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Synthesis of chitooligomer-based glycoconjugates and their binding to the rat natural killer cell activation receptor NKR-P1†

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NKR-P1 protein is an important activating receptor at the surface of the rat natural killer cells. GlcNAc and chitooligomers were identified as strong activation ligands *in vitro* and *in vivo*. Their clustering brings about increase of their affinity to the NKR-P1 by 3–6 orders. Here we describe novel methodology for preparation of neoglycoproteins based on BSA carying the chitooligomers (n = 2–5). Further on we developed novel methodology of the coupling of glycosylamines *via* aromatic-SCN activated linker both to protein or synthetic cores. Inhibition studies of chitooligomer glycoconjugates with the NKR-P1 receptor show that our neoglycoproteins are very strong ligands with high binding affinity ($-\log IC_{50}$ = 13–15). In analogy with our previous observations with GlcNAc clustered on protein or PAMAM backbones the synthetic chitooligomer clusters should provide considerably better ligands in the *in vivo* antitumor treatment.

Keywords: **chitooligomer, BSA, neoglycoprotein, natural killer cell, NKR-P1**

Abbreviations: **GlcNAc, 2-Acetamido-2-deoxy-D-glucopyranose; GalNAc, 2-Acetamido-2-deoxy-D-galactopyranose; ManNAc, 2-Acetamido-2-deoxy-D-mannopyranose; TalNAc, 2-Acetamido-2-deoxy-D-talopyranose; PAMAM, Polyamidoamine (Starburst®); BSA, Bovine serum albumin; NK cell, Natural killer cell; NKR-P1, Natural killer rat cell protein-1; *p*NP-, *p*-Nitrophenyl, MS, Mass spectrometry; MALDI, Matrix-assisted laser desorption/ionization; TOF, Time of flight; ESI, Electrospray ionization; NMR, Nuclear magnetic resonance; COSY, Correlated spectroscopy; HMQC, Heteronuclear multiple quantum correlation; HMBC, Heteronuclear multiple bond correlation; TLC, Thin layer chromatography; PBS, Phosphate buffered saline.**

Introduction

NKR-P1 protein is an important activating receptor at the surface of the rat natural killer cells [1,2]. This protein, which has been cloned [1] and characterized [3,4], belongs to a superfamily of animal C-lectins. We have recently determined structural requirements of the recombinant soluble dimeric form of NKR-P1 for its optimal carbohydrate ligands [5]. The best monosaccharidic ligand known for NKR-P1 is ManNAc; the affinity of other N-acetylhexosamines towards NKR-P1 decreases in the order GalNAc > GlcNAc ≫ TalNAc. Length of the carbohydrate chain is crucial for the binding as determined using a series of $(\beta(1\rightarrow6)\text{GalNAc})_n$ oligomers (n = 2–6) and a series of chitooligomers $(\beta(1\rightarrow4)\text{GlcNAc})_n$; n = 2–9) [5,6]. Optimum length of the oligosaccharidic chain is four carbohydrate units, β-O glycosidic linkage 1→4 being the best. Although the affinity of GlcNAc is 20 × lower than that of ManNAc, the affinity of chitotriose $(\text{GlcNAc})_3$ is comparable to that of ManNAc and chitotetraose $(\text{GlcNAc})_4$ even binds 3 × stronger than $(\text{GlcNAc})_3$ to the NKR-P1 receptor [5]. We have found that the affinity of respective β-C- and β-N-glycosides is considerably inferior to the β-O-glycosides; α-glycosidic linkages inhibit the binding [5].

Chitooligomers were chosen from practical point of view (price, availability from chitin) as a lead structure for the development of glycomimetics usable as a stimulant against NK cell-resistant malignant tumors.

† Dedicated to Prof. Joachim Thiem on the occasion of his 60th birthday.

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Multivalency was found to be crucial in the binding of carbohydrate structures to NKR-P1 receptor *in vitro* [7]. This was also proved in the *ex vivo* experiments with isolated NK cells against NK-resistant tumor cell lines [2], and in the *in vivo* experiments with colon cancer treatment with activated NK cells [8]. Here, trivalent, tetravalent, hexavalent and octavalent glycodendrimer based on the PAMAM (Starburst®) cores with thiourea-bridged β-GlcNAc units were used [7]. Affinity of the glycoclusters for NKR-P1 receptor, namely those carrying eight β-GlcNAc units, is more than 10^4 times better than those substituted with the bare monosaccharide. The activity of the glycoclusters was high despite the use of rather suboptimal ligand for the clustering, e.g. 2-acetamido-2-deoxy-β-D-glucopyranosyl unit connected with β-*N*-glycosidic linkage. We assume that much better results should be achieved using clustered chitooligomers. We have already tested binding affinity of multivalent synthetic neoglycoprotein $GlcNAc_{17}BSA$ for NKR-P1 receptor resulting in 10^7 times stronger binding compared to the affinity of GlcNAc itself and 10^5 times stronger binding when compared to chitotetraose, which is the best ligand from linear chitooligomers. $GlcNAc_{17}BSA$ has been commercially available neoglycoprotein from Sigma, where the GlcNAc residues (as *p*NP-glycosides) were linked to BSA aminogroups through thioureic linkers. This compound is, unfortunately, no more available from Sigma and, therefore, we also had to found other multivalent ligand suitable for the inhibition studies of NKP-P1 protein.

The main target of this work was to prepare neoglycoconjugates based on the chitooligomers ($n = 2$–5), which would be far better ligands for the binding. BSA was used as a model core. Besides that we aimed at the development of (novel) suitable methodology of preparation of respective synthons—activated chitooligomer-based carbohydrates that would be useful for clustering to the synthetic cores in the preparation of antitumor glycomimetics.

Materials and methods

Materials

Chitin, GlcNAc, *p*NP-β-GlcNAc and $GlcNAc_{17}BSA$ were purchased from Sigma. *p*-Nitrophenyl isothiocyanate was purchased from Merck. BSA (97%, fraction V) was purchased from Fluka. *p*-Nitrophenyl β-chitobioside was prepared enzymatically according to the published procedure [9]. NKR-P1 protein was prepared according to a published procedure [5]. It was radiolabeled with $Na^{125}I$ using Iodogen (Pierce, Rockville, IL, USA).

