Challenges in Nucleic Acid-Lipid Films for Transfection

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The use of surface-based methods for the delivery of therapeutics has recently generated increasing interest. These platforms have tremendous potential to minimize detrimental side effects associated with systemic delivery by localizing the therapeutic vehicle, and thus provide higher local doses for improved efficacy. Cationic lipids are one of the most commonly used synthetic carriers for the delivery of genetic cargo, such as DNA and RNA. However, reports on the use of lipid-based films for gene delivery are scarce. Here we investigate the use of a lipid-based film for the in vitro *delivery of plasmid DNA. Solid DNA-lipid films show very low levels of transfection, while identical complexes prepared for bolus delivery provide high levels of transfection when used directly. We investigate the mechanism, whereby the activity of these solid-state films is lost and suggest methods for circumventing these challenges and restoring the efficacy of these films as gene delivery platforms.* © 2013 American Institute of Chemical Engineers *AIChE J*, 59: 3203-3213, 2013

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Introduction

Synthetic transfection vectors have tremendous potential, allowing for straightforward preparation while bypassing many of the immunogenic and mutational concerns associated with viral vectors.[1] Nonviral transfection reagents are typically prepared via the electrostatic complexation of negatively-charged nucleic acids (DNA or RNA) with positively-charged lipids (lipoplexes) or polymers (polyplexes). While the majority of studies have utilized bolus delivery of these complexes as nanoparticles in solution,[2–5] there are an increasing number of applications that would benefit from a more localized transfection strategy, such as thin films or surface-mediated delivery. The goal of many of these strategies is to increase the transfection efficiency of these nonviral methods in order to compete with the performance of viruses,[6,7] or provide both localized and controlled release.[8]

Traditional transfection methods, commonly termed bolus delivery, utilize solution-based delivery of nanoparticles directly to cells. In this method, the nanoparticle containing solution is layered on top of a cultured cell monolayer and allowed to incubate. During incubation the nanoparticles can then diffuse through the solution and interact with cells. However, these interactions are random, and thus the efficiency of

Additional Supporting Information may be found in the online version of this article.

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this process is very low, with only a fraction of the delivered dose effectively interacting with cells.[9] One way to overcome this limitation is to increase the concentration of nanoparticles, so as to increase the probability of interaction. However, this increased frequency of cell-nanoparticle interaction comes at a cost both in terms of delivered material and overall efficiency, as well as the potential for increased toxicity.

Localized delivery strategies have the potential to enable higher transfection efficiencies compared to solution-based methods. By confining transfection reagents to a surface that is in contact with cells, a higher local concentration of the reagent, relative to bolus delivery, can be generated, leading to potentially higher and more efficient cellular uptake and gene expression while utilizing lower overall concentrations of transfection reagents.[1,9,10] Furthermore, layered films have the potential to provide both controlled release of transfection reagents over time and/or sequential release of various factors for more complex experiments.[2–5,8] While a variety of strategies for surface-mediated delivery have been reported, the vast majority have utilized polyplexes,[6–8,11–13] with very few reports of lipoplex-based strategies.[8,14–17]

Previously, we reported on the detailed structural analysis of a solid film composed of DNA and the cationic lipid dimethyldidodecylammonium bromide (DDAB).[9,18–20] These films are prepared first by the electrostatic complexation of DNA and lipid in water. The resulting water-insoluble complex is then dried and cast as a thin film using an organic solvent. The properties of similar bulk films have been reported previously for a variety of applications including coatings, delivery of therapeutics, nanoelectronics, photonics, and optoelectronics.[16,21–24] A unique feature demonstrated for DNA-DDAB films is that, in the dry state, the DNA is maintained in a single-stranded conformation which renders it resistant to degradation by nucleases. However, upon hydration, the structure of the film switches, restoring the DNA to its biologically active double-stranded form.[19,20] This structural transition would allow for the easy storage and use of films in a clinical setting, as the dry film could be stored indefinitely and then activated by hydration upon implantation at the site of therapeutic interest within the body.

Although surface-mediated transfection has the ability to increase the rate of cellular uptake by increasing local concentration and cellular contact, the use of a solid film can also present significant challenges related to the size of particles available for uptake via endocytosis. For instance, the size of the submicron particles used in bolus delivery can be controlled during preparation. Delivery from a film, however, requires the spontaneous formation and/or release of particles from a larger monolith. It then becomes an interesting materials challenge to design a system that enables release of particles from a film within a size range amenable to endocytosis. While some reports have utilized bolus-type particles tethered to a surface,[12,25–27] strategies which either encapsulate particles heterogeneously into a matrix[28–31] or use a monolithic solid typically utilize carefully designed polymers that degrade in a controlled manner that has been tailored to release small particles.

Additionally, the release of DNA from polymer-based complexes (polyplexes) in the cytoplasm after endocytosis can also be a challenge. The multivalent interactions of polymers with DNA which provide high stability during delivery must be disrupted to allow for the genetic cargo to be accessed.[7,11,13,32–43] In fact, because of this high level of stability conferred by long polymer chains, gene delivery by polyplexes has the potential to be less efficacious than with lipoplexes where disassembly can occur more easily.[43–46] However, these weaker interactions have also resulted in significant challenges in attempting to develop film-based methods for lipoplex delivery. Here we examine the challenges associated with lipoplex-based films for gene therapy. We first compare bolus delivery of lipoplexes with film-based transfection methods. Based on these results we determine the bottleneck for gene delivery from lipid films and correlate these results with a closer examination of the disassembly of these films under cell culture conditions.

Materials and Methods

Plasmid DNA

Plasmid DNA (pDNA) used in this study was pAAV CMV luc SN coding Firefly luciferase under the control of an CMV promoter.[47] The plasmid was expanded in *Escherichia coli* and purified using a Qiagen MaxiPrep kit. The plasmid was dissolved in Milli-Q water and stored at −80°C until use.

