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High molecular weight neoglycoconjugates for solid phase assays

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Adsorption of a carbohydrate on solid phase is the necessary stage of the immunosorbent assay (ELISA) and analogous methods of the study of carbohydrate-protein interaction. Usually physical adsorption on polystyrene requires a high concentration of conjugated carbohydrate and, thus, enormous consumption of it. In this study, we explored two approaches allowing more rational use of oligosaccharide (Glyc). The first of them is based on the covalent immobilization of neoglycoconjugates on the NH_2-modified polystyrene; the second one is based on the elevated adherence of high m.w. neoglycoconjugates to polystyrene. Covalent immobilization of polyacrylamide conjugates, Glyc-PAA, provided a possibility to solve the problem, but the nonspecific binding of antibodies in ELISA proved to be unacceptably high. At the same time, the increase of the Glyc-PAA m.w. from 30 kDa to 2,000 kDa allowed a 10–20 fold decrease of its consumption, when using physical adsorption, whereas the assay background remained at the low level. The amount of 2,000 kDa Glyc-PAA that is sufficient for the coating of a standard 96-well plate corresponds to the nanomole level of oligosaccharide, this providing a possibility to use saccharides that are available in a very limited amount when studying the carbohydrate-protein interaction with solid-phase techniques.
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Keywords: **ELISA, glycoconjugates, carbohydrate-binding proteins**

Abbreviations: **BSA, bovine serum albumin; DMSO, dimethylsulfoxide; pNPA^{30}, poly(4-nitrophenyl acrylate), 30 kDa; pNSA^{2000}, poly(*N*-oxysuccinimidyl acrylate), 2000 kDa; PAA, poly(*N*-2-hydroxyethylacrylamide); A_{tri}, GalNAcα1-3(Fucα1-2)Gal; B_{tri}, Galα1-3(Fucα1-2)Gal; ELISA, enzyme-linked immunosorbent assay; OD, optical density; Glyc, oligosaccharide residue; Ig, immunoglobulin.**

Introduction

Adsorption of carbohydrate antigens, usually as Glyc-BSA or Glyc-PAA conjugates, on the surface of polystyrene plate is the necessary stage during the study of carbohydrate-protein interaction using conventional routine solid-phase assays. Physical adsorption is simple, well reproducible, and demonstrates a low signal/background level. It is also important that a wide range of glycoconjugates is commercially available. The only disadvantage of the method, based on physical adsorption, is the large consumption of neoglycoconjugates, about 100 μg ($\sim$50 nmols of a saccharide) per a standard 96-well plate, even in the case of polystyrene plates with an increased adsorption capacity [1–3]. This value is quite acceptable when using synthetic saccharides. However, the investigations requiring complex natural glycans, in particular carbohydrate chains of hardly accessible glycoproteins, are encountered more and more often. Thus, when a natural glycoprotein is isolated as a new receptor for the lectin in study, it is necessary to determine what particular oligosaccharide chain takes part in the functional receptor binding. A typical situation occurs when this glycoprotein is heavily glycosylated and glycosylation heterogeneity is high, moreover, a minor chain could be responsible for a biological function. Therefore, it is necessary to use design assays that are more sensitive. Our goal is bridging of analytical HPLC and routine solid-phase assay in order to coat polystyrene plates with the OS, separated and identified with the help of analytical HPLC (nanomoles and less). Within this goal the study is related to working out of the methods of carbohydrate immobilization. At

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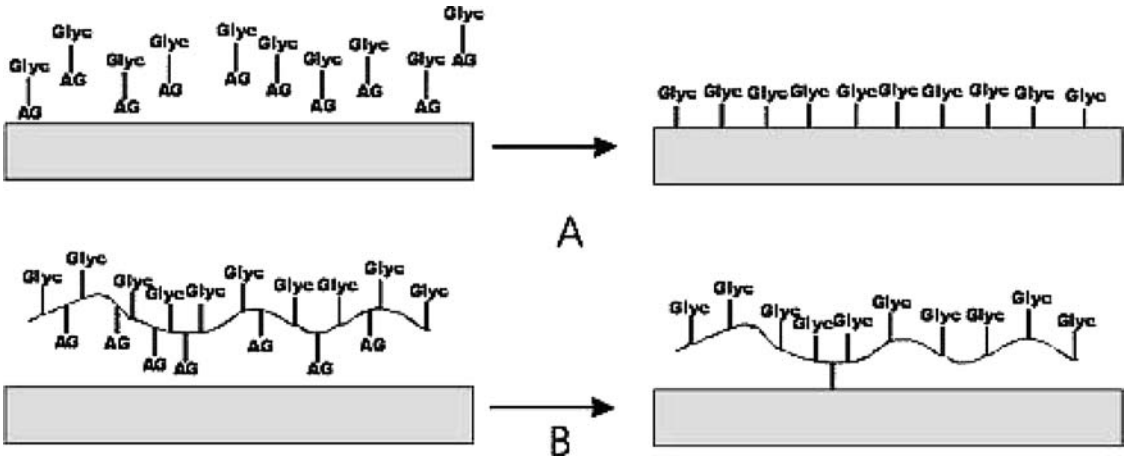


Figure 1. Prerequisite advantage of chemical immobilization of polymeric *vs.* monomeric glycoconjugates: Formation of several chemical bonds (A) is necessary for immobilization of several Glyc residues, whereas formation of single covalent bond (B) is enough for immobilization of numerous Glyc residues. AG is an active group.

this stage we have used synthetic OS and antibodies to them; in future it is supposed to use the developed method for the search of natural ligands of mammalian lectins.