General procedures

NMR spectrometry

NMR spectra were recorded on a Varian ^{UNITY}*Inova*-400 MHz spectrometer (399.90 MHz for ^1H, 100.55 MHz for ^{13}C) in D_2O (99.95% D, Chemtrade) at 30°C. The assignment was based on COSY, HMQC, HMBC and differential NOE experiments performed using the manufacturer's software. Acetone (δ_H 2.030 ppm, δ_C 30.5 ppm) was used as internal standards. Chemical shift of carbon at 127.2 ppm in compound **1d** was established from HMBC spectra.

MALDI-TOF mass spectrometry

A saturated solution of sinapic acid (Sigma) in aqueous 30% acetonitrile/0.1% TFA was used as a MALDI matrix. Sample (2 μl) and the matrix solution (2 μl) were premixed in an Eppendorf tube, 0.5 μl of the mixture was placed on the sample target and allowed to dry at the ambient temperature. Positive ion MALDI-TOF mass spectra were recorded on a Bruker BIFLEX time-of-flight mass spectrometer (Bruker-Franzen, Bremen, D) equipped with a multiprobe sample inlet, a gridless delayed extraction ion source and a nitrogen laser (337 nm) Laser Science (Cambridge, MA, USA). The instrument was operated in the linear mode and the ion acceleration voltage was set to 19 kV. Spectra were calibrated externally using $[M + H]^+$ and $[M + 2H]^{2+}$ ions of protein standards (cytochrome c and BSA, Sigma).

ESI mass spectrometry

Positive-ion mass spectra of synthesized derivatives of oligosaccharides were recorded on an LCQ DECA ion trap mass spectrometer (Finnigan, San Jose, CA, USA) equipped with ESI ion source. Spray voltage was set at 5.5 kV, tube lens voltage was 30 V. The flow of the sheath gas (nitrogen 99.999%) was set at 55 arb units and the heated capillary was kept at 320°C, with a potential of 30 V. Samples dissolved in 20% aqueous acetonitrile were continuously infused into the ion source *via* linear syringe pump at a flow rate of 3 μl/min. Full scan spectra were acquired over the m/z range 100–1500 Da. Ultramark 1621 (PCR, Inc., Gainesville, FL, USA) (0.1% solution in MeCN) was used to calibrate the m/z scale of the instrument.

Chromatography

Flash column chromatography was performed on Silica Gel 60 (40–63 μm, 230–400 mesh, Merck). TLC was run on a precoated Silica Gel 60 F_{254} aluminium sheets; detection by UV light (254 nm) and charring with 5% H_2SO_4 in EtOH. For all mobile phases 25% ammonium hydroxide solution (Fluka) was used. Gel chromatography was performed on a Bio-Gel P-2 (Fine, 45–90 μm, BioRad, USA) and Bio-Gel P-4 (Fine, 45–90 μm, BioRad) columns (100 × 2.5 cm and 100 × 5 cm, respectively) eluted with water.

SDS PAGE

The molecular size of the neoglycoproteins was estimated by SDS-PAGE electrophoresis on 10% polyacrylamide minigels (70 × 50 × 0.75 mm) using the discontinuous buffer system of

Table 1. Separation of chitooligomers on charcoal column

EtOH in eluent (v/v)	*Composition of fraction*	*Volume (ml)*
N/A (water)	Salts (>95%), GlcNAc (<5%)	500
4%	**GlcNAc** (>95%), chitobiose (<5%)	1000
8%	**chitobiose** (>85%), chitotriose (<15%)	1000
10%	**chitotriose** (>85%), chitotetraose (<15%)	1000
13%	**chitotetraose** (>85%), chitopentaose (<15%)	1000
30%	**chitopentaose** (70%), $(GlcNAc)_n$, $a = 6$–9	1500

Laemmli [10]. Gels were fixed in 35% ethanol and 10% acetic acid, and stained by Coomassie Brilliant Blue R-250.

Overlays of neoglycoproteins with NKR-P1A receptor on nitrocellulose sheets

Binding of the rat NKR-P1A receptor (protein construct used was denoted NKR-358, [5]) was performed according to the published procedure [2] with minor modifications. Neoglycoproteins (10 μg/lane) were separated on 10% polyacrylamide gels as above, and electrotransferred onto nitocellulose membranes (0.45 μm pores, BioTrace^{TM} NT, Pall Corporation, Ann Arbor, MI, USA). Duplicate nitrocellulose sheets were either stained with amidoblack 10B in 3% acetic acid, or blocked in 2% BSA in TBS buffer containing 10 mM $CaCl_2$ and overlayed with ^{125}I-NKR-358 (5×10^6 cpm/ml, specific radioactivity 10^7 cpm/μg protein) in BSA/TBS buffer. Sheets were incubated overnight at 4°C, washed 3 × 30 sec with TBS buffer, dried, and exposed onto Kodak XAR-5 film with intensifying screens at room temperature.

Inhibition assays

Binding and inhibition assays were performed as described previously [2], with minor modifications. Briefly, 96 well polyvinylchloride microplates (Titertek Immuno Assay-Plate, ICN Flow, Irvine, Scotland) were coated overnight at 4°C with 50 μl of $GlcNAc_{17}$BSA (Sigma) in PBS buffer (with 1 mM NaN_3). Plates were blocked with 2% BSA in PBS for 2 h at 4°C, incubated with the concentration of the radiolabeled protein corresponding to the half of the saturating amount (0,3 μg/ml) and various dilutions (10^{-14}–10^{-1} mM) of the inhibitors (total reaction volume 100 μl), washed three times with PBS, and dried. Scintillation solution (100 μl) was added, and the radioactivity in the individual wells was counted by β-counting (Microbeta, Wallac, Turku, SF). All experiments were performed three times in duplicates and the inhibition degree was calculated relative to the wells containing no inhibitor. Because of rather high reproducibility of these assays Figure 3 shows no standard deviation error bars which were within 2%.

