Polymerized Liposome Assemblies: Bifunctional Macromolecular Selectin Inhibitors Mimicking Physiological Selectin Ligands†

Richard E. Bruehl,‡ Falguni Dasgupta,§,|| Tamiko R. Katsumoto,‡ Jennifer H. Tan,⊥ Carolyn R. Bertozzi,‡,@ Wayne Spevak,⊥,# Dong June Ahn,▽ Steven D. Rosen,‡ and Jon O. Nagy*,⊥

Department of Anatomy and Program in Biomedical Sciences, University of California, San Francisco, California 94143, Life Sciences Division and Center for Advanced Materials, Lawrence Berkeley National Laboratory, Berkeley, California 94720, Glycomed, Inc., Alameda, California 94501, and Department of Chemical Engineering, Korea University, Seoul 136-701, Korea

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ABSTRACT: Monomeric sialyl Lewis^X (sLe^x) and sLe^x-like oligosaccharides are minimal structures capable of supporting selectin binding in vitro. However, their weak binding interactions do not correlate with the high-affinity binding interactions witnessed in vivo. The polyvalent display of carbohydrate groups found on cell surface glycoprotein structures may contribute to the enhanced binding strength of selectin-mediated adhesion. Detailed biochemical analyses of physiological selectin ligands have revealed a complicated composition of molecules that bind to the selectins in vivo and suggest that there are other requirements for tight binding beyond simple carbohydrate multimerization. In an effort to mimic the high-affinity binding, polyvalent scaffolds that contain multicomponent displays of selectin-binding ligands have been synthesized. Here, we demonstrate that the presentation of additional anionic functional groups in the form of sulfate esters, on a polymerized liposome surface containing a multimeric array of sLe^x-like oligosaccharides, generates a highly potent, bifunctional macromolecular assembly. This assembly inhibits L-, E-, and P-selectin binding to GlyCAM-1, a physiological ligand better than sLe^x-like liposomes without additional anionic charge. These multivalent arrays are 4 orders of magnitude better than the monovalent carbohydrate. Liposomes displaying 3′-sulfo Lewis^X-like oligosaccharides, on the other hand, show slight *loss* of binding with introduction of additional anionic functional groups for E- and P-selectin and negligible change for L-selectin. The ability to rapidly and systematically vary the composition of these assemblies is a distinguishing feature of this methodology and may be applied to the study of other systems where composite binding determinants are important for high-affinity binding.

Localized recruitment of leukocytes into tissues at sites of injury, infection, or disease is central to an inflammatory response. This is achieved through sequential adhesion events involving leukocyte and endothelial cell adhesion molecules belonging to the selectin, integrin, and immunoglobulin gene families (reviewed in refs *1*−*15*). Leukocyte tethering to and rolling on vascular endothelium is the first in this series of adhesion events and is critical for a successful inflammatory response. Three calcium-dependent carbohydrate-binding proteins, L-, E-, and P-selectin, mediate this initial step and have attracted significant attention as potential targets for anti-inflammatory therapeutics (*4*, *14*, *16*). L-Selectin is constitutively expressed on most circulating leukocytes and binds to sulfated carbohydrate ligands on activated endothelium and to carbohydrate ligands on leukocytes. E-Selectin is synthesized by activated endothelium in response to inflammatory mediators. P-Selectin exists preformed in storage granules and is translocated to the surface of activated platelets and endothelium during inflammation. E- and P-selectin both bind to carbohydrate ligands on leukocytes.

Five well-characterized selectin ligands, GlyCAM-1 (*17*), CD34 (*18*, *19*), Podocalyxin (*20*), PSGL-1 (*21*), and MadCAM-1 (*22*), are cell surface or secreted glycoproteins containing sialomucin domains that are heavily O-glycosylated and present multivalent arrays of sLe^x,[1] sulfated sLe^x, or sulfo-Le^x capped oligosaccharides (*23*−*26*). Although all three selectins bind to sLe^x in vitro (*27*), and early selectin inhibitors were modeled after this structure (*28*), detailed biochemical analyses of selectin ligands have illustrated a much more complicated composition of the

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* To whom correspondence should be addressed: LigoCyte Pharmaceuticals, Inc., 920 Technology Blvd., Bozeman, MT 59718. Telephone: (406) 585-2733. Fax: (406) 585-2766. E-mail: jon.nagy@ligocyte.com.

‡ University of California.

§ Glycomed, Inc.

|| Present address: Biomarin Pharmaceuticals, Inc., Novato, CA 94949.

⊥ Lawrence Berkeley National Laboratory.

@ Present address: Department of Chemistry, University of California, Berkeley, CA 94720.

Present address: Telik, Inc., South San Francisco, CA 94080.

▽ Korea University.

[1] Abbreviations: sLe^x, sialyl Lewis X; 3′-sulfo-Le^x, 3′-sulfated Lewis X; GlyCAM-1, glycosylation-dependent cell adhesion molecule 1; PSGL-1, P-selectin glycoprotein ligand 1; MAdCAM-1, mucosyl addressin cell adhesion molecule 1; PCDA, 10,12-pentacosadiynoic acid; EtOAc, ethyl acetate; IPA, isopropyl alcohol; THF, tetrahydrofuran; MeOH, methyl alcohol; TEA, triethylamine; PDA, polydiacetylene; FAB, fast atom bombardment; Le^a, Lewis^a; Le^x, Lewis^x; NHS, *N*-hydroxysuccinimide.

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physiological molecules that bind the selectins in vivo. For example, L- and P-selectin ligands present a composite of carbohydrate and sulfate ester binding determinants (*24*, *29*−*33*), and while E-selectin binding is not sulfate dependent, binding to 3′-sulfo Le^a or 3′-sulfo Le^x is well documented (*34*). Although L- and P-selectin will bind to either nonsulfated oligosaccharides or sulfated polymers (*35*), high-affinity binding of these receptors requires a combination of carbohydrate and sulfate ester binding determinants (*30*, *36*, *37*). The spatial separation of sLe^x and tyrosine sulfate in PSGL-1 has lead to the notion that there are distinct carbohydrate and sulfate ester binding sites in the ligand recognition domain of P-selectin, a result that has now been confirmed by solution of the crystal structure for the P-selectin/PSGL-1 complex (*38*). Although the carbohydrate ligands for L-selectin are themselves sulfated, the requirement for both carbohydrate and sulfate ester for high-affinity L-selectin binding suggests that the ligand recognition domain of L-selectin may also accommodate carbohydrate and sulfate esters independently. It is worth noting P-selectin will bind to the peripheral lymph node addressin (PNAd), a complex of several carbohydrate-sulfated glycoprotein ligands for L-selectin, despite the absence of tyrosine sulfate. Since both PNAd and PSGL-1 display sLe^x oligosaccharides but differing supports for sulfate esters, this cross reactivity suggests the carbohydrate and anionic recognition sites may be not only distinct but also permissive.

To improve the efficacy of weakly binding selectin inhibitors, multimerization of carbohydrates and carbohydrate mimetics has been a practical strategy (reviewed in refs *14* and *39*−*45*). Indeed, selectin antagonists presenting multivalent forms of sLe^x or sulfo-Le^x have demonstrated enhanced binding to all three selectins relative to their monomeric derivatives in a myriad of cellular and cell free binding inhibition assays (reviewed in ref *14*). To extend this approach and see the effect of introducing anionic sites on the scaffold bearing multimeric sLe^x mimetics, we engineered ionic functionality into the design of multivalent oligosaccharide selectin inhibitors. We report herein the design and synthesis of self-assembling, bifunctional, polymerized liposomes that present a polyvalent array of sLe^x-like or 3′-sulfo Le^x-like oligosaccharides in a background of anionic, neutral, or cationic functional groups (Figure 1) and compare the ability of these assemblies to inhibit L-, E-, and P-selectin binding to GlyCAM-1. GlyCAM-1 is a putative physiological ligand for L-selectin (*17*) that supports binding of all three selectins (*46*); thus, the binding interactions assessed here are likely to be physiologically relevant.

