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Enzymatic synthesis of *N*-linked oligosaccharides terminating in multiple sialyl-Lewis^x and GalNAc-Lewis^x determinants: clustered glycosides for studying selectin interactions

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Abstract

Galactosyltransferase, sialyltransferase, and fucosyltransferase were used to create a panel of complex oligosaccharides that possess multiple terminal sialyl-Le^x (NeuAcα2–3Gal[Fucα1–3]β1–4GlcNAc) and GalNAc-Le^x (GalNAc[Fucα1–3]β1–4GlcNAc). The enzymatic synthesis of tyrosinamide biantennary, triantennary, and tetraantennary *N*-linked oligosaccharides bearing multiple terminal sialyl-Le^x was accomplished on the 0.5 μmol scale and the purified products were characterized by electrospray MS and ^{1}H NMR. Likewise, biantennary and triantennary tyrosinamide oligosaccharides bearing multiple terminal GalNAc-Le^x determinants were synthesized and similarly characterized. The transfer kinetics of human milk α3/4-fucosyltransferase were compared for biantennary oligosaccharide acceptor substrates possessing Galβ1–4GlcNAc, GalNAcβ1–4GlcNAc, and NeuAcα2–3Galβ1–4GlcNAc which established NeuAcα2–3Galβ1–4GlcNAc as the most efficient acceptor substrate. The resulting complex oligosaccharides were chemically tethered through the tyrosinamide aglycone to the surface of liposomes containing phosphatidylthioethanol, resulting in the generation of glycoliposomes probe which will be useful to study relationships between binding affinity and the micro- and macro-clustering of selectin ligand. © 1998 Elsevier Science Ltd. All rights reserved

Keywords: *N*-linked oligosaccharides; Enzymatic synthesis; Sialyltransferase; Fucosyltransferase; Liposomes; Selectins

1. Introduction

N- and *O*-linked oligosaccharides serve numerous specific biological functions in mammals such as mediating glycoprotein trafficking, participating in cellular adhesion, and facilitating protein folding [1–3]. The specificity of carbohydrate/protein binding often relies on clustered arrays of non-reducing residues that are organized by the peptide backbone for *O*-linked oligosaccharides or by a carbohydrate scaffolding for *N*-linked oligosaccharides. These

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multivalent glycoconjugates bind with high affinity to a complementary set of binding sites on multivalent lectins [4]. The C-type lectins are a family of multivalent mammalian lectins involved in a broad range of biological functions. Several members of this lectin family, including the asialoglycoprotein receptor and the mannose receptor, possess multiple carbohydrate recognition domains (CRDs) that are clustered into a high affinity binding site on cell surfaces [5].

The selectins (E, P, and L) are a subfamily of C-type lectins that are involved in mediating inflammation [6]. Presently, there is little information regarding the cell surface clustering of E-, L-, and P-selectin. However, E and P selectin do bind multivalent ligands with greater avidity than monovalent ligands, providing circumstantial evidence that these C-type lectins also reside in an oligomeric state on the cell surface.

Evidence that E-selectin recognizes microclustered arrays of sialyl-Le^x on *N*-linked oligosaccharides was provided by characterizing glycoproteins isolated from HL60 cells which lost their high affinity binding when treated with *N*-glycosidase F [7,8]. More direct evidence comes from the analysis of a purified biantennary *N*-linked oligosaccharide isolated from recombinant human protein C which bound E-selectin with high affinity by virtue of its two terminal GalNAc-Le^x determinants [9]. Even artificial bivalent sialyl-Le^x neoglycopeptides were found to possess a five-fold increase in affinity for E-selectin relative to a monovalent ligand [10], whereas a tetravalent sialyl-Le^x *O*-linked oligosaccharide achieved an IC_{50} of 50 n*M* in a Stamper–Woodruff assay [11,12].

Macroclustering of sialyl-Le^x determinants is also likely to be an important contributor to the binding affinity and specificity between glycoproteins and individual members of the selectin family [2]. Studies using neoglycoproteins detected a 1000-fold increase in binding affinity to E-selectin upon increasing the sialyl-Le^x valency from 1 to 16 mol:mol on BSA [13]. Liposomes displaying sialyl-Le^x (5 mol%) possessed an IC_{50} of 2 n*M* for P-selectin compared to a monovalent ligand which inhibits in the m*M* range [14]. Collectively, these studies suggest that E- and P-selectin oligomerize to recognize multivalent ligands that are both microclustered and macroclustered.

To further probe the macrocluster and microcluster effect of selectin ligand binding, it is necessary to design suitable probes possessing controlled multimeric arrays of sialyl-Le^x, GalNAc-Le^x, or other selectin ligands. Common *N*-linked oligosaccharides terminating in NeuAc, Gal, GlcNAc, and Man can be purified from commercial sources [15]. However, the isolation of appreciable quantities of unique structures possessing sialyl-Le^x or GalNAc-Le^x is problematic due to their rare occurrence in nature [2,16,17]. One alternative to their isolation is the enzymatic remodelling of common *N*-linked oligosaccharides into these rare structures.

In the present study we have used glycosyltransferases to remodel di, tri, and tetravalent *N*-linked oligosaccharides into microclustered arrays of sialyl-Le^x and GalNAc-Le^x. These putative selectin ligands are then macroclustered on the surface of liposomes, providing useful tools to assess the oligomeric state of the selectins on cell surfaces.

2. Experimental

Materials.—CM Sephadex C50, Sephadex G-25 and G-50, ATP, sodium cacodylate, iodoacetic acid *N*-hydroxysuccinimide ester, and ganglioside G_{M1} were purchased from Sigma (St. Louis, MO). Alkaline phosphatase (EC 3.1.3.1) and cytidine-5′-monophospho-*N*-acetylneuraminic acid (CMP-NANA) were purchased from Boehringer Mannheim (Indianapolis, IN). Guanosine-diphosphate-β-L-fucose (GDP-Fuc) was synthesized according to the method of Nunez et al. [18]. Phosphatidylcholine, cholesterol, and phosphatidylthioethanol were purchased from Avanti Lipids (Alabaster, AL). Dowex ion-exchange resins AG 1-X2 and AG 50W-X2 were purchased from Bio-Rad (Richmond, CA). Human milk was donated by the neonatal intensive care unit of Children's Hospital, Columbus, OH. Reverse phase (C8 and C18) HPLC columns (5 μm, 0.47×25 cm) were purchased from Rainin (Emeryville, CA). Polymer PRP-1 (10 μm) reverse phase HPLC columns (0.47×25 cm) were purchased from Baxter Scientific (McGraw Park, IL). α2,3-Sialyltransferase (EC 2.3.99.6) was a kind gift from Dr. James Paulson, Cytel Corp., San Diego, CA.

