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Synthesis of polymeric neoglycoconjugates based on *N*-substituted polyacrylamides

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Several types of polymeric glycoconjugates, *N*-substituted polyacrylamides, have been synthesized by the reaction of activated polymers with ω-aminoalkylglycosides: (i) (carbohydrate-spacer)$_n$-polyacrylamide, 'pseudopolysaccharides'; (ii) (carbohydrate-spacer)$_n$-phosphatidylethanolamine$_m$-polyacrylamide, neoglycolipids, derivatives of phosphatidylethanolamine; (iii) (carbohydrate-spacer)$_n$-biotin$_m$-polyacrylamide, biotinylated probes; (iv) (carbohydrate-spacer)$_n$-polyacrylamide-(macroporous glass), affinity sorbents based on macroporous glass, covalently coated with polyacrylamide. An almost quantitative yield in the conjugation reaction makes it possible to insert in the conjugate a predetermined quantity of the ligand(s).

Pseudopolysaccharides proved to be a suitable form of antigen for activation of polystyrene and poly(vinyl chloride) plates (ELISA) and nitrocellulose membranes (dot blot), being advantageous over traditional neoglycoproteins. Polyvalent glycolipids insert well in biological membranes: their physical properties, particularly solubility, can be changed in a desired direction. Biotinylated derivatives were used as probes for detection and analysis of lectins.

Abbreviations: sp, spacer arm; PAA, polyacrylamide; PE, phosphatidylethanolamine; Biot, biotin; MPGlass, macroporous glass 2000 Å; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; AIBN, azodiisobutyronitrile; BSA, bovine serum albumin; DMG, 3,6-di-*O*-methyl-D-glucose; TLC, thin-layer chromatography; DMF, dimethylformamide; DMSO, dimethylsulfoxide; RI, refractive index; PBS, phosphate buffered saline (0.14 M NaCl, 0.01 M sodium phosphate, pH 7.3).

Glycoconjugates on the cell surface or in soluble form take part in many vitally important processes of cell recognition. Synthetic analogues of glycoconjugates, such as neoglycoproteins [1], neoglycolipids [2] and pseudopolysaccharides [3] have been used successfully for studying and modelling these processes. An advantage of synthetic glycoconjugates is the possibility of creating molecules with predetermined properties, e.g., solubility, molecular weight, ligand density, and capability of biodegradation.

Special methods of synthesis are known for each class of compound mentioned. Here we suggest a methodically simple and universal approach for the synthesis of various glycoconjugates built using the same scheme, e.g., carbohydrate groups and other non-sugar ligand (lipid, biotin, etc.) are attached to a polyacrylamide core, via a spacer-arm. Polyacrylamide-based glycoconjugates have a number of essential differences from traditionally used BSA-based neoglycoproteins, such as chemical and immunological inertness (low nonspecific interaction with proteins) and also the flexibility of the polymer, which is a random coil, allowing the haptens to rearrange themselves and to interact with the carbohydrate-binding protein in an optimal way.

To synthesize polyacrylamide pseudopolysaccharides, the strategy of introducing an olefinic group (allyl or acryloyl) in a carbohydrate was employed earlier, followed by copolymerization with acrylamide [4 and the papers cited therein]. Our experience in performing double and even triple copolymerization [6–8], and literature data analysis [4], showed the essential limits of this approach: the main problems are low reproducibility of copolymerization (regarding yield, ratio of ligands inserted into polymers and molecular weight of co-polymers) and technical difficulties when performing the reaction with only about 0.1–0.5 mg of material. An alternative approach to obtaining the same or analogous macromolecular derivatives of carbohydrates was therefore sought.

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This work deals with an approach based on condensation of poly(4-nitrophenylacrylate) (e.g., fully activated polyacrylic acid) with aminoalkyl glycosides. A simultaneous or subsequent introduction of nonsugar aminoligands makes it possible to provide a polymer molecule with a series of additional properties. The possibilities of this approach are illustrated by a number of examples.

Materials and methods

General methods

HPLC was carried out using an LKB 2150 HPLC pump with a Rapid Spectral detector, fluorometer (Gilson, model 121) and RI detector (Altex, model 156). TLC was performed on 60F-254 silica gel plates (Merck); substances were detected by 5% sulfuric acid solution in water at 150 °C (carbohydrates), ninhydrin (aminocompounds) and molybdate reagent [prepared by solution of 1 g ammonium molybdate in 10 ml H_2O at 40 °C; then 5 ml 60% HCl, 2 ml conc. HCl and acetone (up to 40 ml) were added to the solution] for detection of phosphorus-containing compounds.

Optical rotation was measured on polarimeters Polamat M (GDR) and Jasco DIP 360 at 20–25 °C. Optical density was measured using a Hitachi 150-20 spectrophotometer. ELISA results were registered on photometer Titertec Multiskan MCC at a wavelength of 492 nm. ELISA assay was performed on polystyrene or poly(vinyl chloride) plates (Nunc); dot blotting was made on nitrocellulose membranes (pore size 45 μm, Schleicher & Schull), quantitative results were obtained using a laser densitometer, Gel Scan XL (LKB). ^{1}H-NMR spectra were obtained on a Brucker WM-500 spectrometer. Melting points were measured with a Boetius device (GDR). Crystalline compounds had the correct element composition.

Phosphatidylethanolamine (PE) was obtained from eggs as described [5]. PE labelled with ^{14}C (Amersham) was used. BSA, cytochrome *c*, d-biotin (Sigma), DMSO and acetonitrile (Merck) were also used. Dimethylformamide was distilled over ninhydrin. Dexal (periodate oxidated dextran with an aldehyde group content of 1900 μmole g^{-1}) was purchased from Biolar (Olaine, Latvia). TLC was performed in the following systems: EtOH–BuOH–Pyridine–H_2O–AcOH, 100:10:10:10:3 by vol (A), and $CHCl_3$–EtOH–H_2O, 3:8:2 by vol (B).

