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Multiplexed supramolecular self-assembly for non-viral gene delivery

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ABSTRACT

Recently, there has been success in applying a semi-rational approach to non-viral gene delivery vector development using a combinatorial/parallel synthesis approach to construct libraries of materials with unique molecular structures. In this approach, it is hoped that the random incorporation of various hydrophobic and hydrophilic domains in the library will yield candidates with the appropriate balance of DNA binding strength and endosomolytic properties to yield efficient gene delivery. Herein we describe a library approach to gene delivery vector development that relies on the supramolecular self-assembly of individual components instead of chemical reaction. Each component in the described system is capable of performing a single and well-defined purpose—DNA binding (dioleylspermine), membrane permeation (oligoarginine) or targeting (folic acid). A combination of electrostatic attraction and the hydrophobic effect is used to bring the individual groups together to form nanoscale complexes with DNA. Because the components responsible for DNA binding, membrane permeation and targeting are separate, it is possible to alter the balance between hydrophilic and hydrophobic groups by varying the relative amounts in the final formulation. By doing so, we can readily identify cell-specific formulations that have greater transfection efficiency than the individual components and have superior transfection efficiency to lipofectamine 2000 under similar conditions.

1. Introduction

Gene therapy attempts to treat and ultimately cure some of the world's most challenging diseases by addressing the underlying genetic causes. However, nearly 20 years since the technique was first applied in a human clinical trial, its successes have been few [1]. The greatest single factor responsible for the slow progress is the lack of a safe and efficient method to deliver genetic material to patient cells. While viral vectors have been the dominant vehicles for gene delivery because of their in vivo efficiency, clinical events within the last decade have revealed that their safety is questionable [2–4]. More likely, it seems, future gene delivery will utilize synthetic non-viral vectors. While safer than viruses, these non-viral alternatives currently suffer from poor performance. As such, effort has been made to improve non-viral gene delivery efficiency through rational and semi-rational design of vectors capable of overcoming the various extra- and intracellular barriers that inhibit their performance.

A variety of materials are being considered for non-viral gene delivery applications. These include a wide range of polyamines and lipids that are able to electrostatically bind and condense DNA for delivery to cells [5–9]. Compared to viral gene delivery systems, these lipids and polyamines are generally safer as their genetic cargos are explicitly non-viral and are not integrated into the host genomes, thereby avoiding the immunogenic and oncogenic tendencies of some viruses. They are also comparatively cheap and easy to produce [10]. The primary disadvantage of non-viral vectors, however, is poor gene delivery efficiency—typically orders of magnitude below that found in viruses. The inefficiency largely lies with extra- and intracellular barriers existing between the site of administration and the nucleus of target cells. Cellular association, endocytosis, vector escape from the endosomal pathway, disassociation of the non-viral carrier and the plasmid DNA, migration of the plasmid DNA to the nucleus and finally transcription all stand as obstacles to overcome [11].

Considerable effort has been put into the rational design of vectors that are able to contend with a small, well-defined subset of the identified extra- and intracellular barriers to gene delivery. For example, PEG and other steric shielding materials have been attached to polymers to promote serum stability and sustained in vivo circulation [12–14]. Small molecules, proteins and antibodies have been incorporated into the design to permit receptor-mediated uptake by particular target cells [15,16]. Polymers and lipids with various pH-sensitive and endosomolytic moieties have been produced to facilitate escape from the endosomal pathway [17–19]. Polymers have been made to be degradable or modified in such

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a way as to promote unpackaging within the cell cytosol [20–22]. Nuclear localization signals have also been attached to DNA in attempts to aid nuclear delivery [23,24]. While these strategies have been successful in tackling the various individual barriers, the designs are, in general, too complicated or too molecule-specific to allow them to be utilized in conjunction with one another. Moreover, changing one aspect of a gene delivery vehicle can have implications on other areas of its performance. For these reasons, a single vector that is able to effectively incorporate all of the rational designs in one package that addresses all of the critical barriers mentioned above has not been developed.

Recently, there has been success in applying a semi-rational approach to the design of non-viral gene delivery vectors using a combinatorial/parallel synthesis approach to construct large libraries of materials with unique molecular structures [25–27]. Often, the only rational design strategy in the library approach is the inclusion of amines in the molecule to impart a net positive charge that facilitates DNA binding and condensation. Other concerns, such as endosomal escape and vector unpackaging, are not explicitly addressed. It is hoped that the random incorporation of various structurally unique domains in the library will yield a few materials with the appropriate balance of DNA binding strength and endosomolytic properties to yield efficient gene delivery. Anderson et al. used Michael addition reactions between various diacrylates and monoamines to generate a library of over two-thousand unique molecules [25]. Using automated screening technology, they identified 46 compounds with gene delivery efficiency superior to lipofectamine 2000 (LFA) and 25 kDa polyethylenimine (PEI). In a similar approach, Barua et al. created a library of 80 unique compounds using the ring-opening polymerization of diglycidyl ethers by amines to identify a compound with transfection efficiency that was also superior to 25 kDa PEI [26]. In both of the aforementioned library approaches, a chemical reaction was used to generate large numbers of distinct polymer molecules with unique balances of hydrophobic and hydrophilic domains. The amine-containing hydrophilic domains are used to bind and condense plasmid DNA while the hydrophobic domains serve other unspecified roles ranging from endosomal escape to enhanced vector unpackaging. When the two groups are present in the appropriate amounts, as demonstrated by Anderson et al. and Barua et al., the resulting polymer is able to mediate effective gene delivery.

In this article we report a multiplexed supramolecular assembly (MSA) strategy that integrates features of both rational design and combinatorial selection to identify promising non-viral gene delivery vectors. Unlike previous combinatorial approaches in which polydisperse polymer vectors are derived from a library of random building blocks, our systems are built upon a few (3–4) low molecular weight substrates with discrete molecular structures and rationally selected specific functions. In contrast to rational design approaches that typically involve multi-step conjugation chemistry to produce materials with discrete composition, our lead vectors were identified via the combinatorial selection of substrates mixed at various ratios to form supramolecular structures without chemical reaction. By screening a variety of formulations with differing amounts of substrates, we identify particular combinations such that each component works synergistically and performs its intended role without impairing the remaining components.

In the MSA system described here, the domains responsible for DNA condensation and membrane spermine and nona-arginine (Arg9), respectively—are bound to negatively charged DNA via electrostatic attraction. By conjugating monounsaturated C18 oleyl groups onto both terminal primary amines of spermine to yield dioleylspermine (DOS), we provide a means to decorate the complex with additional lipid groups via the hydrophobic effect. For this study, we chose to incorporate a folate-targeting component—oleyl-PEG-folate (OPF)—into the MSA complex via the hydrophobic effect (Fig. 1). Rather than using chemical reaction between various monomer units to alter the balance between hydrophilic and hydrophobic groups, we vary the relative amounts of individual components in the final MSA. By doing so, we identify two cell-specific formulations that have greater transfection