Purification of fucosyltransferase.—α3/4-Fucosyltransferase was partially purified from human milk using a modification of the method described by Palcic et al. [19]. Fucosyltransferase activity was determined by reacting 2 nmol of tyrosinamide

biantennary oligosaccharide in 10 μl of enzyme buffer A (25 m*M* cacodylate, pH 6.5, containing 8 m*M* MnCl*2* and 1.6 m*M* ATP) with 2 μL of enzyme, 40 nmol GDP-Fuc, and 1 U of alkaline phosphatase at 37 °C. The reaction was quenched after 10 min with 180 μL of cold 0.1% acetic acid and analyzed by injecting 10 μL on RP-HPLC. The C18 column (50 °C) was eluted isocratically at 1 mL/min with 0.1% acetic acid containing 11% acetonitrile, while monitoring fluorescence at an excitation of 275 nm and emission of 305 nm. Gal-biantennary oligosaccharide eluted at 20 min whereas a monofucosylated biantennary oligosaccharide eluted at 18 min. The area of the monofucosylated biantennary oligosaccharide was integrated relative to the substrate peak to quantify the units of activity, defined as μmols of fucose transferred to biantennary oligosaccharide per minute. The specific activity of the fucosyltransferase was typically 155 μU/mg following protein determination by the method of Bradford [20].

Synthesis of sialyl-Le^x terminated oligosaccharides.—Gal-biantennary, triantennary, and tetra-antennary oligosaccharides were isolated from bovine fetuin and human orosomucoid as described previously [21,22]. Each oligosaccharide possessed a *tert*-butoxycarbonyl-tyrosine (Boc-Tyr) linked to GlcNAc through a β-glycosylamide linkage.

Sialyl-oligosaccharides were prepared from Gal-terminated oligosaccharides by reacting 1 μmol of substrate with 50 mU of recombinant α2,3-sialyltransferase, 10 μmol CMP-NANA, and 10 U of alkaline phosphatase in 1 mL of enzyme buffer B (50 m*M* cacodylate, 50 m*M* NaCl, 30 μ*M* $MnCl_2$, and 0.1% Triton CF-54 at pH 6.0) at 37 °C for 72 h. Sialyltransferase reactions were monitored by RP-HPLC eluting with 0.1% trifluoroacetic acid and 11% acetonitrile. Sialyl-oligosaccharides were purified using a Sephadex G-25 column (1.5×120 cm) eluted with water while detecting $A_{280\,nm}$. The oligosaccharide peak eluting in the void of the column (60 mL) was collected and freeze dried.

Sialyl-Le^x oligosaccharides were prepared from sialyl-biantennary oligosaccharide (500 nmol) by reaction with 1.25 mU of fucosyltransferase, 7.5 μmol GDP-Fuc, and 10 U of alkaline phosphatase in 500 μL of enzyme buffer A. Alternatively, sialyl-triantennary oligosaccharide (500 nmol) was treated with 4.25 mU of enzyme, 20 μmol GDP-Fuc, and 15 U of alkaline phosphatase prepared in 500 μL of enzyme buffer A. Sialyl-tetraantennary oligosaccharide (500 nmol) was reacted with 7.5 mU of fucosyltransferase, 25 μmol GDP-Fuc, and 20 U alkaline phosphatase in 1 mL of enzyme buffer A. Each reaction was incubated at 37 °C for 72 h. The resulting sialyl-Le^x oligosaccharides were chromatographed on a CM Sephadex C50 column (1.5×35 cm) eluted with water while monitoring $A_{280\,nm}$. The oligosaccharide eluted in the void of the column and was collected and freeze dried. Further purification was achieved on a polymer PRP-1 RP-HPLC column (50 °C) eluted at 2 mL/min with 25 m*M* ammonium acetate pH 8.0 and a gradient of acetonitrile (9–11% over 30 min) while monitoring $A_{280\,nm}$. Fractions were pooled and freeze dried with an overall yield of 60%.

Monovalent sialyl-Le^x was prepared from Boc-Tyr-β-1-*N*-acetyllactosamine which was synthesized by coupling Boc tyrosine-*N*-hydroxysuccinimide ester to the glycosylamine of β-D-galactosyl-(1→4)-*N*-acetyl-β-D-glucosamine (*N*-Acetyllactosamine) using a method described previously [21]. The complete sialylation of 1 μmol Boc-Tyr *N*-acetyllactosamine required 50 mU of α2,3-sialyltransferase, 5 μmol CMP-NANA, 10 U alkaline phosphatase in 200 μL of enzyme buffer B at 37 °C for 72 h and the product was purified by gel filtration as described above. Fucosylation was achieved by reacting 500 nmol of sialylated substrate with 2.5 mU of fucosyltransferase, 12 μmol of GDP-Fuc, and 10 U alkaline phosphatase in 500 μL of enzyme buffer A at 37 °C for 72 h and Boc-tyrosinamide sialyl-Le^x lactosamine was purified by HPLC as described above.

Synthesis of GalNAc-Le^x terminated biantennary and triantennary oligosaccharides.—The preparation of GalNAc-Le^x terminated biantennary and triantennary oligosaccharides utilized a multi-step enzymatic remodelling scheme. Gal-biantennary and triantennary oligosaccharides were treated with β-galactosidase and then reacted with bovine milk galactosyltransferase and UDP-GalNAc to produce GalNAc-biantennary and triantennary oligosaccharides as described previously [23]. Optimized fucosyltransferase reactions were achieved by reacting 500 nmol of GalNAc-biantennary oligosaccharide with 1.5 mU of fucosyltransferase, 17 μmol GDP-Fuc, and 10 U of alkaline phosphatase prepared in 500 μL of enzyme buffer A. In addition, a core fucosylated GalNAc-biantennary oligosaccharide was reacted with fucosyltransferase under identical conditions in order to prepare a trifucosylated GalNAc-Le^x

biantennary oligosaccharide. The complete conversion of GalNAc-triantennary oligosaccharide (200 nmol) to GalNAc-Le^x required 4 mU of enzyme, 20 μmol GDP-Fuc and 8 U alkaline phosphatase in 200 μL of enzyme buffer A.

GalNAc-Le^x terminated biantennary and triantennary oligosaccharides were chromatographed on a mixed bead ion-exchange column (1.5×35 cm: top, AG50WX2 acid form; bottom, AG-1-X2 acetate form) eluted with water while monitoring $A_{280\,nm}$. The oligosaccharide eluting in the void volume was freeze dried, reconstituted in water, and further purified on a PRP-1 RP-HPLC column (50 °C) eluted at 3 mL/min with 0.1% acetic acid and 13% acetonitrile while detecting at $A_{280\,nm}$. Each product was >95% pure and isolated with a 60–70% yield.

Structural analysis of oligosaccharides.—Remodeled oligosaccharides (0.2–1 μmol) were prepared for ^{1}H NMR by freeze drying twice in D_2O (100 μL, 99.96%) then dissolved in 0.5 mL of D_2O containing 0.01% acetone as an internal standard and analyzed on a Bruker 500 MHz NMR spectrometer at 23 °C. Samples were processed with resolution enhanced parameters using WIN-NMR (Bruker, CA).

