Glycoconjugate Journal 16, 271–281 (1999)

Preparation and isolation of neoglycoconjugates using biotin-streptavidin complexes

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Glycoproteins commercially available in multi-gram quantities, were used to prepare milligram amounts of neoglycoproteins. The glycoproteins bromelain and bovine γ-globulin were proteolyzed to obtain glycopeptides or converted to a mixture of glycans through hydrazinolysis. The glycan mixture was structurally simplified by carbohydrate remodeling using exoglycosidases. Glycopeptides were biotinylated using *N*-hydroxysuccinimide activated-long chain biotin while glycoprotein-derived glycans were first reductively aminated with ammonium bicarbonate and then biotinylated. The resulting biotinylated carbohydrates were structurally characterized and then bound to streptavidin to afford neoglycoproteins. The peptidoglycan component of raw, unbleached heparin (an intermediate in the manufacture of heparin) was similarly biotinylated and bound to streptavidin to obtain milligram amounts of a heparin neoproteoglycan. The neoglycoconjugates prepared contain well defined glycan chains at specific locations on the streptavidin core and should be useful for the study of protein-carbohydrate interactions and affinity separations.

Keywords: **neoglycoconjugates, neoglycoprotein, neoproteoglycan, biotin, streptavidin, heparin, bromelain, γ-globulin**

Abbreviations: **MWCO, molecular weight cut-off; ANTS, 8-amino-1, 3, 6-napthalenetrisulfonate; DMSO, dimethyl sulfoxide; ESI, electrospray ionization; NHS, *N*-hydroxysuccinimide; LC, long chain; CE, capillary electrophoresis; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate; BSA, bovine serum albumin; SAX, strong-anion exchange.**

Introduction

Glycoproteins and proteoglycans are glycoconjugates of increasing interest to biologists, biotechnology companies and the pharmaceutical industry because of their wide range of important biological activities [1,2,3]. The protein cores of glycoproteins are typically decorated with one or more branched, neutral or sialated oligosaccharide chains, corresponding to 1–50 wt % of the molecule [4,5] while the core protein of a proteoglycan contains one or more linear, highly charged polysaccharide chains often comprising >50 wt % of the molecule [6]. There are many difficulties associated with the large scale preparation and purification of these glycoconjugates. Glycoproteins are not normally biosynthesized by procaryotes. Eucaryotes such as yeast and insect cells do not fully elaborate their glycan structure and it is often difficult to consistently obtain uniformly glycosylated proteins even in mammalian cell culture [7,8] Glycoprotein heterogeneity, corresponding to unoccupied glycosylation sites and the presence of partially elaborated glycan structures can result, either from incomplete biosynthesis or glycoprotein catabolism [4]. Many glycoproteins and virtually all proteoglycans are prepared from animal tissue often increasing their glycan heterogeneity. While a small number of purified glycoproteins can be purchased in milligram to gram quantities, no proteoglycans are commercially available. Thus, studies requiring multi-milligram quantities of homogeneous, structurally defined glycoproteins and proteoglycans have been slowed.

Neoglycoconjugates have been prepared and used successfully to study protein-carbohydrate interactions as well as affinity separations [9–12]. The most direct approach to prepare neoglycoconjugates relies on the random chemical glycation of proteins at reactive lysine, aspartate, glutamate or cysteine residues [12,13]. Such neoglycoconjugates are used in routine lectin blot [14] and microtiter plate assays [15] as well as in sophisticated bioassays [17] and in protein-carbohydrate interaction studies [9,11]. The use of such neoglycoconjugate mixtures is complicated in studies requiring structurally defined molecules, such as the development of new affinity separation methods aimed at the purification of glycoprotein glycoforms [10].

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The biotin-avidin system holds great potential for the preparation of homogenous neoglycoconjugates having both a defined valancy and defined glycan location [18]. Streptavidin is a non-glycosylated microbial protein (MW 60,000) that is comprised of 4 identical tetrameric subunits each containing a site for the high affinity ($K_d \sim 10^{-15}$ M) binding of biotin [18]. The most commonly used approach for the preparation of neoglycoproteins is to bind biotinylated glycopeptides to streptavidin. These glycopeptides are typically obtained by proteolysis of commercially available glycoproteins. Biotinylated glycopeptides are prepared through the reaction of glycopeptides with various reagents including *N*-hydroxysuccinimide (NHS) activated esters of biotin [18–20]. Such neoglycoproteins, however, can also contain heterogeneity associated with variable amino acid residues present in the glycopeptide component. Glycans, prepared from glycoprotein using endoglycosidases or hydrazine [5] can be biotinylated by reductive amination with an aminobiotin or biotin hydrazide [21,22]. Alternatively, glycans can be reductively aminated with ammonium bicarbonate [23] and then reacted with a NHS activated ester of biotin. Biotinylated glycans have been prepared with sufficient purity to permit their full characterization using NMR spectroscopy and mass spectrometry [24,25].

Recently, our laboratory developed an affinity based reversed micellar extractive separation (ARMES) method for the fractionation of glycoproteins [10]. ARMES can potentially resolve glycoprotein glycoforms on a preparative scale making this method of great importance to the biotechnology and pharmaceutical industries. Such separations are difficult to achieve using more conventional affinity separation methods such as lectin chromatography [26]. The absence of milligram quantities of structurally defined glycoprotein glycoforms has precluded studies to examine the effect of process parameters (*i.e.,* pH, surfactant, ionic strength, temperature, etc.) critical for the high resolution separation of glycoforms [10]. The current study examines the preparation of milligram quantities of both neoglycoproteins and a neoproteoglycan of value in the refinement of the ARMES method