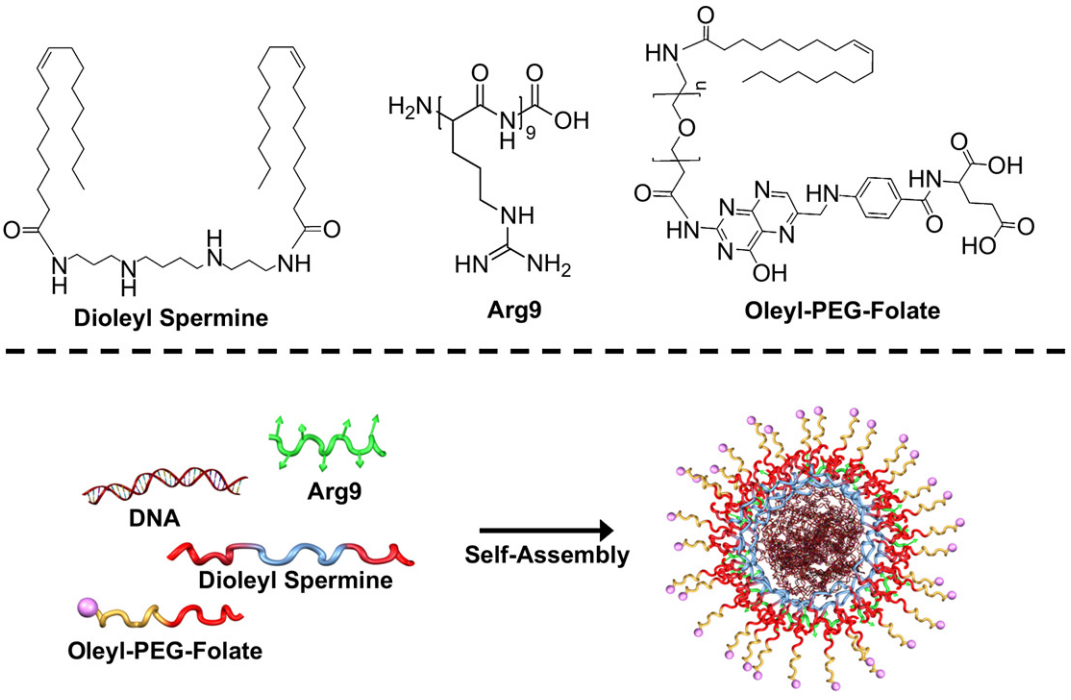


Fig. 1. Schematic representation of the supramolecular self-assembly of individual vector components with plasmid DNA.

efficiency than any of the individual components and have superior transfection efficiency to LFA under similar formulations conditions.

2. Materials and methods

2.1. Materials

Spermine, oleoyl chloride, folic acid, *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide (EDC) and branched polyethylenimine (PEI, MW = 25 kDa) were obtained from Sigma–Aldrich (St. Louis, MO). *N,N′*-diisopropylethylamine (DIEA) was purchased from Fisher Scientific (Bridgewater, NJ). Oligoarginine (Arg9, H-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-OH trifluoroacetate salt) was purchased from Bachem (Torrance, CA). NH_2-PEG-COOH (MW = 3400 Da) was purchased from Laysan Bio (Arab, AL). DC protein assay kits were purchased from Biorad (Hercules, CA). Luciferase assay reagent was purchased from Promega (Madison, WI). Lipofectamine 2000 and the fluorescent dyes rhodamine and YOYO-1 were purchased from Invitrogen (Carlsbad, CA).

2.2. Cells and plasmids

COS-7 African green monkey kidney cells and HeLa human cervical cancer cells were obtained from ATCC. The cells were cultured according to their ATCC protocols at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM). The growth medium was supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The expression vector pCMV-Luc coding for the luciferase gene was obtained from Elim Biopharmaceuticals (Hayward, CA).

2.3. Synthesis of oleyl-NHS

NHS (11.6 mmol, 1.33 g) and DIEA (7.8 mmol, 1.35 mL) were mixed in THF (50 mL). The mixture was placed in an ice bath and cooled to 0 °C. Oleoyl chloride (7.8 mmol, 3 mL) was added drop-wise over the course of 1 h. The mixture was allowed to stir at 0 °C for 2 h and then overnight at room temperature. The resulting precipitate was discarded by centrifugation. THF was removed under reduced pressure and the resulting yellowish oil was dissolved in methylene chloride (10 mL). The mixture was washed with 5% HCl (10 mL × 2) and then evaporated under reduced pressure to give the crude product. Oleyl-NHS was crystallized from cold ethanol and characterized by ^{1}H NMR. ^{1}H NMR ($CDCl_3$): δ 5.34 (m, 2H), 2.83 (s, 4H), 2.594 (t, 2H), 2.004 (m, 4H), 1.74 (m, 2H), 1.38 (m, 20H) and 0.874 (t, 3H).

2.4. Synthesis of DOS

Spermine (0.60 mmol, 120 mg) and oleyl-NHS (1.9 mmol, 459 mg) were dissolved in a 2:1 mixture of water and THF (6 mL). The reaction was allowed to proceed overnight at room temperature. The resulting dioleylspermine (DOS) was crystallized from a 4:1 mixture of ethanol and concentrated HCl, and characterized by FD mass spectrometry (expected $m/z = 731.2$, obtained $m/z = 731.8$).

2.5. Synthesis of oleyl-PEG-COOH and oleyl-PEG-folate

Oleyl-PEG-COOH was obtained by reacting NH_2-PEG-COOH (3.4 kDa, 0.07 mmol, 240 mg) with oleyl-NHS (0.07 mmol, 52 mg) in a 2:1 mixture of THF and water (6 mL). The reaction was allowed to proceed overnight at room temperature, after which it was dialyzed against water using a 2000 MWCO dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA). After dialyzing for two days, the product was lyophilized and characterized by MALDI mass spectrometry. Individual peak shifts (relative to unmodified NH_2-PEG-COOH) corresponding to the addition of one oleyl group per PEG were observed ($m/z = 266$). OPF was synthesized by reacting oleyl-PEG-COOH (0.04 mmol, 140 mg) with EDC (0.04 mmol, 7.6 mg) and NHS (0.04 mmol, 4.6 mg) in DMSO (2 mL) to form oleyl-PEG-NHS. The crude product in DMSO (1 mL) was then reacted with folic acid (0.024 mmol, 10.5 mg) in 20 mM HEPES (3 mL). The reaction was allowed to stir overnight at room temperature, after which it was dialyzed against water for two days using a 2000 MWCO dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA). The resulting product was lyophilized and characterized via MALDI mass spectrometry. Individual peak shifts (relative to oleyl-PEG-COOH) corresponding to the addition of one folic acid group per PEG were observed ($m/z = 438$).

2.6. Gel retardation studies

A solution of DNA (1 μg/10 μL) was prepared in double-distilled water. Appropriate amounts of spermine, DOS or oligoarginine dissolved in double-distilled water were added to the DNA solution (10 μL) to achieve the desired spermine:DNA, DOS:DNA or Arg9:DNA weight ratio. Complexes were incubated at room temperature for 15 min, after which loading dye was added and the solution (10 μL) was run on a 1% agarose gel (70 V, 70 min). DNA was stained with ethidium bromide and visualized on a Gel Doc imaging system (Biorad, Herclues, CA).

2.7. Complex formation and transfection

DNA/lipid complexes were prepared at room temperature by dissolving DNA (0.35 μg) in double-distilled water (175 μL). An equal volume of lipofectamine 2000 or DOS in double-distilled water was added to achieve the desired N/P or weight ratio. When forming multi-component complexes, Arg9 was added to DNA first. The Arg9:DNA complexes were allowed to incubate for approximately 10 min before a mixture of DOS and oleyl-PEG-COOH or OPF was added. The final complexes were incubated at room temperature for 15 min before further use. Cells (COS-7 or HeLa) were cultured in DMEM supplemented with 10% horse serum and 1% penicillin-streptomycin according to ATCC protocols and plated in 96-well plates at 1×10^4 cells/well 24 h prior to transfection. Immediately before transfection, the growth medium was replaced with fresh serum-supplemented DMEM—with or without folic acid or chloroquine, as indicated—and polyplex solution (50 μL) was added to each well (0.05 μg DNA/well). The transfection medium was replaced with fresh serum-supplemented growth medium 4 h post-transfection. Luciferase expression was quantified 24 h post-transfection using the Promega Bright-Glo luciferase assay system (Promega, Madison, WI). Luciferase activity was measured in relative light units (RLU) using a PerkinElmer plate reader with luminescence capabilities (Waltham, MA). Results were normalized to total cell protein using the DC protein assay kit (Biorad, Hercules, CA). Transfections were performed in triplicate.