Physical adsorption is not the only way of surface coating; see for instance use of streptavidin-biotin system for construction of a glycoarray [4]. Other coating methods have been described in the literature [5,6], including the chemical immobilization of saccharides on modified polystyrene (see Discussion). Chemical immobilization is attractive only on the condition that the reaction proceeds with a high yield despite low concentration of the saccharide. We supposed that polymeric glycoconjugates having multiple activated groups and multiple oligosaccharide residues suit this criterion much better than small molecules, because even single-point junction of a polymer to the surface leads to the immobilization of multiple oligosaccharide residues (Figure 1).

Returning to physical adsorption, it seems quite obvious that it would increase in the case of glycoconjugate (*e.g.* Glyc-PAA) size increase as the larger molecule has more potency to adhere to solid phase, and thus adsorption efficiency (proportion of adsorbed material) should be higher. There is another reason for the attractiveness of high m.w. glycoconjugates for the solution of the given problem. It is known that the distance between two Fab-fragments of an antibody depends on its class and flexibility, being as a rule 100–150 Å [7]. This means that antibody is incapable of bivalent binding with one molecule of glycoconjugate when the glycoconjugate size is less than 100 Å; similar reasoning is also basically true for various bivalent or paucivalent lectins. Thus, it is possible to drastically improve protein/glycoconjugate binding due to multivalency by the synthesis of conjugates larger in size. It is desirable to use such an assay allowing multivalent interaction (see also Discussion) for the study of new lectins with unknown subunit organization.

In this study, we compared 2000 kDa PAA-glycoconjugates with commonly used 30 kDa ones as coating reagents in ELISA and demonstrated that the use of the high m.w. variant gives rise to 10–20 fold economy of carbohydrate antigen. There is no reason to doubt that the proposed approach is also compatible with microarrays actively developed at present [8].

Materials and methods

Murine IgG, BSA, diisopropylethylamine, Tween 20 were from Sigma (USA), ^{3}H-labeled leucine (40 Ci/mmol) was from IICH (USA), polyvalent secondary anti-mice Ig antibodies covalently bound with peroxidase were from Boehringer-Manheim (Germany), Sephadex LH-20 was from Pharmacia Biotech (Austria). All the rest chemicals of highest grade available were from Fluka (Switzerland). Polystyrene 8-well immunological modules and 96-well plates MaxiSorp were from NUNC (Denmark), NH_2-modules were from Costar (USA). Poly(4-nitrophenyl acrylate) m.w. 30 kDa was synthesized as described earlier [9]. B_{tri}-$OCH_2CH_2CH_2NH_2$ and A_{tri}-$OCH_2CH_2CH_2NH_2$ were prepared as described in [10]; B_{tri}-PAA^{30} (upper index is molecular weight of polymer) containing 20% mol of B_{tri} was from Lectinity (Moscow). Monoclonal IgM antibodies A16 and B8 specifically agglutinating erythrocytes of blood group A or B donors were obtained from Hematological Center, Moscow. Radioactivity was measured on Beckman LS6000SC (USA) counter; optical density was measured using Multiscan MCC 340 reader (Labsystems, Finland) at 420 nm.

Synthesis of poly(N-oxysuccinimidyl acrylate), m.w. 2000 kDa

N-acryloyloxysuccinimide (1 g, 5.9 mmol) synthesized according to the method described in [11] and 2,2′-azodiisobutyronitrile (5 mg) were dissolved in 3 ml DMSO. The reaction vessel was vacuum pumped, and the mixture was heated for 24 h at 70°C. The obtained polymer was precipitated by diethyl ether (10 ml), filtered off, and dried. Average molecular weight was determined as described earlier [12]. The

same polymer batch was used for preparation of B_{tri}-PAA^{2000}, B_{tri}-$pNSA^{2000}$ and A_{tri}-PAA^{2000}; the glycoconjugates were synthesized as described for low m.w. conjugates [9], carbohydrate content in all the cases was 20% mol.

Microscale synthesis of PAA^{2000}-conjugates for ELISA

Solution of 85 μg $pNSA^{2000}$ (0.5 μg-eq) in 25 μl DMSO and 0.5 μl diisopropylethylamine was added to solution of 70 μg (0.1 μmol) B_{tri}-$OCH_2CH_2CH_2NH_2$ in 25 μl DMSO, the mixture was kept at 37°C for 48 h, 5 μl of 2-ethanolamine was added and solution was kept for 18 h at room temperature. The reaction mixture was neutralized with AcOH to pH 7.0 and diluted with water to 0.1 ml volume corresponding to 1 mM B_{tri} concentration.

Chemical immobilization of glycoconjugate B_{tri}-PAA^{30} on aminated polystyrene

Solution of 1.9 mg (10 μg-eq.) $pNPA^{30}$ in 500 μl DMSO followed by 10 μl diisopropylethylamine was added to solution of 1.4 mg (2 μmol) of B_{tri}-$OCH_2CH_2CH_2NH_2$ in 500 μl DMSO and the mixture was kept at 37°C for 48 h. For immobilization the neoglycoconjugate solution (2 μg/ml) was added to the first well of the module, followed by serial double dilutions with DMSO (solutions were transferred to the next wells) and incubation at room temperature for 24 h. Finally, 10 μl of 3 M aqueous ammonia was added followed by incubation at room temperature for 24 h and washings with water.