Materials and methods

Materials

Bovine γ-globulin was obtained from Pel-Freez Biologicals (USA). Crude stem bromelain, pronase P *(Steptomyces griseus)* and Amberlite IR-120 H^+ resin were from Sigma (USA). Semi-purified raw, unbleached sodium heparin (150 U/mg) was from Celsus Laboratories (USA). Hydrazine (anhydrous), biotinylation reagents and Gelcode blue staining reagent were from Pierce (USA). β-D-Galactosidase (exoglycosidase, EC 3.2.1.23 from *Esherichia coli),* and β-N-acetyl-D-glucosaminidase (exoglycosidase, EC 3.2.1.30 from beef kidney), were from Boehringer Mannheim Biochemical (USA). Neuraminidase (exoglycosidase, EC 3.2.1.18 from *Clostridium perfringens*) was purchased from Oxford GlycoSciences, (USA). Heparin lyase I (EC 4.2.2.7) was from Seikagaku (USA). BioGel P-4, SM-2 absorbent BioBeads and PVDF membrane were from Bio-Rad (USA). Diaflo ultra filters (YM-3) and Centri filters were from Amicon (USA). Spectropore dialysis tubing (1000 MWCO) was from Spectrum Medical Industries, (USA). All the other chemicals and organic solvents were of reagent grade, obtained from Aldrich Chemicals, (USA).

Preparation of glycopeptides

Bovine γ-globulin (2.5 g) was dissolved in water dialyzed (1000 MWCO) exhaustively against water at 4 °C and lyophilized to obtain 2.4 g glycoprotein. Bromelain (4 g) obtained as an acetone powder containing 50% mannitol was similarly dialyzed and lyophilized affording 2 g glycoprotein. Glycoprotein (2 g in 30 mL of 100 mM ammonium acetate at pH 7.9, containing 10 mM calcium chloride) was denatured by heating at 95 °C for 5 min, then cooled and treated for 6 h at 40 °C with gentle shaking with pronase P (40 mg). A second and third portion (30 mg each) of pronase P were added at 6 h and 18 h, before stopping the reaction at 24 h. The pronase digestion mixture from bromelain was concentrated to 12 mL by rotary evaporation, passed over an Amberlite IR-120H^+ column (1.5 × 25 cm) and eluted with 5 column volumes of water. The combined eluent was concentrated by rotary evaporation to 10 mL and desalted on a 2.5 × 46 cm BioGel P-4 column. Fractions collected at and immediately following the void volume were pooled, concentrated and passed again over Amberlite IR-120H^+ column (as above) and the eluent (50 mL) subjected to pressure filtration (YM-3 membrane, 3000 MWCO). The clear transparent permeate was concentrated and passed over a hydrophobic SM-2 (1 × 10 cm) column and eluted with 10 column volumes of water. Lyophilization yielded 34 mg of dry bromelain glycopeptides. **1.** The pronase digestion mixture from γ-globulin was heated at 95 °C for 5 min and dialyzed (1000 MWCO) for 2 days at 4 °C. The retentate was concentrated by rotary evaporation and lyophilized, affording 200 mg of dry γ-globulin glycopeptides.

Preparation of glycans

The dried γ-globulin glycopeptide mixture (200 mg) was subjected to hydrazinolysis by heating in anhydrous hydrazine (10 ml) at 98 °C for 8 h under Argon using an oil bath heated with steam *(Caution: hydrazine represents an explosion hazard and hydrazinolysis should be performed in a hood behind a shield).* Hydrazine was removed by rotary evaporation at 25 °C. Toluene was added and rotary evaporation was repeated to remove residual hydrazine. The deacetylated glycan hydrazones obtained were dissolved in

10 mL of ice cold 1 M sodium bicarbonate, immediately followed by the addition of 500 μL of acetic anhydride at 4 °C, with stirring for 30 min. A second aliquot of acetic anhydride was added and stirred for an additional 10 min at 4 °C after which the reaction mixture was slowly brought to room temperature and left for stirring for 1 h. The mixture was concentrated, centrifuged and supernatant was loaded onto a (1.5 × 45 cm) Sephadex G-25 column. Fractions eluting in void volume, having UV absorbence at 215 nm, were pooled and lyophilized. The re-*N*-acetylated glycan hydrazones were then converted to reducing glycans by incubation (for 6–8 hrs with gentle agitation) with 5 ml of 1 mM cupric acetate in 1 mM acetic acid at room temperature. The reaction was passed through a Dowex Ag 50-X 12 H^+ resin (1 × 40 cm) column and rinsed with 10 column volumes of water. The eluant and washings were combined, concentrated by rotary evaporation, passed over SM-2 column (5 ml). The eluant and washings were combined and lyophilized. The recovered dried material weighed 67 mg.

ANTS tagging and CE analysis

The released glycan mixture (100 μg) or purified glycan **4** (10 μg) was dried and taken in 1 ml vial and reductively aminated by mixing with 50 μL of 0.2 M ANTS solution (acetic acid: water, 3:17) and 50 μL of 1.0 M sodium cyanoborohydride in dimethylsulfoxide. The reaction mixture was mixed by vortexing to bring the reagents and glycans to the bottom of the vial, incubated at 45 °C for 12 h, diluted to 750 μL and loaded onto a Sephadex G-25 column to separate labeled glycans from the excess ANTS reagent. Glycan purity was determined using CE under reverse polarity conditions at pH 3.5 in 20 mM sodium phosphate buffer.

Carbohydrate remodeling

All exoglycosidase digestions were carried out in the presence of 0.02% sodium azide and in the presence of 300 μg of BSA. The γ-globulin glycan mixture **3** (14 mg) was dissolved in 200 μL of 50 mM sodium acetate buffer (pH 5.0). Neuraminidase (0.1 U, 5 U/ml) was added and the glycan mixture digested at 37 °C with gentle shaking. Enzyme (0.1 U) was added two additional times at 48 h intervals. Incubation was stopped by heating at 95 °C for 5 min and centrifuged to removed the denatured enzyme. The supernatant was desalted on a Sephadex G-25 column to obtain the desialylated γ-globulin glycan mixture which was freeze dried.

