DOI: 10.1002/cmdc.201100259

Hydrophobic Oxime Ethers: A Versatile Class of pDNA and siRNA Transfection Lipids

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The manipulation of the cationic lipid structures to increase polynucleotide binding and delivery properties, while also minimizing associated cytotoxicity, has been a principal strategy for developing next-generation transfection agents. The polar (DNA binding) and hydrophobic domains of transfection lipids have been extensively studied; however, the linking domain comprising the substructure used to tether the polar and hydrophobic domains has attracted considerably less attention as an optimization variable. Here, we examine the use of an oxime ether as the linking domain. Hydrophobic oxime ethers were readily assembled via click chemistry by oximation of hydrophobic aldehydes using an aminooxy salt. A facile ligation reaction delivered the desired compounds with hydrophobic domain asymmetry. Using the MCF-7 breast cancer, H1792 lung cancer and PAR C10 salivary epithelial cell lines, our findings show that lipoplexes derived from oxime ether lipids transfect in the presence of serum at higher levels than commonly used liposome formulations, based on both luciferase and green fluorescent protein (GFP) assays. Given the biological compatibility of oxime ethers and their ease of formation, this functional group should find significant application as a linking domain in future designs of transfection vectors.

Introduction

Cationic lipids and their derived liposomes have become the most well-studied and widely used synthetic, nonviral gene delivery vehicles since Felgner et al.[1] first disclosed DOTMA-mediated gene transfer in 1987.[2,3] Due to the major limitations of viral vectors, such as associated immune responses, limited polynucleotide carrying capacity and high cost,[4,5] cationic lipids remain an attractive alternative. The advantages of low immunogenicity, the ability to transfect RNA or DNA of nearly unlimited size,[6,7] and the relative ease of cationic lipid–plasmid DNA (pDNA) or RNA complex (lipoplex) formulation[8] continue to attract interest aimed at developing safer and more efficient cationic lipids for use as transfection agents.[9]

Cationic lipid molecules, such as the prototypical dual chain lipids DOTMA[1] and DOTAP[10] (Figure 1), contain a polar, positively charged (DNA binding) head group connected to a hydrophobic domain via a linking functionality. These three principal structural components of cationic glycerol-type lipids have been extensively studied in efforts to improve lipid-mediated intracellular delivery of polynucleotides to mammalian cells.[11] Many structure–activity relationships have been determined,[12,13] particularly for the hydrophobic domain. Indeed, the hydrophobic domain's structural variables of chain length, degree of unsaturation, and domain asymmetry are among the strongest contributors to transfection efficacy.[14] Fewer direct structural comparisons of changes in the cationic lipid backbone, or linking domain, have been reported, with the most well-known comparison being that of the diether DOTMA versus the diester DOTAP.[10] The linking functionality, and to a lesser extent the cationic head group, seem to be the principal determinants of toxicity.[15–17] The linker determines conformational flexibility, degree of stability, and biodegradability. Among the most studied chemical functionalities comprising the linking domain of transfection lipids are the ether, ester, ortho ester,[18,19] carbamate,[20] amide,[21,22] and phosphono[23] moieties.

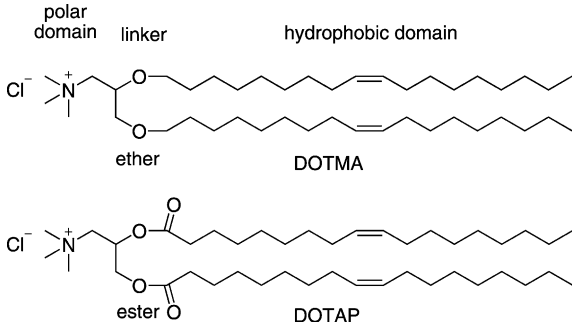

Figure 1. Structural domains of common transfection lipids.

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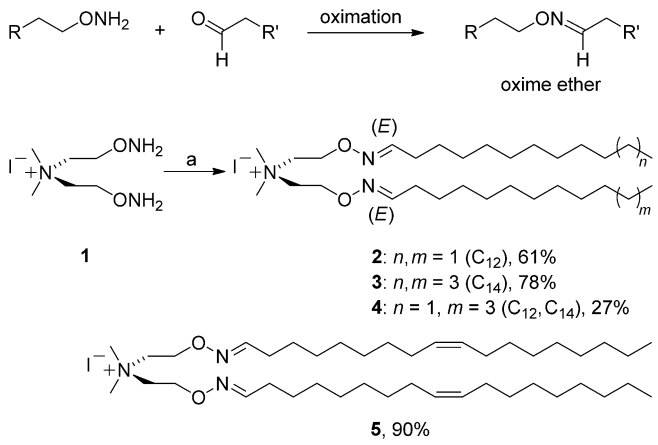
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.201100259.