*Hydrolysis of chitin and separation of chitoligomers (**1–5**)*

With regard to rather high price of chitooligomers used for our research we decided to develop a simple technique for their preparation in a large scale. Hydrolysis of chitin was performed according to a published procedure [11]. Crude mixture of chitooligomers was desalted by electrodialysis (4–6 h, 20 V, 30°C) using ion-selective membrane. Separation was finished after specific conductance decreased to 0.5 mS/cm. For the separation of chitooligomers ($(\beta(1\rightarrow4)GlcNAc)_n$, $n = 2$–5) a glass column (300 × 40 mm) packed with 1 : 1 mixture of charcoal (Norrit, Sigma) and Cellite 545 (Lachema, Brno, Czech Republic) slurred in 30% aqueous ethanol was used. Column was then washed with 1500 ml of water using peristaltic pump P1 (Pharmacia, Sweden) with a steady flow of 60 ml/h. Up to 7 g of the desalted chitooligomer mixture dissolved in 50 ml of water was loaded on the column. Individual chitooligomers were washed out by ethanol gradient (4, 8, 10, 13, 16, 30%). Composition of respective fractions (1000 ml) was checked by TLC (2-propanol/H_2O/NH_3 *aq.*, 7 : 1 : 2). Desorption was quite specific yielding fractions containing each chitooligomer ($(\beta(1\rightarrow4)GlcNAc)_n$, $n = 2$–5) with minor amounts of other impurities (Table 1).

Fractions were concentrated at 50°C under vacuum and final separation was performed on Bio-Gel P-4 Fine (BioRad) column (100 × 5 cm) eluted with water. Separation of 7 g of crude chitooligomer mixture gave 850 mg of chitobiose, 1050 mg of chitotriose, 930 mg of chitotetraose and 690 mg chitopentaose and minor amounts of higher chitooligomers (up to 200 mg of $(\beta(1\rightarrow4)GlcNAc)_n$, $n = 6$–7).

*Preparation of glycosylamines (**1a–5a**)*

Glycosylamines of GlcNAc and chitooligomers ($(\beta(1\rightarrow4)GlcNAc)_n$, $n = 1$–5) were synthesized according to previously published procedure [12] using saturated solution of ammonium hydrogencarbonate (30°C, 7 d). After removal of the bulk of ammonium hydrogencarbonate with multiple lyophilization, sugar amines were purified using ion exchange resin Dowex 50 W 1X2 H^+(Fluka). The resin was added to the solution of the sugar amine until pH of the mixture dropped to 4. Then the resin with absorbed glycosylamines was filtered off and washed with ice-cold water. Pure product was eluted with 2M ammonia solution in methanol. Quality of glycosylamines was checked by TLC (2-propanol/NH_3 *aq.*, 7 : 3). Negligible amounts of impurities (>5%) were detected. Glycosylamines **1a–5a** were obtained in the yields 72%, 62%, 78%, 81% and 89%, respectively.

Dry products protected from air humidity could be stored at −60°C for ca 50 days.

*Preparation of glycosylisothiocyanates (**1b–5b**)*

Glycosylamine (**1a–5a**, 1 mmol) prepared according to the previous procedure was dissolved in 2 ml of water and within 3 minutes added dropwise to a mixture of $CSCl_2$ (400 μl, 5 mmol) and $NaHCO_3$ (200 mg, 2.4 mmol) in acetone (2 ml). Reaction was carried out at ambient temperature in a sealed flask. The reaction was monitored by TLC (2-propanol/H_2O/NH_3 *aq.*, 7 : 1 : 2). After 45 minutes 2 ml of water were added and acetone was removed by prompt evaporation under vacuum at 30°C. Unreacted $CSCl_2$ was extracted with chloroform. Resulting aqueous solution of glycosylisothiocyanates (**1b–5b**) was then either directly used for the conjugation reaction with BSA, or stored frozen at −60°C.

Preparation of glycoconjugates of BSA with glycosylisothiocyanates

Aqueous solution of glycosylisothiocyanates (**1b–5b**, 1 mmol) prepared according to the previous procedure was lyophilized, dissolved in 10 ml of water (NANOpure) and filtered. pH of the solution was adjusted to 7–7.5 by addition of 0.1 M $NaHCO_3$. BSA (15 mg) in 1 ml of water was added dropwise under stirring. Solution was then kept at room temperature for 5 h and pH was then adjusted to 8–8.5. Clear yellow solution was left overnight at room temperature and then 72 h dialyzed in dialysis tubings (Sigma, cut-off 15 000) against 10% acetonitrile and 1% acetic acid solution in water (NANOpure) at 4°C. Lyophilized preparations **1A–5A** were stored at −60°C.

*Preparation of N-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-N′-(p-nitrophenyl) thiourea (**1c**)*

2-Acetamido-2-deoxy-β-D-glucopyranosylamine (**1a**) was synthesized according to the published procedure [12]. Compound **1a** (500 mg, 2.3 mmol) was dissolved in 10 ml of water and added dropwise to a solution of *p*-nitrophenyl isothiocyanate (500 mg, 2.8 mmol) in dioxane (20 ml) under stirring. After 1 h the reaction mixture was analyzed by TLC (CH_2Cl_2/MeOH, 3 : 1) and another portion of *p*-nitrophenyl isothiocyanate (200 mg in 5 ml of dioxane) was added. After another 3 h the reaction mixture was mixed with equal amount of water (unreacted *p*-nitrophenyl isothiocyanate, which precipitated as a yellow solid was filtered off) and dioxane was evaporated under vacuum at 35–40°C. Product **1c** was obtained in 92% yield, no impurities were detected. Lyophilized mixture was purified on silica gel using CH_2Cl_2/MeOH (3 : 1) as eluent. Yellow solid was stored at −12°C.

^{1}H NMR: δ 1.838 (s, 3H, C**H**$_3$CONH), 3.293 (dd, 1H, J = 8.8, 9.8 Hz, H-4), 3.370 (ddd, 1H, J = 2.2, 5.0, 9.8 Hz, H-5), 3.462 (dd, 1H, J = 8.8, 10.2 Hz, H-3), 3.572 (dd, 1H, J = 5.0, 12.4 Hz, H-6a), 3.706 (dd, 1H, J = 2.2, 12.4 Hz, H-6b), 3.710 (dd, 1H, J = 9.7, 10.2 Hz, H-2), 5.458 (br s, 1H, H-1), 7.367 (2H, m, H-*ortho*), 8.065 (2H, m, H-*meta*).

^{13}C NMR: δ 22.33 (**C**H$_3$CONH), 54.72 (C-2), 60.91 (C-6), 69.93 (C-4), 74.41 (C-3), 77.84 (C-5), 83.51 (C-1), 125.26 (2 × C-*ortho*, 2 × C-*meta*), 143.90, 145.33 (C-*ipso*, C-*para*), 175.24 (CH$_3$**C**ONH), 182.55 (C=S).