The molecular weight of the polymers was determined on a HEMA 1000 Bio column (Tessek, CSFR) in water with a flow rate of 1.0 ml min^{-1} and RI detection. Ultrafiltration was performed on PM 30, XM 100 and XM 300 membranes (Amicon) in water with polymer concentration 1 mg ml^{-1} and RI detection.

The spacer derivative of muramyl-dipeptide (MDP) (**4**) was synthesized as described in [9]; Neu5Acα2-$OCH_2C_6H_4NHCOCH_2NH_2$ (**9**) was synthesized as described in [10]; the blood group A-trisaccharide spacer (**3**) was synthesized as described in [11]; synthesis of the Le^x and Le^a trisaccharide-spacers was as described in [8]; 3,6-di-*O*-methyl-D-glucose was synthesized by N. E. Byramova [12].

Monoclonal anti-A antibodies were obtained from All-Union Hematological Scientific Centre (Moscow), sera of leprosy patients from Institute of Leprae Research (Astrakhan, Russian Federation), crude lectin from *B. frondosa* was obtained as in [13].

Poly(4-nitrophenylacrylate)

AIBN, corresponding to 3% (w/w) of the monomer, was added to a 1 M solution of 4-nitrophenylacrylate in dry benzene under a stream of nitrogen and kept for 50 h at 70 °C. The benzene solution was decanted and the viscous brown residue on the flask walls was dissolved in DMF or DMSO to obtain a 1–2% solution. The polymer was re-precipitated with five volumes of methanol. Precipitation was repeated and the clean white residue was washed with methanol and dried. The polymer did not contain residual monomer, short oligomers or AIBN according to TLC (EtOH–Et_2O, 2:1 by vol).

Oligo(4-nitrophenylacrylate)

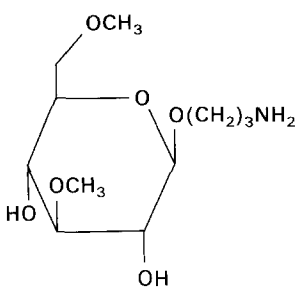
AIBN, corresponding to 3% (w/w), was added to a 1 M solution of 4-nitrophenylacrylate in DMF under a stream of nitrogen and was kept for 5–6 h at 70 °C. The solution was cooled and the oligomers were re-precipitated with 10 volumes of water. The clean white residue was washed with water and dried. The reaction product did not contain residual monomer or AIBN according to TLC (EtOH–Et_2O, 2:1 by vol).

*3-Aminopropyl 3,6-di-*O*-methyl-β-D-glucopyranoside* (**1**)

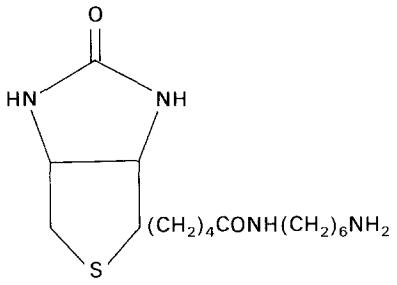
5 ml acetic anhydride was added to a solution of 3,6-di-*O*-methyl-D-glucose (600 mg, 2.9 mmol) in 10 ml pyridine; after 16 h, methanol (0.2 ml) was added and the solution was then evaporated to dryness. The residue was applied to a silica gel column and eluted with the mixture of toluene–ethyl acetate, 9:1 by vol. 950 mg (98%) of the peracetate mixture was obtained (α/β ratio 2:3 according to anomeric proton signal intensities in ^{1}H-NMR: at 5.65 ppm with $J_{1,2}$ = 8.5 Hz, and at 6.30 ppm with $J_{1,2}$ = 3.5 Hz, respectively), R_F 0.60 (toluene–ethyl acetate, 1:1 by vol).

Acetyl bromide (4 ml) was added to a solution of 670 mg (2 mmol) peracetate mixture in 30 ml chloroform at 0 °C, and then 0.8 ml methanol in 10 ml chloroform was added. The mixture was kept for 30 min at the same temperature, then washed with water, $NaHCO_3$ solution, water, dried, and evaporated to dryness. 700 mg syrupy glycosyl bromide, homogeneous according to TLC, was obtained.

A mixture of $HgBr_2$ (360 mg, 1 mmol), $Hg(CN)_2$ (460 mg, 2 mmol), 1.6 g molecular sieves (4 Å) and



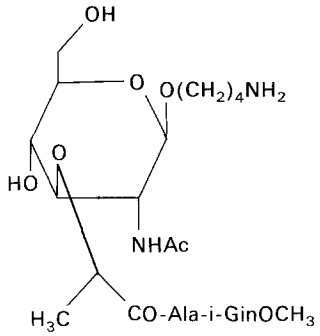
(1)



(2)

GalNAcα1 → 3
GalNAcα1 → 3Galβ1 → O(CH2)3NH2
Fucα1 → 2

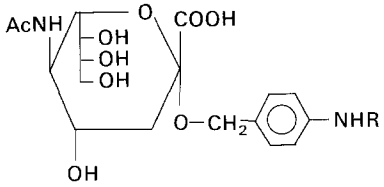
(3)



(4)

GalNAcα1 → 3Galβ1 → o(CH2)3NH2
GalNAcα1-3Galβ1-o(CH2)3NH2

(5)



(6) R = H
(7) R = $COCH_3$
(8) R = $COCH_2NHCOOC(CH_3)_3$
(9) R = $COCH_2NH_2$

Galβ1 → 4
GlcNAcα1 → 3Galβ1 → o(CH2)3NH2
Fucα1 → 3

(10)

Fucα1 → 4
GlcNAcα1 → 3Galβ1 → o(CH2)3NH2
Galβ1 → 3

(11)