Neuraminidase treated glycan mixture was dissolved in 400 μL of 0.1 M sodium phosphate buffer (pH 7) and galactosidase 50 U was added three times over a period of 48 h, heated for 5 min at 95 °C, centrifuged, the supernatant was desalted on a Sephadex G-25 column and the sample freeze-dried. The peak eluting in the included volume (Kavg ~ 0.53) contained 8.5 mg of glycan **4** as demonstrated by MS and NMR analysis.

Sialidase and galactosidase treated glycan **4** (2.0 mg) was next subjected to β-glucosaminidase treatment. Substrate was dissolved in 100 μL of 0.1 M citrate buffer (pH 4.5) containing 5 nmol of α-mannosidase inhibitor, deoxymannojiromicine. β-glucosaminidase (1 U) was added to the glycan solution at 24 h intervals for 6 days after which the reaction was terminated by heating at 95 °C for 5 min, desalted on a Sephadex G-25 column and freeze-dried to afford 900 μg of glycan **5** as demonstrated by MS and NMR analysis.

Reductive amination and biotinylation of glycans

Octasaccharide **4** (1 mg) and pentasacharide **5** (450 μg) were dissolved in 1 ml of saturated ammonium bicarbonate and 1 mL of sodium cyanoborohydride solution (10 mg/mL) was added. After stirring for 6 days at room temperature the reductively aminated glycans were purified using size exclusion chromatography on a Sephadex G-25 column (1 × 45 cm) eluted with 10 mM ammonium bicarbonate. The eluent containing products were collected and freeze-dried. Aminitol sugars were dissolved in 500 μL of 100 mM, sodium phosphate buffer (pH 7) and 10-fold molar excess sulfo-NHS-LC-biotin was added at 4 °C and kept at this temperature with stirring for 30 min. After additional stirring overnight at room temperature the biotinylated glycans were purified using a Sephadex G-25 column eluted with water and freeze-dried to obtain biotinylated glycans **8** and **9.**

Biotinylation of glycopeptides

Bromelain glycopeptides **1** (1 mg) were dissolved in 1 ml of 100 mM sodium phosphate buffer at pH 7 and reacted with a 10-fold molar excess of sulfo-NHS-LC biotin at 4 °C added in 3 equal portions over 3 h and then stirred overnight at room temperature. The reaction mixture was purified on a Sephadex G-25 column eluted with water and freeze-dried to obtain **2.**

Preparation of neoglycoproteins

Streptavidin (500 μg) was dissolved in 200 μL of 10 mM potassium phosphate buffer (pH 8.0). The solution containing biotinylated glycan (200 μg/200 μL) was added to the streptavidin solution and kept at room temperature for 30 min followed by overnight incubation at 4 °C. The reaction mixture was purified on a Sephadex G-100 column to separate neoglycoproteins from excess non-complexed biotinylated γ-globulin glycans **8** and **9.** Fractions eluting ahead of excess glycans was corresponding to the neoglycoprotein were pooled, concentrated using a Centricon filter (10,000 MWCO) and lyophilized.

Biotinylation of peptidoglycan heparin

Semi-purified heparin (10 mg, 0.71 μM) was dissolved in 400 μL of 0.1M sodium bicarbonate and incubated with NHS-LC-biotin (4.8 mg, 10.7 μM) dissolved in 40 μL of dimethylformamide (DMF) at room temperature for 15 h [16]. The reaction mixture was dialyzed (3,500 MWCO) and lyophilized. The product was purified by low pressure SAX chromatography on a Dowex macroporous anion exchange resin column (1 × 7 cm) eluted with 3 column volumes of water, 2 column volumes of 50% aqueous methanol, 3 column volumes of 3% and 16% aqueous sodium chloride solution. Fractions obtained in the 3% and 16% sodium chloride washes were exhaustively dialyzed against distilled water (3,500 MWCO) and freeze-dried to afford 6.2 mg of a mixture of glycosaminoglycan heparin and biotinylated peptidoglycan heparin **10.**

Complex formation of biotinylated peptidoglycan heparin with streptavidin

The mixture of glycosaminoglycan heparin and biotinylated peptidoglycan heparin **10** (5 mg, 3.5×10^{-7} M) dissolved in water was combined with streptavidin (0.375 mg, 0.5×10^{-8} M) and incubated at room temperature 0.5 h, then at 4 °C overnight. PAGE analysis was performed on the product using a 14 × 16 cm gel prepared with a 12% non-reducing gel and GAG was visualized by Alcian blue staining [27].

Purification of neoproteoglycan by Sepharose CL-6B size exclusion chromatography

The neoproteoglycan and glycosaminoglycan solution prepared above was fractionated by size exclusion chromatography on Sepharose CL-6B column (1.0 × 107 cm) eluted with 30 mM Tris HCl pH 7.5 buffer containing 4 M guanidine hydrochloride and 0.5% CHAPS [28]. Fractions (1 ml) were collected and monitored by absorbance at 280 nm for proteins and by DMMB assay at 525 nm for GAG chains [29]. Fractions containing neoproteoglycan (showing both protein and GAG) were combined, dialyzed (6,000–8,000 MWCO) and lyophilized. The resulting neoproteoglycan was analyzed by PAGE on an 8% non-reducing gel in the presence of SDS stained first for protein with Gelcode blue stain reagent and then for GAG with Alcian blue [27].

Heparin lyase treatment of neoproteoglycan

The heparin neoproteoglycan (15 μg dissolved in 10 μL of 50 mM, pH 7.1 sodium phosphate buffer containing 100 mM sodium chloride) was treated with 150 mU of heparin lyase I for 24 h. The reaction mixture was immediately analyzed on a 8% non-reducing PAGE gel stained first with Gelcode blue stain reagent and then with Alcian blue [27].

Spectroscopy

NMR spectroscopy was performed on a UNITY-Varian instrument at 500 MHz in D_2O after exchanging the sample by freeze-drying it 3-times with D_2O. ESI-MS was performed on a Micromass Autospec instrument equipped with an electrospray interface. Samples dissolved in 1:1 water acetonitrile with 0.05% NH_4OH were injected at a flow rate of 20 μL/min.