In this study, the major observation is that the addition of anionic groups to the liposome matrix containing polyvalent displays of sLe^x-like oligosaccharide groups has an enhancing effect on the inhibitory potency. Carboxylate groups decreased the IC_{50} values 2- and 3-fold for L- and P-selectin binding, respectively. Incorporation of highly anionic sulfate esters (along with the sLe^x-like oligosaccharide groups) further decreased the IC_{50} values, resulting in 5- and 8.5-fold enhancement for L- and P-selectin binding, respectively. Up to a maximum tested carbohydrate concentration of 2.5 μM, polymerized liposomes did not show any E-selectin inhibition unless the sLe^x-like carbohydrate groups were displayed in a matrix containing sulfate esters. Here the IC_{50} value of 425 nM is observed. Liposomes displaying 3′-sulfo

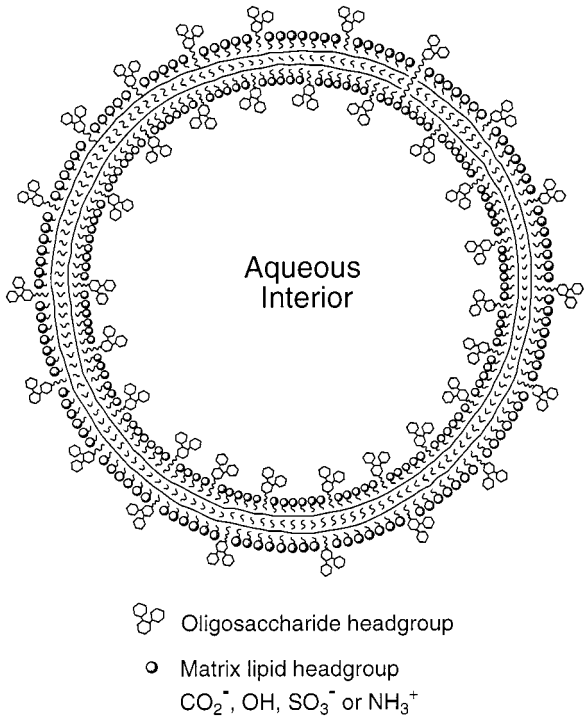


FIGURE 1: Schematic of interior and exterior surfaces of a polymerized bilayer liposome displaying oligosaccharide and matrix headgroups.

$Lewis^X$-like oligosaccharides, on the other hand, show slight *loss* of binding with introduction of additional anionic functional groups for E- and P-selectin and negligible change for L-selectin.

EXPERIMENTAL PROCEDURES

General Procedures. Materials were obtained from commercial suppliers: 10,12-pentacosadiynoic acid (PCDA) (GFS Chemicals, Powell, OH), cystamine, ethanolamine, ethylenediamine, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC), and *N*-hydroxysuccinimide (NHS) (Aldrich, Milwaukee, WI), and Dowex 50 resin (H^+ form) (Bio-Rad, Richmond, CA). The silica gel used in column chromatography was Kieselgel 60 (Merck, 230−400). Thin layer chromatography (TLC) was performed using Analtech silica gel GHLF coated on glass plates. TLC plates were visualized using molybdate stain. Microtiter plates (Immulon II) were from Dynatech (Alexandria, VA); biotinylated goat anti-human Ig Fc [$F(ab')_2$] and the streptavidin−alkaline phosphatase conjugate were from Caltag (Burlingame, CA). The L-, E-, and P-selectin−IgG chimeras have been described previously (*27*, *47*). The anti-GlyCAM peptide antibody CAM02 was generated as described previously (*48*). Mouse serum was from Pel-Freez (Rodgers, AR). ^1H NMR spectra were determined at either 400, 500, or 200 MHz as indicated. Chemical shifts are reported in parts per million (δ), positive values indicating shifts downfield of tetramethylsilane. ^1H NMR spectra determined in D_2O and d_6-DMSO are reported relative to 4.61 and 2.49 ppm, respectively. The ^1H NMR spectra determined in $CDCl_3$ are reported relative to 7.25 ppm. The ^1H NMR spectra determined in CD_3OD are reported relative to the HOD signal at 4.65 ppm. ^1H NMR data are tabulated in the following order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet;

m, multiplet; br, broad), number of protons, and coupling constant(s) in hertz. ^{13}C NMR spectra were proton decoupled and measured at either 100 or 125 MHz. Chemical shifts are reported relative to the central peak of $CDCl_3$ as 77.0 ppm. Fast atom bombardment (FAB^+) mass spectra and electron ionization (EI^+) mass spectra were recorded at the University of California Berkeley Mass Spectral Laboratory. Elemental analyses were performed by the microanalytical laboratory operated by the College of Chemistry at the University of California (Berkeley, CA).

*N-[O-(3-O-(2-Acetic acid)-β-D-galactopyranosyl)]-(1→4)-O-[(α-L-fucopyranosyl)-(1→3)]-O-(β-D-glucopyranosyl)-1-acetamido-6-(10,12-pentacosadiynamide)-3-thiohexane (**1**)*. To 0.120 g (0.187 mmol) of *N*-[*O*-(3-*O*-(methyl 2-acetate)-β-D-galactopyranosyl)]-(1→4)-*O*-[(α-L-fucopyranosyl)-(1→3)]-*O*-(β-D-glucopyranosyl)-3-acetamido-1-propene (*49*) in a 0.5 mL quartz cuvette was added 400 μL of deoxygenated H_2O followed by 0.050 g (0.440 mmol) of 2-aminoethanethiol hydrochloride (deoxygenated H_2O was prepared by passing a stream of N_2 through refluxing H_2O for 10 min and then allowing the H_2O to cool to room temperature with a constant N_2 stream). The reaction cuvette was covered with a septum, and a N_2 flow was introduced with a needle. The solution was irradiated at 254 nm from a UVP mineralight lamp at a distance of 1 cm for 2 h. TLC (1:2:1 EtOAc/IPA/H_2O mixture) indicated that most of the starting allylic compound had reacted to give compound **3**. The volatiles were removed by high-vacuum rotary evaporation, and the resulting residue was dissolved in 1 mL of a 1:2:0.5 THF/MeOH/H_2O mixture. To this solution was added a solution of 0.399 g (0.847 mmol) of *N*-succinimidyl-10,12-pentacosadiynate (*50*) in 2 mL of THF followed by 177 μL (1.27 mmol) of TEA. The reaction solution was allowed to stir at ambient temperature overnight, giving crude **3a**. The volatiles were removed by rotary evaporation, and residue was dissolved in methanol and brought to pH 12 with aqueous 1 N sodium hydroxide. The solution was stirred for 3 h, and then just enough Dowex 50 resin (H^+ form) was added to neutralize the base and the mixture filtered; then the volatiles were removed by rotary evaporation. The residue was purified by flash column chromatography, eluted first with a 10:3:1 EtOAc/IPA/H_2O mixture, then with a 5:3:1 EtOAc/IPA/H_2O mixture, and finally with a 3:2:1 EtOAc/IPA/H_2O mixture. The fractions containing the desired material were combined, and the volatiles were removed by high-vacuum rotary evaporation to afford 0.092 g (46%) of compound **1** as a white solid: ^1H NMR (200 MHz, $CDCl_3$, CD_3OD, D_2O) δ 0.89 (t, 3 H, $J = 6.7$ Hz), 1.02 (d, 3 H, $J = 6.4$ Hz), 1.24 (br m, 26 H), 1.49 (m, 4 H), 1.60 (m, 2 H), 1.90 (m, 2 H), 2.20 (m, 9 H), 2.57 (m, 2 H), 2.65 (m, 2 H), 3.34 (m, 2 H), 3.50 (m, 2 H), 3.65−4.15 (m, 18 H), 4.48 (d, 1 H, $J = 7.3$ Hz), 4.81 (m, 1 H), 5.41 (d, 1 H, $J = 3.7$ Hz); ^{13}C NMR (50 MHz, $CDCl_3$, CD_3OD, D_2O) δ 14.05, 16.21, 19.13, 19.17, 22.18, 22.65, 22.68, 22.83, 24.09, 25.68, 26.41, 28.25, 28.31, 28.73, 28.81, 28.84, 29.01, 29.03, 29.71, 29.79, 29.93, 30.05, 30.91, 31.12, 33.91, 35.26, 37.61, 44.78, 62.64, 66.08, 67.53, 69.01, 69.21, 70.23, 71.00, 72.00, 72.47, 72.94, 73.16, 75.81, 78.23, 78.42, 78.70, 79.01, 82.75, 83.54, 88.04, 99.39, 102.66, 174.65, 176.18, 177.23; mass spectrum (FAB^+) *m/z* 1083 ($M + Na^+$), 1106 ($M + 2Na^+$).