2.8. Uptake

DNA complexes were formed at their respective optimum transfection weight ratios as described above. The intercalating dye YOYO-1 was added at the ratio 15 nL YOYO-1 per 1 μg of DNA. Cells (COS-7 or HeLa) were cultured in DMEM supplemented with 10% horse serum and 1% penicillin-streptomycin according to ATCC protocols and plated in 24-well plates at 5×10^4 cells/well 24 h prior to transfection. Immediately before transfection, the growth medium was replaced with fresh serum-supplemented medium and polyplex solution (50 μL) was added to each well (0.25 μg DNA/well). Four hours post-transfection, the cells were rinsed with PBS (0.5 mL × 2) to remove surface-bound complexes. Next, trypsin in PBS (0.05%, 100 μL) was added to each well. The cells and trypsin were allowed to incubate for approximately 10 min before formaldehyde (4%, 400 μL) was added to each well. The cells were then collected and FACS analyses were performed on a BD Biosciences LSR II flow cytometer (Franklin Lakes, NJ). Data were analyzed using the FCS Express software package (De Novo Software, Los Angeles, CA). Transfections and uptake measurements were performed in triplicate.

2.9. Dynamic light scattering

DNA complexes were formed in double-distilled water at various compositions of DNA, Arg9, DOS and OPF as indicated above. Following incubation at room temperature for 15 min, the complexes were diluted in water or PBS (1.8 mL) and subjected to size measurement on a Brookhaven Instruments Corporation 90 Plus Particle Size Analyzer Holtsville, NY. Five sets of measurements were performed for each sample.

2.10. Cytotoxicity measurements

The cytotoxicity of the components was characterized using the MTT cell viability assay (Sigma–Aldrich, St. Louis, MO). Cells (COS-7 or HeLa) were seeded in 96-wells plates at 1×10^4 cells/well and grown overnight at 37 °C, 5% CO_2 in medium containing 10% horse serum and 1% penicillin-streptomycin. Approximately 24 h after seeding the medium was replaced with serum-supplemented DMEM and the uncomplexed material was added to the cells at final concentrations between 0 and 50 μg/mL. After 4 h of incubation, the medium was replaced with serum-containing medium and grown for another 20 h, after which reconstituted 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, 10 μL) was added. The plates were then incubated for another 4 h and MTT solubilization solution (100 μL, Sigma–Aldrich, St. Louis, MO) was added and the absorbance at 570 nm was read using a PerkinElmer plate reader (Waltham, MA). The background absorbance of cells killed with ethanol was subtracted from the viable cell absorbance and normalized to cells grown in DMEM. Each experiment was repeated four times at each concentration.

2.11. Confocal microscopy

DNA complexes were formed at the previously determined optimum transfection weight ratio for HeLa transfection as described above save for the use of YOYO-1 labeled DNA (one YOYO-1 molecule per 50 DNA base pairs) and rhodamine-labeled oligoarginine (one rhodamine molecule per oligo). HeLa cells were cultured in DMEM supplemented with 10% horse serum and 1% penicillin-streptomycin according to ATCC protocols and plated in 6-well plates containing coverslips at 25×10^4 cells/well 24 h prior to transfection. Immediately before transfection, the growth medium was replaced with fresh serum-supplemented medium with or without 50 μM chloroquine and polyplex solution (100 μL) was added to each well (0.5 μg DNA/well). Ninety minutes post-transfection, the cells were rinsed with PBS

(2 mL × 2) to remove surface-bound complexes and formaldehyde (4%, 1 mL) was added to each well. Following 10 min incubation, the cells were rinsed with PBS (2 mL × 2) and mounted on glass slides. Cells were visualized with an Olympus Model BX60 confocal microscope equipped with a 100x oil immersion lens with Argon and Krypton lasers for visualizing the YOYO-1 ($\lambda_{ex} = 488$ nm) and rhodamine ($\lambda_{ex} = 568$ nm) signals, respectively.

3. Results

3.1. Synthesis and characterization of individual components

Spermine with two oleyl tail groups (dioleylspermine, DOS) was synthesized by conjugating amine-reactive oleyl-NHS to the two primary amines of spermine (Fig. 2A). The successful conjugation of oleyl to spermine was verified by ^{1}H NMR. Field desorption mass spectrometry was used to confirm that no mono-substituted product was obtained and DOS has the expected molecular weight (m/z calc. = 731.2, m/z obtained = 731.8, Fig. 2B). A gel retardation assay confirmed the ability of DOS to bind and condense DNA (Fig. 2C). Interestingly, DOS is able to retard DNA at much lower weight ratios (2.5:1 DOS:DNA) than unmodified spermine (>90:1 spermine:DNA, Fig. 2D). It is likely that DOS behaves as a cationic lipid and self-assembles into aggregates such that the oleyl groups are hydrophobically packed. In this manner, multiple spermine units are displayed on the surface of the aggregate, giving rise to a multivalent effect that permits stronger DNA binding than unmodified spermine. Although gene delivery complexes formed in solutions of high ionic strength (e.g., in OptiMEM as suggested for LFA/DNA formulation) tend to yield increased transfection over those prepared in solutions of low ionic strength, the complexes are generally too large (micrometer in size) for potential in vivo applications. Thus, we chose to form MSAs and LFA/DNA in water. Doing so yielded complexes with diameters between 150 nm and 250 nm (Fig. 2E). Based on the gel retardation assay in Fig. 2C, complexes formed at a 1:1 DOS:DNA weight ratio are not completely condensed, thus explaining the large diameter measured for complexes at that weight ratio.

Due to the positively charged guanidinium groups of arginine, nona-arginine (Arg9) is able to electrostatically interact with negatively charged plasmid DNA (Fig. 3A). Thus, it can be incorporated into complexes with DOS and DNA without the addition of a hydrophobic oleyl group. Dynamic light scattering revealed that the addition of Arg9 to complexes of DOS and DNA (10:1 DOS:DNA weight ratio) has nearly negligible effect on the MSA particle size (Fig. 3B).

Oleyl-PEG-COOH was synthesized by conjugating amine-reactive oleyl-NHS to the primary amine of NH_2-PEG-COOH. The carboxylic acid group of the resulting oleyl-PEG-COOH was subsequently conjugated to the primary amine of folic acid through EDC/NHS chemistry (Fig. 4A). Matrix-assisted laser desorption ionization (MALDI) mass spectrometry verified the successful conjugation of oleyl and folic acid groups to NH_2-PEG-COOH (Fig. 4B). Individual peak shifts corresponding the addition of a single oleyl group ($m/z = 266$) were noted following reaction of NHS-oleyl and NH_2-PEG-COOH to form oleyl-PEG-COOH. Subsequent activation of oleyl-PEG-COOH with EDC and NHS to form oleyl-PEG-NHS and reaction with folic acid resulted in oleyl-PEG-folate (OPF). An additional peak shift corresponding to the addition of single molecule of folic acid ($m/z = 438$) in MALDI-MS confirmed the composition of OPF. Dynamic light scattering revealed that the addition of OPF to complexes formed between DNA and DOS (10:0:1 DOS:Arg9:DNA weight ratio) did not significantly alter the measured complex diameter. However, the incorporation of OPF at 10 and 20 mol% slowed the aggregation of complexes in PBS relative to complexes of DOS and DNA without PEG groups (Fig. 3C).

