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A Combinatorial Library of Unsaturated Lipidoids for Efficient Intracellular Gene Delivery

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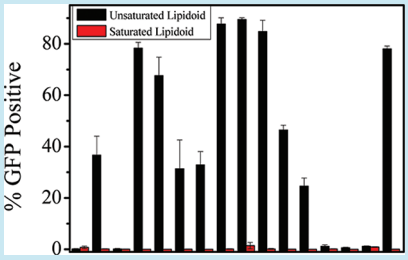
Supporting Information

ABSTRACT: A combinatorial library of unsaturated lipidoids was synthesized through the Michael addition of amines to oleyl acrylamide. Their capability in facilitating in vitro gene delivery was evaluated by transfecting HeLa cells with EGFP-encoding plasmid DNA and mRNA. The preliminary screening results indicated that lipidoids with unsaturated oleyl tails are superior transfection agents compared to saturated lipidoids with *n*-octadecyl tails under the same conditions. The different transfection abilities of the unsaturated and saturated lipidioids were ascribed to the large, tightly packed lipoplexes of saturated lipidoids. The potential applications of the library of lipidoids were further expanded by looking at their ability to transfect fibroblasts as well as different cancerous cell lines.



KEYWORDS: *lipidoid, gene delivery, combinatorial library*

The lack of a safe and efficient gene delivery vehicle remains a bottleneck in the genetic treatment of inherited and acquired diseases.[1−8] A combinatorial strategy of constructing libraries of lipid-like materials termed "lipidoids" has been developed for efficient in vitro and in vivo gene delivery.[9−15] These lipidoids can be synthesized via solvent- and catalyst-free conjugation reactions of amines and α,β-unsaturated carbonyl compounds (e.g., acylates, acrylamides) or epoxides. These simple and mild reactions have enabled us to refine the structure of the lipidoids and improve their transfection capability. This was accomplished by reacting structurally diverse amines, α,β-unsaturated carbonyl compounds, or epoxides. It has been demonstrated that the tail length and head amine group of the lipidoid play significant roles in identifying the efficient carriers for gene delivery. For example, lipidoids incorporating tails in the range of 8−12 carbons as well as a secondary amine were reported to have high efficiency for siRNA delivery,[9] whereas lipidoids containing two tails of 14 carbons each were superior at delivering DNA.[14]

To further explore the structure−activity relationship of lipidoids in gene delivery, we have designed a new library of lipidoids with unsaturated hydrophobic tails. We compared this new group to saturated lipidoids containing the same amine group and tail length and evaluated their ability to facilitate in vitro gene delivery. Lipids with lower saturation levels usually have higher gene transfection efficiencies due to increased membrane fluidity;[16−19] however, the effect tail saturation levels of lipidoids plays on gene delivery has not been investigated. As shown in Figure 1, a library of unsaturated and saturated lipidoids was prepared through the reaction of amines with oleyl acrylamide (Figure 1B, I) and *n*-octadecyl acrylamide (Figure 1B, II), respectively. This was completed in the absence of any solvents or catalysts, and the simple and mild reactions allowed us to generate a structurally diverse library of 32 lipidoids. Their capability to facilitate intracellular gene delivery was first evaluated by delivering enhanced green fluorescent protein (EGFP)-encoding DNA and mRNA (mRNA) into human cervical carcinoma cells (HeLa). The preliminary screening results indicated that unsaturated lipidoids are the superior gene transfection agent compared to saturated lipidoids under the same conditions. Such differences can be ascribed to the large, tightly packed lipoplexes of the saturated lipidoids, which prohibit efficient cellular uptake of lipidoid/gene complexes and intracellular gene releases. The potential applications of the unsaturated lipidoids for gene therapy were further determined by looking at their ability to transfect fibroblasts as well as different cancerous cell lines.