*N-[(3-Sulfo-β-D-galactopyranosyl)]-(1→4)-O-[(α-L-fucopyranosyl)-(1→3)]-O-(β-D-glucopyranosyl)-1-acetamido-6-amino-3-thiohexane (**4**)*. To a solution of 600 mg (0.89 mmol) of *N*-[(3-sulfo-β-D-glactopyranosyl)-(1→4)]-*O*-[(α-L-fucopyranosyl)-(1→3)]-*O*-(β-D-glucopyranosyl)-3-acetamido-1-propene (*49*) in 0.2 mL of deoxygenated H_2O in a 10 mL quartz cuvette was added 350 mg (3.08 mmol) of 2-aminoethanethiol hydrochloride (deoxygenated as described in the synthesis of compound **3**). The cuvette was fit with a septum, and a N_2 purge was introduced with a syringe needle. The reaction mixture was irradiated (as described in the synthesis of compound **3**) at 254 nm for 240 min. TLC (3:2:1 EtOAc/IPA/H_2O mixture) indicated complete consumption of the starting allylic compound. The product was partially purified by ion exchange column chromatography with Dowex 50 resin (NH_4^+ form) eluting with water followed by a gradient of 0.05 to 0.2 M NH_4OH. The product was further purified by silica gel chromatography (eluting with a 3:2:1 to 1:2:1 EtOAc/IPA/H_2O gradient) to give 466 mg (70%) of compound **4** as a white powder, after lyophilization: ^1H NMR (400 MHz, D_2O) δ 1.03 (d, 3 H, $J = 6.0$ Hz), 1.65−1.81 (bm, 2 H), 2.08 (s, 3 H), 2.45 (t, 2 H, $J = 7.0$ Hz), 2.70 (t, 2 H, $J = 6.5$ Hz), 3.06 (t, 2 H, $J = 6.5$ Hz), 3.42−3.85 (m, 17 H), 4.17 (app t, 2 H, $J = 12.2$ Hz), 4.40 (d, 1 H, $J = 7.8$ Hz), 5.29 (d, 1 H, $J = 3.5$ Hz); mass spectrum (FAB^+) *m/z* 727 ($M + H^+$), 749 ($M + Na^+$).

*N-[(3-Sulfo-β-D-galactopyranosyl)]-(1→4)-O-[(α-L-fucopyranosyl)-(1→3)]-O-(β-D-glucopyranosyl)-1-acetamido-6-(10,12-pentacosadiynamide)-3-thiohexane (**2**)*. A solution of 466 mg (0.62 mmol) of compound **4** was dissolved in 5 mL of anhydrous DMF containing 4 g of 4 Å molecular sieves. A solution of 334 mg (0.71 mmol) of *N*-succinimidyl-10,12-pentacosadiynate (*50*) in 1 mL of DMF was added followed by 100 μL (0.71 mmol) of TEA. The reaction solution was allowed to stir at ambient temperature for 8 h, giving crude **2**. The solution was filtered, and the volatiles were removed by rotary evaporation; the residue was dissolved in methanol and brought to pH 9 with aqueous 1 N sodium hydroxide. The volatiles were removed by rotary evaporation, and the residue was dissolved in $CHCl_3$ and MeOH (1:1) and filtered. The residue was purified by flash column chromatography, eluted first with a 5:2 $CHCl_3$/MeOH mixture and finally with a 2:1 $CHCl_3$/MeOH mixture. Compound **2** was isolated as an off-white solid, 550 mg (80%): ^1H NMR (400 MHz, CD_3OD) δ 0.66 (t, 3 H, $J = 6.8$ Hz), 0.95 (d, 3 H, $J = 7.0$ Hz), 1.02−1.20 (br m, 26 H), 1.27 (m, 4 H, $J = 7.3$ Hz), 1.37 (m, 2 H), 1.69 (m, 2 H), 1.96 (m, 5 H), 2.01 (t, 4 H, $J = 6.8$ Hz), 2.32 (t, 2 H, $J = 7.3$ Hz), 2.41 (t, 2 H, $J = 7.0$ Hz), 2.99 (q, 3 H, $J = 7.3$ Hz), 3.28 (m, 3H), 3.4−3.75 (m, 12 H), 4.01 (m, 2 H), 4.30 (d, 1 H, $J = 7.6$ Hz), 4.60 (d, 1H, $J = 7.4$ Hz), 5.20 (d, 1H, $J = 3.8$ Hz); mass spectrum (FAB^+) *m/z* 1081 ($M - H^+$). Compound **2** was dissolved in a chloroform solution for long-term storage.

*N-(2-Hydroxyethyl)-10,12-pentacosadiynamide (**6**)*. To a solution of 1.21 g (2.6 mmol) of *N*-succinimidyl-10,12-pentacosadiynate (*50*) in 50 mL of CH_2Cl_2 was added 0.20 mL (2.9 mmol) of ethanolamine followed by 0.35 mL (2.5 mmol) of TEA, and the reaction solution was allowed to stir for 2 h at ambient temperature. The reaction mixture was diluted with chloroform, washed with 1 N HCl and saturated aqueous sodium bicarbonate, dried over magnesium sulfate, and filtered. The solvent was removed by rotary evaporation, and the residue was purified by silica gel column chromatography eluting with a 10:1 chloroform/MeOH

mixture to give 0.99 g (93%) of **6** as a rapidly polymerizing white solid: mp 88 °C; IR (mineral oil) 3297, 2922, 2852, 1732, 1645, 1563, 1469, 1422, 1262, 1059, 1035 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 0.88 (t, 3 H, $J = 6.8$ Hz), 1.23 (br m, 26 H), 1.49 (m, 4 H), 1.61 (m, 2 H), 2.16−2.24 (two overlapping t, 6 H), 3.41 (m, 2 H), 3.71 (t, 2 H, $J = 4.8$ Hz), 5.97 (br s, 1 H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 14.10, 19.16, 19.18, 22.66, 25.63, 28.26, 28.33, 28.71, 28.84, 28.87, 29.08, 29.11, 29.15, 29.32, 29.46, 29.58, 29.60, 29.62, 31.89, 36.61, 42.46, 62.63, 65.18, 65.27, 77.20, 77.44, 174.50; high-resolution mass spectrum (FAB^+) calcd for $C_{27}H_{47}NO_2$ (MH^+) 417.3607, found 417.3611. Anal. Calcd for $C_{27}H_{47}NO_2$: C, 77.64; H, 11.34; N, 3.35. Found: C, 77.60; H, 11.33; N, 3.32. Compound **6** was dissolved in a chloroform solution to prevent polymerization and for long-term storage.

N-(2-Aminoethyl)-10,12-pentacosadiynamide (7). To a solution of 3.8 g (8.2 mmol) of *N*-succinimidyl-10,12-pentacosadiynate (*50*) in 40 mL of dichloromethane was added 10.45 mL (156 mmol) of ethylenediamine. The reaction solution was allowed to stir for 10 h at ambient temperature. The resulting fluffy white solid was dissolved in a large volume of chloroform containing ~5% methanol. The solution was washed with water and saturated aqueous bicarbonate, then dried over magnesium sulfate, and filtered. The solvent was removed by rotary evaporation, and the residue was dissolved in a small amount of 20% methanol in chloroform and purified by silica gel column chromatography eluting with a 6:1 to 3:1 chloroform/methanol gradient. Compound **7** (2.9 g, 85%) was isolated as a rapidly polymerizing white solid: ^1H NMR (500 MHz, $CDCl_3$) δ 0.89 (t, 3 H, $J = 6.6$ Hz), 1.23 (br m, 26 H), 1.50 (m, 4 H), 1.60 (m, 2 H), 1.72 (bs, 2 H), 2.17−2.24 (two overlapping t, 6 H), 2.81 (t, 2 H, $J = 5.2$ Hz), 3.28 (t, 2 H, $J = 5.2$ Hz), 6.1 (br s, 1 H). Compound **7** was dissolved in a 10% methanol in a chloroform solution to prevent polymerization and for long-term storage.

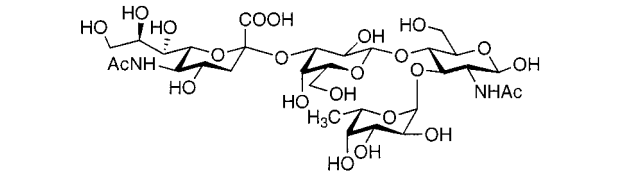
N-(2-Sulfoethyl)-10,12-pentacosadiynamide, Sodium Salt (8). To a solution of 0.21 g (0.5 mmol) of *N*-(2-hydroxyethyl)-10,12-pentacosadiynamide (**6**) in 2 mL of pyridine was added 0.6 g (4.3 mmol) of $Me_3N{\cdot}SO_3$ (sulfur trioxide−trimethylamine complex; *51*). The solution was allowed to stir for 2 days at ambient temperature, and the reaction was followed by TLC (10:1 $CHCl_3$/MeOH mixture). The solvent was removed by rotary evaporation, and the residue was triturated with water several times and dried by vacuum. The crude product was purified by flash silica gel chromatography with chloroform and methanol (4:1) as the eluent. The purified residue was dissolved in 10 mL of a 1:1 $CHCl_3$/MeOH mixture, treated with 2.5 g of the cation-exchange resin [Dowex 50 (Na^+ form)], filtered, and evaporated, to give 0.08 g (30%) of **8** as a white solid: ^1H NMR (400 MHz, 1:1 $CDCl_3/CD_3OD$) δ 0.85 (t, 3 H, $J = 6.9$ Hz), 1.23 (br m, 26 H), 1.49 (t, 4 H, $J = 7.7$ Hz), 1.59 (m, 2 H), 2.16−2.21 (two overlapping t, 6 H, $J = 6.9$, 7.8 Hz), 3.45 (t, 2 H, $J = 5.3$ Hz), 4.05 (t, 2 H, $J = 5.3$ Hz); mass spectrum (FAB^+) *m*/*z* 496 (free acid $M - H^+$). Compound **8** was dissolved in a 50% methanol/50% chloroform solution to prevent polymerization and for long-term storage.

