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A biomimetic lipid library for gene delivery through thiol-yne click chemistry

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ABSTRACT

The delivery of nucleic acids such as plasmid DNA and siRNA into cells is a cornerstone of biological research and is of fundamental importance for medical therapeutics. Although most gene delivery therapeutics in clinical trials are based on viral vectors, safety issues remain a major concern. Non-viral vectors, such as cationic lipids and polymers, offer safer alternatives but their gene delivery efficiencies are usually not high enough for clinical applications. Thus, there is a high demand for more efficient and safe non-viral vectors. Here, we present a facile two-step method based on thiol-yne click chemistry for parallel synthesis of libraries of new biomimetic cationic thioether lipids. A library of novel lipids was synthesized using the developed method and more than 10% of the lipids showed highly efficient transfection in different cell types, surpassing the efficiency of several popular commercial transfection reagents. One of the new lipids showed highly efficient siRNA delivery to multiple cell types and could successfully deliver DNA plasmid to difficult-to-transfect mouse embryonic stem cells (mESC). Analysis of structure–activity relationship revealed that the length of the hydrophobic alkyl groups was a key parameter for efficient cell transfection and was more important for transfection efficiency than the nature of cationic head groups. The correlation of the size and surface charge of liposomes with transfection efficiency is described.

1. Introduction

The delivery of nucleic acids such as plasmid DNA and siRNA into cells is a cornerstone of biological research and is of fundamental importance for medical therapeutics. Indeed, since the first FDA-approved gene therapy experiment in 1990 [1], over 1700 clinical trials have been conducted for gene delivery [2]. Although most gene delivery therapeutics in clinical trials are based on viral vectors, safety issues remain a major concern [3]. Non-viral vectors, including cationic lipids [4,5], polymers [6,7], dendrimers [8], cationic proteins [9] and inorganic nanoparticles [10,11], offer safer alternatives but their gene delivery efficiencies are usually not high enough for clinical applications. Although lipid-based vectors have only 4.8% of all gene therapy clinical trials [12], they are already the most commonly used systems for in vitro delivery of nucleic acids into cells [4,13–15]. However, most of the lipid-based delivery systems are synthesized using a multi-step synthesis route, requiring protecting groups and excessive purifications [15], thus limiting the possibility for successful and fast structural optimizations. Recently Anderson et al. reported two important combinatorial approaches to synthesize libraries of alkyl amines for siRNA delivery using aza-michael addition [16] and epoxide-amine [17] reactions. However, there are still no convenient combinatorial methods that could lead to lipid-like molecules structurally similar to natural phospholipids, the main lipid components of the cell membrane.

Here we report a facile modular and scalable approach employing thiol-yne "click" chemistry [18–21] for the parallel synthesis of a library of cationic thioether lipids with two hydrophobic tails of variable lengths and possessing a linker group structurally mimicking the glycerol core of the phospholipids. We used the method to synthesize more than 100 novel lipids and found that more than 10% of all lipids showed highly efficient transfection in different cell types. Both siRNA delivery and transfection of difficult-to-transfect cell lines, such as mESC, was analyzed. Analysis of structure–activity relationship and the correlation of the size and surface charge of liposomes with transfection efficiency are described.

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2. Materials and methods

2.1. Chemicals, plasmids and siRNAs

1-Hexanethiol (95%), 1-heptanethiol (98%), 1-octanethiol (≥98.5%), 1-nonanethiol (95%), 1-decanethiol (96%), 1-undecanethiol (98%), 1-dodecanethiol (≥98%), 1-hexadecanethiol (≥95%), 2,2-dimethoxy-2-phenylacetophenone (99%), 4-pentynoic acid (95%), 5-hexynoic acid (97%), 4-(2-aminoethyl)morpholine (99%), 1-(2-aminoethyl)pyrrolidine (98%), N′,N′-diethylethane-1,2-diamine (99%), N′,N′-diethylpropane-1,3-diamine (≥99%), N′,N′-dimethylpropane-1,3-diamine (≥98%) and 1-[2-(dimethylamino)ethyl]piperazine (≥98%) were bought from Sigma–Aldrich (Steinheim, Germany). Dichloromethane (99.8%), tetrahydrofuran (99.8%) and *n*-hexane (98.5%) were bought from Merck (Darmstadt, Germany). Dimethylformamide (99.8%) was bought from Merck (Hohenbrunn, Germany). N′,N′-diisopropylcarbodiimide (99%) and N′,N′-dimethylethane-1,2-diamine (97%) were bought from Alfa Aesar (Karlsruhe, Germany); anhydrous hydroxybenzotriazole (≥95%) was bought from Molekula (Shaftesbury, Dorset, United Kingdom). 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine (DOPE) was bought from Corden Pharma Switzerland LLC (Liestal, Switzerland). Plasmid DNA consisting of 67.5 ng pCS2 + β-galagtosidase and 7.5 ng of pEGFP-1 (Clontech) was used per well of a 96-well plate. For siRNA transfection experiments siGENOME SMARTpool targeting human LRP6 (Dharmacon) was used at a final concentration of 40 nM to assess functional delivery of siRNA molecules in 24-well plates.

