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Toward creating cell membrane glyco-landscapes with glycan lipid constructs

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ABSTRACT

Synthetic glycolipid-like constructs dispersible in biological media and capable of incorporating into cell membranes have the ability to create novel artificial glyco-landscapes on living cells. Using a variety of different glycans ranging from disaccharides to polysaccharides, together with different lengths and high hydrophilicity spacers, we created a series of synthetic glycolipid-like constructs. Contacting these constructs with live cells gave modified cells with controlled glycan density and/or altered biological function. The ability to also use these constructs as solutions to inhibit antibodies, toxins, and virions extends the potential diagnostic and therapeutic uses for these synthetic glycolipid-like constructs.

1. Introduction

Glycosphingolipids by nature of their lipid tail have a natural ability to self-insert into cell membranes,[1–3] but often the requirement to use solvents to maintain them in a solution-phase suitable for insertion can limit their compatibility with live cells. Equally important is the complex chemical steps needed to synthesize glycosphingolipids bearing ceramide lipid tails. Recently synthetic glycolipid-like constructs, which have a spacer between a synthetically friendly lipid (as a rule, dioleoylphosphatidylethanolamine, DOPE) and the glycan head have been constructed (Chart 1).[4] These constructs are now known as Function-Spacer-Lipid constructs (FSLs) while construct modified cells are termed 'kodecytes'.[5–9] All FSLs have been designed to disperse as a solution directly in organic solvents free water, saline and biological media (one function of the selected spacers).[5] Like natural glycolipids,[1–3] all FSLs have the ability to self-insert into cell membranes.[5] Because FSLs are synthetically made, their structure can be fully controlled and engineered and they can be designed to carry glycans not naturally present as glycolipids, for example hyaluronic acid. Since their introduction in 2003 a range of biological applications for carbohydrate based FSL constructs have been reported (Table 1). These constructs can be attached to live cells in a controlled and precise manner[5–9] making them powerful tools for the study and manipulation of glycoconjugates at the cell surface. This paper describes in detail the synthesis of FSL constructs, including variations of spacer arms between glycan and lipid tails, and gives new evidence of biological activity/applications relating to these synthetic glycolipid-like constructs.

2. Experimental procedures

2.1. Materials

2.1.1. Synthesis

1,2-*O*-Dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) was from Northern Lipids Inc. (NLI, Vancouver, Canada). All CMG (*N*-carboxymethyglycine) linkers,[†] $MCMG_2$, CMG_2 and CMG_4, were prepared as described.[15] Hyaluronic acids HA_{5kDa}, and HA_{17kDa} were obtained from Lifecore Biomedical, USA, HA_{8kDa} was from Sigma. Immunological reagents were monoclonal anti-A (Epiclone, CSL, Melbourne) and affinity purified polyclonal sheep anti-hyaluronic acid (Biogenesis 5029-990, Poole UK). TLC chromatography was performed using Silica Gel 60 F_{254} aluminum sheets with visualization via either 7% H_3PO_4 or ninhydrin.

2.2. Synthesis of activated Spacer-Lipid units

Various activated spacers conjugated to DOPE were synthesized.

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[†] Structures of all three linkers are shown on Scheme 2.

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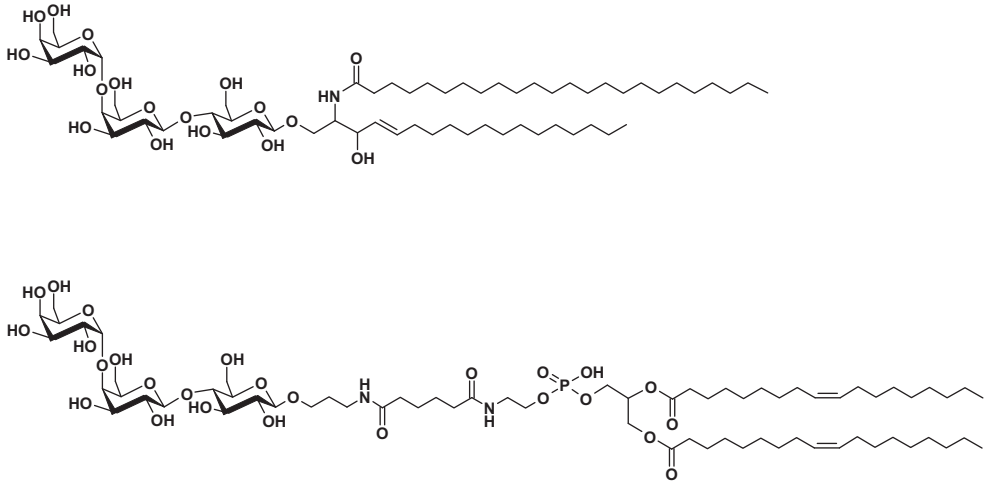


Chart 1. Natural glycosphingolipid versus its FSL synthetic analog. Schematic diagram contrasting: (upper image) a natural Gb3 globotriaosylceramide (lignoceric acid) with (lower image) FSL-Gb3 with the same glycan conjugated to spacer-DOPE.

Table 1
Published biological applications of FSL constructs

Function-Spacer-Lipid[a] construct		Biomedical application(s)
Functional group	Spacer	
GalNAcα1-3 Galβ	Ad	ABO quality control[4–6] Flow cytometry controls[7]
Fucα1-2		Mimicking ABO transfusion reactions[8,9] In vivo ABO antibody neutralization[9]
Galα1-3 Galβ	Ad	ABO quality control[4–6] Flow cytometry controls[7]
Fucα1-2		
Galα1-4Galβ1-4Glcβ	Ad	HIV virus inhibition with Gb3[10]
Galα1-4Galβ1-4Glcβ	Ad	Toxin neutralization with Gb3[10]
Galα1-3 Galβ	Ad	Lewis antibody identification[4]
Fucα1-4		
Galβ1-3 Galβ	Ad	Acquired-B determination[4]
Fucα1-2		
Galα1-3Galß1-4GlcNAcß	Ad	Galili xenotransplant mimics[11]
Biotin	CMG_2	Biotinylation of cells[5,8,9] and virions[5,12]
Fluorophores	Various	Labeling of cells[4,5] and virions[5,12]
Peptides	CMG_2	Peptide modification of cells[13,14]

[a] All constructs have DOPE as their lipid tail.