Liposome Preparation. Polymerized poyldiacetylene (PDA) liposomes were prepared according to the method previously described (*52*). Briefly, polymerizable lipids and neoglycolipids were mixed and evaporated to a film. Deionized water was added to the films to give a 1 mM (total lipid) suspension. The suspension was heated to between 70 and 80 °C and probe sonicated for 30 min. The resulting clear solution was then cooled to 5 °C for 20 min and polymerized by UV light irradiation (254 nm). The deeply colored solutions were syringe filtered through 0.45 μm cellulose acetate filters to remove trace insoluble aggregates. Essentially all of the lipid material (>98%) is incorporated into the soluble liposomes. Dionex Analysis (Glyko, Inc., Novato, CA) quantitatively determined the carbohydrate contents in the polymerized liposome assemblies. The polymerized liposome preparations containing carbohydrate were digested with 2 N HCl for 2 h at 100 °C. The solution was dried after freezing with a Speedvac; the residue was redissolved in a known volume of water, and the solution was centrifuged to separate the polymer particles. An aliquot was injected into the Dionex instrument for monosaccharide identification and quantification. The monosaccharides were identified by comparison to D-galactose and L-fucose standards. Each sample was run in duplicate.

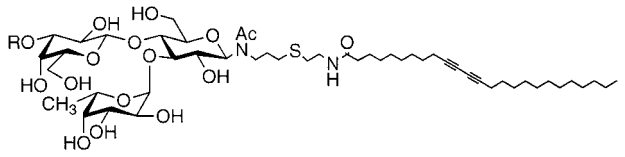
Langmuir Isotherm Characterizations. The lipid components were compressed on an air−water interface using a Langmuir film balance (KSV Corp.). By plotting surface pressure versus mean molecular area, we generated an isotherm curve. This was done for several matrix lipid mixtures with increasing percentages of the neoglycolipid component. The smoothness of the isotherm curve and number of collapse points indicate the mixing state (ideal vs phase-separated) of the multilipid film.

Preparation of GlyCAM-1 for Inhibition Binding Assays. Partially purified GlyCAM-1 was prepared as described previously (*53*). Briefly, mouse serum was extracted with four volumes of a 2:1 $CHCl_3$/MeOH mixture, and the layers were separated by centrifugation at 2000*g*. The upper aqueous layer was separated form the organic and precipitated protein layers, concentrated to half of the original serum volume by boiling, and dialyzed against 20 volumes of Dulbecco's PBS with two changes. This preparation was then diluted with PBS back to the original serum volume and used as a semipure source of GlyCAM-1 for the binding inhibition assays described below.

Selectin−GlyCAM-1 Inhibition Assay. The selectin−GlyCAM-1 inhibition assays used here have been described previously (*46*). Microtiter plates were coated with a 1.6 μg/mL solution of a polyclonal anti-GlyCAM-1 antibody specific for the peptide core (CAM02) in PBS at 100 μL/well, overnight at 4 °C. After being washed with 0.1% Tween 20 in PBS, the wells were blocked with 3% BSA (200 μL/well) for 2 h at ambient temperature. After washing, GlyCAM-1 was captured onto the microtiter wells by incubation with the partially purified GlyCAM-1 preparation described above, 100 μL/well for 1 h at ambient temperature. The plate was then washed with PBS Tween 20 in preparation for incubation with the selectin−IgG chimeras and serial dilutions of the liposomes. The L-selectin−IgG chimera was diluted to 2 μg/mL in 2% BSA in PBS, and the E- and P-selectin−IgG chimeras were diluted to 4 μg/mL in 0.2% BSA in PBS. The chimeras were then complexed with biotinylated goat anti-human IgG Fc $F(ab')_2$ diluted 1:2000 and the streptavidin−alkaline phosphatase conjugate diluted 1:2000 for 25 min at ambient temperature. Naïve rabbit serum was added to a concentration of 5% (v/v), and the



sialyl Lewis X



1. R = -CH_2CO_2H (sLe^x analog)

2. R = -SO_3Na (sulfo-Le^x analog)

FIGURE 2: Sialyl Lewis X (sLe^x) and polymerizable neoglycolipid analogues for sLe^x and sulfo-Le^x.

solution was incubated for an additional 5 min. This solution (80 μL) was incubated with 80 μL serial dilutions of the neoglycoliposomes in PBS and incubated for 30 min at 4 °C. The mixture was then transferred in duplicate to the GlyCAM-1-coated wells (100 μL/well), and the wells were incubated for 30 min at ambient temperature. The plate was washed with PBS Tween 20, and the bound selectin−IgG chimera was detected by the addition of phosphatase substrate (*p*-nitrophenyl phosphate, 1 mg/mL, in 10% diethanolamine, with 0.1 mM $MgCl_2$, 100 μL/well). Optical densities were obtained at 405 nm.

Statistics. The concentration of liposome required to inhibit 50% of selectin binding (IC_{50} value) was calculated by linear regression of a plot of optical density versus inhibitor concentration after the background signal was subtracted. The input value was one-half the optical density of the wells where no inhibitor was present. Significant differences between means were calculated at a significance level of $\alpha = 0.05$ using a Student's *t* test when two variables were compared, or one-way analysis of variance when multiple variables were compared. Statistical tests were performed using SigmaStat software (SPSS, Inc., Chicago, IL).

RESULTS

The neoglycolipids **1** and **2** (Figure 2) were synthesized with polymerizable diacetylene groups in the lipid tails, via the strategy depicted in Scheme 1. The *N*-allyl glycosides of the sLe^x and sulfo-Le^x analogues were prepared as previously reported (*49*). The allyl group of each is extended into an amine-terminated linking chain with the reaction of 2-aminoethanethiol hydrochloride (*54*), giving compounds **3** and **4**, respectively. Condensation of these amines with the activated ester of 10,12-pentacosadyinoic acid (PCDA) gave the neoglycolipids **2** and **3a**, respectively. The methyl ester at the C3′ position of the sLe^x-like neoglycolipid **3a** was saponified with base and neutralized with Dowex resin to give neoglycolipid **1** with an acetic acid functionality at the C3′ position.

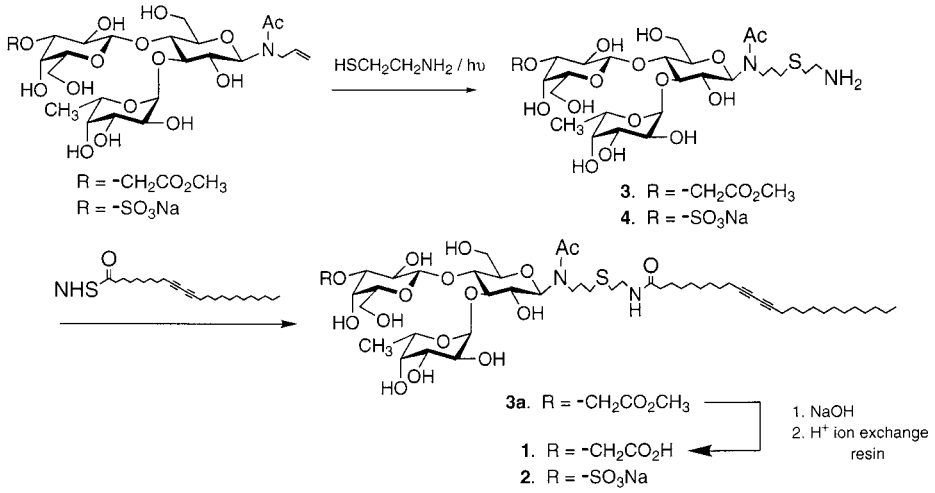
Aqueous dispersions of the polymerizable lipids are sonicated while being heated to a temperature above the lipid phase transition, giving liposomes that appear as clear or slightly opaque solutions at concentrations of 1 mM. To obtain polymerized PDA liposomes, the lipid chains must be in a tightly packed, solid analogous state as part of the membrane bilayer (*55*). Since the packing density is controlled to a large extent by the size of the headgroup, liposomes consisting solely of neoglycolipids such as **1** or **2** cannot be polymerized using this methodology. The bulkiness of the carbohydrate headgroup has prevented the lipid chains from packing densely enough to allow the cross-linking reaction to occur. We found that stable, cross-linked liposomes could be generated, however, if the neoglycolipid content was less than 40% of the total lipid content of the suspension. A second polymerizable lipid (matrix lipid) with a small headgroup is mixed with the neoglycolipid, cosonicated, and irradiated with UV light to initiate a radical polymerization process, resulting in a deeply colored, polydiacetylene liposome solution.