*Preparation of N-chitobiosyl-N′-(p-nitrophenyl) thiourea (**2c**) and of N-chitotriosyl-N′-(p-nitrophenyl) thiourea (**3c**)*

These compounds were synthesized according to the previous procedure using chitobiosylamine (**2a**) and chitotriosylamine (**3a**), respectively. Products **2c** and **3c** were obtained in the yields 94% and 97%, no impurities were detected.

*Preparation of N-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-N′-(p-aminophenyl) thiourea (**1d**)*

Reduction of **1c** was performed according to the published procedure with some modification [13]. Compound **1c** (400 mg, 1 mmol) was dissolved in water (10 ml) and mixed with 50 ml aqueous solution of $FeSO_4 \cdot 7\ H_2O$ (2.8 g, 10 mmol). Reaction mixture was then precipitated under stirring by dropwise addition of slightly excessive amount of ammonia (3 ml), heated to 50°C on a water bath and then cooled to room temperature within 1 h. Black precipitate was filtered off and washed on the paper filter with 50 ml of water a 30 ml of methanol. Methanol was removed at 40°C under vacuum. Crude mixture was analyzed by TLC (CH_2Cl_2/ MeOH/EtOH/H_2O, 8 : 4 : 1 : 0.8) and purified using anion exchange resin Dowex 1X2 – 400 OH^- (Aldrich) to remove sulfate ions, followed by solid-phase extraction on Amberlite XAD-4 resin (BDH Chem. Ltd., UK), eluted with methanol and dried. The white solid was stored at −12°C.

^{1}H NMR: δ 1.811 (s, 3H, C**H**$_3$CONH), 3.226 (dd, 1H, J = 8.9, 9.8 Hz, H-4), 3.325 (ddd, 1H, J = 2.2, 5.1, 9.8 Hz, H-5), 3.408 (dd, 1H, J = 8.9, 10.2 Hz, H-3), 3.530 (dd, 1H, J = 5.1, 12.4 Hz, H-6a), 3.567 (dd, 1H, J = 8.2, 10.2 Hz, H-2), 3.675 (dd, 1H, J = 2.2, 12.4 Hz, H-6b), 5.346 (br d, 1H, J = 8.2 Hz, H-1), 6.656, 6.810 (2× 2H, m, H-*ortho*, H-*meta*).

^{13}C NMR: δ 22.23 (**C**H$_3$CONH), 54.54 (C-2), 60.96 (C-6), 69.98 (C-4), 74.34 (C-3), 77.77 (C-5), 83.80 (C-1), 117.27 (2×C-*ortho* or 2× C-*meta*), 127.2 (C-*ipso* or C-*para*), 128.13 (2× C-*ortho* or 2× C-*meta*), 146.70 (C-*ipso* or C-*para*), 174.95 (CH$_3$**C**ONH), 182.02 (C=S).

*Preparation of N-chitobiosyl-N′-(p-aminophenyl) thiourea (**2d**) and N-chitotriosyl-N′-(p-aminophenyl) thiourea (**3d**)*

These compounds were synthesized according to the previous procedure using **2c** and **3c** respectively.

*Preparation of N-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-N′-(p-isothiocyanato-phenyl) thiourea (**1e**) and its chitobiosyl (**2e**), or chitotriosyl (**3e**) analogues*

Reaction was performed under the same conditions as in the preparation of glycosylisothiocyanates (**1b–5b**) described heretofore with minor modifications. Reaction was carried out in the mixture of water/acetone with molar ratio of these solvents 1 : 2 and $NaHCO_3$ was not added. During evaporation of acetone under vacuum the product precipitated as an off-white solid.

*Preparation of neoglycoconjugates of BSA with glycosylisothiocyanates comprising thioureic linker (**1B–3B**)*

Lyophilized preparations (100 mg) of glycosylisothiocyanates comprising thioureic linker (**1e–3e**) prepared as described in the previous procedure were dissolved in 4 ml of water (NANOpure) and 2.5 ml of acetone and filtered over a cotton. pH of the solution was adjusted to 7–8 by the addition of 0.1 M $NaHCO_3$. BSA (15 mg) in 1 ml of water was added dropwise under stirring. Solution was then kept at room temperature overnight and then 72 h dialyzed in dialysis tubings (cut-off 15 000) against mixture of acetone/water 1 : 2 (v/v). Lyophilized preparations (**1B–3B**) were stored at −60°C.

*Preparation of p-isothiocyanatophenyl-β-GlcNAc (**1h**) and p-isothiocyanatophenyl-β-chitobioside (**2h**)*

*p*NP Derivatives of GlcNAc and chitobiose (**1f, 2f**) were reduced with H_2 to respective amines at ambient temperature for 4 h in methanol using 15–20% Pd/C as a catalyst. Reaction was monitored using TLC (CH_2Cl_2/MeOH/EtOH/H_2O, 7 : 4 : 1 : 0.8). After methanol evaporation and lyophilization amines (**1g, 2g**, 0.32 mmol) obtained were dissolved in 2 ml of water and added dropwise to stirred solution of $CSCl_2$ (200 μl, 2.6 mmol) in 4 ml of acetone. Reaction was finished after 30 min and to the colourless solution water (2 ml) was added (precipitation may occur). Acetone was evaporated under vacuum at 30°C. Lyophilized preparations (**1h, 2h**) were stored at −60°C.

*Preparation of neoglycoconjugates of BSA with p-isothiocyanatophenylβ-GlcNAc and p-isothiocyanatophenyl-β-chitobioside (**1C–2C**)*

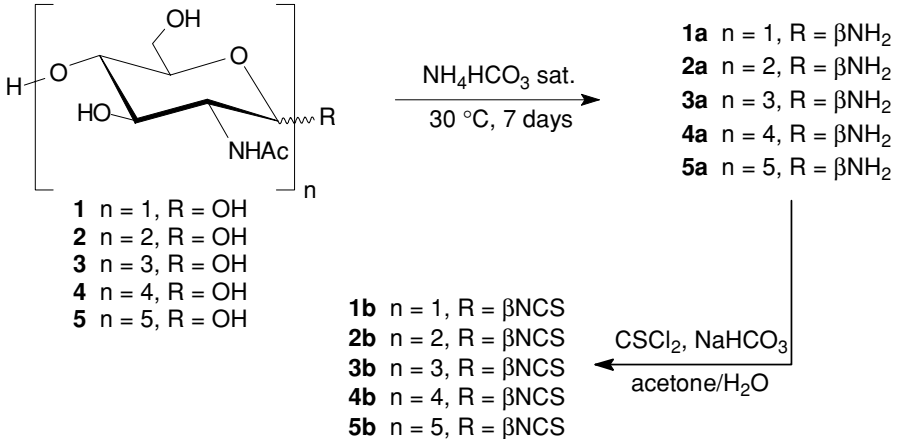
p-Isothiocyanatophenyl glycoside (**1h, 2h**) prepared according to the previous procedure was dissolved in mixture of acetone (2.5 ml) and water (4 ml, NANOpure) and filtered over cotton. pH of the solution was adjusted to 7–8 by addition of 0.1 M $NaHCO_3$. BSA (15 mg) in 1 ml of water was added dropwise under stirring. Solution was then kept at room temperature overnight and then 72 h dialyzed in dialysis tubings (cut-off 15 000) against mixture of acetone/water 1 : 2 (v/v). Lyophilized preparations (**1C, 2C**) were stored at −60°C.