The use of oxime ether linkages for applications in chemical biology and medicinal chemistry research has increased dramatically in the past several years as the benefits of chemoselectivity have become better understood. Chemoselective click ligation[24] of an aldehyde or ketone carbonyl group with an aminooxy counterpart to form an oxime ether linkage (i.e., oximation, Scheme 1) is the key step in the synthesis of numerous



Scheme 1. Oximation route to hydrophobic oxime ethers. *Reagents and conditions*: a) RCHO, MeOH, RT, 4 h.

bioconjugates.[25–27] Oxime ether linkages are also used as a mechanism for prodrug generation,[28] and are present in US Food and Drug Administration (FDA)-approved pharmaceuticals, such as fluvoxamine.[29] Although oximation has been used to attach ligands to transfection lipids, such as the ligation of carbohydrates,[30] and although an oxime-ether-based assembly for siRNA delivery has been reported,[31] no studies have examined the use of the robust yet biodegradable oxime ether linkage as the backbone feature of cationic lipids for DNA delivery.

Our program in developing lipid-based gene delivery vectors[32] and interest in click chemistry[33,34] inspired us to use oximation as a facile approach to prepare low toxicity transfection-active oxime ethers (Scheme 1). Here, we report the physicochemical characterization of this new class of lipids and in vitro evaluation of a hydrophobic oxime ether panel in pDNA and siRNA delivery experiments using epithelial cell lines, including two human cancer cell lines.

Results and Discussion

Synthesis of hydrophobic oxime ethers

We prepared a panel of oxime ethers by reacting bis-(aminooxy) ammonium salt **1**[35] with hydrophobic aldehydes dodecanal, tetradecanal, or (*Z*)-octadec-9-enal (oleyl aldehyde) in methanol at room temperature to afford oxime ethers **2**, **3** and **5**, respectively (Scheme 1). In each case, the lipid was obtained as a mixture containing two stereoisomers (diastereomeric ratio ranging from approximately 2.3:1 to 5.3:1). The major diastereomer has (*E*)-stereochemistry about each oxime C=N bond (as depicted). The minor diastereomer has an (*E*)-oxime ether as well as a (*Z*)-oxime ether linkage. The (*E*,*E*):(*E*,*Z*) ratio was readily measured by integration of the well-separated oximyl proton shifts in the ^{1}H NMR spectra,[36] and the ratio was found to be susceptible to a variety of factors including exposure to mild acid and heat. The stereochemical integrity of the oxime ether lipids is inconsequential in that isomerization can be expected in vivo. Of greater interest, however, is that the oximation approach used to prepare these compounds delivers the lipids with intrinsic asymmetry in the hydrophobic domain. We have recently noted the beneficial influence of unsymmetrical hydrophobic domains in cationic-lipid-mediated DNA transfection.[32] Consequently, we used the oxime ether lipids as obtained directly in transfection experiments. To probe further the influence of an unsymmetrical hydrophobic domain, we also prepared unsymmetrical analogue **4** (Scheme 1) by successively condensing **1** with dodecanal and tetradecanal. Hydrophobic oxime ethers **2**–**5** were formulated as cationic liposomes using equimolar quantities of co-lipid dioleoylphosphatidylethanolamine (DOPE) for subsequent studies.

Size and charge characterization

Particle size and zeta potential measurements on the oxime ether liposome suspensions revealed that the particles were net positively charged with electrostatic potentials in the range of 45–66 mV (Figure 2). Liposomes derived from bis-C_{18} lipid **5** were the largest, with particle diameters exceeding 200 nm, and the most highly charged. Not unexpectedly, the liposomes derived from the shortest oxime ether lipids **2** and **4**—i.e., lipids prepared using dodecanal—had the smallest mean diameters, near 150 nm. The trend in particle size versus particle charge correlates well for this series of lipids.

Of significance as a predictor of transfection activity is the net charge of the lipoplex formed on mixing a liposome suspension with pDNA.[13,37] Complexation of the oxime ether/DOPE liposomes with pDNA (pCMV-Luc) at different ammoni-

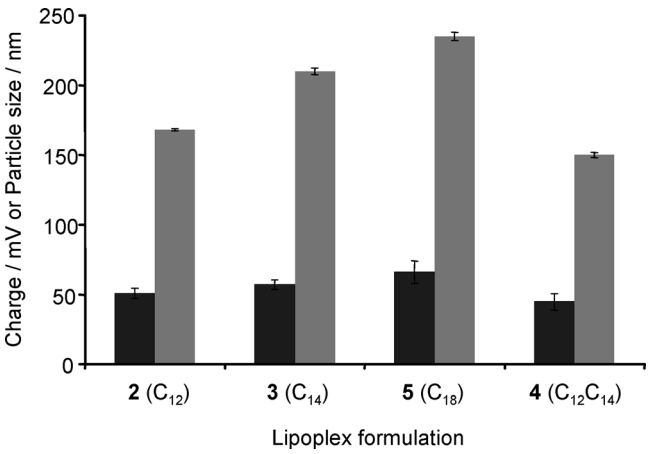


Figure 2. Zeta potential (■) and mean particle size (■) measurements for oxime ether: DOPE liposomes (0.1 mg cationic lipid per mL H_2O).

um nitrogen/DNA phosphate ratios (N:P ratio) gave the corresponding lipoplexes. Zeta potential measurements of the oxime-ether-derived lipoplexes at N:P ratios of 3, 5 and 7 showed high positive values in the range of 31–53 mV (Figure 3). As can be expected at these electrostatic potentials,