2.2. Library synthesis

First step: solutions of either 4-pentynoic or 5-hexynoic acid (0.5 mmol) in 0.5 ml tetrahydrofuran (THF), an alkyl thiol (2 eq.) in 0.5 ml of THF and 2,2-dimethoxy-2-phenylacetophenone (5 mg, 0.02 mmol) in 0.2 ml of THF were combined in a 20 ml glass vial protected from light and degassed by ultrasonication and purging with argon for 3 min. The samples were then irradiated with UV (365 nm, 1.87 mW/cm^2) for 1 h.

Second step: After evaporation of THF, the residue was redissolved in 8 ml of dichloromethane (DCM). The solution was separated into seven 1 ml batches for subsequent reactions with different amines. N,N′-Diisopropylcarbodiimide (DIC) (12 μl 0.075 mmol), an amine (0.063 mmol) and a 20 μl solution of 1-hydroxybenzotriazole (HOBt) in dimethylformamide (DMF) (c 0.5 g/ml) were added to each sample and vortexed. The solutions were protected from light with aluminum foil and shaked for 16 h under argon atmosphere. After evaporation of the solvent, 2 ml of *n*-hexane was added to each sample for extraction followed by centrifugation at 10,000 g for 5 min and separation of supernatant. Evaporation of hexane from supernatants gave final lipids as clear yellowish oils, which were then used for the screening. The average yield in library synthesis is 80%, and the estimated purity is about 70–80%.

2.3. ESI-MS and HRMS characterization

Electrospray ionization mass spectrometry (ESI-MS) was performed using an API 4000 Quadrupol mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo V™ electrospray ion source (TurboIonSpray® probe, Applied Biosystems) operating in positive ion mode at a source temperature of 400 °C. Nitrogen was used as nebulizer, curtain, collision and auxiliary gas. Instrument controlling and data acquisition were carried out using the Analyst Software V 1.4 (Applied Biosystems, Foster City, CA, USA). All parameter settings were optimized by flow injection experiments with standard solutions infused into the mass spectrometer using a syringe pump (Harvard Apparatus Inc, South Natick, USA) at an infusion flow rate of 10 μl/min. Lipids C6–C12 were dissolved in methanol, and tested in ESI-MS at concentration 5 μg/ml.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was performed on a 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA). Peak lists were generated using Data Explorer Software 4.0 (Applied Biosystems). 2,5-Dihydroxybenzoic acid (DHB, 10 mg in 1 ml tetrahydrofuran containing 0.1% trifluoroacetic acid) was used as a matrix for C16 lipids. 10 μl lipids (1 mg in 1 ml THF) were mixed with 10 μl matrix, and 1 μl mixed solution was spotted on a stainless steel MALDI carrier.

2.4. NMR characterization

NMR spectra were obtained on a Bruker AMX 500 spectrometer, chemical shifts are reported in parts per million (ppm) on the δ scale, and were referenced to residual protonated solvent peaks.

2.5. Cationic liposome preparation in the initial screening

A 20 μl solution of DOPE (0.0067 mol/l) and 10 μl solution of a cationic lipid (0.0175 mol/l) in ethanol (for C16-lipids, 1:1 ratio of ethanol and THF was used to dissolve lipids) were mixed and vortexed. Then 70 μl of a 200 mM sodium acetate buffer (pH 5.0) was added and the solution was vortexed for 30 s and sonicated for 5 min to form liposomes.

2.6. Cationic liposome preparation during optimization of hits

Stock solutions of DOPE (7.44 mg/ml, 0.01 mol/l) and cationic lipids (0.01 mol/l) were made in absolute ethanol. Stock solutions of DOPE and lipids were mixed to produce 300 μl solutions with three different lipid/DOPE mole ratios (2:1, 1:1 and 1:2) in Eppendorf tubes, followed by vortexing for 10 s and mixing with 700 μl of 200 mM sodium acetate buffer (pH 5.0). The solution was then vortexed again for 10 s.