Results and discussion

Preparation of neoglycoconjugates from linear chitooligomers (*1A–5A*)

For the synthesis of the target neoglycoconjugates GlcNAc (**1**) and chitooligomers composed of 2–5 units (**2–5**) were converted to their respective glycosylamines (**1a–5a**) in 62–89% yield (higher conversion for compound **4** and **5**) followed by the reaction with $CSCl_2$ furnishing isothiocyanates **1b–5b** in 70–90% yield (Scheme 1).

Glycosylisothiocyanates were easily detectable on TLC plates by UV light (254 nm). Acetone was found to be very good solvent both for glycosylamines and glycosylisothiocyanates unlike methanol or ethanol that underwent side addition reactions with isothiocyanate group. Very good yields were achieved by the addition of $NaHCO_3$ to the reaction mixture. Finely powdered $CaCO_3$ that is commonly used for this reaction has lower buffering capacity and moreover contaminates the products with the Ca^{2+} ions that are often unacceptable for consequent conjugation with proteins. Because of its better buffering capacity $NaHCO_3$ suppress quick decrease of pH at the beginning of the reaction and promotes thus amino group conversion to the isothiocyanate. The amount of $NaHCO_3$ added must be kept low because increase of pH to values of 8–9 would promote undesirable side reaction of respective



Scheme 1. Synthesis of glycosylamines from chitooligomers and conversion of glycosylamines to glycosylisothiocyanates.

Table 2. Spectral characterization of prepared neoglycoconjugates by MALDI-TOF

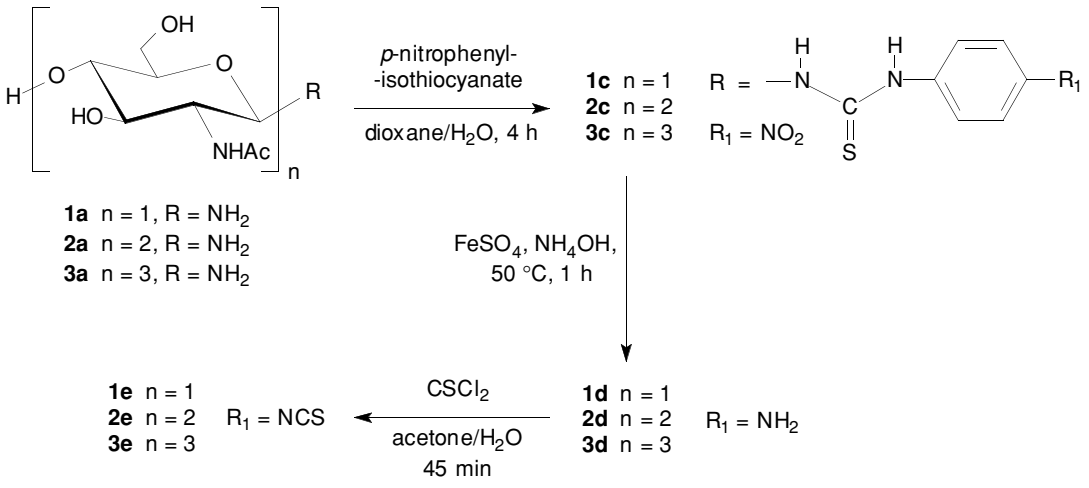
Neoglycoprotein	*Substituent (glycosylisothiocyanate)*		*Average mass (MALDI-TOF)*	*Peak width at half height*	*Average substitution (No. of saccharidic residues)*
BSA std.	–	–	66 400	1 000	–
1A	GlcNAc	(**1b**)	70 900	2 700	20
2A	Chitobiose	(**2b**)	76 600	8 900	22
3A	Chitotriose	(**3b**)	73 200	11 400	10
4A	Chitotetraose	(**4b**)	74 600	9 500	9
5A	Chitopentaose	(**5b**)	75 400	9 500	8
1B	*N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-*N′*-(*p*-nitrophenyl) thiourea	(**1e**)	72 100	11 400	14
2B	*N*-chitobiosyl-*N′*-(*p*-nitrophenyl) thiourea	(**2e**)	73 700	18 300	13
3B	*N*-chitotriosyl-*N′*-(*p*-nitrophenyl) thiourea	(**3e**)	82 200	18 200	19
1C	*p*NP-GlcNAc	(**1h**)	75 700	10 600	26
2C	*p*NP-chitobioside	(**2h**)	84 000	21 700	32

glycosylisothiocyanate with the glycosylamine. Lyophilized compounds **1b–5b** were then coupled with BSA in slightly alkaline (pH 7.5–8) aqueous solution. Higher values of pH led to conversion of glycosylisothiocyanate to unidentified adduct probably with water or other solvent that did not further react with the protein. To achieve high degree of substitution 50 fold excess of glycosylisothiocyanates (**1b–5b**) was used for the coupling. New neoglycoconjugates (**1A–5A**) were characterized by MALDI-TOF mass spectrometry (Table 2).