2.2.1. DOPE-Ad-ONSu

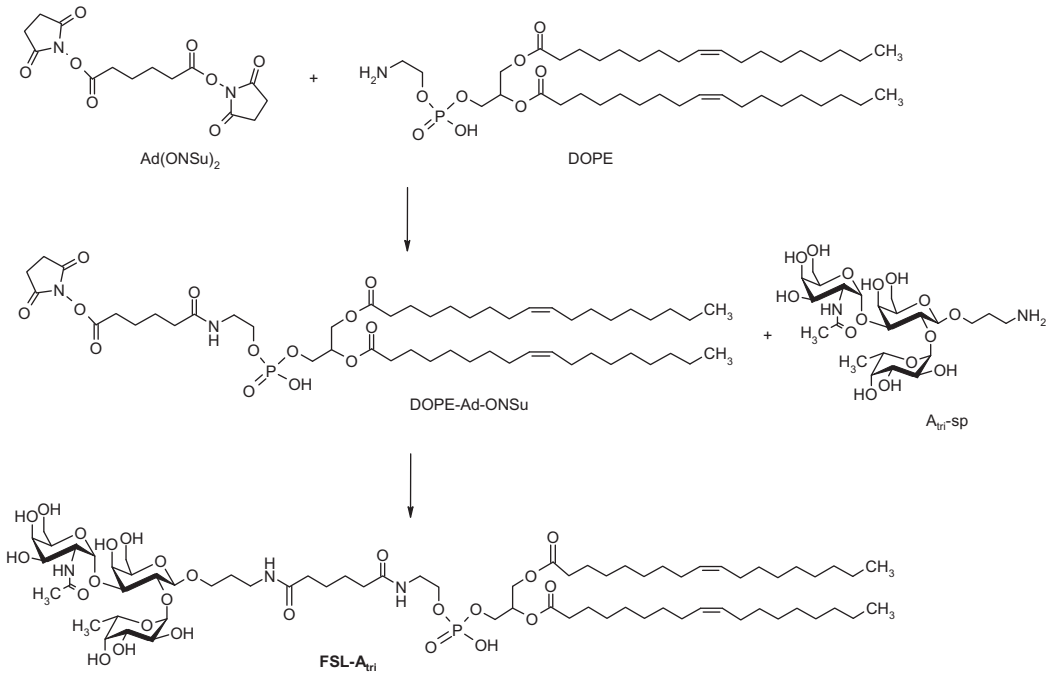
To a solution of *bis*(*N*-hydroxysuccinimidyl) adipate (700 mg, 2.05 mM) in dry *N*,*N*-dimethylformamide (30 mL) were added DOPE (300 mg, 0.403 mM) in chloroform (15 mL) and triethylamine (50 μL) (Scheme 1). The mixture was stirred for 2 h at room temperature, then neutralized with acetic acid and concentrated in vacuo. Column chromatography (Sephadex LH-20, 1:1 chloroform–methanol, 0.2% acetic acid) of the residue yielded the activated lipid (370 mg, 95%) as a colorless syrup; TLC (chloroform–methanol–water, 6:3:0.5): R_f = 0.5. ^{1}H NMR according to Supplementary data.

2.2.2. $MCMG_2$-Ad-DOPE

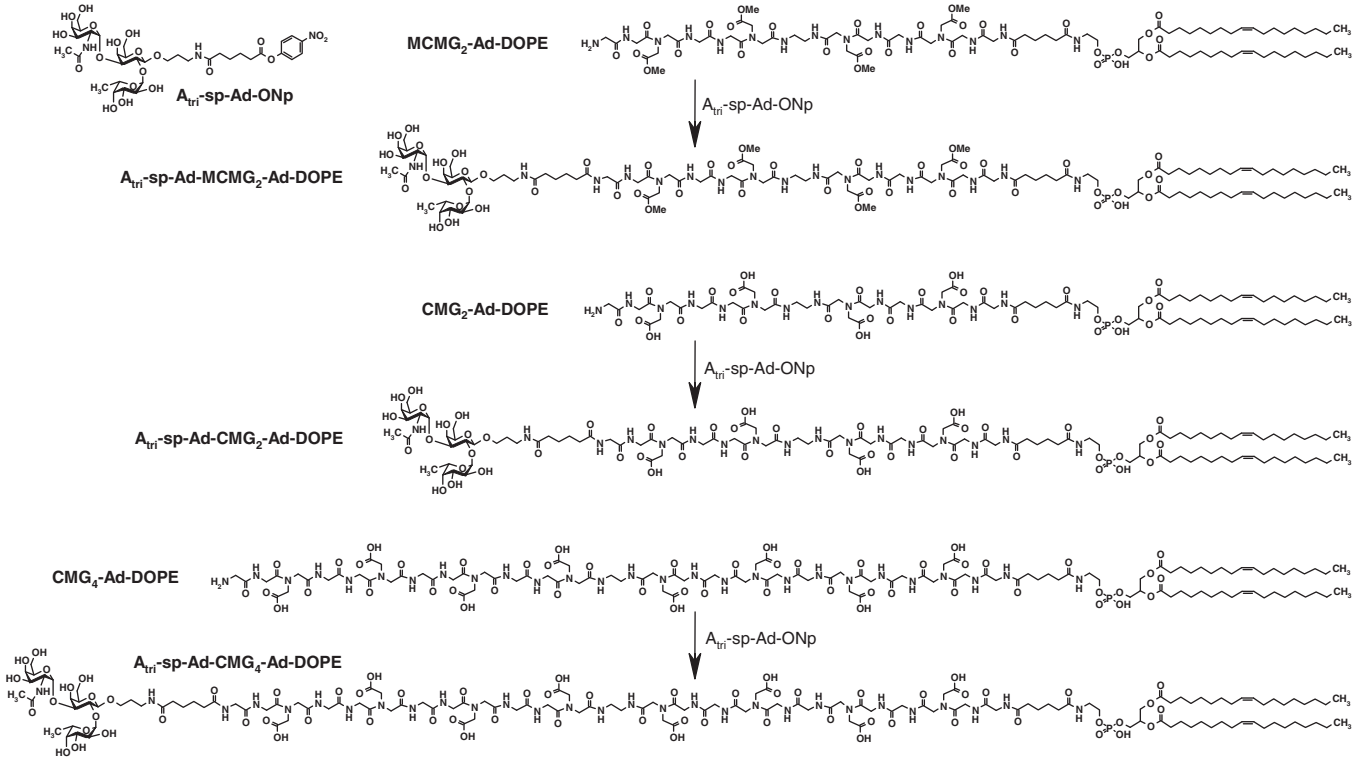
To an intensively stirred solution of $MCMG_2$ (378 mg of wet material with ~10% of water, ~0.27 mM) in pyridine (5 mL) were added Et_3N (0.1 mL, 0.72 mM) and a solution of DOPE-Ad-ONSu (174 mg, 0.18 mM) in dichloroethane (0.87 mL). The reaction mixture was stirred for 2 h and then acidified with 0.2 mL of AcOH and evaporated to minimal volume at 35 °C. The residue was dried in vacuum (solid foam). The obtained mixture was separated on silica gel column (2.8 × 33 cm, ~200 mL of silica gel in $CHCl_3$/MeOH 5:1). The mixture was placed on column in $CHCl_3$/MeOH (5:1) and the components were eluted with $CHCl_3$/MeOH (1:1) and then with alteration of MeOH/$CHCl_3$/water composition from 7:7:1 to 6:6:1. The first elute was DOPE-Ad-$MCMG_2$-Ad-DOPE (R_f = 0.72, MeOH/$CHCl_3$/water 6:6:1) while the second was aimed $MCMG_2$-Ad-DOPE (R_f = 0.34, MeOH/$CHCl_3$/water 6:6:1). $MCMG_2$ was eluted with MeOH/1 M Py·HOAc (3:2, R_f = 0.47). Fractions, containing pure $MCMG_2$-Ad-DOPE amine were combined, evaporated, and dried in vacuum. The residue was dissolved in 5% AcOH and freeze-dried. Yield of $MCMG_2$-Ad-DOPE (Scheme S1, Supplementary data) was 132 mg (26% on $MCMG_2$). MS, *m/z*: 1887 [M+H], 1909 [M+Na], 1925 [M+K] and ^{1}H NMR according to Supplementary data.