Chemical immobilization of glycoconjugate B_{tri}-PAA^{2000} on activated polystyrene

Solution of 1.7 mg $pNSA^{2000}$ (10 mg-eq) in 500 μl DMSO followed by 10 μl diisopropylethylamine was added to solution of 1.4 mg (2 μmol) B_{tri}-$OCH_2CH_2CH_2NH_2$ in 500 μl DMSO, the mixture was kept at 37°C for 48 h. The further procedures were analogous to those described above.

ELISA

Plates were coated with B_{tri}-PAA conjugates, 10 μg per ml (30 kDa conjugate) or 1.25 μg per ml (2000 kDa conjugate) in 0.05 M Na-carbonate buffer, pH 9.6, for 24 h at room temperature. Alternatively, NH_2-modules were coated with B_{tri}-$pNPA^{30}$ or B_{tri}-$pNSA^{2000}$ conjugates as described above. Plates or modules were blocked with 3% BSA in PBS for 1 h at 37°C and washed three times with PBS containing 0.1% Tween-20 (washing buffer). Anti-B_{tri} antibodies (100 μl) were added and the plates were incubated for appropriate time at 37°C, washed three times with washing buffer, incubated with anti-mouse Ig-HRPO conjugate (1/1000 in PBS containing 0.3% BSA) and washed. Finally, the color was developed by incubation with 0.1 M sodium phosphate/0.1 M citric acid buffer, containing 0.04% *o*-phenylenediamine and 0.03% H_2O_2, the reaction was stopped with the addition of 50 μl 1 M H_2SO_4 and absorbance was recorded.

Synthesis of ^{3}H-labelled glycoconjugates

The solution of 0.038 μmol ^{3}H-leucine in 200 μl of acidic methanol (2 μl of conc. HCl in 1 ml of methanol) was added to solution of 1.7 mg $pNSA^{2000}$ (10 μg-eq) or 1.9 mg $pNPA^{30}$ (10 μg-eq) in 200 μl DMSO followed by 3 μl diisopropylethylamine, and the mixture was kept at 37°C for 24 h. Solution of 1.4 mg (2 μmol) B_{tri}-$OCH_2CH_2CH_2NH_2$ in 200 μl DMSO and 2 μl diisopropylethylamine was added, the mixture was kept at 37°C for 24 h followed by addition of 60 μl 3 M aqueous ammonia or 2-ethanolamine. The mixture was kept for 15 h at room temperature. The resulting conjugate was isolated on Sephadex LH-20 column (1.2 × 25, acetonitrile:water 1:1), evaporated and stored in water at 4°C. Activity of B_{tri}-[^{3}H-Leu]-PAA^{30} was 0.15 mCi/μmol B_{tri}, activity of B_{tri}-[^{3}H-Leu]-PAA^{2000} was 0.23 mCi/μmol B_{tri}.

Quantification of physical adsorption

Solution of B_{tri}-PAA in 0.05 M carbonate buffer, pH 9.6 (buffer A) was added to polystyrene module followed by two-fold serial dilutions and incubation. Starting concentration of conjugate was 20 μg/ml, final concentration was 2.5 μg/ml. After incubation the solution from each well was transferred to the corresponding vial containing 5 ml of scintillation "Universal cocktail" (Fluka, Switzerland). Plate was washed by 0.1 M phosphate buffer solution, pH 7.4 (buffer B), containing 0.05% Tween 20, each portion of the washing was also placed to a vial with scintillation cocktail. The washed modules were cut to individual wells and each well was placed in the scintillation cocktail. Finally, radioactivity of solutions, washings, and wells was measured.

Serial dilutions from 10 to 0.2 μg/ml and from 16 to 2 μg/ml were used in case of B_{tri}-PAA^{2000} and B_{tri}-PAA^{30}, respectively; incubation time was 24 h at room temperature.

Agglutination of erythrocytes

Reaction of agglutination of human erythrocytes B (III) by antibodies B8 was performed according to [13]; commercial antibody solutions were taken in dilution 1:4.

ELISA, time dependence

Plates were coated with B_{tri}-PAA conjugates, 10 μg per ml for B_{tri}-PAA^{30} or 1.25 μg per ml for B_{tri}-PAA^{2000} in 0.05 M Na-carbonate buffer, pH 9.6, for 24 h at room temperature. Plates were blocked with 3% BSA in PBS for 1 h at 37°C and washed three times with PBS containing 0.1% Tween-20 (washing buffer). Anti-B_{tri} antibodies (100 μl) were added and the plates were incubated for appropriate time at 37°C, washed three times with washing buffer, incubated with anti-mouse Ig-HRPO conjugate (1/1000 in PBS containing 0.3% BSA) and

washed. Finally, the color was developed by 30 min incubation with 0.1 M sodium phosphate/0.1 M citric acid buffer, containing 0.04% *o*-phenylenediamine and 0.03% H_2O_2, the reaction was stopped with the addition of 50 μl 1 M H_2SO_4 and absorbance was recorded.

