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A Flexible Method for the Conjugation of Aminooxy Ligands to Preformed Complexes of Nucleic Acids and Lipids

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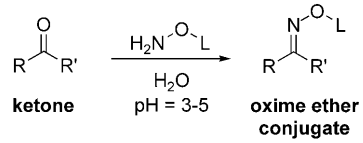
Attachment of targeted ligands to nonviral DNA or RNA delivery systems is a promising strategy that seeks to overcome the poor target selectivity generally observed in systemic delivery applications. Several methods have been developed for the conjugation of ligands to lipids or polymers, however, direct conjugation of ligands onto lipid– or polymer–nucleic acid complexes is not as straightforward. Here, we examine an oximation approach to directly label a lipoplex formulation. Specifically, we report the synthesis of a cationic diketo lipid DMDK, and its use as a convenient ligation tool for attachment of aminooxy-functionalized reagents after its complexation with DNA. We demonstrate the feasibility of direct lipoplex labeling by attaching an aminooxy-functionalized fluorescent probe onto pre-formed plasmid DNA–DMDK lipoplexes (luciferase, GFP). The results reveal that DMDK protects DNA from degradation on exposure to either DNase or human cerebral spinal fluid, and that simple mixing of DMDK lipoplexes with the aminooxy probe labels the complexes without sacrificing transfection efficiency. The biocompatibility and selectivity of this method, as well as the ease of bioconjugation, make this labeling approach ideal for biological applications.

Introduction

Since Felgner's seminal publication on cationic lipid-mediated gene transfer,[1] considerable effort has been devoted to improving nonviral methods of gene delivery to mammalian cells.[2–4] These efforts have intensified as the limitations of using viral vectors have become better understood.[5,6] Cationic lipid-mediated transfection has a number of advantages over viral vectors, most notably low immunogenicity, and the ability to transfect vectors of nearly unlimited size.[3,7] Furthermore, facile preparation of a cationic lipid–plasmid DNA or RNA complex (lipoplex), and the protection from DNase I or RNase digestion afforded by lipoplex formulation,[8,9] continue to drive the development of new cationic lipids. Recent advances in the field include directing lipoplexes to particular cell types via targeting strategies.[10,11] The attachment of a targeting element, such as a peptide fragment[12] or antibody,[13] to a lipid followed by its incorporation into a lipoplex formulation is one means of targeting lipoplexes.[14] However, this strategy is highly dependent on the ability of the labeled lipid to incorporate into the lipoplex without compromising the formation and stability. If a labeled lipid interferes with efficient compaction of DNA or RNA, then lipoplex disassembly and enzymatic digestion may become problematic. Our interests in lipid-mediated gene delivery into the DNase and RNase rich cerebral spinal fluid (CSF) and in the brain[15,16] led us to develop a versatile method for directly labeling preformed lipoplexes. Herein, we report the synthesis and application of a new cationic keto-lipid, DMDK (**5**), exploiting the aminooxy-ketone ligation reaction (Scheme 1) as a convenient tool for directly labeling lipoplexes. A commercially available, aminooxy-functionalized, fluorescent probe (Alexa Fluor 488, Invitrogen, Carlsbad, CA) was attached to a preformed plasmid DNA–DMDK lipoplex as a representative example of this new labeling approach. Transfection efficacy was demonstrated using DNA vectors that express luciferase and GFP.



Scheme 1. Chemoselective ligation (oximation) of an aminooxy-functionalized ligand (**L**) to a ketone under aqueous conditions.

Results

DMDK synthesis

N-Benzyl-*N*-methylamino acetone (**1**) was prepared according to a literature protocol[17] and was reacted immediately with triethylorthoformate in ethanol to give the corresponding diethyl acetal. Subsequent hydrogenolysis of the benzyl group gave amino acetal **2**. Following the diepoxide cleavage procedure established in the synthesis of the transfection lipid