2.2.3. CMG_2-Ad-DOPE

To an intensively stirred solution of CMG_2 (425 mg, 0.44 mM of internal salt) in isopropyl alcohol (IPA)/water mixture (IPA/water 3:2, 10 mL) were added the 1 M aq solution of $NaHCO_3$ (0.44 mL, 0.44 mM) and then the solution of DOPE-Ad-ONSu (211 mg, 0.22 mM) in dichloroethane (0.4 mL). The reaction mixture was stirred for 2 h, acidified with 0.2 mL of AcOH and evaporated in vacuo to minimal volume at 35 °C. The solid residue was dried in vacuum (solid foam) and then thoroughly extracted with $CHCl_3$/MeOH mixture ($CHCl_3$/MeOH 4:1, several times with 10 mL, TLC control). The extracted residue consisted of unreacted CMG_2 and salts (about 50% of CMG_2 was recovered by desalting of combined residue and fractions after chromatography on silica gel according to the procedure described in the CMG_2 synthesis.). The combined $CHCl_3$/MeOH extracts (solution of CMG_2-Ad-DOPE, DOPE-Ad-CMG_2-Ad-DOPE, N-oxysuccinimide and some CMG_2 were evaporated in vacuum and dried. The obtained mixture was separated on silica gel column (2.8 × 33 cm, ~200 mL of silica gel in $CHCl_3$/MeOH 5:1). The mixture was placed on column in MeOH/$CHCl_3$/water mixture (MeOH/$CHCl_3$/water 6:3:1 + 0.5% of pyridine) and the components were eluted in a stepwise ternary gradient: MeOH/$CHCl_3$/water composition from 6:3:1 to 6:2:1 and then to 6:2:2 (all with 0.5% of pyridine). DOPE-Ad-CMG_2-Ad-DOPE was eluted first (R_f = 0.75, MeOH/$CHCl_3$/water 3:1:1), followed by desired DOPE-Ad-CMG_2 (R_f = 0.63, MeOH/$CHCl_3$/water 3:1:1), last eluted was CMG_2 (R_f = 0.31, MeOH/$CHCl_3$/water 3:1:1). Fractions, containing pure CMG_2-Ad-DOPE amine were combined and evaporated to dryness.



Scheme 1. Synthesis of DOPE-Ad-ONSu and FSL-A_{tri}.



Scheme 2. Synthesis of CMG variations of FSL-A.

To remove any low molecular weight impurities and solubilized silica gel the residue was dissolved in IPA/water 1:2 mixture (2 mL), and was passed through a Sephadex LH-20 column (column volume 130 mL, eluent—IPA/water 1:2 + 0.25% of pyridine). Fractions containing pure CMG_2-Ad-DOPE were combined and evaporated (~20% of IPA was added to prevent foaming) to dryness, the residue was dissolved in water (~4 mL) and freeze-dried. Yield of CMG_2-Ad-DOPE (Scheme S1, Supplementary data) was 270 mg (68% on DOPE-Ad-ONSu or 34% on CMG_2). MS, *m/z* 1831 [M+H] and ^{1}H NMR according to Supplementary data.