Mass spectral analysis was acquired using ES-MS by preparing 1 nmol of oligosaccharide in 100 μL of 50:50 CH_3CN:H_2O. Samples were infused to the electrospray ionization source at 5–10 μL/min by a syringe pump. Spectra were acquired in positive and negative mode on a VG (Micromass) Platform single quadrupole mass spectrometer (0–3000 m/z).

Kinetics of fucosyltransferase acting on biantennary oligosaccharide substrates.—The kinetics of α3/4-fucosyltransferase-mediated conversion of Gal-biantennary, sialyl-biantennary, and GalNAc-biantennary oligosaccharide into Le^x (Gal[Fucα1–3]β1–4GlcNAc), sialyl-Le^x, and GalNAc-Le^x were studied at a fixed enzyme (5 μU, 48 μg of protein) and a saturating GDP-Fuc (8 mM) concentration while varying oligosaccharide acceptor concentration. Each reaction contained 1 U of alkaline phosphatase and 7.5 μL of buffer A, resulting in a total volume of 10 μL. After incubation for 30 min at 37 °C, less than 15% of the substrate was converted into the monofucosylated product as determined by RP-HPLC as described above. The data were analyzed according to Lineweaver–Burk to determine K_m and V_{max} [24].

Preparation of glycoliposomes.—Liposomes were formulated by combining phosphatidylcholine (PC, 5.3 mg), cholesterol (CH, 1.35 mg), and phosphatidylthioethanol (PE-SH, 0.82 mg) at a molar ratio of 2:1:0.3 in a total volume of 1 mL of chloroform [25]. Lipids and cholesterol were dried to a thin film and then hydrated in 10 mM sodium phosphate, pH 8.0. The resulting multilamellar liposomes were subjected to four cycles of freeze-thaw, then extruded two times using a 200 nm pore polycarbonate membrane (Corning Costar, Cambridge, MA). Liposomes were further processed by four extrusions through a 50 nm pore polycarbonate membrane to prepare unilamellar liposomes with a mean diameter of 85±15 nm as determined by quasielastic light scattering on a Nicomp 370 particle sizer [26]. The final concentration of PE-SH-liposomes was determined colorimetrically using PC as a standard [27]. The *N*-linked oligosaccharides were then tethered to PE-SH-liposomes using a heterobifunctional reagent as described below.

Sialyl-Le^x biantennary oligosaccharide (15 nmol) was dried and then treated with 50 μL of TFA for 10 min to remove the Boc group. The dried oligosaccharide was then reacted (1 h) with 150 nmol of VS-PEG-NHS (Shearwater Polymers Inc., Huntsville, AL) prepared in 50 μL of 10 mM phosphate buffer, pH 8.0. VS-PEG derivatized sialyl-Le^x biantennary oligosaccharide (15 nmol) was purified by gel filtration, then reacted with PE-SH-liposomes (0.64 mg) for 12 h in 200 μl of 10 mM phosphate, pH 7.0. The glycoliposomes were purified on a Sephadex G-50 column (1.5×30 cm) eluted with 10 mM sodium phosphate, pH 8.0. The oligosaccharide coupling efficiency was determined by analyzing an aliquot of glycoliposome by quantitative glucosamine analysis on HPAEC [28] relative to a control in which a Boc-protected oligosaccharide was subjected to the derivatization procedure.

Alternatively, liposomes containing PC, CH, PE-SH, and mono-sialoganglioside-G_{M1} at a molar ratio of 2:1:0.3:0.2 were prepared as described above. The Boc group was removed from the oligosaccharide (15 nmol) with TFA after which the primary amine was derivatized with 1.2 μmol of iodoacetic acid *N*-hydroxysuccinimide ester in 100 μL of 200 mM sodium bicarbonate buffer, pH 8.0, then purified by gel filtration chromatography on Sephadex G-50. The resulting iodo-oligosaccharide (15 nmol) was then reacted for 12 h with

0.64 mg of PE-SH-G_{M1}-liposomes in 200 μL of 10 m*M* sodium phosphate, pH 8.0, then purified by gel filtration chromatography.

3. Results

Synthesis and characterization of sialyl-Le^x and GalNAc-Le^x N-linked oligosaccharides.—Tyrosinamide biantennary, triantennary, and tetraantennary oligosaccharides were used as substrates for recombinant α2,3-sialyltransferase which transferred a Neu5Ac to the 3 position of Gal on each antenna as depicted in Fig. 1(A). The transfer of Neu5Ac was monitored by RP-HPLC which identified two isomeric monosialyl-biantennary oligosaccharides at an intermediate time point (Fig. 2(B)) which converted into disialyl-biantennary oligosaccharide over 48 h (Fig. 2(C)). Sialyl oligosaccharides were purified by gel filtration chromatography to remove buffer and sialyltransferase, resulting in a >90% yield. The structures of sialyl-biantennary, triantennary, and tetraantennary oligosaccharides were determined using 500 MHz ^{1}H NMR and ES-MS (data not shown) which established their close identity to previously reported spectroscopic data for these oligosaccharides [29–31].

Fucosylation of the sialyl oligosaccharides was performed using a partially purified human milk fucosyltransferase (Fig. 1(B)). The fucosylation of sialyl-biantennary oligosaccharide resulted in the formation of one unresolved intermediate peak on RP-HPLC that possessed two monofucosylated biantennary oligosaccharide isomers (Fig. 2(D), peak 5). The complete conversion of sialyl-biantennary to sialyl-Le^x biantennary oligosaccharide was achieved in 48 h and resulted in a single peak on RP-HPLC (Fig. 2(E)). Attempts to drive the reaction in a shorter period of time with GDP-Fuc in excess of 15 m*M* or enzyme concentration greater than 0.1 U/mL caused inhibition. Comparison of biantennary, triantennary, and tetraantennary oligosaccharide substrates established that both Fuc and Neu5Ac were transferred less efficiently as the oligosaccharide valency increased, whereas the generation of sialyl-Le^x *N*-acetyl lactosamine (Fig. 5, structure **I**) was the least efficient, presumably due to interference from the aglycone.

The synthesis of biantennary and triantennary GalNAc-Le^x oligosaccharides utilized the multistep enzymatic reaction scheme outlined in Fig. 1 (Steps A′, B′, and C′) and demonstrated chromatographically in Fig. 3. Agalactosyl biantennary and triantennary oligosaccharides served as a substrate for galactosyltransferase and UDP-GalNAc to prepare GalNAc-oligosaccharides [23] which were then used as synthons for the transfer of Fuc onto the subterminal GlcNAc using α3/4-fucosyltransferase and GDP-Fuc. The complete transfer of Fuc to GalNAc-triantennary oligosaccharide was less efficient than to sialyl-triantennary oligosaccharide such that the reaction was only successful on the 0.2 μmol scale. Mix-bed ion-exchange chromatography was used to resolve the neutrally charged GalNAc-Le^x oligosaccharides from proteins in the reaction mixture, and the oligosaccharides were then brought to final purity by isolation from RP-HPLC.