Cell Culture

HeLa cells were obtained from ATCC. The cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco), supplemented with 10% (v/v) fetal bovine serum (FBS, ATCC) and 1% (v/v) penicillin and streptomycin (Life Technologies) at 37°C in a humidified atmosphere with 5% CO_2.

Preparation of Lipoplexes

The cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and the zwitterionic lipids 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were obtained from Avanti Polar Lipids and used without further purification. Liposome and lipoplexes were prepared using methods published elsewhere.[48] Briefly, solutions of small unilamellar liposomes were prepared by first dissolving the individual lipids in chloroform at a concentration of 30 mM. The resultant solutions were mixed and dried in a glass vial first under nitrogen to form a uniform film on the inside walls of the vial and then overnight *in vacuo* to ensure removal of the chloroform. The liposomes were reconstituted at 1 mM by adding Milli-Q water and allowing the lipid films to rehydrate at 37°C for at least 6 h, and then tip-sonicated for 7 min and filtered through a 0.2 μm nylon syringe filter to remove metallic particles from the tip sonicator. This procedure was used for the creation of not only standard liposomes, but also fluorescently labeled and liposomes incorporating poly(ethylene glycol) (PEG, Sigma) and PEG-lipids such as 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N—[amino(poly(ethylene glycol))−2000] (DSPE PEG, with either maleimide or methoxy termination of the PEG chain, Avanti Polar Lipids) of varying PEG molecular weights.

Lipoplexes were prepared in microcentrifuge tubes (Eppendorf) by diluting both DNA and liposome solutions separately in OptiMem (Gibco). Typical experiments utilized 1 μg of DNA and 100 μL of total solution per sample, prepared in triplicate. Lipid solutions were then added to the

dilution containing the DNA via pipette and immediately mixed by pipetting up and down. Solutions were not allowed to stand longer than 1 h before use.

Positive control samples were prepared using either the lipid-based Lipofectamine 2000 (Invitrogen) or the polymer-based Exgen 500 (Fermentas Life Sciences) according to the manufacturer's specifications.

Preparation of films and film substrates

Once prepared, the lipoplexes were dried *in vacuo* and then rehydrated using a 54:46 (v/v) mixture of isopropyl alcohol and water. The higher water content was necessary for dissolution of the OptiMem buffer salts. Films were then cast either directly into the cell culture well plate (24-well, BD Falcon) or onto squares of treated polydimethylsiloxane (PDMS, Sylgard, Dow) and allowed to dry completely before being added to cells in culture. All work was done in a biological safety cabinet.

The PDMS substrates were prepared using a standard kit. One part crosslinking agent was combined with 10 parts monomer by mass, mixed well using a tongue depressor, and then degassed *in vacuo*. The resulting clear liquid was cast onto a glass slide in a Petri dish and allowed to cure overnight at 60°C. Small squares, typically 1 cm^2, were cut out using a razor blade. The PDMS was then washed twice for 12 h in hexanes, ethanol, and Milli-Q water, respectively, to remove small-chain components that have been shown to have an adverse effect on cell culture.[49] All substrates were all sterilized using an autoclave before use.

Transfection and cell viability studies

For transfection, two types of experiments were performed: (1) transfection reagents were added on top of preseeded cells, or (2) cells were seeded on top of precast films. A seeding density of 100,000 HeLa cells per well was used for all experiments unless otherwise specified.

For experiments involving preseeded cells, the cells were passaged, counted, and allowed to settle for 24 h in a 24-well BD Falcon well plate. Before starting the experiments the cells were washed once with 0.5 mL phosphate buffered saline (PBS, Gibco). The transfection reagents were then added directly onto the cells. In addition to the transfection reagent or film, OptiMem was added to the wells to a final volume of 1 mL. For experiments involving chloroquine 1.8 μL of a 6 mg/mL solution of chloroquine was added along with the transfection reagents. After 6 h the wells were washed with 1 mL of 1X PBS and then covered with 1 mL of 10% FBS in DMEM. Experiments were typically read out 24 h after the addition of transfection reagents.

For experiments where cells were seeded directly onto transfection reagents, the films were precast into the well plate and allowed to dry overnight. For bolus delivery, reagents were pipetted into the wells directly before the addition of cells. Whereas transfection for preseeded cells was done in the presence of OptiMem, it was necessary to perform these transfection studies in DMEM with 10% FBS in order to facilitate attachment of the cells to the surface.

Quantification of transfection was performed using Firefly luciferase as a reporter gene. Luciferase expression was assayed using a standard kit (Invitrogen SteadyGlo® Luciferase Assay System). The strength of the observed luminescence signal was normalized with respect to total cellular protein, as determined by a Bradford assay (Bio-Rad). Luminescence and colorimetric absorbance measurements were taken with an Infinite M200 plate reader using i-Control software version 1.6 (Tecan).

Cell viability studies were performed using an Alamar Blue assay (Invitrogen). The cells were transfected as above. After 24 h they were first washed with 1 mL of 1X PBS. 1 mL of 10% Alamar Blue reagent in DMEM was then added to the wells and allowed to incubate for 4 h at 37°C. Fluorescence intensity was measured using an Infinite M200 plate reader and was normalized to the cell control.

Dynamic light scattering (DLS)

Lipoplexes were first prepared in microcentrifuge tubes (Eppendorf). The lipoplex solutions were then dried, dissolved in isopropyl alcohol/water to simulate film casting, dried again, and then rehydrated in water. Measurements of particle size were taken of the initially prepared lipoplex solutions and of the reconstituted films using a Viscotek 802 dynamic light scattering instrument using Omnisize software version 3.0 (Malvern). All measurements were taken at an angle of 90°. Particle sizes were calculated using a single-exponential fit of the correlation curve. All data below 20 nm and above 10,000 nm was rejected for this analysis. A similar analysis was performed on the initial liposome solutions before complexation with DNA.

