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High Molecular Weight Blood group A Trisaccharide-Polyacrylamide Glycoconjugates As Synthetic Blood Group A Antigens For Anti-A Antibody Removal Devices

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Abstract

Specific immunoadsorption of blood group antibodies by synthetic antigens immobilized on support matrices in the peri-transplantation period provides a promising solution to hyperacute rejection risk following ABO-incompatible transplantation. In this study, we investigated binding interactions between anti-A antibodies and synthetic blood group A trisaccharide conjugated with polyacrylamide of different molecular weights (30 and 1000 kDa). The glycopolymers were equipped with biotin tags and deposited on streptavidin-coated sensor chips. The affinity and kinetics of anti-A antibodies binding to glycoconjugates were studied using surface plasmon resonance (SPR). The high molecular weight conjugate (Atri-PAA¹⁰⁰⁰-biotin) enhanced antibody binding capacity by two to three fold compared to the low molecular weight conjugate (Atri-PAA³⁰-biotin), whereas varying the carbohydrate content in Atri-PAA¹⁰⁰⁰-biotin (20 mol% or 50 mol%) did not affect antibody binding capacity of the glycoconjugate. The obtained results suggest that immunoadsorption devices, especially hollow fiber-based antibody filters which are limited in available surface area for antigen immobilization, may greatly benefit from the new synthetic high molecular weight polyacrylamide glycoconjugates.

Keywords

ABO-incompatible transplantation; Glycoconjugates; Hyperacute rejection; Immunoadsorption; Kinetics; Surface Plasmon Resonance

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Introduction

In ABO-incompatible organ transplantation, pre-existing anti-A and/or anti-B antibodies of the transplant recipient bind to the blood group A and/or B antigens present on the graft endothelium.^{1,2} Binding of antibody activates complement pathways, resulting in endothelial cell disruption, platelet adhesion, intravascular thrombosis, and hyperacute organ rejection due to insufficient blood circulation in the graft.³ The donor pool could be enhanced by 30-40% by overcoming this immunological barrier.⁴ ABO-incompatible kidney and liver transplantation appears feasible by removing anti-A and/or anti-B antibodies from circulating blood of the organ recipient in the peri-transplant period.⁵⁻¹² Following initial successful ABO-mismatch renal transplantations reported by Alexandre et al. in 1985¹³, and Bennett et al. in 1987¹⁴ and the subsequent successful long term outcomes^{5,15,16}, several protocols have been developed and used in ABO-mismatch kidney, liver and heart transplantation in a few transplantation centers around the world.⁵⁻¹²

Traditionally, ABO-incompatible transplantation is preceded by several sessions of plasmapheresis (with membrane filters or centrifugal separators) followed by infusion of fresh frozen donor plasma or replacement albumin solution. In many cases, the procedure has been coupled with intensive immunosuppressive protocols and splenectomy.^{8,9} Total plasma exchange with fresh frozen donor plasma is limited by donor supply and is also associated with the risks of infection transmission and immunological reactions to non-autologous plasma. Replacement with albumin solution depletes the recipient of requisite proteins, including clotting factors.^{17,18} Alternatively, in some cases, specific immunoadsorption of anti-A/B antibodies from plasma has been performed.^{6,7} Synsorb® from Chembiomed (Alberta, Canada), BioSorbent A and B developed by Rieben et al.¹⁹ and Glycosorb® from Glycorex transplantation AB (Lund, Sweden) are examples of affinity columns specifically made for removing anti-A/B antibodies from human plasma. These devices incorporate synthetic A or B antigens immobilized on porous beads. Following plasmapheresis, separated plasma passes through the affinity columns and anti-A/B antibodies are specifically adsorbed by immobilized A or B antigens. Although in most cases plasmapheresis is coupled with immunoadsorption, minimal replacement fluid is required as plasma is returned to blood circulation.^{6,7,10-12} Our group previously reported development of a fiber based anti A/B immunosorption device (SAF) in which the synthetic A or B antigens were covalently attached to the luminal surface of hollow fiber membranes²⁰. Hollow fibers were perfused with whole blood and the SAF device selectively removed anti-A/B antibodies in a one step process through high affinity interaction between immobilized A or B antigens and the corresponding antibodies.^{20,21}

Specificity, biocompatibility, high affinity, and proper accessibility for target binding antibodies are the keys to successful immunosorption in hemoperfusion devices. Both the specificity and biocompatibility of synthetic A and B antigens conjugated to short aliphatic hydrocarbon chains (up to 6 carbon atoms in length) or to poly-N hydroxyethylacrylamide (50 kDa) have been previously established in several studies.^{19,22-24} Affinity and accessibility of small haptens (such as blood group A and B trisaccharides) for target binding molecules (such as anti-A and anti-B antibodies) could be greatly enhanced by using a flexible spacer in linking the small molecule to the support matrix.²⁵⁻²⁶ Flexible spacers

increase lateral mobility of immobilized haptens and facilitate the binding interaction.²⁵ The use of spacers is even more crucial when hollow fibers are used as support matrices because of their limited surface area for ligand immobilization. Polymeric spacers such as polyethylene glycol (PEG), poly-L-Lysine (PLL) and polyacrylamide (PAA) have been previously proposed as linkers to enhance binding affinity and removal capacity of surface.^{19,20,25,26} Synthetic A and B antigens have been used both with relatively short aliphatic chains (6-17 carbon atoms in length) as in Synsorb® and Glycosorb® columns^{23,27} or with longer multivalent PAA spacers (30-50 kDa).^{19,20} Nevertheless, limited antibody binding capacity is still the major challenge especially with the hollow fiber based antibody removal devices. In the previous work by Gautam et al.,²⁰ blood group A trisaccharide antigens coupled to PAA spacers of 30 kDa molecular weight (Atri-PAA³⁰) were attached to the luminal surface of hollow fibers in a small scale SAF device. Initial evaluation of anti-A antibody removal capacity and capture rate in this small scale SAF device (scaled down from a full size dialysis cartridge with total surface area of about 1m²) indicated that a 4 to 5-fold increase in removal capacity per unit surface area of the module was required to achieve 100% antibody removal in a clinically relevant set up.