2.2.4. CMG_4-Ad-DOPE

To an intensively stirred solution of CMG_4 (340 mg, 0.14 mM of hexa-Et_3N salt) in IPA/water mixture (IPA/water 3:2, 7 mL) were added 1 M aq solution of $NaHCO_3$ (0.23 mL, 0.23 mM) followed by the solution of DOPE-Ad-ONSu (110 mg, 0.11 mM) in dichloroethane

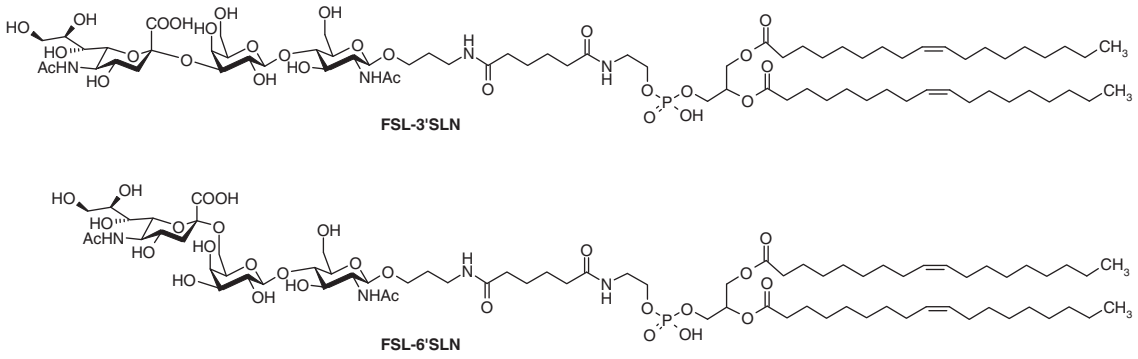


Chart 2. Structures of two sialyl FSL constructs.

(0.55 mL). The reaction mixture was stirred for 2 h, acidified with 0.2 mL of AcOH and evaporated to minimal volume at 35 °C. The solid residue was dried in vacuum (solid foam). The obtained mixture was separated on silica gel column (2.6 × 25 cm, ~130 mL of silica gel in IPA/water 5:1). The mixture was placed on column in IPA/water mixture (3:1) and the components were eluted with alteration of IPA/water composition from 3:1 to 2:1 and then to 3:2 (all with 0.5% of pyridine). First eluted was DOPE-Ad-CMG_4-Ad-DOPE (R_f = 0.61, MeOH/$CHCl_3$/water 3:1:1), while the second elute was aimed at CMG_4-Ad-DOPE (R_f = 0.38, MeOH/$CHCl_3$/water 3:1:1), last eluted was CMG_4 (R_f = 0.12, MeOH/$CHCl_3$/water 3:1:1). Fractions, containing pure CMG_4-Ad-DOPE amine were combined and evaporated to dryness and purified as above. Yield of CMG_4-Ad-DOPE (Scheme S1, Supplementary data) was 108 mg (29% on CMG_4). MS, *m/z* 2748 [M+H] and ^{1}H NMR according to Supplementary data.

2.3. Synthesis of blood group related FSLs

Although an almost unlimited number of glycans can be created as FSL constructs, for the sake of brevity and comparative discussion, the blood group A trisaccharide, GalNAcα1-3(Fucα1-2)Galß, is described here as the benchmark construct. Other related blood group FSL constructs are shown in Chart S1 Supplementary data.

2.3.1. Preparation of FSL-A_{tri} (with adipic (Ad) linker)

To a solution of aminopropyl glycoside of A trisaccharide (176 mg, 0.30 mM) in dry *N,N*-dimethylformamide (3 mL) were added 50 μL of triethylamine and the solution of DOPE-Ad-ONSu (320 mg, 0.33 mM) in dichloromethane (1 mL) (Scheme 1). The mixture was stirred for 2 h at room temperature. Column chromatography (the first on Sephadex LH-20, 1:1 chloroform–methanol, and the second on silica gel, dichloromethane–ethanol–water, 6:5:1) of the mixture yielded 390 mg (90%) of the synthetic molecules designated FSL-A_{tri}. MS, *m/z* 1442 [M+H] and ^{1}H NMR according to Supplementary data.

2.3.2. Preparation of FSL-A_{tri}-$MCMG_2$, FSL-A_{tri}-CMG_2 and FSL-A_{tri}-CMG_4 (Scheme 2)

FSL-A-trisaccharide constructs based on a variety of carboxymethylglycine spacers were synthesized by the conjugation of GalNAcα1-3(Fucα1-2)Galβ-O$(CH_2)_3$NHCO$(CH_2)_4$COO(*p*-C_6H_4)NO_2 (A_{tri}-sp-Ad-ONp) glycoside with corresponding amine ($MCMG_2$-Ad-DOPE, CMG_2-Ad-DOPE or CMG_4-Ad-DOPE) (Scheme 2). The desired pure products were isolated by gel-permeation chromatography (Sephadex LH-20, IPA/water 1:2 + 0.25% pyridine).

2.3.3. FSL-A_{tri}-$MCMG_2$

DMF was used as solvent for conjugation (+4 equiv of Et_3N). Yield 85% on $MCMG_2$-Ad-DOPE or 65% on A trisaccharide (30% excess was needed). TLC: R_f = 0.48 (MeOH/$CHCl_3$/water 6:6:1). MS, *m/z* 2606 [M+Na], 2622 [M+K and ^{1}H NMR according to Supplementary data.

2.3.4. FSL-A_{tri}-CMG_2

Solvent for conjugation—pyridine/water (4:1), 5 equiv of Et_3N as base. Yield 78% on CMG_2-Ad-DOPE or 39% on A trisaccharide glycoside (100% excess was needed). TLC: R_f = 0.28 (IPA/acetonitrile/water 4:3:2). MS, *m/z* 2551 [M+Na], 2567 [M+K] and ^{1}H NMR according to Supplementary data.

2.3.5. FSL-A_{tri}-CMG_4

Solvent for conjugation—pyridine/water (2:1), 5 equiv of aq 1 M $NaHCO_3$ as base. Yield 96% on CMG_4-Ad-DOPE or 32% on A trisaccharide glycoside (200% excess was needed). TLC: R_f = 0.49 (MeOH/$CHCl_3$/water 3:1:1). MS, *m/z* 3467 [M+Na], 3483 [M+K] and ^{1}H NMR according to Supplementary data.

2.4. Synthesis of sialic acid based FSLs

Derivatives of 6′SLN (Neu5Acα2-6Galβ1-4GlcNAc), and 3′SLN (Neu5Acα2-3Galβ1-4GlcNAc) trisaccharides (Chart 2), with adipic linker, were synthesized similarly to A trisaccharide construct (Scheme 1). NMR data are specified in Supplementary data.