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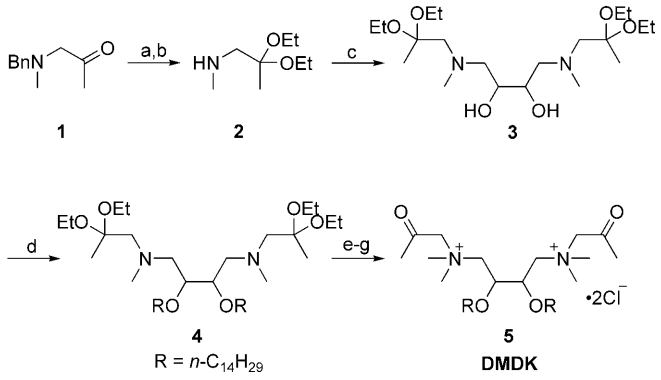
Tfx,[18,19] we reacted (±)-1,3-butanediepoxide (Sigma–Aldrich) with **2** in the presence of lithium perchlorate to obtain bis-adduct **3**. As expected,[20] the dialkylation of **3** was sluggish; however, prolonged reaction of the diol with NaH and myristyl iodide in a DMF/HMPA mixture gave diether **4** in modest yields. Acetal hydrolysis, amine quaternization and counterion exchange (I to Cl) afforded the dimyristyl diketo lipid DMDK (**5**) (Scheme 2).



Scheme 2. Synthesis of keto-lipid DMDK. Reagents and conditions: a) EtOH, $(EtO)_3CH$, *p*-TsOH, reflux, 8 h, 65%; b) H_2, Pd/C, EtOH, 60 h, 68%; c) 1,3-butanediepoxide, $LiClO_4$, EtOH, 55 °C, 13 h, 78%; d) i: NaH, HMPA, DMF, 0 °C to RT, 1 h, ii: $CH_3(CH_2)_{13}I$, DMF, 0 °C to RT, 2.5 d, 57%; e) *p*-TsOH, acetone, RT, 5 h; f) MeI, RT, 16 h; g) Dowex 1X8-400 (chloride exchange resin), 83% (3 steps).

DNA protection assays

To demonstrate that the novel diketo lipid DMDK (**5**) could protect plasmid DNA from degradation by DNase, or in human cerebral spinal fluid (hCSF), we carried out experiments similar to those previously described for other lipids.[16] Briefly, luciferase- or GFP reporter protein-expressing plasmid DNA (pND.Luc and pND.eGFP, respectively) was formulated as a nucleic acid lipoplex using established methods,[15,16] and then incubated with commercially available DNase, or in hCSF in vitro. Following incubation, DNA was recovered from the lipoplex using a phenol/chloroform extraction procedure, and run on an agarose gel to confirm intact DNA of an appropriate size (Figure 1),

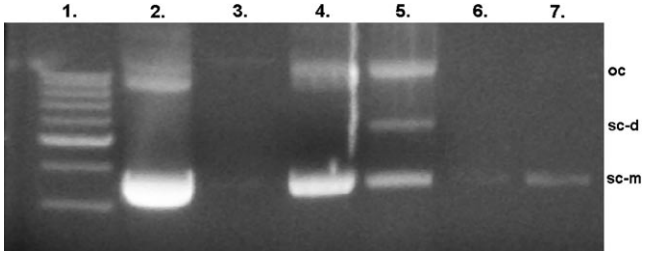


Figure 1. Agarose gel showing stability of the pND.Luc–DMDK lipoplex after incubation with DNase, and in hCSF in vitro. Lane 1: DNA ladder. Lane 2: pND.Luc alone. Lane 3: pND.Luc treated with RQ1 RNase-free DNase. Lane 4: pND.Luc isolated from pND.Luc–DMDK lipoplex. Lane 5: pND.Luc isolated from pND.Luc–DMDK lipoplex after exposure to RQ1 RNase-free DNase. Lane 6–7: Organic (Lane 6) and aqueous (Lane 7) phenol/chloroform extraction phases from pND.Luc–DMDK lipoplex following incubation in hCSF.

or was used for transfection to demonstrate that functional protein could still be transcribed and translated.

The agarose gel shown in Figure 1 clearly shows the DNA protective properties of the lipoplex (Lanes 5–7) compared to controls (Lanes 1–4). Naked DNA (pND.Luc) was completely degraded by DNase (Lane 3) or recovered intact from the untreated pND.Luc–DMDK lipoplex (Lane 4), however, enzymatic degradation did not occur when the pND.Luc–DMDK lipoplex was incubated with DNase (Lane 5) or incubated in hCSF (Lane 7).