SEM measurements

Films were prepared as for transfection studies and were cast on small silicon chips that had been cleaned beforehand by rinsing in ethanol and drying under nitrogen. After drying, the solution-exposed films were immersed in 1 mL of water, OptiMem, or another buffer for approximately 30 min before beginning preparations for imaging. Films were then fixed using standard procedures. Briefly, the films were first soaked in a 2% gluteraldehyde solution in 0.1 M sodium cacodylate buffer pH 7.2 for 1 h, then rinsed twice for 10 min in 0.1 M sodium cacodylate buffer. They were then stained with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h, rinsed 3 times for 15 min in 0.1 M sodium cacodylate buffer, and gradually dehydrated by immersion in a series of water/ethanol mixtures (5 min in 35, 50, 70 and 80% ethanol followed by 10 min in 95% and twice in 100% ethanol). Finally the samples underwent critical point drying and were sputter-coated with ~2–25 nm gold before mounting on a copper mount with adhesive tape and imaging with a Hitachi S-5000 scanning electron microscope.

Flow cytometry

Transfection was performed as described perviously using Cy5-labeled pDNA (Mirius Label IT® Nucleic Acid Labeling Reagents kit) DOTAP mixed with 5% Texas Red-DHPE (Invitrogen). After 24 h the films were removed, cells were washed with 1X PBS and then released from the well plate using trypsin, and analyzed by flow cytometry in a Beckman-Coulter FC500 flow cytometer in an experiment similar to that described in the literature.[50] Gates and compensations were set against nontransfected and singly labeled cell standards. The average percentage of gated cells was calculated across triplicate samples.

Confocal microscopy

HeLa cells were seeded for 24 h at 10,000 cells per well in 8-well boxes for microscopy measurements. The amounts

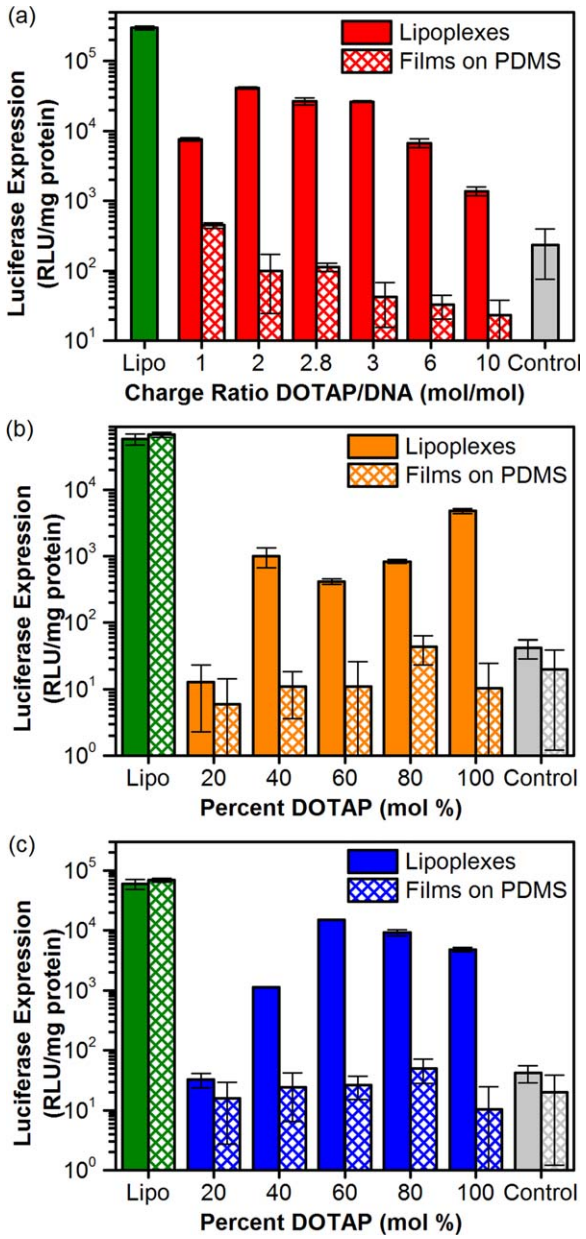

Figure 1. Analysis of gene expression after 24 h in HeLa cells using a luciferase assay for both lipoplexes (bolus delivery, solid bars) and films (hashed bars) as a function of (a) the ratio of cationic lipid DOTAP to DNA, (b) the ratio of the cationic lipid DOTAP to the zwitterionic lipid DOPC or (c) DOPE.

Results shown in (b) and (c) are at a DOTAP:DNA ratio of 2:1. Lipofectamine (Lipo) is shown as a positive control. Hashed bars shown for the positive and negative controls in (b) and (c) are shown because experiments for bolus and film delivery were performed separately, rather than indicating film delivery of these controls. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

of each transfection reagent were scaled to account for the smaller well. Samples were prepared typically using Cy5-labeled pDNA (Mirius Label IT® Nucleic Acid Labeling Reagents kit) and 1% NBD-DOTAP (Avanti Polar Lipids). 24 h after addition of the transfection reagents all films were removed and wells were washed with 200 μL 1X PBS. The cells were then stained with DAPI (nuclear stain) and/or AlexaFluor 594-labeled wheat germ agglutinin (AF 594 membrane stain, Invitrogen) as appropriate, followed by fixing in 4% formaldehyde. Imaging was performed at 40× magnification on an Optimus 710 inverted laser scanning confocal microscope (Carl Zeiss). All images were taken at a focus plane near the bottom of the cell. The intensity of the signal was corrected using Zen 2011 software (Carl Zeiss), but was adjusted to "best fit" settings for the red, green, and blue channels and the full range for the yellow channel for display purposes in figures.

Results

Transfection and cell viability

We first performed a series of parallel transfection experiments to both optimize the conditions for transfection (see Supplemental Information online for Figures S1–S4) and compare the efficiency of gene delivery from our DNA-lipid films as compared to bolus delivery. Gene expression levels were determined using luminescence from Firefly luciferase as a quantitative measure, normalized to the total amount of cellular protein present. The commercial transfection reagent Lipofectamine 2000 was used as a positive control.