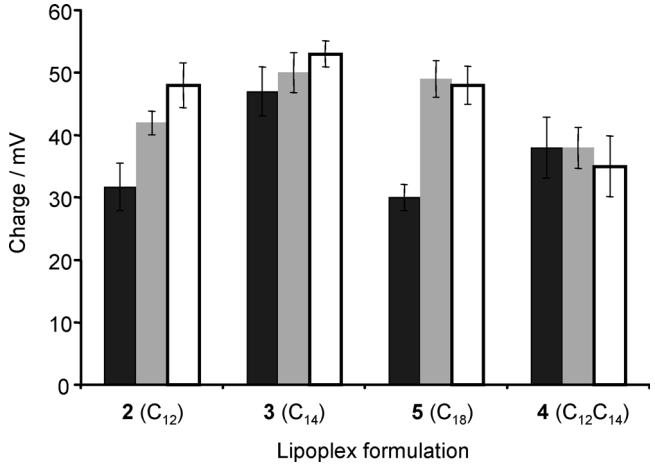


Figure 3. Lipoplex zeta potential measurements. Lipoplexes were formulated at 0.1 mg cationic lipid per mL H_2O with pDNA (pCMV-Luc) at different N:P charge ratios: N/P = 3 (■); NP = 5 (■); N/P = 7 (<?QI>). Results are expressed as the mean values of $n = 3$ measurements.

minimal lipoplex aggregation ensued—the oxime ether/DNA lipoplex suspensions were stable at concentrations up to 0.33 mg oxime ether lipid per mL beyond one week. Particle size measurements of the lipoplexes (data not shown) indicated that the mean effective diameter for all N:P formulations remained under 250 nm. Lipoplex size mirrored closely the precursor liposome, with the lipoplex generally having an increase in diameter by 20–50 nm.

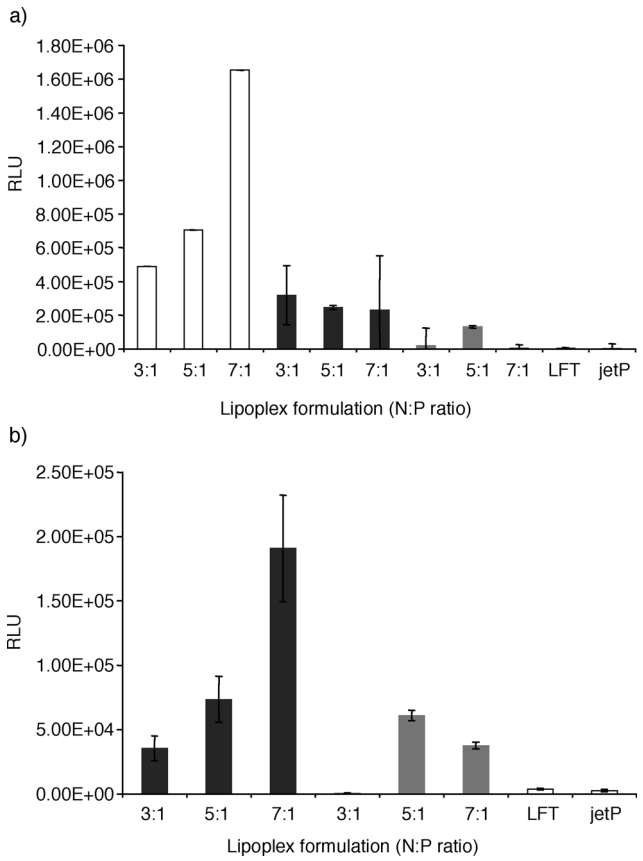


Figure 4. Transfection of cells using lipoplexes formulated at different N:P charge ratios: a) MCF-7 cells (lipid **3**: □; lipid **4**: ■; lipid **5**: ■) and b) H1792 cells (lipid **5**: □; lipid **4**: ■; control: □). Results are expressed as total relative light units (RLU). Transfections were performed in 24-well tissue culture plates using luciferase reporter construct (pCMV-Luc; 0.025 μg per well) with 18 h transfection time. Each data point represents the mean value of three separate transfections. Error bars show the standard deviation (SD) from the mean. LFT = lipofectamine 2000, jetP = jetPRIME.

Transfection activity

We evaluated the transfection activity of the dual-chain oxime ether lipid panel in human breast cancer (MCF-7) and human lung cancer (H1792) cells using a CMV-luciferase reporter plasmid in the presence of serum (Figure 4). Whereas the shortest-chain lipid **2** was essentially ineffective at all N:P formulations examined in MCF-7 cells (data not shown), we were gratified to find that the transfection efficiency of lipid **3** in this cell line at an N:P ratio of 7 was notably superior (greater than 2.5 orders of magnitude higher activity) to the commercial transfection standard lipofectamine 2000 (3500 ± 990 RLU) as well as to the popular[38] transfection formulation jetPrime (2500 ± 209 RLU) (Figure 4a). The unsaturated lipid **5** was also found to be efficacious in MCF-7 cells, although we observed greater deviation in the measurement of transgene expression at the higher N:P ratio using this lipid (Figure 4a). We did not observe an additional boost in transfection activity on further amplifying the hydrophobic domain asymmetry by using dissymmetric lipid **4**, relative to the activities of lipids **2** and **5** in this cell line.