ELISA, exhaustion experiment

The first row on the plate was coated with A_{tri}-PAA conjugates (in two-fold dilutions starting from 8 μg per ml for A_{tri}-PAA^{30} and 1 μg per ml for A_{tri}-PAA^{2000}) in 0.05 M Na-carbonate buffer, pH 9.6. After 1 h incubation at 37°C the content of each well was transferred into the well of the next row and PBS was added instead. After 1 h incubation the content was transferred into the next well, etc. for six times. Then the plate was blocked with 3% BSA in PBS for 1 h at 37°C and washed three times with PBS containing 0.1% Tween-20 (washing buffer). Anti-A_{tri} antibodies (100 μl) were added and the plates were incubated for 1 h at 37°C, washed three times with the washing buffer, incubated with anti-mouse Ig-HRPO conjugate (1/1000 in PBS containing 0.3% BSA), washed and developed as described above.

Results

Physical adsorption of B_{tri}-PAA^{30} and B_{tri}-PAA^{2000} on polystyrene

PAA-conjugates of principally different molecular weights, about 30 kDa and 2000 kDa, hereinafter denoted as Glyc-PAA^{30} and Glyc-PAA^{2000} were used in this study. Optimization of adsorption conditions on polystyrene, *i.e.* process duration, temperature, pH and ionic strength was performed for both conjugates. Optimal conditions for B_{tri}-PAA^{30} were the following: 1 h at 37°C or 16 h at 4°C in any buffer solution. The degree of adsorption remained constant in various buffer solutions at pH range 5.0–9.6; only in pure water the adsorption was two times less efficient (data not shown). Thus it is possible to avoid the use of the conventional alkaline buffer pH 9.6 [1,2] that can be of principal importance when alkali-labile saccharides, such as *O*-acetates are coated onto plastic. The commonly used range of Glyc-PAA^{30} concentrations is 1–10 μg/ml, *i.e.* 0.1–1 μg/well, optimal concentration is selected depending upon the affinity of antibodies or lectins tested and the plastic grade [14]. In the case of B_{tri}-PAA^{30} and in the ELISA version used (see Materials and Methods) an acceptable value proved to be 5–10 μg/ml (4–8 pmoles B_{tri}/ml). Decrease of glycoconjugates concentration lower than 1 μg/ml led to proportional decrease in assay sensitivity, which could not have been compensated by an increase of adsorption time. Optimal conditions selected for physical adsorption of the conjugate B_{tri}-PAA^{2000} were 24 h at room temperature, the required quantity of the high m.w. material is reported below. Further increase in incubation time insignificantly affected the adsorption (data not shown).

Chemical immobilization of B_{tri}-$pNPA^{30}$ and B_{tri}-$pNSA^{2000}$ on NH_2-polystyrene

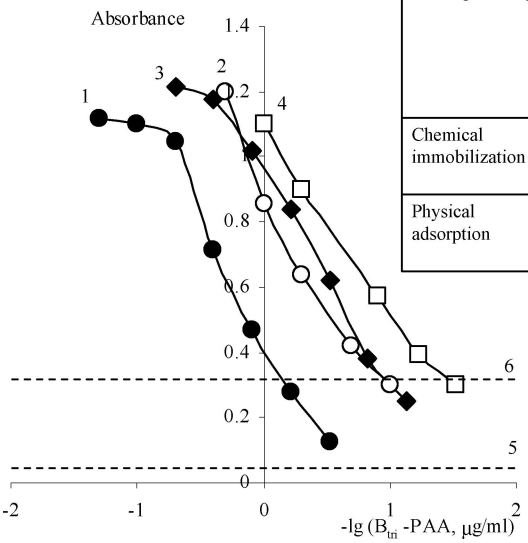
Chemical immobilization of a carbohydrate was performed using glycoconjugates of m.w. 30 kDa (B_{tri}-$pNPA^{30}$) and 2,000 kDa (B_{tri}-$pNSA^{2000}$). Both polymers contained 20% mol. of oligosaccharide exactly as is in Glyc-PAA, but in contrast to the latter one, these polymers were polyacrylic acid derivatives where 80% of the carboxy groups were activated either by *p*-nitrophenol or *N*-hydroxysuccinimide. Immobilization was performed using NH_2-polystyrene that according to the manufacturer's data contains 2×10^{13} NH_2 group/cm^2. Activated groups of B_{tri}-$pNPA^{30}$ or B_{tri}-$pNSA^{2000}$ are capable of interacting with amino groups on the surface forming covalent amide bond. At the final stage excessive active groups are amidated with aqueous ammonia or 2-ethanolamine, *i.e.* finally the chemically immobilized polymers are the same substituted polyacrylamides as the physically adsorbed ones. The nature of side groups on the backbone, *i.e.* $-CONH_2$ or $-CONHCH_2CH_2OH$ is not essential for the further immunochemical tests. Immobilization in a wide range of starting polymer concentrations successfully takes place only in DMSO, whereas in aqueous buffers B_{tri}-$pNPA^{30}$ and B_{tri}-$pNSA^{2000}$ can be immobilized only at a relatively high concentration, 200 and 100 ng/well, correspondingly.