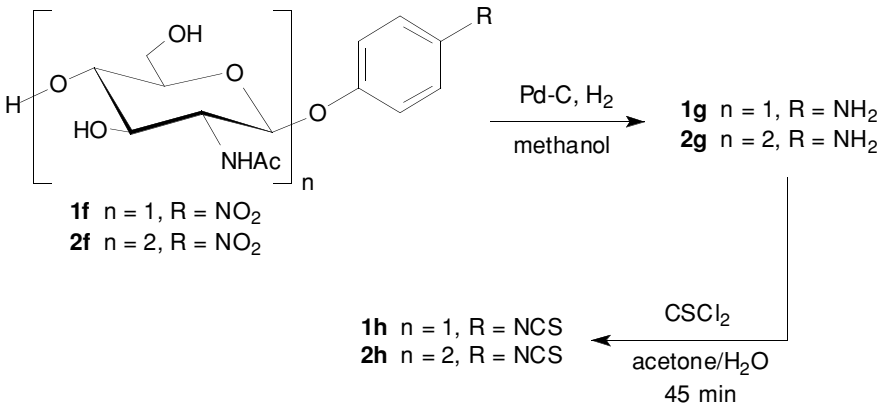
Preparation of novel chitooligomer-based activated ligands and their coupling with BSA

Our studies of binding preferences of NKR-P1 protein demonstrated that a presence of the aromatic core in the ligand molecule considerably increases its affinity towards receptor [5]. Therefore, we focused our attention on the synthesis of neoglycoconjugates from *p*NP-glycosides of chitooligomers (*p*NP-β-GlcNAc (**1f**) and *p*NP-β-chitobioside (**2f**)) and also on the synthesis of novel chitooligomer-based ligands comprising aromatic linker. In order to prepare such derivatives, the reaction of glycosylamines (**1a–3a**) with *p*-nitrophenyl isothiocyanate was designed (Scheme 2).

Conjugation of **1a–3a** with *p*-nitrophenyl isothiocyanate was performed in a dioxane/water (2 : 1) mixture at neutral pH and yielded **1c–3c** (over 90% yield). Reduction of nitro-group in the compounds **1c–3c** cannot be achieved using standard Pd/C hydrogenation techniques (presence of sulphur in the molecule inhibits the catalyst). Therefore, we used reduction of **1c–3c** with Fe^{2+} that afforded compounds **1d–3d** quantitatively. Compound **1c**: ESI-MS: $[M + H]^+ = 401.1$, compound **1d** (MALDI-TOF): $[M + H]^+ = 371.2$, $[M + Na]^+ = 393.2$. The structure of **1c** and **1d** was established by NMR. Resulting aromatic amines (**1d–3d**) were soluble in water and were more stable than glycosylamines even at elevated temperatures. Consecutive synthesis of respective isothiocyanates **1e–3e** (Scheme 2) furnished these compounds in nearly quantitative yield (<99%). Coupling of **1e–3e** with BSA gave



Scheme 2. Synthesis of thiourea-bridged glycosides.



Scheme 3. Synthesis of *O*-glycosides.

neoglycoconjugates **1B–3B** that were characterized by MS MALDI-TOF (Table 2).

Preparation of neoglycoconjugates from pNP-β-GlcNAc and pNP-β-chitobioside (***1C–2C***)

When considering that saccharidic derivatives comprising β-*O* glycosidic linkage 1→4 and aromatic core in its molecule are superior for the binding to NKR-P1 receptor [5], we aimed our attention to respective *p*NP-glycosides, e.g., *p*NP-β-GlcNAc (1f) and *p*NP-β-chitobioside (**2f**). Both compounds were quantitatively reduced with H_2 on Pd/C in MeOH to give respective amines **1g** and **2g**, which were treated with $CSCl_2$ in aqueous 65% acetone to furnish isothiocyanates **1h** and **2h**, quantitatively (Scheme 3).

Despite expected high reactivity of **1h** and **2h** the formation of side-products was not observed, these compounds were quite stable and could be characterized by ESI MS: **1h** $[M + Na]^+ = 377.1$; **2h** $[M + Na]^+ = 580.2$. Neoglycoconjugates prepared by coupling of **1h** and **2h** with BSA (**1C, 2C**) were characterized by MALDI-TOF mass spectrometry (Table 2).

Figure 1 shows MALDI-TOF mass spectra of selected neoglycoconjugates. When compared to molecular weight of parent BSA (approx. 66 400) the spectra demonstrate that prepared neoglycoproteins are highly glycosylated forming a population of relatively uniform degree of substitution. Although a high excess (more than 1 000%) of appropriate isothiocyanate was used for coupling reaction with BSA, the degree of substitution cannot be increased in some cases (**4A, 5A**). This may be due to a lower reactivity of respective glycosylisothiocyanates (**4b, 5b**) or sterical problems. High substitution of **1C** and **2C** demonstrates the significance of *O*-glycosides for preparation of well defined glycoclusters and glycodendrimers.

Binding studies of prepared neoglycoproteins (immunoblotting and plate analyzes)

Final neoglycoprotein preparations were analyzed by SDS polyacrylamide gel electrophoresis, electrotransferred onto nitrocellulose membranes and incubated with ^{125}I-NKR-358 (soluble recombinant NKR-P1). Figure 2 shows exposed sheet after 6 hours of exposition and duplicate sheet stained with amidoblack 10B as a control. The results summarised in Figure 2 show strong binding of all neoglycoprotein preparations to NKR-P1, including commercial $GlcNAc_{17}BSA$, except neoglycoprotein **1A**. We assume that *N*-glycosidic linkage restricts binding of monosaccharidic residue of the bare GlcNAc to NKR-P1.

As it is also evident from the Figure 2, preparations of varying heterogenity have been obtained. Notably, neoglycoproteins **1B** and **2B** were both homogenous (Figure 2ii), and displayed high affinity for NKR-P1 receptor (Figure 2i). On the other hand, dimeric forms of neoglycoprotein are evident in some preparations (**4A, 5A**). The dimeric forms often displays higher affinity for NKR-P1 receptor (**5A**).

Inhibition studies (Figure 3) demonstrated high binding affinities of synthetic neoglycoconjugates **1B–3B** and **1C–2C** derived from the novel chitooligomer-based carbohydrates comprising thioureic linker (**1c–3c**) and from *p*NP derivatives of chitooligomers, even when compared to commercial preparative $GlcNAc_{17}BSA$. Highly glycosylated neoglycoproteins **3B** ($-\log IC_{50} = 15$) and **2C** ($-\log IC_{50} = 14.9$) bind more than 10 times stronger to NKR-P1 than does $GlcNAc_{17}BSA$ ($-\log IC_{50} = 13.7$). Our synthetic analogue of $GlcNAc_{17}BSA$, neoglycoprotein **1C**, binds approx. 3 times better to NKR-P1, mainly due to its higher degree of substitution. This observation is consistent with previous findings that the binding affinities of neoglycoconjugates for NKR-P1 receptor depend not only on degree of substitution (multivalency) but also on type of sugar linkage (β-*O*-(1→4) being the best), on the length of oligosaccharidic chain and on the presence of aromatic core in the substituents (lower affinities of **1A–5A**) [5,6].