2.7. A1C11 cationic liposome preparation for mESC D3 transfection

Stock solutions of DOPE and the A1C11 lipid were mixed in a round-bottom glass flask to produce 300 μl solution with 1:1 lipid/DOPE mole ratio, followed by slow evaporation of ethanol using a rotary evaporator. Sodium acetate buffer (1000 μl, 200 mM, pH 5.0) was added to a clear lipid film formed on glass walls, followed by gentle rotation of the flask for 2 h to produce liposomes.

2.8. In vitro transfection assay in HEK293T and Hela cell with eGFP-pDNA

For the HEK293T cell transfection screen, 0.4 μl liposomal reagent, 75 ng plasmid DNA (67.5 ng pCS-LacZ and 7.5 ng pCMV-EGFP) and 4×10^4 cells were used per well in 96-well plates using a one-step (also termed reverse transfection) protocol. Both the liposomal reagent and DNA were diluted in a final volume of 10 μl with 50 mM sodium acetate buffer (pH 5.0), combined and mixed with pipette action to give 20 μl lipoplex solution and left at room temperature for 30 min. 80 μl of freshly trypsinized cell suspension in DMEM supplemented with 10% FCS was then added, mixed gently with pipette and immediately transferred to the 96 wells. After 16 h incubation at 37 °C in 5% CO_2, Hoechst 33342 was added to a final concentration of 1 μg/ml by pipetting 10 μl of a 10 μg/ml solution. After a further 30 min incubation to allow staining of DNA within nuclei, cells were transferred to an Olympus IX81 automated fluorescent imaging microscope and images for bright field, Hoechst and GFP acquired. Cell transfection efficiencies were estimated using the free software programme CellProfiler after counting the number of GFP positive cells and dividing by the total number of cells (nuclei). A detailed manual analysis of several images after overlaying the Hoechst and GFP channels in Photoshop showed that both CellProfiler as well as Image J software programmes underestimated the true transfection efficiency due to the inability of the software to always discern individual GFP transfected cells that were closely adjacent. This did not effect the overall results however as the error was constant for various transfection efficiencies. Relative transfection efficiency was determined by dividing the transfection efficiencies of lipids with transfection efficiency of Lipo2000.

2.9. In vitro transfection assay with siRNA

For siRNA mediated gene silencing experiments 1 μl liposomal reagents A1C11 and A7C11 and 20 pmol (1 μl of 20 μM) standard control or LRP6 siRNA (Genome SMARTpool, Dharmacon) were used per well in 24-well plates. Liposomes and siRNA were diluted in a final volume of 50 μl with 50 mM sodium acetate buffer (pH 5.0), combined and mixed with pipette action to give 100 μl lipoplex solution and left at room temperature for 20 min. 400 μl of DMEM supplemented with 10% FCS was then added, mixed gently with pipette and immediately transferred to the 24 wells containing mouse embryonic fibroblast (MEF) cells at 50% confluency, after first removing the existing media from the wells.

2.10. Western blot

48 h after siRNA transfection, MEF cells were lysed in 100 μl of 1% Triton X-100 buffer (50 mM Tris, pH 7.0, 0.15 M NaCl plus protease/phosphatase inhibitors). After centrifugation, clarified supernatants from these total cellular lysates were denatured in SDS-loading buffer and 5 μl loaded on a 10% SDS-PAGE gel. Proteins separated by SDS-PAGE were transferred to PVDF membrane and Western blot performed using a polyclonal rabbit antibody for total LRP6 protein (anti-T1479; at 1:2000 dilution in 5% BSA). Beta-Actin was used as a loading normalization control.

2.11. mESC D3 transfection assay

Mouse D3 Embryonic Stem (mES) Cell transfection was conducted in a 6-well plate coated with 0.1% gelatine. Effectene was chosen as a positive control. Cells were cultured in ES cell medium (DMEM-GlutaMAX™-I medium supplemented with 15% FCS, 0.1 mM β-mercaptoethanol, 1% penicillin/streptomycin and 1000 U/ml LIF) in culture dishes. 4 μl liposomal reagent, 1 μg plasmid DNA (0.9 μg pCS-LacZ and 0.1 μg pCMV-EGFP) and 2.5×10^5 cells were used per well using a one-step (also termed reverse transfection) protocol. Both the liposomal reagent and DNA were diluted in a final volume of 100 μl with 50 mM sodium acetate buffer (pH 5.0), combined and mixed with pipette action to give 200 μl lipoplex solution and left at room temperature for 30 min. 500 μl of freshly trypsinized cell suspension in ES cell medium was then added, mixed gently with pipette and immediately transferred to one well of a 6 well plate. 1300 μl of ES cell medium was further added to each well.