Analysis of GalNAc-Le^x biantennary and triantennary oligosaccharides by ^{1}H NMR established the presence of anomeric and *N*-acetyl resonances consistent with the addition of multiple GalNAc and Fuc residues attached to the *N*-linked oligosaccharide substructure (Fig. 5, **II** and **III**). GalNAc-Le^x biantennary oligosaccharide possessed two new anomeric protons at 5.126 and 5.133 ppm assigned to Fuc residues A and B in structure **II** (Fig. 5). The spectrum of **II** revealed that the anomeric proton signals for GalNAc residues 9 and 9′ were shifted upfield by 0.07 ppm relative to the same resonances identified previously for GalNAc-biantennary oligosaccharide [23], resulting in the near chemical shift equivalence of these with the Gal anomeric protons reported previously for Le^x-biantennary oligosaccharide [32]. Similarly, the *N*-acetyl resonances assigned to GalNAc 9 and 9′ in **II** were shifted downfield by approximately 0.03 ppm and became chemical shift equivalent as a result of the neighboring Fuc residues (Fig. 4, **II**). The chemical shifts reported for **II** were also in close agreement with those determined previously for a GalNAc-Le^x biantennary oligosaccharide isolated from a natural source [16]. A second GalNAc-Le^x biantennary oligosaccharide containing a core fucose was also prepared using the biantennary oligosaccharide from porcine fibrinogen [33]. The NMR spectrum of this oligosaccharide was identical to that of **II** with the exception of an additional doublet at 4.874 ppm arising from core Fuc 1 (data not shown).

The proton NMR spectrum of the GalNAc-Le^x triantennary oligosaccharide possessed resonances that were readily assignable to GalNAc and Fuc

residues (Fig. 4, **III**). Three anomeric protons between 5.133 and 5.109 ppm were assigned as Fuc residues A, B, and C in structure **III** (Fig. 5) [32]. As in GalNAc-Le^x biantennary oligosaccharide, the resonance frequencies of the GalNAc anomeric protons in **III** were significantly influenced by the presence of the neighboring Fuc residues which resulted in chemical shifts that were almost identical to those found previously in Le^x-triantennary oligosaccharide [32].

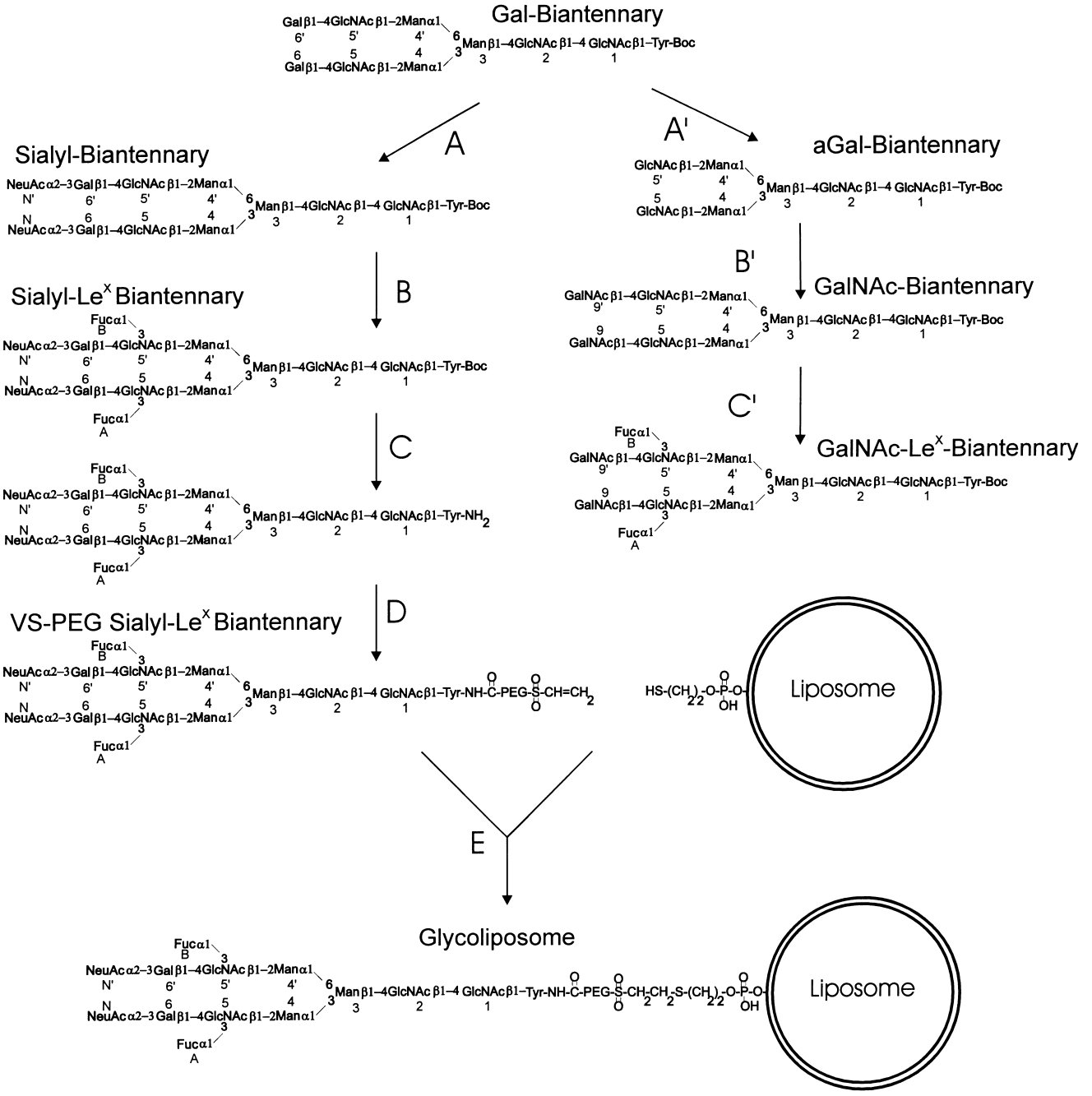

Fig. 1. Reaction schemes for enzymatic synthesis of sialyl-Le^x and GalNAc-Le^x biantennary oligosaccharides. The conversion of Gal-biantennary into sialyl-biantennary oligosaccharide (A) using α2,3-sialyltransferase was followed by fucosylation of subterminal GlcNAc using an α3/4-fucosyltransferase (B) to prepare sialyl-Le^x biantennary oligosaccharide. In the second pathway Gal-biantennary oligosaccharide was treated with β-galactosidase (A′) to prepare agalactosyl-biantennary oligosaccharide. This product was transformed into GalNAc-biantennary oligosaccharide using galactosyltransferase and UDP-GalNAc (B′). The GalNAc-biantennary oligosaccharide served as a substrate for α3/4-fucosyltransferase (C′) to prepare GalNAc-Le^x terminated biantennary oligosaccharide. Glycoliposomes were prepared by removing the Boc group from sialyl-Le^x biantennary oligosaccharide (C) and reacting the terminal amine with VS-PEG-NHS (D) to prepare VS-PEG sialyl-Le^x biantennary oligosaccharide. The oligosaccharide was reacted with PE-SH-liposomes resulting the formation of glycoliposomes (E).