On a scale of 1 μg DNA per well, transfection of MCF-7 cells in a six-well format using lipoplexes derived from lipid **3** at an N:P ratio of 7:1 gave approximately a 70-fold higher luciferase expression (as assessed by RLU per μg protein) than lipofectamine 2000-derived lipoplexes (see Supporting Information). This result shows that the oxime ether lipid **3** is effective over a wide range of DNA doses.

Only lipids **4** and **5** led to high transgene expression in H1792 cells (Figure 4b). In agreement with previous studies on lung cell transfections,[14,39] the presence of unsaturation in the hydrophobic domain, such as is present in lipid **5**, proved to be beneficial for activity. Lipoplexes derived from lipid **5** effectively transfected H1792 cells relative to the commercial controls (> 60-fold higher activity at N:P = 7). Interestingly, the enhanced asymmetry of lipid **4** resulted in improved transfection of this cell line relative to the other saturated lipids examined.

To further examine the gene-transfer capability of lipids with an oxime ether linker domain, we used a green fluorescent protein (GFP) reporter vector to estimate the transfection efficiency in MCF-7 cells (Figure 5). Examination of cells by fluorescence microscopy using an EVOS fluorescent microscope de-

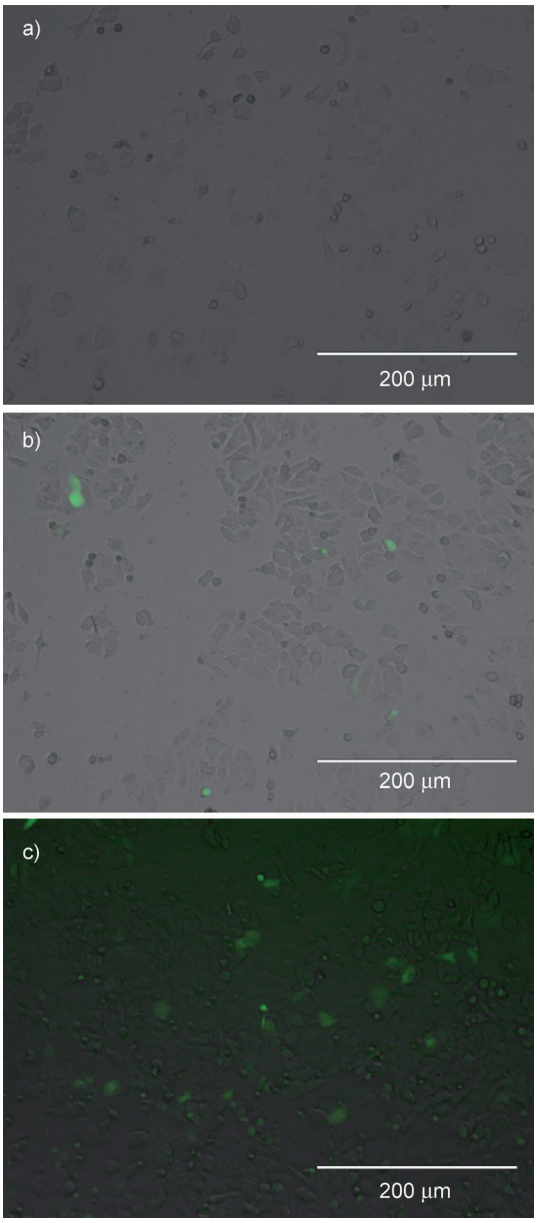


Figure 5. MCF-7 cells were transfected with a GFP expression plasmid (0.1 μg per well in a 24-well plate). Figure are presented as an overlay of green fluorescence (visualized by fluorescent microscopy after 20 h of transfection) over the phase contrast image: a) cells treated with pDNA (negative control); b) cells transfected with lipoplexes derived from lipofectamine 2000 (positive control; vendor-recommended dose = 0.25 μL); c) cells transfected with lipoplexes derived from lipid **3** at N:P = 7.