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After 48 h incubation at 37 °C in 5% CO_2, Hoechst 33342 was added to a final concentration of 1 μg/ml by pipetting 200 μl of a 10 μg/ml solution. After a further 30 min incubation to allow staining of DNA within nuclei, cells were transferred to an Olympus IX81 automated fluorescent imaging microscope and images for bright field, Hoechst and GFP acquired.

2.12. DLS analysis

Dynamic light scattering was performed on a Malvern Zetasizer Nano ZS (Malvern, Germany). Cationic liposome samples were prepared by diluting 32 μl lipids with 768 μl 50 mM NaOAc/HOAc buffer (pH 5.0). Lipoplex samples were prepared as follows: 16 μl lipids were diluted with 384 μl 50 mM NaOAc/HOAc buffer (pH 5.0), and 30 μl DNA (GFP:LacZ = 1:10; 0.1 μg/μl) were diluted with 370 μl 50 mM NaOAc/HOAc buffer (pH 5.0). Diluted lipids and DNA solutions were mixed to form 800 μl lipoplex solution. Particle size (hydrodynamic diameter) and surface charge (zeta potential) were measured at different time points sequentially by using a standard operating procedure.

3. Results and discussions

3.1. Synthesis of thiol-yne lipids

In order to produce a library of cationic lipids parallel synthesis was performed using 8 different alkyl thiols (alkyl chain length from C6 to C16), 2 alkynyl carboxylic acid linkers bearing a terminal triple bond, and 7 different cationic amines (Fig. 1). The synthesis of lipids is based on two consecutive modular steps. First, the thiol-yne click reaction of an alkyl thiol with an alkynyl carboxylic acid gives a lipid molecule with two hydrophobic tails and one carboxylic head group. In the second step, a cationic head group is attached to the lipid through the amide coupling, leading to a cationic thioether lipid (Fig. 1). Both reactions are very efficient and can be completed in just one day. In addition, since the lipids are built from three functional blocks sequentially (hydrophobic tail, linker and a cationic head group), every block can be varied separately and both reaction steps can be used to easily multiply the library. This makes this approach convenient for fast modular synthesis of libraries of structurally diverse phospholipid-like molecules for gene- or drug-delivery applications.

3.2. In vitro pDNA delivery

3.2.1. Initial screen

It is known that unsaturated neutral phospholipids (co-lipids) are often required for effective transfection by cationic lipids [22–25]. We used the phosphatidylethanolamine DOPE as a co-lipid as it has been shown to be one of the most effective [26]. A small set of the cationic lipids synthesized were first selected to determine the optimal cationic lipid/DOPE ratio as well as optimal DNA/liposome ratio for transfection. An optimal cationic lipid/DOPE molar ratio was found to be 1.3:1 and all 112 cationic lipids were then mixed with DOPE at this ratio in ethanol before dilution in a pH 5.0 buffer to yield liposomal suspensions containing 30% ethanol and 2 mg/ml total lipid. The optimal amount of DNA required per μg of liposomes was found to be only 150 ng. Cell based screening of transfection was performed using a one-step method, where freshly resuspended HEK293T cells were added directly to the lipoplexes, followed by a transfer to culture wells (see Supporting Information). The following day cell nuclei were stained with Hoechst, imaged automatically with a fluorescent microscope (Fig. 2B), and analyzed by CellProfiler [27].