The proton NMR spectrum of sialyl-Le^x biantennary oligosaccharide possessed readily identifiable Fuc, Gal, GlcNAc and Man anomeric protons (Fig. 4, **IV**). The presence of two NeuAc residues linked α-(2→3) to Gal is evident from the chemical shift of the Gal anomeric protons relative to the same resonances in Le^x-biantennary oligosaccharide [32] and by the resonance frequency of the 3a/3e protons of each Neu5Ac residue (Table 1), leading to the proposed structure (**IV**) shown in Fig. 5.

The triantennary oligosaccharide possessing three terminal sialyl-Le^x determinants displayed unresolved anomeric resonances for Fuc A, B, and C but did illustrate 3a/3e and *N*-acetyl protons consistent with three Neu5Ac residues linked α-(2→3) to Gal (Fig. 4, **V**). Likewise, the sialyl-Le^x tetraantennary oligosaccharide possessed four unresolved Fuc anomeric resonances in addition to resonances consistent with four Neu5Ac residues linked α-(2→3) to Gal, as well as GlcNAc and Gal anomeric protons which overlapped severely (Fig. 4, **VI**). These data were used to support the proposed structures of **V** and **VI** as shown in Fig. 5.

Each oligosaccharide was analyzed by ES-MS which produced a single deconvoluted m/z of 1082, 2278, 2831, 2778, 3581, and 4383 for **I–VI**, respectively, which agreed to within one mass unit with the calculated mass (M + 1) for each structure shown in Fig. 5, further supporting the proposed structural assignments.

Fucosyltransferase kinetics.—Biantennary oligosaccharides possessing terminal Galβ1–4GlcNAc, NeuAcα2–3Galβ1–4GlcNAc, and GalNAcβ1–4GlcNAc were compared as acceptor substrates for fucosyltransferase (Fig. 6). The K_m and V_{max} were determined for these three substrates using a single

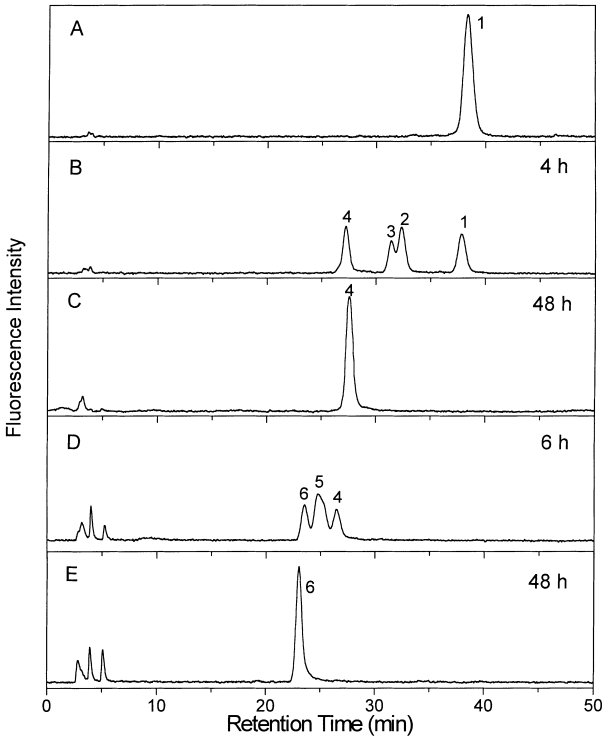

Fig. 2. RP-HPLC analysis of sialyl-Le^x biantennary oligosaccharide synthesis. The synthesis of sialyl-Le^x biantennary oligosaccharide was monitored by RP-HPLC using fluorescence detection. Reaction of Gal-biantennary oligosaccharide (panel A, peak 1) with sialyltransferase and CMP-NANA for 4 h resulted in two earlier eluting monosialylated peaks (panel B, peaks 2 and 3) detected on RP-HPLC. At 48 h the complete conversion to sialyl-biantennary oligosaccharide is evident from a single peak eluting at 27 min (panel C, peak 4). Partial fucosylation of sialyl-biantennary oligosaccharide by reaction with α3/4-fucosyltransferase and GDP-Fuc for 6 h produced an unresolved intermediate (panel D, peak 5) and product (peak 6), whereas continuing the reaction for 48 h resulted in the complete conversion to the sialyl-Le^x biantennary oligosaccharide product eluting at 23 min (panel E, peak 6).

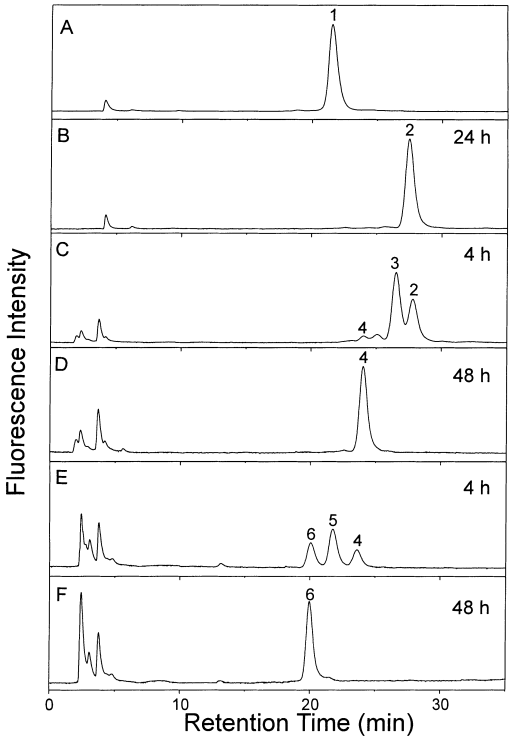

Fig. 3. RP-HPLC analysis of GalNAc-Le^x biantennary oligosaccharide synthesis. Gal-biantennary oligosaccharide (panel A, peak 1) was de-galactosylated with β-galactosidase to produce (panel B, peak 2). The product was treated with galactosyltransferase and UDP-GalNAc resulting in the formation of intermediate 3 at 4 h (panel C) and the GalNAc-biantennary oligosaccharide product at 48 h (panel D, peak 4). This oligosaccharide was used as a substrate for α3/4-fucosyltransferase to produce an intermediate product (panel E, peak 5) at 4 h and a final GalNAc-Le^x biantennary oligosaccharide in 48 h as shown in panel F.