We then investigated the level of transfection observed over a range of cationic lipid (DOTAP) to DNA ratios (Figure 1a). A maximum was seen in the observed transfection from bolus delivered DOTAP-only lipoplexes at a DOTAP:DNA ratio of 2:1. This ratio was used in all subsequent experiments. We also tested the efficacy of the zwitterionic "helper" lipids DOPC and DOPE as have been used previously in the literature.[48,51–54] The addition of DOPC showed no benefit (Figure 1b), while a 60/40 mixture of DOTAP/DOPE showed significantly higher levels of transfection (Figure 1c). The transfection levels for all films were at least an order of magnitude lower than for bolus delivery and were comparable to the levels observed in the negative control samples. The transfection levels for all samples were significantly lower than those observed in the Lipofectamine positive control. A similar decrease in activity is shown for films prepared from Lipofectamine (Figure 3).

An analysis of cell viability (see online for Figure S2) shows no correlation between the viability of cells grown in the presence of either DOTAP-based films or lipoplexes and the observed levels of transfection, while a significant decrease in viability is observed for cells treated with Lipofectamine.

Transfection experiments were also performed in the presence of the small molecule chloroquine, which can be used to eliminate the barrier of endosomal escape by facilitating endosomal rupture.[1,7,51,54] As can be seen in Figure 2, the addition of chloroquine had little or no effect on samples where cells were seeded directly onto films. For films that are applied to preseeded films, some benefit was seen for samples containing 60/40 DOTAP/DOPE and a tremendous improvement in transfection was seen for the delivery of naked DNA. The addition of chloroquine was highly detrimental to the transfection efficiency of Lipofectamine. In all cases, the observed transfection levels for films were at least an order of magnitude smaller than those for bolus delivery of lipoplexes, regardless of the presence of chloroquine.

To investigate the effect of various steps in film preparation on the observed decrease in transfection for films

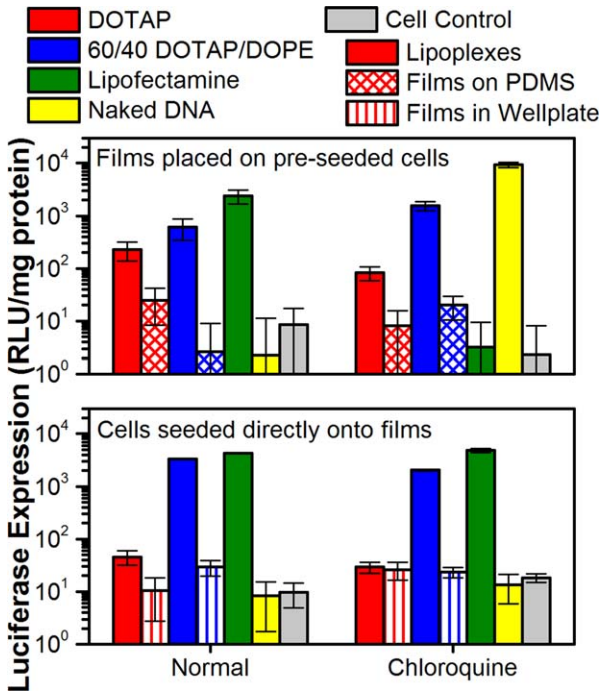

Figure 2. Analysis of gene expression after 24 h in HeLa cells on the effect of both chloroquine, to facilitate endosomal escape, and the method of seeding cells/adding transfection reagent.

Both lipoplexes (bolus delivery, solid bars), and films (hashed bars) were tested. For preseeded cells, films were cast onto PDMS and then inverted onto the cells. For cells seeded onto films, the films were cast directly onto the well plate and cells were allowed to seed directly on the films. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

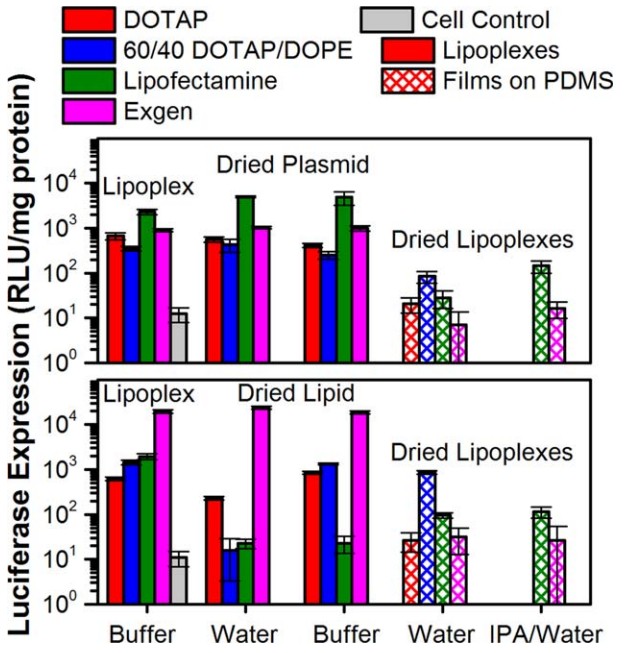

Figure 3. Analysis of the effect of drying of the various components (pDNA, lipid, and lipoplexes) from water, buffer, and an isopropyl alcohol/water mixture on luciferase expression in HeLa cells.

The data compares bolus delivery of lipoplexes (solid bars) to film exposure (hashed bars). OptiMem was used for all mixtures of lipids and pDNA while 150 mM NaCl was used for Exgen, as per the manufacturer's instructions. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

compared to lipoplexes, we ran a series of experiments where the individual components of the films, i.e., the pDNA and the lipid or a polymer-based transfection reagent (Exgen 550) were dried from either water or OptiMem buffer, rehydrated, and then reconstituted for use in the formation of lipoplexes. For film samples, lipoplexes were formed and then dried either directly from water or from the standard isopropyl alcohol/water mixture (Figure 3). While drying of the pDNA appeared to have a minimal effect on the observed transfection, drying of the lipid tended to cause a decrease in the observed transfection levels. Interestingly, the polymer-based transfection reagent Exgen 550 showed minimal loss of activity when dried separately. However, for all reagents, drying of the assembled lipoplex displayed the lowest transfection levels, regardless of whether water or an isopropyl alcohol/water mixture was used to cast the films.