The matrix lipids define the nature of the liposome surface that surrounds the carbohydrate group. We have chosen four matrix lipids (**5**−**8**) that range in charge from acidic to neutral to basic, to mix with the carbohydrate lipids in the liposome preparations (Scheme 2). Because of the strong ionic character of sulfate esters, liposomes containing only matrix lipid **8** proved to be unpolymerizable. The sphere of hydration around the sulfate group may be sufficiently large to prevent this compound from being an effective matrix lipid. However, when a matrix lipid such as compound **6** is also included, liposomes containing up to approximately 70% sulfated lipid **8** can be efficiently polymerized. Therefore, we used an equimolar ratio of lipids **6** and **8** to generate liposomes with a negatively charged surface of sulfate esters. The active ester of compound **5** (PCDA) is used to prepare compound **6** or **7** from ethanolamine or ethylenediamine, respectively (Scheme 3). The hydroxyl group of compound **6** is sulfated to give compound **8**.

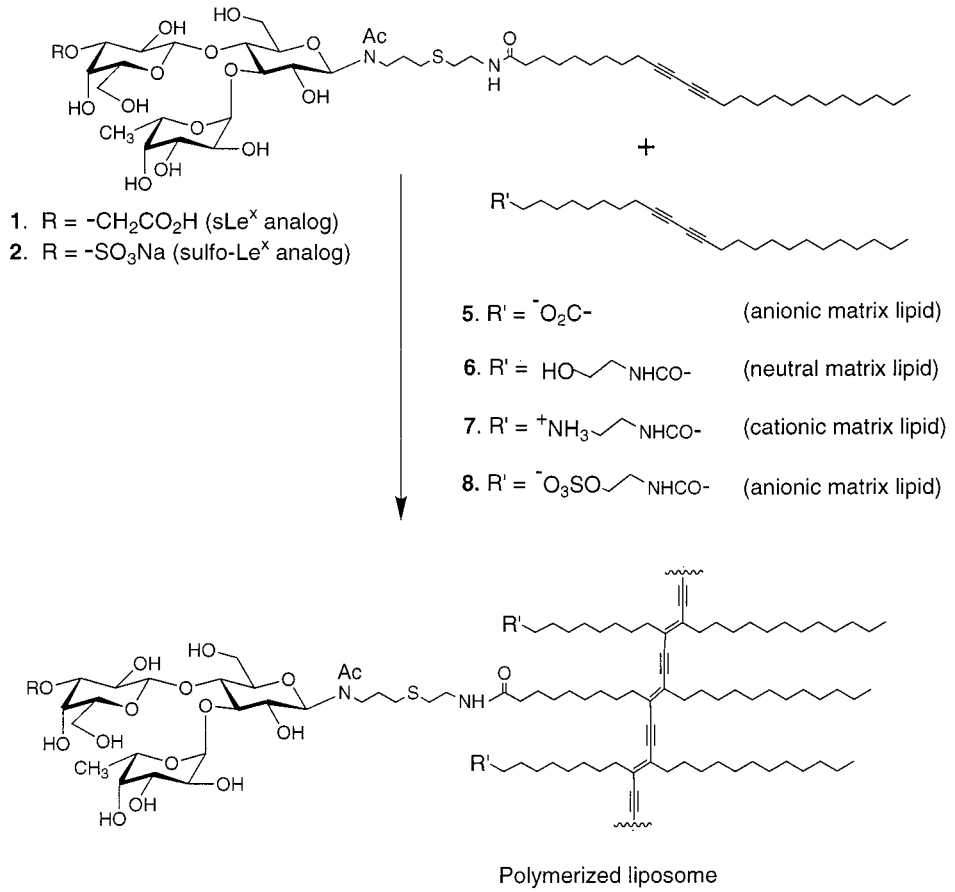
Surface isotherms (*56*) obtained from Langmuir film studies were measured to determine the extent of lipid phase separation in the monolayers formed from mulicomponent mixtures. The neoglycolipid component is incrementally introduced into the matrix mixture in known amounts. As the mole fraction of neoglycolipid lipid is increased, a proportional expansion of the monolayer film with a single collapse pressure indicates ideal mixing of the components. Conversely, the presence of multiple collapse pressures and nonproportion expansion of the film indicates phase separation. The isotherms were measured for the increasing, incremental additions of glycolipid component **1** or **2** into a matrix lipid mix of 50% sulfated lipid **8** and 50% neutral lipid **6**. This study revealed proportional expansion and a single collapse pressure indicating that the components mixed very well over the ratio of lipids observed (up to 40% **1** or **2**) and that only ideal mixtures were being created (data not shown). These data tend to support the notion that ideal mixing would also be observed during liposome formation with similar lipid component ratios.

In a previous study using analogous sLe^x-like liposomes to inhibit P-selectin binding to HL-60 cells, we showed assemblies prepared with 5% neoglycolipid afforded the best inhibition (*52*). In this current study, we used this 5% glycolipid:95% matrix lipid ratio for all the glycoliposome preparations. Monosaccharide analysis by Dionex was carried out (described earlier) to determine the efficiency of introducing the synthetic glycolipids into the cross-linked polymer

Scheme 1



Scheme 2



particles. The concentration of L-fucose liberated from the polymerized liposome preparations containing a starting carbohydrate concentration of neoglycolipid **2** of 50 μM (5% glycolipid in a 1 mM total lipid solution prior to polymerization) with matrix lipid **5**, **6**, or **7** was found to be 48.6, 52.7, or 53.7 μM, respectively.

To evaluate the reactivity of the liposomes with L-, E-, and P-selectin, we assayed their ability to competitively inhibit binding of recombinant selectin−Ig chimeras to GlyCAM-1. As expected, multimerization of the sLe^x-like oligosaccharide had a profound effect on lowering the IC_{50} value relative to the monomer (Table 1). When the IC_{50} values of the sLe^x-like liposome are compared to those of the free monomer, the multimerized form achieved an IC_{50} value 4 orders of magnitude lower for L- and P-selectin ($p < 0.001$). Although the sLe^x-like monomer inhibited E-selectin binding with an IC_{50} of 1.7 mM, the sLe^x-like liposome without additional anionic charge was a surprisingly weak inhibitor for E-selectin. The multivalent form did not inhibit E-selectin binding to GlyCAM-1 at concentrations of <2.5 μM.

The general trends for each selectin are summarized as follows.

L-Selectin Inhibition. With sLe^x-like carbohydrate compound **1**, the matrix lipid has a marked effect on the binding strength of the assembly. The strength of binding increases

Scheme 3

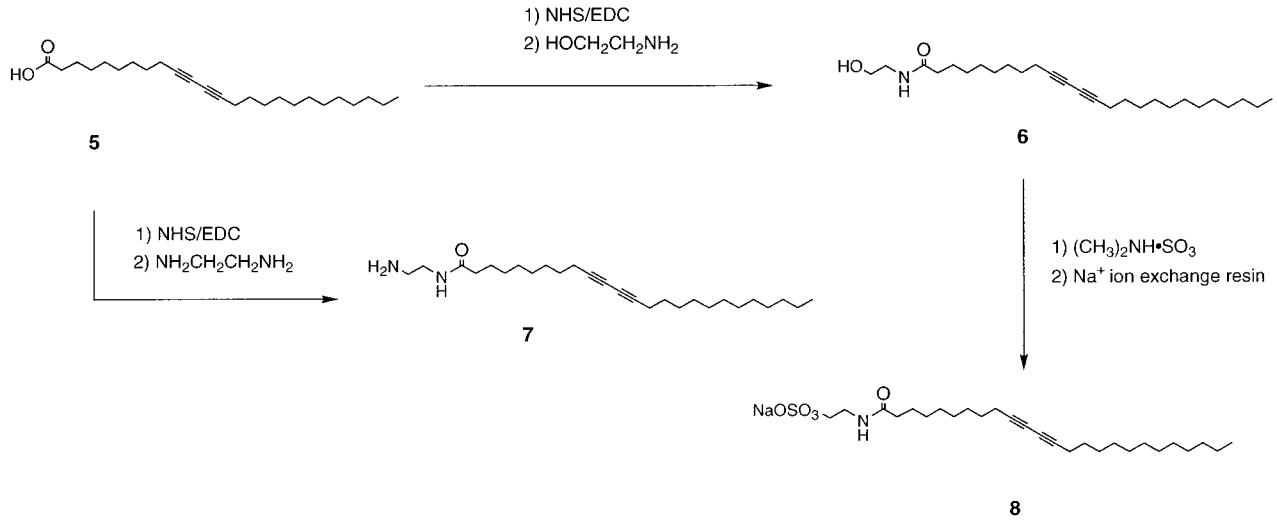


Table 1: Selectin Inhibition by Polymerized Liposomes

		IC_{50} *nM*					
	Surface functionality	L-selectin Mean	SD	P-selectin Mean	SD	E-selectin Mean	SD
Liposomes with embedded oligosaccharide:							
A sLe^x-like (**1**)	SO_3^-	217	29	175	66	425	87
	CO_2^-	500	71	467	58	>2,500	---
	OH	1,150	123	1487	278	>2,500	---
	NH_3^+	>12,500	---	>12,500	---	>12,500	---
B 3'-sulfo Le^x-like (**2**)	SO_3^-	200	20	ND[b]		ND[b]	
	CO_2^-	257	45	280	27	683	64
	OH	240	42	181	38	379	48
	NH_3^+	>12,500	---	>12,500	---	>12,500	---
C None[a]	SO_3^-	7,500	66	4,320	58	>50,000	---
	CO_2^-	>250,000	---	>50,000	---	>50,000	---
	OH	>250,000	---	>50,000	---	>50,000	---
	NH_3^+	>250,000	---	>50,000	---	>50,000	---
Monovalent sLe^x-like oligosaccharide (**1**)		12*mM*		7.5*mM*		1.7*mM*	