Results

The preparation of neoglycoproteins first requires that glycopeptides or glycans be obtained from commercially available glycoproteins. Stem bromelain is an inexpensive glycoprotein protease obtained from pineapple, which contains only one glycosylation site and one type of glycan (Fig. 1) [30]. Bromelain (2 g) was thermally inactivated, treated with pronase and purified of contaminating peptides to afford glycopeptide product in 51% yield. High field NMR spectroscopy confirmed a single glycan structure, identical to that already determined and fully assigned **1** [30]. ESI-MS analysis, however, indicated that the bromelain glycopeptide product was a mixture containing at least 4 different species having peptides portions ranging from tripeptide to pentapeptide (Table 1). Reaction of this glycopeptide mixture with NHS-LC biotin afforded **2,** a mixture of biotinylated glycopeptides. ESI-MS gave molecular-ions in the positive-ion mode consistent with the expected structures of biotinylated glycopeptides in this mixture (Table 1).

Next, bovine γ-globulin was examined as a source of glycan for the preparation of neoglycoproteins. Bovine γ-globulin, the major glycoprotein component of cow blood, was proteolyzed to obtain a mixture of glycopeptides (Fig. 1). Large scale hydrazinolysis afforded nearly quantitative yield [8] of glycan product. Fluorescent labeling with ANTS and analysis using CE demonstrated the presence of multiple glycan structures (Fig. 2). This result is consistent with previous studies [31] showing that γ-globulin affords a glycan mixture containing a predominately fucosylated biantenary dodecasaccharide, **3.** (Fig. 1). Carbohydrate remodeling [32] by treatment of this glycan mixture with α-neuraminidase and β-galactosidase and subsequent purification using gel permeation chromatography afforded an octasaccharide product **4** as the major product (Fig. 1). Its purity was confirmed following ANTS tagging as ~85% by CE analysis (Fig. 2). Treatment of **4** with excess β-glucosaminidase resulted in the loss of the two terminating *N*-acetylglucosamine residues as well as the surprising loss of the core fucose residue (presumably due to the result of a contaminating α-L-fucosidase activity) affording a pentasaccharide **5.** The structures of **4** and **5** were confirmed by NMR spectroscopy and ESI-MS analysis (Tables 2 and 3). Reductive amination of both **4** and **5** with ammonium bi-

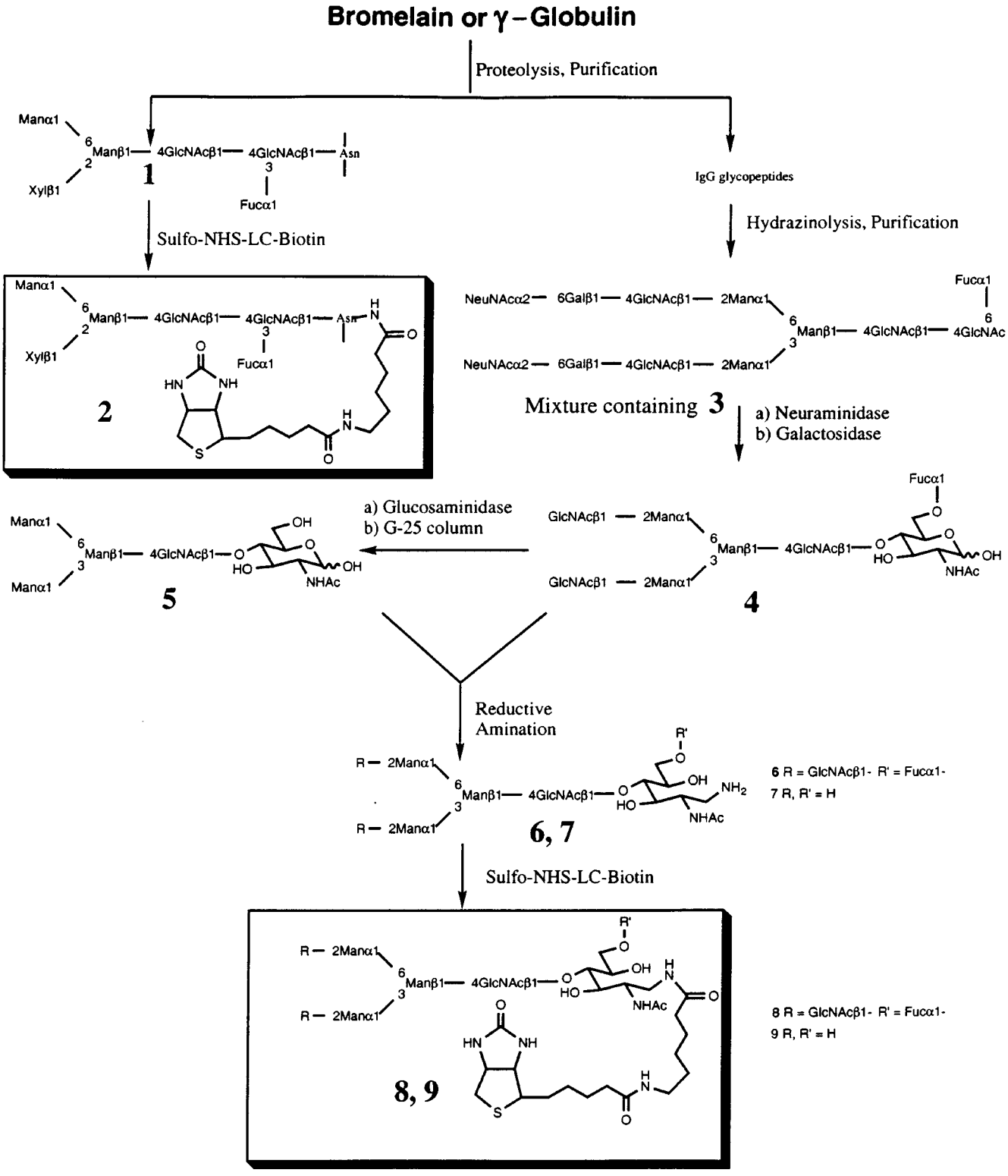


Figure 1. Scheme for the preparation of biotinylated glycopeptides and glycans from bromelain and γ-globulin.