Particle size and film disassembly studies

We utilized dynamic light scattering (DLS) to investigate the size of lipoplex or film-based particles available for cellular uptake. We compared the size of initial liposomes, the resulting lipoplexes formed with pDNA, and particles released from a reconstituted film using pDNA-DOTAP as a test case. A single-exponential fit of the resulting correlation curves showed a trend of increasing size going from liposome to lipoplex, followed by nearly an order of magnitude increase in size for particles released from the film (Figure 4).

We then used scanning electron microscopy (SEM) to visualize the disassembly of films in the presence of buffer. Films were cast on silicon chips using the same amount and preparation method of dissolved complex as used for transfection studies, after which the treated films were immersed in 1 mL of the appropriate solution for 30 min before processing the films for imaging. Untreated (virgin) films have a

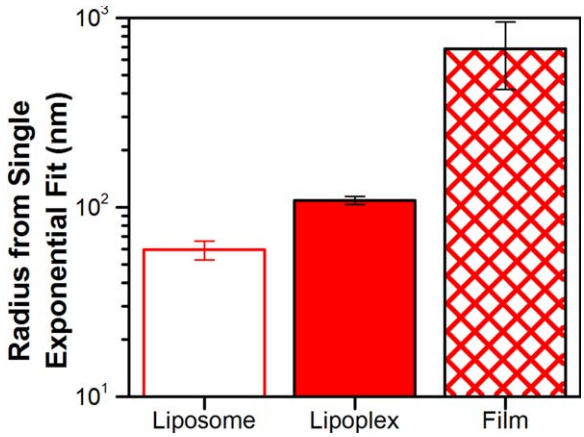

Figure 4. Size of DOTAP liposomes, pDNA-DOTAP lipoplexes, and particles released from pDNA-DOTAP films cast from isopropyl alcohol/water and reconstituted in water. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

All samples were prepared initially in OptiMem.

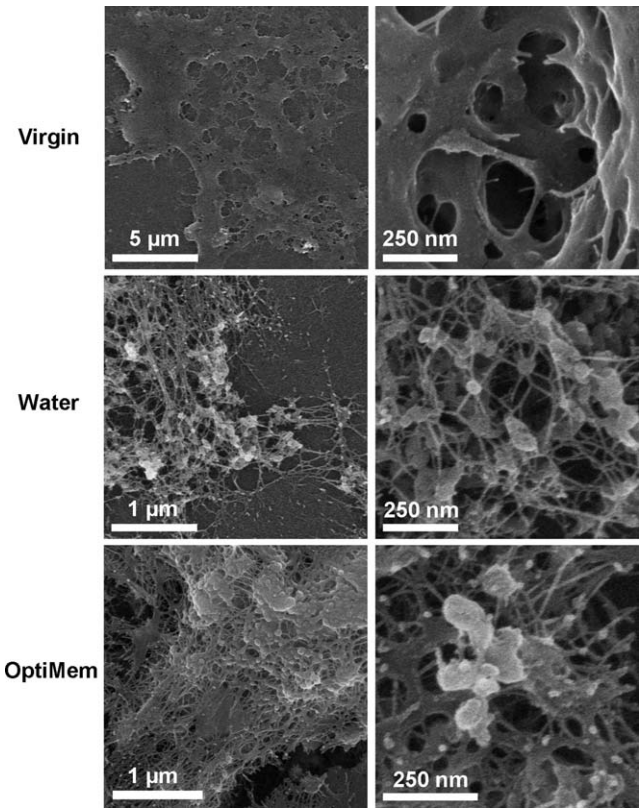

Figure 5. SEM images of a virgin (top), water-exposed (middle) and OptiMem-exposed (bottom) pDNA-DOTAP film.

Resolution of the images increases from left to right.

smooth appearance (see Supplementary Information online for Figures S6 and S7) with some areas where sheets coating an underlying scaffold can be seen (Figure 5, top). Upon immersion in either water or OptiMem, dense fibrous mats, presumably of DNA, connected by globules of lipid become visible (Figure 5, middle and bottom). Untreated films have a similar appearance to lipid-only films; while those films exposed to buffer closely resemble samples where pDNA was cast directly onto the surface (see online for Figure S5).

Determination of particle uptake and component colocalization

Having observed low levels of transfection from films and tested the disassembly of these materials under cell culture conditions, we sought to visualize and quantify the uptake of these materials via confocal microscopy and flow cytometry. For confocal microscopy we utilized Cy5-labeled pDNA (red) and incorporated 1% NBD-DOTAP (green) into our films. In addition to these components, samples were fixed and then typically stained for the cell nucleus (DAPI, blue) and the outer cell membrane (wheat germ agglutinin AF 594, yellow).

Confocal imaging of cells treated via bolus delivery of lipoplexes show the presence of punctate red fluorescence due to Cy5 labeled pDNA near the cell nucleus and within the boundaries of the cell membrane (Figure 6a and online for Figure S14a). Unlike lipoplexes from bolus delivery, no punctate red fluorescence corresponding to pDNA is observed within cells (Figure 6b and online for Figure S14b). We also observe mixing of the NBD-DOTAP signal with the cell membrane as well as a more diffuse signal present throughout the cytoplasm. These observations correlate well with results from control experiments where each of the individual components were delivered separately to cells (see online for Figures S11–13).

Analysis also indicates a significant amount of signal carryover between the NBD, Cy5, and AF 594 channels. In addition to the strong signal seen in samples where all four dyes are present, we also see apparent signal from these fluorophores where the dyes were not present (see online for Figures). For this reason we will focus our analysis on locations in the image where the signal from Cy5-pDNA and/or NBD-DOTAP does not overlap with the AF 594 membrane stain.