[a] Liposome shell only, no embedded oligosaccharide, IC_{50} based on the concentration of total lipid headgroup. [b] ND, not determined.

with the acidity of the matrix. The order of activity for the matrix groups (with 5% compound **1**) is SO_3^- > CO_2^- > OH, with no activity (IC_{50} > 12.5 μM) with the NH_3^+ group (Table 1, section A). The most active formulation in this series is found for the sLe^x-like carbohydrate displayed in a matrix of sulfate esters (IC_{50} = 217 nM). When sulfo-Le^x carbohydrate compound **2** is presented, the binding strength seems to be independent of the type of matrix headgroup as long as the charge is not basic (Table 1, section B). For all of the acidic or neutral matrices, the IC_{50}'s are in the range of 200 nM. Simple polymerized liposomes containing no saccharide groups (section C) show moderate activity with L-selectin only when sulfated groups are present.

P-Selectin Inhibition. In the case of P-selectin glycoliposomes prepared from the sLe^x-like carbohydrate **1**, increasing the acidity of the matrix group (SO_3^- > CO_2^- > OH) (Table 1, section A) leads to a stronger inhibitor, with no activity (IC_{50} > 12.5 μM) with NH_3^+ groups, similar to that observed for L-selectin. The most active formulation in this series, like L-selectin, is found for the sLe^x-like carbohydrate displayed in a matrix of sulfate esters (IC_{50} = 175 nM). However, when sulfo- Le^x carbohydrate compound **2** (section

B) is presented, the additional matrix anionic groups seem to have a slightly weakening effect. The neutral matrix leads to an inhibitor slightly better than the carboxylate (IC_{50} = 181 nM). Simple polymerized liposomes containing no saccharide groups show moderate activity with P-selectin only when sulfated groups are present (Table 1, section C). This is consistent with the observation that sulfated polymers bind avidly to this selectin (*57*).

E-Selectin Inhibition. For E-selectin, the only assemblies containing sLe^x-like carbohydrates (**1**) (section A) to show any activity at the concentrations that were tested (up to 2.5 μM) are glycoliposomes where the carbohydrate is presented in a highly sulfated matrix (IC_{50} = 425 nM). As opposed to L- and P-selectin, where carboxylic acid matrices enhance the activity of glycoliposomes, E-selectin appears to specifically require the anionic nature of sulfate groups to possess sub-micromolar inhibitory activity. The presence of an sLe^x-like carbohydrate appears to be a much more rigid requirement for activity with E-selectin than it is with P- or L-selectin. Sulfated groups alone on polymerized liposomes are not sufficient to lead to binding analogues (Table 1, section C).

Interestingly, if the sulfate is part of the carbohydrate, as in glycoliposomes containing compound 3′-sulfo Le^x-like analogue **2**, E-selectin inhibition seems to be slightly weakened by additional anionic charge. The most potent assembly is the sulfated carbohydrate in a neutral matrix (IC_{50} = 379 nM). Like the other selectins, presentation of carbohydrates in a matrix of amine groups creates glycoliposomes with no E-selectin binding affinity up to tested concentrations of 12.5 μM.

DISCUSSION

The search for the structural features that are required for high-affinity selectin binding has been a focus of research in many laboratories. Selectin binding to physiological ligands is estimated to be 10^4-10^5 times stronger than binding to monomeric sLe^x (*58*), and carbohydrate avidity may play a key role in this enhanced binding. Indeed, a common feature among the selectin ligands characterized to date is the presence of at least one sialomucin domain displaying multimeric arrays of sLe^x- and sulfo sLe^x-capped oligosaccharides. Furthermore, it is likely that L- and P-selectin exist as oligomers which would allow them to take advantage of polyvalent ligands. L-Selectin is found clustered on the tips of leukocyte microvilli (*59*, *60*), and there is evidence suggesting L-selectin exists as a tetramer (*61*) and P-selectin exists as a pentamer (*58*) in the plasma membrane. The oligomeric state for E-selectin, however, is not clear. A recent study suggests that E-selectin displays two carbohydrate binding sites that simultaneously accept two sLe^x ligands (*62*). If receptor and ligand oligomerization is the general case for L- and P-selectin (and possibly E-selectin), then high-affinity binding to physiological ligands may be aided by multipoint attachments of oligomeric receptors to polyvalent ligands. Examples of these types of interactions have been recently extensively reviewed (*45*).

Detailed affinity studies and crystallographic examination of the N-terminal domain of PSGL-1 bound to P-selectin has revealed an interesting dual component nature to the ligand. The simultaneous binding of both an sLe^x-terminated oligosaccharide and several sulfate ester groups occurs on neighboring sites in the lectin binding domain and are required for high-affinity P-selectin interaction (*38*). We sought to determine if this bicomponent requirement could be used to design new inhibitors that have enhanced P-selectin binding capabilities. In addition, given the large similarities in the ligands that bind to all three selectins, we examined if bifuctional scaffolds would show any enhancement with L- or E-selectin as well.

We chose polymerized polydiacetylene liposomes as the form for ligand multimerization because the size and composition of these particles are easily controlled and their surface chemistry can be tailored to allow the exploration of composite receptor binding sites. Because these liposomes are polymerized, they are considerably more stable than conventional phospholipid liposomes. They have no propensity to fuse with themselves or other bilayer membranes and remain stable in solution for extended periods of time. In addition, the polymerizable lipid precursor is relatively inexpensive and easy to functionalize, compared to the corresponding phosphatidylcholine analogue. The results from the Langmuir isotherm study show that the lipid components mix well at the percent compositions that were tested. Therefore, it can be inferred that the liposome surface is composed of randomly disbursed lipid types rather than a phase-separated mosaic. By measuring the starting verses final mass recovery, we find the starting amount of lipid sonicated together closely compares with the final amount of material in the polymer. The results of the Dionex carbohydrate analysis confirm that the theoretical amount of carbohydrate at the beginning of each preparation becomes incorporated into the polymerized liposome bilayer surface.

The oligosaccharide ligands used here are Le^x-like in nature because they maintain the same carbohydrate moieties and linkages that define the natural $Lewis^X$ trisaccharide [Galβ1→4(Fucα1→3)GlcNAc]. In the analogues used here, the C2 position of glucose is not *N*-acetylated. This conservative alteration results in a negligible change in the inhibitory potency of the oligosaccharide (*63*, *64*). The sLe^x-like compound is analogous to sialyl $Lewis^X$ in that the C3 position of galactose is substituted with acetic acid rather than sialic acid. The carboxylic acid functionality of the acetic acid substitution serves to mimic the C1 carboxylic acid of sialic acid in sLe^x and is two carbon atoms away from the C3 hydroxyl of galactose as it would be in the α2−3 linked sialic acid of genuine sLe^x (Figure 2). This exchange results in only a modest decrease in the inhibitory potency of the trisaccharide relative to sLe^x (*2*). Substitution of the 3′-sialic acid of sLe^x with a 3′-sulfate ester has been shown to produce an active inhibitor of selectin binding (*34*, *65*, *66*). It is possible that like the acetic acid substitution, the 3′-sulfate ester compensates for the anionic contribution of the carboxylic acid of sialic acid. While the 3′-sulfo Le^x trisaccharide has not been identified on GlyCAM-1 (*24*, *67*), this structure along with the isomeric form, 3′-sulfo Le^a, has been found on epithelial glycoproteins and ovarian cystadenoma cells, and has E-selectin binding activity (*32*, *68*−*71*).