Table 1. Electro-spray mass spectrometry data for bromelain glycopeptides.

Glycopeptides **1**[a]	*Calculated mass*	*M-H*	*Observed mass (negative-ion)* *1M-2H + Na*	*M-2H*	*M-3H + Na*
Sugar-Asn Glu Ser Asn	1356	1355.46	1377.40	677.02	688.03
Sugar-Asn Glu Sugar-Asn Glu Ser Ser Asn	1470	1469.46	1492.10	734.05	745.06
Sugar-Asn Glu Ser Ser Asn	1443	1442.86	1464.89	720.85	731.88
Sugar-Asn Glu Ser Ser	1557	1556.51	1579.37	777.53	—

Biotinylated glycopeptides **2**[a,b]	*Calculated mass*	*Observed mass (positive-ion)* *M + 2H*	*M + H + N*	*M + Na*
Sugar-Asn-B Glu Ser Asn-B	1695	849.00	860.00	871.00
Sugar-Asn Glu Sugar-Asn-B Glu Ser Ser Asn-B	1809	905.77	916.78	927.78
Sugar-Asn Glu Sugar-Asn-B Glu Ser Ser Asn-B	1782	892.71	903.71	914.71
Sugar-Asn Glu Ser Ser	1896	949.53	960.53	971.53

[a] See Figure 1 for structure
[b] B = LC-biotin linked to the *N*-terminus of the peptide.

carbonate afforded aminitol derivatives **6** and **7** that were biotinylated using sulfo-NHS-LC-biotin affording biotinylated glycans **8** and **9** (Fig. 1).

Efforts next focused on the preparation of peptidoglycans required for the synthesis of a neoproteoglycan. Proteoglycans are generally only available in microgram quantities. Heparin, an anticoagulant drug prepared from porcine intestinal mucosa, is prepared commercially in metric ton quantities [33]. Semi-purified heparin, containing approximately 9:1 GAG:peptidoglycan [33], was reacted with NHS-LC-biotin to biotinylate the amino group of the peptidoglycan component [16]. Previous studies, using an amino reactive fluorescent label, had demonstrated that such reactions could be targeted specifically at amino group of the peptide portion of peptidoglycan component [34]. Furthermore, these previous studies had demonstrated that the labeled peptidoglycan had both an average molecular weight and anticoagulant activity that was comparable to that of the semi-purified heparin starting material. A representative structure for the biotinylated peptidoglycan heparin **10** is shown in Figure 3.

Neoglycoconjugates were next prepared through the addition of a 2-fold excess (8 moles biotinylated species/1 mole streptavidin) biotinylated glycan, biotinylated gly-

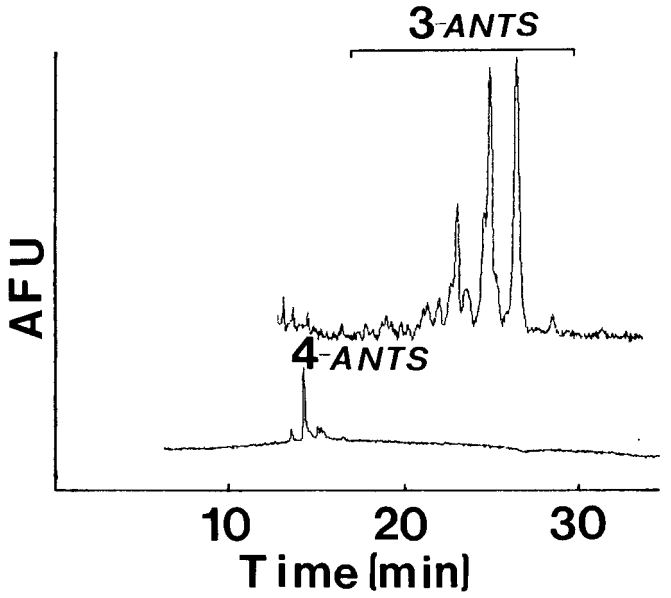


Figure 2. CE analysis of oligosaccharides fluorescently labeled with ANTS. The γ-globulin derived oligosaccharides showed multiple peaks (20–30 min) that on treatment with exoglycosidasis (Fig. 1) afforded a single major octasaccharide **4** having reduced migration time (15 min).

copeptide or biotinylated peptidoglycan to streptavidin (Fig. 4). Neoglycoproteins and neoproteoglycan were easily purified on the basis of size using gel permeation chromatography (Fig. 5A and B). The presence of glycosaminoglycan chains in the neoproteoglycan was conclusively demonstrated in a series of PAGE experiments (Fig. 6). Biotinylated-peptidoglycan and GAG mixture ran as a smear in the bottom third of the polyacrylamide gel (Fig. 6, lane a). Addition of streptavidin resulted in a second smear, in the upper third of the gel, tentatively assigned to the neoproteoglycan (lane b). Since this gel contained no SDS,

Table 2. Proton NMR chemical shifts of constituent monosaccharides for γ-globulin glycans.

Residue	*Reporter group*	*Chemical shift*[a] *Octasaccharide* **4**	*Pentasaccharide* **5**
Man-3	H-1	4.750	4.751
	H2	4.221	4.226
Man-4	H-1	5.153	5.158
	H-2	4.154	4.038
Man-4′	H-1	4.886	4.886
	H-2	4.076	3.943
GlcNAc-5,5	H-1	4.524	—
Fuc	H-1	4.866	—
	CH3	1.185	

[a]Chemical shifts are reported in ppm relative to HOD signal(4.75ppm) at 25 °C.