3-(trifluoroacetamido)propanol (510 mg, 3 mmol) in 7 ml acetonitrile was stirred for 4 h, the solution of glycosyl bromide in 5 ml methylene chloride was then added, and the mixture was stirred for 2 h at 20 °C. The mixture was diluted with chloroform and filtered. The filtrate was washed with KI solution, then water, dried and evaporated. The residue was applied to a silica gel column and eluted with a mixture of ethyl acetate and benzene (with an ethyl acetate gradient of 0–50%), giving 3-Trifluoroacetamidopropyl 2,4-di-*O*-acetyl-3,6-di-*O*-methyl-β-D-glucopyranoside, 750 mg (84%), MP 73–74.5 °C ether–hexane; $[\alpha]_D$ −60° in $CHCl_3$; R_F 0.44 in ethylacetate–toluene 2:1 by vol; ^{1}H-NMR: 1.85 m (2H, CH_2N), 2.13 c (6H, 2Ac), 3.34 c and 3.42 c (6H, $2CH_3O$), 4.20 d (1H, $J_{1,2} = J_{2,3} = 8$ Hz, H-2), 4.94 dd (1H, $J_{4,5} = J_{3,4} = 10$ Hz, H-4).

50 mg Amberlyst A-26 (OH-form) resin was added to the solution of 0.1 mmol of the latter compound in 10 ml ethanol–H_2O, 2:1 by vol, and the mixture was stirred for 24 h. The reaction was followed with TLC in the system EtOH–aq.NH_3, 4:1 by vol, development with ninhydrin. The resin was removed, the solution was evaporated and 0.1 mmol of compound **1** (R_F 0.2) was obtained.

(N-*Biotinyl*)*hexamethylenediamine trifluoracetate* (*BiotNH*$_2$) (**2**)

Boc-hexamethylenediamine hydrochloride (126 mg; 0.5 mmol) [14] in 5 ml 0.1 M $NaHCO_3$ solution, pH 8.5, was added to a solution of 170 mg (0.5 mmol) of the succinimide ester of biotin in 5 ml DMF, the mixture was stirred for 4 h at 20 °C, the solvents were removed *in vacuo*, the residue was washed twice on a filter with 5 ml water and dried. 200 mg (90%) light-yellow crystals of Boc-substituted *N*-biotinylhexamethylenediamine was obtained with R_F 0.65 ($CHCl_3$–MeOH–DMF, 8:1:3 by vol), 0.64 (BuOH–AcOH–H_2O, 4:1:1 by vol). The Boc-group was removed by the action of 7 ml 95%

CF_3COOH–$CHCl_3$, 1:3 by vol, for 0.5 h at 20 °C. The solvents were removed *in vacuo* and the oily residue was converted into a solid product by dispersion in ether and drying with P_2O_5. 205 mg (93%) of **2** was obtained, MP 82–84 °C, R_F 0.62 (BuOH–Pyridine–H_2O, 4:1:1 by vol), 0.15 ($CHCl_3$–MeOH–DMF, 8:1:3 by vol).

Synthesis of pseudopolysaccharides

Attachment of A-trisaccharide (**3**). 1, PAA with 5% substitution of oligosaccharide. A solution of 0.27 mg (0.5 μmol) trisaccharide **3** in 0.5 ml DMF and 10 μl triethylamine was added to a solution of 1.93 mg (10 μg equivalents) poly(4-nitrophenylacrylate) in 0.5 ml DMF, the resulting solution was kept for 16 h at 25 °C, and a 30 to 50-fold molar excess of aq. NH_3 was added and left for 16 h. The solution was applied to a Sephadex LH-20 column and the conjugate was eluted with a mixture of acetonitrile–water, 1:1 by vol, with RI control of fractions. The yield of lyophilized conjugate was more than 90%. 2, PAA with 30% substitution of oligosaccharide. A solution of 1.64 mg (3 μmol) trisaccharide **3** in 0.5 ml DMF and 10 μl diisopropylethylamine was added to 1.93 mg (10 μg-equivalents) polymer in 0.5 ml DMF, the solution was then kept for 48 h at 37 °C, and treated as described in 1. 3, Series of PAA conjugates with 5, 10, 15, 20, 25, 30, and 35% substitution of A-trisaccharide (**3**). To aliquots of 1.93 mg polymer in 1.0 ml DMF (seven 0.1 ml aliquots of 1 μg equivalent each) were added solutions of A-trisaccharide in DMF containing from 0.027 mg (0.05 μg equivalents) to 0.19 mg (0.35 μg equivalents) trisaccharide **3** and 1 μl diisopropylethylamine. The solutions were kept for 48 h at 50 °C, then 10 μl ethanolamine were added and the solutions were kept at 25 °C for 16 h, after which they were used directly, without any purification.

Series of polymeric sialosides. Solutions of 1.75, 3.5, and 5.25 μmol sialoside **9** in 0.2 ml DMF as well as DMF itself were mixed each with 70 μl 10% (35 μg equivalents) solution of polymer in DMF, 20 μl triethylamine were then added to each mixture and the mixtures were kept at 20 °C for 48 h. The resulting polymers were then modified by addition of 1 ml 10% ethanolamine in DMF. 72 h later, nitrophenol and ethanolamine were removed by gel-filtration on a Sephadex LH-20 column (1 cm × 25 cm, acetonitrile–H_2O, 1:1 by vol), and the resulting polymers lyophilized. Yields were about 95%.

Coupling of dimethylglucose derivative (**1**) *and* Le^x (**10**) and Le^a (**11**) *trisaccharide-space with polymer.* 10% conjugates were obtained as described for the A-trisaccharide in example 1.

Dextran conjugate with MDP. 12 mg 4-aminobutyl glycoside of MDP methyl ether hydrochloride (**4**) were added to 50 mg dexal solution in 1 ml $NaHCO_3$ and stirred for 12 h at pH 8–8.5, then 10 mg $NaBH_4$ were added in portions keeping the pH between 8 and 9 with 5% acetic acid. The solution was then kept for 16 h before desalination was performed on a Sephadex G-15 column in water. The yield of lyophilized conjugate was 90%.

