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Synthesis and Formulation of Neoglycolipids for the Functionalization of Liposomes and Lipoplexes

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Novel carbohydrate-based agents for the stabilization of ternary liposome:mu:DNA (LMD) nonviral vector systems are described. LMD vector systems comprise plasmid DNA (pDNA; D,7.5 kb) expressing a reporter gene (in this instance β-galactosidase expressing gene) that is precondensed with the adenoviral core peptide μ (mu, M; MRRAHHRRRRASHRRMRGG) and then further packaged by means of DC−Chol:DOPE (3:2; *m*/*m*) cationic liposomes. Final optimized lipid:mu:pDNA ratio is typically 12:0.6:1 (w/w/w). We report the synthesis of a series of nine neoglycolipids prepared by coupling completely unprotected sugar monomers or oligomers (mannose, glucose, galactose, glucuronic acid, maltose, lactose, maltotriose, maltotetraose, and maltoheptaose) through their reducing-residue termini to an aminoxy-functionalized cholesterol-based lipid. Characterization of these novel neoglycolipids by ^{1}H NMR reveals that the coupling reaction has a major configurational preference for the β-anomer. Unusually, even mannose coupling results in a neoglycolipid product with a predominantly β-anomeric conformation (>85%). Formulation of neoglycolipids into LMD vector systems by incubation of LMD particles with neoglycolipid micelles results in the formation of a range of potential stabilized-LMD (sLMD) vector systems. Those potential sLMD systems prepared with longer chain neoglycolipids are found to have enhanced stabilities, with respect to aggregation in high ionic strength buffers, and enhanced transfection efficacies in comparison to the transfection properties of the naked first generation LMD vector system (i.e., gene delivery and expression). By contrast, when LMD vector systems are incubated with poly(ethylene glycol) DSPE-PEG micelles, resulting PEG-LMD vector systems are very stable with respect to colloidal instablility and aggregation in high ionic strength buffers and in serum, but are completely refractory to transfection. These data suggest that oligosaccharides could represent an alternative to PEG as a stealth polymer able to stabilize synthetic nonviral vector systems in some fluids but without impairing transfection efficiency. Furthermore, sLMD systems prepared with longer chain neoglycolipids appear to have sufficient useful characteristics to form the basis of viable second-generation LMD vector systems after further development.

INTRODUCTION

With the wealth of data generated by the human genome project, the use of genes for therapeutic purposes (gene therapy) is increasingly expected to revolutionize medical treatment. Well aware of the problems and high risks associated with the efficient delivery of transgenes by means of virus particles, various researchers, including chemists (*1*), have been searching for safer synthetic nonviral alternatives to mediate nucleic acid delivery into host cells including the use of histone proteins (*2*), lipids (*3*−*5*) and/or liposomes (*6*−*8*), dendrimers (*9*−*11*), or polymers (*12*−*14*). Although the transfection efficiencies of synthetic nonviral vector systems are remote from the efficiencies of viral vectors, this technology should ultimately have clinical applications given the genuine prospects that nonviral systems should have with respect to superior safety and manufacturing profiles compared to viral systems (*15*−*18*). In the drive for clinically viable, synthetic nonviral vector systems, we recently developed a synthetic nonviral platform system known as liposome:mu:DNA (LMD) based upon cationic liposomes (L), the adenoviral core peptide μ (mu, M) (*19*), and plasmid DNA (D) (*20*). This system showed several promising features including long-term storage characteristics, enhanced stability with respect to aggregation compared with binary cationic liposome−plasmid DNA (lipoplex, LD) systems (*21*).

However, despite these useful characteristics, naked first generation LMD is not clinically viable owing to several clear limitations that have since come to light in more recent experiments. First, a mechanistic study comparing LD and LMD systems demonstrated that the nuclear localization sequence (NLS) of the adenoviral peptide mu is not properly exploited by the LMD system. In other words, the peptide was apparently unable to promote the active uptake of the DNA into the nucleus although mu peptide clearly harbors strong NLS characteristics (*22*). Second, LMD systems were found to possess inadequate stability toward aggregation in serum and other biological fluids rendering them unsuitable for routine use in vivo, even though inclusion of the mu

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[1] Abbreviations: Boc, *tert*-butoxycarbonyl; br, broad; chol, cholesterol; DMF, *N*,*N*-dimethylformamide, DMSO, dimethyl sulfoxide, TFA, trifluoroacetic acid; THF, tetrahydrofuran.

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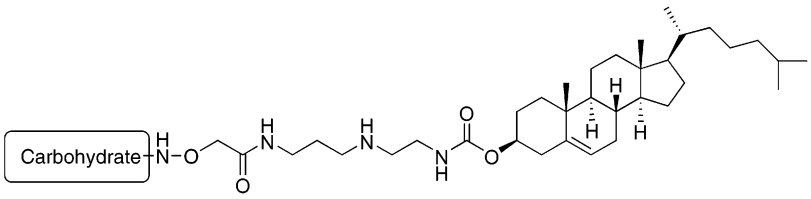


Figure 1. General structure of neoglycolipids. Completely unprotected mannose, glucose, galactose, glucuronic acid, maltose, lactose, maltotriose, maltotetraose, and maltohepatose were coupled with high yields to the aminoxy functional group of aminoxy lipid **11**.

peptide had been found to confer some elements of stability over and above the stability of simple LD systems.

In the latter respect, LMD differs little from most current synthetic nonviral vector systems (*23*−*28*), with the notable exception of vector systems such as the stabilized plasmid−lipid particle (SPLP) system that takes advantage of surface-associated poly(ethylene glycol) (PEG) to provide a "stealth" barrier to serum-induced aggregation and opsonization (*29*−*34*). In general, stealth vectors are obtained by either PEGylated lipids, or surface PEGylation of existing lipo- or polyplexes. PEG is a nontoxic, neutral polyether with a large exclusion volume for most macromolecules (*35*). In general, PEGylation of synthetic nonviral vector systems can be carried out on condensed DNA particles using a PEG lipid insertion procedure or by means of direct surface modification. Either procedure has been shown to produce nonviral vector systems that are completely resistant to aggregation in the presence of physiological salt concentrations (150 mM NaCl) or aggregation and opsonization in the presence of serum components (*25*, *36*−*38*). Unfortunately, the levels of PEG that are necessary to stabilize adequately nonviral vector system particles against aggregation in biological fluids also appear to completely inhibit the transfection process (*39*). This may be due to the shielding of the cationic surface of particles from interaction with the negatively charged plasma membrane of cells. However, recent evidence now suggests that PEG coats may be sufficiently interactive with plasma membranes to induce rapid PEG-particle cell entry by endocytosis, thereby implying that PEG may actually inhibit cellular transfection not by blocking cell entry but by blockading pDNA transport within the intracellular environment once cell entry has taken place (*40*, *41*). In the first case, nontransfecting PEG particles would be cleared by macrophages postopsonization, or else in the second case by intracellular degradation post endocytosis (*42*).

Given the shortcomings of PEG and the lack of an obvious surrogate, we speculated that an alternative approach to escape the destructive effect of biological fluids could be to coat the surface of our lipid-based nonviral vector systems such as LMD, with oligosaccharides (*43*, *44*). The biological functions of carbohydrates are diverse and we hoped to exploit their combined targeting potential and stabilization properties (*45*−*47*). Previous work on liposomes demonstrated the potential of neoglycolipids (*48*−*50*), and we anticipated that LMD stability in biological fluids might be enhanced by inserting such molecules into the outer cationic lipid bilayer of LMD particles. This was expected to lead to an increase in system stability under high serum and salt conditions without causing a loss in transfection efficiency (*51*).

Here we describe the development of a facile synthetic procedure for the preparation of a variety of diverse neoglycolipids necessary to evaluate this proposition by exploiting the high reactivity between aminoxy functional groups and a wide range of aldehydes, a reaction affinity that is well documented in the literature (*52*−*57*) (Figure 1). In sharp contrast to other methods found in the literature describing the synthesis of neoglycolipids (*58*, *59*), this procedure allows for the retention of the cyclic nature of the sugar unit and is far more simple and cost-effective than traditional methods applying tedious protection and deprotection steps during syntheses. Transfection and stability studies are then described in which a direct comparison is made between neoglyco-LMD vector systems and PEG-LMD vector systems prepared in a parallel fashion by inserting poly(ethylene glycol) grafted distearoylphosphatidylethanolamine (DSPE-PEG). Data suggest that oligosaccharide coats do indeed confer a combination of enhanced LMD particle stability under high salt concentrations (but not in serum) without impairing transfection efficiency.

EXPERIMENTAL PROCEDURES

Materials and Reagents. All organic solvents and chemicals were purchased from Sigma-Aldrich Company LTD (Poole, Dorset, UK). Dried CH_2Cl_2 was distilled with phosphorus pentoxide before use. DOPE and DSPE-PEG^{2000} were purchased from Avanti Polar Lipid (Alabaster, AL). 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl]-cholesterol (DC−Chol) was synthesized in our laboratory according to published procedures (*6*). The adenoviral core peptide mu (NH_2-MRRAHHRRRRASHRRMRGG-OH) was synthesized by the standard 9-fluorenylmethoxycarbonyl Fmoc procedure based the Wang resin and using standard Merrifield solid-phase peptide chemistry (*19*, *60*).

Plasmid DNA pUMVC1 (pDNA; 7528bp) was obtained from the University of Michigan Vector Core (http://www.med.umich.edu/vcore/Plasmids/), amplified, and purified by Bayou Biolabs (LA). The concentration of pDNA was determined spectrophotometrically ($A_{260} = 1 \cong 50$ μg/mL), and pDNA molar concentration was determined using an average nucleotide base pair molecular weight of 660 Da. OptiMEM and FCS were purchased from Gibco BRL (Lexington, KY).

Analytical Techniques. ^{1}H NMR spectra were recorded at ambient temperature on either Brucker DRX400, DRX300 or JEOL GX-270Q spectrometers, with residual nonisotopicaly labeled solvent (e.g., $CHCl_3$, δ_H 7.26) as an internal reference. ^{13}C NMR spectra were recorded on the same range of spectrometers at 100, 75, and 68.5 MHz, respectively, also with residual nonisotopically labeled solvent (e.g., $CHCl_3$, δ_C 77.2) as an internal reference. Infrared spectra were recorded on a Jasco FT/IR 620 using NaCl plates and mass spectra (positive ions electrospray) were recorded using VG-7070B or JEOL SX-102 instruments. Chromatography refers to flash column chromatography, which was performed throughout on Merck-Kieselgel 60 (230−400 mesh) with appropriate solvent. Thin-layer chromatography (TLC) was performed on precoated Merck-Kieselgel 60 F254 aluminum backed plate and visualized with ultraviolet light,

iodine, acidic ammonium molybdate(IV), acidic ethanol, vanillin, or other agents as appropriate. Neoglycolipids purity was assessed by analytical high-pressure liquid chromatography (HPLC) using a Purospher RP-18 end-capped column (5 μm) attached to an Hitachi HPLC system. Elution was performed on RP-18 at an isocratic flow rate of 1 mL/min with CH_3CN/H_2O (60:40), and fractions were detected at A_{205} before collection and analysis by mass spectrometry.

Synthesis of 2-(Cholesteryloxycarbonylamino)-ethanol (2). A solution of cholesteryl chloroformate (99.89 g, 0.218 mol) in CH_2Cl_2 (600 mL) was added to a stirred solution of 2-aminoethanol (29.5 mL, 0.489 mol, 2.2 equiv) in CH_2Cl_2 (450 mL) at 0 °C over a period of 2 h. The reaction was allowed to warm to room temperature and stirring continued for a further 14 h. The reaction mixture was washed with saturated $NaHCO_3$ (2 × 200 mL) and water (2 × 200 mL) and dried ($MgSO_4$), and the solvents were removed under reduced. The solid obtained was recrystallized (CH_2Cl_2/MeOH) to give **2** as a white solid. Yield: 99.67 g (97%); mp: 168 °C; R_f = 0.26 (acetone/ether 1:9); IR (CH_2Cl_2): ν_{max}= 3353, 2942, 2870, 1693, 1674, 1562, 1467, 1382, 1264 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$): δ=5.35 (d, J = 6.5 Hz, 1H, H6′), 5.25−5.29 (m, 1H, NH), 4.42−4.57 (1H, m, H3′), 3.70−3.62 (m, 2H, H1), 3.25−3.35 (m, 2H, H2), 3.12 (s, 1H, OH), 2.28−2.38 (m, 2H, H4′), 1.77−2.03 (m, 5H, H2′, H7′, H8′), 1.59−0.96 (m, 21H, H1′, H9′, H11′, H12′, H14′−H17′, H22′−H25′), 1 (3H, s, H-19′), 0.9(d, J = 6.5 Hz, 3H, H21′), 0.87 (d, J = 6.5 Hz, 6H, H26′ and H27′) and 0.67 (s, 3H, H18′); MS (FAB^+): m/z = 496 $[M + Na]^+$, 474 $[M + H]^+$, 369 $[chol]^+$, 255, 175, 145, 105, 95, 81, 43.