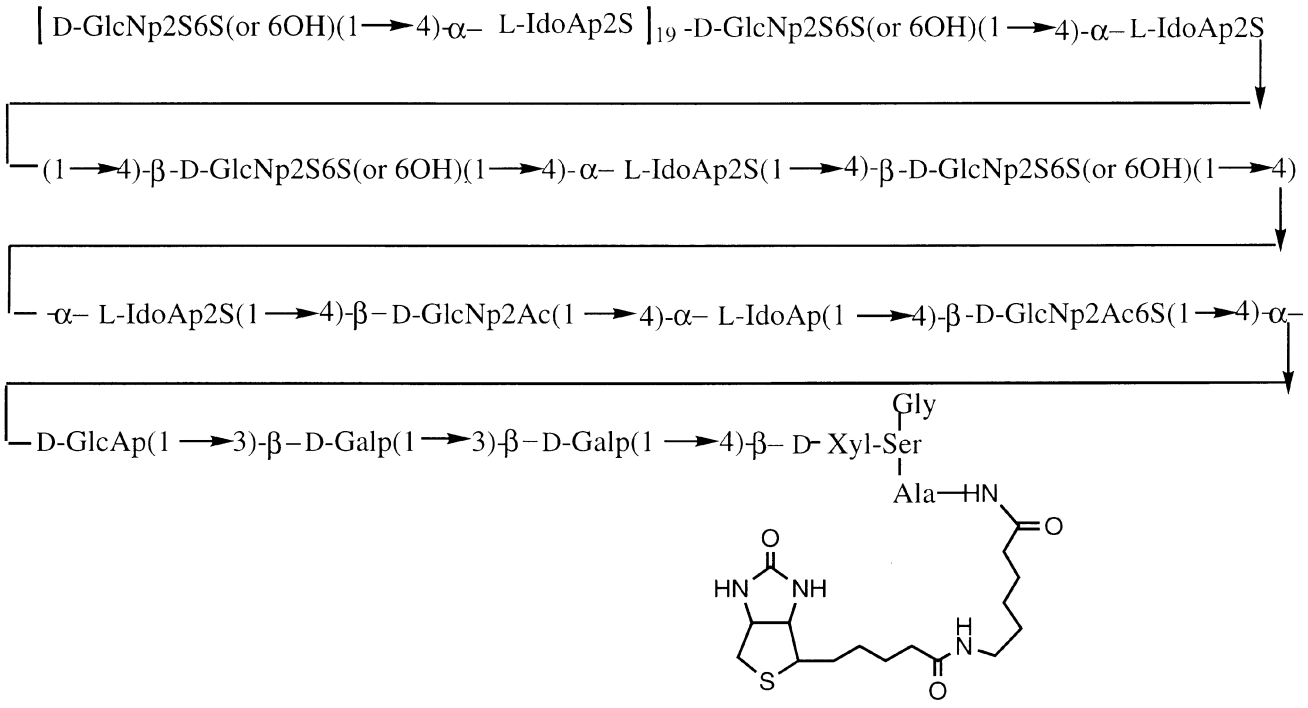
Table 3. Positive-ion electro-spray mass spectrometry data for γ-globulin glycans

Glycan	*Calculated mass*	*Observed mass* (M + H)	(M + Na)
Octasaccharide **4**	1462	1463.2	1485.5
Pentasaccharide **5**	932	933.4	—

streptavidin alone would not be expected to migrate into the gel. Furthermore, streptavidin alone can not be visualized in this gel as it was stained only for GAG [35]. Purification of the neoproteoglycan by gel permeation chromatography, in the presence of guanidine hydrochloride to prevent aggregation, afforded the neoproteoglycan (a smear in the upper third of the gel) free of contaminating biotinylated peptidoglycan and GAG (Fig. 6, lane d). The size of the neoproteoglycan determined 114 kDa using protein markers (lane c). Treatment of the neoproteoglycan with heparin lyase I resulted in a disappearance of the neoproteoglycan (absence of a smear in the upper third of the gel) and the appearance of protein bands from added enzyme and streptavidin (lane e).

Discussion

The increasing importance of carbohydrate containing products in biotechnology has focused attention on improving methods for the purification and bioassay of glycoconjugates. The general lack of pure, well characterized glycoproteins and proteoglycans has plagued the rapid advancement of glycobiology. Neoglycoconjugates are often used in bioassays [15]. Most frequently neoglycoproteins are prepared as a mixture from a non-glycosylated protein, such as bovine serum albumin, by chemical coupling to glycoprotein-derived glycans [5,13]. Conjugation of glycan to protein usually relies on using amino-reactive carbodimides, NHS active esters or sulfhydryl reactive disulfides, introduced into the reducing-end of the glycan [21,22,24,36]. The protein core of resulting neoglycoprotein is glycated at random sites on its surface and often contains a highly variable glycan content [13]. Moreover, the glycans prepared from readily available glycoproteins are often heterogeneous increasing the heterogeneity of these neoglycoproteins [5]. The preparation of neoproteoglycans has encountered even greater difficulties. Glycosaminoglycans (GAGs), such as heparin, have a high molecular weight (MWavg ~ 14,000), polydispersity (MW 5,000–50,000) and sequence microheterogeneity [33] that greatly complicates their conjugation to proteins. This results in multiple site attachment of GAG to protein that can be detrimental in affinity separations [15].



10

Figure 3. Representative structure of biotinylated heparin peptidoglycan.

The introduction of streptavidin as a carrier of biotinylated glycans offers an alternative approach, facilitating the preparation of neoglycoconjugates carrying a fixed number of glycan chains at defined positions [18]. While early studies relied on biotinylating glycopeptides released from commercially available glycoproteins through the proteolysis [18], the increased availability of *N*-glycanase has made it easier to prepare glycans for biotinylation. The addition of a chromophore (or fluorophore) into the biotin facilitates sensitive detection and has been important for the microanalysis of glycans [21,22,24].