Synthesis of neoglycolipids

1, 2.24 mg (4 μmol) Le^x-trisaccharide-spacer (**10**) was added to a solution of 4.6 mg (24 μg equivalents) poly(4-nitrophenylacrylate) in 0.5 ml DMF and then 3 mg (4 μmol) PE in 0.2 ml $CHCl_3$–MeOH, 1:1 by vol, and 10 μl diisopropylethylamine were added. The mixture was then kept for 24 h at 25 °C, the reaction was checked by the absence of a carbohydrate component (TLC, system A, development with ninhydrin) and absence of PE (TLC, system B, development with ninhydrin and molybdate reagent on phosphorus, see General methods section). Aq. NH_3 (50-fold excess) was then added to the reaction mixture and the conjugate was obtained in 24 h as described above for pseudopolysaccharides. The yield was about 90%. 2. The synthetic blood-group A substance was obtained in a similar way from 25 mg (125 μg equivalents) oligo(4-nitrophenylacrylate), 7.1 mg (13 μmol) A-trisaccharide dissolved in 0.5 ml DMF and 19 mg (26 μmol) PE dissolved in 1 ml $CHCl_3$–MeOH, 1:1 by vol. The yield was quantitative.

Synthesis of probe

A-Disaccharide derivative (**5**). A solution of 5 mg (11 μmol) disaccharide derivative (**5**) [11] in 0.6 ml DMF was added to 11 mg (55 μg equivalents) poly(4-nitrophenylacrylate) in 0.4 ml DMF, then 20 μl triethylamine and 1.3 mg (2.8 μmol) $BiotNH_2$ in 0.1 ml DMF were added. The mixture was kept for 24 h at 40 °C until the absence of carbohydrate and $BiotNH_2$ was shown by TLC (B). 0.05 ml ethanolamine was added, and the mixture was kept for 24 h at room temperature. The conjugate was obtained as described for pseudopolysaccharides (1). The yield was 90%.

Synthesis of glycosorbents

A-Trisaccharide derivative (**3**). Activation of MPGlass is described in [15] and [16]. Briefly, 0.5 g aminopropylated MPGlass (100 μmol of NH_2 groups per g) was added to a solution of 25 mg poly(4-nitrophenylacrylate) in 2.5 ml DMF and kept for 24 h at room temperature with periodical shaking, washed three times with DMF, ether and dried *in vacuo*. The capacity of activated carrier was more than 100 μmol nitrophenyl groups per g (capacity was determined by measuring spectrophotometrically the quantity of 4-nitrophenol yielded after hydrolysis with an excess of aq. NH_3).

The carrier obtained was suspended in 2 ml DMF and the solution of 2 μmol glycoside **3** in 0.5 ml DMF was added, the mixture was kept for 24 h with periodical shaking, then 0.2 ml ethanolamine was added and kept for 15 h, washed

again with DMF and ether, then dried *in vacuo*. According to TLC (complete disappearance of aminopropylglycoside) and monosaccharide analysis of product, the attachment of 2 μmol proceeded quantitatively.

Derivative of the A-disaccharide **5** was obtained as described for the A-trisaccharide, from 0.5 g activated MPG and 5 μmol glycoside.

ELISA

100 μl of a solution of DMG conjugated with polyacrylamide (in different concentrations from 0.5 mg ml^{-1} to 1.0 mg ml^{-1}) in sodium carbonate buffer, pH 9.6, were added to each well of a 96-well polystyrene or poly(vinyl chloride) plate, the plates were kept for 2 h at 37 °C, then for 15 h at 4 °C, and washed with sodium phosphate buffer, pH 7.4, containing 0.05% Tween 20. Blood sera from leprosy patients, tuberculous and normal donors were diluted in PBS–Tween 1:200, then the solution and its subsequent two-fold dilutions were added in the amount of 100 μl per well, and kept for 1 h at 37 °C. After washing with PBS–Tween, horseradish peroxidase conjugated with anti-human IgM was added, the samples were kept for 1 h at 37 °C and washed again. Hydrogen peroxide and *o*-phenylenediamine were used as substrate.

Dot blot

10 μl aqueous solutions of A-trisaccharide conjugated with PAA (containing different amounts of saccharide, 5–35 mol %) in subsequent two-fold dilutions beginning with a concentration of 0.1 mg ml^{-1} were placed on a nitrocellulose membrane, the membrane was dried, washed for 30 min at 37 °C, then kept for 1 h at 20 °C in a monoclonal anti-A antibody solution (haemagglutination titre 1:64) on a rotation shaker, then washed four times with PBS containing 0.5% Tween 20 for 5 min. After washing, the membrane was incubated for 2 h at 20 °C in horseradish peroxidase conjugated with antibodies against mouse immunoglobulins (BioS, Russian Federation, dilution 1:500 in PBS), washed as described above and treated for 30 min with a solution of peroxidase substrate (0.25% hydrogen peroxide and 0.05% 4-chloro-1-naphthol in PBS).

Hybridoma production with Le^x neoglycolipid as immunogen

Insertion of neoglycolipid in *Salmonella minnesota* was performed according to the method developed by Hakomori *et al.* [17] for gangliosides and neutral glycolipids. Briefly, neoglycolipid (2 mg) was dissolved in ethanol (0.5 ml), mixed with a suspension of acid-treated *Salmonella minnesota* strain R595 (2 mg in 10 ml saline buffer), vortexed for 30 s, then incubated 10 min at 50 °C.

Immunization protocol of BALB/c mice. 15 μg neoglycolipid complexed with *S. minnesota* on day 0, 30 μg on day 4, 45 μg on day 7, 60 μg on day 12, and 60 μg on day 77. Spleen cells were harvested and fused with mouse myeloma 653A using standard procedures.

Hybridoma screening. Pseudopolysaccharides of Le^x and Le^a specificity were used as the antigens in ELISA for the analysis of mouse sera (titres were 1:400 before booster immunization) and culture supernatants for cloning hybridomas. Sixteen clones secreting anti-Le^x (but not Le^a) antibodies were chosen from 302 clones obtained in the fusion.