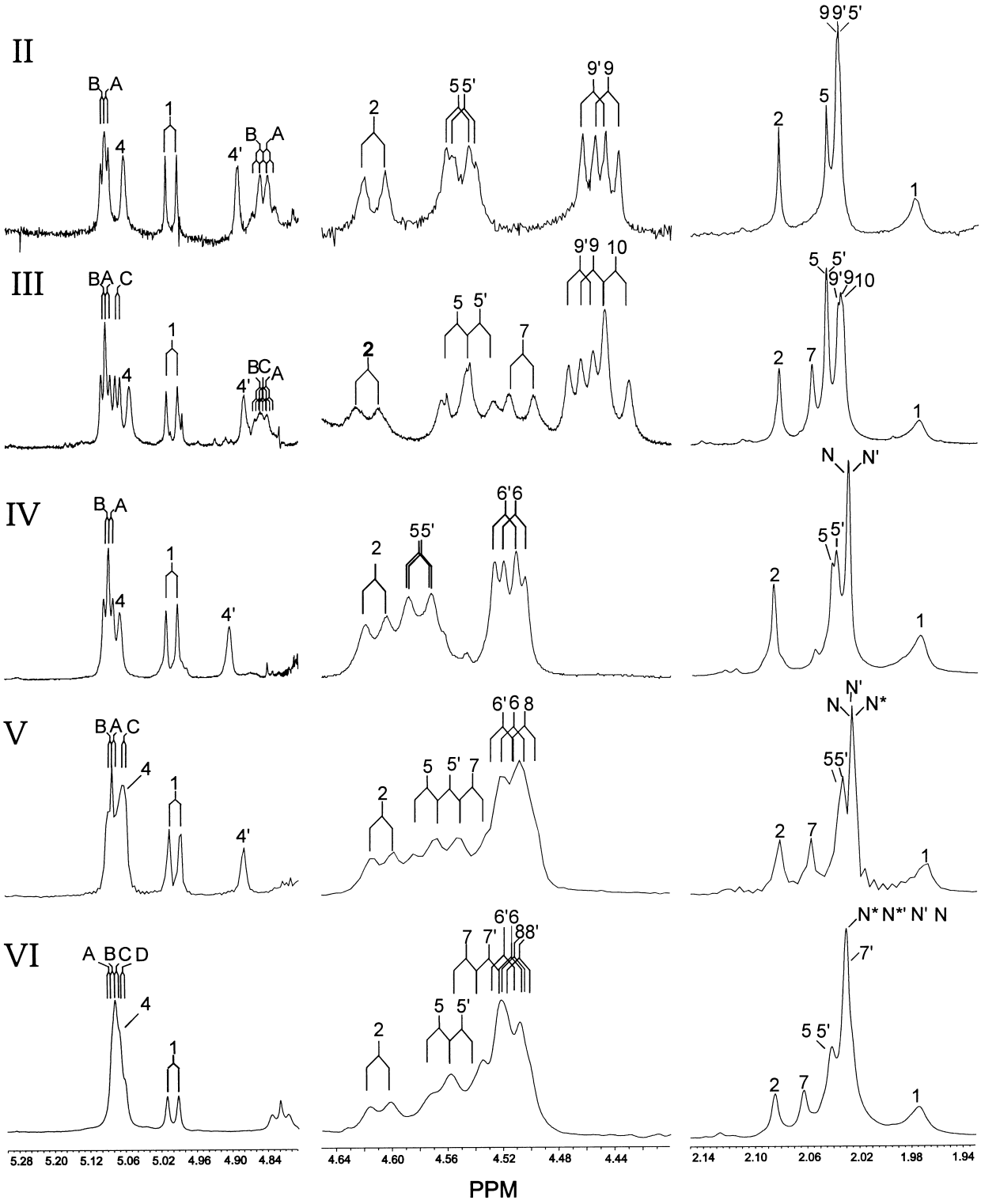

Fig. 4. Partial proton NMR spectra of sialyl-Le^x and GalNAc-Le^x terminated *N*-linked oligosaccharides. The Man, Fuc, and GlcNAc 1 anomeric region (5.28–4.84 ppm) for oligosaccharides **II–VI** are shown in the left panels. The middle panels compare the GlcNAc, Gal, and GalNAc anomeric proton (4.64–4.44 ppm) resonance patterns. The panels at the right display the *N*-acetyl protons for GlcNAc, GalNAc and Neu5Ac (2.14–1.94 ppm). The resonance assignments in each spectrum refer to sugar residue nomenclature shown in Fig. 5.

enzyme preparation, thereby avoiding complications resulting from potential heterogeneity in individual enzyme preparations (Fig. 6). The initial velocity was measured while the acceptor concentration was varied from 10 μM to 1.5 mM. GDP-Fuc and fucosyltransferase concentration were held constant at 8 mM and 0.5 mU/mL, respectively. The rate of fucose transfer was plotted against oligosaccharide substrate concentration to establish saturation curves and the double