The capability of the lipidoids in transfecting HeLa cells was evaluated by counting GFP-positive cells after DNA or mRNA delivery. As summarized in Figure 2, unsaturated lipidoids 8-I, 9-I, 10-I, and 16-I (the Arabic numeral indicates the amine number in Figure 1C, the Roman numeral indicates unsaturated (I) or saturated tails (II)) were effective in facilitating DNA delivery (Figure 2A). More than 40% cells were positive for GFP expression, whereas less than 10% GFP expression was observed for cells transfected with the saturated lipidoid/DNA complexes. Additionally, the unsaturated lipidoids were more efficient in facilitating mRNA delivery, as shown in Figure 2B. A majority of the unsaturated lipidoids facilitated intracellular mRNA delivery; 4-I, 5-I, 8-I, 9-I, 10-I, and 16-I can transfect the cells with efficiency greater than 50%, which was even higher than that obtained with the commercial

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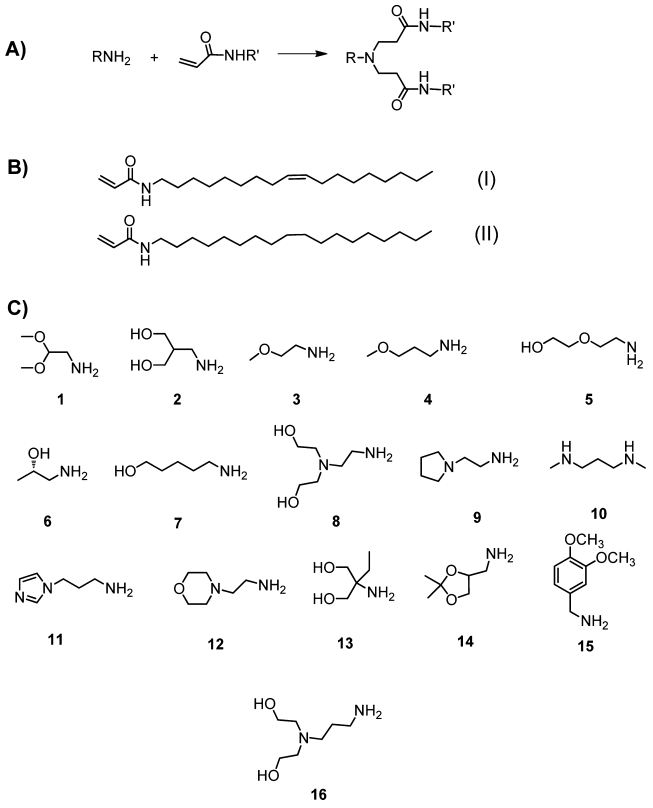

Figure 1. (A) Synthesis route of lipidoids. (B) Chemical structures of acrylamides used. (C) Amines used for lipidoids synthesis.