For flow cytometry we again utilized Cy5-labeled pDNA, but used 5% Texas Red-DHPE instead of the NBD-DOTAP so as to better match the fluorescent channels available in the instrument. The results reveal the percentage of live cells that are associated only with an individual fluorophore (Cy5 or Texas Red), both fluorophores (+ +), or neither (- -). As can be seen in Figure 7, only bolus delivery of DNA-DOTAP and DNA-60/40 DOTAP/DOPE lipoplexes shows a strong colocalization of both the Cy5 and Texas Red signals. The quantity of gated cells showing colocalized fluorescence for film-based delivery is lower by more than a factor of two, with much higher signals observed for both Cy5 alone and nonfluorescent cells. Control experiments testing the delivery of fluorescently labeled lipid alone show similar levels of lipid colocalization when compared to the double-positive signal for transfection complexes; i.e., high levels of Texas Red fluorescence for bolus delivery with significantly lower levels for film-based delivery. Additional samples that tested for the delivery of Cy5-labeled DNA indicate that while Lipofectamine performs as well or better than the DOTAP-based lipoplexes, the delivery of naked DNA produced levels of fluorescence similar to those seen for the total Cy5 signal present for film samples.

Addition of PEG-lipids to enhance transfection

Polyethelene glycol (PEG) grafting has been shown to act as a stabilizing agent when added to a variety of therapeutic delivery systems, including liposomes and lipoplexes.[1,55] The protective corona of neutral PEG chains helps both to distance particles from one another while also minimizing the electrostatic interactions between particles. This has proven particularly useful in generating "stealth" particles for drug delivery where the corona limits the aggregation of serum proteins with the particle.[56–58] Here we tested the incorporation of both PEG alone and PEG tethered to a lipid tail for both bolus and film delivery.

As can be seen from the graph in Figure 8, the addition of PEG or PEG-lipids to DNA/DOTAP lipoplexes for bolus delivery results in a decrease in transfection with increasing PEG molecular weight. For the addition of PEG 2000 this represents a 46% decrease in luciferase activity compared to lipoplexes without PEG. However, the addition of either PEG or PEG-lipids to film samples actually improved the observed transfection levels. Films that incorporated PEG with a molecular weight of 2000 actually surpassed the transfection levels of the equivalent bolus-delivered lipoplex. In fact, from an initial difference between bolus and film transfection levels of nearly 14x, the addition of PEG improved performance such that a comparison of the initial lipoplexes with a film containing DSPE PEG 2000

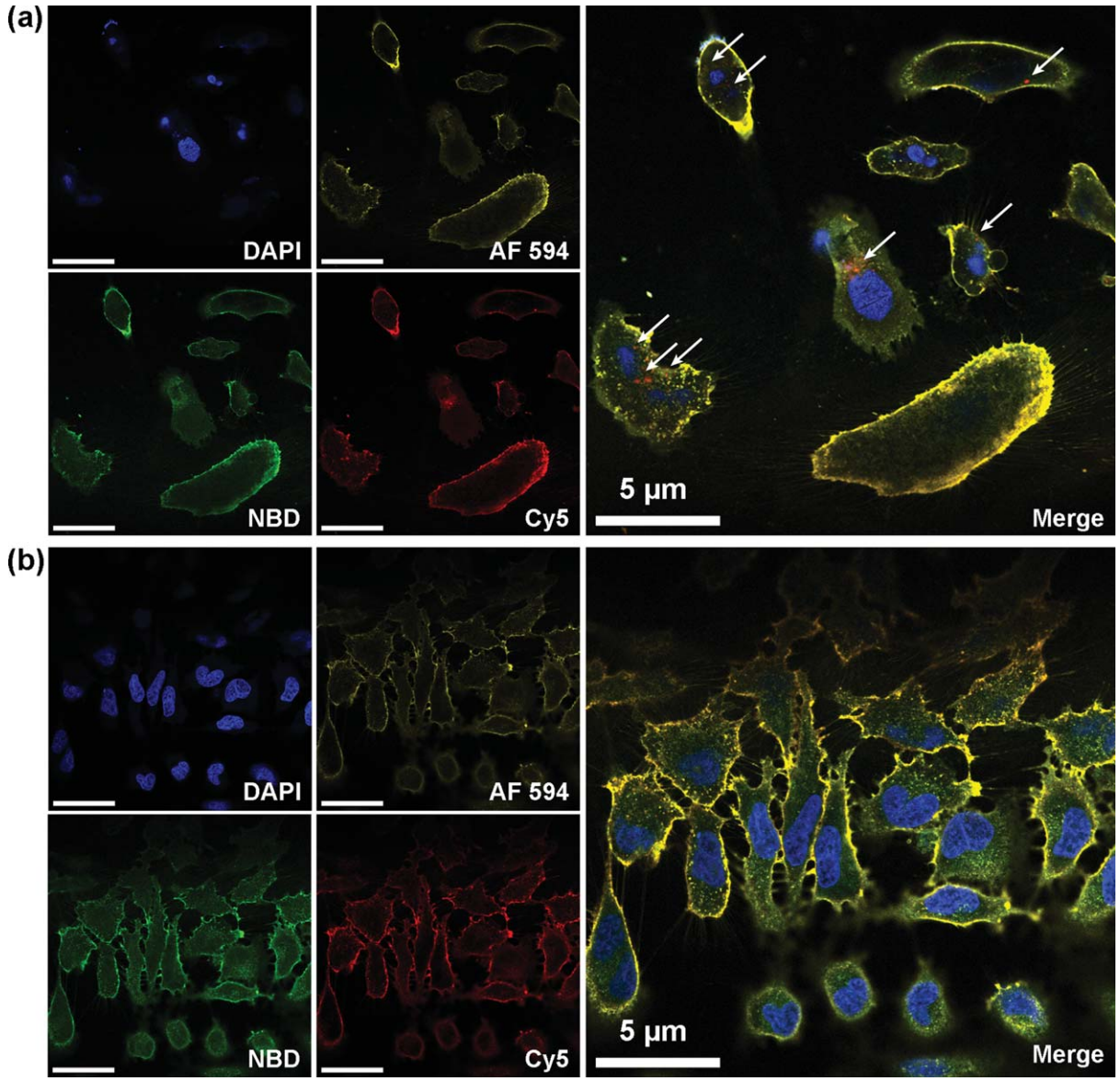

Figure 6. Confocal microscopy image of HeLa cells exposed to (a) Cy5-pDNA/NBD-DOTAP lipoplexes, and (b) Cy5-pDNA/NBD-DOTAP films.