Accordingly, multivalent ligand mimetics offer a practical approach to the design of potent selectin antagonists and the study of selectin binding requirements. We generated two panels of liposomes displaying a multimeric array of Le^x-

like oligosaccharides in a background of ionic functional groups. These assemblies were assayed for their ability to inhibit L-, E-, and P-selectin binding to GlyCAM-1 (Table 1). GlyCAM-1 contains two sialomucin domains that present multivalent clusters of sulfated sLe^x oligosaccharides (*23*, *67*).

By displaying sLe^x-like oligosaccharides on a polymerized liposome composed of a neutral matrix, we observed a 4 order of magnitude lowering of the IC_{50} value for P- and L-selectin, over the monovalent carbohydrate. Liposomes devoid of carbohydrate groups did not inhibit any of the selectins except for the assemblies prepared with sulfate ester matrix lipids. These displayed moderate inhibition for L- and P-selectin binding (Table 1, section C). This is consistent with the observation that highly sulfated polymers bind avidly to L- and P-selectins (*35*, *57*). As the amount of sulfate ester was decreased, the ability of the liposome to inhibit L- and P-selectin binding was diminished (data not shown). It is worth noting that the liposome with the carboxylic acid surface did not inhibit L-, E-, or P-selectin binding despite having twice the negative charge as the sulfated liposome.

To explore whether the inclusion of additional anionically charged groups has an enhancing effect on binding, liposomes containing combinations of carbohydrates and charged matrix groups were created and tested. In the case of sLe^x-like liposomes tested with L- and P-selectin (Table 1, section A), the introduction of a negative charge in the form of a high density of carboxylic acids increased its inhibitory potency 2-fold for L-selectin ($p < 0.001$) and 3-fold for P-selectin ($p = 0.007$), over that of the neutral matrix. Introduction of negative charge into the liposome matrix as a sulfate ester enhanced the inhibitory potency 5- and 8.5-fold for L- and P-selectin, respectively ($p < 0.001$ and $p = 0.004$, respectively), relative to that of the sLe^x-like liposome with a neutral matrix. The only liposome containing the sLe^x-like carbohydrate to show E-selectin inhibition is where the carbohydrate is displayed in a matrix of sulfate esters. Inclusion of cationic charged groups (amines) in the matrix eliminated any binding activity of glycoliposomes to all three selectins. A possible rationalization for these observations is that the carbohydrate and anionically charged ligands are acting synergistically when binding to the selectin, enhancing the overall inhibition potency of the construct. Another possibility is that the charged matrix groups can act to modify the orientation of the carbohydrate moiety presented to the selectin. The cationically charged amine matrix can, for example, charge pair with the anionically charged carbohydrate group and reduce the ability of the carbohydrate to bind to the lectin. This study cannot distinguish between the two possibilities.

L-Selectin ligands present sulfate esters attached directly to carbohydrate, and high-affinity binding requires both carbohydrate and sulfate ester determinants to be present. However, the observed ability of the receptor to interact with either independently suggests that the ligand recognition domain could in fact maintain independent binding sites for carbohydrate and sulfate esters. However, the enhancing effect of anionic matrix lipids on the inhibitory potency of the liposomes was not observed for L-selectin with the 3′-sulfo Le^x-like liposomes, and there were no significant differences in the IC_{50} values among liposomes bearing a neutral or acidic surface ($p = 0.469$).

For P- and E-selectin inhibition, there were unexpected matrix effects with the liposomes displaying the 3′-sulfo Le^x-like glycoform. For both selectins, the 3′-sulfo Le^x carbohydrate gave the best inhibition when presented in a neutral matrix. Liposomes displaying the 3′-sulfo Le^x-like oligosaccharide in a background of sulfate esters were not tested for either P- or E-selectin. As was the case for the sLe^x-like liposome, introduction of a positive charge into the outer layer of the 3′-sulfo Le^x-like liposome greatly diminished the inhibitory activity for all three selectins.

The inhibition trends in this study lend themselves to support of the proposed two-site model for P-selectin (*38*, *72*) and a possible two-site model for L-selectin (*73*); i.e., both a carbohydrate recognition domain and an area that forms ionic bonds may be required for strong binding. In E-selectin, the two-site model is less distinct. Clearly, for a given unsulfated carbohydrate such as sLe^x analogue **1**, inclusion of sulfate groups in the matrix increases activity. This augmentation of binding to E-selectin by inclusion of anionically charged groups in multivalent inhibitors has been previously observed (*41*, *74*). However, unlike L- and P-selectin, highly sulfated liposomes that do not display sLe^x or sulfo-Le^x-like carbohydrates do not show binding to E-selectin. The inability of any of the charged liposomes without carbohydrate to inhibit E-selectin binding in combination with the lack of a sulfate requirement for high-affinity E-selectin binding suggests that the ligand recognition domain of this selectin may not possess independent recognition sites for sulfate esters and carbohydrate.

The results from a comparison of static and dynamic shear flow assays with several mono- and disulfated trisaccharides and polyvalent polymers displaying them were reported (*75*). Many of the inhibitors that were tested showed potent inhibition under static conditions, but gave little or no inhibition of binding under shear flow. We are observing similar inconsistencies (*76*) between the activity of monovalent inhibitors under static and dynamic flow assay conditions. However, to a first approximation, a static assay comparing polymers such as the analogues reported here gives a good starting point with which to develop a structure−activity relationship. Studies, now underway, are demonstrating that various glycoliposome constructs are very potent inhibitors of leukocyte rolling on activated endothelial, platelet, and neutrophil cell monolayers in shear flow assay systems. The results of this work will be reported shortly (*76*).

Since site specific sulfation of carbohydrates is synthetically challenging and impractical on a large scale, the ability to introduce sulfate functionality independent of the carbohydrate groups is a significant advance in the design of selectin inhibitors. The ability to rapidly and systematically vary the composition of these liposomes is a distinguishing feature of this methodology and may be applied to study other systems where composite binding determinants are important for high-affinity binding.