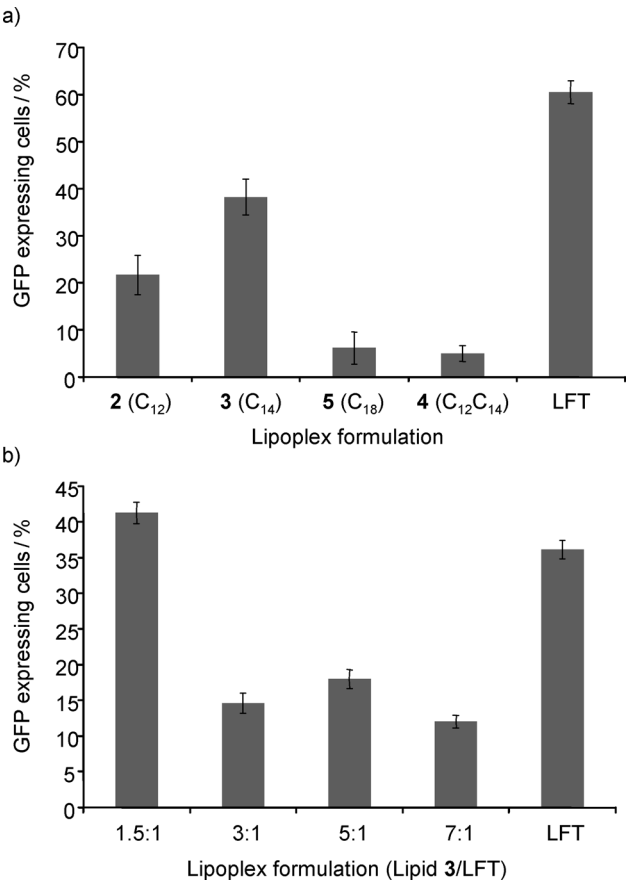


Figure 6. GFP expression in PAR C10 cells. a) Cells were transfected (0.2 μg GFP reporter construct) with lipoplex formulations derived from lipids **2**–**5** (N:P = 1.5) and excess lipofectamine 2000 (LFT; positive control; 1.6 μL); b) Comparison of lipid **3** formulations at the indicated N:P ratio with LFT at the vendor-recommended dose (0.5 μL). The transfection percentage of GFP expressing cells was analyzed by flow cytometry 48 h post-transfection. Each data point reflects the mean value ± standard deviation (SD) of three independent experiments.

termined that the results were in agreement with those of the luciferase assay.

MCF-7 and H1792 cells are both cancer cell lines. To test the lipids in a noncancer epithelial cell line, GFP was expressed in the salivary gland cell line PAR C10.[40] Lipoplexes were formulated at an N:P ratio of 1.5, the optimal ratio determined for lipids **2**–**5** in this cell line. GFP expression was evaluated 48 h post-transfection. The fraction of transfected cells was quantitated by flow cytometry. Lipid **3** transfected approximately 40% of the cells, lipid **2** transfected about 20%, while lipids **4** and **5** exhibited less than 10% cell transfection (Figure 6a). Lipofectamine was slightly more effective in this cell line when used in excess (>3× recommended dose; Figure 6a) and comparable to lipid **3** when used at the vendor-recommended dose (Figure 6b). These results suggest that lipids with an oxime ether linker domain are suitable for the pDNA transfection of epithelial cell lines and, depending on hydrophobic domain composition, competitive with the transfection standard lipofectamine.

Transfection with siRNA

In addition to pDNA transfection, we tested the ability of lipid **3** to transfect epithelial cells with siRNA. The cellular "housekeeping" gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as a target, and transfection efficiency was determined by measuring the reduction in GAPDH activity. Lipid **3** reduced GAPDH activity by 55 ± 9% (mean ± SEM), while the control lipid, lipofectamine RNAiMAX, reduced GAPDH activity by 71 ± 6% ($n = 9$). This difference was not stat-

istically significant ($p = 0.23$). These results suggest that lipid **3** effectively introduces siRNA into epithelial cells.

Cytotoxicity measurements

To measure the cytotoxicity of the lipids in MCF-7 and H1792 cells, we performed cell viability studies using the vital dye trypan blue to stain dead cells. The number of live cells remaining after treatment was calculated as a percentage over the untreated control (Figure 7). Our lipids were found to be less toxic than lipofectamine and jetPrime in both cell lines, with cell viability comparable to untreated cells at all N:P formulations examined.

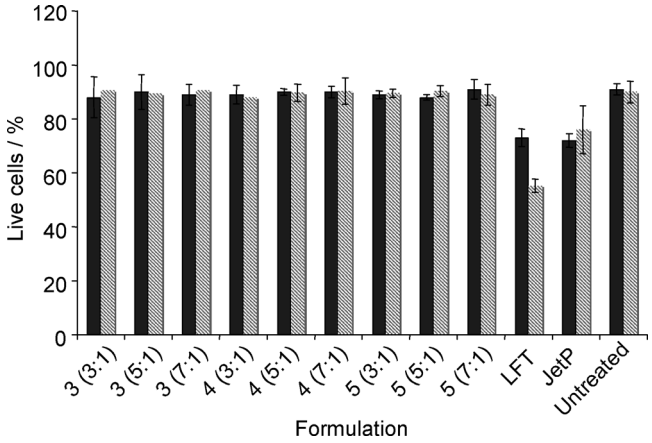


Figure 7. Relative cytotoxicity. MCF-7 (■) and H1792 cells (■) were transfected using either lipofectamine 2000 (LFT), jetPrime (jP) or oxime ether lipoplexes at the specified N:P ratio (value in parenthesis). Transfections were performed in a 96-well format. Dead and live cells were counted 18 h post-transfection using a trypan blue stain ($n = 3$).

For PAR C10 cells, the viability was determined by reduction of alamar blue (Figure 8). None of the lipids reduced cell viability significantly compared with untreated cells, confirming the low toxicity of lipids with an oxime ether linker domain.

Conclusions

We have demonstrated that the facile aminooxy carbonyl click reaction can be used to rapidly assemble transfection lipids furnished with asymmetric hydrophobic domains. The data show that lipids containing an oxime ether linking domain are well tolerated by both cancerous and noncancerous epithelial cells. Furthermore, with appropriate tuning of hydrophobic domain composition and N:P formulation, lipoplexes derived from oxime ether lipids readily transfect representative epithelial cell lines with excellent activity relative to established transfection agents. The present study shows that oxime ethers are a suitable choice for linker domain. Given the biological compatibility of oxime ethers and their ease of formation, this functional group should find much application in the future design of transfection vectors.