Study of lectin specificity

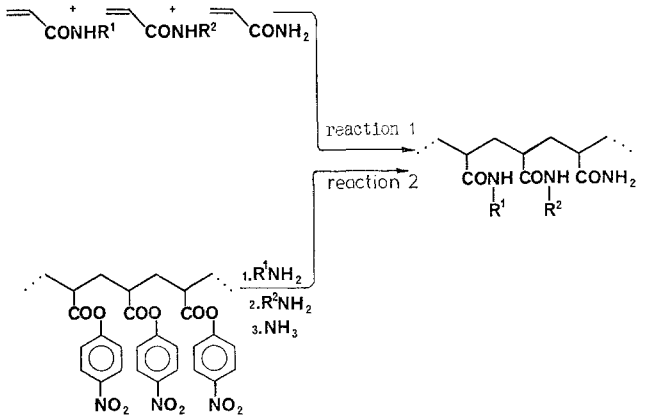
100 μl of a solution of lectin from *Butea frondosa* seeds (family Leguminosae) [13] in sodium carbonate buffer (pH 9.6, 10 mg ml^{-1}) were added to each well of 96-well plates, incubated for 1 h at 37 °C then 16 h at 4 °C. After washing (PBS + 0.1% Tween 20), the plates were incubated with 1% gelatin solution in PBS, then carbohydrate inhibitors in twofold dilutions were added and incubated for 1 h at 37 °C. After the inhibitor, the probe $(GalNAc\alpha 1\text{-}3Gal\beta spacer)_{4n}Biotin_{1n}PAA$ at a concentration of 6 μg ml^{-1} was added and incubated under the same conditions. After washing, avidin-peroxidase conjugate (Sigma, dilution 1:1000) was added, and development was performed using *o*-phenylenediamine.

Anti-Rh serum preparation

1, By using glycosorbent A. 1 ml of donor blood serum containing anti-Rh and anti-A antibodies (the latter in titre 1:64) was kept for 1 h at 20 °C with 100 mg glycosorbent A with periodical shaking. The serum containing the initial titre of anti-Rh, but free of anti-A antibodies, was separated by decanting. 2, By using pseudopolysaccharide A. 4 ml synthetic blood group substance A (pseudopolysaccharide A) was added to 1 ml donor blood serum containing the two antibodies anti-Rh and anti-A (the latter in titre 1:64) and kept at 0–20 °C. After 20 min, anti-A antibodies were not detectable in the serum using saline agglutination, but the titre of anti-Rh antibodies remained unchanged.

Results and discussion

Water soluble PAA-derivatives of carbohydrates (pseudopolysaccharides) have proved to be suitable tools for studying carbohydrate-binding proteins [18–21]. However, the method for their synthesis by radical copolymerization of olefinic glycosides described earlier [4 and papers cited therein, 20] has a number of restrictions (see below). We therefore developed an alternative approach (reaction 12) involving condensation of activated polyacrylic acid with aminocompounds, the reaction resulting in the formation of substituted polyacrylamide by radical copolymerization of acrylamide monomers (reaction 1).



Initial polymers

The attempts to activate polyacrylic acid or its copolymers with *N*-vinylpyrrolidone by *N*-hydroxysuccinimide, 4-nitrophenol or pentafluorophenol in the presence of carbodiimides gave a low degree of activation (less than 10%). Therefore, the activation was performed at the monomer stage, e.g., to synthesize an activated ester of acrylic acid and to polymerize it for complete activation of an acrylic acid. Poly(4-nitrophenylacrylate) was obtained by radical polymerization of 4-nitrophenylacrylate in benzene in the presence of AIBN. The results of the polymerization were reproducible with amounts of monomer from 100 mg to 10 g, yields after reprecipitation being about 80%. Lower molecular weight compounds (oligomers) were obtained when the reaction was carried out in DMF. The polymers and oligomers are nonhygroscopic white powders, easily weighed and the polymer is very soluble in DMF, DMSO, nitrobenzene and nitromethane. The oligomer is also soluble in chloroform–methanol mixtures.

Condensation of polymers with amines

The pre-activation of all carboxylic groups in the initial polymers opens many possibilities for their further modification (reaction 2) with aminocompounds. Complete substitution of the $-COOC_6H_4NO_2$ groups by –CONHR, where R is a bulky oligosaccharide group, does not occur, but the reaction proceeds quantitatively up to around 30–35% modification. As a rule, we worked with polymers with 5–10% substitution: the large number of residual activated groups in the polymers allowed condensation with additional (nonsugar) amines, also in quantitative yield. The 15–20% substitution proceeds quantitatively at room temperature and without a catalyst. For higher degrees of substitution, heating to 40–60 °C and catalysis with triethylamine were used. The reaction proceeds faster in DMF than in DMSO. The attachment can be monitored easily by TLC and detecting nonreacted amine with ninhydrin. A quantitative attachment may be detected directly, with UV-absorbing amines, for example, with the

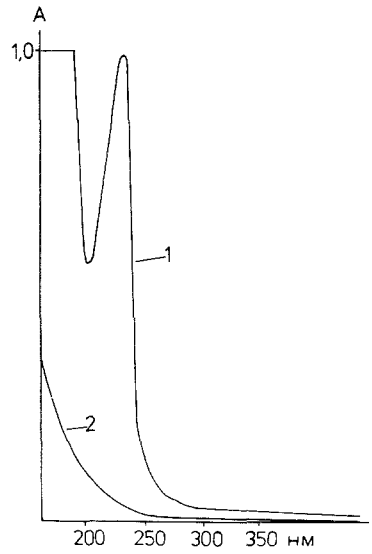


Figure 1. Absorbance of polymer-bound Neu5Acα2-$OCH_2C_6H_4NHCOCH_2NH$- (curve 1), and control polyacrylic acid free of ligand (curve 2). The concentration of ligand was 61 μmol, and the concentration of acrylic units was in both cases 610 μmol.

substituted benzyl glycoside of *N*-acetylneuraminic acid (**6**). A calibration curve was obtained using the corresponding *N*-acetyl derivative (**7**). Absorption spectra are in the range 200–400 nm, absorption maximum is 248 nm and the molar extinction coefficient coincides for *N*-acetyl (**7**) and Boc (**8**) derivatives. The control polymer without a ligand absorbs very little in this range (Fig. 1). The amounts of amine (**6**) corresponding to 0, 10, 20 and 30% of the number of nitrophenyl groups were added to poly(4-nitrophenylacrylate), the remaining nitrophenyl groups were removed by ethanolamine, conjugates were applied to the Sephadex LH-20 column to remove low molecular weight compounds, and their absorption at 248 nm was measured in aqueous acetonitrile (1:1 by vol). The results are given in Table 1.