The current study has focused on the large scale (milligram) preparation of neoglycoconjugates using the streptavidin-biotin system. Bromelain, initially selected as a glycan source, is one of the few glycoproteins having a single type of glycan chain located at a single glycosylation site [30]. Bromelain is also available in multi-gram quantities. We expected that proteolysis of bromelain using pronase would afford a pure glycopeptide. Surprisingly, while a single glycoform was obtained, it was contained in a heterogenous mixture of 4 different glycopeptides (Table 1). Biotinylation with NHS-LC biotin and conjugation with streptavidin, yielded an inexpensive, albeit heterogenous source of neoglycoprotein. While the elimination of peptide heterogeneity might be possible using a mixture of proteases, the unusual (plant-like) structure of the bromelain glycan suggested the necessity for developing an alternative approach.

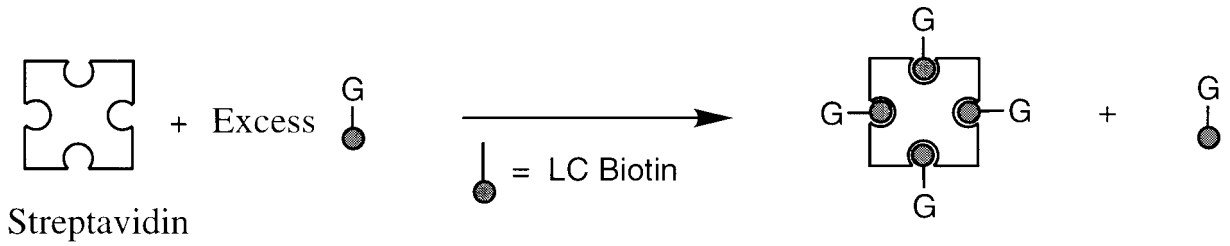


Figure 4. A schematic depiction of the preparation of fully occupied neoglycoconjugate. G corresponds to glycan or GAG.

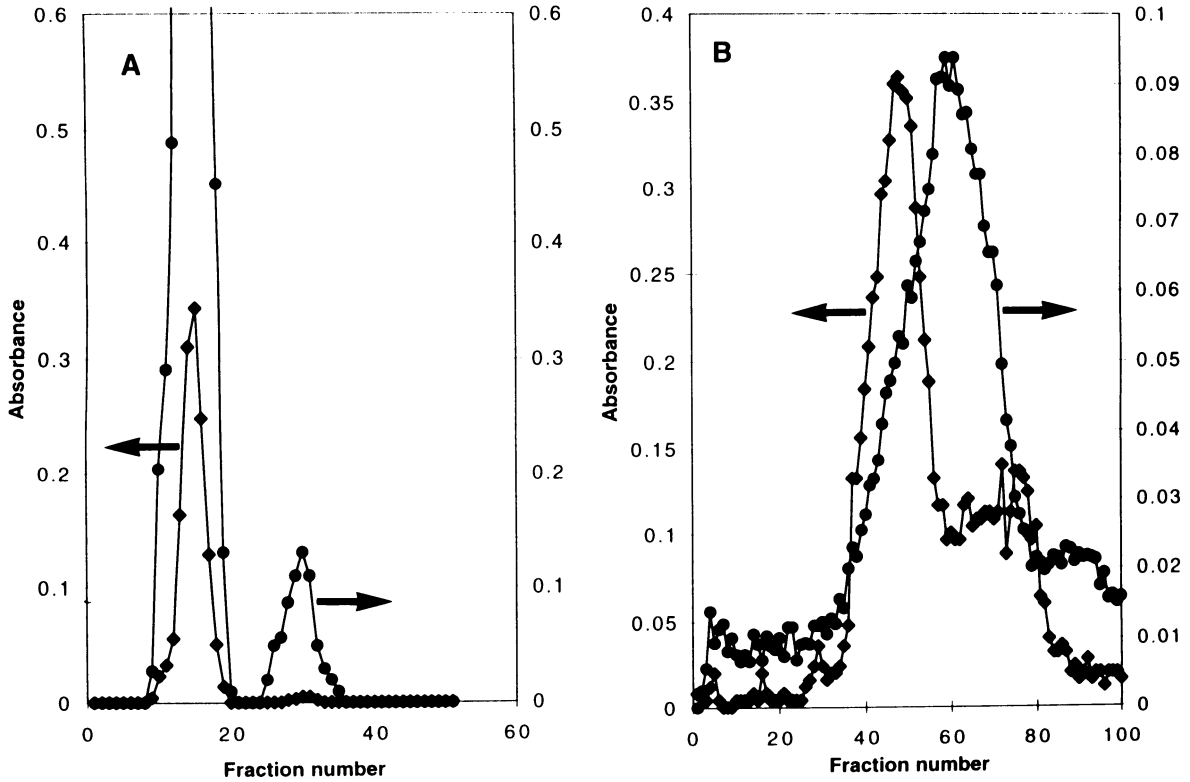


Figure 5. Purification of neoglycoconjugates. **A.** Sephadex G-100 purification (eluted with water) of neoglycoprotein prepared through binding biotinylated glycans, prepared from γ-globulin, to streptavidin. The peak in the void volume (fractions 10–20) contained neoglycoprotein, demonstrated by the presence of both protein (measured at 280 nm) and glycan (measured at 215 nm). The peak eluting in the total volume (fractions 25–35) contained only excess biotinylated glycan. The first peak was collected for analysis. **B.** Sepharose CL-6B separation (eluted using 30 mM Tris HCl pH 7.5 buffer containing 4 M guanidine hydrochloride and 0.5% CHAPS) of neoproteoglycan from biotinylated heparin. The first peak contained both protein (measured at 280 nm) and GAG (measured at 525 nm following DMMB assay [30]) while the second peak contained only GAG. The first peak was collected for analysis. The arrows indicate the Y-axis corresponding to each trace.