3.2. In vitro transfection

After preparing the individual components responsible for DNA condensation (DOS), membrane permeation (Arg9) and targeting (OPF), we performed transfection assays using the luciferase marker gene to study the effect of varying the relative amounts of these components in the resulting structures. Transfections were first performed using complexes formed with only DNA and DOS to obtain an estimate of the range of DOS:DNA weight ratios that are able to successfully mediate gene delivery. Although gene delivery complexes formed in solutions of high ionic strength (PBS, OptiMEM, etc.) tend to yield increased transfection over those prepared in solutions of low ionic strength, the complexes are also substantially larger [12,28]. Complexes with diameters of 1 μm or larger were typically obtained for lipofectamine 2000:DNA and DOS:DNA complexes formed in OptiMEM and 150 mM NaCl, 20 mM HEPES, respectively. As particles of micrometer size are unsuitable for endocytosis through the folate receptor and for in vivo applications, we chose to form complexes—including LFA—in pure water to reduce particle sizes. Although such conditions result in sub-optimal performance under routine in vitro transfection conditions where targeting is not considered, it is necessary to obtain the small particle diameters reported in Figs. 2E and 3B. Thus, following complex preparation in pure water, COS-7 and HeLa cells were transfected with complexes of DOS and DNA. In COS-7 and HeLa cells, the optimum N/P ratio was found to be 50:1 and 20:1, respectively, corresponding to weight ratios of approximately 25:1 and 10:1 (DOS:DNA). At these weight ratios, DOS:DNA complexes outperformed LFA by 4–5 fold in both cell lines (Fig. 5A and B). When high ionic strength solutions (OptiMEM or 150 mM NaCl with 20 mM HEPES) were used for the complexation, DOS and LFA performed comparably (data not shown).

The optimum amount of OPF and Arg9 was determined simultaneously by testing the transfection efficiency of formulations made with the optimum amount of DOS for each respective cell line (25:1 DOS:DNA weight ratio for COS-7 and 10:1 for HeLa) and various amounts of OPF and Arg9. In addition to looking for merely the highest level of gene expression, we also sought to identify formulations capable of targeted delivery. Thus, we examined the data for mixtures which showed increased performance with the addition of OPF at constant Arg9. The reason for us to seek increased performance with the addition of OPF at constant Arg9 was because, to a first approximation, this suggests that complexes are entering cells in a receptor-specific manner. The formulation satisfying our aforementioned conditions in COS-7 cells was found to be 25:10:1 (DOS:Arg9:DNA weight ratio) with 10 mol% OPF (Fig. 5C). Gene delivery MSAs prepared in this manner outperformed the baseline—25:0:1 weight ratio (DOS:Arg9:DNA)—by approximately 2-fold. To verify receptor-specific uptake, a competitive inhibition experiment was performed. Transfections with complexes formed at a 25:10:1 weight ratio (DOS:Arg9:DNA) in the presence of various amounts of free folic acid. The results of Fig. 5E indicate that the addition of 300 μM folic acid results in a greater than 2-fold decrease in transfection with complexes formed with 10 mol% of OPF while no change is observed in complexes formed with 10 mol% of the untargeted carboxylic acid-terminated analog oleyl-PEG-COOH. This indicates that transfection with complexes formed at a 25:10:1 DOS:Arg9:DNA weight ratio with 10 mol% OPF are proceeding via a receptor-specific path. In HeLa cells, the incorporation of Arg9 at a 1:1 Arg9:DNA with 20 mol% OPF resulted in a 10-fold improvement over the baseline formulation lacking Arg9 and OPF (Fig. 5D). A competitive inhibition assay was performed to verify targeted

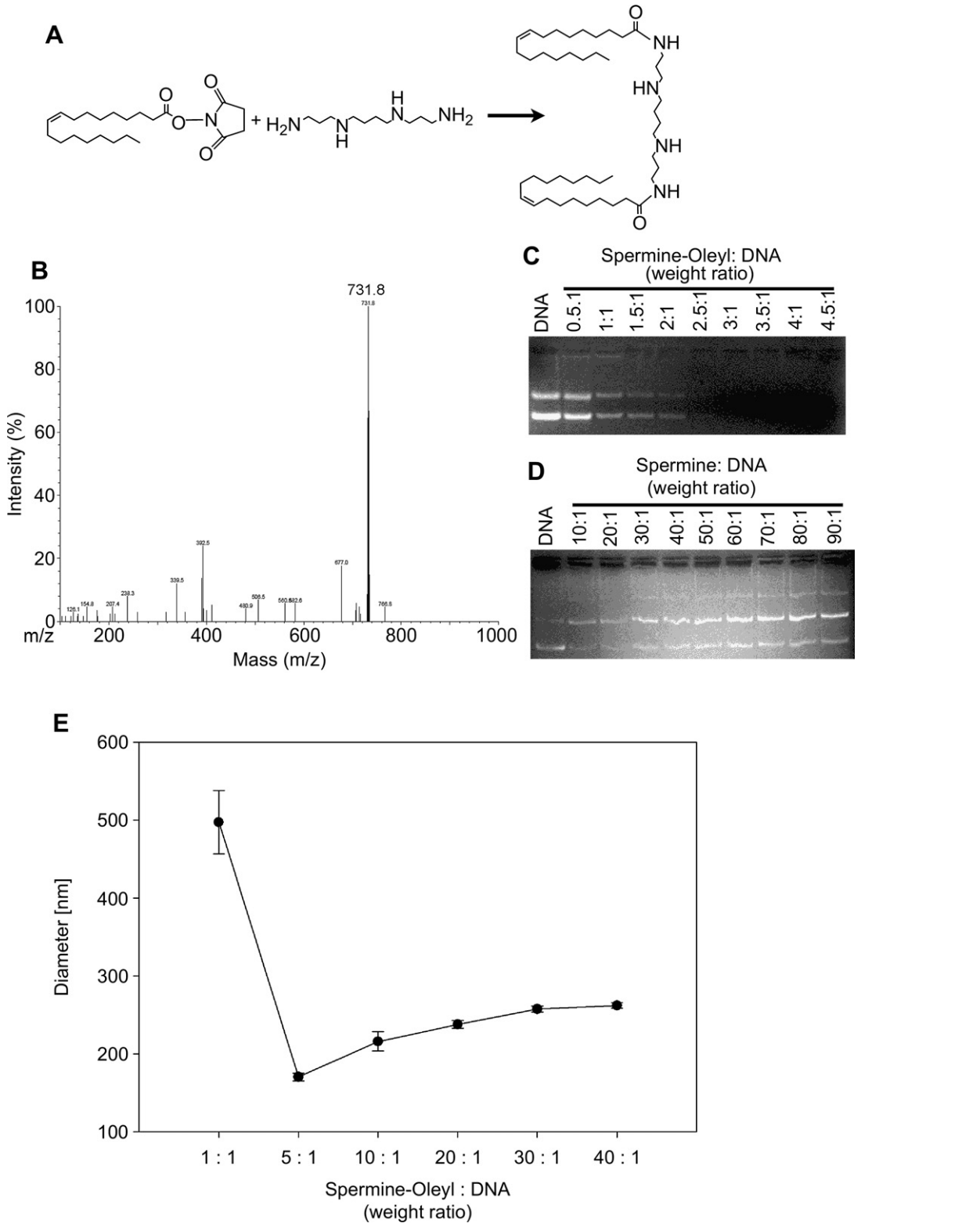
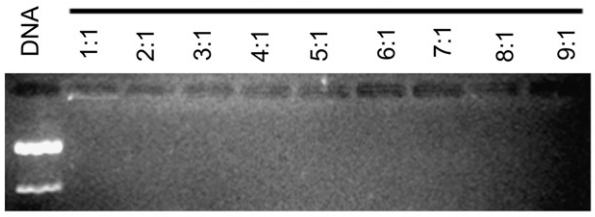