Table 1. Concentration of amino ligand (**6**) used in the reaction and attached to poly(4-nitrophenylacrylate).

Concentration of Neu5Acα2-$OCH_2C_6H_4NHCOCH_2NH_2$ in reaction		*Concentration of attached ligand*
μM	*% molar quant. of active groups on polymer*	*μM*
0	0	0.0
1.75	10	1.8
3.5	20	3.3
5.25	30	5.0

After RNH_2 was attached to the polymer, the remaining activated groups were converted to amides by the action of 2-ethanolamine or NH_3 (in aqueous or DMF solution), these small amines taken in 10–100-fold excess, convert activated esters quantitatively to amides. The aqueous ammonium treatment (but not of 2-ethanolamine) results in partial hydrolysis to carboxylic groups (as shown on model compounds using elemental analysis) that gives a small negative charge to the conjugate, which does not affect subsequent use. Hydrolysis with 0.1 M NaOH leads to substituted polyacrylic acids [22].

The purification of the conjugate consists of its separation from nitrophenol and excess of aq. NH_3 (ethanolamine), and was carried out by filtration on TSK-gel or Sephadex LH-20 columns with a visual detection of nitrophenol. In some cases no purification is required. For example, when the conjugate is used for activation of plates or nitrocellulose membranes, the conjugate is diluted only with an appropriate buffer solution. The approach permits preparation of series of conjugates with different content of carbohydrate ligands.

Molecular weights of PAA conjugates

Apparent molecular weights (MW) were estimated for three water-soluble polymers: (i) nonsubstituted PAA obtained from poly(4-nitrophenylacrylate) by reaction with aq. NH_3, (ii) poly(*N*-2-hydroxyethylacrylamide) obtained by reaction with ethanolamine in DMSO, and (iii) PAA-conjugates of A-trisaccharide (**3**) (5–35 mole % trisaccharide). Two approaches were used: 1, size-exclusion chromatography (HPLC on column with working interval 80–250 kDa) with column calibration by poly(ethylene glycols) with MW 0.3–200 kDa; and 2, ultrafiltration on membranes with protein exclusion limits of 30, 100 and 300 kDa. The above approaches gave consistent results: polymers not substituted with carbohydrate ligands (i, ii) had MW within 30–100 kDa with a maximum MW distribution 36 kDa for the ethanolamine derivative, and 60 kDa for PAA-derivatives; in the latter case, higher apparent MW may be explained by expansion of the polymer due to a small negative charge (unlike case (ii), ammonolysis was carried out in aqueous solution).

Synthesis of neoglycolipids, probes, MDP derivatives and sorbents

This includes an additional stage, e.g., attachment of R^2NH_2 to an acrylic core, where R^2 is an additional (nonsugar) ligand. Neoglycolipids were synthesized by coupling phosphatidylethanolamine to the polymer next to the carbohydrate ligand. PE should be attached in the presence of triethylamine or diisopropylethylamine because of the presence of the phosphate group. Natural, rather than synthetic, PE is more convenient to use due to its greater solubility. To determine that PE attachment proceeded quantitatively, TLC (development with ninhydrin) and ^{14}C labelling were used. The molar ratio of initial reagents was aminoalkyl glycoside–PE–monomeric unit = 1:1:6; when it was necessary to increase the solubility in nonpolar organic solvents and to lower solubility in water, the ratio was adjusted to 1:2:10. In all these cases (more than 10), quantitative attachment of the ligand and PE was observed. Synthetic radioactively labelled derivatives were used for quantitative estimation of insertion of neoglycolipids into membranes. Neoglycolipids obtained from poly- and oligo(4-nitrophenylacrylate) differed considerably in their physicochemical properties: oligomeric derivatives had poor solubility in water and good solubility in organic solvents, incuding ethanol and $CHCl_3$/MeOH (like glycosphingolipids), whereas polymeric derivatives were more soluble in water, aqueous methanol, and in methanol, than in ethanol and $CHCl_3$/MeOH. This may be explained by the formation of hydrophobic regions in polymeric conjugates and by the absence of internal structuring in oligomers.

The neoglycolipids described here differ significantly from neoglycolipids obtained by reductive amination of free oligosaccharides with phosphatidylethanolamine [23]. The compounds described in [23] have a low molecular weight and only one oligosaccharide fragment, making them similar to natural glycosphingoolipids. The compounds described here have a high molecular weight and they have several oligosaccharide fragments, thus resembling segments ('splinters') of biological membranes. Neoglycolipids designed similarly and synthesized using oxidized dextran are described in [21].

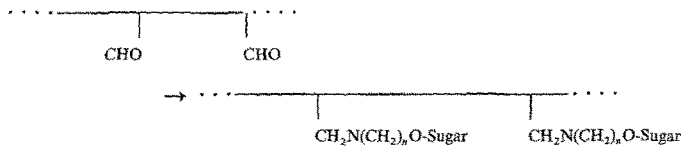
To obtain carbohydrate probes, i.e., reagents for carbohydrate-binding proteins (such as lectins, glycosyltransferases and perhaps glycosidases), a biotin label was also introduced in the polymeric molecule. An aminohexyl derivative of biotin, $BiotNH_2$ (**2**) was obtained by the reaction of *N*-hydroxysuccinimide-activated biotin with mono-Boc-hexamethylenediamine. The Sugar-$OCH_2CH_2CH_2NH_2$:$BiotNH_2$ ratio in the probe synthesis was 4:1. The biotin groups did not affect the solubility in water.

