

Chapter 5

Biotinylated Multivalent Glycoconjugates for Surface Coating

Alexander A. Chinarev, Oxana E. Galanina, and Nicolai V. Bovin

Abstract

Systematic studying of biological processes driven by multipoint high-cooperative carbohydrate recognition requires application of multivalent carbohydrates as tools. In this regard polyacrylamides with various pendant carbohydrate residues and labels are probably the most well advanced class of carbohydrate multimerics. Here we describe a synthetic approach to polyacrylamide-based glycoconjugates with biotin tag, with special emphasis to development of carbohydrate biosensors and arrays.

Key words: Glycoarrays, glycopolymers, radical polymerization, biotin.

Abbreviations

AC	6-aminocaproyl
AIBN	α,α' -azoisobutyronitrile
biot	biotin
B _{tri}	Gal α 1-3(Fuc α 1-2)Gal-
DP	average degree of polymerization
ELISA	enzyme-linked immunosorbent assay
Glyc	glycoside residue
GPC	gel-permeation chromatography
M_n	number average molecular mass
M_w	weight average molecular mass
NPA	4-nitrophenylacrylate
pHEAA	poly(<i>N</i> -(2-hydroxyethyl)acrylamide)
pNPA	poly(4-nitrophenylacrylate)
Str	streptavidin.

1. Introduction

Multivalent glycoconjugates based on linear poly(acrylamides) with attached side carbohydrates groups (Glyc) have turned to routine instruments for glycobiology research (1, 2). The latter often demand modification of surfaces with complex

carbohydrates in multivalent form (3). A convenient approach of glycopolymer deposition on surface is based on application of biotin–streptavidin (Str) system, when glycopolymers bearing biotin tags are anchored to Str-coated surface (**Fig. 5.1**). This “click”-like procedure is known to give quantitative yield.

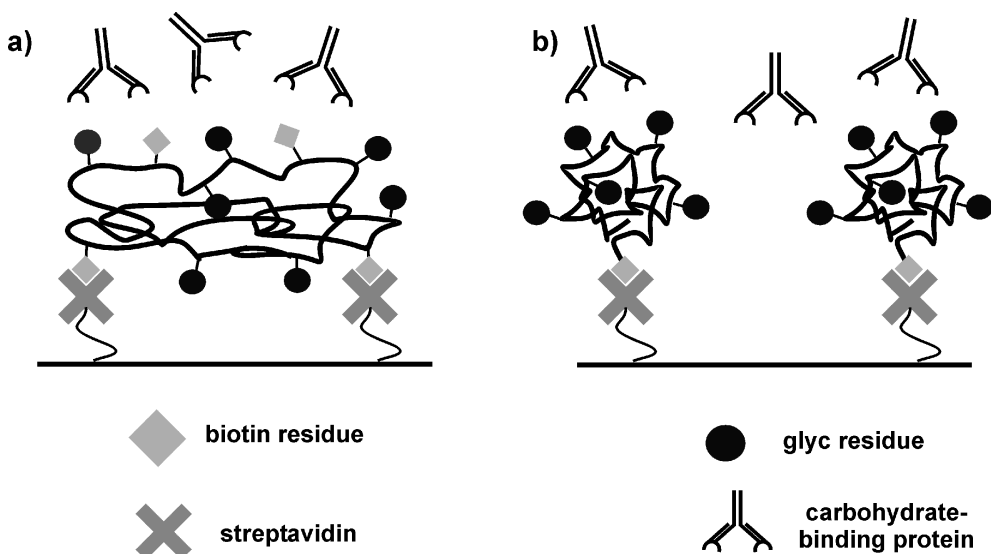


Fig. 5.1. Construction of glycoarrays on Str-coated surface using glycopolymers carrying several side biotin groups (a) or one end biotin group (b) to study carbohydrate specificity of antibodies, lectins, enzymes, cells, pathogens.