Synthesis of 2-(Cholesteryloxycarbonylamino)-ethyl Methanesulfonate (3). To a solution of **2** (25 g, 52.3 mmol) and triethylamine (22 mL, 0.16 mol, 3 equiv) in CH_2Cl_2 (500 mL) at 0 °C was added dropwise a solution of methanesulfonyl chloride (10.5 mL, 0.13 mol, 2.5 equiv). The reaction mixture was allowed to warm at room temperature and stirred for 90 min. After thin-layer chromatography analysis has indicated that the reaction had gone to completion, ice was added to quench the reaction. The reaction mixture was added to saturated aqueous NH_4Cl (600 mL) and extracted with ether (3 × 300 mL). The combined organic layers were washed with water (2 × 300 mL) and brine (250 mL) and dried (Na_2SO_4). Solvent was removed under reduced pressure to give a white solid, which on purification by chromatography (ether) gave **3**. Yield: 28.3 g (98%); IR (CH_2Cl_2): ν_{max} = 3453, 3342, 1716, 1531, 1377, 1137, 798 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$): δ = 5.34 (d, J = 6.5 Hz, 1H, H6′), 5−5.1 (m, 1H, NH), 4.41−4.53 (1H, m, H3′), 4.29−4.25 (t, J = 5 Hz, 2H, H1), 3.47−3.52 (m, 2H, H2), 3.01 (s, 3H, H3), 2.24−2.36 (m, 2H, H4′), 1.74−2 (m, 5H, H2′, H7′, H8′), 0.9−1.6 (m, 21H, H1′, H9′, H11′, H12′, H14′−H17′, H22′−H25′), 0.98 (3H, s, H-19′), 0.84(d, J = 6.5 Hz, 3H, H21′), 0.83 (d, J = 6.5 Hz, 6H, H26′ and H27′) and 0.65 (s, 3H, H18′); MS (FAB^+): m/z = 1104 $[2M + H]^+$, 574 $[M + Na]^+$, 552 $[M + H]^+$, 369 $[chol]^+$, 255,175,145,-95,81.

Synthesis of 4-Aza-N^6-(cholesteryloxycarbonylamino)hexanol (4). To a stirred solution of **3** (28.3 g, 51 mmol) dissolved in a minimum amount of THF was added 1-aminopropan-3-ol (160 mL, 2 mol, 39 equiv). After completion of the reaction (12 h) as monitored by thin-layer chromatography, $CHCl_3$ (350 mL) and K_2CO_3 (20 g) were added, and the solution was vigorously stirred for 30 min. The suspension was then filtered through a short pad of Celite, washing thoroughly with $CHCl_3$. This was washed with a saturated solution of sodium hydrogen carbonate and dried (Na_2CO_3). The solvent was removed to give **4** as a white solid. Yield: 26.1 g (96%); IR (CH_2Cl_2): ν_{max} = 3350−3210, 2937, 2850, 1531, 1460, 1380, 1220, 1120, 1040 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$): δ = 5.33−5.35 (m, 1H, H6′), 4.92−4.96 (m, 1H, NH), 4.42−4.51 (1H, m, H3′), 3.7−3.83. (m, 2H, H5), 3.23−3.29 (m, 2H, H1), 2.73−2.57 (m, 6H, H2, H3, H4), 2.2−2.36 (m, 2H, H4′), 1.7−2 (m, 5H, H2′, H7′, H8′), 0.85−1.58 (m, 21H, H1′, H9′, H11′, H12′, H14′−H17′, H22′−H25′), 0.98 (3H, s, H-19′), 0.84(d, J = 6.5 Hz, 3H, H21′), 0.8 (d, J = 6.5 Hz, 6H, H26′/H27′) and 0.61 (s, 3H, H18′); MS (FAB^+): m/z = 543 $[M + Na]^+$, 530 $[M + H]^+$, 485 $[M - CO_2]^+$, 369 $[chol]^+$, 144 $[M - chol]^+$.

Synthesis of 4-Aza-(Boc)-N^6-(cholesteryloxycarbonylamino)hexanol (5). To a solution of **4** (26.1 g, 49 mmol) were added Et_3N (8.3 mL, 1.1 equiv) and Boc_2O (10.7 g, 1 equiv) in CH_2Cl_2 (200 mL), and the resulting solution was followed by TLC. On completion, the reaction mixture was poured into saturated aqueous NH_4Cl (100 mL), washed with water, and dried (Na_2SO_4). The solvent was removed in vacuo to give the white solid **5**. The solvent was removed under reduced pressure to give a white solid, which on purification by chromatography (CH_2Cl_2/MeOH/NH_3 92:7:1) gave **3**. Yield (27.9 g, 90%); IR (CH_2Cl_2): ν_{max} = 3352, 3054, 2937, 1675, 1530, 1455, 1380, 1220, 1120; ^1H NMR (270 MHz, $CDCl_3$): δ = 5.33−5.35 (m, 1H, H6′), 4.86 (m, 1H, NH), 4.42−4.5 (1H, m, H3′), 3.62−3.7 (m, 2H, H5), 3.27−3.38 (m, 6H, H1, H2, H3), 2.18−2.33 (m, 2H, H4′), 1.73−2 (m, 5H, H2′, H7′, H8′), 1.45 (s, 9H, Boc), 1−1.65 (m, 23H, H4, H1′, H9′, H11′, H12′, H14′−H17′, H22′−H25′), 0.97 (3H, s, H-19′), 0.93 (d, J = 6.5 Hz, 3H, H21′), 0.8 (d, J = 6.5 Hz, 6H, H26′ and H27′) and 0.65 (s, 3H, H18′); MS (FAB^+): m/z = 654 $[M + Na]^+$, 543 $[M - Boc]^+$, 369 $[chol]^+$, 145, 121, 95, 69,57.

Synthesis of 4-Aza-(Boc)-N^6-(cholesteryloxycarbonylamino)hexyl Methanesulfonate (6). This experiment was carried out in a manner similar to the preparation of 2-(cholesteryloxycarbonylamino)ethyl methanesulfonate **3** on 44 mmol scale giving **6**. Yield (28 g, 90%); IR (CH_2Cl_2): ν_{max} = 3305, 2980, 2900, 2865, 1675, 1530, 1455, 1350, 1150; ^1H NMR (270 MHz, $CDCl_3$): δ = 5.33−5.35 (m, 1H, H6′), 4.86 (m, 1H, NH), 4.35−4.55 (m, 1H, H3′), 4.22 (t, 2H, J = 6.5 Hz, H5), 3.2−3.4 (m, 6H, H1, H2, H3), 3.01(s, 3H, H6), 2.15−2.33 (m, 2H, H4′), 1.73−2 (m, 5H, H2′, H7′, H8′), 1.44 (s, 9H, Boc), 1−1.67 (m, 23H, H4, H1′, H9′, H11′, H12′, H14′−H17′, H22′−H25′), 0.97 (3H, s, H-19′), 0.94 (d, J = 6.5 Hz, 3H, H21′), 0.8 (d, J = 6.5 Hz, 6H, H26′ and H27′) and 0.65 (s, 3H, H18′); MS (FAB^+): m/z = 722 $[M + Na]^+$, 609 $[M - Boc]^+$, 369 $[chol]^+$, 145, 121, 95, 69,55.

Synthesis of 4-Aza-(Boc)-N^6-(cholesteryloxycarbonylamino)hexanamine (7). Anhydrous DMF (200 mL) was added to **6** (25 g, 35 mmol), sodium azide (11.49, 175.7 mmol, 5 equiv), and sodium iodine (5 g, 35 mmol, 1 equiv) under nitrogen while stirring. Heating at 80 °C for 2 h resulted in completion of the reaction. The reaction mixture was allowed to cool to room temperature, the DMF removed under reduced pressure, and the residue dissolved in ethyl acetate. This was washed with water (2 × 100 mL) and brine (100 mL) and dried (Na_2SO_4) to give after purification by chromatography (hexane/ether 1:1) **7** as a white solid. Yield (22 g, 95%); ^1H NMR (270 MHz, $CDCl_3$): δ = 5.34−5.36 (m, 1H, H6′), 4.35−4.55 (m, 1H, H3′), 4.25 (t, 2H, J = 6.5 Hz, H5), 3.2−3.5 (m, 6H, H1, H2, H3), 2.25−2.33 (m, 2H, H4′), 1.7−2.05 (m, 5H, H2′, H7′, H8′), 1.45 (s, 9H, Boc), 1−1.72 (m, 23H, H4, H1′, H9′, H11′, H12′, H14′−H17′, H22′−H25′), 0.98 (3H, s, H-19′), 0.94 (d, J = 6.5 Hz, 3H, H21′), 0.83 (d, J = 6.5

Hz, 6H, H26′ and H27′) and 0.64 (s, 3H, H18′); MS (FAB^+): m/z = 568 [M + Na − Boc]$^+$, 556 [M − Boc]$^+$, 369 [chol]$^+$, 145, 121, 95, 69, 57.

Synthesis of 4-Aza-(Boc)-N^6-(cholesteryloxycarbonylamino)hexylamine (8). To a round-bottomed flask charged with **7** (22.75 g, 34,6 mmol) in THF (230 mL) was added trimethylphosphine in THF (1 M, 40 mL, 1.15 equiv), and the reaction was monitored by TLC. On completion the reaction was stirred with water (3 mL) and aqueous ammonia (3 mL) for 1 h, and the solvent was removed under reduced pressure affording a white crystalline powder. Yield (19.1 g, 88%); IR (CH_2Cl_2): ν_{max} = 3689, 3456, 3155, 2948, 2907, 2869, 2253, 1793, 1709, 1512, 1468, 1381, 1168; ^{1}H NMR (270 MHz, $CDCl_3$): δ = 5.32−5.35 (m, 1H, H6′), 4.35−4.51 (m, 1H, H3′), 3.45−3.05 (m, 8H, H1, H2, H3, H5), 2.18−2.4 (m, 2H, H4′), 1.8−2.1 (m, 5H, H2′, H7′, H8′), 1.46 (s, 9H, Boc), 1.01−1.72 (m, 23H, H4, H1′, H9′, H11′, H12′, H14′−H17′, H22′−H25′), 0.97 (3H, s, H-19′), 0.85 (d, J = 6.5 Hz, 3H, H21′), 0.82 (d, J = 6.5 Hz, 6H, H26′ and H27′), 0.64 (s, 3H, H18′); MS (FAB^+): m/z = 630 [M + H]$^+$, 530 [M − Boc]$^+$, 369 [chol]$^+$, 145, 121, 95, 69,57.

Synthesis of (Boc)aminoxyacetic Acid (9). *O*-(Carboxymethyl)hydroxylamine hemihydrochloride (1.16 g, 5.3 mmol) was dissolved in CH_2Cl_2 (40 mL), and the pH was adjusted to 9 by addition of triethylamine (3 mL). Then, di-*tert*-butyl dicarbonate (2.36 g, 10.6 mmol, 2.0 equiv) was added, and the mixture was stirred at room temperature until TLC indicated completion of reaction. The pH was lowered to 3 by addition of diluted HCl. The reaction mixture was partitioned between saturated aqueous NH_4Cl (20 mL) and CH_2Cl_2 (30 mL). The aqueous phase was extracted with CH_2Cl_2 (3 × 100 mL). The combined organic extracts were washed with H_2O (2 × 100 mL) and dried (Na_2SO_4). The solvent was removed in vacuo to afford **9** as a white solid. Yield (1.86 g, 97%); IR (CH_2Cl_2): ν_{max} = 3373, 2983, 2574, 2461, 1724, 1413, 1369, 1235; ^{1}H NMR (270 MHz, $CDCl_3$): δ = 4.48 (s, 2H, CH_2), 1.48 (s, 9H, Boc); MS (FAB^+): m/z =214 [M + Na]$^+$, 192 [M + H]$^+$, 135, 123, 109, 69.