Fig. 2. (A) Reaction scheme for the synthesis of DOS. (B) FD mass spectrometry results confirming the di-substituted DOS product (expected $m/z = 731.2$, obtained $m/z = 731.8$). (C) Gel retardation assay confirming the condensation of plasmid DNA with DOS. The numbers above the gel reflect the DOS:DNA mass ratio used. (D) Gel retardation assay confirming the inability of unmodified spermine to condense plasmid DNA at the ratios tested. The numbers above the gel reflect the spermine:DNA mass ratio used. (E) Plot of measured diameter of DOS and DNA complexes formed at a variety of DOS:DNA weight ratios.

uptake. According to Fig. 5F, the addition of as little as 100 μM free folic acid results in a greater than 2-fold decrease in transfection of complexes formed with 20 mol% of OPF while no change is observed in complexes formed with 20 mol% of oleyl-PEG-COOH. Based on the data of Fig. 5D and F, it appears that the formulation resulting in effective gene delivery as well as successful targeting in HeLa cells is a 10:1:1 weight ratio (DOS:Arg9:DNA) with 20 mol% OPF.

A



B

DOS : Arg9 : DNA (weight ratio)	OPF (mol%)	Diameter (nm) t=0 min	Diameter (nm) t=60 min, PBS	Diameter (nm) t=90 min, PBS
10:0:1	0	180.2 ± 2.9	398.1 ± 6.3	481.5 ± 3.1
10:5:1	0	187.9 ± 2.6	—	—
10:10:1	0	204.8 ± 4.6	—	—
10:20:1	0	218.5 ± 1.5	—	—
10:0:1	10%	213.9 ± 3.0	213.9 ± 4.9	247.1 ± 2.2
10:0:1	20%	214.9 ± 3.5	243.2 ± 4.2	286.9 ± 6.2

Fig. 3. (A) Gel retardation assay confirming the condensation of plasmid DNA with oligoarginine. The numbers above the gel reflect the Arg9:DNA mass ratio used. (B) Diameter measurements for complexes formed at the indicated DOS:Arg9:DNA weight ratios.

3.3. Uptake

Oligoarginine has traditionally been incorporated into drug delivery design to enhance cellular uptake of molecules or vectors unable to passively diffuse through the cell membrane. Flow cytometry experiments were performed to explore the effects of Arg9 incorporation on the uptake of DOS:DNA complexes. As shown in Fig. 6A, the incorporation of oligoarginine in complexes of DOS and DNA does not enhance uptake beyond what is achieved solely with DOS. In fact, Arg9 appears to slightly decrease complex uptake in COS-7 cells. Given that the addition of Arg9 increases the measured gene delivery efficiency in COS-7 and HeLa cells even without the addition of OPF, it is likely that the beneficial effect of Arg9 occurs intracellularly. Although Arg9 covalently conjugated to cargo like DNA, peptides, and proteins has been reported to remain in endosomes in the absence of an endosomolytic agent, free Arg9 has been shown to rupture membranes and allow endosomal escape following a pH dependent process [29–32]. As the system presented in this article makes exclusive use of free and unconjugated Arg9, it is likely that enhanced endosomal escape is the source of Arg9's beneficial impact. To explore this, we performed transfections in COS-7 and HeLa cells in the presence or absence of 50 μM chloroquine. The membrane permeation of unconjugated penetratin—a cell-penetrating peptide similar to Arg9—has been shown to be dependent upon the formation of transmembrane pH gradients [33]. Thus, prohibiting the development of endolysosomal pH gradients by chloroquine treatment should reduce the corresponding endosomal escape and transfection efficiency of complexes containing free Arg9. Supporting the notion that Arg9 aids gene delivery by enhancing endosomal escape, the results of Fig. 6B and C reveal that chloroquine treatment has no significant effect on the transfection efficiency of DOS:DNA complexes lacking Arg9 but results in a 50% and 80% decrease in COS-7 and HeLa cells, respectively, by complexes containing Arg9. Furthermore, confocal microscopy of HeLa cells transfected with fluorescent DOS:Arg9:DNA complexes reveal a diffuse staining pattern in the absence of chloroquine, suggesting efficient endosomal escape. The punctuated staining pattern observed in the presence of chloroquine suggests inefficient endosomal escape (Fig. 6D).

The incorporation of OPF in complexes of DOS, Arg9 and DNA is intended to promote uptake through the folate receptor. It is interesting to note that although the incorporation of OPF in DOS:Arg9:DNA complexes results in improvement in gene delivery (Fig. 5C and D), it actually reduces uptake by 20% in COS-7 cells and 40% in HeLa cells relative to complexes of only DOS, Arg9 and DNA. The decreased uptake is likely due to masking of the cationic surface charge of the PEGylated complexes. The increased gene expression with folate-targeted complexes suggests that endocytosis through the folate receptor leads to more favorable intracellular processing than a non-specific mode of cell entry, as has been reported elsewhere [34].

3.4. Cytotoxicity

Many gene delivery vectors have toxic effects due to their high cationic charge. Generally, the same electrostatic interactions that draw cationic materials to negatively charged DNA also induce a lytic event in cells. To this end, we determined the cytotoxicity of the various gene delivery components used in this paper. Based on the results (Fig. 7), it appears that none of the synthetic materials display any significant toxicity at the concentrations used. For COS-7 cells, the best performing complexes result in a DOS concentration of 12.5 μg/ml, an OPF concentration of 12.7 μg/ml and an Arg9 concentration of 5.0 μg/ml. For HeLa cells, the optimum formulation results in a DOS concentration of 5 μg/ml, an OPF concentration of 5.1 μg/ml, and an Arg9 concentration of 0.5 μg/ml. None of these concentrations results in a significant decrease in cell viability. At times the measured viability of treated cells is greater than unity. This is likely due to the increased metabolic activity of injured but still viable cells as they repair damaged cell membranes.