Following DNase inactivation, aliquots of the pND.luc–DMDK lipoplex incubated with DNase (Lane 5) or incubated in hCSF (Lane 7) were used to transfect Chinese hamster ovary (CHO) cells. Using an enhanced luciferase assay (BD PharMingen), cells transfected with pND.luc–DMDK without DNase treatment emitted 22×10^6 (22,030,216) Relative Light Units (RLUs), while cells transfected with pND.luc–DMDK incubated with DNase emitted 27×10^6 (27,023,812) RLUs, and cells transfected with pND.luc–DMDK incubated in hCSF emitted 12.4×10^6 (12,367,732) RLUs.

Transfection using the DNA–DMDK–Alexa Fluor 488 conjugate

DNA–DMDK lipoplexes were labeled with a fluorescent probe (Alexa Fluor 488) under the appropriate conditions. CHO cells were transfected with the DNA–DMDK–Alexa Fluor 488 conjugate to demonstrate that transfection efficacy was not impaired. Again, using an enhanced luciferase assay, CHO cells transfected with pND.luc–DMDK (positive control) were compared to CHO cells transfected with the DNA–DMDK–Alexa Fluor 488 conjugate under the same conditions. An average luminescence of 30×10^6 ($29{,}984{,}445 \pm 327{,}712$, $n = 3$) RLUs was measured for the positive control (pND.luc–DMDK), and 30×10^6 ($30{,}227{,}672 \pm 7800$, $n = 3$) RLUs (DNA–DMDK–Alexa Fluor 488 conjugate) clearly showing efficient enzyme transcription/translation.

Fluorescence imaging of the DNA–DMDK–Alexa Fluor 488 conjugate

Figure 2 shows the imaging results of CHO cells transfected with the DNA–DMDK–Alexa Fluor 488 conjugate. Figure 2A shows a fluorescence image, while Figure 2B shows a Brightfield image of the same CHO cells. An overlay of these two images (Figure 2C) clearly shows that fluorescence, and thus DNA–DMDK–Alexa Fluor 488 conjugate, is localized in the cells. Figure 3 shows CHO cells transfected with Alexa Fluor 488 hydroxylamine only; there is no uptake of label in the absence of the lipoplex (Figure 3A), further confirmed by the brightfield image of the same cells (Figure 3B).

Discussion

We have described a method for the direct labeling of a lipoplex formulation by simple mixing of the DNA–lipid complex with an aminooxy-functionalized reagent. This same lipid and labeling procedure also can be used to form RNA lipoplexes