We have suggested two approaches to biotinylated glycopolymers. In accordance with the first one, activated polyacrylic acid is initially prepared by radical polymerization of 4-nitrophenylacrylate (NPA). Then, a biotin derivative containing amino group in linker, biot-NH(CH₂)₆NH₂, and an ω -aminoalkyl glycoside, Glyc-O(CH₂)₃NH₂, are consequently coupled to poly(4-nitrophenylacrylate), pNPA. Finally, the remaining active ester groups in the polymer are quenched by treatment with ethanolamine. Resultant substituted poly(*N*-(2-hydroxyethyl)acrylamide), pHEAA-Glyc_x-biot_y, contains several Glyc and biotin residues (**Fig. 5.2a**). In the second method, biotin residue is introduced into a polymer scaffold as end group with a fragment of biotinylated initiator. After that, like in the first case, biotinylated poly(4-nitrophenylacrylate), biot₁-pNPA, is treated with Glyc-O(CH₂)₃NH₂ followed by ethanolamine (**Fig. 5.2b**). The obtained glycopolymer, biot₁-pHEAA-Glyc_x, with end biotin group possesses a pronounced advantage: in immobilized macromolecules the single end biotin is crypted inside streptavidin matrix, thus does not impact on potential unspecific interactions. Both synthetic routes are considered in detail below. Also we describe a deposition procedure of the obtained biotinylated glycopolymers on the surface

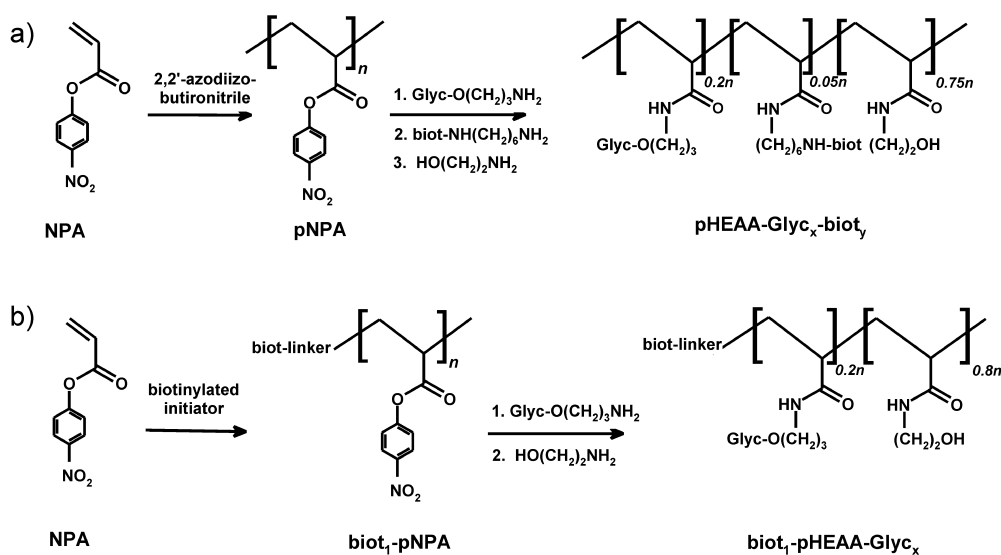


Fig. 5.2. Preparation of biotinylated polyacrylamide glycoconjugates. Synthetic approach leading to glycopolymer with several pendant biotins (2, 4) (a); an approach suggests incorporation of biotin group into pNPA as the fragment of biotinylated initiator followed by ligation with Glyc-O(CH₂)₃NH₂ (5) (b); x, y – average numbers of Glyc and biotin groups per polymer molecule (see **Notes 1** and **2**).

of Str-covered plates and the use of these plates in enzyme-linked immunosorbent assay (ELISA).

2. Materials

2.1. Chemicals

1. Acrylic acid 4-nitrophenyl (NPA) ester was synthesized from acryloyl chloride and 4-nitrophenyl as described in (7) (see **Notes 3, 4** and **5**).
2. α, α' -Azobisisobutyronitrile (AIBN) (Fluka).
3. [biot-AC-NH(CH₂)₅Co(7-Me-salen)(en)]Br] was synthesized as described in (5) (see **Note 6**).
4. B_{tri}-O(CH₂)₃NH₂ and B_{tri}-O(CH₂)₃NH-biotin (Lectinity Inc., Russia).
5. Biot-NH(CH₂)₆NH₂ (Lectinity Holding, Inc., Russia).
6. Kiesselgel 60 (Merck).
7. NEt₃ (Fluka) was kept for 5 days over BaO (Aldrich), and then distilled under fresh BaO.
8. DMF (Fluka) was distilled in vacuum under BaO.
9. Et₂O (Fluka) was distilled with CaH₂ (Sigma).