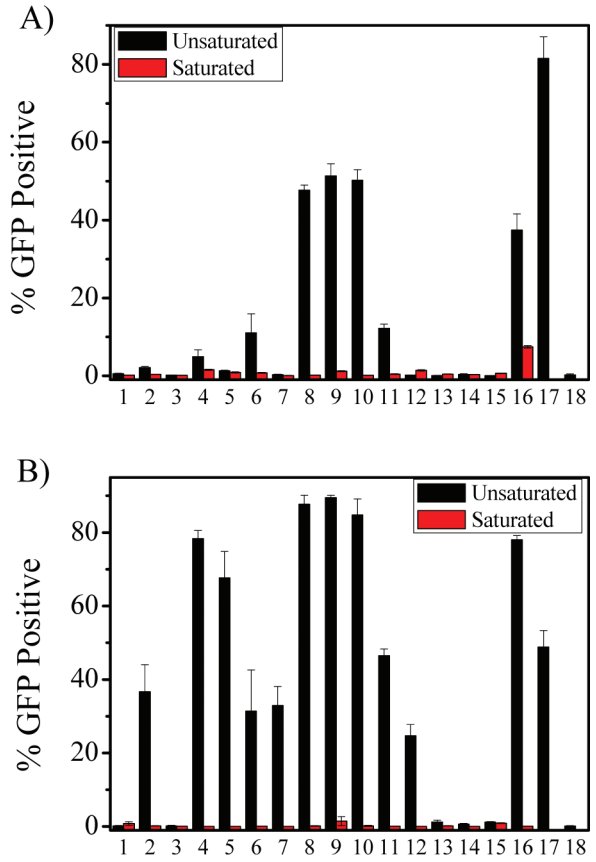

Figure 2. Initial *in vitro* screening results of lipidoid efficiency at delivering EGFP encoding (A) DNA and (B) mRNA into HeLa. (1−16: DNA or mRNA was delivered by lipidoids with different amine headgroups as indicated in Figure 1C; 17: DNA or mRNA was delivered using Lipofectamine 2000; 18: DNA or mRNA only). The transfection efficiency was determined by counting GFP-positive cells.

transfection agent Lipofectamine 2000. However, none of the saturated lipidoids displayed any significant ability to deliver mRNA. The screening results clearly demonstrated that for lipidoids with 18 carbon tails, the introduction of unsaturated bonds can effectively improve their performance in gene delivery. Furthermore, we observed a higher GFP expression level for the HeLa cells transfected with mRNA compared with those dosed with DNA combined with unsaturated lipidoids. This difference is understandable when the different protein transcription mechanisms of intracellular mRNA and DNA delivery are considered. For DNA-based gene delivery and therapy, DNA released into the cytoplasm has to enter the nucleus, be translated into mRNA, and then be released back into the cytoplasm. The mRNA must then be transcribed as the GFP that is then expressed in the cell. In contrast, mRNA is released intracellularly and can initiate the transcription process directly, omitting entering the nucleus. This shorter process leads to the higher transient GFP expression levels observed with lipidoid/mRNA transfection.[20,21] These results agree with previous reports for mRNA delivery using cationic lipids and polymers as carriers.[22]

To probe the mechanism that leads to the different transfection capabilities of unsaturated and saturated lipidoids, lipidoids 16-I and 16-II were selected and purified for further investigation. The DNA condensation abilities of 16-I and 16-II were evaluated first by Picogreen assay. By fixing a lipidoid/DNA ratio (5:1 w/w), the DNA condensation efficiency of 16-I was determined to be 68.4%, whereas only 38.2% DNA was condensed by 16-II. The different DNA condensation abilities of unsaturated and saturated lipidoids may result from the rigid and long hydrophobic chains of the saturated lipioids, which comprise the water solubility of lipoplexes and thus affect DNA condensation efficiency.[19,23] Negative-staining transmission electron microscopy (TEM) was then used to characterize the self-assembled structures of lipidoids and genes. As shown in Figure 3, multilamellar nanostructures (~ 50 nm) with genes intercalated between the lipidoid bilayers formed when 16-I was bound to either DNA or mRNA at a 5:1 (w/w) ratio. For lipidoid 16-II however, shapeless and large aggregation complexes formed upon DNA or mRNA binding (Supplementary Figure S1). The hydrodynamic sizes of lipidoid/DNA and lipidoid/mRNA complexes were also analyzed by dynamic light scattering (DLS). Unsaturated lipidoid 16-I/DNA and 16-I/mRNA formed narrow distributed nanoparticles of 131.9 ± 10.8 and 84.6 ± 3.8 nm, respectively. However, saturated lipidoid 16-II formed particles in the size range of 100−600 nm following DNA or mRNA binding. In addition, the large, tightly packed saturated lipidoid/genes complexes may prohibit efficient intracellular gene release and result in low transfection efficiency. The intracellular gene release from the lipidoid/gene complexes was then simulated by a heparin competition assay. Heparin is a biopolymer with a high negative charge and can compete with DNA or mRNA in binding to the lipidoids. These competitive interactions can be assayed by gel electrophoresis and ethidium bromide (EB) exclusion assay. By using a fixed ratio of lipoidoid to genes (5:1 w/w), the amount of heparin required to displace genes from the lipidoid/gene complexes is a good indicator of the degree of intracellular