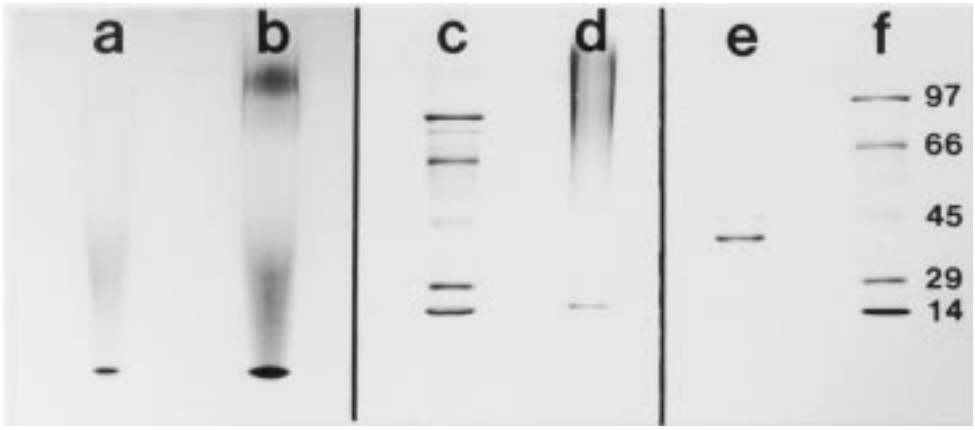


Figure 6. PAGE analysis of neoproteoglycan. A composite of three gels of identical size is shown. The first (no SDS present, stained only for GAGs) shows (a) a mixture of GAG and biotinylated peptidoglycan **10** and (b) GAG and biotinylated peptidoglycan **10** added to streptavidin. The second (stained for protein and GAG) shows (c) protein MW markers and (d) purified neoproteoglycan (the first peak in Fig. 5). The third (stained for protein and GAG) shows (e) neoproteoglycan treated with heparin lyase I and (f) protein MW markers: bovine lactalbumin, 14 kDa; bovine erythrocyte carbonic anhydrase, 29 kDa; ovalbumin, 45 kDa; bovine serum albumin, 66 kDa; and rabbit muscle phosphorylase b, 97 kDa.

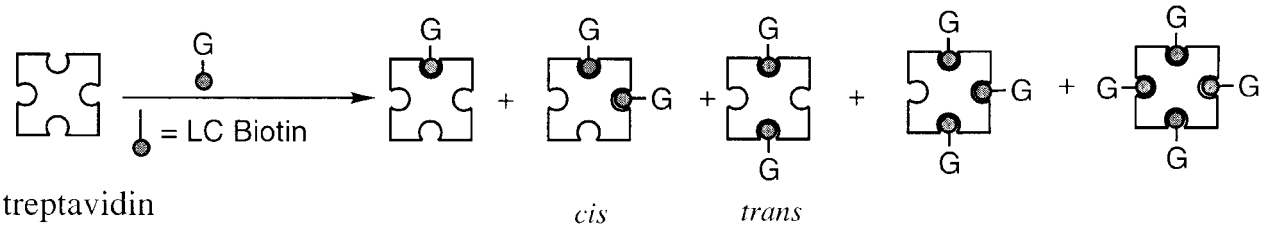


Figure 7. A schematic depiction of different glycoforms of glyconjugates that can be prepared using the streptavidin-biotinylated glycan system. G corresponds to glycan or GAG.

Bovine γ-globulin, known to contain multiple glycoforms and various glycosylation sites [31], was next examined as an inexpensive source of glycan. Glycan from γ-globulin glycopeptides was released by large scale hydrazinolysis and simplified glycan structures were prepared by carbohydrate remodeling. (Fig. 1). Reductive amination with ammonium bicarbonate and sodium cyanoborohydride and biotinylation using sulfo-NHS-LC-biotin afforded good yields of spectroscopically pure biotinylated octasaccharide **8** and pentasaccharide **9** that were conjugated with streptavidin to obtain neoglycoproteins (Fig. 4).

Next, our attention turned to the preparation of neoproteoglycans. Since proteoglycan heparin was not available, semi-purified heparin was used as a source of peptidoglycan heparin. Biotinylation and conjugation to streptavidin afforded a neoproteoglycan that behaved similar to other proteoglycans on PAGE analysis (Fig. 6) [35]. Treatment of neoproteoglycan with heparin lyase I removed the GAG chains from the streptavidin core in an identical fashion as is conventionally observed on treatment of other proteoglycans [35]. This neoproteoglycan also has GAG chains that are correctly (naturally) oriented (*i.e.,* reducing-end attached to core protein). A similar method has been applied in our laboratory to the surface assembly of a heparin neoproteoglycan on a biosensor used in surface plasman resonance to probe heparin interaction with heparin binding proteins [16].

This study provides several new approaches to the assembly of neoglycoconjugates for protein interaction and affinity separation studies. Excess biotinylated glycan, glycopeptide and peptidoglycan were used to ensure full occupancy of streptavidin (Fig. 7). Indeed, assessment of occupancy of the heparin neoproteoglycan, based on its size, suggests that it is composed of species containing 1 to 4 peptidoglycan chains with an average occupancy of 3. Full occupancy may be difficult in the case of large biotinylated peptidoglycan chains due to steric factors. Studies are currently underway to examine the use of sub-stoichiometric quantities of biotinylated glycan or biotinylated peptidoglycan to prepare glycoform mixtures (Fig. 7). Glycoform mixtures should be useful for studying the separation efficiency of affinity fractionation methods such as lectin affinity chromatography [26], heparin-binding protein affinity chromatography [37] and ARMES [10]. Furthermore, neoglycoconjugates containing one or more unoccupied biotin binding sites might be useful in immobilization to biotinylated surfaces [15,21], biotinylated external membrane of cells [38], or in receptor mediated glycotargeting [39].

Acknowledgments

The authors are grateful for financial support from the National Institutes of Health in the form of grant R01-GM49056. The authors also thank Dr. Youmie Park for her helpful suggestions.

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Received 9 February 1999, revised 10 May 1999, accepted 13 May 1999