Comparison of physical adsorption and chemical immobilization

The dependence of absorbance in ELISA from glycoconjugates coating/chemosorption concentration was standard under the selected conditions. It was found that in the case of the 30 kDa polymer, the chemical immobilization was more effective (Figure 2) as compared to physical adsorption, *i.e.* absorbance value 1.0 was achieved at conc. 2 μg/ml of B_{tri}-$pNPA^{30}$, whereas 5 μg/ml of B_{tri}-PAA^{30} was necessary for this. In the case of high m.w. polymers, both methods demonstrated comparable results (Figure 2, curves 2, 4).

Comparing chemical immobilization of high m.w. polymer, B_{tri}-$pNSA^{2000}$ (Figure 2, curve 4) and low m.w. one, B_{tri}-$pNPA^{30}$ (Figure 2, curve 2), it can be seen that the results are better in the first case. However, it is necessary to note a high background level (0.3–0.5 OD) of the used aminated polystyrene, *i.e.* absorbance value in the wells not containing the antigen or containing it, but without antibodies added; the background does not disappear after glycoconjugate immobilization. It was not possible to lower the background of aminated plates using neither physical blocking (BSA, gelatin, Tween) nor chemical modification with the help of acetic anhydride. Oppositely, physical adsorption led to low background values of 0.03–0.05 OD.

When physical adsorption of the Glyc-PAA conjugates was performed at a coating concentration of 5–10 μg/ml, the low and high m.w. variants did not differ from each other with respect to absorbance values after the binding with antibodies in



Coating technique		Conjugate concentration (μg/ml), resulting absorbance value ~ 1.0	Background (conjugate is omitted)
Chemical immobilization	B_{tri}–pNPA	2.0	0.3 – 0.5
	B_{tri}–pNAS	0.5	
Physical adsorption	B_{tri}–PAA^{30}	5.0	0.03 – 0.05
	B_{tri}–PAA^{2000}	0.5	

Figure 2. Binding of anti-B_{tri} antibodies to physically adsorbed B_{tri}-PAA^{30} (1), physically adsorbed B_{tri}-PAA^{2000} (2, in optimal conditions), chemically immobilized B_{tri}-PAA^{30} (3), and chemically immobilized B_{tri}-PAA^{2000} (4). Plots 5 and 6 correspond to background for normal plates and aminated plates, respectively.

ELISA. However, when decreasing the coating concentration of B_{tri}-PAA^{30}, absorbance value decreased sharply, whereas in the case of high m.w. polymer notable absorbance deterioration was observed only in the case of a concentration decrease of one order of magnitude (Figure 2). The detailed comparison in concentration range further used for ELISA provides the following result: a high m.w. coating solution in a concentration of 0.5 μg/ml is equivalent to a low m.w. coating solution in a concentration of 5 μg/ml.

Measurement of the absolute value of physically adsorbed glycoconjugate

In order to reveal what part of Glyc-PAA introduced to a polystyrene plate well is really adsorbed on plastic and what part is washed and practically thrown away after the procedure finishing, we have synthesized ^{3}H-Leu-labeled B_{tri}-PAA^{30} and -B_{tri}-PAA^{2000}. The amount of introduced Leu was so small that it did not change adsorption properties of the polymers (data not shown). Adsorption was performed in optimal conditions selected for each conjugate (see above). Radioactivity of both solid phase and solutions both before and after adsorption was measured, allowed the evaluation of the absolute amount of the adsorbed substance. It can be seen (Figure 3) that only two percent of total B_{tri}-PAA^{30} is really adsorbed from the solution on plastic, whereas this value reaches 21% for B_{tri}-PAA^{2000}.

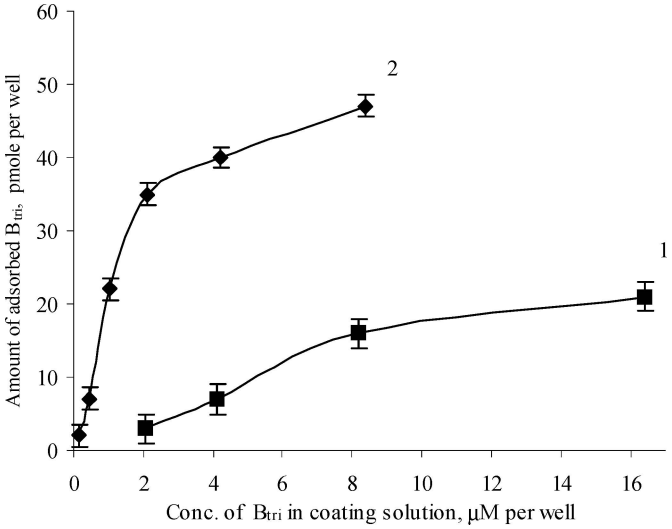


Figure 3. Dependence of physical adsorption of radioactively labeled B_{tri}-PAA^{30} (1) and B_{tri}-PAA^{2000} (2) on polystyrene from solution concentration, see immobilization conditions in Experimental.

Inhibition of erythrocyte agglutination

In order to compare B_{tri}-PAA^{30} and B_{tri}-PAA^{2000} in respect to their avidity to antibodies *in solution* the standard hemagglutination assay in inhibitory mode was used. Agglutination tests were performed with human blood group III (B) erythrocytes and monoclonal IgM antibodies B8 known to be B_{tri} specific. B_{tri}-PAA^{2000} in concentration 5 μg/ml completely inhibited erythrocyte agglutination whereas minimal concentration of B_{tri}-PAA^{30} causing inhibition was 500 μg/ml, *i.e.* one hundred times higher. A_{tri}-PAA^{2000} in concentration 500 μg/ml taken as a control did not affect agglutination, this confirming the test specificity.