2.2. Other Commercial Reagents and Solvents

1. Globular protein calibration kit, $M_w = 12.4\text{--}450$ kDa (Serva, USA).
2. Sephadex LH-20 (Pharmacia BioTech, Austria).
3. TSK-2000SW column, 7.5×300 mm (Ultrapack, Sweden).
4. Reacti-Bind Streptavidin Coated High Binding Capacity Black 96-Well Plates (Pierce, USA).

2.3. Buffers and Coating Solutions

1. Coating solution: 200 mg/mL pHEAA- $B_{tri}(20)$ -biot (5) or biot₁-pHEAA- $B_{tri}(20)$ in PBS (pH 7.4, Sigma).
2. Washing buffer: 0.1% Tween-20 (Sigma) in PBS.
3. Blocking buffer: 3% BSA (Sigma) in PBS.
4. Carbonate buffer, Na_2CO_3 – $NaHCO_3$, pH 9.6, was prepared as described in (6).

2.4. Antibodies

1. Mouse monoclonal antibodies B8 against B_{tri} were obtained from All-Russian Hematology Research Center (Moscow, Russia).
2. Anti-mouse IgG+IgM (H+L)-alkaline phosphatase conjugate (Ig-AP) was the product of AP Biotech Inc. (UK).

3. Methods

Two methods of NPA polymerization induced by a typical radical azo-initiator, AIBN, are described. In the first method adopted from literature (7), the polymerization is performed in benzene. The growing macromolecules become insoluble when reached certain molecular weight; thus, the forming polymer is steadily evolved from the reaction mixture during the course of polymerization. Further growth of pNPA molecules presumably takes place in the polymer gel phase, until diffusion of the monomer to the active centers is allowed. Effective control of such occlusion polymerization process is rather difficult, the resultant pNPA possess relatively low meanings of number average molecular weight ($M_n = 15\text{--}45$ kDa) and wide molecular mass distribution ($M_w/M_n = 2\text{--}7$). Moreover, the molecular weight characteristics of the polymer are poorly reproducible in this method, and different batch of pNPA may noticeably differ in this respect.

The second method of NPA polymerization is original; the polymerization is carried on in solution, i.e., in homogeneous conditions; DMSO is used as the solvent (8). In this case, molecular mass characteristic of the obtained polymer can be easily controlled. Thus, in consistency with the theory of

radical polymerization, a decrease in AIBN concentration ($[I]_0$) caused an increase in pNPA number average molecular weight ($M_n = 110$ – 145 kDa). The growth of M_n values is also observed upon increase in the monomer concentration ($[M]_0$), although this dependence is less pronounced. Noteworthy, the polymer obtained by this method has narrower molecular mass distribution than pNPA prepared in accordance with the first approach (*see Table 5.1*).

Table 5.1

Molecular weight characteristics of activated polyacrylic acids depending on the amount of initiator taken for polymerization of 4-nitrophenylacrylate ($[I]_0$). Starting concentration of monomer in polymerizing mixture ($[M]_0$) was 1 mol/L for all experiments

	$[I]_0$, mass % on monomer weight	M_n , kDa	M_w/M_n
pNPA, Method 1	5	~15–45	2–7
pNPA, Method 2	0.5	110	1.85
	0.25	135	2.01
	0.1	145	2.03
Biot ₁ -pNPA	3	35	1.88
	1.5	40	1.93
	0.5	47	2.15

Finally, we describe a method leading to an active ester homopolymer with end biotin group, biot₁-pNPA. To accomplish this biotinylated organocobalt(III) chelate, [biot-NH(CH₂)₅Co(7-Me-salen)(en)]Br], is used as initiator of NPA polymerization in DMSO. Homolytic splitting of the chelate Co–C bonds taking place with heating of the reaction mixture leads to carbon-centered radicals, biotin-AC-NH(CH₂)₅ (*see Fig. 5.3*). The latter triggers polymer formation and provides incorporation of biotin end group into pNPA molecule. Starting concentration of the initiator in reaction mixture may be varied to get the polymers of different molecular weights (*see Table 5.1*).