Synthesis of (Boc)aminooxy Compound (10). *N*-Hydroxysuccinimide (0.36 g, 3.13 mmol, 1 equiv), **9** (0.6 g, 3.13 mmol, 1 equiv), and *N*,*N*′-dicyclohexylcarbodiimide (0.68 g, 3.13 mmol, 1 equiv) were dissolved in EtOAc (90 mL), and the heterogeneous mixture was allowed to stir at room-temperature overnight. The mixture was then filtered through a pad of Celite to remove the dicyclohexyl urea, which was formed as a white precipitate (rinsed with 60 mL ethyl acetate), and added to a solution of **8** (1.97 g, 3.13 mmol, 1 equiv) in THF (10 mL). A pH of 8 was maintained for this heterogeneous reaction by addition of triethylamine (6 mL). The resulting mixture was allowed to stir at room-temperature overnight. On completion the mixture was filtered, and the solvent was removed under reduced pressure to give after purification by flash-chromatography (CH_2Cl_2/MeOH/NH_3 92:7:1) **10** as a white solid. Yield (2.3 g, 90%); ^{1}H NMR (270 MHz, $CDCl_3$): δ = 5.33−5.35 (m, 1H, H6′), 4.4−4.52 (m, 1H, H3′), 4.3 (s, 2H, H90, 3.2−3.42 (m, 8H, H1, H2, H4, H6), 2.23−2.35 (m, 2H, H4′), 1.7−2.1 (m, 7H, H2′, H7′, H8′, H5), 1.44−1.46 (m, 18H, 2 Boc), 1−1.73 (m, 21H, H1′, H9′, H11′, H12′, H14′−H17′, H22′−H25′), 0.98 (3H, s, H-19′), 0.85 (d, J = 6.5 Hz, 3H, H21′), 0.83 (d, J = 6.5 Hz, 6H, H26′/H27′), 0.65 (s, 3H, H18′); MS (FAB^+): m/z =803 [M + H]$^+$, 703 [M − Boc]$^+$, 647, 603 [M − 2Boc]$^+$, 369, 279, 255, 235, 204, 145, 95, 69.

Synthesis of Aminoxylipid (11). To a solution **10** (1.1 g, 1.36 mmol, 1 equiv) in CH_2Cl_2 (10 mL) was added TFA (2 mL, 20.4 mmol, 15 equiv) at 0 °C. The solution was allowed to stir at room temperature for 5 h. On completion toluene was added to azeotrope TFA from the reaction mixture. The solvents were removed in vacuo to afford after purification by chromatography (CH_2Cl_2/MeOH/NH_3 92:7:1 to 75:22:3) **11** as a white solid (709 mg, Yield: 86%); IR ($CHCl_3$): ν_{max} = 3306, 2948, 2850, 2246, 1698, 1647, 1541, 1467, 1253, 1133; ^{1}H NMR (270 MHz, $CDCl_3$): δ =5.26−5.4 (m, 1H, H6′), 4.4−4.52 (m, 1H, H3′), 4.12 (s, 2H, H9), 3.34−3.41 (m, 2H, H2), 3.15−3.3 (m, 2H, H4), 2.6−2.74 (m, 4H, H1/H6), 2.14−2.39 (m, 2H, H4′), 1.62−2.1 (m, 7H, H2′, H7′, H8′, H5), 1.02−1.6 (m, 21H, H1′, H9′, H11′, H12′, H14′−H17′, H22′−H25′), 0.96 (3H, s, H-19′), 0.86 (d, J = 6.5 Hz, 3H, H21′), 0.83 (d, J = 6.5 Hz, 6H, H26′ and H27′), 0.66 (s, 3H, H18′); MS (FAB^+): m/z = 603 [M + H]$^+$, 369 [chol]$^+$, 160, 137, 109, 95, 81, 69, 55.

Synthesis of Aminoxy-mannopyranose Compound (12a). A solution of D-mannose (266 mg, 4.8 mmol) in acetic aqueous buffer (sodium acetate/acetic acid 0.1 M, pH 4, 7 mL) and a solution of **11** (290 mg, 0.48 mmol, 10 equiv) in DMF (7 mL) was mixed and stirred for 3 days at room temperature. The solvent was removed in vacuo by freeze-drying and chromatography (CH_2Cl_2/MeOH/NH_3 75:22:3) afforded the product **21** a white solid (233 mg, yield: 65%). The purity was further confirmed by HPLC. The final product contained of the β-pyranose (82%) form and α-pyranose (18%) form that were not separated but characterized in the mixture. MS (FAB^+): m/z = 765 [M + H]$^+$, 787 [M + Na]$^+$, 397, 369 [chol]$^+$, 322, 240, 121, 109, 95, 81, 69,57; *β-pyranose form*: ^{1}H NMR (400 MHz, CD_3OD/$CDCl_3$ [75/25]): δ = 7.64−7.62 (d, $^3J_{1a-2a}$ = 7 Hz, 1H, H1a), 5.35−5.36 (m, 1H, H6′), 4.45−4.5 (s, 2H, H9), 4.35−4.5 (m, 1H, H3′), 4.19−4.24 (dd, 1H, $H2_a$, $^3J_{1a-2a}$ = 7.4 Hz, $^3J_{2a-3a}$ = 7.7 Hz), 3.81−3.9 (m, 1H, H3?), 3.73−3.8 (m, 2H, H4a, $H6_{ax}$a), 3.63−3.71 (m, 2H, H5a, H_{eq}6a), 3.34−3.42 (m, 2H, H2), 3.27−3.30 (m, 2H, H4), 3−3.08 (m, 2H, H1), 2.9−2.98 (m, 2H, H6), 2.25−2.35 (m, 2H, H4′), 1.78−2.07 (m, 7H, H2′, H7′, H8′, H5), 1.03−1.65 (m, 21H, H1′, H9′, H11′, H12′, H14′−H17′, H22′−H25′), 1.01 (3H, s, H-19′), 0.91 (d, J = 6.5 Hz, 3H, H21′), 0.85 (d, J = 6.5 Hz, 6H, H26′/H27′), 0.69 (s, 3H, H18′); ^{13}C NMR (400 MHz, $CDCl_3$/ CD_3OD [25/75]): 12.33 (C18′), 19.20 (C21′), 19.74 (C19′), 21.91 (C11′), 22.91 (C27′), 23.17 (C26′), 24.67 (C23′), 25.07 (C15′), 27.37 (C5), 28.85 (C25′), 28.96 (C2′), 29.07 (C12′), 32.76 (C7′), 32.87 (C8′), 36,38 (C2), 36.78 (C20′), 37.09 (C1) 37.76 (C22′),37.95 (C1′), 38.4 (C4), 39.36 (C4′), 40.41 (C24′), 40.76 (C16′), 46.16 (C6), 51.19 (C9′), 57.19 (C17′), 57.75 (C14′), 64.62 (C6a), 70.19 (C2a), 70.58 (C4a), 72.12 (C3a), 72.37 (C5a), 73.11 (C9), 75.91 (C3′), 123.39 (C6′), 140.72 (C5′), 155.02 (C1a), 158.69 (NHCOOChol), 173.1 (C8); α-*pyranose form*: identical data except, ^{1}H NMR (400 MHz, CD_3OD/CDCl3 [75/25]): δ = 6.90−6.88 (d, $^3J_{1a-2a}$ = 7 Hz, 1H, H1a), 5−5.05 (d×d, 1H, H2α, $^3J_{1a-2a}$ = 7.3 Hz, $^3J_{2a-3a}$ = 7.6 Hz); ^{13}C NMR (400 MHz, $CDCl_3$/CD_3-OD [25/75]): 65.33 (C2a), 155.79 (C1a).^{1}H NMR (400 MHz, CD_3OD/$CDCl_3$ [75/25]): (m, 1H, H3′) missing, underneath solvent peak; confirmed by ^{1}H NMR (300 MHz, DMSO): δ = 4.67−4.82 (m, 1H, H3′). ^{13}C NMR (400 MHz, $CDCl_3$/CD_3OD [25/75]): C1 missing, underneath MeOH peak confirmed by ^{1}H/^{13}C correlation at 400 MHz, around 49. Proton resonance assignments were confirmed using ^{1}H gradient type DQF−COSY and TOCSY; ^{1}H/^{13}C correlation and DEPT 135 were used to assign unambiguously the carbon resonances. α-pyranose form gave $^1J^{13}$C1a-H1a = 177 Hz and β-pyranose form gave $^1J^{13}$-C1a-H1a = 167 Hz. ^{1}H phase-sensitive NOESY confirmed the observed conformation.

Synthesis of Aminoxy-glucopyranose Compound (12b). This was prepared with a solution of D-glucose (150 mg, 0.82 mmol) and **11** (100 mg, 0.16 mmol) in a similar way to the preparation of **12a**, stirred for 1 day, and purified by chromatography (CH_2Cl_2/MeOH/NH_3 75:22:3) to afford the product **12b** as a white solid (103 mg, yield: 82%). The purity was further confirmed by HPLC. The final product contained the α-pyranose (11%) anomer and the *β*-pyranose (89%) anomer that were not isolated but characterized in the mixture. (FAB$^+$): m/z = 765 [M + H]$^+$, 787 [M + Na]$^+$, 391, 369 [chol]$^+$, 309, 290, 171, 152, 135, 123, 109, 95, 81, 69; *β-pyranose form*. (300 MHz, $CDCl_3$/CD_3OD [90/10]): δ = 7.53−7.56 (d, J = 5.6 Hz, 1H, H1a), 5.26−5.36 (m, 1H, H6′), 4.2−4.45 (m, 3H, H9, H3′), 4.05−4.15 (m, 1H, H2a), 3.45−3.85 (m, 5H, H6a, H3a, H5a, H4a), 2.9−3.4 (m, H2, H4, MeOH), 2.9−3.15 (m, 4H, H1, H6), 2.15−2.3 (m, 2H, H4′), 1.65−2 (m, 5H, H2′, H7′, H8′), 0.95−1.55 (m, 23H, H5, H1′, H9′, H11′, H12′, H14′−H17′, H22′−H25′), 0.93 (3H, s, H-19′), 0.84 (d, J = 6.5 Hz, 3H, H21′), 0.78 (d, J = 6.5 Hz, 6H, H26′ and H27′), 0.62 (s, 3H, H18′); α-*pyranose form*: identical data except, ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD [90/10]): δ = 7.22−7.24 (d, J = 6,61 Hz, 1H, H1a), 4.95−5.07 (m, 1H, H2a); ^{1}H NMR (300 MHz, CD_3OD): (m, 1H, H3′) missing, presumably underneath solvent peak; confirmed by ^{1}H NMR (300 MHz, DMSO): δ = 4.7−4.86 (m, 1H, H3′).