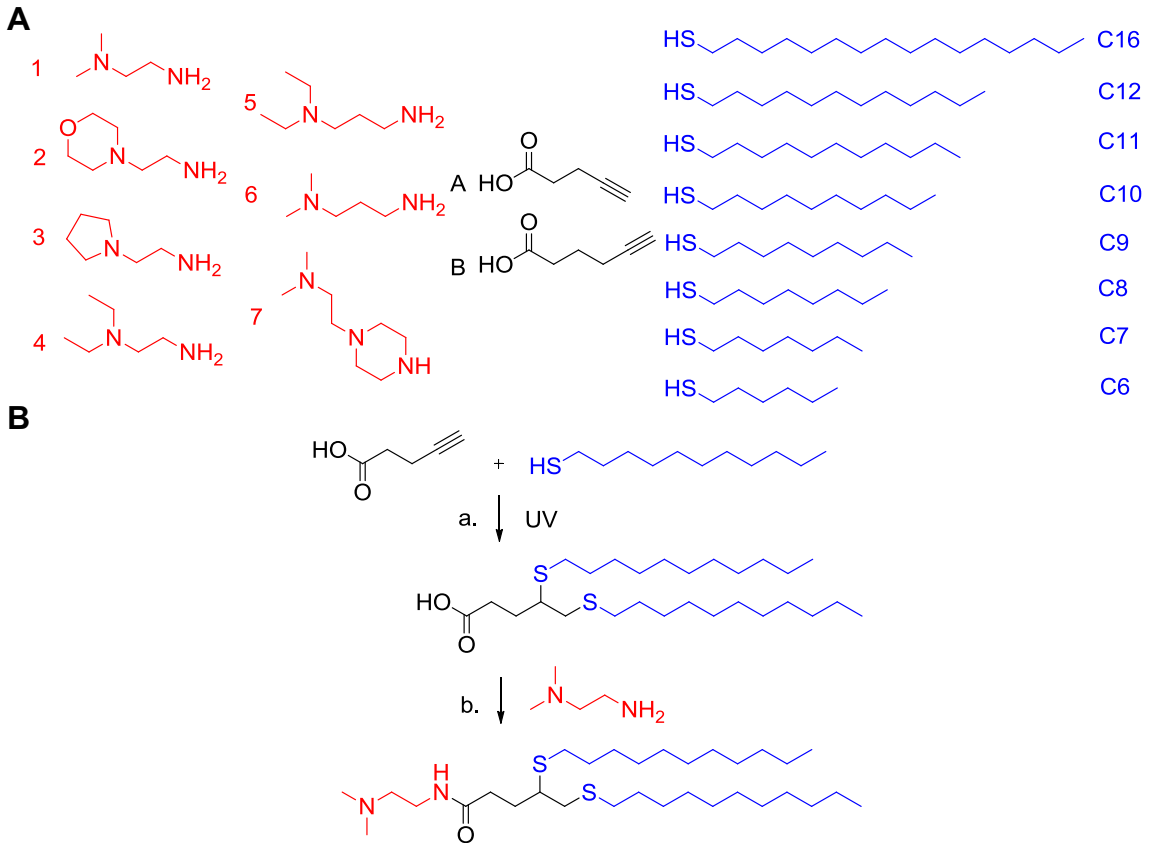

Fig. 1. Combinatorial synthesis of cationic thioether lipids via thiol-yne chemistry. (A) Seven amines, two alkynes, and eight alkyl thiols (C6–C12, C16) were used in the synthesis. (B) Synthesis scheme of a typical lipid from the library. (a) UV 365 nm, 5% DMPA, THF, 1 h (b) DIC, HOBt, DCM, 16 h.

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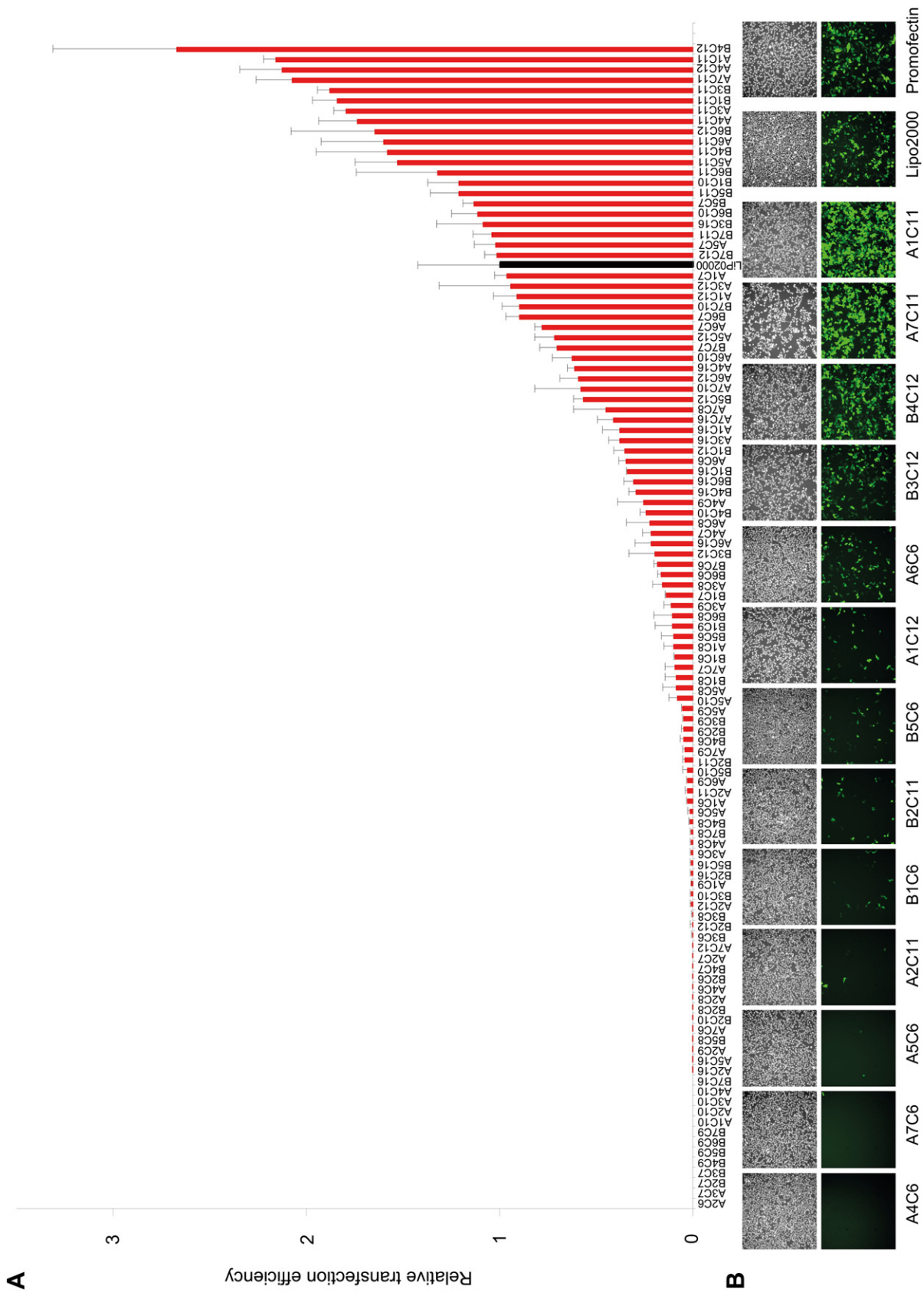