Conclusions

This paper describes novel methodology for convenient preparation of neoglycoproteins based on the chitooligomers ($n = 2$–5) and chitooligomer-based activated ligands using cheap and fast techniques with a good yield. Our compounds comprising

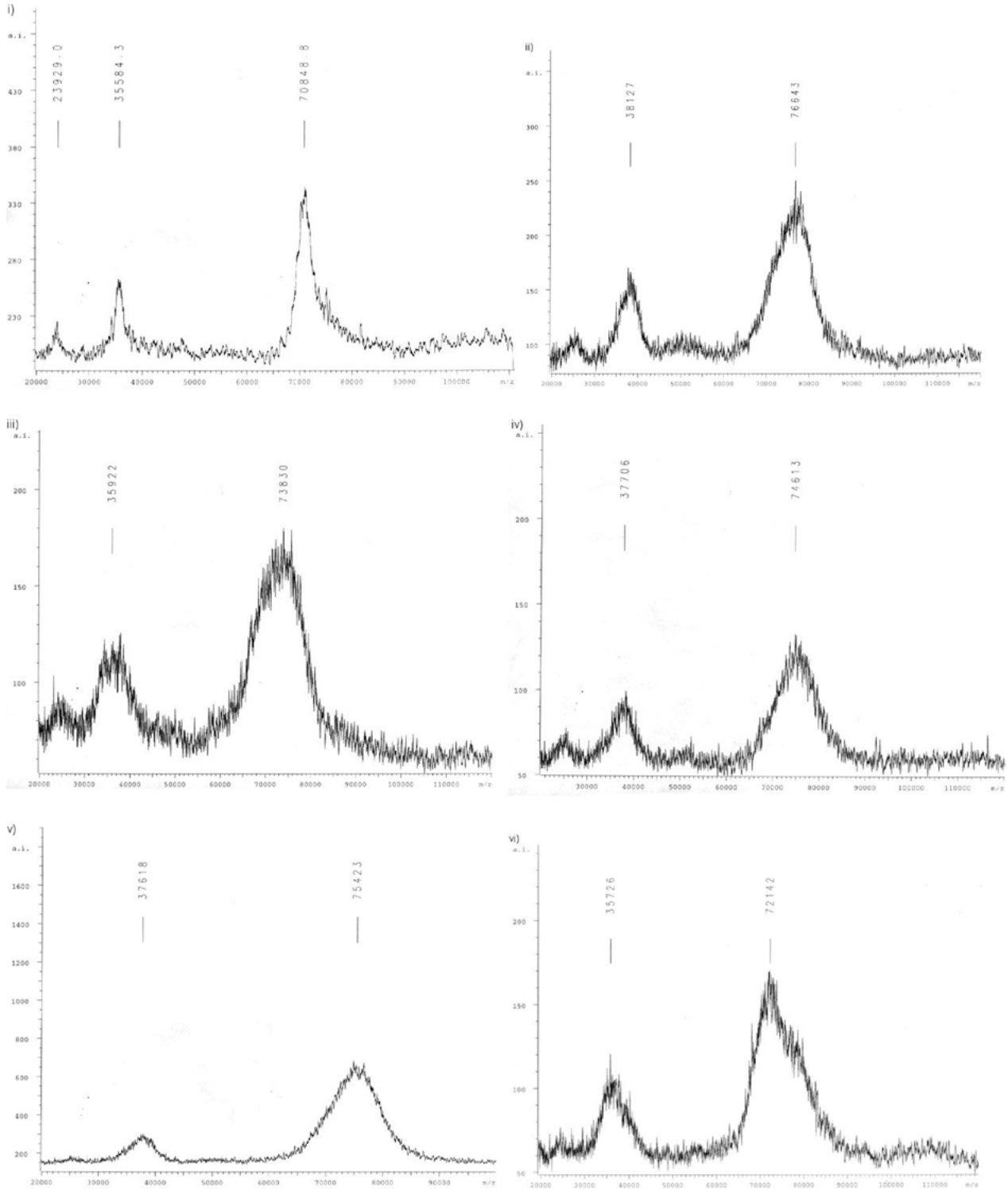


Figure 1. MALDI-TOF spectra of prepared neoglycoproteins (for details see Table 2): (i) preparation **1A**—conjugate of BSA with GlcNAc (16 residues), (ii) **2A**—conjugate of BSA with chitobiose (22 residues), (iii) **3A**—conjugate of BSA with chitotriose (13 residues), (iv) **4A**—conjugate of BSA with chitotetraose (9 residues), (v) **5A**—conjugate of BSA with chitopentaose (8 residues), (vi) **1B**—conjugate of BSA with *N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-*N*′-(*p*-nitrophenyl) thiourea (14 residues), (vii) **2B**—conjugate of BSA with *N*-chitobiosyl-*N*′-(*p*-nitrophenyl) thiourea (9 residues), (viii) **3B**—conjugate of BSA with *N*-chitotriosyl-*N*′-(*p*-nitrophenyl) thiourea (19 residues), (ix) **1C**—conjugate of BSA with *p*NP-GlcNAc (26 residues), with BSA, (x) **2C**—conjugate of BSA with *p*NP-chitobioside (32 residues), and (xi) native BSA (std.).