Synthesis of Aminoxy-galactopyranose Compound (12c). This was prepared with a solution of D-galactose (50 mg, 0.27 mmol) and **11** (40 mg, 0.066 mmol in a similar way to the preparation of **12a**, stirred for 1 day, and purified by chromatography (CH_2Cl_2/MeOH/NH_3 75:22:3) to afford the product **12c** as a white solid (35 mg, yield: 70%). The purity was further confirmed by HPLC. The final product contained of the α-pyranose (15%) form and *β*-pyranose (85%) form that were not isolated but characterized in the mixture. MS (FAB$^+$): m/z = 765 [M + H]$^+$, 588, 391, 369 [chol]$^+$, 322, 290, 165, 152, 135, 121, 109, 95, 81, 69; *β-pyranose form*: ^{1}H NMR (270 MHz, DMSO): δ = 7.78−7.82 (m, 1H, NHCO of C8), 7.55−7.58 (d, J = 7.2 Hz, 1H, H1a), 6.95−7.1 (m, 1H, NHCOOChol), 5.25−5.37 (m, 1H, H6′), 4.2−4.43 (m, 3H, H9, H3′), 3.2−3.9 (m, H2a, H6a, H3a, H5a, H4a, OH), 2.9−3.18 (m, 4H, H2, H4), 2.4−2.65 (m, 4H, H1, H6), 2.15−2.3 (m, 2H, H4′), 1.67−2 (m, 5H, H2′, H7′, H8′), 0.92−1.6 (m, 23H, H5, H1′, H9′, H11′, H12′, H14′−H17′, H22′−H25′), 0.96 (3H, s, H-19′), 0.89 (d, J = 6.5 Hz, 3H, H21′), 0.84 (d, J = 6.5 Hz, 6H, H26′/H27′), 0.65 (s, 3H, H18′); α-*pyranose form*: identical data except, ^{1}H NMR (270 MHz, DMSO): 6.86−6.88 (d, J = 6 Hz, 1H, H1a)

Synthesis of Aminoxy-glucopyranuronic Acid Compound (12d). This was prepared with a solution of D-glucuronic acid, sodium salt monohydrate (30 mg, 0.128 mmol, 1.5 equiv), and **11** (50 mg, 0.08 mmol) in a similar way to the preparation of **12a**, stirred for 1 day, purified by chromatography (CH_2Cl_2/MeOH/NH_3 75:22:3) to afford the sodium salt of **12d** as a white solid (41 mg, Yield: 60%). The purity was further confirmed by HPLC. The final product contained of the α-pyranose (85%) form and *β*-pyranose (15%) form that were not isolated but characterized in the mixture. MS (FAB$^+$): m/z = 779 [M + H]$^+$, 733, 588, 411, 369 [chol]$^+$, 336, 290, 240, 214, 159, 145, 135, 121, 109, 95, 81, 69, 55; *β-pyranose form*: ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD [75/25]): δ = 7.51−7.53 (d, J = 5.9 Hz, 1H, H1a), 5.25−5.33 (m, 1H, H6′), 4.2−4.45 (m, 3H, H9, H3′), 3.8−4.1 (m, 3H, H2a, H3a, H4a), 3.6−3.75 (m, 1H, H5a), 3.2−3.55 (m, H2, H4, MeOH), 2.7−3.15 (m, 4H, H1, H6), 2.18−2.32 (m, 2H, H4′), 1.62−2 (m, 5H, H2′, H7′, H8′), 0.9−1.6 (m, 23H, H5, H1′, H9′, H11′, H12′, H14′−H17′, H22′−H25′), 0.93 (3H, s, H-19′), 0.83 (d, J = 6.5 Hz, 3H, H21′), 0.77 (d, J = 6.5 Hz, 6H, H26′/H27′), 0.6 (s, 3H, H18′); α-*pyranose form*: identical data except, ^{1}H NMR (300 MHz, CD_3OD): δ = 7.22−7.24 (d, J = 6.3 Hz, 1H, H1a), 5−5.1 (m, 1H, H2a).

Synthesis of *β*-D-Lactose Compound (12e). A solution of *β*-D-lactose, containing 25−30% of α (1.13 g, 3.3 mmol) and **11** (200 mg, 0.33 mmol) in 14 mL of DMF/acetic aqueous buffer was stirred for 4 days at room temperature. The solvent was removed in vacuo by freeze-drying, and chromatography (CH_2Cl_2/MeOH/NH_3 75:22:3) afforded the product **12e** as a white solid (145 mg, yield: 47%). The purity was further confirmed by HPLC. The final product contained of the α-pyranose (15%) form and *β*-pyranose (85%) form (containing itself around 25% of α-lactose) that were not isolated but characterized in the mixture. MS (FAB$^+$): m/z = 927 [M + H]$^+$, 588, 482, 369 [chol]$^+$, 290, 243, 216, 178, 152, 135, 121, 109, 95, 81, 69, 55; *β-pyranose form*: ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD [20/80]): δ_H = 7.69−7.71 (d, $^3J_{1a-2a}$ = 5.8 Hz, 1H, H1a of *β* lactose), 7.66−7.68 (d, $^3J_{1a-2a}$ = 6.2 Hz, 1H, H1a of α lactose), 5.35−5.37 (m, 1H, H6′), 4.37−4.6 (m, 4H, H9, H3′, H2a), 4.2−4.37 (m, 1H, H1a), 3.65−4.05 (m, 7 H, H3a, H4a, H5a, H4b, H5b, H6b), 3.25−3.6 (m, 8H, H2, H4, H6a, H2b, H3b, MeOH), 3−3.2 (m, 4H, H1, H6), 2.25−2.42 (m, 2H, H4′), 1.8−2.15 (m, 5H, H2′, H7′, H8′), 1−1.65 (m, 23H, H5, H1′, H9′, H11′, H12′, H14′−H17′, H22′−H25′), 1.01 (3H, s, H-19′), 0.91 (d, J = 6.5 Hz, 3H, H21′), 0.85 (d, J = 6.5 Hz, 6H, H26′/H27′), 0.69 (s, 3H, H18′); ^{13}C NMR (400 MHz, $CDCl_3$/CD_3OD [20/80]): 12.32 (C18′),19.2 (C21′), 19.76 (C19′), 21.94 (C11′), 22.91 (C27′), 23.17 (C26′), 24.7 (C23′), 25.1 (C15′), 27.22 (C5), 28.89 (C25′), 29 (C2′), 29.1 (C12′), 32.8 (C7′), 32.92 (C8′), 36.29 (C22′),36.81 (C10′), 37.12 (C1′), 37.99 (C6), 38.11 (C1), 39.48 (C2), 40.45 (C24′), 40.80 (C16′), 46.13 (C4′), 51.23 (C9′), 57.22 (C17′), 57.80 (C14′), 62.41 (C6a), 63.4 (C6a), 70.02 (C5b), 70.63 (C2a), 72.8 (C3a), 73 (C3′), 73.18 (C9), 74,75 (C2b), 76.8 (C3a), 81 (C4b), 92.39 (C1a), 105.2 (C3′), 123.42 (C6′), 140.72 (C5′), 154.8 (C1a), 156.2 (NHCOOChol), 173.17 (C8); α-*pyranose form*: identical data except, ^{1}H NMR (400 MHz, CD_3OD/$CDCl_3$ [80/20]): δ_H = 7.04−7.05 (d, $^3J_{1a-2a}$ = 5.6 Hz, 1H, H1α), 5.05−5.07 (m, 1H, H2a), 4.09−4.11 (m, 1H, H3a);^{1}H NMR (270 MHz, CD_3OD): (m, 1H, H3′) missing, presumably underneath solvent peak; confirmed by ^{1}H NMR (300 MHz, DMSO): δ = 4.7−4.85 (m, 1H, H3′). Proton resonance assignments were confirmed using ^{1}H gradient type DQF−COSY and TOCSY; ^{1}H/^{13}C correlation and DEPT 135 were used to assign unambiguously the carbon resonances. ^{1}H phase-sensitive NOESY confirmed conformation.

Synthesis of Maltose Compound (12f). This compound was prepared with a solution of D-maltose monohydrate (30 mg, 1.8 mmol, 5 equiv) and **11** (100 mg, 0.16 mmol) in a similar way to the preparation of **12e**, stirred for 1 day, and purified by chromatography (CH_2Cl_2/MeOH/NH_3 75:22:3) to afford **12f** as a white solid (100 mg, yield: 65%). The purity was further confirmed by HPLC. The final product contained of the α-pyranose (87%) form and *β*-pyranose (13%) form that were not isolated but characterized in the mixture. MS (FAB$^+$): m/z = 927 [M + H]$^+$, 765, 588, 559, 484, 369 [chol]$^+$, 322, 290, 213, 167, 161, 143, 135, 121, 109, 95, 81, 69, 55; *β-pyranose form*: ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD [80/20]): δ = 7.55−7.57 (d, $^3J_{1a-2a}$ = 5.3 Hz, 1H, H1a), 5.3 (s, 1H, H6′), 4.85−5.02 (m, 1H, H3′), 4.09−4.22 (m, 1H, H1b, 3.57−4 (m, 7 H, H3a, H4a, H5a, H4b, H5b, H6b), 3.2−3.6 (m, 8H, H2, H4, H6a, H2b, H3b, MeOH), 2.8−

3.1 (m, 4H, H1, H6), 2.1−2.36 (m, 2H, H4′), 1.6−2.05 (m, 5H, H2′, H7′, H8′), 1−1.6 (m, 23H, H5, H1′, H9′, H11′, H12′, H14′−H17′, H22′−H25′), 0.93 (3H, s, H-19′), 0.83 (d, J = 6.5 Hz, 3H, H21′), 0.78 (d, J = 6.5 Hz, 6H, H26′/H27′), 0.6 (s, 3H, H18′); α-*pyranose form*: identical data except, ^{1}H NMR (300 MHz, $CD_3OD/CDCl_3$ [80/20]): δ = 6.92−6.94 (d, J = 4.62 Hz, 1H, H1a), 5.02−5.15 (m, 1H, H2a), 4.04−4.08 (m, 1H, H3a)

Synthesis of Maltotriose Compound (12g). This was prepared with a solution of maltotriose (246.4 mg, 0.46 mmol, 7 equiv) and **11** (40 mg, 0.066 mmol) in a similar way to the preparation of **12e**, stirred for 5 days, and purified by chromatography (CH_2Cl_2/MeOH/NH_3 75:22:3) to afford **12f** as a white solid (61 mg, yield: 85%). The purity was further confirmed by HPLC. The final product contained of the α-pyranose (15%) form and β-pyranose (85%) form that were not isolated but characterized in the mixture. MS (FAB$^+$): m/z = 1111 [M + Na]$^+$, 1089 [M + H]$^+$, 588, 423, 391, 369 [chol]$^+$, 240, 171, 159, 145, 121, 105, 95, 81, 69; *β-pyranose form*: ^{1}H NMR (300 MHz, $CDCl_3$/MeOH[20/80]): δ = 7.56−7.58 (d, J = 6 Hz, 1H, H1a), 5.2−5.27 (m, 1H, H6′), 4.9−4.95 (m, 1H, H3′), 4.2−4.45 (m, 4H, H9, H3′, H2a), 4.05−4.2 (m, 2H, H1b, H1c), 2.95−4 (m, 21H, H2, H4, H6a, H3a, H5a, H4a, H2b-6b, H2g-6 g, MeOH), 2.85−2.95 (m, 4H, H1, H6), 2.2−2.3 (m, 2H, H4′), 1.8−2.1 (m, 5H, H2′, H7′, H8′), 0.98−1.6 (m, 23H, H5, H1′, H9′, H11′, H12′, H14′−H17′, H22′−H25′), 0.94 (3H, s, H-19′), 0.84 (d, J = 6.5 Hz, 3H, H21′), 0.78 (d, J = 6.5 Hz, 6H, H26′/H27′), 0.61 (s, 3H, H18′); α-*pyranose form*: identical data except, ^{1}H NMR (300 MHz, $CDCl_3$/MeOH[20/80]): δ = 6.85 (d, J = 5.6 Hz, 1H, H1a).

Synthesis of Maltotetraose Compound (12h). This was prepared with a solution of D-maltotetraose (200 mg, 0.3030 mmol) and **11** (80 mg, 0.133 mmol, stirred for 5 days and purified by chromatography (CH_2Cl_2/MeOH/NH_3 75:22:3) to afford **12h** as a white solid (67.5 mg, yield: 41%). The purity was further confirmed by HPLC. The final product contained of the α-pyranose (15%) form and β-pyranose (85%) form that were not isolated but characterized in the mixture. MS (FAB$^+$): m/z = 1273 [M + Na]$^+$, 1251 [M + H]$^+$, 588, 369 [chol]$^+$, 159, 145, 121, 109, 95, 81, 69; HRMS (FAB$^+$) $C_{59}H_{102}N_4O_{24}Na$: [M + Na]$^+$ calcd 1273.6782, found 1273.6821. *β-pyranose form*: ^{1}H NMR (300 MHz, $CDCl_3$/MeOH[20/80]): δ = 7.56−7.58 (d, 1H, H1a), 5.15−5.25 (m, 1H, H6′), 4.95−5.1 (m, 1H, H3′), 4.38−4.5 (m, 4H, H9, H3′, H2a), 4.04−4.22 (m, 3H, H1b, H1g, H1d), 3.1−3.95 (m, 27H, H2, H4, H6a, H3a, H5a, H4a, H2b-6b, H2g-6g, H2d-6d, MeOH), 2.85−3.1 (m, 4H, H1, H6), 2.2−2.33 (m, 2H, H4′), 1.75−2.1 (m, 5H, H2′, H7′, H8′), 1−1.6 (m, 23H, H5, H1′, H9′, H11′, H12′, H14′−H17′, H22′−H25′), 0.92 (3H, s, H-19′), 0.82 (d, J = 6.5 Hz, 3H, H21′), 0.78 (d, J = 6.5 Hz, 6H, H26′/H27′), 0.68 (s, 3H, H18′); α-*pyranose form*: identical data except, ^{1}H NMR (300 MHz, $CDCl_3$/MeOH[20/80]): δ = 7 (d, 1H, H1a).