2.5. Synthesis of FSL-HA (hyaluronic acid)

A heterogeneous range of hyaluronic acid (HA) fragments were prepared from chromatographic purified hyaluronidase digested hyaluronic acid[16] and used to manufacture FSL-HA constructs (Chart 3). HA oligomers were converted to HA-glycamines by reductive amination. Acylation of HA-glycamines with N-oxysuccinimide ester DOPE-Ad-ONSu and subsequent purification resulted in FSL-HA-Ad-DOPE (Scheme 3).

2.5.1. Fsl-HA_{8kda}

Hyaluronic acid oligomer HA ~8 kDa (36 mg) was dissolved in 5 M NH_4OAc (3.6 mL), and the solution was kept for 21 h at 40 °C. After addition of aqueous 2 M $NaCNBH_3$ in five sequential portions the mixture was kept at 40 °C (40 μL of 2 M $NaCNBH_3$—for 3 h, 80 μL—18 h, 160 μL—8 h, 160 μL—21 h, 160 μL—21 h). Desalting of the reaction mixture by gel-permeation chromatography on Sephadex G-10 column (1.8 × 40 cm, eluent—aqueous 0.1 M Py·AcOH) and freeze-drying gave 32.8 mg of HA-glycamine (with admixture having no amine group HA oligomer). TLC (eluent IPA/MeOH/MeCN/water 4:3:6:4): HA-glycamine (ninhydrine-positive) R_f = 0.2; HA oligomer R_f = 0.31. HA-glycamine (32.8 mg) was dissolved in the mixture of IPA (1.5 mL) and water (1.5 mL). To the rapidly stirred solution of HA-glycamine the solution of N-oxysuccinimide ester DOPE-Ad-ONSu (34 mg, 35 μM) in CH_2Cl_2

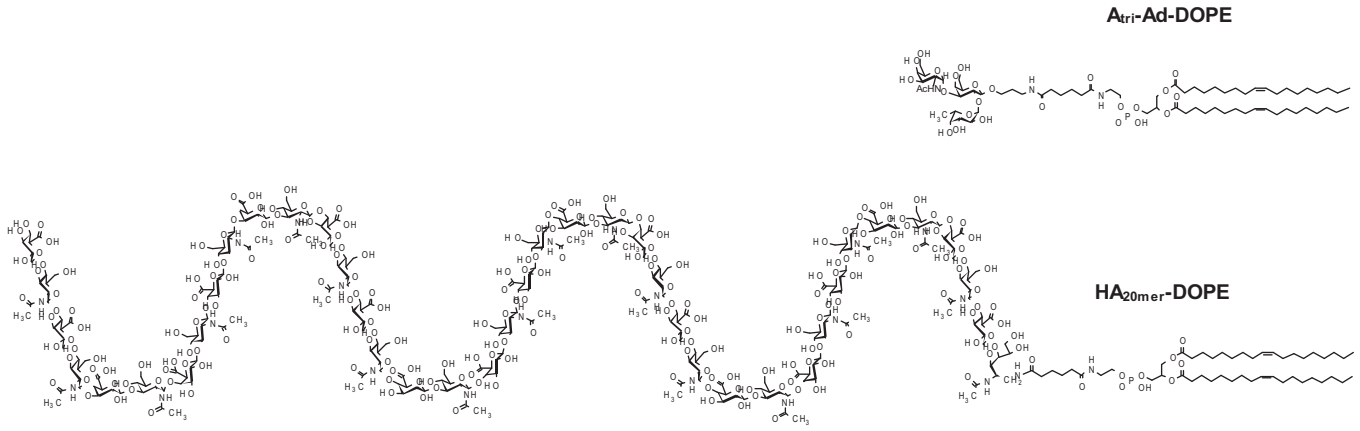
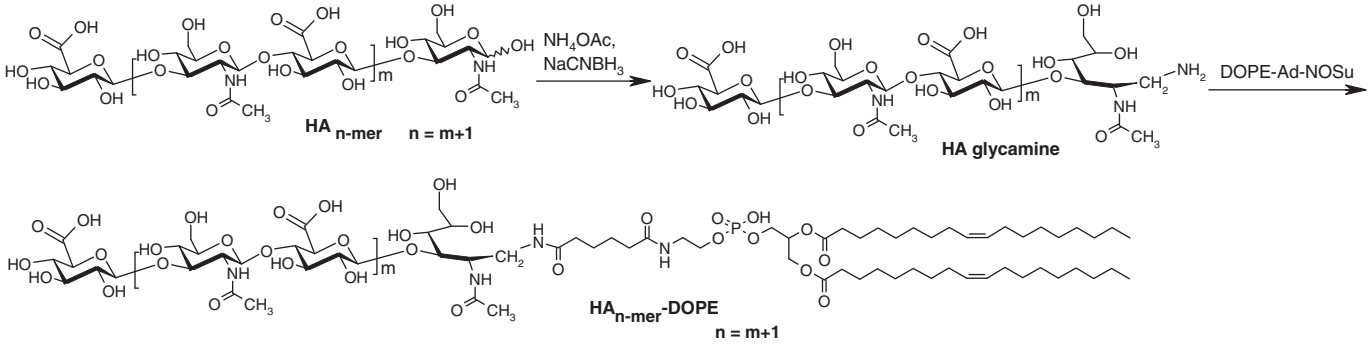


Chart 3. Scale comparison of FSL-A with FSL-HA. FSL-HA is 40 oligosaccharide (~8 kDa), whereas FSL-A_{tri} is a trisaccharide.



Scheme 3. Synthesis of HA-Ad-DOPE.

(0.2 mL) and then aqueous 1 M Na_2CO_3 in two portions (85 and 45 μL) with 45 min interval were added. The mixture was stirred for 45 min then acidified with AcOH (30 μL). Gel-permeation chromatography of reaction mixture on Sephadex LH-20 column (1.8 × 35 cm, eluent—MeOH/water 2:1, 0.03 M Py·AcOH) gave 42.5 mg of FSL-HA_{8kDa} (with admixture of HA oligomer).