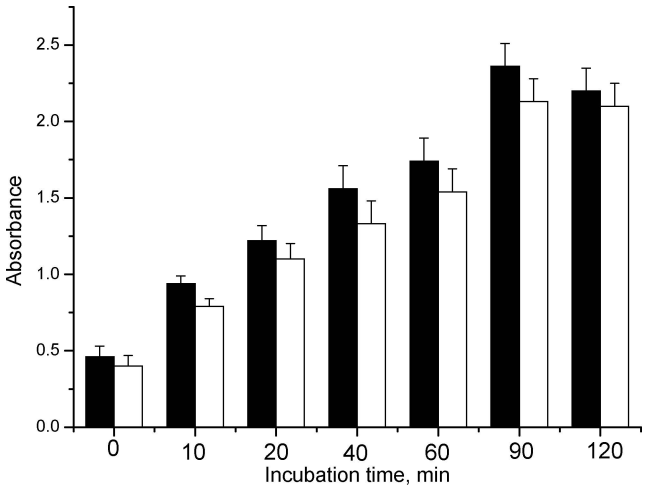


Figure 4. Kinetics of anti-B antibodies/coating antigen interaction in ELISA. Comparison of B_{tri}-PAA^{30} (white bars) and B_{tri}-PAA^{2000} (black bars), antigen loading 26 ng/well.

Rate of formation of antigen-antibody complex, comparison of B_{tri}-PAA^{30} and B_{tri}-PAA^{2000}

Time optimization for the binding of antigen with antibody is necessary for obtaining the better signal/background value in ELISA and to minimize the procedure duration. Adsorption conditions and antigen concentrations were selected in such a way that the amount of the really adsorbed low and high m.w. conjugates was 26 ng/well (24 h at room temperature, B_{tri}-PAA^{30} coating concentration of 20 μg/ml, that of B_{tri}-PAA^{2000} 1.25 μg/ml). It can be seen (Figure 4) that absorbance reaches the reasonable value of 1.5–2.0 for 40–60 min. It was also interesting (taking into consideration agglutination data, see above) to reveal whether high m.w. conjugate more avid to antibody binding on the surface comparing to 30 kDa analog. Actually, no difference between the coated low and high m.w. conjugates taken in the given concentration was observed (Figure 5).

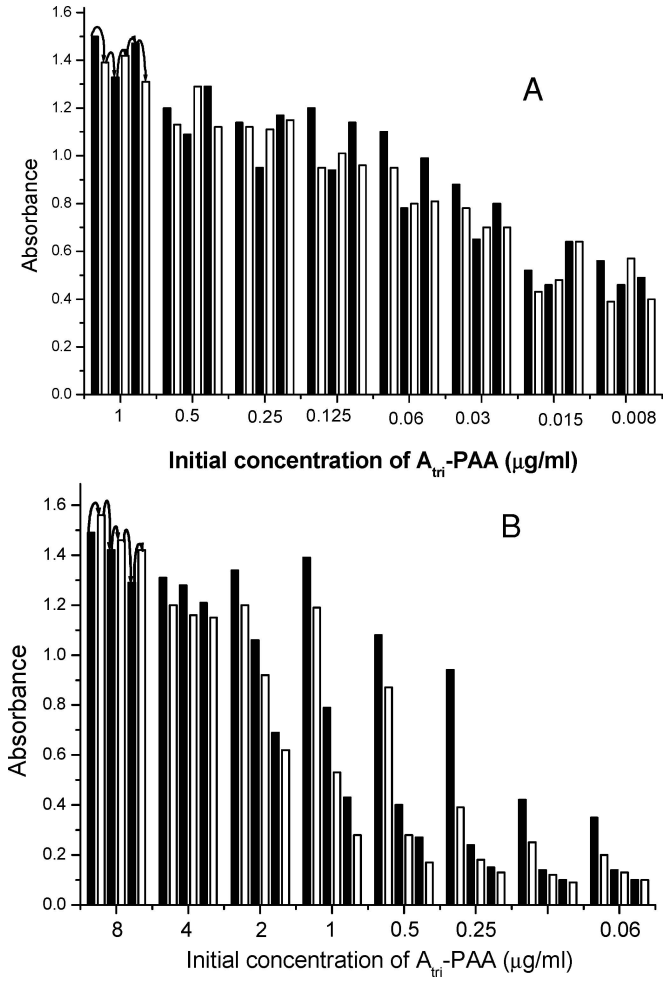


Figure 5. 'Exhaustion' experiments: the conjugate in the noted initial concentration was coated onto well. After 1 h incubation the solution was transferred to the next well, incubated, etc. Finally, anti-A antibodies were added followed by standard development (see Experimental). (A) A_{tri}-PAA^{2000} conjugate; (B) A_{tri}-PAA^{30} conjugate.

Antigen exhaustion experiment

As showed the experiment with radiolabeled Glyc-PAA, only a small part of substance in solution is adsorbed on surface. This result can be explained by at least three reasons: firstly, only a small fraction of polymer is capable of good adsorption; secondly, a limited number of adsorption active sites is present on plastic surface; thirdly, adsorption proceeds slowly. To select between these options we performed an exhaustion experiment, *i.e.* after standard incubation the Glyc-PAA solution was transferred to the next well, etc.; identical procedure was performed with eight different concentrations of Glyc-PAA (Figure 5).