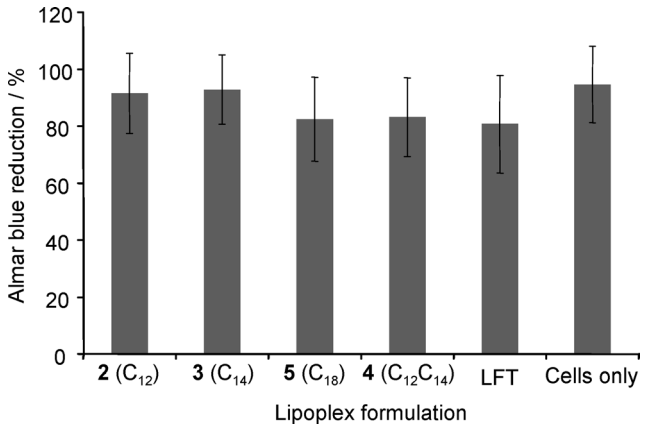


Figure 8. Cell viability assay. PAR C10 cells were transfected (N:P = 1.5) with lipoplex formulations derived from lipids **2**–**5** and lipofectamine 2000 (LFT; 1.6 μL). After 48 h, the cell viability was measured by calculating the percentage reduction of alamar blue dye. The data are shown as the mean value ± standard deviation (SD) of three independent experiments.

The ability to transfect epithelial cells could have clinical applications, as epithelial surfaces are readily accessible and attrative targets for gene therapy protocols that aim to express theraputic proteins in vivo.[41] The ability to simultaneously transfect pDNA and siRNA with a single reagent now opens potential applications to express therapeutic proteins in cells that are simultaneously modified by gene knockdown.

Experimental Section

Chemistry

All ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded in CD_3OD (Varian 400 MR NMR spectrometer). High-resolution mass spectrometry (HRMS) was performed by the CREAM facility at the University of Louisville (Kentucky, USA) using electrospray ionization (ESI) (Thermo LTQ-FT). Lipid **3** was prepared as previously described.[35]

***N*,*N*-Dimethyl-*N*-2-((dodecylideneamino)oxy)-(2-((dodecylideneamino)oxy)ethyl)ethanaminium iodide (2)**: Compound **1** (26.5 mg, 0.091 mmol) and dodecanal (35.4 mg, 0.19 mmol) were dissolved in MeOH (3 mL) and stirred at RT. After 3.5 h, the solvent was removed in vacuo, and the residue was purified by column chromatography (SiO_2; MeOH:CH_2Cl_2, 3%) to afford oxime ether lipid **2** as a white solid (34.6 mg, 61%; (*E*, *E*):(*E*, *Z*), 5.3:1): $R_f = 0.52$ (CH_2Cl_2/MeOH, 9:1); mp: 155 °C (dec); ^{1}H NMR (400 MHz, CD_3OD): (major diastereomer) $\delta = 0.86$ (t, $J = 6.8$ Hz, 6H), 1.20–1.39 (m, 28H), 1.43–1.48 (m, 4H), 2.17 (dd, $J = 6.8$, 7.2 Hz, 4H), 3.20 (s, 6H), 3.73–3.75 (m, 4H), 4.40–4.41 (m, 4H), 7.46 ppm (t, $J = 6.0$ Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD): $\delta = 14.6$, 23.9, 27.1, 27.3, 27.7, 30.4, 30.6, 30.9, 33.2, 53.4, 64.9, 68.0, 68.3, 155.0, 155.5 ppm; IR (neat): $\tilde{\nu} = 1735$, 1468, 966, 920 cm^{-1}; HRMS: *m/z* $[M]^+$ calcd for $C_{30}H_{62}N_3O_2^+$: 496.4837, found: 496.4839.

***N*,*N*-Dimethyl-*N*-2-((dodecylideneamino)oxy)-(2-((tetradecylideneamino)oxy)ethyl)ethanaminium iodide (4)**: Compound **1** (22.6 mg, 0.078 mmol) and dodecanal (7.2 mg, 0.034 mmol) were dissolved in MeOH (1 mL) and stirred at RT. After 14 h, the solvent was removed in vacuo, and the residue was purified by column chromatography (SiO_2; MeOH:CH_2Cl_2, 5%) to afford the intermedi-

ate mono-oxime ether as a yellow oil (14.0 mg, 39%; (*E*, *E*):(*E*, *Z*), 1.7:1): $R_f = 0.25$ (CH_2Cl_2/MeOH, 9:1); ^{1}H NMR (400 MHz, CD_3OD): (major diastereomer) $\delta = 0.85$ (t, $J = 6.8$ Hz, 3H), 1.20–1.38 (m, 16H), 1.45 (dd, $J = 6.4$, 6.8 Hz, 2H), 2.16 (dd, $J = 6.4$, 7.4 Hz, 2H), 3.17–3.20 (m, 6H), 3.65–3.74 (m, 4H), 4.04 (m, 2H), 4.40 (m, 2H), 7.45 ppm (t, $J = 6.0$ Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD): $\delta = 14.6$, 23.9, 27.1, 27.3, 27.6, 30.4, 30.6, 30.8, 30.9, 33.2, 53.3, 64.7, 68.0, 68.4, 70.0, 134.0, 155.0, 155.5 ppm; HRMS: *m/z* $[M]^+$ calcd for $C_{18}H_{40}N_3O_2^+$: 330.3115, found: 330.3118.