Fig. 2. In vitro cell based screening of the lipid library. (A) Graph showing relative transfection efficiency of 112 lipids from the library. Transfection efficiency was determined by calculating the ratio of fluorescent to total cells after transfection with a plasmid containing the green fluorescent protein (GFP) gene. Relative transfection efficiency is a ratio of the transfection efficiency of a lipid to that of the positive control (Lipo2000). (B) Examples of fluorescent microscopy images of HEK293T cells transfected with different lipids from the library. Images show nuclei stained with the DNA dye Hoechst (upper row, showing total cell number) and GFP positive cells (bottom row, transfected cells). Lipo2000 and Promofectin were used as positive controls.

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Lipofectamine 2000 (lipo2000) and Promofectin were used as positive controls. Surprisingly, the results showed that over 90% of all lipids in the library displayed positive cell transfection and over 10% showed efficiencies significantly higher than that of the positive controls (Fig. 2A).

3.2.2. Structure–activity relationship

The synthesized library of new cationic lipids contained both different cationic head groups as well as hydrophobic tails with various lengths. When transfection efficiencies were correlated with the nature of the functional groups of the lipids (lipid tails C6–C16, amine head groups 1–7 and linker A or B), important patterns became obvious (Fig. 3). First, lipids containing undecyl (C11) and dodecyl (C12) hydrophobic tails showed enhanced cell transfection performance in comparison to lipids with shorter or longer tails. Furthermore, all cationic heads apart from the morpholine head group (Fig. 1A, amine 2) showed high activity when combined with hydrophobic chains of C11 or C12. This result clearly indicates that the length of the hydrophobic alkyl groups is a key parameter for cell transfection and can be used for an intelligent design of new or improvement of existing gene-delivery vectors.

3.2.3. Correlation of particle size and surface charge with transfection efficiency

In addition to the chemical nature of lipids, particle size, surface charge as well as stability of liposomes and lipoplexes are important parameters for the efficient gene-delivery. Lipoplexes are complexes of liposomes with DNA or RNA while liposomes are empty vesicles. To correlate these properties with transfection efficiency we selected 11 of the most active lipids as well as 2 inactive ones from the original library (Fig. S8). The selected compounds were used to make liposomes at lipid/DOPE molar ratios of 2:1, 1:1 and 1:2. Transfection efficiency was then tested on HEK293T and Hela cells and correlated with the particle size and zeta potential of both liposomes and lipoplexes (Fig. 4, Figs. S4, S6). The results showed that the optimal particle size before mixing with plasmid DNA lies between 100 and 200 nm (Fig. 4A, Fig. S6A). The observed correlations also suggest that addition of DOPE to liposomes increased their ability to ensheath DNA by aggregation of several liposomes around added nucleic acid molecules, which helped to successfully deliver DNA into cells. The correlation of surface charge of lipoplexes with their transfection efficiency (Fig. 4B and Fig. S6B) showed the importance of a stable positive charge (above +50 mV) of lipoplexes after complexation with DNA for achieving highly efficient cell transfection.