A solution of the crude product in water was slowly loaded onto a C_{18} reverse phase column (1.2 × 7 cm, water). Elution with water and water/MeOH 10:1 gave no lipid HA oligomer (12.3 mg). Elution with water/MeOH 1:3 and then with water/MeOH/$CHCl_3$ 5:15:1 gave FSL-HA_{8kDa} with little admixture of eluted C_{18} phase. This product was evaporated and the residue (thin film on the flask walls) was extracted with hexane (2 × 2 mL) followed by ether (2 × 2 mL), then dissolved in water (1.5 mL) and freeze-dried. Yield of pure FSL-HA_{8kDa} was 20.6 mg (~50%). TLC: R_f = 0.33, eluent IPA/MeOH/MeCN/water 4:3:6:4. NMR data are as specified in Supplementary data.

For stability and storage FSL-HA_{8kDa} was converted to mono-Na salt (by phosphate) and $(NH_4)_n$-salt (by glucuronic acids). FSL-HA was dissolved in water and a calculated quantity of aqueous $NaHCO_3$ and NH_3 were added followed by freeze-drying.

By the analogous procedure but starting from commercially available fractions of hyaluronic acid (Lifecore Biomedical, USA) FSL-HA_{5kDa} (yield 49%) and FSL-HA_{17kDa} (yield 66%) were synthesized.

2.6. Synthesis of FSL-biotin-CMG_2

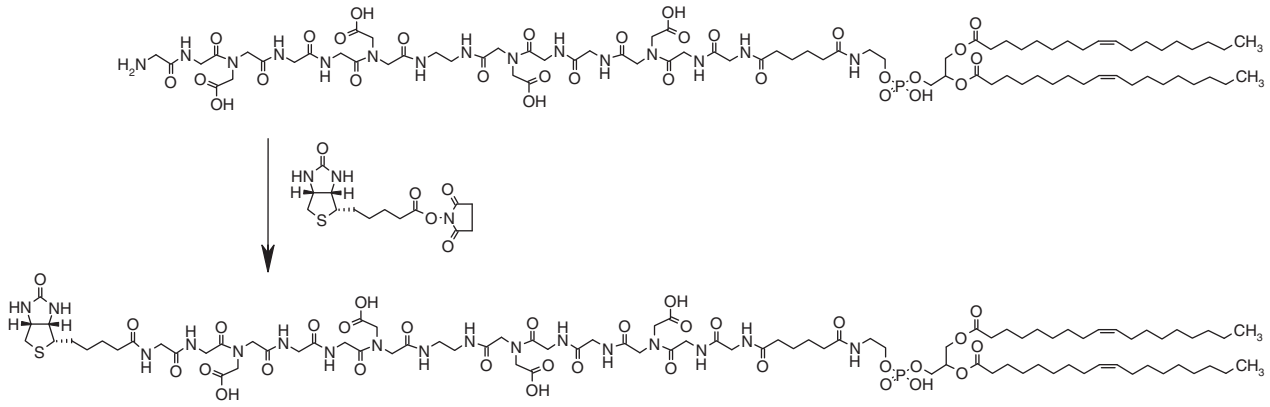
To an intensively stirred solution of CMG_2-Ad-DOPE (366 mg, 0.20 mM) in IPA/water mixture (IPA/water 1:1, 15 mL) were added 1 M aq solution of $NaHCO_3$ (1.2 mL, 1.20 mM) and a solution of biotin N-oxysuccinimide ester (75.1 mg, 0.22 mM) in DMF (1.5 mL). The reaction mixture was stirred for 2.5 h and then acidified with 0.15 mL of AcOH and evaporated to minimal volume (beginning of a precipitate formation) at 35 °C. The residue was diluted with water (1.5 mL) and 0.05 mL of pyridine was added. The resulting solution was passed through Sephadex LH-20 column (column volume 300 mL, eluent—IPA/water 1:2 + 0.5% of pyridine and 0.25% of AcOH). Fractions, containing pure FSL-biotin-CMG_2 were combined and evaporated (~20% of IPA was added to prevent foaming) to dryness, the residue was dissolved in water (~4 mL, containing 0.05 mL of pyridine) and freeze-dried. Yield of FSL-biotin-CMG_2 (pyridinium salt) was 384.5 mg (~0.18 mM). The product was dissolved in water (3 mL) containing $NaHCO_3$ (0.18 mL of 1 M aqueous solution, 0.18 mM) and freeze-dried. Yield of FSL-biotin-CMG_2 (sodium salt) was 374.3 mg (90% on CMG_2-Ad-DOPE)—Scheme 4. TLC: R_f = 0.64 ($MeOH/CHCl_3$/water 6:2:1. MS, *m/z*: 2079 [M+Na], 2095 [M+K], 2101 [MNa+Na] and NMR data are as specified in Supplementary data.

2.7. Preparation of biotinylated blood group A trisaccharides

Two mono-biotinylated forms of blood group A trisaccharides, one, A_{tri}-sp-AC-biotin, with a short 3-carbon spacer and aminocaproic unit (AC), and the other on long polyacrylamide spacer, A_{tri}-PAA-biotin (MW ~30 kDa), were prepared as described.[17,18]

2.8. FSL membrane insertion creating kodecytes

The generic established method for inserting FSLs into cell membranes was used to create kodecytes. This involves simply contacting one volume of cells (either 100% packed or in solution) with one



Scheme 4. Synthesis of FSL-biotin.

volume of FSL solution (usual range 10–1000 μg/mL of FSL in phosphate buffer saline (PBS)) and incubation at 37 °C for 1–2 h.[4–9] Still based primarily on creating kodecytes but this time glycoconjugates were attached through an alternative 'sandwich' technique. First FSL-biotin kodecytes were obtained with a solution of 100 μg/mL of FSL-biotin. The resultant biotin kodecytes were then reacted with an excess of avidin (1 mg/mL) and washed to give rise to avidin kodecytes. Equal volumes of avidin kodecytes were then incubated with an excess of biotinylated glycan at room temperature for 30 min. The resultant A kodecytes, with either A_{tri}-sp-AC-biotin or A_{tri}-PAA-biotin attached via an avidin bridge to the FSL-biotin kodecytes were then suspended in PBS and used in serologic assays.

2.9. Red cell serology

The detection of A antigen on the kodecytes was via direct serological agglutination against IgM monoclonal anti-A.[4] The detection of hyaluronic acid by indirect (anti-IgG bridging) agglutination of HA-kodecytes with polyconal anti-HA.