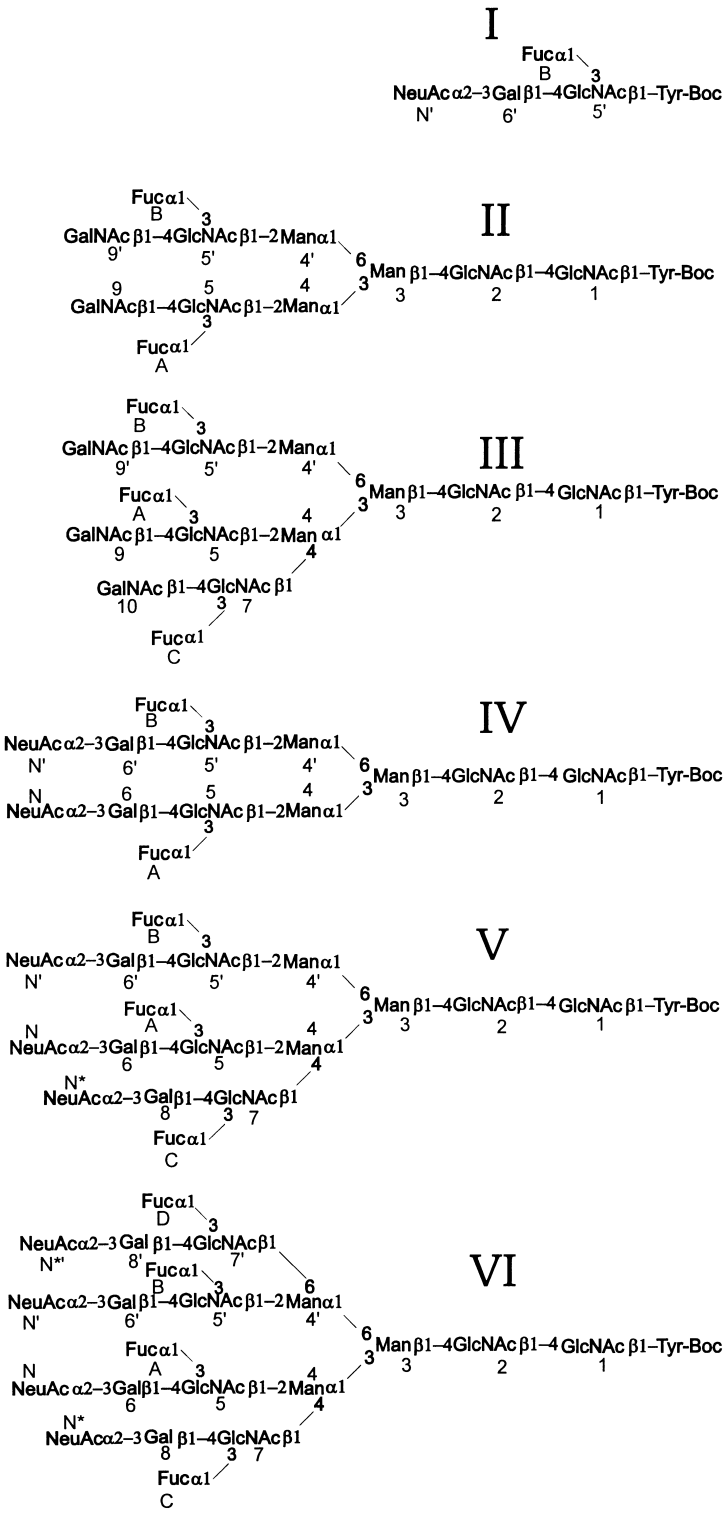

Fig. 5. Proposed structures of sialyl-Le^x and GalNAc-Le^x *N*-linked oligosaccharides. The structure of sialyl-Le^x and GalNAc-Le^x oligosaccharides **I–VI** are shown. The residue nomenclature shown corresponds to NMR assignments in Table 1.

reciprocal plots were used to derive V_{max}, K_m, and V_{max}/K_m (Table 2). Sialyl-biantennary oligosaccharide possessed a three-fold lower K_m relative to Gal and GalNAc terminated substrates indicating it binds to the enzyme with higher affinity. However, a lower V_{max} for sialyl-biantennary oligosaccharide established that its turnover was less efficient than the other substrates. On the basis of the relative V_{max}/K_m data, the relative order of substrate acceptor efficiency was NeuAcα2–3Galβ1–4GlcNAc > Galβ1–4GlcNAc > GalNAc-β1–4GlcNAc.

Clustering oligosaccharides on liposomes.—A key factor in the covalent attachment of sialyl-Le^x biantennary oligosaccharide to liposomes is the preservation of the Neu5Ac and Fuc residues

Table 1
Proton NMR chemical shift[a] analysis for *N*-linked oligosaccharides

Structure[b]	I	II	III	IV	V	VI
H1 of						
1		5.015	5.015	5.015	5.015	5.015
2		4.612	4.610	4.612	4.608	4.609
4		5.097	5.089	5.104	5.099	5.098
4′		4.903	4.892	4.918	4.898	n.d.[c]
5		4.547	4.546	4.580	4.578	4.565
5′	5.103	4.554	4.530	4.580	4.563	4.549
6				4.512	4.515	4.515
6′	4.543			4.519	4.525	4.518
7			4.502		4.548	4.545
7′						4.527
8					4.507	4.515
8′						4.510
9		4.446	4.454			
9′		4.545	4.562			
10			4.437			
A		5.126	5.125	5.119	5.116	5.118
B	5.107	5.133	5.133	5.127	5.124	5.118
C			5.109		5.099	5.110
D						5.110
H-3a/3e of						
N				1.798/2.764	1.798/2.759	1.801/2.761
N′	1.797/2.765			1.798/2.764	1.798/2.759	1.801/2.761
N*					1.798/2.759	1.801/2.761
N*′						1.801/2.761
NAc of						
1		1.977	1.975	1.975	1.972	1.972
2		2.082	2.082	2.087	2.085	2.084
5		2.045	2.046	2.042	2.037	2.042
5′	1.973	2.036	2.046	2.039	2.037	2.042
7			2.057		2.061	2.063
7′						2.026
9[d]		2.036	2.035			
9′[d]		2.036	2.035			
10[d]			2.035			
N				2.030	2.030	2.031
N′	2.032			2.030	2.030	2.031
N*					2.030	2.031
N*′						2.031
CH_3 of						
A		1.268	1.266	1.173	1.167	1.170
B	1.174	1.260	1.261	1.164	1.167	1.170
C			1.271		1.167	1.170
D						1.170

[a] Chemical shifts in ppm relative to an internal standard of acetone.
[b] See Fig. 5 for residue labeling.
[c] Indicates resonance was not detected.
[d] Refer to ref. [23] for chemical shift values for GalNAc-oligosaccharides.

during Boc removal. These residues were retained by deprotection in neat TFA, as previously established for α-(2→6) linked Neu5Ac residues [21]. HPAEC analysis of the sialyl-Le^x oligosaccharides following removal of Boc also established the stability of the α-(2→3) Neu5Ac residues in these structures (data not shown).

Sterically stabilized glycoliposomes were prepared using two different strategies. The first approach utilized VS-PEG-NHS as a linker that was