Cell nuclei were stained with DAPI. Cell membranes were stained with AF 594. Fluorescent microscope images are split to show the individual channels and the merged image. Arrows indicate the presence of pDNA inside cell near the nucleus, shown as punctate red fluorescence. Images were taken at a magnification of 40×.

(maleimide) showed only a 3.9× difference. These increases in transfection efficiency can also be correlated to trends in particle size (see online for Figure S15).

Discussion

In this study, we sought to determine the mechanism whereby the transfection levels for films were nearly two orders of magnitude lower than for lipoplexes of identical composition (Figure 1). The goal in understanding this decrease is to better understand the challenges associated with the delivery of genetic material or other nucleic acid, macromolecular, or polymeric therapeutics from lipid-based films.

During transfection there are a series of bottlenecks that must be overcome for gene expression to occur: (1) cellular uptake, (2) endosomal escape, and (3) nuclear entry.[1,59] Of these bottlenecks, endosomal escape is the simplest to test for. However, experiments performed with the small molecule chloroquine (Figure 2) demonstrated that while endosomal escape may represent a minor bottleneck, it is not the major challenge accounting for the difference in activity between lipoplexes and films.

An analysis of the effect of drying various components of the films gave useful insights into the mechanism behind the decreased transfection levels for films. We observed a loss of activity when the lipid portion of the film was dried during preparation, but not if the lipid was replaced with a cationic polymer analogue (Figure 3). This correlates with reports of successful transfection using polymer-based films in the literature.[8,11–13,32–42] This difference in behavior upon drying is most likely due to the difference in the way in which lipids and

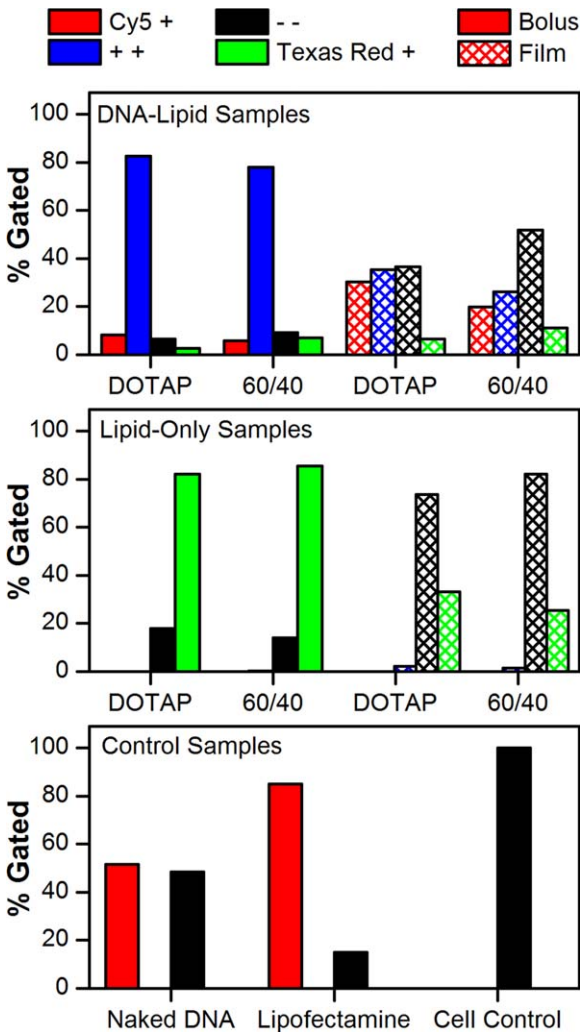

Figure 7. Plot of the percent of gated cells from flow cytometry for various bolus (solid bars) and film (hashed bars) delivered samples showing the gating of cells based on their association with Cy5-labeled pDNA Texas Red-labeled DOTAP lipid, both (+ +), or neither (− −).

The top plot shows data for DNA-containing lipoplex or film transfection samples. The middle plot shows control data for lipid-only liposome or film samples. The bottom plot shows the data for DNA-containing control samples including the direct delivery of DNA and the use of unlabeled Lipofectamine 2000 as positive controls. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

polymers form nanoparticles in solution. Lipids self-assemble into liposomal structures due to hydrophobic driving forces and are, thus, more prone to aggregation in water than are charged polymers, which are highly soluble. Furthermore films of DNA and polymers tend to be more stable due to the multivalent nature of the interaction while cationic lipids are limited to monovalent interactions. We suggest that this weaker univalent interaction between cationic lipids and DNA, coupled with the tendency for structural rearrangements due to hydrophobically driven self-assembly allows for changes in the structure of the film during dehydration and subsequent rehydration. The results of this rearrangement can be observed as aggregation and an increase in the size of particles released from the film (Figure 4). The size of these larger particles is such that cellular uptake via endocytosis may be a major limitation for transfection.[10,59–67]

In addition to particle aggregation, rearrangements of film structure may also result in separation of the film components and release of DNA from films. Based on SEM imaging (Figure 5), and analysis of component colocalization using both confocal microscopy and flow cytometry (Figures 6 and 7) exposure of a film to cell culture medium such as OptiMem or even water, appears to cause the disassembly of the films by the release of lipids from the surface, leaving the majority of the DNA behind. These findings agree with previously published results from our group in which we exposed a DNA-DDAB film to concentrated PBS buffer and monitored the results using atomic force microscopy (AFM) and fluorescence microscopy.[20] We observed that the nucleic acid and DDAB appeared to physically separate in a layer-by-layer fashion due to the presence of salts in the buffer.