2.10. Influenza virus binding assay

Human red blood cells (native) were first desialylated with sialidase (neuraminidase from *Vibrio cholera,* Sigma), 0.1 UN for 1 mL packed red cells in acetate buffer solution (pH 5.0), containing 0.15 M sodium chloride and 4 mM calcium chloride; 1 h, 37 °C. Desialylated red blood cells were then inserted with FSL-sialyl constructs (1 mg/mL—2 h 37 °C) to produce 6′SLN kodecytes and 3′SLN kodecytes. The capability of sialyl kodecytes to bind three different strains of influenza viruses (avian H5N2/Mallard/10218, swine H9N2/9/98, and human B/HK/54800) was determined in a hemagglutination reaction assay (50 μL of double dilutions of virus in 96-wells microtiter round bottom plate, 50 μL of 0.5% cell suspension, 40 min at 4 °C). Native red blood cells were used as a positive control while the same desialylated cells, but FSL unmodified, were tested in parallel as a negative control.

2.11. Supporting information available

NMR data of a number of synthesized compounds; Scheme S1. Synthesis of DOPE with $MCMG_2$, CMG_2 and CMG_4 Spacers; Chart S1: Other Blood Group Related FSL Constructs; Chart S2. Spacer Defined Distances Between Cell Membrane and Ligand. This material is available free of charge via the Internet at http://elsevier.com.

3. Results and discussion

3.1. FSL synthesis

A variety of different and successful approaches were taken to build a range of FSL constructs bearing glycans (Schemes 1–3). The design of all FSL constructs was deliberately constrained by an absolute requirement to be dispersible in water (without organic co-solvents); a feature, which it is believed will favor biocompatibility and potential therapeutic use. Constructs were further constrained by a requirement that they are able to spontaneously and stably incorporate into cell membranes and that the spacer be inert with serum. Enabling all of these features required the designing of a series of different spacers, in particular CMG variations, and abandoning alternatives such as ethylene glycol, which although able to bring about most features, was not able to achieve inertness with undiluted serum particularly, binding of PEG with serum antibodies of numerous healthy donors.[19] The diacyl nature of the lipid tail was also important for normal cell retention as exemplified by a construct with a single lipid chain (octadecanoic acid) which although dispersible in saline did not efficiently insert and/or be retained in cell membranes (results not shown). Notably, all synthesized glyco-FSL constructs were water dispersible and capable of inserting in cell membrane quantitatively[4] when constructed in the simplest version, with $-OCH_2CH_2CH_2NHC-OCH_2CH_2CH_2CH_2CO-$ spacer (Scheme 1) between glycan and DOPE fragments. Insertion of more sophisticated CMG or MCMG spacers were also utilized to make glycan constructs (Scheme 2). These spacers though not required for glycans (although they did bring about increased sensitivity) were required in the case of hydrophobic F groups, as exemplified here by biotin derivatives (Scheme 4). The data presented here are primarily with a 1,2-*O*-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) as the FSL lipid tail, however other lipids such as sterol instead of an oleoyl moiety, can cause cell labeling, albeit much less efficiently (not shown). The ability to change the lipid tail of FSLs, for instance to ceramide, is expected to alter the function of some FSLs and potentially determine where they may partition within the cell membrane.[20] Multiple DOPE tails on the polymeric glycoconjugates (based on polyacrylamide backbones) give rise to insertion complications due to intramolecular assembling of the tails,[21] so only monomeric DOPE constructs were used here. As the lipid DOPE was compatible with all current biological assays (Table 1) it was chosen as the default lipid for FSL synthesis. Stability trials have established that FSL constructs are stable for periods exceed-

Table 2
Serologic reactions of A kodecytes created with biotinylated A trisaccharide constructs

Biotinylated saccharide[a]			Anti-A serology
Description	A trisaccharide valency[b]	Spacer length nm[c]	A kodecytes[d]
A_{tri}-sp-AC-biotin	1	~1.4	++
A_{tri}-PAA-biotin	~20	~10	++++

[a] Two forms of biotinylated saccharide constructs of differing spacer length and valency loaded onto avidin kodecytes to create A kodecytes.
[b] Number of A trisaccharide residues on each biotinylated construct.
[c] Total length of the construct (taking into consideration coil conformation of PAA and stretched one for sp-AC moiety) excluding avidin and FSL-biotin.
[d] Serologic scoring as per Table 4.

Table 3
Sialyl kodecyte differentiation of human, swine, and avian influenza viruses

Test cells	Agglutination[a] of kodecytes with viruses		
	Human B/HK/54800	Avian H5N2/Mallard/10218	Swine H9N2/9/98
6′SLN kodecytes[b,c]	++++ (1:80)	–	++++ (1:25600)
3′SLN kodecytes[c]	–	++++ (1:800)	++++ (1:1600)
Desialylated red cells[d]	–	–	–
Native red cells[e]	++++ (1:640)	++++ (1:1600)	++++ (1:25600)

[a] Serologic scores as reported in Table 4.
[b] Red cells were de-sialylated and then converted into sialyl kodecytes with specific sialic acid bearing FSLs.
[c] FSL sialyl constructs used to make the kodecytes are as shown in Chart 2.
[d] The same desialylated cell as used to make the kodecytes as a negative control.
[e] Untreated red cells as a positive control (also the same cell as used to make the kodecytes).

ing 2 years when dry or at least 1 year in sterile saline solutions at 4 °C (or frozen).

3.2. Biological analysis

In addition to the already published applications for FSL constructs (Table 1) we extended the range of applications and opportunities by creating three new series of constructs; one utilizing FSL-biotin + avidin + biotinylated saccharides, another based on sialic acid residues and another utilizing polysaccharide fragments of hyaluronic acid.