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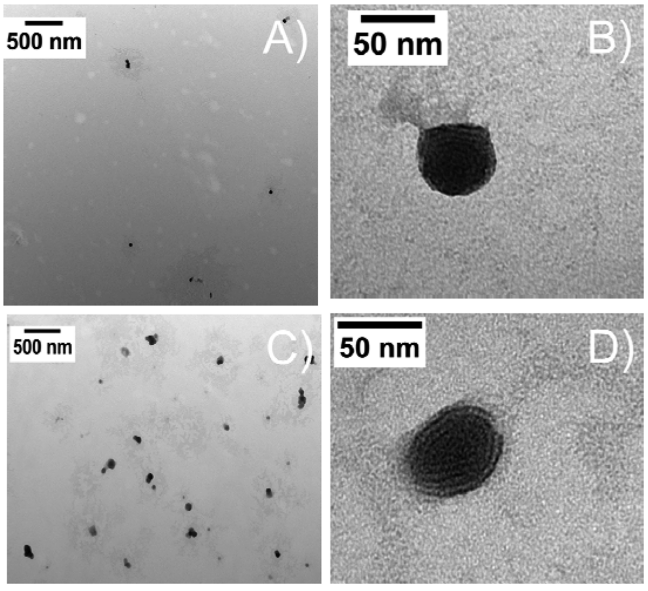

Figure 3. TEM images of (A,B) lipidoid 16-I/DNA complex and (C,D) 16-II/mRNA complex.

gene release.[24,25] As shown in Figure 4, both 16-I and 16-II can bind with DNA or mRNA effectively. By increasing

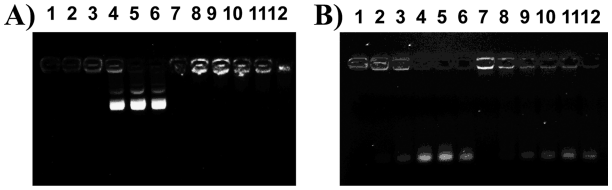

Figure 4. Heparin competition assay of lipidoid/gene complexes. (A) 16-I/DNA(1−6) and 16-II/DNA (7−12) in the absence (1, 7) and presence of heparin (2−6, 8−12). (B) 16-I/mRNA(1−6) and 16-II/mRNA (7−12) in the absence (1, 7) and presence of heparin (2−6, 8−12). The lipidoid/gene ratios were fixed at 5:1 (w/w); the concentration of heparin in lane 2 and 8 was 4 mg/mL; in 3 and 8, 8 mg/mL; in 4 and 9, 16 mg/mL; in 5 and 10, 32 mg/mL; in 6 and 11, 50 mg/mL.

concentration of heparin from 4 to 50 mg/mL, DNA is released gradually from the lipidoids. It was observed that the DNA was completely released from the 16-I/DNA complex in the presence of 16 mg/mL heparin. For 16-II with DNA, no DNA release was observed in the presence of same amount of heparin. The EB exclusion assay also demonstrated the different DNA release behavior of 16-I/DNA and 16-II/DNA complexes in the presence of heparin. As shown in Supplementary Figure S2, the relative fluorescence intensity of DNA/EB complex without any lipidoid was set to be 100%, and the binding of DNA with 16-I or 16-II quenched the emission. However, with the addition of heparin, the EB fluorescence restored gradually. The addition of 16 μg/mL heparin restored 85% of emission for 16-I/DNA, whereas only 55% was restored for 16-II/DNA complex. The gel retardation and EB exclusion assay demonstrated that the genes could be easily released from the lipoplexes of unsaturated lipidoids. The lipidoid/mRNA complexes displayed a mRNA release behavior similar to that of the lipidoid/DNA complexes, in which mRNA is released easier from 16-I/mRNA complex than from the 16-II/mRNA complexes. The heparin competition assay results correlate very well with the cellular transfection experiments. They showed that poor transfection ability of the saturated lipidoids is likely due to the large, tightly packed aggregation complexes formed, resulting in the ineffective intracellular gene release.