Small part of the activated polymers, pNPA and biot₁-pNPA, was converted into pHEAA and biot₁-PHEAA, correspondingly, and number average molecular mass (M_n) was defined by analytical gel-permeation chromatography (GPC). This is an important

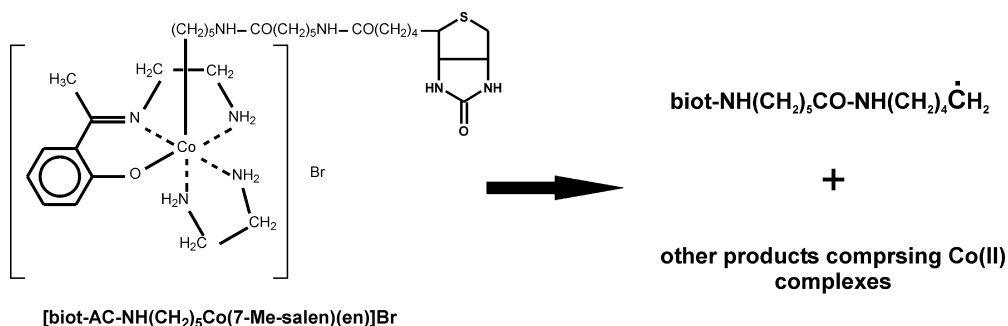


Fig. 5.3. Structure of the biotinylated organocobalt initiator and the scheme of its decomposition due to splitting of the alkyl–Co bond. Noteworthy, only one radical is released under the decomposition, which diminishes the “cage” effect and enhances the initiator efficiency.

characteristic of the polymer scaffold as it defines number of pendant side groups in glycoconjugate and hence its capacity to bind carbohydrate recognizing proteins. Data on molecular mass properties of pNPA and biot₁-pNPA obtained under different conditions are given in **Table 5.1**.

A universal approach used earlier to get a wide variety of label-free neoglycoconjugates as well as probes bearing different label types, neoglycolipids, adsorbents, etc. (1, 2), is adopted here for biotinylated polyacrylamide glycoconjugate synthesis (**Fig. 5.2**). Consequent condensation of the aminoligands, biot-NH(CH₂)₆NH₂ and B_{tri}-O(CH₂)₃NH₂, with the activated polyacrylic acids, pNPA ($M_n \sim 23$ kDa, Method 1) and biot₁-pNPA ($M_n \sim 47$ kDa), is carried on in DMSO at 40°C with quantitative yield (*see* **Notes 1** and **2**). Then, the non-reacted activated acryloyl groups of the polymer are quenched by treatment with an excess of ethanolamine giving rise to inert *N*-(2-hydroxyethyl)acrylamide form. The resultant glycopolymers bear Glyc residues statistically distributed along the backbone; biotins are attached to the polymer scaffold as side (for pHEAA-Btri(20)-biot (5)) or end groups (for biot₁-pHEAA-Btri(20)).

The prepared biotinylated polyacrylamide glycoconjugates are used to coat the surface of commercial Str-covered plates for ELISA. The coating procedure is simple and consists just in incubation of the plates with the biotinylated glycopolymers in buffer solution and washing. In the similar way we coated commercial fluorescent beads for suspension assay, modified surface of red blood cells aimed at designing serological reagents with controllable hapten density and printed the polymers onto microchip (5).

Binding of monoclonal antibodies (mAbs) specific to B_{tri} to the glycopolymers, pHEAA-Btri(20)-biot (5) and biot₁-pHEAA-Btri(20), coated onto surface of polystyrene plates, was tested using ELISA. For comparison, mAbs binding with the plates coated with the monomeric glycoside, B_{tri}-O(CH₂)₃NH-biotin,

was determined. The intensity of the signal detected in ELISA was plotted against glycoconjugate amount (Fig. 5.4).