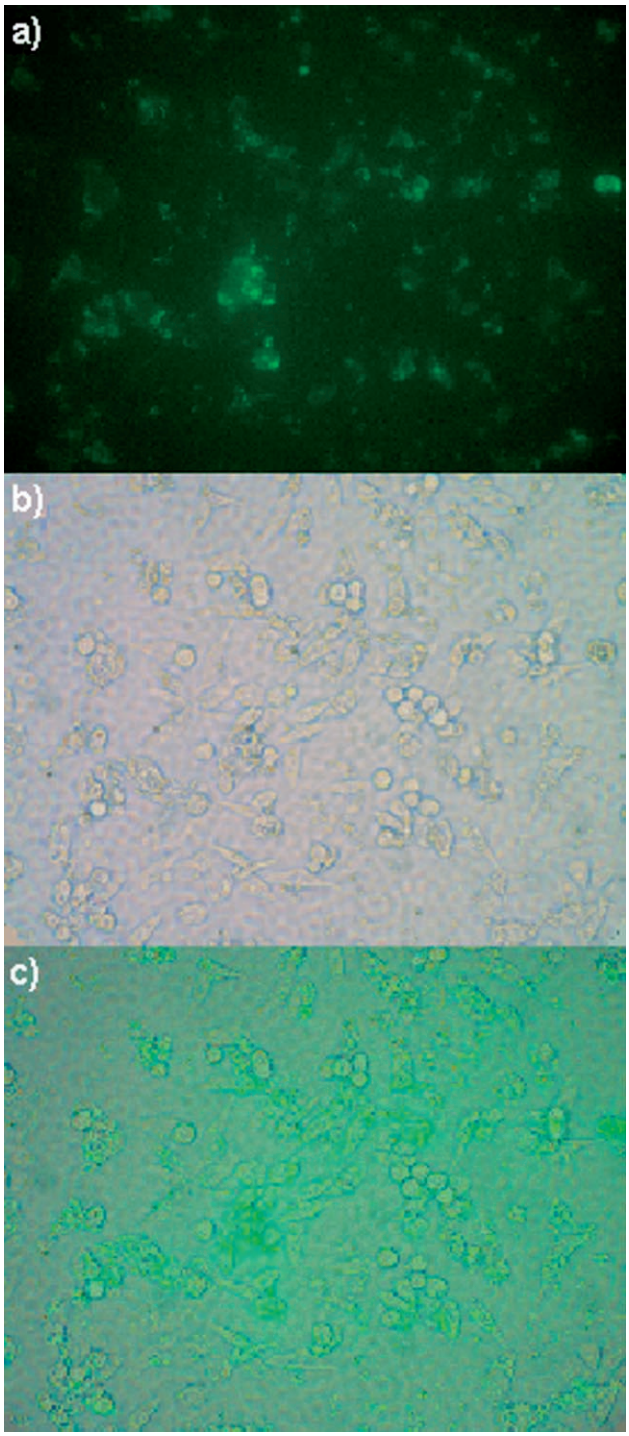


Figure 2. a) Fluorescent image of CHO cells transfected by DNA–DMDK–Alexa Fluor 488 conjugate. b) Brightfield image of cells shown in 2a; c) image overlay of 2a and 2b.

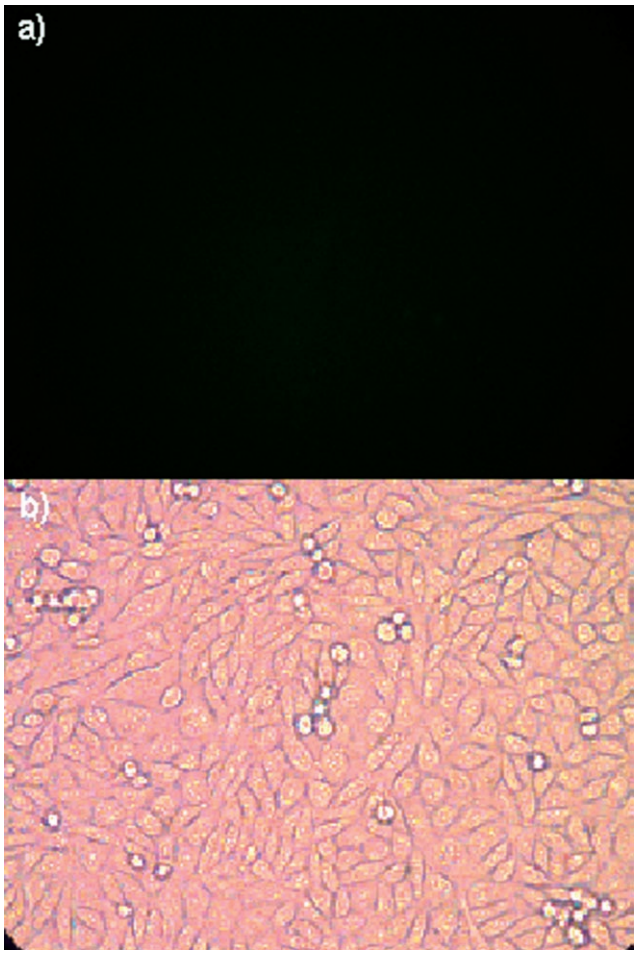


Figure 3. a) Negative control; CHO cells transfected with Alexa Fluor 488 hydroxylamine only; b) Brightfield image of CHO cells in 3a, showing cell density for comparison.

with mRNA,[16] siRNA, shRNA, or microRNA, for RNA applications. The labeling procedure is straightforward, requiring only a brief incubation period under mildly acidic conditions (pH 5), and the resulting labeled lipoplex formulation can be directly transfected into cells without an intermediate purification step. This demonstration is an important step in the development of lipoplex formulations as the need for versatile methods of labeling and/or targeting lipid–DNA or lipid–RNA complexes[21] continues to increase.