High m.w. A_{tri}-PAA^{2000} was not exhausted during five subsequent transfers even in the lowest starting concentration of 8 ng/ml; gradual decrease of absorbance during the passage from 1 μg/ml to 8 ng/ml is explained by slowed down diffusion of macromolecules in gradually diluted solutions. Another pattern was observed in case of low m.w. A_{tri}-PAA^{30}; already at starting concentration of 2 μg/ml the distinct exhaustion effect was observed. The obtained data correlate well with the first suppositions: only a small fraction of Glyc-PAA^{30} is capable of efficient adsorption, whereas in composition of Glyc-PAA^{2000} the adsorption-active fraction is dominating.

Discussion

Much effort was done in order to improve the coating of carbohydrate antigen onto a solid phase. Chemical immobilization of saccharides on aminated polystyrene using divinylsulfone, was described [15]. The method is characterized by execution simplicity; grafted saccharides obtained in such a way can be used for the study of carbohydrate-binding proteins, however there is a weak point: attachment of divinylsulfone to polyols

proceeds statistically, *i.e.* hydroxyl group principal for biointeraction could be involved into the tethering. Moreover, quite a large amount of saccharide (~10 nmol/well) is necessary due to low yield of conjugation. Chemical immobilization of polysaccharides on polystyrene using photoactivated anthraquinone attached to 1-OH of polysaccharide has been described in [16]. It has also been proposed to precoat polystyrene with copolymer of methylvinyl ester and maleinic anhydride. Saccharides were attached by 1-OH to reactive groups of this copolymer using bifunctional spacer containing hydrazide or amino functions. Despite the simplicity of the method, not all saccharides were immobilized successfully with the copolymer and not all of them displayed biological activity after immobilization [17,18]. 1-OH derivatives of mono- and simple oligosaccharides were covalently linked to the solid surface via long hydrophobic cyanurchloride-activated linker [19].

In addition, large amount of saccharide, 20–200 nmol/well, was necessary in all the described examples. It should be also noted that the hydrophobic linker between saccharide and the surface increases the risk of nonspecific interactions in the further bioassay.

The cited examples illustrate a generic contradiction of the covalent immobilization strategy: to decrease the consumption of the immobilized substance it is necessary to lower its concentration to micromole level, this leading to decrease in reaction rate due to critically low concentration of this substance. This contradiction can be overcome by the use of high m.w. conjugate bearing multiple (hundred or more) groups active for immobilization in composition of one molecule. It is sufficient for such a conjugate to be attached to the surface by a single covalent bond resulting in simultaneous grafting of dozens or even hundreds of carbohydrate ligands (Figure 1).

In this study we have demonstrated that polymers containing multiple activated groups can be chemically immobilized on aminated surface with reasonable yield. Considerable gain in conjugate consumption is observed in case of B_{tri}-pNPA^{30} (Figure 2). This distinguishes favorably the proposed approach from cited above chemical methods. However, due to high background level observed in the followed bioassay, we had to refuse chemical immobilization. It seems that the procedure of polystyrene chemical modification leads to increase of its nonspecific interaction with proteins that can not be abolished by usual "blocks", such as albumin or Tween; anyhow, we failed to find such a method (unpublished results), although, perhaps other surface modification chemistries could allow this (see, for example, [8]).

Importantly, conventional physical adsorption methodology demonstrated dramatic improvement when heavy conjugate of 2000 kDa was used instead of that with m.w. 30 kDa, *i.e.* getting the same absorbance values in ELISA required one order of magnitude less amount of high m.w. polymer. Moreover, in this case the background value remained very low. We believe that physical adsorption has a generic advantage over chemical immobilization: it does not generate new potential sites of interaction with proteins (as chemical approaches do), moreover, it seems to mask the already existing "extra" active sites on high-capacity polystyrene. Ten-fold decrease of carbohydrate antigen consumption means that ~microgram amount of saccharide is sufficient for performing of several solid-phase assays when studying anti-carbohydrate antibodies or lectins. It should be noted that recently developed methodology [20] gives possibilities to synthesize quantitatively microamounts of Glyc-PAA from the corresponding aminospacered derivatives, as well as glycopeptides and glycosyl amines. Herein (see Materials and Methods) we describe a protocol for conjugation 100 nmol of spacered saccharide with PAA^{2000}, *e.g.* quantity sufficient for coating of 10–20 plates. Thus, at present it becomes real to design carbohydrate-binding assays with oligosaccharides available only in minute amounts, in particular, glycoprotein N-chains separated on analytical HPLC column, for the study of carbohydrate-binding proteins using normal 96- or 384-well polystyrene plates.

However, it remained unclear whether the limit of glycoconjugate consumption had been reached using Glyc-PAA^{2000} coating, *i.e.* it was necessary to find out which part of the taken Glyc-PAA was adsorbed on the surface and which one rested unbound. To reveal this the adsorption of radioactively labeled conjugates was tested. It was found that only about 2% of B_{tri}-PAA^{30} (when coating concentration was 5 μg/ml) was strongly adsorbed on plastic. In case of B_{tri}-PAA^{2000} (0.5 μg/ml) this value was 21% (Figure 3). When increasing the mentioned concentrations of coating conjugate, the absolute amount of adsorbed material increases weakly, whereas adsorption yield (per cent of adsorbed substance) correspondingly decreases.