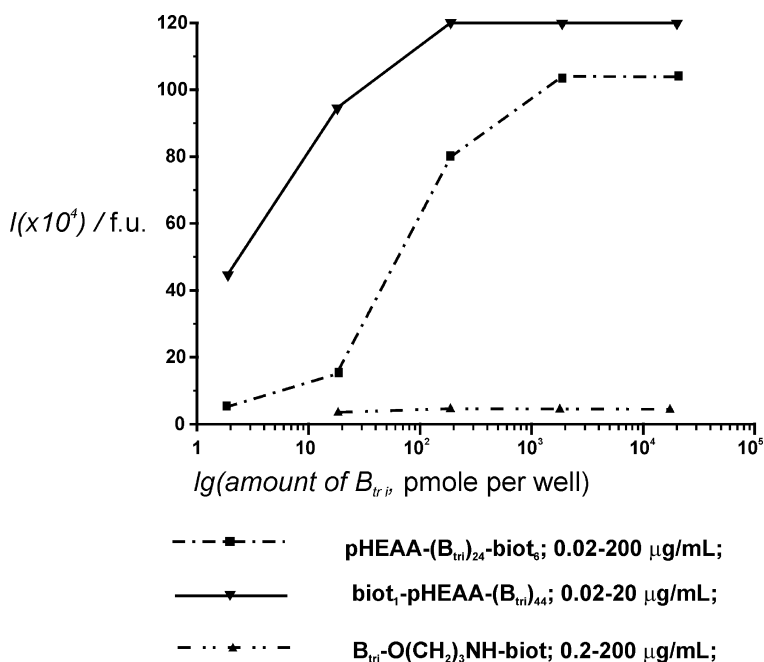


Fig. 5.4. Dependence of fluorescent signal in ELISA on the amount of B_{tri} added per well as the biotinylated glycoconjugates. The plot displays higher binding of mAbs with the glycopolymers, whereas the lack of binding is observed with the monomeric conjugate. Our earlier data showed that the difference in binding affinity between the glycopolymers cannot be attributed to the observed minor disparity in molecular weight and valence (1, 2). It is rather connected with their different anchoring on surface and, correspondingly, favored presentation of glycoligands on surface (5).

3.1. Chromatography

3.1.1. Thin Layer

Chromatography (TLC)

1. TLC was performed on silica gel covered plates Kiesselgel 60.
2. Spots were visualized by charring a plate with 7% H_3PO_4 , by a plate exposure to NH_3 vapor (for NPA) or by ninhydrin treatment (for aminocompounds).

3.1.2. Preparative GPC

1. GPC was performed on Sephadex LH-20 in $\text{MeCN}/\text{H}_2\text{O}$, 1:1, using 75×1.5 cm column.

3.1.3. Analytical GPC

1. Analytical gel-permeation chromatography of pHEAA and biot₁-pHEAA was carried out using HPLC TSK-2000SW column, 7.5×300 mm; mobile phase 0.2 M NaCl; flow rate ~ 1 mL/min; and UV detection at 210 nm.
2. The column was calibrated with a globular protein calibration kit, $M_w = 12.4\text{--}450$ kDa.

3. Based on the polymer elution profiles, molecular weight distribution differential curves were plotted by the Schultz method and analyzed using standard approaches (9) to enable the calculation of the polymer molecular weight characteristics M_n , and M_w/M_n (see Table 5.1).

3.2. Preparation of Activated Poly(acrylic) Acids

3.2.1. Poly (4-Nitrophenylacrylate), pNPA. Method 1

1. Apparatus consisting of a two-neck round-bottomed 50 mL flask equipped with magnetic stirrer, inlet tube (not too narrow in diameter) provided with current of nitrogen, and a water condenser was assembled. The apparatus was closed by a calcium chloride tube; care should be taken before the experiment to ensure that there is a free gas passage through the tube.
2. 1 g (5.18 mmol) NPA, 50 mg (0.3 mmol) AIBN, and 12 mL benzene were placed in the flask.
3. The mixture was stirred under slow nitrogen flow (extra pure) for 1 h and then heated at 70°C for 24 h.
4. Precipitate evolved from the reaction mixture was collected on a glass filter, dissolved in 10 mL DMF and re-precipitated by adding 30 mL MeOH.
5. The product was washed on a glass filter with 3×30 mL MeOH and dried in vacuum.
6. Yield of the product ~60%; fine yellowish powder (see Note 7).