The potential of the library of lipidoids for gene therapy was further expanded by optimizing the lipidoid/gene ratio and transfecting different cell lines. As shown in Figure 5, GFP

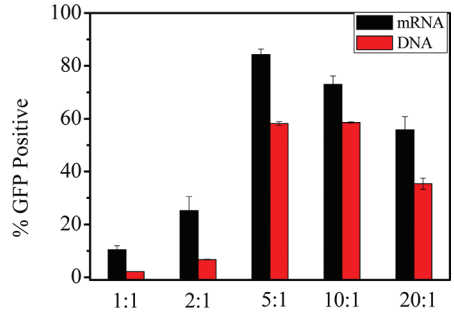

Figure 5. Relationships of lipidoid 16-I to DNA or mRNA ratios and delivery efficiency. The delivery efficiency was monitored by counting GFP-positive cells.

expression was enhanced by increasing the ratio of 16-I to DNA or mRNA. This increase in expression was seen with ratios from 1:1 to 5:1 (lipidoid:DNA/mRNA), while any further increase led to a lower transfection efficiency. Furthermore, different cell lines including the cancerous NIH3T3, MCF-7, HepG2, and MDA-MB-231 cell lines and non-cancerous fibroblasts (BJ cells) were dosed with 16-I/DNA or 16-I/mRNA to test the versatility of the lipidoids. As shown in Figure 6, lipidoid 16-I facilitates both DNA and mRNA delivery

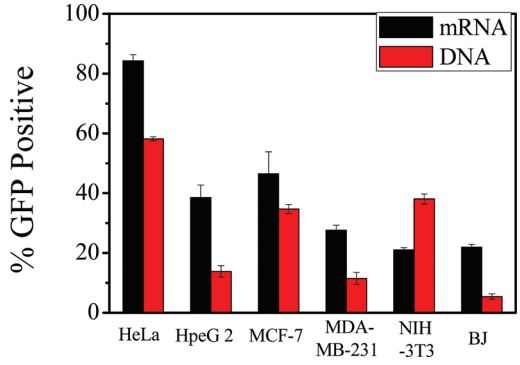

Figure 6. Gene delivery efficiency of lipidoid 16-I in different cancerous and non-cancerous cell lines.

in all of the tested cell lines. It was observed that HeLa cell can be transfected at the highest efficiency (greater than 50%) of the tested cells. For MCF-7, HepG2, MDA-MB-231, and BJ cells, mRNA transfection led to higher GFP expression compared to DNA transfection. For NIH-3T3 cells, DNA delivery was more efficient than mRNA delivery.

In conclusion, we have used a combinatorial approach to design and construct a lipidoid library consisting of lipidoids with different saturation levels to facilitate intracellular gene delivery. The investigation of the structure−activity relationships indicates that the introduction of unsaturated bonds can improve the transfection ability of the lipidoids. This is most likely due to the increased cellular uptake and enhanced intracellular gene release of the unsaturated complexes.

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Meanwhile, the amine groups in the lipidoids play critical roles in determining the transfection efficiency, and a secondary amine and a hydrophilic group incorporated in a lipidoid can facilitate the gene delivery. The potential applications of these unsaturated lipidoids for gene therapies were expanded by evaluating their ability to transfect different cells lines. Furthermore, we have reported the first application of lipidoids for in vitro mRNA delivery. This application has the potential to be beneficial for cancer vaccines[26] and cellular reprogramming[27] due to the advantages of using mRNA rather than DNA. These advantages include pharmaceutical safety as well as the ability to transiently transfect cells.

METHODS

Materials. Compounds in the library were synthesized and characterized as previously described by our group as well as several others.[9,14] All chemicals were purchased from Sigma-Aldrich or Alfa-Aesar and used directly. EGFP encoding Plasmid DNA was purchased from Elim Biopharmaceuticals, Inc. (Hayward, CA). Messenger RNA encoding EGFP was purchased from Stemgent Inc. (Cambridge, MA). Picogreen assay kit was purchased from Invitrogen (Carlsbad, CA), and the DNA condensation efficiency was determined according to the manufacturer's instruction.