4. Discussion

Although seemingly full of potential, the rational design of gene delivery vectors has failed to yield a suitable candidate that displays

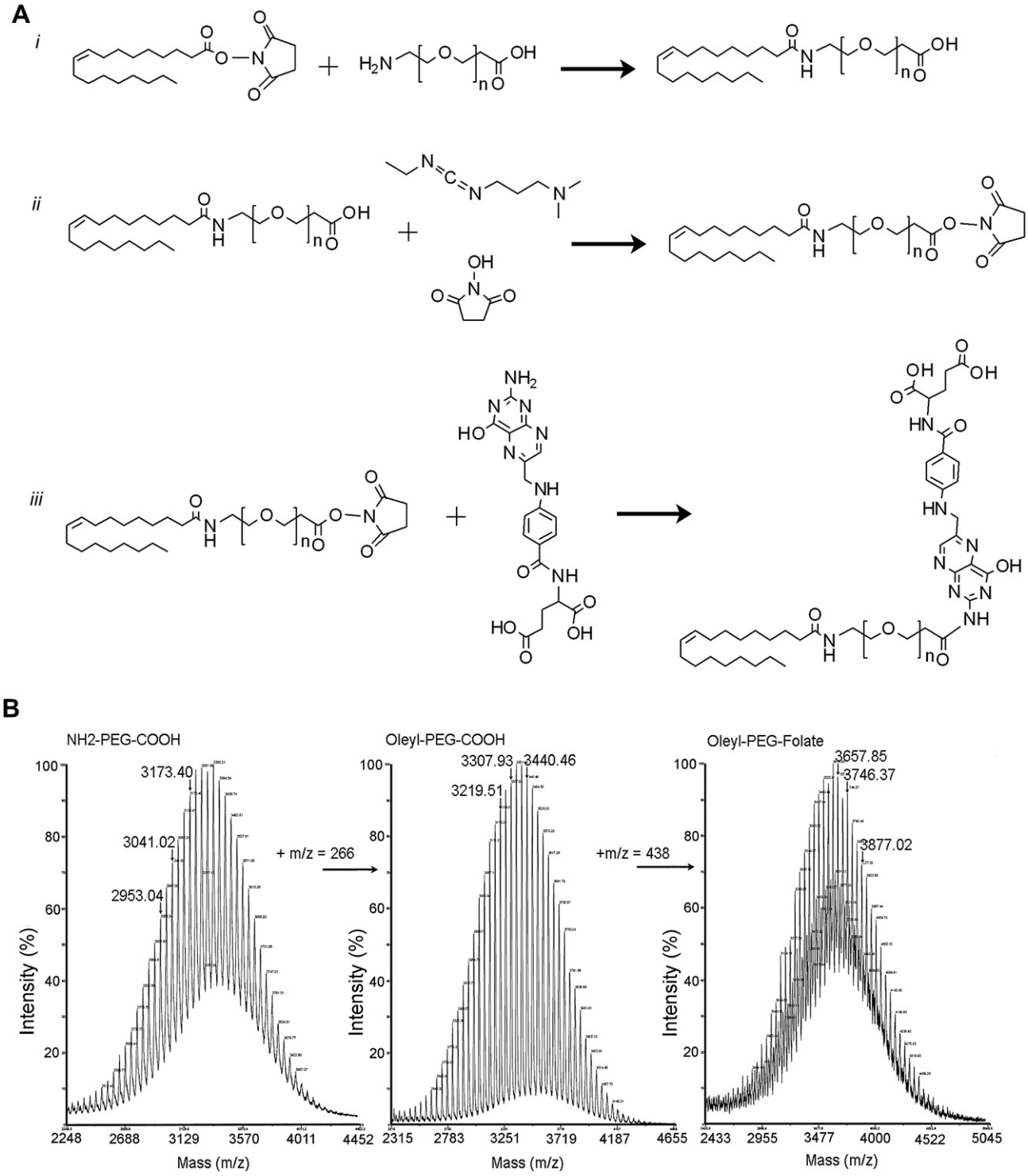


Fig. 4. (A) Reaction scheme for the synthesis of oleyl-PEG-COOH and oleyl-PEG-folate (OPF). (B) MALDI mass spectrometry results confirming the successful synthesis of oleyl-PEG-COOH (individual peak shift of $m/z = 266$ relative to PEG) and OPF (individual peak shift of $m/z = 438$ relative to oleyl-PEG-COOH).

both high transfection efficiency and biocompatibility. This is likely due to the lack of a clear understanding of all the intracellular processing steps involved in successfully ushering DNA-containing complexes from the extracellular environment, through the endo-lysosomal pathway and into the cell nucleus. However, there has been recent success in applying a semi-rational approach to the design of non-viral gene delivery vectors using a combinatorial/parallel synthesis approach to construct large libraries of polymeric materials [25–27]. Following material synthesis, it is possible to sift through the multitude of complexes and identify candidates that can effectively mediate gene delivery.

In this article we describe our application of the library approach for the identification of lipid-like materials for gene delivery. Following our strategy, we generated a library of gene delivery materials using the supramolecular self-assembly of individual components, each with a well-defined purpose—DNA