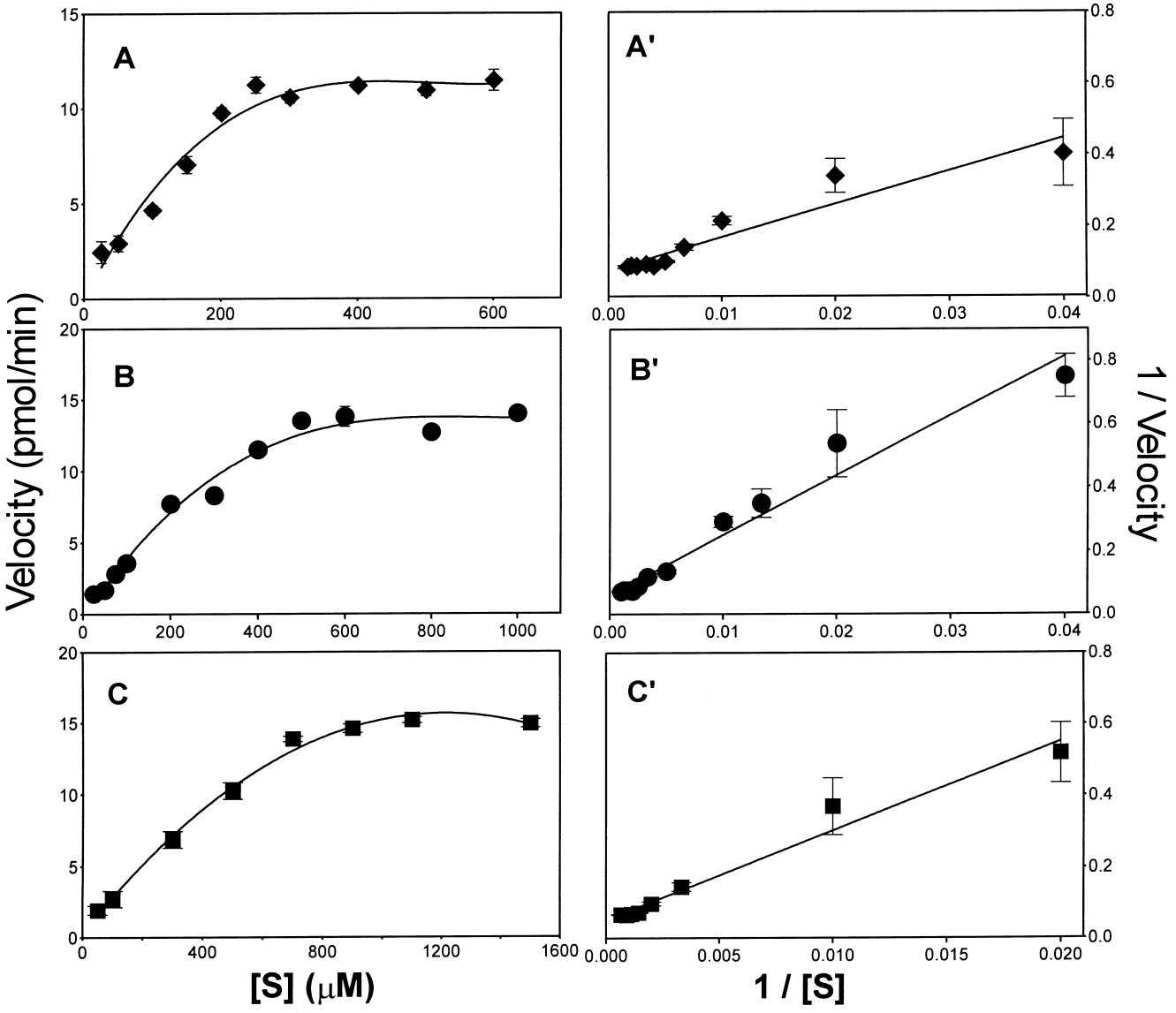

Fig. 6. Kinetics of α3/4-fucosyltransferase acting on biantennary oligosaccharides. The plots illustrate the initial velocity of fucosyltransferase acting on three different biantennary *N*-linked oligosaccharides possessing either terminal NeuAcα2–3Galβ1-4GlcNAc (A), Galβ1–4GlcNAc (B), or GalNAcβ1–4GlcNAc (C). The substrate concentration was varied from 10 μM to 1.5 mM to reach saturation. Panels A′–C′ show the double reciprocal plots that were used to derive the K_m and V_{max} for each substrate (Table 2).

Table 2
Transfer kinetics for α3/4-fucosyltransferase

Biantennary oligosaccharide substrates	V_{max} (pmol/min/mg protein)	K_m (μM)	V_{max}/K_m
Galβ1-4GlcNAc	421 ± 39[a]	365 ± 81	1.15
GalNAcβ1-4GlcNAc	466 ± 52	563 ± 150	0.83
NeuAcα2–3Galβ1–4GlcNAc	318 ± 33	155 ± 44	2.05

[a] Calculated standard error.

covalently attached to the amine on tyrosinamide sialyl-Le^x biantennary oligosaccharide, then coupled to the sulfhydryl group on PE-SH-liposomes (Fig. 1, steps D and E). In the second approach, ganglioside G_{M1} was included in the lipid cocktail during extrusion as the surface masking agent, resulting in PE-SH-G_{M1}-liposomes which were then reacted with iodo-oligosaccharides. Both strategies required purging of the lipids with nitrogen, extruding the liposomes with argon, and the conjugation to be conducted within several hours to avoid the oxidation of thiol groups. This consistently yielded comparable amounts of covalently attached oligosaccharide (3.6 nmol per μmol of lipid) which failed to dissociate on gel filtration chromatography. The yield was below saturation of the liposome surface which had a calculated 65 nmols of PE-SH but was similar to that reported previously for attaching other biomolecules to the surface of liposomes [34,35]. It was further established that the size of liposomes was not influenced by either coupling approach.

4. Discussion

The preparation of multivalent complex oligosaccharides possessing unique terminal sugar residues is important for advancing the understanding of the function of complex oligosaccharides in glycoproteins [4,36,37]. It is typically the non-reducing ends of oligosaccharides that interact with endogenous receptors, whereas the underlying oligosaccharide acts as scaffolding to present multiple copies of the terminal residues in an optimal conformation for binding to distally located binding pockets. Isolated *N*-linked oligosaccharides often bind to their target receptor with affinity comparable to glycoproteins. Thus, it is attractive to remodel purified oligosaccharides for studies aimed at probing the binding properties of their target receptors.

Sialyl-Le^x has been identified as an important ligand for binding to the selectins [38,39]. Although numerous studies have advanced the hypothesis that natural selectin ligands contain a clustered array of sialyl-Le^x [40,41], only a few studies have systematically analyzed the binding between homogenous glycoconjugates and the selectins. Since no previous studies have prepared *N*-linked oligosaccharides possessing sialyl-Le^x, we developed a chemoenzymatic approach that would produce a panel of natural multivalent *N*-linked oligosaccharides. A key feature of our study was the use of well-characterized tyrosinamide oligosaccharides as synthons for enzymatic transferase reactions. These modified *N*-linked oligosaccharides have many properties that make them attractive as biological probes in addition to being efficient enzyme substrates that are easily resolved on RP-HPLC and can be detected by fluorescence. This feature of the tyrosinamide oligosaccharides allowed monitoring of the transferase reaction and development of optimal conditions to ensure complete transfer of both Neu5Ac and Fuc.

Remarkably, fucosyltransferase is not significantly sterically restricted from transferring four Fuc residues onto sialyl-tetraantennary oligosaccharide resulting in a complex oligosaccharide possessing 21 sugar residues. This establishes that Nature does not preclude the synthesis of such complex oligosaccharides by allowing the antennae sufficient flexibility to gain access to the enzyme active site. Thus milk fucosyltransferase is capable of producing sialyl-Le^x bearing *N*-linked triantennary and tetraantennary oligosaccharides that have not previously been detected as components of glycoproteins.

Similarly, GalNAc-Le^x has only recently been identified as a rare oligosaccharide component found on certain serum glycoproteins [16]. A biantennary oligosaccharide possessing two GalNAc-Le^x determinants was proposed to be an E-selectin ligand [42]. Using a second enzymatic remodelling strategy we have transformed both biantennary and triantennary oligosaccharides into multivalent oligosaccharides possessing this determinant. This approach allows access to very rare oligosaccharides, such as GalNAc-Le^x triantennary oligosaccharide, that have not yet been discovered in Nature but which are also biosynthetically feasible.

The fucosyltransferase secreted in human milk is a mixture of enzymes that are collectively referred to as α3/4-fucosyltransferase. The transfer rate of fucose onto the terminal antennae of different biantennary oligosaccharide substrates closely parallels similar studies on disaccharides [19], indicating that the enzymes utilize a wide range of structures with similar but not identical efficiency. This provides the opportunity to synthesize moderate amounts (0.5 μmol) of sialyl-Le^x terminated *N*-linked oligosaccharides using a single preparation of partially purified enzyme from 300 mL of human milk.

The application of remodeled tyrosinamide oligosaccharides to prepare glycoliposomes will allow analysis of the macrocluster ligand binding effect exerted by the selectins. Sterically stabilized glycoliposomes, possessing long circulatory half-life, are ideal reagents for testing the potency of variably clustered ligands for inhibiting selectin mediated interactions in vivo.

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