Despite, or perhaps because of this separation of components, very little lipid is observed to colocalize with cells, while pDNA does (Figure 7). In fact, analysis by flow cytometry showed that the level of association of fluorescently labeled pDNA was similar for both films and bolus delivery of naked pDNA. While the uptake of pDNA may be successful in these cases, lipid or polymer carriers are used to provide protection for the pDNA against intracellular nucleases.[63,68,69] Therefore, it is unlikely that high levels of transfection will result from the uptake of even large quantities of pDNA.

In an effort to improve the activity of our films, we investigated the possibility of incorporating PEG or PEG-lipids to limit the aggregation and/or rearrangement of the lipids during the film casting process. PEG has been used previously to limit particle aggregation for suspensions of drug delivery particles, including lipoplexes,[1,55] but it was necessary to test whether this protective ability would extend to the casting of films from organic solvents. The addition of PEG to lipoplexes for bolus delivery resulted in a decrease in transfection levels (Figure 8), as has been reported elsewhere.[55] This decrease is most likely the result of decreased interactions between the lipoplexes and the cell membrane due to the protective PEG corona. The fact that we do not see the same sort of "stealth" behavior from particles released from PEG-containing DNA-lipid films may be a result of different interactions between the PEG, particle, and cellular membrane. We suggest, however, that the presence of PEG in film based transfection helps to minimize aggregation and/or rearrangement of lipids during film casting and reconstitution, thereby improving the potential for cellular uptake. Additional investigations are necessary, both to elucidate the exact mechanism, whereby PEG enhances the transfection of film-based particles while decreasing the transfection efficiency for bolus delivery, as well as to improve upon the transfection efficiencies of the DNA-lipid films described here.

Conclusions

While one goal of localized delivery strategies for transfection is to provide localized therapies, these platforms also have tremendous potential to raise the efficiency of synthetic carriers to a level that is comparable with viral methods while integrating advanced functionalities such as controlled or sequential release. There are significant opportunities for this kind of localized, surface-mediated gene delivery in

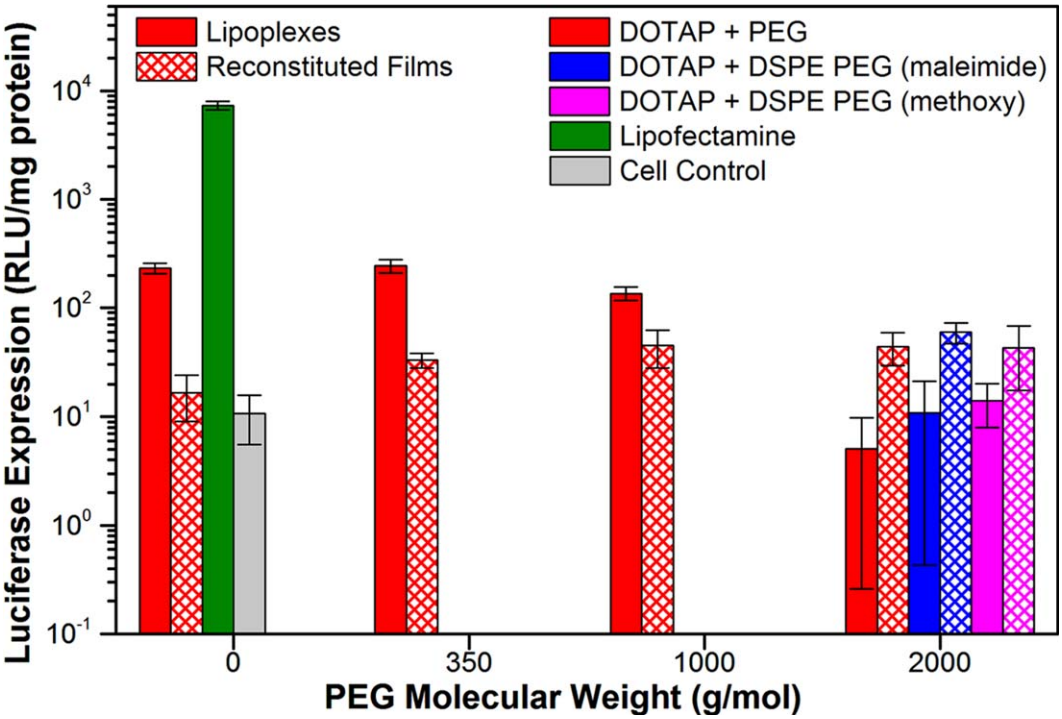

Figure 8. Analysis of gene expression after 24 h in HeLa cells using a luciferase assay for both lipoplexes (bolus delivery, solid bars), and films (hashed bars) for the incorporation of both PEG and various DSPE PEG-lipids of increasing chain length into DNA-DOTAP complexes and films.

[Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

applications ranging from wound healing to medical implants.[36,70–73] However, we have shown that the use of a solid film rather than bolus delivery of nanoparticles can present significant challenges in terms of film disassembly and cellular uptake.

Here, we have investigated the bottlenecks associated with monolithic DNA-lipid films for gene therapy. The major limitation for gene delivery appears to be related to cellular uptake. The process of film casting produces a monolithic solid, which may not disassemble into small enough particles for uptake via endocytosis. Furthermore, exposure of these films to cell culture media or even water appears to result in the disassembly of the film; with lipids going into solution, leaving DNA behind on the surface. While flow cytometry suggests that cells are able to take up this residual DNA, it is no longer protected against degradation from nucleases and is most likely destroyed before reaching the nucleus. Despite these challenges we suggest that methods such as the incorporation of PEG could be used to circumvent these challenges and help in the realization of lipid-based films for gene delivery or other applications where lipid-polymer films could be of use.

Figure 9. Picture of all of the Department Heads from Chemical Engineering and Materials Science at the University of Minnesota from 1949 to the present.

From left to right: Frank S. Bates, 1999-present; Matthew V. Tirrell, 1995-99; H. Ted Davis; 1981-95; Kenneth H. Keller, 1978-81; Rutherford Aris, 1974-78; Neal R. Amundson, 1949-1974. Photograph was taken on Oct. 16, 1999 at Rutherford Aris' 70th birthday celebration.

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