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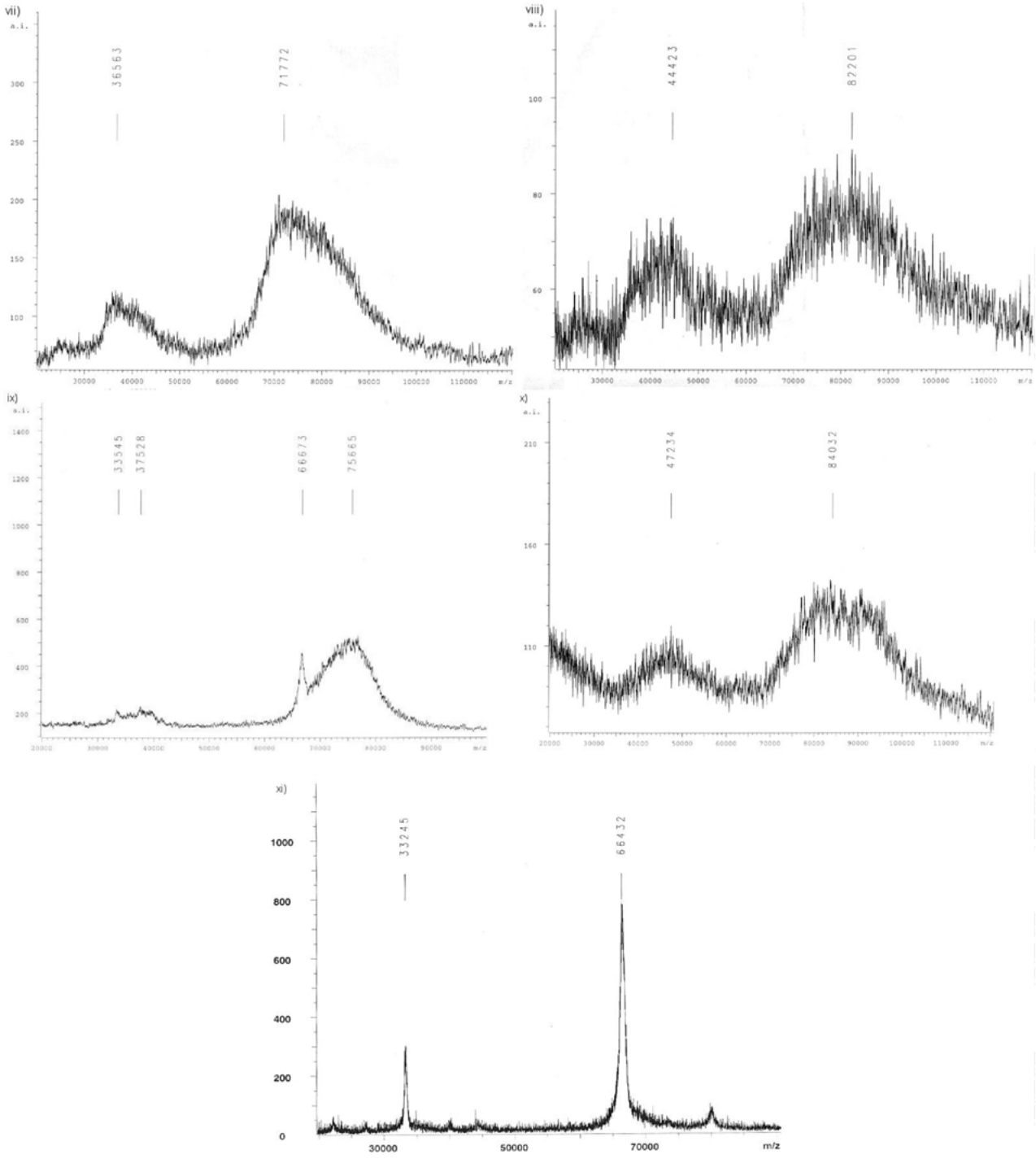

Figure 1. (*Continued.*)

aromatic linker can be further used for preparation of the glycodendrimers based on the synthetic cores, e.g. PAMAM-based or other neoglycoconjugates. The results of binding studies to NKR-P1 receptor show that our neoglycoproteins are very strong ligands, although they are not homogenous. We conclude from this observation that synthesis of well defined glycodendrimers from chitooligomers and appropriate core molecules (e.g. PAMAM) characterized by good geometric properties and biological acceptability could be crucial for potential *in vivo* applications of antitumor glycomimetics [14,15]. However, in analogy with our previous observations with GlcNAc clustered on protein or PAMAM backbones [7], synthetic chitooligomer clusters should provide considerably better ligands in these applications.

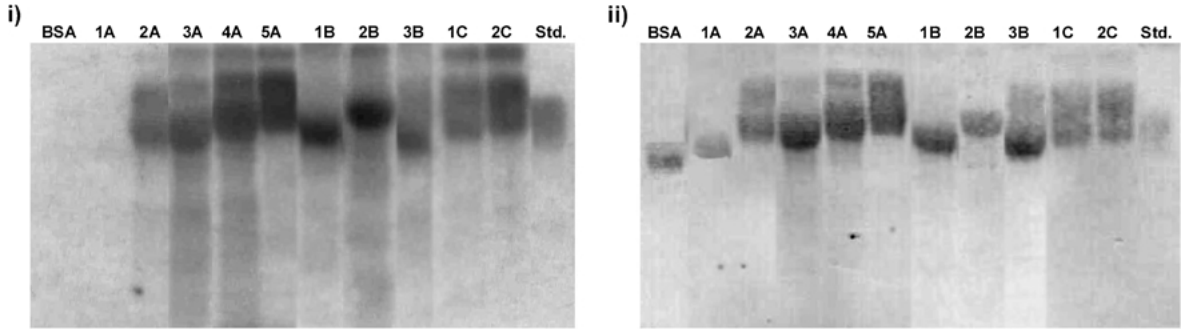

Figure 2. Eastern blotting of synthesized neoglycoproteins (for details see Table 2) on duplicate PVDF membranes, (i) sheets were incubated with ^{125}I-NKR-358 and exposed onto Kodak XAR-5 film with intensifying screens (6 hours) and (ii) sheets were stained with amidoblack 10B and dried. *Std. represents $GlcNAc_{17}BSA$ (Sigma).

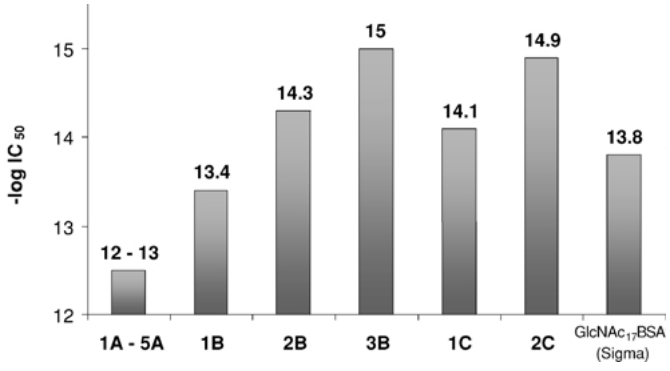

Figure 3. Inhibition of binding of NKR-P1 to $GlcNAc_{17}BSA$ (Sigma) with different neoglycoproteins (see Table 1).

Acknowledgments

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