Carbohydrate sorbents were designed in a similar way to the above compounds, but aminopropylated (with aminogroup content about 100 μmol g^{-1}) macroporous glass with a pore diameter of 2000 Å functioned as R^2NH_2. The sorbents were obtained as follows: the aminopropylated matrix was treated with poly(4-nitrophenylacrylate) solution, resulting in an activated carrier with a $-COOC_6H_4$-*p*-NO_2 group capacity up to 150 μmol g^{-1}, to which the aminoalkyl glycoside solution (0.5–20 μmol g^{-1}) was added. The remaining active groups were then converted to amides by the action of ethanolamine. These sorbents are of interest because of their mechanical strength and hydrophilic surfaces.

Practically all the types of neoglycoconjugates discussed here can also be obtained from aminoalkylglycosides and polyaldehyde-dextran partially oxidized with periodate, by

reductive amination in the presence of sodium borohydride or cyanoborohydride (reaction 3, development of the approach described in [24]; in this approach, dextran was first acylated with myristinic acid, oxidized with periodate and a Schiff base with a peptide hapten was then obtained).

Reaction 3



We tested the approach by synthesizing polymeric forms of muramyldipeptide. It is known that 'macromolecular' muramyldipeptides appear to be strong immunomodulators [15]. Condensation of **4** with commercially available oxidized dextran (dexal) proceeded in two stages: the aminoligand was first mixed with polymer, then the Schiff base and nonreacted aldehyde groups were reduced with sodium borohydride. At CHO:RNH_2 ratio of 5:1, condensation proceeded quantitatively. This was confirmed by the absence of aminoligand in the reaction mixture (TLC, development with ninhydrin), and quantitative analysis of conjugate amino acid composition after hydrolysis.

This approach makes it possible to obtain series of conjugates with different molecular weights ranging from 70,000 to 1,000,000. We obtained conjugates containing up to 17 mol % sialic acid [as glycoside (**9**)] by periodate oxidation of dextrans followed by attachment of **6** to them in the presence of sodium cyanoborohydride.

Applications of polymeric neoglycoconjugates

The application of neoglycoconjugates based on polyacrylate, the synthesis of which was discussed above, is described briefly. Pseudopolysaccharides obtained by the copolymerization process were described earlier as reagents for coating polystyrene plates for ELISA [3]. We compared two conjugates with specificity to *Mycobacterium leprae* obtained in two different ways. The first way was copolymerization of 3-acrylamidopropyl 3,6-di-*O*-methyl-β-D-glycopyranoside with acrylamide (under conditions described in [8]), and the second way was attachment of the 3-aminopropylglycoside, DMG (**1**) to poly(4-nitrophenylacrylate). The two conjugates were identical from the point of view of revealing antibodies specific to the carbohydrate antigen. It should be mentioned that they proved to be more successful in many aspects than the BSA-conjugate of the corresponding specificity with the same content of hapten groups. 1, The quantity of PAA-antigen necessary is within the 0.01–0.1 μg ml^{-1} range, while the quantity of BSA-conjugate needs to be two orders higher (see Fig. 2). 2, When working with the former

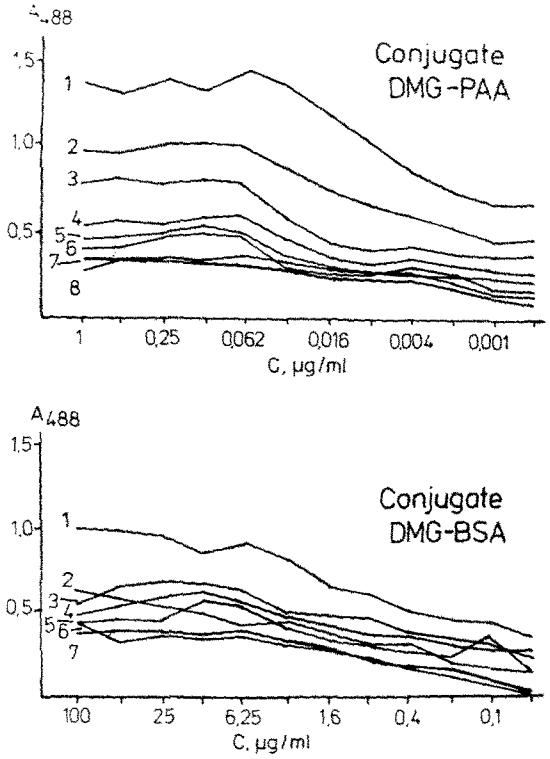


Figure 2. Binding of anti-DMG antibodies (IgM) from active leprosy patient (LL) serum with DMG-PAA and DMG-BSA as determined by ELISA. Doubling dilutions of serum begin at 1:800 (curve 1) to 1:102400 (curve 8). On the *x* axis is given the concentration of the coating antigen.

conjugate it is possible to dilute the serum more, the absolute optical density being higher. 3, The specificity of the reaction is much higher; sera from patients with leprosy (LL) or tuberculosis (T) and from healthy donors differ clearly from each other by intensity of the reaction with PAA-DMG, but not with BSA-DMG (Fig. 3). PAA-conjugate gives fewer false positive reactions.

Pseudopolysaccharides proved to be good targets for dot blot assays (see the 'Materials and methods' section) when studying epitope specificity of monoclonal antibodies. Binding of anti-A MA 44F9 with 10, 20, 30, and 35% PAA conjugates of A-trisaccharide is shown in Fig. 4. The results obtained demonstrate strict dependence of antibody 44F9 binding with epitope density of antigen (trisaccharide content). Thus, the 30% conjugate has greater affinity than other antigens tested, including the 35% conjugate and natural A substance.