3.2.2. Poly (4-Nitrophenylacrylate), pNPA. Method 2

1. A solution containing 1 g (5.18 mmol) NPA and 1–5 mg AIBN in 5.2 mL DMSO was placed in a long-stem glass 10 mL ampoule.
2. The mixture was degassed by 3–5 times repeating of freeze–pump–thaw cycles; the ampoule was flame-sealed under vacuum.
3. The mixture was heated at 70°C for 24 h.
4. The ampoule was opened, and the reaction mixture was poured into flask with 30 mL intensively stirred anhydrous Et₂O to wash from DMSO. At times Et₂O was replaced with fresh portions, until the polymer did not turn into glassy state.
5. Pieces of the glassy polymer were pounded and then stirred with Et₂O to form fine powder.
6. The product was collected on a glass filter and dried in vacuum.

7. Yield of the product 50–70%; fine yellowish powder (*see* **Note 7**).

**3.2.3. Poly
(4-Nitrophenylacrylate)
with End Biotin Group,
biot₁-pNPA**

1. A solution containing 1 g (5.18 mmol) NPA and 5–30 mg [biot-NH(CH₂)₅Co(7-Me-salen)(en)]Br⁺ in 5.2 mL DMSO was placed in a long-stem glass 10 mL ampoule.
2. The mixture was degassed by 3–5 times repeating of freeze–pump–thaw cycles; the ampoule was flame-sealed under vacuum.
3. The mixture was heated at 70°C for 24 h.
4. The further treatment was the same as for pNPA (*see* **Sect. 3.2.2**).
5. Yield of the product –50 to 70%; fine olive colored powder (*see* **Notes 7 and 8**).

**3.3. Preparation of
Poly (N-(2-
Hydroxyethyl)acrylamide),
pHEAA and
biot₁-pHEAA**

1. One hundred microliters of ethanolamine (#411000, Aldrich) was added to a solution of 30 mg pNPA or biot₁-pNPA in 1 mL DMSO and the mixture was kept for 48 h under 70°C.
2. Product was purified by GPC, fractions with product were evaporated, and the remains were dried in vacuum.
3. Yield 93–95%; white solids.

**3.4. Preparation of
Polyacrylamide-
Based
Glycoconjugates**

**3.4.1. Preparation of
Glycopolymers with Side
Biotin Groups,
pHEAA-B_{tri}(20)-biot (5)**

1. To a solution of 4.7 mg (27.5 μmol) pNPA in 400 mL DMSO (*see* **Note 7**) were added 0.5 mg (1.3 μmol) biot-NH(CH₂)₆NH₂ and 0.4 mL (2.6 μmol) NEt₃ (*see* **Notes 9 and 10**).
2. A solution containing 3 mg (5.5 μmol) B_{tri}-O(CH₂)₃NH₂ and 1.5 μL (11 μmol) NEt₃ in 100 mL DMSO was mixed with the solution of pNPA.
3. The mixture was kept for 12 h at 40°C, absence of the free glycoside was confirmed by TLC (*see* **Note 11**).
4. An excess of ethanolamine (5 equiv.) was added to the reaction and it was kept for 24 h at 40°C.
5. The product was purified by GPC, fractions containing the product were evaporated and the residue was dried in vacuum.
6. Yield 85–95%; white solid.

**3.4.2. Preparation of
Glycopolymers with End
Biotin Group,
biot₁-pHEAA-B_{tri}(20)**

1. To a solution of 4.7 mg (27.5 μmol) biot₁-pNPA in 500 mL DMSO were added 3 mg (5.5 μmol) B_{tri}-O(CH₂)₃NH₂ and 1.5 μL (11 μmol) NEt₃.

2. The further treatment was the same as for pHEAA-B_{tri}(20)-biot (5) (*see Sect. 3.4.1*)
3. Yield 85–95%; white solids.