3.3. In vitro siRNA delivery

Delivery of small interfering RNA (siRNA) molecules to cells for targeted loss-of-function experiments has become one of the most widely used methods for reverse genetic screening approaches in the last decade [28]. Although plasmid DNA transfection requires delivery of the nucleic acid cargo to the cell nucleus, siRNA molecules require delivery to the cytoplasm, where they interact with their target mRNA. As a consequence, only few reagents (e.g. Lipo2000) are capable of mediating effective delivery of both siRNA and DNA. We tested siRNA delivery using two similarly potent DNA transfection reagents from the library, A1C11 and A7C11, with Lipo2000 as a positive control. siRNA targeting mRNA for the Wnt receptor protein, low-density lipoprotein receptor related protein 6 (LRP6) was used and immuno-blot analysis performed to visualize the levels of endogenous LRP6 in cell lysates (Fig. 4C) [29]. Difficult to transfect mouse embryonic fibroblast (MEF) as well as HEK293T cells were used. The results showed that, although A7C11 was less efficient than the positive control (Fig. 4C), A1C11 maintained high transfection efficiency to deliver siRNA, indeed surpassing the efficiency of Lipo2000.

3.4. In vitro pDNA delivery into difficult to transfect cells

Finally, we tested whether A1C11 could efficiently deliver DNA plasmid into very difficult to transfect cell line D3 mouse embryonic stem cells (mESC). Effectene was chosen as a positive control as it has been reported to achieve high transfection efficiencies in

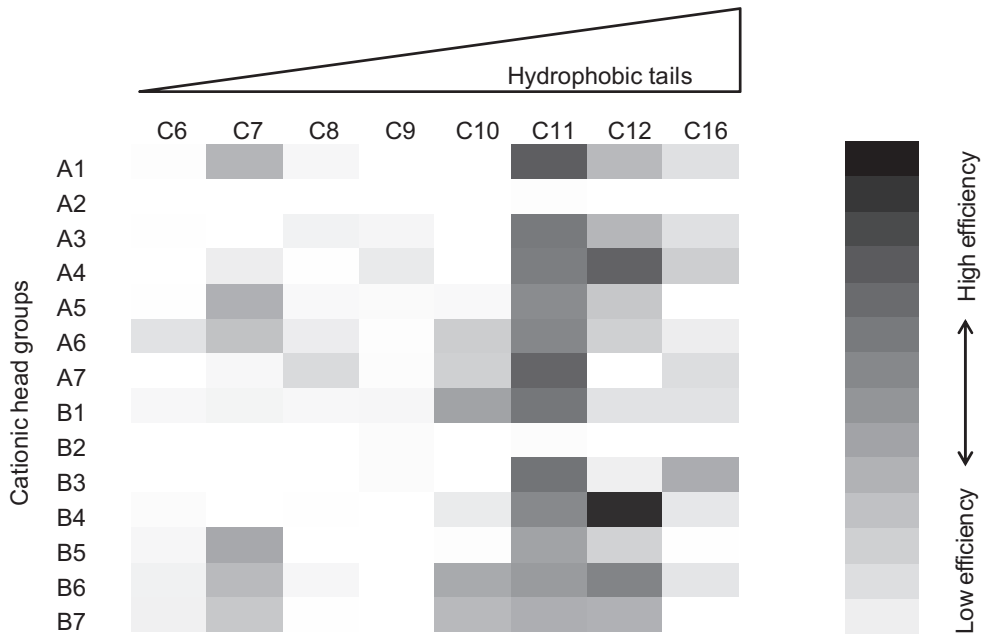

Fig. 3. Graph showing structure–activity relationship within the lipid library. Lipids with C11 and C12 hydrophobic tails show the highest transfection efficiencies. All lipids with A2 and B2 (morpholino) head groups were inactive independently of the length of their hydrophobic tails.