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REFERENCES

1. Rosen, S. D., and Bertozzi, C. R. (1994) *Curr. Opin. Cell Biol. 6*, 663.
2. Dasgupta, F. (2000) in *High Throughput Screening for Novel Anti-Inflammatories* (Kahn, M., Ed.) pp 123−144, Birkhäuser Verlag, Basel.
3. Lasky, L. A. (1995) *Annu. Rev. Biochem. 64*, 113.
4. Welply, J. K., Keene, J. L., Schmuke, J. J., and Howard, S. C. (1994) *Biochim. Biophys. Acta 1197*, 215.
5. Lawrence, M. B. (1999) *Curr. Opin. Struct. Biol. 3*, 659.
6. Feizi, T. (1993) *Curr. Opin. Struct. Biol. 3*, 701.
7. Springer, T. A. (1994) *Cell 76*, 301.
8. Varki, A. (1994*) Proc. Natl. Acad. Sci. U.S.A. 91*, 7390.
9. Crottet, P., Kim, Y. J., and Varki, A. (1996) *Glycobiology 6*, 191.
10. Kansas, G. S. (1996) *Blood 88*, 3259.
11. Bertozzi, C. R. (1995) *Chem. Biol. 2*, 703.
12. McEver, R. P., Moore, K. L., and Cummings, R. D. (1995) *J. Biol. Chem. 270*, 11025.
13. Zak, I., Lewandowska, E., and Gnyp, W. (2000) *Acta Biochim. Pol. 47*, 393.
14. Simanek, E. E., McGarvey, G. J., Jablonowski, J. A., and Wong, C.-H. (1998) *Chem. Rev. 98*, 833.
15. Berg, E. L., McEvoy, L. M., Berlin, C., Bargatze, R. F., and Butcher, E. C. (1993) *Nature 366*, 695.
16. Yarema, K. J., and Bertozzi, C. R. (1998) *Curr. Opin. Chem. Biol. 2*, 49.
17. Lasky, L. A., Singer, M. S., Dowbenko, D., Imai, Y., Henzel, W., Fennie, C., Watson, S., and Rosen, S. D. (1992) *Cold Spring Harbor Symp. Quant. Biol. 57*, 259.
18. Baumhueter, S., Singer, M. S., Henzel, W., Hemmerich, S., Renz, M., Rosen, S. D., and Lasky, L. A. (1993) *Science 262*, 436.
19. Puri, K. D., Finger, E. B., Gaudernack, G., and Springer, T. A. (1995) *J. Cell Biol. 131*, 261.
20. Sassetti, C., Tangemann, K., Singer, M. S., Kershaw, D. B., and Rosen, S. D. (1998) *J. Exp. Med. 187*, 1965.
21. Moore, K. L., Stults, N. L., Diaz, S., Smith, D. F., Cummings, R. D., Varki, A., and McEver, R. P. (1992) *J. Cell Biol. 118*, 445.
22. Streeter, P. R., Berg, E. L., Rouse, B. T. N., Bargatze, R. F., and Butcher, E. C. (1988) *Nature 331*, 41.
23. Hemmerich, S., and Rosen, S. D. (1994) *Biochemistry 33*, 4830.
24. Hemmerich, S., Leffler, H., and Rosen, S. D. (1995) *J. Biol. Chem. 270*, 12035.
25. Shailubhai, K., Streeter, P. R., Smith, C. E., and Jacob, G. S. (1997) *Glycobiology 7*, 305.
26. Moore, K. L., Eaton, S. F., Lyons, D. E., Lichenstein, H. S., Cummings, R. D., and McEver, R. P. (1994) *J. Biol. Chem. 269*, 23318.
27. Foxall, C., Watson, S. R., Dowbenko, D., Fennie, C., Lasky, L. A., Kiso, M., Hasegawa, A., Asa, D., and Brandley, B. K. (1992) *J. Cell Biol. 117*, 895.
28. Edgington, S. M. (1992) *Bio/Technology 10*, 383.
29. Zhou, Q., Moore, K. L., Smith, D. F., Varki, A., McEver, R. P., and Cummings, R. D. (1991) *J. Cell Biol. 115*, 557.
30. Pouyani, T., and Seed, B. (1995) *Cell 83*, 333.
31. Sako, D., Comess, K. M., Barone, K. M., Camphausen, R. T., Cumming, D. A., and Shaw, G. D. (1995) *Cell 83*, 323.
32. Green, P. J., Tamatani, T., Watanabe, T., Miyasaka, M., Hasegawa, A., Kiso, M., Yuen, C.-T., Stoll, M. S., and Feizi, T. (1992) *Biochem. Biophys. Res. Commun. 188*, 244.
33. Green, P. J., Yuen, C.-T., Childs, R. A., Chai, W., Miyasaka, M., Lemoine, R., Lubineau, A., Smith, B., Ueno, H., Nicolaou, K. C., and Feizi, T. (1995) *Glycobiology 5*, 29.
34. Yuen, C.-T., Lawson, A. M., Chai, W., Larkin, M., Stoll, M. S., Stuart, A. C., Sullivan, F. X., Ahern, T. J., and Feizi, T. (1992) *Biochemistry 31*, 9126.
35. Shailubhai, K., Abbas, S. Z., and Jacob, G. S. (1996) *Biochem. Biophys. Res. Commun. 229*, 488.
36. Wilkins, P. P., Moore, K. L., McEver, R. P., and Cummings, R. D. (1995) *J. Biol. Chem. 270*, 22677.
37. Hemmerich, S., Butcher, E. C., and Rosen, S. D. (1994) *J. Exp. Med. 180*, 2219.
38. Somers, W. S., Tang, J., Shaw, G. D., and Camphausen, R. T. (2000) *Cell 103*, 467.
39. Sakagami, M., Horie, K., Nakamoto, K., Kawaguchi, T., and Hamana, H. (1998) *Bioorg. Med. Chem. Lett. 8*, 2783.
40. Sears, P., and Wong, C.-H. (1999) *Angew. Chem., Int. Ed. 38*, 2300.
41. Thoma, G., Patton, J. T., Magnani, J. L., Ernst, B., Öhrlein, R., and Duthaler, R. O. (1999) *J. Am. Chem. Soc. 121*, 5919.
42. Stahn, R., Schäfer, H., Kernchen, F., and Schreiber, J. (1998) *Glycobiology 8*, 311.
43. Roy, R. (1996) *Curr. Opin. Struct. Biol. 6*, 692.
44. Kiessling, L. L., and Pohl, N. L. (1996) *Chem. Biol. 3*, 71.
45. Mammen, M., Choi, S.-K., and Whitesides, G. M. (1998) *Angew. Chem., Int. Ed. 37*, 2754.
46. Bertozzi, C. R., Singer, M. S., and Rosen, S. D. (1997) *J. Immunol. Methods 203*, 157.
47. Watson, S. R., Imai, Y., Fennie, C., Geoffroy, J. S., Rosen, S. D., and Lasky, L. A. (1990) *J. Cell Biol. 110*, 2221.
48. Lasky, L. A., Singer, M. S., Dowbenko, D., Imai, Y., Henzel, W. J., Grimley, C., Fennie, C., Gillett, N., Watson, S. R., and Rosen, S. D. (1992) *Cell 69*, 927.
49. Spevak, W., Dasgupta, F., Hobbs, C. J., and Nagy, J. O. (1996) *J. Org. Chem. 61*, 3417.
50. Spevak, W., Nagy, J. O., Charych, D. H., Schaefer, M. E., Gilbert, J. H., and Bednarski, M. D. (1993) *J. Am. Chem. Soc. 115*, 1146.
51. Nicolaou, K. C., Bockovich, N. J., and Carcanague, D. R. (1993) *J. Am. Chem. Soc. 115*, 8843.
52. Spevak, W., Foxall, C., Charych, D. H., Dasgupta, F., and Nagy, J. O. (1996) *J. Med. Chem. 39*, 1018.
53. Singer, M. S., and Rosen, S. D. (1996) *J. Immunol. Methods 196*, 153.
54. Roy, R., and Tropper, F. D. (1988) *J. Chem. Soc., Chem. Commun.*, 1058.
55. Day, D., and Ringsdorf, H. (1978) *J. Polym. Sci. Polym. Lett. Ed. 16*, 205.
56. Gaines, G. L., Jr. (1966) in *Insoluble Monolayers at Liquid Gas Interfaces*, Wiley, New York.
57. Kretzschmar, G., Toepfer, A., Hüls, C., and Krause, M. (1997) *Tetrahedron 53*, 2485.
58. Ushiyama, S., Laue, T. M., Moore, K. L., Erickson, H. P., and McEver, R. P. (1993) *J. Biol. Chem. 268*, 15229.
59. Von Andrian, U. H., Hasslen, S. R., Nelson, R. D., Erlandsen, S. L., and Butcher, E. C. (1995) *Cell 82*, 989.
60. Bruehl, R. E., Springer, T. A., and Bainton, D. F. (1996) *J. Histochem. Cytochem. 44*, 835.
61. Crommie, D. (1994) Ph.D. Dissertation, University of California, San Francisco.
62. Thomas, V. H., Yang, Y., and Rice, K. G. (1999) *J. Biol. Chem. 274*, 19035.
63. Tyrrell, D., James, P., Rao, N., Foxall, C., Abbas, S., Dasgupta, F., Nashed, M., Hasegawa, A., Kiso, M., Asa, D., Kidd, J., and Brandley, B. K. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10372.
64. Brandley, B. K., Kiso, M., Abbas, S., Nikrad, P., Srivasatava, O., Foxall, C., Oda, Y., and Hasegawa, A. (1993) *Glycobiology 3*, 633.
65. Sanders, W. J., Katsumoto, T. R., Bertozzi, C. R., Rosen, S. D., and Kiessling, L. L. (1996) *Biochemistry 35*, 14862.
66. Koenig, A., Jain, R., Vig, R., Norgard-Sumnicht, K. E., Matta, K. L., and Varki, A. (1997) *Glycobiology 7*, 79.
67. Hemmerich, S., Bertozzi, C. R., Leffler, H., and Rosen, S. D. (1994) *Biochemistry 33*, 4820.
68. Yuen, C.-T., Bezouska, K., O'Brien, J., Stoll, M., Lemoine, R., Lubineau, A., Kiso, M., Hasegawa, A., Bockovich, N. J., Nicolaou, K. C., and Feizi, T. (1994) *J. Biol. Chem. 269*, 1595.

69. Tsujishita, H., Hiramatsu, Y., Kondo, N., Ohmoto, H., Kondo, H., Kiso, M., and Hasegawa, A. (1997) *J. Med. Chem. 40*, 362.
70. Chai, W., Feizi, T., Yuen, C.-T., and Lawson, A. M. (1997) *Glycobiology 7*, 861.
71. Galustian, C., Lubineau, A., le Narvor, C., Kiso, M., Brown, G., and Feizi, T. (1999) *J. Biol. Chem. 274*, 18213.
72. Rosen, S. D., and Bertozzi, C. R. (1996) *Curr. Biol. 6*, 261.
73. Malhotra, R., Taylor, N. R., and Bird, M. I. (1996) *Biochem. J. 314*, 297.
74. Lin, C.-C., Kimura, T., Wu, S.-H., Weitz-Schmidt, G., and Wong, C.-H. (1996) *Bioorg. Med. Chem. Lett. 6*, 2755.
75. Sanders, W. J., Gordon, E. J., Dwir, O., Beck, P. J., Alon, R., and Kiessling, L. L. (1999) *J. Biol. Chem. 274*, 5271.
76. Bargatze, R. F., Palecanda, A., Benson, E., Watts, G., Perry, V., Warwood, S., and Nagy, J. O. (2001) manuscript in preparation.

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