage of peptide released ($\pm$ S.E.M.) over the course of 48 hours. Inset shows peptide release over the course of 7 days.

Figure 4. Peptide delivery to hTCEpi cells via liposomes

hTCEpi cells were incubated with **A)** FITC-labelled peptide alone, **B)** FITC-labelled peptide with digitonin incubation or **C)** liposomes containing the FITC-labelled peptide. Size bar = 10 μ m. **D)** Quantification of FITC positive cells is displayed in the bar graph. Data represent the mean $\pm$ S.E.M. from three experiments in which 60–100 cells were counted in each experiment. **(E–G)** To assess the mechanism of liposome uptake, 30 min after incubation with liposomes, the cells were fixed and stained with an antibody against EEA1 to mark

early endosomes. Representative micrographs are shown. **E**) FITC-labelled peptide (green), **F**) EEA1 (red). **G**) Merge of (E) and (F) with DAPI stained nuclei (blue). Size bar = 10 μ m.

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Samples	Incubation time (h)	Mean FL1-H (SPR2)
Cells only	0 h	6.34
Liposome	0.5 h	27.7 ± 1.3
FITC-peptide	2 h	25.8 ± 0.6
	16 h	46.7 ± 1.9

Figure 5. FACS analysis: peptide-encapsulated liposomes demonstrated enhanced uptake into hTCEpi cells
hTCEpi cells were incubated with liposomes containing FITC-peptide for 0, 30 min, 2 h, or 16 h. **A)** Histograms of the fluorescence intensity at each time point. **B)** Table showing the mean fluorescence intensity $\pm$ S.D. for each time point. Data are from a representative experiment repeated twice.

Figure 6. hTCEpi cells were transfected with lipoplexes

hTCEpi cells were transfected with cDNA encoding RFP-labelled Histone 2B using Ca^{2+} alone [(A) and (B)] or liposomes [(C) and (D)]. After 72 h transfection, cells were fixed, stained with DAPI, and mounted on coverslips. Cells were visualized with fluorescence microscopy. Panels A) and C) show the red channels (RFP-Histone 2B); panels B) and D) are the merge of Histone 2B (red) and DAPI (blue). White arrows indicate transfected cells illustrated by the presence of red nuclei. Size bar = 10 μm .