Synthesis of Lipidoids. Oleyl acrylamide and *n*-octadecyl acrylamide were synthesized according to the published methods.[28] In a 5-mL Telfon-lined glass screw-top vial, oleyl acrylamide or *n*-octadecyl acrylamide was added to amine at a molar ratio of 1:2.4 (amine:acrylamide). The mixture was then stirred at 90 °C for 2 days. After cooling, the lipidoid mixtures were used without purification unless otherwise noted. Representative lipidoids 16-I and 16-II were purified through flash chromatography on silica gel and characterized by ^{1}H NMR.

Cell Culture. All cell lines used in this paper (HeLa, MCF-7, HepG2, MDA-MB-231, NIH-3T3, BJ) were purchased from ATCC (Manassas, VA) and cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in the presence of 5% CO_2. For gene transfection experiments, cells were seeded in 96-well plates at a density of 10,000 cells per well 1 day prior to transfection.

In Vitro Gene Transfection. To facilitate high-throughput screening, the lipidoid/DNA or lipidoid/mRNA complexes were prepared simply by adding lipidoid to the sodium acetate buffer solutions (25 mM, pH = 5.5) of DNA or mRNA at ratios of 5:1 (w/w), followed by 15 min of incubation at room temperature. After the addition of the lipidoid/gene complexes to the cells (200 ng DNA or 100 ng mRNA complexed with lipidoids per well), HeLa cells were incubated at 37 °C for an additional 48 h. Control experiments were performed by complexing DNA or mRNA with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in OPTI-MEM as per the manufacturer's instructions. The percentage of GFP expressing cells was evaluated by a flow cytometer (BD FACS LSRII) attached with a high-throughput system. All transfection experiments were performed in quadruplicate.

Cytotoxicity of Unsaturated Lipidoids (MTT Assay). Cytotoxicity of unsaturated lipidoids was assessed by incubating lipidoid (7 μg/μL) with cells seeded on 96-well plate at a density of 10,000 cells per well. Lipidoids were incubated with cells for 24 h, and the cell viability was evaluated through MTT assay according to previous reports.[29]

Transmission Electron Microscopy (TEM). Lipidoid/DNA or lipidoid/mRNA complexes were prepared with the same protocol as in vitro transfection experiment. One drop of the samples (10 μL) was applied to hydrophilic carbon-covered copper grids (300 meshes) for 10 min. The grids were subsequently rinsed with contrasting materials (1% uranyl acetate solution at pH 4.5). Any remaining staining solution was removed with filter paper and air-dried. TEM microstructure was determined using a Tecnai FEG TEM (FEI tecnai 12 spirit Biotwin, FET company, Hillsboro, OR) operating at 80 kV.

Gel Retardation and Heparin Competition Assay. Lipidoid/DNA and Lipidoid/mRNA complexes were prepared by mixing 0.5 μg of DNA or 0.25 μg of mRNA with lipidoids at a ratio of 1:5 (w/w), respectively. For the heparin competition assay, heparin solutions with concentrations increasing from 0 to 50 mg/mL were added to the lipidoid/DNA or lipidoid/mRNA complexes, followed by 15 min of incubation before loading into gels. The electrophoresis gels were prepared by dissolving 0.4 g of agarose in 50 mL of TBE with 0.1 μg/mL ethidium bromide.

Ethidium Bromide (EB) Exclusion Assay. Briefly, 2 μg of DNA and 2 μL of EB solution (0.01%) were mixed in 500 μL of sodium acetate buffer solution (25 mM, pH = 5.5) and incubated for 10 min, and the fluorescence intensity at 605 nm was recorded and set as 100%. For the heparin competition assay, the lipidoid 16-I or 16-II (10 μg), 2 μg of DNA, and 2 μL of EB solution (0.01%) were incubated in 500 μL of sodium acetate buffer solution (25 mM, pH = 5.5) for 10 min, followed by the addition of different amount of heparin and 10 min of incubation. The emission intensity was monitored and compared with that of the lipidoid/DNA mixtures.

ASSOCIATED CONTENT

Supporting Information

Cytotoxicity assay of unsaturated lipidoid; TEM images of 16II/DNA and 16II/mRNA complexes; ethidium bromide exclusion assay for heparin competition binding of lipidoid/DNA complexes. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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