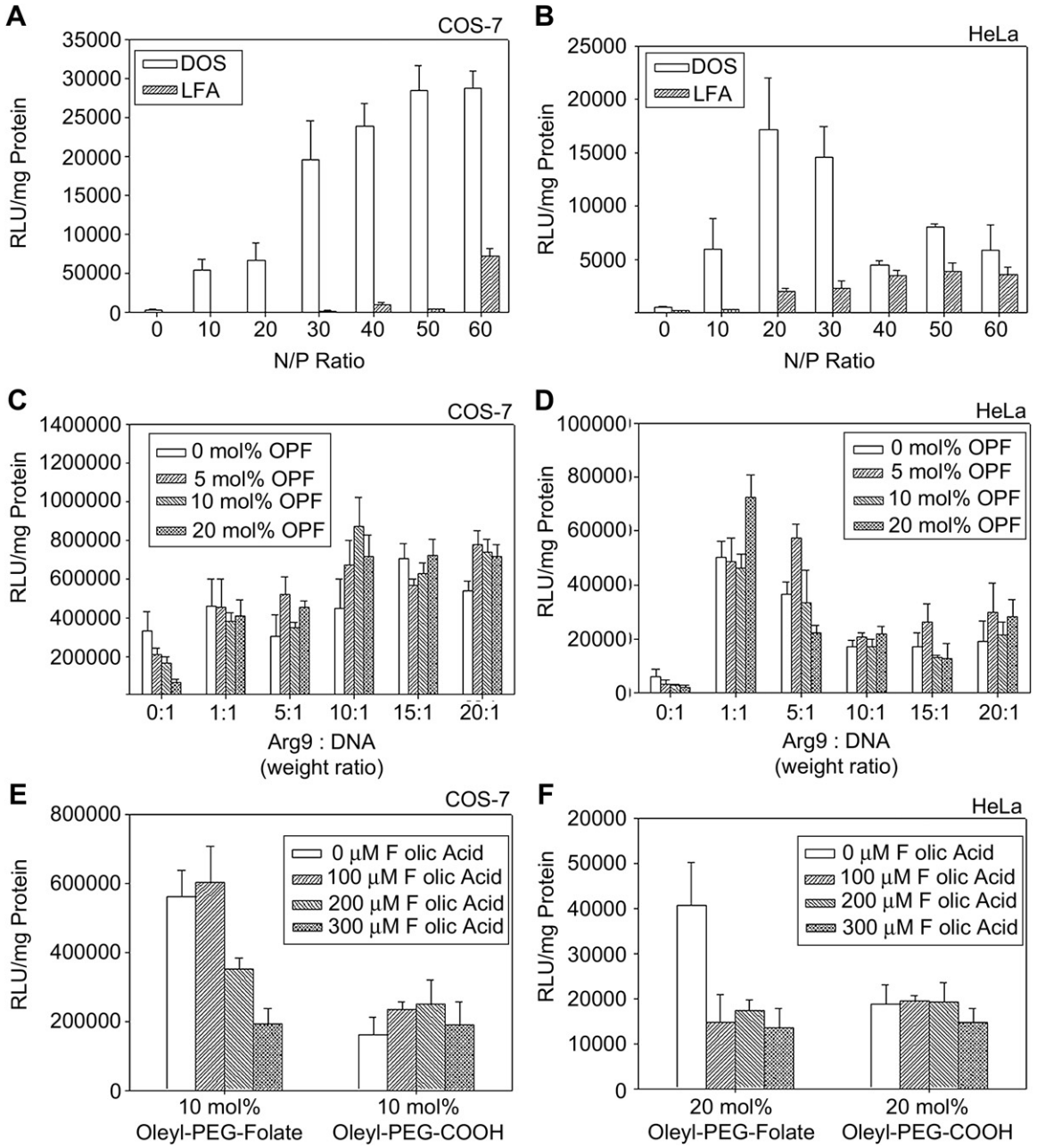


Fig. 5. (A) In vitro transfection of COS-7 cells with complexes formed at various N/P ratios. Lipofectamine 2000 (LFA) was used as a control. (B) In vitro transfection of HeLa cells with complexes formed at various N/P ratios. Lipofectamine 2000 (LFA) was used as a control. (C) In vitro transfection of COS-7 cells with complexes formed at a 25:1 DOS:DNA weight ratio and various Arg9:DNA weight ratios. OPF was incorporated at the indicated mol% relative to DOS. (D) In vitro transfection of HeLa cells with complexes formed at a 10:1 DOS:DNA weight ratio and various Arg9:DNA weight ratios. OPF was incorporated at the indicated mol% relative to DOS. (E) In vitro transfection of COS-7 cells with the optimized vector formulation (i.e. 25:10:1 DOS:Arg9:DNA weight ratio) and 10 mol% of either OPF or oleyl-PEG-COOH in the presence of various concentrations of free folic acid. (F) In vitro transfection of HeLa cells with optimized vector formulation (i.e. 10:1:1 DOS:Arg9:DNA weight ratio) and 20 mol% of either OPF or oleyl-PEG-COOH in the presence of various concentrations of free folic acid. All complexes—including lipofectamine 2000—were prepared in double-distilled water. Transfections were performed in serum-supplemented media.

binding (DOS), membrane permeation (Arg9) and targeting (OPF). In much the same way as other groups generated a library of materials by varying the structural properties of their gene delivery vectors, we generate a library by combining the individual components in various relative ratios. The various components are held together in nanoscale complexes by a combination of electrostatic attraction and the hydrophobic effect. With this approach, we are able to generate a variety of formulations, each with different DNA binding strength, cell membrane penetration and targeting capabilities.

The individual components used to generate the various formulations in the study presented here were chosen for their simplicity, efficacy and biocompatibility. The naturally occurring polyamine spermine was chosen as the basic component of DNA binding because it has been shown to neutralize DNA phosphate charges and aid condensation into compact forms such as rods, toroids, and spheroids [35,36]. Oleyl groups were selected as the lipid component because, in addition to being naturally present in a variety of animal and vegetable sources, oleic acid is capable of promoting membrane destabilization [37,38]. In the context of gene

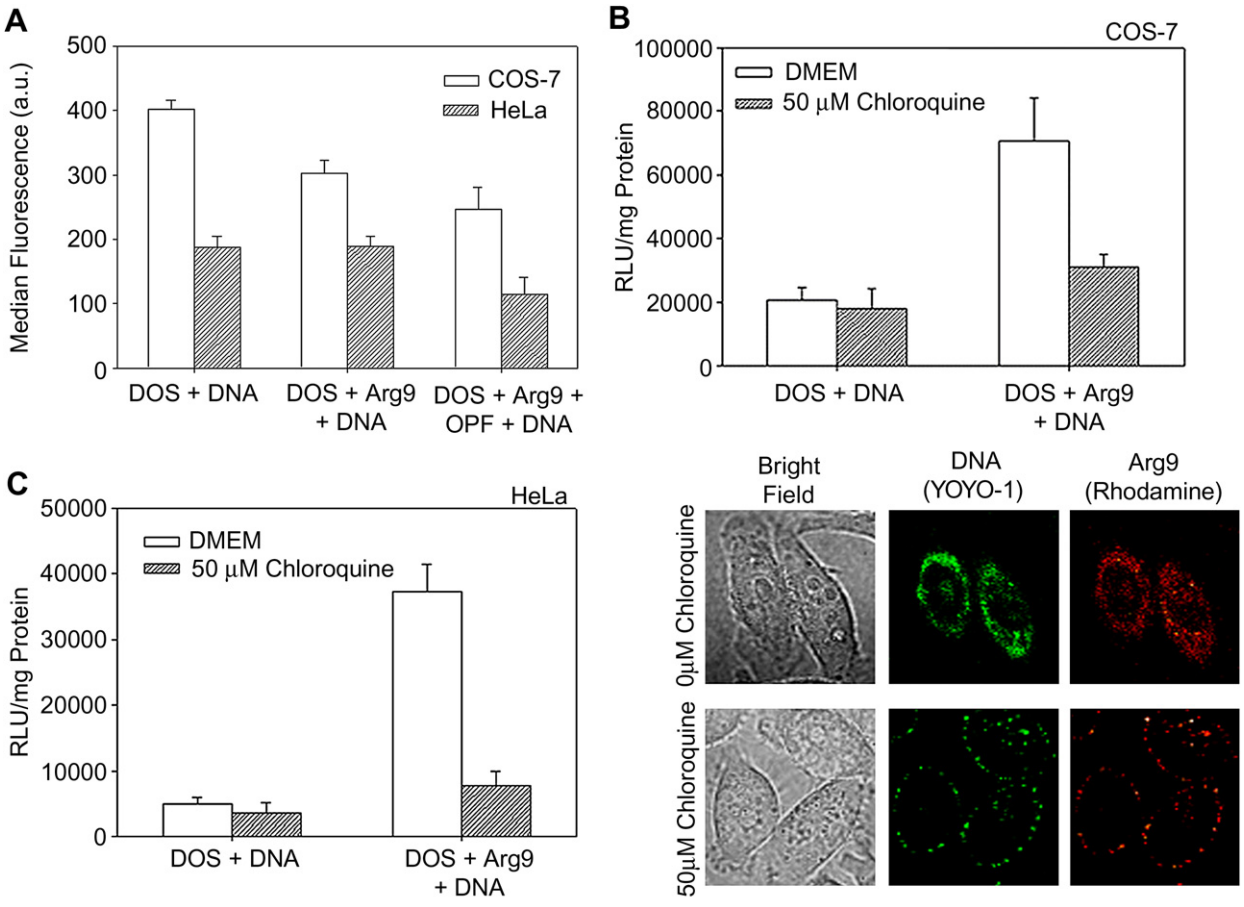


Fig. 6. (A) Uptake of fluorescently-labeled complexes formed with the indicated components in COS-7 and HeLa cells. For COS-7 cells, the previously determined optimized weight ratio of 25:10:1 DOS:Arg9:DNA with 10 mol% OPF was used. For HeLa cells, the previously determined optimized weight ratio of 10:1:1 DOS:Arg9:DNA with 20 mol% OPF was used. (B) In vitro transfection efficiency of COS-7 cells in the presence and absence of chloroquine with complexes formed with the indicated components. The previously determined optimized weight ratio of 25:10:1 DOS:Arg9:DNA was used. (C) In vitro transfection efficiency of HeLa cells in the presence and absence of chloroquine with complexes formed with the indicated components. The previously determined optimized weight ratio of 10:1:1 DOS:Arg9:DNA was used. (D) Confocal microscopy of HeLa cells transfected in the presence and absence of chloroquine. Complexes were formed of DOS:Arg9:DNA (10:1:1 weight ratio).