The C_{12} mono-oxime ether intermediate (13.1 mg, 0.029 mmol) and tetradecanal (9.1 mg, 0.043 mmol) were dissolved in MeOH (1 mL) and stirred at RT. After 14 h, the solvent was removed in vacuo, and the residue was purified by column chromatography (SiO_2; MeOH:CH_2Cl_2, 3%) to afford unsymmetrical lipid **5** as a yellow oil (13 mg, 69%; (*E*, *E*):(*E*, *Z*), 2.3:1): $R_f = 0.48$ (CH_2Cl_2/MeOH, 9:1); ^{1}H NMR (400 MHz, CD_3OD): (major diastereomer) $\delta = 0.84$ (t, $J =$ 6.8 Hz, 6H), 1.23–1.26 (m, 36H), 1.44 (dd, $J = 6.8$, 7.2 Hz, 4H), 2.15 (dd, $J = 6.4$, 7.2 Hz, 4H), 3.18 (s, 6H), 3.69–3.72 (m, 4H), 4.38–4.40 (m, 4H), 7.44 ppm (t, $J = 6.2$ Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD): $\delta = 14.6$, 23.9, 27.1, 27.3, 27.6, 30.4, 30.6, 30.7,.30.8, 30.9, 33.2, 53.3, 64.9, 68.0, 68.4, 155.0, 155.5 ppm; IR (neat): $\tilde{\nu} = 1728$, 1467, 964, 942 cm^{-1}; HRMS: *m/z* $[M]^+$ calcd for $C_{32}H_{66}N_3O_2^+$: 524.5150, found: 524.5172.

***N*,*N*-Dimethyl-*N*-2-((((*Z*)-octadec-9-en-1-ylidene)amino)oxy)-(2-((((*Z*)-octadec-9-en-1-ylidene)amino)oxy)ethyl)ethanaminium iodide (5)**: Compound **1** (19.7 mg, 0.068 mmol) and *cis*-9-octadecenal (37.8 mg, 0.142 mmol) were dissolved in MeOH (3 mL) and stirred at RT. After 3.5 h, the solvent was removed in vacuo, and the residue was purified by column chromatography (SiO_2; MeOH:CH_2Cl_2, 3%) to afford oxime ether lipid **4** as a yellow oil (45.8 mg, 90.0%; (*E*, *E*):(*E*, *Z*), 3.0:1): $R_f = 0.51$ (CH_2Cl_2/MeOH, 9:1); ^{1}H NMR (400 MHz, CD_3OD): (major diastereomer) $\delta = 0.84$ (t, $J =$ 6.8 Hz, 6H), 1.23–1.27 (m, 40H), 1.42–1.47 (m, 4H), 1.96–1.97 (m, 8H), 2.15 (dd, $J = 6.4$, 7.2 Hz, 4H), 3.16 (s, 6H), 3.68–3.71 (m, 4H), 4.38–4.39 (m, 4H), 5.27–5.29 (m, 4H), 7.43 ppm (t, $J = 6.0$ Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD): $\delta = 14.6$, 23.9, 27.6, 28.3, 30.4, 30.5, 30.6, 30.8, 31.0, 33.2, 53.3, 64.9, 68.0, 130.9, 131.1, 155.0 ppm; IR (neat): $\tilde{\nu} = 1730$, 1464, 964, 942 cm^{-1}; HRMS: *m/z* $[M]^+$ calcd for $C_{42}H_{82}N_3O_2^+$: 660.6401, found: 660.6395.

Zeta potential and particle size: Measurements of liposome and lipoplex zeta potential and particle size were performed using a ZetaPALS dynamic light scattering detector (Brookhaven Instruments Corporation; Model 90 Plus). All measurements were taken in water. Lipoplexes were formulated at a final concentration of 0.1 mg oxime ether lipid per mL water. To formulate lipoplexes at different N:P ratios, 100 μL of the liposome formulation was added to the required quantity of pDNA (taken from a stock solution of 0.1 mg pDNA per mL) in an Eppendorf tube. After incubating each lipoplex for 15 min, the solutions were diluted to 1 mL by adding ultra-pure water. Measurements were taken within minutes of formulation.

Biology

Tissue culture: Human breast cancer cells (MCF-7) and lung tumor cells (H1792) were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were grown to 50–60% confluency prior to transfection. MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA), 1% Pennstrep (cat. no.: 30-002-CI, Mediatech Inc., Manassas, VA, USA), 10% fetal bovine serum (FBS; Valley Biomedical, Winchester, VA, USA). H1792 cells were cultured in RPMI (Invitrogen), 1% Pennstrep, 10% FBS.