3.5. Coating of Str-Plates with Biotinylated Glycopolymers

1. Reacti-Bind Streptavidin Coated High Binding Capacity Black 96-Well Plates were rinsed twice with PBS.
2. Serial 10-fold dilution of coating solution in PBS (containing 0.02–200 mg/mL biotinylated glycoconjugate) was added to the plates (100 mL per well); the plates were kept for 1 h at 37°C.
3. The plates were washed (hereinafter, the washing buffer, 200 mL per well).
4. The plates were blocked (the blocking buffer, 200 mL per well) and washed.

3.6. ELISA

1. B8 mAbs (1:100 in PBS containing 0.3% BSA) were added to the plates (100 mL per well).
2. The plates were incubated for 1 h at 37°C and washed.
3. Ig-AP (1:5000 in PBS containing 0.3% BSA) was added to the plates (100 mL per well); the plates were incubated for 1 h at 37°C and washed.
4. A solution of 10^{-4} M 4-methylumbelliferyl phosphate disodium salt in the carbonate buffer was added to the plates (100 mL per well); the plates were incubated for 30 min at room temperature.
5. Fluorescence intensity (355 nm/460 nm) was measured by Victor² multilabel counter. Each assay was done in duplicate, and blank reaction was performed by omitting mAb. The blank reading was subtracted from the final fluorescence to provide the corrected fluorescence intensity values.

4. Notes

1. Routinely, molar fractions of Glyc and biotin in pHEAA-Glyc_x-biot_y are 20 and 5%, respectively; this optimal loading be changed if necessary. In glycopolymer notations, meanings of the molar fractions are given in brackets for the corresponding ligands; for example, pHEAA-Glyc(20)-biot (5) or biot₁-pHEAA-Glyc(20).
2. Obviously, the average number of pendant Glyc and biotin groups (x , y) depends not only on the substitution rate of the acrylate units with corresponding residues, but also on

the molecular weight of the polymer. Polyacrylamide with higher molecular weight can bear larger number of the pendant residues.

3. Some characteristics of NPA are given below. NPA: yellow needle crystals; m.p. 44–47°C; TLC: toluene/Me₂CO, 4:1, *R_f* 0.68; ¹H NMR (δ, CDCl₃, 303 K): 6.11 (dd, 1H, *J*_{cys} 1 Hz, *J*_{trans} 10.6 Hz, CH₂ = CHCO), 6.35 (dd, 1H, *J*_{hem} 17.4 Hz, *J*_{trans} 10.6 Hz, CH₂ = CH), 6.67 (dd, 1H, *J*_{cys} 1 Hz, *J*_{hem} 17.4 Hz, CH₂ = CH), 7.35 and 8.30 (d, *J* 7.6 Hz, 4H, Ar).
4. The monomer taken for polymerization could contain up to 20% of oligomeric admixtures, this does not affect the polymerization course.
5. The monomer should be kept at 2–8°C avoiding contact with moisture and amines.
6. The biotinylated organocobalt chelate should be kept in darkness at –20°C avoiding contact with acids.
7. pNPA and biot₁-pNPA should be kept at 2–8°C avoiding contact with moisture and amines.
8. Biot₁-pNPA is slightly impure with cobalt-containing products of [biot-NH(CH₂)₅Co(7-Me-salen)(en)]Br] decomposition. Nevertheless, it proved fit for further modifications without additional purification.
9. Concentration of pNPA or biot₁-pNPA solutions in DMSO taken for glycoconjugates preparation should be in the range 10–20 mg/mL, otherwise gelation of the polymers can occur.
10. Stock solution of pNPA-biot_x can be prepared, stored at 5°C for a long time without decomposition of the active ester groups, and used to obtain a glycoconjugate if necessary.
11. To control coupling of the aminoligands with pNPA and biot₁-pNPA, TLC may be used. Recommended eluents: MeOH/1 M Py·HOAc 3:1, *R_f* 0.54, for biot-NH(CH₂)₆NH₂; EtOH/BuOH/Py/H₂O/AcOH 10:1:1:1:0.3, *R_f* 0.3, for B_{tri}-O(CH₂)₃NH₂.

Acknowledgments

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