Synthesis of Maltoheptaose Compound (12i). This was prepared with a solution of D-maltoheptaose (100 mg, 0.08673 mmol) and **11** (30 mg, 0.0497 mmol) stirred for 7 days and purified by chromatography (CH_2Cl_2/MeOH/NH_3 75:22:3) to afford **12i** as a white solid (46 mg, yield: 53%). The purity was further confirmed by HPLC. The final product contained of the α-pyranose (15%) form and β-pyranose (85%) form that were not isolated but characterized in the mixture. MS (FAB$^+$): m/z = 1759 [M + Na]$^+$, 1737 [M + H]$^+$, 369 [chol]$^+$, 145, 121, 109, 95, 81; *β-pyranose form*: ^{1}H NMR (300 MHz, $CDCl_3$/MeOH[20/80]): δ = 7.53−7.58 (d, 1H, H1a), 5.35−5.37 (m, 1H, H6′), 4.97−5.12 (m, 1H, H3′), 4.45−4.6 (m, 4H, H9, H3′, H2a), 4−4.5 (m, 6H, H1b, H1c−g), 3.1−3.9 (m, 45H, H2, H4, H6a, H3a, H5a, H4a, H2b-6b, H2c-6c, H2d−6d, H2e-6e, H2f−6f, H2g-6 g, MeOH), 2.7−3 (m, 4H, H1, H6), 2.15−2.35 (m, 2H, H4′), 1.7−2.1 (m, 5H, H2′, H7′, H8′), 1−1.6 (m, 23H, H5, H1′, H9′, H11′, H12′, H14′−H17′, H22′−H25′), 0.94 (3H, s, H-19′), 0.84 (d, J = 6.5 Hz, 3H, H21′), 0.77 (d, J = 6.5 Hz, 6H, H26′/H27′), 0.63 (s, 3H, H18′); α-*pyranose form*: identical data except in ^{1}H NMR (300 MHz, $CDCl_3$/MeOH[20/80]): δ = 6.9 (d, 1H, H1a).

Preparation of Liposomes DC−Chol (7.5 mg, 15 μmol) and DOPE (7.5 mg, 10 μmol) were combined in dichloromethane. The solution was transferred to a round-bottomed flask (typically 50 mL) and organic solvent removed under reduced pressure (rotary evaporator) giving a thin-lipid film that was dried for in vacuo. Following this, 4 mM HEPES buffer, pH 7.2 (3 mL) was added to the round-bottomed flask so as to hydrate the thin-lipid film. After brief sonication (2−3 min) under argon, the resulting cationic liposome suspension (lipid concentration of 5 mg/mL) was extruded 10 times through two stacked 100 nm polycarbonate filters (Millipore, Ireland) to form small unilamellar cationic liposomes (average diameter 105 nm according to PCS analysis). Lipid concentrations (approximately 4−4.8 mg/mL) were determined by inorganic phosphorus assay (*61*).

Preparation of LMD and LD Systems. First, mu:DNA (MD) particles were prepared by mixing as follows: plasmid DNA stock solutions (typically 1.2 mg/mL) were added to a vortex-mixed, dilute solution of mu peptide (1 mg/mL) in 4mM HEPES buffer, pH 7.2. The final mu:DNA ratio was 0.6:1 (w/w), unless otherwise stated, and final plasmid DNA concentration was 0.27 mg/mL. MD containing solutions were then added slowly under vortex conditions to suspensions of extruded cationic liposomes (typically approximately 4.5 mg/mL), prepared as described above, resulting in the formation of small LMD particles with narrow size distribution (120 ± 30 nm) as measured by PCS. Final lipid:mu:DNA ratio 12:0.6:1 w/w/w. A solution of sucrose (100%, w/v) in 4 mM HEPES buffer, pH 7.2, was then added to obtain LMD particle suspensions in 4 mM HEPES buffer, pH 7.2, containing 10% w/v sucrose at the desired DNA concentration (final DNA concentration typically 0.14 mg/mL) and the whole stored at −80 °C. LD systems were prepared for experiments with a lipid:DNA ratio of 12:1 (w/w) following the same vortex protocol without the addition of mu peptide.

Preparation of Stabilized LMD Systems. Premodified, stabilized LMD were prepared by adding the defined molar percent of the stealth molecule with DC−Chol and DOPE in dichloromethane/methanol (3:1) followed by the described preparation of liposome and LMD. Postmodified stabilized LMD were prepared by incubating unmodified LMD with the required volume of an aqueous solution (1 mM) of stealth molecule (DSPE-PEG or neoglycolipid) for 2 h at room temperature.

Particle Size Measurement. The sizes of all LMD and potential sLMD particles were evaluated after 30 min exposure at 37 °C to biological media by photon correlation spectroscopy (N4 plus, Coulter). The particular chosen pDNA concentration was selected for compatibility with in vitro conditions (1 μg/mL of DNA). The parameters used were: 20 °C, 0.089 cP, reflexive index of 1.33, angle of 90°, λ = 632.8 nm. Unimodal analysis was used to evaluate the mean particle size in OptiMEM. The size distribution program using the CONTIN algorithm was utilized to separate the subpopulation of small serum particles of less than 50 nm and to extracted the

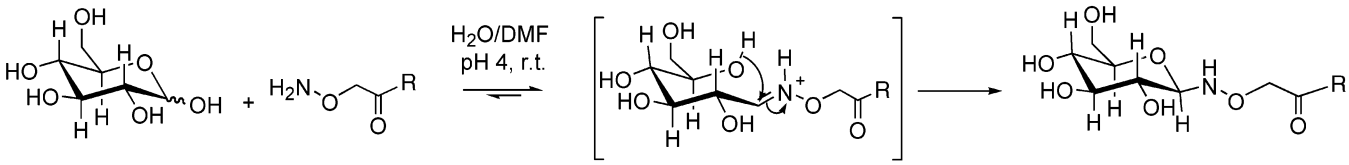


Figure 2. Suggested mechanism of the reaction of glucose (and other carbohydrates with reducing termini) with aminoxy lipid **11** (R = 4-aza-N^6-(cholesteryloxycarbonylamino)hexylamine). After the formation of an oxime bond, intramolecular acetalization affords the cyclic carbohydrate to generate closed-ring glycolipids.

calculated size of lipoplexes in OptiMEM in the presence of 10% FCS.

Critical Micelle Concentration. Micellar forms of the neoglycolipids or PEG-lipids were prepared by sonication of a solution of neoglycolipids (5 mM) or DSPE-PEG (1 mM) in HEPES (4 mM, pH 7) containing 10^{-7} M pyrene. These stock solutions were used to generate different concentrations by serial dilutions with the same buffer. Each sample was incubated with pyrene for 24 h in darkness at 4 °C prior to measurement of fluorescence intensity on a Shimadzu RF-5301PC (Milton Keynes, UK) spectrofluorophotometer, excitation wavelength was 332 nm and emission wavelength was 392 nm.

Turbidity Measurement. Sixty microliters of LMD of different compositions at 100 μg/mL were mixed with 240 μL of serum, and the mixtures were incubated at 37 °C with gentle shaking. The absorbance at 600 nm was then recorded at different times with serum alone as blank reference.

EtBr Exclusion Assay. EtBr solution (2 μL, 0.1 mg/mL) was added to a sample of 1.5 μg ($\approx$1.5 μL) of DNA and 60 μL of FCS, incubated for different lengths of time before complemented with HEPES 4 mM pH 7.2 to a final volume of 300 μL. Samples were excited at 310 nm (slit widths of 5 nm for both the excitation and emission) and emission intensity measured at 590 nm in order to determine the effect of serum upon pDNA-intercalation by EtBr. The fluorescence emission intensity, I_{590}, usually stabilized in less than 3 min and was reported as a fraction of the maximum fluorescence obtained when EtBr was added to free plasmid DNA alone.

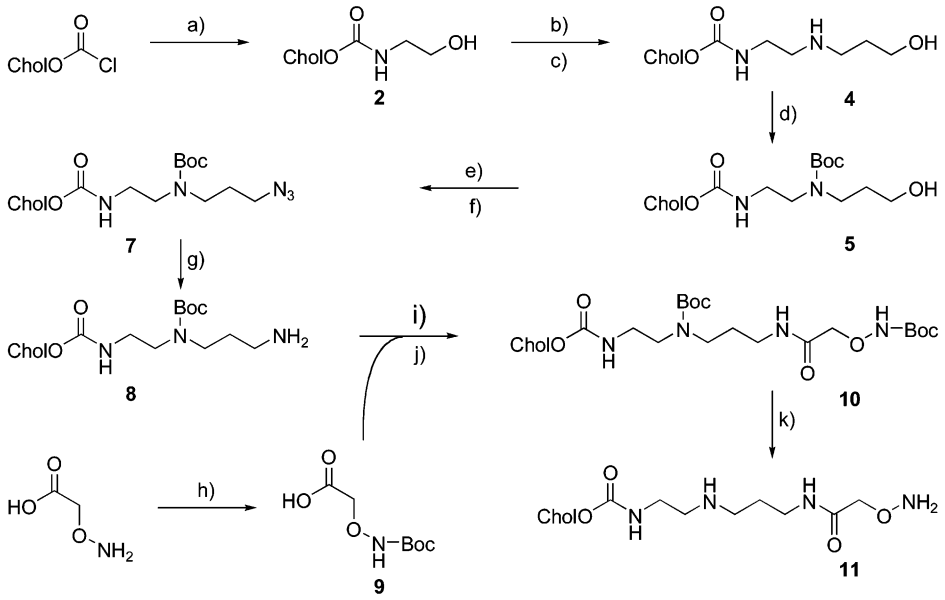
Transfection of HeLa Cells. Cells ($\approx$50 000) were seeded in a 24-well culture plate in DMEM supplemented with 10% FCS and grown to approximately 70% confluence for 24 h at 37 °C in the presence of 5% CO_2. The cells were washed with PBS before the transfection media was added to each well (0.5 mL of solution with 0, 50, or 100% FCS in OptiMEM). LMD or potential sLMD systems as appropriate (5 μL, 100 μg/mL pDNA) were added to each well containing the HeLa cells and incubated for a transfection time of 1 h. Cells were then rinsed three times with PBS and incubated for a further 24 h in DMEM supplemented with 10% FCS prior to final determination of β-galactosidase (β-Gal) enzyme activity in cells post transfection by means of a chemioluminescent reporter gene assay kit (Roche).

RESULTS AND DISCUSSION

Synthesis of Neoglycolipids. Highly convergent syntheses were devised involving first the preparation of a cholesterol-based cationic lipid containing an aminoxy functional group, followed by the chemoselective coupling of this lipid to commercially available, completely unprotected monosaccharides or oligosaccharides. Chemoselective coupling of the lipid to such unprotected sugars was enabled by the preference of the aminoxy functional group to react only with the aldehyde functional group revealed in the open-chain form of aldose monosaccharides or in the open chain form of terminal glycose residues (reducing termini) of oligosaccharides (Figure 2). The synthesis of Boc-protected amino-lipid **8** was based upon our published methodologies for the syntheses of other cationic cholesterol-based lipids from readily available amino-alcohols as starting materials employing complementary blocking group strategies to protect secondary amino functional groups originated during syntheses (*6*, *22*). Hence the synthesis of Boc-protected amino-lipid **8** was accomplished without major difficulty although requiring a few simple modifications to existing protocols. Compound **8** was then converted into Boc-protected aminoxylipid **10** by means of carboxylic acid **9** (Scheme 1). Acid **9** was prepared by Boc-protection of commercially available *O*-(carboxymethyl)-hydroxylamine hydrochloride and then activated by means of *N*-hydroxysuccinimide (NHS) and *N*,*N*′-dicyclohexylcarbodiimide (DCC) in order to promote clean acylation of aminolipid **8** in THF in situ affording **10** in good yield. Thereafter, synthesis of aminoxy lipid **11** was accomplished by Boc-deprotection of **10** with aqueous trifluoroacetic acid. Finally, the combination of aminoxy lipid **11** with a variety of aldose monosaccharides and the glucose (reducing) terminal residue of oligosaccharides was performed in order to realize a corresponding variety of neoglycolipids with a similar general formula (Figure 1). Coupling reactions were conducted under mild conditions making use of a solvent buffer system comprising DMF and aqueous acetic acid (1:1, v/v), pH 4, selected to optimize simultaneously both the combined solubilities of sugars and aminoxy lipid **11**, together with aminoxy functional group reactivity. At pH 4, the proton concentration is approximately optimal for the addition−elimination mechanism that leads to alkoxime functional group formation (Figure 2). Acid-catalyzed ring closure (acetalization) then completes the process leading to the generation of mono- and oligosaccharidoneoglycolipid products with an aminoxy-glycopyranose residue attached to the lipid moiety. Reaction times and yields of all coupling reactions carried out are given (Table 1). Further optimization could reduce the reaction times required. Analytical HPLC and NMR studies of all isolated aminoxyglycopyranose products were carried out revealing that each product was comprised of two isomers (major and minor) after workup and purification. In the case of each product, these two isomers were presumed to result from the formation of both α and β anomers of the aminoxy glycopyranose residue during the ring closure.