The PAR C10 cell line was immortalized from rat parotid acinar cells.[40] Experiments were performed on cells of passage number 40–60. The cultures were grown to 50–60% confluency in DMEM/Ham's F12 media (1:1; GIBCO BRL, Gaithersburg, MD, USA) containing 2.5% (*v/v*) FBS (GIBCO BRL, Gaithersburg, MD, USA) and the following supplements: 0.1 μM retinoic acid, 80 ng mL^{-1} epidermal growth factor, 2 nM triiodothyronine, 5 mM glutamine, 0.4 μg mL^{-1} hydrocortisone, 5 μg mL^{-1} insulin, 5 μg mL^{-1} transferrin, 5 ng mL^{-1} sodium selenite and 50 μg mL^{-1} gentamicin (all from Sigma, St. Louis, MO, USA). Cells were cultured at 37 °C in a humidified atmosphere of 95% air/5% CO_2.

Liposome formulation: An equimolar amount of DOPE from a stock solution (10 mg DOPE per mL $CHCl_3$) was added to a solution of oxime ether lipid (1 mg) in $CHCl_3$ (0.20 mL). The solvent was evaporated, and the resultant thin lipid film was dried under vacuum (4 h). Ultra-pure water (3 mL) was added to the dry lipid film, and the suspension was sonicated (bath sonicator) for 1 min at RT to furnish the liposome formulation (0.33 mg oxime ether lipid per mL).

Luciferase expression: Luciferase transfections in MCF-7 and H1792 cells were performed in triplicate using 0.025 μg of pDNA (pCMV-Luc) per well. Cells were seeded up to 1×10^5 cells per well in a 24-well plate to give 50–60% confluency, and 400 μL of media containing 10% FBS was added to each well. Lipoplexes (lipid–pDNA complex) were prepared at N:P charge ratios of 3, 5 and 7 by adding the required volume of liposome solution to a pDNA solution (3 μL, 0.025 μg DNA per μL) and incubated for 5 min. Serum-free DMEM (100 μL) was then added to each lipoplex solution followed by incubation for 20 min at RT with occasional gentle vortex mixing. The lipoplex solutions were diluted to 600 μL with serum-free DMEM, and then an aliquot of the final lipoplex formulation (200 μL) was added directly to each well. No additional media containing FBS was added to the cells during transfection. After 18 h incubation at 37.5 °C, the cells were lysed and luciferase expression was quantified using a commercial kit (Promega Corp., Madison, WI, USA) and luminometer according to the vendor's protocol. Lipofectamine 2000 (Invitrogen) and jetPrime (Polyplus Transfection SA, Illkirch, France) lipoplexes were prepared at the vendor-recommended stoichiometry (2 μL stock per μg DNA).

GFP expression: Transfection studies were performed using a GFP reporter gene in MCF-7 cells using 0.1 μg of pEGFP DNA (Clontech Laboratories Inc., Madison, WI, USA) per well in a 24-well plate with a similar transfection protocol as described for the luciferase assays. Cells were examined 40 h post-transfection by fluorescence microscopy (EVOS, Advanced Microscopy Group, Bothell, WA, USA) and photographed.

GFP expression also was assessed in PAR C10 cells using the plasmid pZsGreen1-N1 (Clontech). Transfection of PAR C10 cells proceeded by plating the cells at a density of 5×10^4 cells cm^{-2} in 24-well plates in complete medium (described above) with pDNA (200 ng per well). Lipolexes were formed at an N:P charge rato of 1.5 in 200 μL of complete medium containg 2.5% FBS. Media was removed, and cells were transfected with lipoplexes. After 3 h, each well was replenished with 250 μL of complete media. Lipofectamine was used as a positive control. After 48 h, GFP-expressing cells were analyzed using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and CellQuest software (version 3.3).

Cytotoxicity assay: Lipid cytotoxicity was evaluated using the vital dye trypan blue to distinguish between live and dead cells. MCF-7 and H1792 cells were seeded at 1×10^4 cells per well and then grown to 50–60% confluency in 96-well plates. Transfections were performed as described above. The media was removed 18 h post-transfection. Cells were then trypsinized by adding 100 μL trypsin to each well and incubated for 5 min at 37.5 °C. Trypsin was neutralized by adding 1X phosphate-buffered saline (1X PBS), and then 10 μL of extract from each well was mixed with 10 μL of trypan blue. Dead and live cells were counted under an inverted microscope to determine the percentage cell viability using a hemocytometer.

Cell viability in PAR C10 cells was assessed using the alamarBlue cell viability reagent (Invitrogen). The PAR C10 cells were transfected as described above using lipoplexes formulated from lipids **2**–**5** at the optimal N:P charge ratio of 1.5, and lipofectamine at 1.6 μL. At 48 h post-transfection, the viability assay was performed as per the manufacturer's protocol. Absorbance was measured at 600 nm and 570 nm using a Synergy HT multimode microplate reader (Bio-Tek, Winooski, VT, USA). The reduction of alamar blue is proportional to the proliferation of cells and is expressed as a percentage.

Acknowledgements

We thank the US National Institutes of Health (NIH) (5R21DE019271, 1P20 RR18733) for support of this work. R.J.K. thanks the University of Louisville (Kentucky, USA) for a graduate research fellowship. We also thank Dr. Olga Baker (University at Buffalo, New York, USA) for donating PAR C10 cells, Mr. Xu Han and Dr. Mahsa Abdolhosseini (University of Minnesota, Minneapolis, USA) for contributions to the PAR C10 experiments.

Keywords: aminooxy salts · gene transfer · nonviral vectors · oximation · transfection agents

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Received: May 25, 2011
Published online on August 31, 2011