Our approach relies on chemoselective oximation of the ketone by an aminooxy ($RONH_2$) reagent under aqueous conditions to give the corresponding oxime ether conjugate (Scheme 1). The biocompatibility and selectivity of this process, and the ease of bioconjugation, make this ligation reaction ideal for biological applications. Oximation of aldehydes or ketones by aminooxy agents has previously been used for bioconjugation, such as ligations of keto-peptides[22] or keto-proteins[23] with aminooxy-carbohydrates, aldo-proteins with aminooxy-poly(ethylene glycol),[24] and keto-carbohydrates with aminooxy-polymers[25] or biotin.[26] Syntheses of aminooxy-[27] and ketone-oligonucleotides[28] have also been developed to exploit the complimentary reactivity of these functional groups. However, this ligation approach had not previously been examined for lipoplex labeling purposes.

To examine the feasibility of direct lipoplex labeling, a short synthesis of the cationic diketo-lipid DMDK (**5**) was designed, closely following the strategy used for transfection lipid Tfx preparation.[18] With DMDK in hand, two crucial questions were next addressed: a) does this lipid bind DNA and afford protec-

tion against DNase digestion?; b) is DMDK capable of mediating gene transfer?

The DNA protection assays show that plasmid DNA is protected from degradation by DNase or in hCSF when conjugated to DMDK, as previously shown for RNA.[16] The agarose gel results (Figure 1) confirm DNA protection. Lane 5 clearly shows intact DNA, with bands corresponding to the oc-form (oc) and ccc-supercoiled monomer form (sc-m) of DNA seen in the control (Lane 4). The third band, seen only in Lane 5, probably represents a ccc-supercoiled dimer form of DNA (sc-d). The ccc plasmid topology is the most compact structure and is expected to be the most active topology. The ccc-supercoiled form represents intact and undamaged DNA. If one strand is nicked, the oc form results. Linear forms are generated if both strands are cleaved at approximately the same position.[29] Lanes 6 and 7 show the extracted DNA of pND.luc–DMDK following incubation in hCSF (organic and aqueous phases, respectively); only single bands corresponding to supercoiled DNA can be seen with weaker intensities compared with the control (Lane 4) or pND.luc–DMDK incubated with DNase (Lane 5). This is consistent with the luciferase assay results, which suggest that although functional DNA is clearly present after both incubation with DNase and in hCSF, more degradation occurs in hCSF at the concentration used. The luciferase assay data is self-explanatory and shows that DNA is protected from DNase degradation and can be extracted in an undamaged state that can be used for transfection.

We have shown that the novel cationic keto-lipid DMDK can be easily labeled with a commercially available probe, such as Alexa Fluor 488, without sacrificing transfection efficiency. This methodology can be used to determine the transfection efficiency in both in vitro and in vivo studies, which otherwise may require cotransfection of the gene of interest with a reporter gene such as luciferase or GFP, or the formation of a fusion protein, requiring more time for the synthesis of additional vectors, inefficient cotransfection, and further imaging.

In conclusion, this study confirms that direct labeling of a preformed lipoplex can be conveniently accomplished using oximation chemistry. The wide availability of radioisotopes, imaging contrast agents, targeting moieties, and chemotherapeutics fitted with aminooxy groups suggests keto-lipids, such as DMDK, may be used to enable a variety of potentially powerful applications in gene transfer.

Experimental section

Chemistry

All ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded in $CDCl_3$. High resolution mass spectrometry was performed by the Mass Spectrometry Service Laboratory of the University of Minnesota (Minneapolis). Elemental analyses were performed by Midwest Microlabs (Indianapolis).

Amino Acetal (2): A solution of **1** (13.5 g, 76.1 mmol) in 1,1,1-triethoxymethane and EtOH (1:1, 120 mL) was treated with *p*-TsOH (23.0 g, 121 mmol) and heated to reflux (8 h). The reaction mixture was cooled to RT, diluted with Et_2O (300 mL) and washed successively with saturated aq Na_2CO_3 (5×), water and brine. The organic phase was then dried (Na_2SO_4), filtered and concentrated in vacuo. Purification by flash chromatography (SiO_2, hexane/EtOAc, 7:1) gave the diethyl acetal as a colorless oil (12.5 g, 65%); R_f = 0.26 (hexane/EtOAc, 7:1); ^{1}H NMR δ = 7.20-7.36 (m, 5H), 3.58 (s, 3H), 3.49 (q, J = 4.0 Hz, 4H), 2.54 (s, 2H), 2.27 (s, 3H), 1.41 (s, 3H), 1.15 ppm (t, J = 4.0 Hz, 6H); ^{13}C NMR δ = 139.8, 128.9, 128.0, 126.7, 102.0, 63.4, 61.7, 55.5, 43.5, 21.7, 15.4 ppm; Anal. calcd for $C_{15}H_{25}NO_2$: C, 71.67; H, 10.02; N, 5.57. Found: C, 71.57; H 9.94; N, 5.45.