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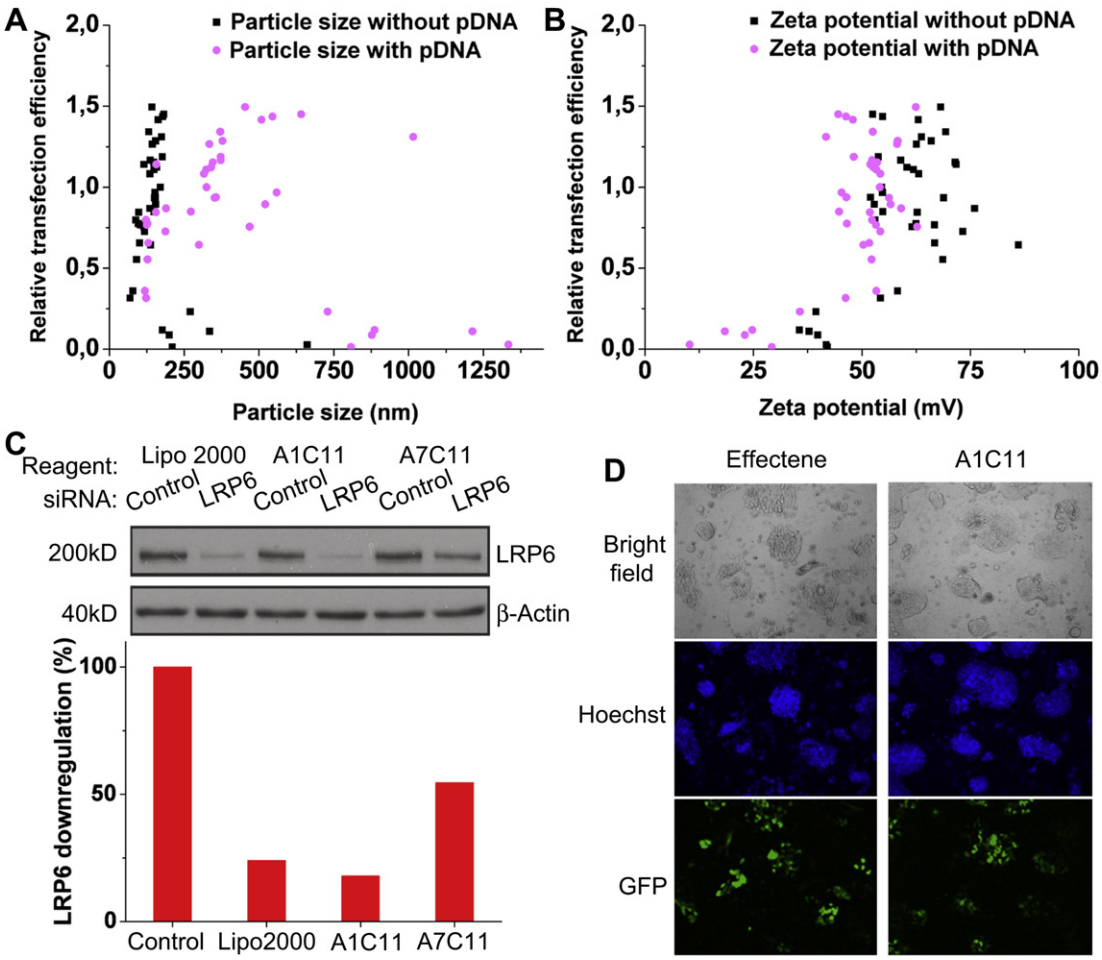

Fig. 4. Correlations between relative transfection efficiencies of selected lipids in HEK293T cells and particle sizes (A) as well as zeta-potentials (B) of liposomes and lipoplexes. Particle size (hydrodynamic diameter, D_h) and zeta potentials of lipoplexes were measured by dynamic light scattering (DLS) 11 min after mixing liposomes with eGFP DNA plasmid. (C) Western blots of endogenous LRP6 and actin proteins from whole cell lysates of mouse embryonic fibroblast (MEF) cells transfected with the indicated siRNA molecules using either Lipo2000 as reference or cationic liposomes prepared from A1C11 and A7C11. (D) DNA plasmid transfection efficiency of A1C11 and Effectene in mES cells.

these stem cells [30]. Similar to Effectene, A1C11 indeed showed efficient transfection of mESC (Fig. 4D).

4. Conclusion

We envision that the developed modular method for the parallel synthesis of cationic biomimetic thioether lipids will greatly help the intelligent design of new efficient gene delivery systems. The simple and rapid synthesis scheme enables efficient parallel synthesis of hundreds of cationic lipids that can be used for in vitro or in vivo delivery of both DNA plasmids and siRNAs. The analysis of structure–activity relationship revealed a particular importance of the length of the hydrophobic lipid tails for cell transfection. Other structural parameters important for efficient cell transfection include liposome size (100–200 nm), stable positive surface charge (60–70 mV) and the ability of liposomes to aggregate around added nucleic acids. We believe that the surprisingly high efficiency of transfection in different cell lines, together with the simple and cost effective way to prepare libraries of cationic thioether lipids, will provide a valuable method for the development of new gene- and drug-delivery systems.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary material

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.biomaterials.2012.07.044.

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