Neoglycolipid Conformations. Conformational analysis of the neoglycolipids was performed by ^{1}H NMR (*62*), with a particular emphasis upon the anomeric conformations of the aminoxy glycopyranose residue in each neoglycolipid product using heteronuclear $^1J_{13C1a-H1a}$ coupling constants to diagnose both presence and relative proportions of α and β anomers associated with the aminoxy anomeric carbon center (C1a) (*63*). The absolute value of $^1J_{13C1-H1}$ coupling constants in typical glycopyranose ring systems depends on a number of factors including the orientation of the C1−H1 bond relative to

Scheme 1. Synthesis of the Lipid Moiety[a]



[a] Reagents: (a) CH_2Cl_2, $HO(CH_2)_2NH_2$ (2.2 equiv), 10 h, 97%; (b) CH_2Cl_2, 0 °C, Et_3N (3 equiv), MsCl (2.5 equiv), 10 min; then 1 h at rt, 98%; (c) THF, $HO(CH_2)_3NH_2$ (10 equiv), 6 h, 96%; (d) CH_2Cl_2, Et_3N, Boc_2O, rt, 5h, 90%; (e) CH_2Cl_2, 0 °C, Et_3N (3 equiv), MsCl (2.5 equiv), 10 min; then 2 h at rt, 90%; (f) DMF, 80 °C, NaN_3 (5 equiv), NaI (1 equiv), 3 h, 95%; (g) (i) THF, PMe_3 (1.15 equiv), rt, 3 h, (ii) H_2O/NH_3, 88%; (h) CH_2Cl_2, Et_3N, Boc_2O, rt, 5 h, 98%; (i), EtOAc, *N*-hydroxysuccinimide (1 equiv), DCC (1 equiv), 10h., rt; (j), EtOAc/THF [95/5], Et_3N (pH 8), 2h, rt, 90%; (k) CH_2Cl_2, TFA (15 equiv), 0 °C, N_2, 5 h, 86%.

Table 1. Yields, Reaction Times, and Diastereoselectivity of the Formation of Neoglycolipids 12a−i

product	carbohydrate	reaction time (days)	yield (%)	β/α
12a	mannose	3	65	82/18
12b	glucose	1	80	89/11
12c	galactose	1	70	85/15
12d	glucuronic acid	1	60	85/15
12e	lactose	4	50	85/15
12f	maltose	1	65	87/13
12g	maltotriose	5	85	85/15
12h	maltotetraose	5	40	85/15
12i	maltoheptaose	7	55	85/15

adjacent lone pairs of the glycopyranose ring oxygen atom and the electronegativity of the other substituent attached to the glycopyranose ring system. Differences between the $^1J_{13C1-H1}$ coupling constants of α- and β-glycopyranose ring systems have been used previously to determine relative ratios of the two anomeric conformations. Typically, the difference of $^1J_{13C1-H1eq}$(α-anomer) minus $^1J_{13C1-H1ax}$(β-anomer) is $\approx +10$ Hz. In our case, taking the aminoxy mannopyranose neoglycolipid **12a** as an example, the major isomer exhibited a $^1J_{13C1a-H1a} =$ 167 Hz and the minor isomer a value of $^1J_{13C1a-H1a} = 177$ Hz, thereby identifying the major isomer as the β-anomer (approximately 80%) and the minor isomer as the α-anomer (approximately 20%). ^{1}H-phase sensitive NOESY confirmed the identification of the major and minor isomers. HPLC analysis further detailed the β:α ratio more precisely as 82:18. Similar anomeric ratios were observed consistently throughout all the neoglycolipid series prepared as part of the studies described here (Table 1). The high β:α ratio of 82:18 determined for the aminoxy mannopyranose neoglycolipid **12a** is unusual. Mannopyranose rings exhibit an axial hydroxyl functional group at C2 that frequently helps to promote α-anomer formation. A possible explanation is that this reaction could be controlled kinetically by a pattern of hydrogen bonding between the sugar and the aminoxy linker, stabilizing the β anomer. For the purposes of the studies described below, we elected to proceed without attempting to resolve and purify each isomer separately by HPLC particularly given that we suspected isomeric mixtures may offer more benefit in terms of stabilizing synthetic nonviral vector systems in biological fluids than not. Obviously, were any of our neoglycolipids required for receptor targeting purposes then the reverse would certainly be true and additional HPLC resolution and purification would be required.

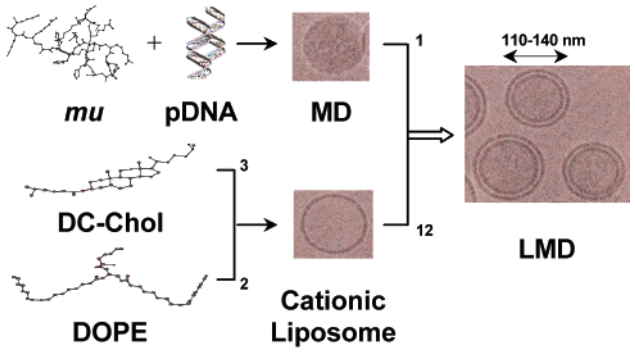


Figure 3. Formulation of LMD systems. Plasmid DNA (pDNA, D) is precondensed with cationic adenoviral peptide μ (mu, M) (mu:pDNA 0.6:1, w/w) to generate MD particles (N/P = 0.7) that are further complexed with extruded cationic liposomes prepared from cytofectin DC−Chol and neutral lipid DOPE to generate LMD vectors. Picture of LMD, MD and liposomes were obtained by cryoelectron microscopy as described previously (*21*).

Formulation of Stabilized LMD Systems (sLMD). First generation LMD systems are prepared as illustrated (Figure 3) (*21*). To generate potential stabilized-LMD (sLMD) systems equipped with a "stealth" barrier sufficient to stabilize these systems against aggregation and inactivation in biological fluids, two alternate formulation procedures were considered as a means of introducing putative stealth molecules such as DSPE-PEG or a neoglycolipid into LMD particles. In the premodification formulation process, putative stealth molecules can be introduced into cationic liposomes prior to potential sLMD particle formation; in postmodifica-

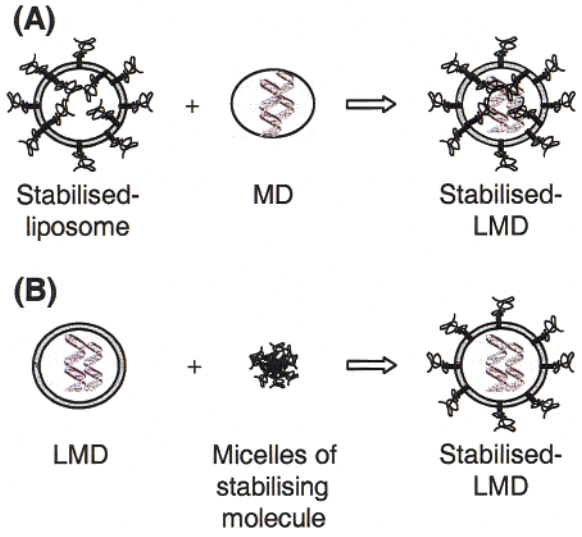


Figure 4. Principles of (**A**) pre- and (**B**) postmodification of LMD particles to generate potential stabilized-LMD (sLMD) system particles.

tion, LMD particles can be prepared first and then combined with putative stealth molecules at the end of the process in order to create potential sLMD particles (Figure 4). Premodification requires that putative stealth molecules are included with the lipid mixture prior to cationic liposome formulation by either dehydration−rehydration (DRV) or reverse-phase evaporation (REV) procedures. Formulation of such a ternary mixture into cationic liposomes then results in "stabilized" cationic liposome (sL) systems wherein putative stealth molecules become distributed on both sides of the cationic liposome bilayer as part of both inner and outer leaflets. Such a distribution was thought to impede the formulation of sLMD particles as illustrated (Figure 4) by creating a steric barrier to plasmid DNA (pDNA) interactions with the cationic liposome bilayer. This is a potentially serious drawback. Hence cationic liposome-forming lipids such as the cationic lipid (cytofectin) 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl]cholesterol (DC−Chol) and the neutral lipid dioleoyl L-α-phosphatidylethanolamine (DOPE) used in our studies here, needed to be combined in a suitable solvent able to cosolubilize the putative stealth molecule as well.

The postmodification process requires that LMD particles are prepared first and stealth molecules may be surface absorbed into the outer leaflet of LMD particles to generate potential sLMD particles. The practical realization of this process was thought possible by combining LMD particles with solutions comprising putative stealth molecules at concentrations in excess of their critical micelle concentration (cmc) (*64*). Amphiphilic monomers such as lipids are known to transfer from one lipid phase to another via the aqueous phase (*65*). Hence, monomers with a relatively low cmc can therefore be expected to adopt a liposome-inserted over a solution-free state if mixed in the presence of liposome systems (*66*, *67*). Such solution-to-liposome transfer processes have been used to generate stabilized liposome systems for drug delivery applications from liposomes and stealth molecule mixtures (*68*, *69*). However, the main drawback of this form of postmodification process is the possibility that the transfer process may be inefficient depending upon the nature of the liposome system involved or the putative stealth molecule under investigation. The incorporation kinetics and equilibrium states of this solution-to-liposome transfer phenomenon

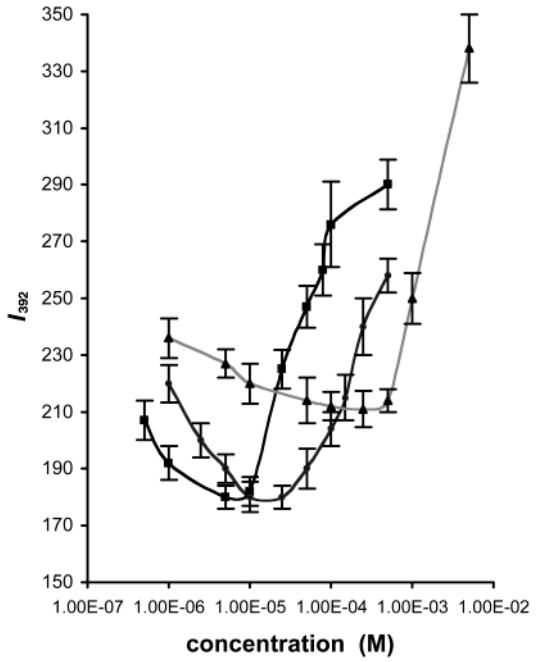


Figure 5. Determination of the cmc for DSPE-PEG (■), **12c** (○), and **12g** (▲). Pyrene (10^{-7} M) fluorescence intensity, I_{392}, in 4 mM HEPES, pH 7, was plotted as a function of concentration with measurements made after samples had been incubated together for 24 h in darkness at 4 °C. Means ± SD, $n = 3$. The cmc was determined from the crossover point of the two straight-lines corresponding to the two domains of each curve.

Table 2. Critical Micelle Concentrations (cmc) of Neoglycolipids Investigated in This Study

product	carbohydrate	cmc
12a	mannose	2×10^{-5} M
12b	glucose	3×10^{-5} M
12c	galactose	2×10^{-5} M
12e	lactose	7×10^{-5} M
12f	maltose	7×10^{-5} M
12g	maltotriose	4×10^{-4} M
12h	maltotetraose	8×10^{-4} M

are not very well understood (*68*); accordingly, applications of this insertion technique in the field of nonviral gene therapy do not appear to be very numerous (*70*).