10% Pd/C (0.72 g) was added to a solution of the diethyl acetal (18.1 g, 72.0 mmol) in EtOH (60 mL) at RT, and the suspension was placed under an H_2 atmosphere and stirred (60 h). The reaction mixture was filtered through a pad of celite and concentrated by distillation. Further distillation of the crude under reduced pressure (bp 32-34 °C, 2.5 mm Hg) gave amino acetal **2** (7.96 g, 68%); ^{1}H NMR δ = 3.42-3.56 (m, 4H), 2.67 (s, 2H), 2.46 (s, 3H), 1.39 (s, 3H), 1.18 ppm (t, J = 7.2 Hz, 6H); ^{13}C NMR δ = 100.5, 57.3, 55.5, 36.8, 21.5, 15.3 ppm; Anal. calcd for $C_8H_{19}NO_2$: C, 59.59; H, 11.88; N, 8.69. Found: C, 59.24; H, 11.95; N, 8.53.

Diol (3): A solution of **2** (7.64 g, 47.4 mmol) and $LiClO_4$ (5.04 g, 47.4 mmol) in EtOH (65 mL) was treated with (±)-1,3-butanediepoxide (1.49 mL, 18.9 mmol) and stirred at 55 °C for 13 h. The reaction solution was cooled to RT, diluted with EtOAc (180 mL) and washed successively with saturated aq $NaHCO_3$ (3×), water (2×) and brine. The organic phase was then dried (Na_2SO_4), filtered and concentrated in vacuo. Purification by flash chromatography (SiO_2, hexane/EtOAc, 2:1, saturated with NH_4OH) gave diol **3** as a colorless oil (6.03 g, 78%); R_f 0.5 (hexane/EtOAc, 2:1); IR $\tilde{\nu}$ = 3460 cm^{-1}; ^{1}H NMR δ = 3.65 (m, 1H), 3.48 (dq, J = 9.4 Hz, J = 2.5 Hz, 4H), 2.53-2.75 (m, 4H), 2.40 (s, 3H), 1.38 (s, 3H), 1.16 ppm (t, J = 9.4 Hz, 6H); ^{13}C NMR δ = 101.6, 69.0, 63.2, 62.3, 55.8, 44.5, 21.6, 15.5 ppm; Anal. calcd for $C_{20}H_{44}N_2O_6$: C, 58.79; H, 10.85; N, 6.86. Found: C, 58.99; H 10.65; N, 6.85.

Dimyristyl Diether (4): A suspension of NaH (0.07 g, 2.94 mmol) and HMPA (0.51 mL, 2.94 mmol) in DMF (1 mL) was treated dropwise with a solution of diol **3** (0.20 g, 0.49 mmol) in DMF (1 mL) at 0 °C, then stirred for 1 h while warming to RT. The reaction was cooled to 0 °C and treated with a solution of myristyl iodide (0.80 g, 2.45 mmol) in DMF (2 mL), then strirred at 0–10 °C for 2.5 d. The reaction was quenched with saturated aq $NaHCO_3$ (1 mL), diluted with Et_2O (20 mL) and washed successively with saturated aq $NaHCO_3$ (2×), water and brine. The organic phase was then dried (Na_2SO_4), filtered and concentrated in vacuo. Purification by flash chromatography (SiO_2, hexane/EtOAc, 12:1, saturated with NH_4OH) gave diether **4** as a light yellow oil (0.24 g, 57%); R_f 0.72 (hexane/EtOAc, 4:1); IR $\tilde{\nu}$ = 2920, 2852 cm^{-1}; ^{1}H NMR δ = 3.55-3.60 (m, 2H), 3.43-3.51 (m, 12H), 2.64 (dd, J = 13.4, 5.3 Hz, 2H), 2.52 (s, 4H) 2.47 (dd, J = 13.3, 5.8 Hz, 2H), 2.32 (s, 6H), 1.54 (m, 4H) 1.38 (s, 6H), 1.25 (s, 40H), 1.16 (t, J = 7.0 Hz, 12H), 0.88 ppm (t, J = 6.8, 6H); ^{13}C NMR δ = 102.3, 77.8, 71.2, 63.1, 59.8, 55.7, 44.1, 32.1, 30.5, 29.6-29.9 (4 signals), 26.5, 22.9, 21.9, 15.7, 14.3 ppm; HRMS (CI/NBA) *m/z* calcd for $C_{49}H_{100}N_2O_6$ $[M + H]^+$ 801.7654, found 801.7691.