delivery, membrane destabilization—in particular endosomal membrane destabilization—aids the endosomal escape of gene delivery complexes [39]. Arginine-rich peptides derived from HIV-1 TAT have attracted considerable attention in drug and gene delivery because they assist cell membrane permeation [40–42]. Free peptides have also been shown to facilitate cytoplasmic release from endosomes following a pH dependent process [43]. By incorporating uncomplexed oligoarginine in our ternary system,

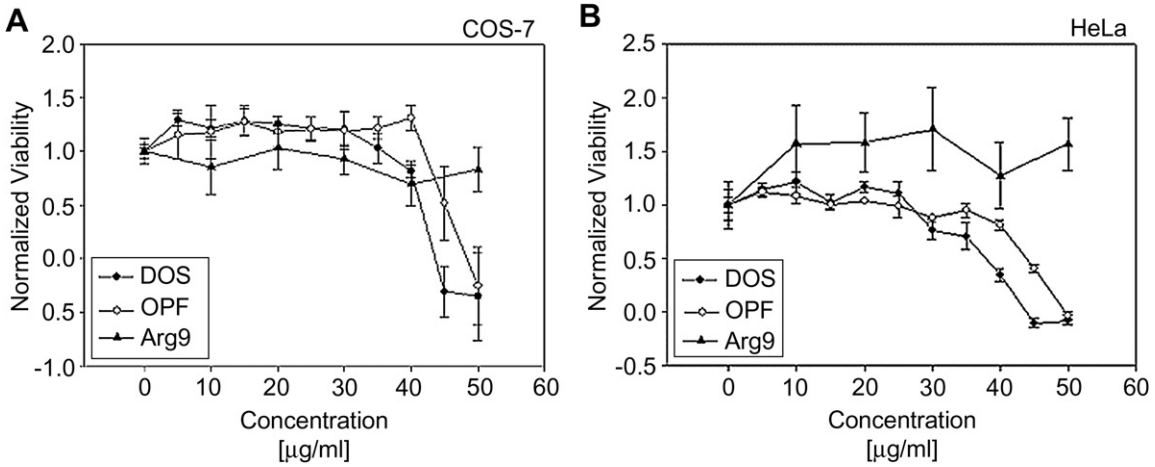


Fig. 7. (A) In vitro toxicity of uncomplexed DOS, OPF and Arg9 at various concentrations in COS-7 cells. (B) In vitro toxicity of uncomplexed DOS, oleyl-PEG-folate and Arg9 at various concentrations in HeLa cells.

we are able to increase endosomal release beyond what is achieved with oleyl groups alone. Lastly, to incorporate targeting functionality, we included folic acid moieties. Folate receptors are overexpressed in a variety of cancers—including the COS-7 and HeLa cells tested here—and have been explored extensively for various drug and gene delivery properties [16,34,44–47]. Furthermore, endocytosis through the caveolae pathway—the endocytic route followed by folate receptors—is believed to be more efficient for gene delivery applications than other uptake pathways [34,47].

On its own, spermine is a relatively poor DNA binding agent (Fig. 2D). However, when conjugated to a lipid tail, hydrophobic interactions drive the assembly of the individual spermine groups into larger, multivalent structures. With its increased charge density, this multivalent spermine structure is an effective DNA binding agent (Fig. 2C) [48,49]. It has previously been shown in polymeric gene delivery systems that increased transfection efficiency can be achieved by optimizing DNA binding strength. Generally, this involves changing the polymer molecular weight to alter its charge density or performing chemical modifications to transform primary and secondary amines—which are readily protonated at physiological pH—into non-protonable groups that do not carry a positive charge (i.e. amides) [20,50]. In the component-based approach presented here, we are able to optimize DNA binding strength by increasing or decreasing the amount of DOS in the formulation. When little DOS is used, the resulting multivalent structures are expected to have low charge density and exhibit weak DNA binding. However, by increasing the amount of DOS present, the resulting multivalent structures have an increased charge density, which results in stronger DNA binding.

Endosomal escape is considered a major source of the inefficiency observed with non-viral gene therapy systems. For liposomal delivery systems, it is speculated that mixing between the cationic lipid and the negatively charged endosomal lipid bilayer results in a reduction of the positive charge density of the multivalent structure formed by the cationic lipid [39,51]. The reduced charge density lowers the affinity between the cationic lipid and the DNA, resulting in vector dissociation. This lipid mixing is also believed to destabilize the endosomal membrane, thus enhancing the release of free DNA from the endosome into the cytosol. Since the lipid groups used in the system described here are covalently attached to the DNA binding group spermine, it is impossible to alter the endosomolytic properties of the vector (i.e. oleyl content) without also changing the DNA binding strength (i.e. spermine content). To this end, oligoarginine was incorporated in the system as a means to separately tune the endosomolytic properties of the formulation. Typically, oligoarginine is used to facilitate cell uptake and not endosomal escape. However, the results of Fig. 6A indicate that the observed increase in transfection efficiency with oligoarginine rests outside of increased uptake. Arginine oligomers have been reported elsewhere to enhance endosomal escape and cytosolic delivery of non-covalently attached cargo via endocytic vesicle membrane destabilization [32]. Further supporting the notion that oligoarginine increases transfection efficiency by enhancing the endosomal escape of complexes, Fig. 6B and C reveals that preventing the acidification of endosomes—a reportedly necessary step in membrane translocation by Arg9 and other cell-penetrating peptides—reduces gene delivery in Arg9-containing complexes in COS-7 and HeLa cells [33,43].

The benefit of the library screening approach—that is, screening multiple formulations and combinations simultaneously—is evident when considering that individual optimization of components would prove troublesome. For example, the addition of OPF groups to DOS:DNA complexes without Arg9 does not appear impart a targeting functionality in either COS-7 or HeLa cells, nor does the addition of OPF groups to the top-performing DOS:Arg9:DNA formulation in COS-7 cells (Fig. 5C and D). However, by screening a variety of formulations it is possible to identify particular combinations such that each component is able to perform its intended function without impairing the function of the remaining components. Furthermore, it is interesting to note that the optimum formulation for COS-7 cells (25:10:1 DOS:Arg9:DNA weight ratio) is distinct from that of HeLa cells (10:1:1 DOS:Arg9:DNA weight ratio). This suggests that individual cell types can be targeted not only through the incorporation of cell-specific ligands, but also by optimizing the vector formulation for the intended cell type. Further exploration of this observation may also provide insight into the different transfection requirements of different cell types, including traditionally hard-to-transfect cell lines.

5. Conclusion

Through application of our supramolecular library approach, we were able to identify a unique formulation strategy to prepare MSAs that out-perform LFA under similar conditions in both HeLa and COS-7 cells. Contrary to its typical use, we demonstrate that free Arg9 can be incorporated into gene delivery complexes through electrostatic interaction to promote endosomal escape. This unique feature allows the endosomolytic capabilities of the vector to be tuned independently from DNA binding strength, an attribute that lends itself particularly well to a library screening approach. Supramolecular assembly has recently been validated in a clinical trial for siRNA delivery [52], showing the great potential of utilizing this strategy for the clinical translation of nanomedicine. We believe the supramolecular assembly approach described here, which is very easy to handle, does not involve tedious chemical reactions and can be further optimized by changing its assembly building blocks, is an important addition to the existing efforts in identifying cell-specific non-viral gene delivery vectors.

Acknowledgements

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Appendix

Figures with essential color discrimination. Figs. 1 and 6 in this article are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.08.024.

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