Cmc Measurements. Prior to the insertion of neoglycolipids or DSPE-PEG into LMD particles, the critical micellar concentrations of all these putative stealth molecules was determined as described previously by means of a fluorescence-based assay (*64*, *71*). Pyrene fluorescence intensity, I_{392}, was measured in HEPES 4 mM, pH 7 at 20 °C and plotted as a function of stealth molecule concentration. A typical data set obtained with DSPE-PEG, **12c**, and **12g** is shown (Figure 5). The cmc was identified by the straight-lines crossover point corresponding to the two domains of each curve and was found to be 8.5 μM for DSPE-PEG. This value agrees well with a DSPE-PEG cmc of 9 μM measured by others at 37 °C in Tris-HCl buffer (*72*). The cmc of DSPE-PEG is significantly higher than for DSPE (more than 10^6 times), consistent with an increase in hydrophilicity by virtue of PEG-conjugation. Cmc values were determined for all the neoglycolipids (Table 2) as well with the exception of the glucuronic acid based compound **12d** and the maltoheptaose-based compound **12i** that were both poorly soluble under the assay conditions used. Compound **12i** in particular was thought to be poorly soluble above millimolar concentrations owing to the peculiar properties of long chain carbohydrates. Inter-oligosaccharide

interactions in water are often cooperative, leading to higher order supramolecular structures. As an example, it is interesting to note the crystallization behavior of maltooligosaccharides; on crystallization from water, maltooligosaccharides ($N > 5$) chains can adopt left-handed parallel stranded double helical conformations, which pack into monoclinic and hexagonal arrays (*73*, *74*). The formation of such supramolecular structures by the carbohydrate headgroups is a possible explanation for the insolubility of these glycolipids. This phenomenon has been observed in different conditions with maltopentaose-based glycolipids (*75*−*77*).

Aggregation of Potential sLMD Systems in Salt Medium. A variety of sLMD systems were prepared by pre- and postmodification procedures as described in the Experimental Section and then studied for stability toward salt-induced aggregation in OptiMEM. OptiMEM is the classical medium of choice for in vitro cell transfection. This serum-free tissue culture medium contains the main salts required for cell functions. It also contains the minimum amino acids and vitamins to guarantee cell survival during the transfection process. Aggregation of potential sLMD systems was followed by photon correlation spectroscopy (PCS). The colloidal instability of first generation LMD particles induced by salt results in aggregates of different sizes depending upon the incubation conditions. The higher the salt concentration, the temperature, and the time of incubation, the larger appear to be the resulting aggregates measured by PCS. Under such conditions, particles depending upon their surface properties are able to interact substantially at short range via attractive van der Waals forces, owing to the shielding of long-range electrostatic forces (*78*). In the case of LMD vectors, the lipids used have a strong tendency to generate hydrophobic interactions and therefore promote the formation of such aggregates. Aggregation propensities of first generation LMD particles and potential sLMD system particles were judged as a function of particle size increases after 30 min incubation at 37 °C in OptiMEM. The quality of the stealth barrier properties of either DSPE-PEG or a given neoglycolipid inserted into the surface cationic liposome bilayer were then assumed to be inversely related to the percentage particle size increase. Data are shown generated with potential sLMD systems prepared by both postmodification (Figure 6A) and premodification procedures (Figure 6B). When premodified, the best stabilization was achieved with formulations using glycolipids containing two glycosidic units. However, we were unable to formulate LMDs with glycolipids containing the longest carbohydrate units (4 or 7 units) at 5 or 10 molar %. Postmodified neoglyco-LMD systems tended to be smaller with increasing chain length of the carbohydrate unit of the glycolipid (Figure 6A). This result is consistent with increased hydrophilicity and steric repulsion (*67*, *79*) expected if these molecules were exposed on the outer layer (molecular brushes). Particle stability induced by the largest maltoheptaose-based neoglycolipid **12i** at 10 mol % was substantial, suggesting that **12i** possessed significant stealth molecule properties in high salt conditions.

As expected, PEG-LMD systems were the most stable above 2−5 mol % PEG included by pre - or postmodification. This result reflects the widespread use of PEG as a stabilization agent in drug delivery. It is interesting to note that PEG does not interfere with the formulation when premodification technique is used even at the highest molar ratio used.

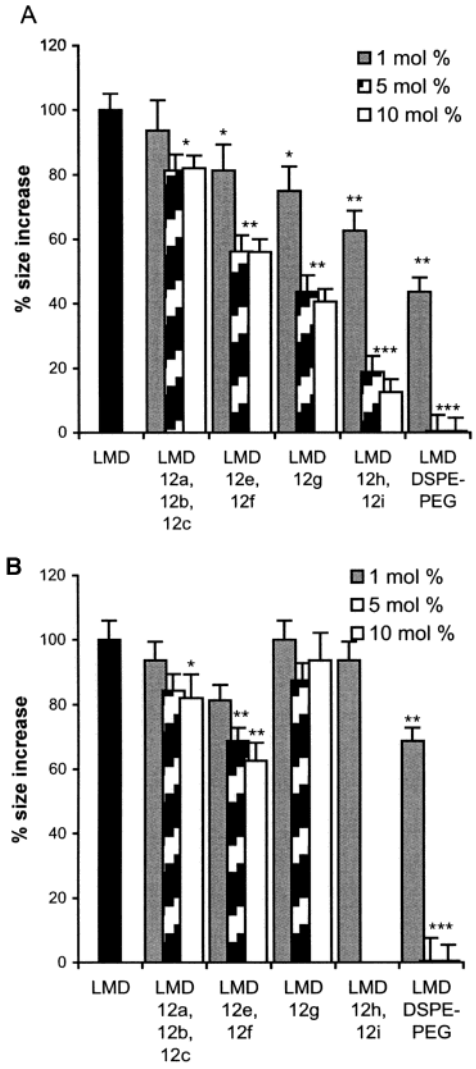


Figure 6. (A, B) Aggregation profile of neoglycolipid formulated stabilized LMD (sLMD) vector systems in OptiMEM. LMD formulations were incubated for 30 min at 37 °C OptiMEM at a DNA concentration of 1 mg/mL. The size increase due to salt-induced aggregation was characterized by photon correlation spectroscopy (PCS), and size increases were normalized with respect to unmodified LMD particle size increases (100%). A; postmodification: sLMD systems were prepared by incubation of LMD particles with a micellar solution of putative stealth molecules at the indicated molar ratios relative to total lipid concentration. B; premodification: sLMD systems were prepared directly from cationic liposomes formulated with the indicated molar ratios of putative stealth molecules relative to total lipid concentration. Means ± SD, $n = 3$. *$P < 0.05$; **$P < 0.005$: ***$P < 0.0005$ (statistical analysis was calculated by Student's *t*-test when stabilized LMD were compared to unmodified LMD).

Aggregation of Potential sLMD Systems in Serum. Serum used in laboratories is usually derived from bovine (BS) or fetal calf (FCS). Media of cell cultures are usually supplemented with 5 to 10% serum to ensure proper cell growth. For in vitro experiments, blood is usually too difficult to handle (mainly due to clotting); therefore, experiments designed to mimic the blood and other biological fluids are frequently performed with serum alone. Serum is a complex mixture of components including serum albumin, lipoproteins, macroglobulins, fibrinogen, heparin, and fatty acids (such as oleic acid). All these components interact with LD or LMD particles rendering them ineffective for transfection either by promoting particle aggregation or disintegration (*80*).

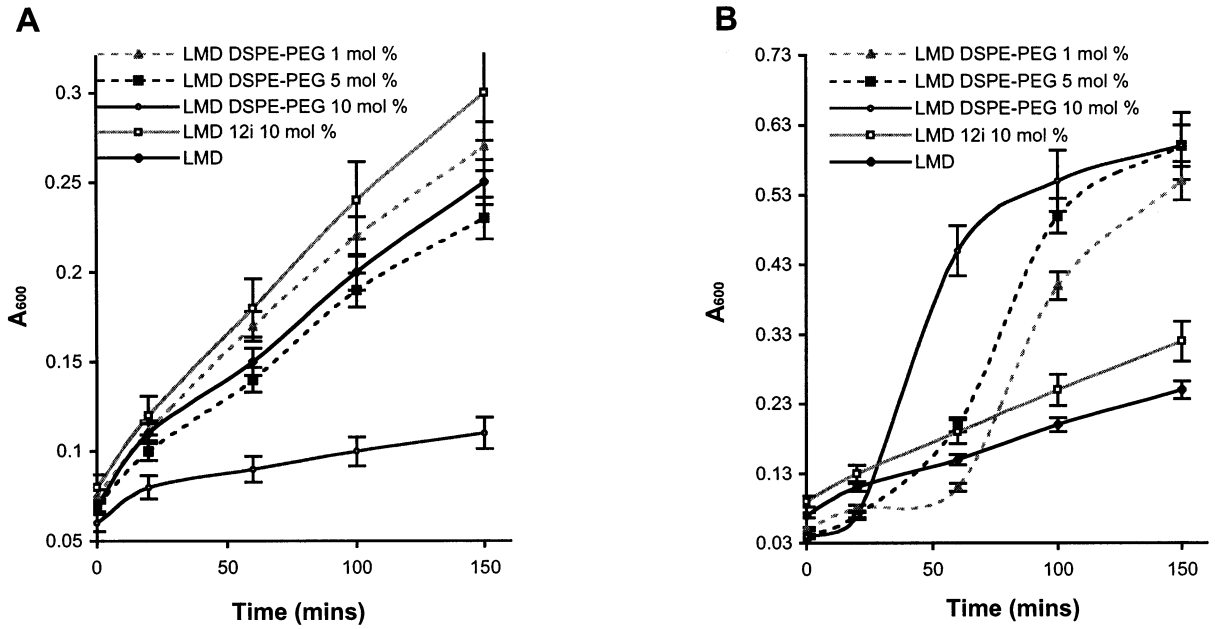


Figure 7. (A, B) Turbidity of sLMD vector systems in FCS. LMD formulations were incubated at 37 °C in FCS at a DNA concentration of 20 mg/mL. The increase in turbidity with time was measured as a function of A_{600}. A; postmodification: sLMD vectors were prepared by incubation of LMD particles with a micellar solution of putative stealth molecules at the indicated molar ratios relative to total lipid concentration. B; premodification: sLMD systems were prepared directly from cationic liposomes formulated with the indicated molar ratios of putative stealth molecules relative to total lipid concentration. Means ± SD, $n = 3$.

Stealth molecules such as DSPE-PEG were originated primarily for the purpose of stabilizing drug delivery liposomes against serum-induced effects. Given the fact that multicomponent serum renders PCS assays unusable owing to random scattering effects, two alternative assays were used to follow potential sLMD system stabilities in the presence of serum, namely turbidity and ethidium bromide (EtBr) exclusion assays.

Turbidity measurements are commonly used to diagnose the appearance of particle aggregation or fibrilization in solution over time. Particle aggregation causes enhanced light scattering particularly at long visible-light wavelengths >600 nm, and this is detected either by an increase in absorbance, A_{600}, above background as a function of time. Data generated does not extrapolate easily to particle size measurements, but aggregation can at least be observed qualitatively. Hence, experiments were conducted in which LMD systems were combined with FCS and changes in A_{600} monitored with time. As above, the quality of the stealth barrier properties conferred by either DSPE-PEG or neoglycolipid inserted into the cationic liposome bilayer of LMD systems were then presumed to correlate inversely with detectable increases in A_{600} over time. Data are shown for LMD systems prepared by both postmodification (Figure 7A) and premodification procedures (Figure 7B). Regrettably, all neoglycolipids, including **12i**, introduced by the postmodification procedure, proved unable to arrest particle aggregation in FCS at all in complete contrast to the effect of DSPE-PEG at 10 mol %. Therefore of the putative stealth molecules used in studies described here, only DSPE-PEG exhibited the full characteristics of a stealth molecule able to generate stable systems with respect to aggregation in both in salt medium and in serum conditions.

sLMD systems prepared from DSPE-PEG by the premodification procedure revealed an inversion of the DSPE-PEG stabilization effect (Figure 7B). While DSPE-PEG inclusion by premodification appeared sufficient to suppress aggregation initially, substantial aggregation effects were observed to take place between 20 and 60 min after mixing with FCS suggesting that potential sLMD particles prepared from DSPE-PEG by this method were substantially metastable with respect to serum effects. It is likely that such sLMD vectors, which are stable under high salt conditions (Figure 6), are destabilized in serum by internally disruptive steric effects deriving from inner particle distributions of DSPE-PEG molecules. This behavior is a direct consequence of the premodification procedure (Figure 4). Hence this data set (Figure 7) suggests that the postmodification procedure is the preferred modus operandi above the premodification procedure for the preparation of future effective sLMD systems.