Dimyristyl Diketone DMDK (5): A solution of **4** (0.25 g, 0.32 mmol) in acetone (3.5 mL) was treated with *p*-TsOH (0.13 g, 0.67 mmol) at RT and stirred (5 h). The reaction mixture was diluted with Et_2O (20 mL) and washed successively with saturated aq $NaHCO_3$ (3×), water and brine. The organic phase was then dried (Na_2SO_4), filtered and concentrated. The residue was dissolved in MeI (4 mL) and stirred for 16 h. Excess MeI was removed in a fumehood using a stream of Ar. The residue was redissolved in $CHCl_3$ and eluted through an ion-exchange resin column (DOWEX 1X8-400, 2.5 g).

The eluent was concentrated under a stream of nitrogen (~1 mL), and the product was precipitated using of Et_2O (8 mL) and collected by centrifugation. The pellet was washed with Et_2O (8 mL) and dried under vacuum to give DMDK (**5**) as a tan solid (0.20 g, 83%); ^{1}H NMR δ = 5.54 (d, J = 17.7 Hz, 2H), 5.24 (d, J = 17.7 Hz, 2H), 4.66 (m, 4H), 3.89 (m, 4H), 3.69 (m, 2H), 3.53 (s, 6H), 3.48 (s, 6H), 2.35 (s, 6H), 1.54 (m, 4H), 1.25 (s, 40H), 0.88 ppm (t, J = 6.5 Hz, 6H); ^{13}C NMR δ = 199.6, 72.6, 71.2, 68.7, 65.5, 52.9, 51.9, 31.8, 30.2, 29.3-29.6 (4 signals), 28.8, 28.7, 26.2, 22.6, 14.0 ppm; HRMS (CI/NBA) *m/z* calcd for $C_{42}H_{86}N_2O_4^{2+}$ $[M]^{2+}$ 341.3288, found 341.3291.

Formulation of DMDK: DMDK (10 mg) was formulated with DOPE (dioleoylphosphatidylethanolamine) in a 1:1 molar ratio and dissolved in $CHCl_3$ (3 mL). The $CHCl_3$ was evaporated under He to give a thin film, which was then dried in vacuo for 3 h. The film was reconstituted in aq NaCl (5 mL, 150 mM) to a final concentration of 3 mM. The solution was vortexed (1 min) and sonicated (5 min) at 50 °C; this process was repeated twice.

Biology

Nucleic acid vectors

Luciferase and eGFP DNA vectors (pND.Luc and pND.eGFP) contain the human CMV immediate early promoter (HCMV IE1) and CMV IE1 intron, and a multiple cloning site (MCS). The luciferase and eGFP coding sequences were inserted into the multiple cloning site followed by the RNA terminator/polyadenylation site derived from bovine growth hormone (BGH),[30] in a pUC19 replicon.

Transfection protocol

Two centrifuge tubes were prepared containing Opti-MEM reduced serum medium (Gibco) (100 μL). One was treated with pND.luc (1 μL, 1 μg μL^{-1}) and the other with DMDK–DOPE formulation (10.9 μL), and then allowed to stand at RT for 10 min. The lipid was then added to the tube containing the pND.luc. After incubation at room temperature for 30 min, lipid/DNA formulations were applied to cell culture and allowed to incubate at 37 °C for 3 h, after which, the lipid/DNA formulations were aspirated off and replaced with F12K medium (Gibco) containing 10% fetal bovine serum (FBS) and 1% L-glutamine. 24-Well plates containing CHO cells were plated at a density of 50000 per well approximately 24 h prior to use. Cell cultures were maintained in a 37 °C incubator and 5% CO_2.