3.2.1. Biotin + avidin + biotinylated glycan composite

By synthesising FSL biotin, a construct successfully used in other applications (Table 1), an alternative approach to glycosylating the surface of cells was enabled. In this approach a three-stage method was used in attaching biotinylated glycans via avidin to FSL-biotin kodecytes. We evaluated the serological reactions of two forms of biotinylated blood group A trisaccharides attached to cells via FSL-biotin + avidin and found them to be clearly recognized by anti-glycan antibodies (Table 2). Increasing the number of glycosylation points and consequent length of the spacer when A_{tri}-sp-AC-biotin is compared with polymeric end-monobiotinylated conjugate A_{tri}-PAA-biotin,[18] resulted in increased sensitivity of the assay. Although compared to the single FSL construct route this method is somewhat cumbersome, it does offer a rapid and flexible alternative approach to 'glycosylating' the surface of cells. However, although glycosylation of the cell clearly occurs, the [biotinylated-spacer-glycan] + [avidin] + [FSL-biotin] glyco-complex is significantly unnatural, and may not necessarily mimic a natural reaction, or that of a single FSL construct.

3.2.2. Sialic acid FSLs

The sialic acid-containing glycans are expressed on host respiratory epithelial cell surfaces and are involved in influenza virus infection.[22] It is known that viruses isolated from human and swine sources prefer binding to sialic acid in a 2–6 linkage (e.g., 6′-sialyl-*N*-acetyllactosamine, 6′SLN), whereas viruses isolated from birds bind more strongly to sialic acid in α 2–3 linkage (e.g., 3′-sialyl-*N*-acetyllactosamine, 3′SLN).[23] Native human red cells react with all influenza species, but when desialylated they become unreactive (Table 3). Re-introduction of specific sialoglycans with FSL constructs to desialylated red cells (Chart 2) creates kodecytes able to differentiate the virus species by their specific sialic acid residues. The 6′SLN kodecytes were reactive with only human and swine viruses while the 3′SLN kodecytes reacted only with avian and swine viruses (Table 3). Because the same desialylated cell is used to create both kodecytes and the negative control, reactivity can be unambiguously assigned to reactivity against the specific sialyl-FSL construct.

3.2.3. Hyaluronic acid FSLs

Hyaluronic acid (HA) is large polysaccharide and relatively large fragments of it can be synthesized into FSL-HA constructs (Chart 3). Using specific antibodies and HA binding proteins it could be established that FSL-HA inserted into the membranes of murine embryos and red cells. Solutions of FSL-HA constructs over the range of 10–250 μg/mL produced maximally strong serological results. HA has been claimed in humans[24] (and mice[25]) to improve embryo implantation. However when we used either free HA, or a range of FSL-HA constructs, we were unable to improve subfertile murine implantation rates, despite demonstrating increased in vitro adherence of HA embryo kodecytes to endometrial cells (unpublished). Although FSL-HA did not affect fertility it remains a potentially promising construct for the modification of cells or liposomes.[26]

3.3. Spacer length

The inclusion of a spacer is integral to the construction of the FSL and brings several important features to the construct, including water dispersibility, and spacing of the glycotope away from the membrane. It had previously not been established what influence the length of the spacer would have on serological reactions. To investigate this we built and tested the serology of the blood group A trisaccharide on three spacers of increasing length

Table 4
Serologic reactions of blood group A kodecytes created with different FSL spacers

FSL-A_{tri}[a]		FSL-A kodecyte serologic reactions[b] against dilutions of monoclonal anti-A										
Spacers[c]	μM	1	2	4	8	16	32	64	128	256	512	1024
Ad	50	++++	++++	++++	++++	++++	++++	+++	++	++	±	—
	10	+++	+++	+++	++	++	++	++	±	—	—	—
	5	++	++	+	±	—	—	—	—	—	—	—
CMG_2	50	++++	++++	++++	++++	++++	++++	+++	+++	++	+	±
	10	++++	++++	++++	+++	+++	+++	+++	++	+	±	—
	5	+++	+++	+++	+++	++	++	++	+	±	—	—
CMG_4	50	++++	++++	++++	++++	++++	++++	+++	+++	++	+	±
	10	++++	++++	++++	+++	+++	+++	+++	++	++	±	—
	5	+++	+++	+++	+++	++	++	++	+	±	—	—

[a] All FSL constructs have A trisaccharide and DOPE as the lipid tail.
[b] Serologic reactions observed against dilutions of monoclonal anti-A in gel reaction cards and scored as ++++ (maximal agglutination), +++ (strong), ++ (medium), + (weak), ± (equivocal) and — (unreactive).
[c] Spacers are as described in Chart S2.

(Scheme 2 and Table 4). It was found that at the 5 μM level blood group A trisaccharide with the 1.9 nm short-spacer showed medium serological reactivity with monoclonal anti-A when diluted 1:2, while with the longer 7.2 nm spacing of FSL-A_{tri}-CMG_2 gave equivalent reactivity at an antibody dilution of 1:64. Further increasing the length of the spacer to 11.5 nm (e.g., A_{tri}-CMG_4) did not improve serological reactivity (Table 4), and is interpreted that a spacer of >7 nm was adequate to induce maximal serological reactivity at the red cell membrane. Neutralizing the charge on the spacer by per-methylation of –COOH groups did not influence the serological results.

4. Final comments

The ability to modify the glyco-landscape of living cells is expected to be a useful tool in understanding the involvement of specific glycoconjugates in biological settings.[27] The glyco-landscape of cells can certainly be modified by the direct use of glycosidases[28] and glycosyltransferases[29] but the actual ability to control the glyco-landscape by these methods is limited, and to some extent incompletely defined. Alternatively genetic manipulation of a cell can either inhibit or induce new glyco-landscapes[30] but as glycosylation is probably the most complex secondary gene event in a cell, the outcome is unpredictable and poorly controlled. Direct covalent labeling of glycoconjugates to the cell surface although possible has significant risks in affecting both the vitality and functionality of the modified cells. The alternative approach as shown here is to use FSL constructs. Because FSL constructs can be controllable and are synthesized and designed to have specific features, they are expected to provide a useful alternative method for altering the glyco-landscape of living cells.

5. Associated content

Supporting Information. ^{1}H NMR data for all FSL constructs. Scheme S1 showing synthesis of DOPE with $MCMG_2$, CMG_2 and CMG_4 Spacers. Chart S1—Schematic diagrams of other FSL blood group related structures and Chart S2 showing spacer defined distances between cell membrane and ligand. This material is available free of charge via the Internet at http://elsevier.com.

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Supplementary data

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