The ethidium bromide exclusion assay is frequently used to study the extent of pDNA charge neutralization, condensation, and encapsulation in response to cationic agents such as cationic liposomes or peptides. Ethidium bromide (EtBr), an otherwise weak fluorophore, exhibits strong fluorescence, I_{590}, following pDNA intercalation. When a saturating amount of EtBr (approximately 6:1 [nucleotide]/[EtBr]) is combined with pDNA in the presence of a cationic pDNA condensing agent, pDNA condensation, and encapsulation results in EtBr displacement (exclusion) from pDNA intercalation sites leading to a substantial fall in I_{590}. Conversely, if a condensed system such as LD, LMD, or a potential sLMD becomes destabilized for whatever reason such as by general interactions with heparin and/or dextran sulfate leading to pDNA displacement, renewed EtBr intercalation may become possible as pDNA becomes reexposed with time leading to an increase in I_{590} from a basal level (*81*). Experiments were conducted once again in which potential sLMD systems were combined with FCS and changes in I_{590} monitored with time. The capacities of cationic bilayer-inserted DSPE-PEG or neoglycolipid to resist particle disintegration mediated by serum components should be inversely correlated with increases in I_{590} over time in a similar way to the turbidity measurements described above. Data are shown for potential sLMD

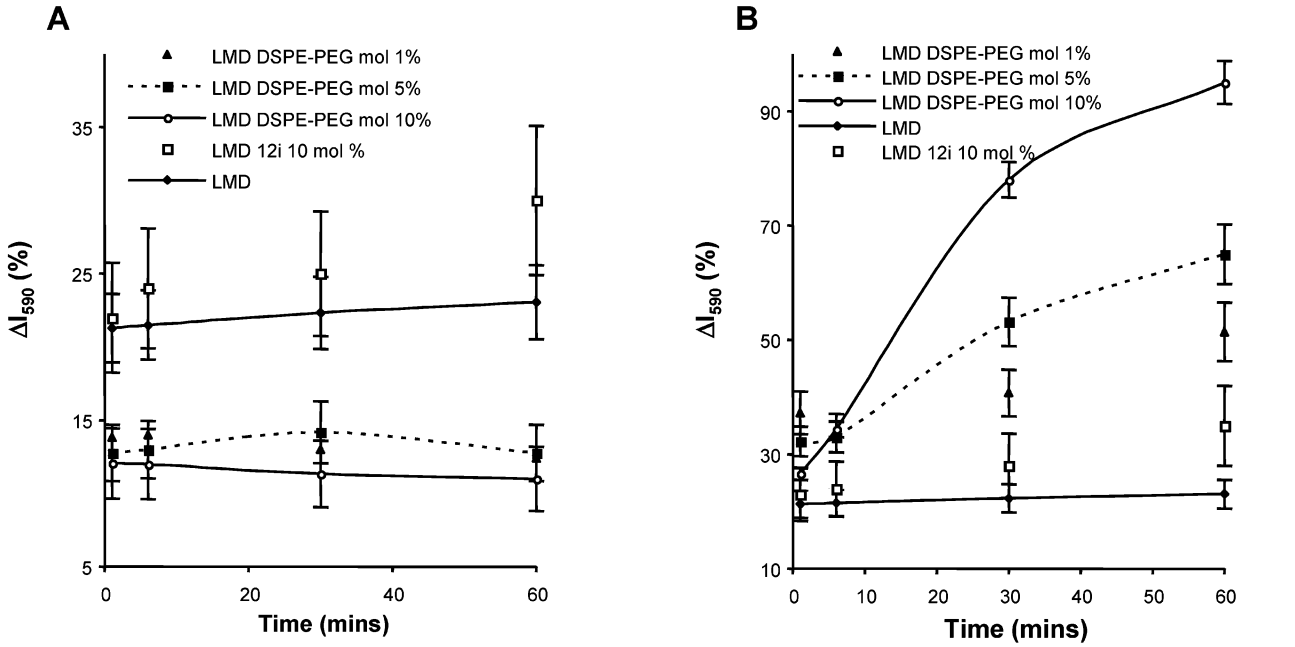


Figure 8. (A and B) EtBr exclusion assay of potential stabilized-LMD (sLMD) in FCS. LMD formulations were incubated at 37 °C in FCS at DNA concentration of 25 mg/mL. Increase in EtBr fluorescence, I_{590}, was monitored as a function of time. Observed intensities, I_{590}, were normalized by dividing through the emission intensity of equivalent levels of EtBr in the presence of equivalent levels of naked pDNA alone under analogous conditions. A; postmodification: sLMD systems were prepared by incubation of LMD particles with a micellar solution of putative stealth molecules at the indicated molar ratios relative to total lipid concentration. B; premodification: sLMD systems were prepared directly from cationic liposomes formulated with the indicated molar ratios of putative stealth molecules relative to total lipid concentration. Means ± SD, $n = 3$.

systems prepared by both postmodification (Figure 8A) and premodification procedures (Figure 8B). Data show that first generation LMD particles were remarkably robust with respect to disintegration, there being no apparent increase in I_{590} over time.

Other aspects of the exclusion assay data set indicated that neoglycolipid **12i** at 10 mol %, introduced by either the postmodification or premodification procedure, marginally impaired particle resistance to disintegration relative to first generation LMD, while DSPE-PEG at 1, 5, and 10 mol % was able to prevent particle disintegration convincingly provided that this stealth molecule was introduced by the postmodification procedure (Figure 8A). By complete contrast, when DSPE-PEG was introduced by the premodification procedure, immediate particle disintegration was observed as demonstrated by the rapid increase in I_{590} over the time period of the assay, most especially when DSPE-PEG at 10 mol % was employed (Figure 8B). This complete inversion of the DSPE-PEG stabilization effect as a direct consequence of the premodification procedure is completely in line with turbidity assay results above (Figure 7B). Furthermore, putting both data sets together suggests a degeneration process in which potential sLMD particles prepared from DSPE-PEG by the premodification procedure are first destabilized with respect to serum-induced particle disintegration by internally disruptive steric effects that derive from inner particle DSPE-PEG molecules (as mentioned above) and then subsequently become prone to aggregation. Both first generation LMD particles and neoglyco-LMD appear only to be very mildly disintegration prone in serum but substantially aggregation prone (Figures 7 and 8), characteristics that would impair their circulation profiles in vivo and result in their rapid clearance by the reticuloendothelial system.

The ability of maltoheptaose chains of **12i** to protect LMD particles from salt-induced destabilization could not be properly exploited to reduce serum protein induced aggregation. One explanation could be that the method of postadsorption does not allow sufficient micellar insertion into the bilayer due to the peculiar cmc of **12i**. Another possibility is that the sugar chains added onto LMD are still too small to actively screen the strongly cationic LMD surface. Further increase of the carbohydrate length may induce better protection but will require the synthesis of more expensive and complex molecules. Moreover, inclusion of such long chain sugar is likely to increase recognition of the carbohydrate motifs by serum amylases, lipopolysaccharide-binding proteins, or cells with specific lectin receptors. This could result in an increased clearance rate of the lipoplexes by complement activation. We believe, therefore, that future research must primarily focus on reducing the excess of cationic charges to generate better stability. Such modification should allow in our view a more favorable harvesting of neoglycolipid properties.

In Vitro Transfection Efficiency. Transfection data gathered with sLMD systems demonstrate the inhibiting effects of PEG-based compounds upon transfection efficiency in marked contrast to the beneficial effects of neoglycolipids (Figure 9) The inclusion of DSPE-PEG was found to abolish transfection irrespective of the transfection conditions, in line with previously published data demonstrating the inhibitory effects of PEG upon the transfection process (*40*, *41*). By contrast, where potential sLMD systems were prepared with neoglycolipids (postmodification), HeLa cell transfection in vitro was found to increase above first generation LMD transfection levels in proportion to the number of glycopyranose residues involved. There is an interesting correlation between increasing transfection efficiency and increasing particle stability with respect to salt-induced aggregation in OptiMEM (Figures 6 and 9). However, in serum, the transfection efficiencies of all potential sLMD systems prepared with neoglycolipids were reduced to the same levels of transfection efficiency as first generation LMD.

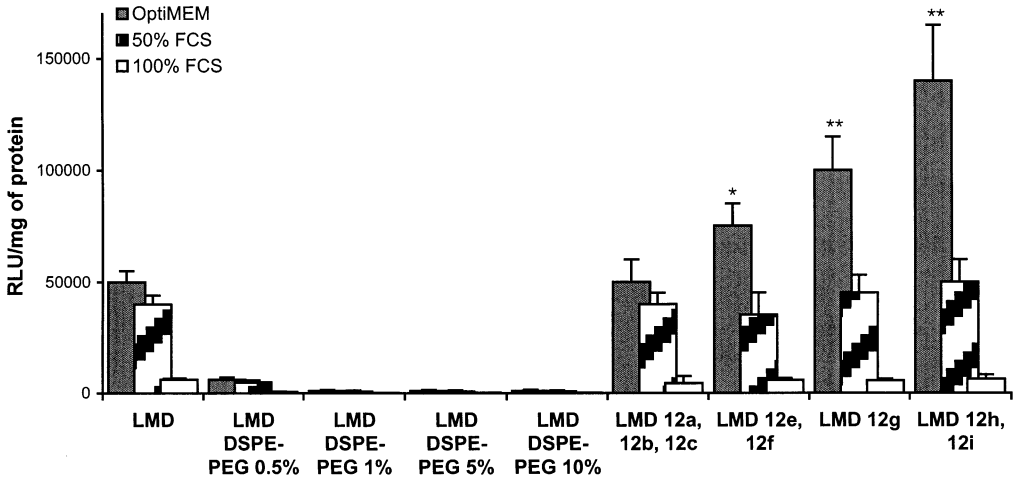


Figure 9. Transfections profile of LMD and potential stabilized LMD systems (sLMD). HeLa cells were transfected in OptiMEM, 50% FCS and 100% FCS for 1 h (48-well plate, 0.25 μg of DNA). Unmodified reference LMD transfection is compared with transfections by potential sLMD systems prepared (postmodification procedure) with either the indicated levels of DSPE-PEG or else 7.5 mol % of each indicated neoglycolipid. Means ± SD, $n = 4$. One representative of three experiments is shown. *$P < 0.05$; **$P < 0.005$ (statistical analysis was calculated by Student's *t*-test when stabilized LMD were compared to unmodified LMD).

This result is consistent with the failure of inserted neoglycolipids to make any controlling impact upon serum-induced particle aggregation (Figure 7A). Hence combining both main observations together there appears to be the suggestion of a possibility that if neoglycolipid modifications to LMD systems can be found that adequately prevent serum-induced particle aggregation, as well as salt-induced aggregation, then the result would be the development of a highly effective sLMD system, with adequate transfection competency and hence with real potential for in vivo applications.

CONCLUSION

We have developed a simple methodology to generate neoglycolipids that we believe could be valuable for drug or gene delivery purposes and may even find more general application in carbohydrate biology. The results of our studies here also suggest that provided neoglycolipids can be identified as able to prevent serum-induced particle aggregation, as well as salt-induced aggregation, then such stealth molecules could be highly beneficial in the quest for clinically viable synthetic nonviral vector systems in view of the positive effects of neoglycolipids upon transfection efficiency. Data presented here show that none of the current neoglycolipids meet this criterion with the partial exception of maltoheptaose-based neoglycolipid **12i**. However, the possibility that alternative neoglycolipids could be developed that do meet this criterion is credible in our view. The negative impact of PEG upon transfection cannot be overstated and makes DSPE-PEG and other PEGylated stealth molecules doubtful components of a clinically viable synthetic nonviral vector system. It is interesting to note in our data reported here that the stabilizing effect of incorporating DSPE-PEG with LMD to prevent salt or serum-induced particle aggregation was only realized with 5−10 mol % of DSPE-PEG (Figures 6 and 7), while transfection was virtually abolished completely with as little as 0.5 mol % of DSPE-PEG (Figure 9). This suggests that the inhibitory effect of PEG is substantially in excess of its stabilizing stealth potential. Recent evidence suggests that PEGylated nonviral vector systems are internalized easily by cells but intracellular PEG then prevents the intramolecular trafficking of both plasmid DNA (*22*) and even small ODNs (*40*).

One alternative way to overcome the clear limitations of these neoglycolipids to confer serum stability to LMD is the postcoupling of sugars onto aminoxylipid containing LMD systems. Postcoupling allows for the stoichiometric control of surface modification without impairing particle integrity, i.e., membrane destabilization. Preliminary results along these lines obtained in our laboratories demonstrated that this novel way of covalently conjugating sugar units onto lipoplex surfaces is successful. Investigations into the serum resistance and potential targeting properties of such postcoupled systems are currently progressing and will be reported in due course.

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