The second rationale for the synthesis of high m.w. Glyc-PAA was the guaranteed realization of multivalent interaction between glycoconjugate and antibody. The distance between Fab-fragments in IgG is ~100 Å (Y-like conformation) or ~150 Å (T-like conformation). It is also important that in T-like conformation the Fab-fragments are oriented oppositely, *i.e.* maximally unfavorably for bivalent binding with one adsorbed Glyc-PAA molecule. On the other hand, the size of Glyc-PAA^{30} is ~90 Å, so the binding of the one molecule with two Fabs of the same IgG (any conformation) is not possible. Oppositely, the size of Glyc-PAA^{2000}, about 500 Å, is deliberately larger than 150 Å between IgG Fabs (Figure 6B). For 10-valent IgM molecule, which size is several times larger the multivalent 1:1, binding is furthermore allowed. As for subunit (multivalent) lectins, the above said should be true for them because the distance between carbohydrate-binding sites of the subunits can exceed 100 Å.

However, despite all the mentioned above concerning the possibility of multivalent binding of immunoglobulins with Glyc-PAA on a surface, the data of Figure 4, *i.e.* the fact that anti-B_{tri} antibodies equally bound both low and high m.w. conjugates seemed surprising for us. Analogous results were obtained using other monoclonal and polyclonal antibodies

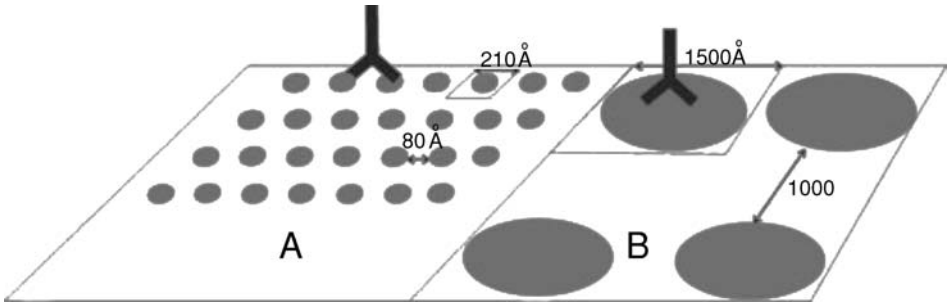


Figure 6. Expected disposition of Glyc-PAA molecules on the polystyrene surface and modes of their binding to bivalent (for simplicity) antibodies; a reconstruction of experimental situation when the weight amount of the adsorbed material is the same (occupies ∼14% of the well surface). The amount of conjugate (both types) adsorbed in one well is about 10^{-8} g, assuming the well area is 90 mm^2 or 9×10^{15} $Å^2$. (A) 30 kDa conjugate, one molecule occupies the area of 45,000 $Å^2$ or a square with dimensions ∼210 × 210 Å, diameter ∼90 Å, distance between molecule peripheries is ∼80 Å; the antibody is able to bind the adsorbed antigen bivalently only by 1:2 manner (to two adjacent molecules) but not 1:1. (B) 2000 kDa conjugate, one molecule occupies the square with dimensions ∼1500 × 1500 Å, diameter ∼500 Å, distance between molecule peripheries is ∼1000 Å; the antibody is able to bind the adsorbed antigen only by 1:1 manner. Electron microscopy data (not shown) give the following approximate average values of molecule size: <90 Å for Glyc-PAA^{30} and 500–600 Å for Glyc-PAA^{2000}.

(data not shown). In contrast, in case of inhibitory ELISA [21] and inhibition of erythrocyte agglutination (see Results), *i.e.* when Glyc-PAA binds the same antibody in solution, the difference between Glyc-PAA^{30} and Glyc-PAA^{2000} can reach factor of 100 and even more. We explain the similarity of surface-immobilized molecules and their principal difference in inhibitory assay (when interacting with the same antibody) by the following. In reality, despite the fractionation, the so called low m.w. polymer contains small amount of high m.w. fraction. If its proportion is several percent, this amount is sufficient for efficient coating, taking into account that only two percent of Glyc-PAA^{30} is really adsorbed onto plastic. The exhaustion experiments (see Figure 5 and comment in Results) are in full agreement with the presence of *minute though essential* fraction of high m.w. polymer in composition of Glyc-PAA^{30}. If this speculation is true, the real situation on surface is described by Figure 6B, independently of starting material, Glyc-PAA^{30} or Glyc-PAA^{2000}.

Concluding remarks

Despite intense development of microarrays [22] there is no doubt that 96- or 384-well-based technique will remain routine and most popular for many years.

The use of high m.w. conjugates Glyc-PAA^{2000} instead of common Glyc-PAA^{30} as coating antigen in ELISA allows reducing 10–20 times the amount of carbohydrate required.

Synthesis of initial activated polymer is simple and well reproducible in respect of molecular weight, its conjugation with oligosaccharides is quantitative and a reliable procedure allowing microscale synthesis.

The next publication will be dedicated to bridging the technology described above with HPLC, *i.e.* conjugation of C1-modified N-glycans with the polymer and use obtained high m/w conjugates for characterization of lectins specificity.

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