DNA protection assay: DNase treatment

Naked DNA and DMDK–DNA complexes were incubated at 37 °C for 30 min with 1 unit of RQ1 RNase-free DNase and 10× reaction buffer (Promega). One unit of RQ1 RNase-free DNase (1 unit μL^{-1}) is defined as the amount required to completely degrade 1 μg of λDNA in 10 min at 37 °C in 50 μL of a buffered solution. After incubation for 30 min, aliquots were removed and used for transfection or phenol/chloroform extraction.

DNA protection assay: hCSF

Naked DNA and DMDK–DNA complexes were incubated at 37 °C for 30 min with hCSF obtained under an approved IRB protocol. The hCSF was collected under sterile conditions during the normal course of preoperative spinal anesthetic administration (Hospital of the University of Pennsylvania).

Phenol/chloroform extraction

An equal volume of phenol/chlororform/isoamyl alcohol (1:1:1) was added to the DMDK–DNA solution. This solution was vortexed (10 s), briefly centrifuged and the aqueous layer separated. NaOAc (3 M) was added and the solution was vortexed. Next, 2.5 volumes of ice-cold EtOH (100%) was added, vortexed and placed in a −70 °C freezer for 15 min. The solution was then microcentrifuged for 5 min at maximum speed and the supernatant was removed. Room temperature EtOH (1 mL, 70%) was added and the solution was centrifuged (5 min) at the maximum speed. The supernatant was removed and the pellet dried. The dry pellet was then dissolved in twice distilled water (30 μL).[31]

Gel electrophoresis

1% agarose gels were made up using 1× tris-acetate-EDTA (TAE) buffer (Invitrogen) and agarose (UltraPure, Invitrogen). Gels were run in 1× TAE buffer at 90 volts for 1 h and imaged using a Kodak Digital Science EDAS 120 camera (Diagnostic Instruments) and Kodak Digital Science 1D software (Kodak).

Formulation of DNA–DMDK–Alexa Fluor 488 conjugate

Alexa Fluor 488 hydroxylamine (1 mg) was reconstituted in RNAse-free water(total volume 1 mL, final concentration 1 μg μL^{-1}). DMDK was optimized for incubation time and charge ratio. Optimization was carried out by varying the charge ratio from 6:1 to 14:1, and varying the incubation time in 15 min intervals up to 45 min at RT. Optimal conditions were a 14:1 charge ratio and 30 min of incubation at RT.

Opti-MEM reduced serum medium (100 μL) was placed in a 2 mL microcentrifuge tube with pND.luc (0.5 μg). A second 2 mL microcentrifuge tube was also made up containing Opti-MEM (100 μL) and the appropriate amount of DMDK. Both tubes were allowed to stand at RT for 10 min and then the lipid was added to the tube containing pND.luc. After 30 min of incubation at RT the pH of the DMDK–DNA mixture was decreased to ~pH 5 using aq HCl (250 mM). The pH was determined using pHydrion Papers 4–9 (Micro Essential Laboratory). Alexa Fluor 488 hydroxylamine (1 μg) was added to the DMDK–DNA lipoplex and allowed to incubate at RT for 2 h in the dark. After incubation with Alexa Fluor 488 hydroxylamine the pH of the cocktails was returned to pH 7 using aq NaOH (250 mM). The labeled DMDK–DNA lipoplexes were applied to cell culture; CHO cells were used at a density of 50,000 per well, as measured using a Bright-Line Hemacytometer (Hausser Scientific) and allowed to incubate at 37 °C. After 3 h the labeled lipoplexes were aspirated off and replaced with F12K medium (Gibco) containing 10% FBS and 1% L-glutamine.

Fluorescence imaging

CHO cells were imaged 24 h post transfection at 40× magnification under brightfield and fluorescein isothiocyanate (FITC) using SPOT Advanced version 4.0.9 (Diagnostic Instruments) with an exposure time manually set to 750 ms. Experiments were repeated in triplicate.

Controls

Controls included transfection of CHO cells with DMDK and DNA, and incubation with Alexa Fluor 488 hydroxylamine alone as per the protocols described above.

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Keywords: gene transfer · keto-lipids · labeling · oximation · targeting

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