A pseudopolysaccharide with blood group A specificity (5%) was used to neutralize anti-A antibodies in blood sera containing anti-A and anti-Rh. Sera were obtained free of anti-A antibodies. A-Trisaccharide immobilized via a polymer on macroporous glass (glycosorbents) was used for the same purpose (see the 'Materials and methods' section).

Neoglycolipids were synthesized as antigenic macro-

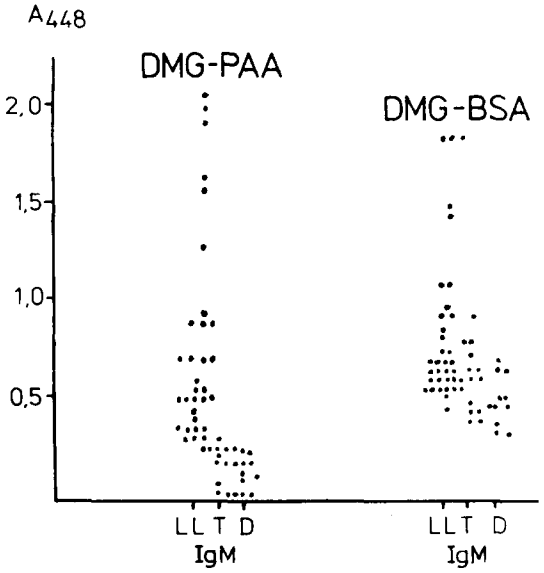


Figure 3. Histograms of IgM anti-DMG response of lepromatose leprosy (LL), tuberculosis (T), and healthy (D) individuals to DMG-PAA conjugate (0.01 μg ml^{-1}, 54 sera) and DMG-BSA conjugate (1 μg ml^{-1}, 59 sera) as determined by ELISA.

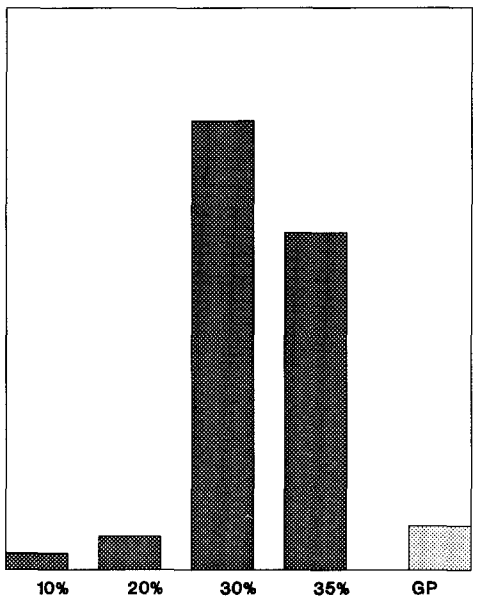


Figure 4. Binding of anti-A monoclonal antibody 44F9 with 10, 20, 30, and 35% PAA-conjugates of A-trisaccharide and natural A substance from human erythrocytes (GP).

molecules that insert easily into membranes. Neoglycolipid carrying the Le^x hapten, absorbed on *Salmonella minnesota* was used as an immunogen for obtaining highly specific monoclonal antibodies reacting only with Le^x but not with Le^a in ELISA.

Table 2. Binding inhibition of two lectins from *Butea frondosa* studied in a solid phase test system with (GalNAcα1-3Gal)$_{4n}$Biot$_{1n}$PAA as lectin binding reagent.[a]

Inhibitor	*Concentration required for 50% inhibition (mM)*	
	BFA I	BFA II
Fucα1-2Galβ-spacer	0.1	0.4
Neu5Acα2-6Lactose	0.4	0.8 (33%)
Fucα1-2Galβ1-3GlcNAcβ-spacer	0.7	ND
GalNAcα1-3Galβ-spacer	0.7	1.2
Galβ1-4Glc	0.74	ND
Galβ1-4GlcNAcβ-spacer	1.7	0.9 (38%)
Galβ1-3GlcNAcβ-spacer	2.0	0.08
GalNAc	2.3	0.2
Gal	8	25.0
GalNAcα1-3GalNAcβ1-spacer	3 (31%)	0.1
GalNAcα1-3 Galβ1-spacer Fucα1-2	NI	NI
Galα1-3 Galβ1-spacer Fucα1-2	NI	NI

[a] ND, not determined; NI, no inhibition.

The probe with biotin as a label and A-disaccharide GalNAcα1-3Galβ- as a specific epitope was used to study the specificity of two lectins from *B. frondosa*, BFA I and BFA II. The following variant of solid phase analysis was developed: wells of polystyrene plates were covered with lectin, interaction of immobilized lectin with the probe was inhibited by a series of mono- and oligosaccharides, and the degree of inhibition was evaluated by subsequent binding to avidin-peroxidase conjugate. It was shown that BFA I and BFA II have distinct oligosaccharide specificity (Table 2). It was found that BFA I prefers structures Fucα1-2Gal and Neu5Acα2-6Lac, while BFA II prefers Galβ1-3GlcNAc and GalNAcα1-3GalNAc.

Conclusion

The above approach for the synthesis of neoglycoconjugates using poly(4-nitrophenylacrylate), which provides pseudo-polysaccharides, neoglycolipids, probes and sorbents for biochemical, immunological and diagnostic purposes has the following advantages: attachment of ligand to polymeric matrix proceeds quantitatively; the ligand content in neoglycoconjugates is set by its initial ratio: it is methodologically convenient to synthesize suitable conjugates to study the influence of the density of the carbohydrate epitope in various biological systems; it is possible to change the physicochemical properties of conjugates by introducing additional, nonsugar ligands;

conjugate purification consists of routine gel chromatography; the condensation conditions (room temperature, absence of requirements of anhydrous conditions), along with a quantitative yield, makes it possible to synthesize conjugates in amounts much less than 1 mg.

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