In this work, we conjugated blood group A trisaccharide antigens to high molecular weight (1000-2000 kDa) PAA. This new, high molecular weight glycoconjugate was investigated as an alternative synthetic A antigen for antibody removal devices. The glycopolymers equipped with biotin tags were deposited on streptavidin covered sensor chips. Antibody removal capacity of the high molecular weight glycoconjugate (Atri-PAA¹⁰⁰⁰-biotin) immobilized surface was 2-3 times higher than with the low molecular weight glycoconjugate (Atri-PAA³⁰-biotin) immobilized surface. The carbohydrate content in Atri-PAA¹⁰⁰⁰-biotin (20 mol% or 50 mol %) did not affect antibody binding capacity of the glycoconjugate. To assess the strength of the binding interactions, the kinetics and affinities in interactions between several clones of anti-A antibodies and (Atri-PAA¹⁰⁰⁰-biotin) were measured and compared to reference values for monoclonal antibody-antigen interactions. The results suggest that immunoadsorption devices, especially hollow fiber-based antibody filters which are limited in surface area for antigen immobilization, may greatly benefit from the new synthetic high molecular weight polyacrylamide glycoconjugates.

Materials and Methods

All experiments were performed using a Biacore® 3000 optical biosensor (Biacore AB, Uppsala, Sweden), equipped with research grade streptavidin-coated sensor chips (SA) (Biacore AB, Uppsala, Sweden). The temperature of the instrument was set at 25°C and data were collected at a collection rate of 5 Hz.

Reagents

All reagents and chemicals were obtained from Biacore AB (Uppsala, Sweden) unless otherwise specified. Filtered and degassed HBS-EP buffer at pH 7.4 contained 0.01 *M* N-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid [HEPES], 0.15 *M* sodium chloride (NaCl), 3 *mM* ethylenediaminetetraacetic acid [EDTA] and 0.005% polysorbate-20 [v/v]. HBS-N buffer contained 0.01 *M* HEPES and 0.15 *M* NaCl, at pH 7.4.

Monoclonal Anti-A Antibodies

Murine monoclonal anti-A antibodies were all of immunoglobulin M (IgM) class; Clone F98 7C6-4 (401 µg/ml) was obtained from Dominion Biologicals, Ltd. (Dartmouth, NS, Canada), Clone A-16 (37 µg/ml) was from Hematological Center (Moscow, Russia), and the Anti-A series1 (47 µg/ml) was from Immucor Inc. (Norcross, GA, USA). The stock concentration of each of the monoclonal antibodies was measured by enzyme linked immunosorbent assay (ELISA) as described before.²⁴

Plasma

Samples of fresh frozen plasma type B from three different healthy donors were obtained from the Central Blood Bank of Pittsburgh. Blood typing and measurement of anti-A/B antibody titers were performed according to the standard blood bank procedure for hemagglutination assay.²⁸ Briefly, plasma samples were serially diluted in 0.9% Saline solution. One drop of either A1 or B reference red blood cells (Immucor Inc., Norcross, GA, USA) was added to each tube and tubes were centrifuged for 1 minute at 1380×g. Tubes were then examined for agglutination of A1 and B red blood cells. Anti-A and anti-B titers were recorded as the reciprocal of the largest dilutions that agglutinated A1 and B reference blood cells, respectively. As expected for type B plasma, all three samples agglutinated type A but not type B blood cells. Anti-A antibody titer in plasma samples (#2) and (#3) were 16 and in sample (#1) the measured titer was 32.

Synthesis of Polyacrylamide Glycoconjugates

Specific determinant fragments of blood group A trisaccharide antigen (Atri), GalNAcα1–3[Fuca1–2]Gal–, and B trisaccharide antigen (Btri), Gala1–3[Fuca1–2]Galβ–, were synthesized as ω-aminopropyl glycosides as previously described.²⁹ 30 kDa poly(4-nitrophenyl acrylate) (pNPA³⁰) and 1000 kDa poly(N-oxysuccinimidyl acrylate) (pNSA¹⁰⁰⁰) were synthesized as previously described.^{30,31} The glycoconjugates containing 20 or 50 mol % of the A or B trisaccharides and 5 mol% of biotin were also prepared according to the previously described method.³⁰ Briefly, aminopropyl glycosides (2 or 5 mmol) and biotin-NH(CH₂)₆NH₂ (0.5 mmol) were added to a solution of 10 mmol activated polymer (pNPA³⁰ or pNSA¹⁰⁰⁰) in DMSO. The reaction mixture was kept at 40°C for 24 hours. The exhaustive immobilization of the aminoligands to the polymers was confirmed by thin layer chromatography (TLC). The remaining active ester groups in the polymer were quenched by treatment with ethanolamine. The synthesized glycoconjugates were purified by gel-permeation chromatography.

Immobilization of Synthetic Antigens on SA Sensor Chips

Flow cells of SA sensor chips were primed with running buffer (HBS-EP), followed by three consecutive injections of 90 µl of 1 M NaCl in 40 mM sodium hydroxide (NaOH) at a flow rate of 90 µl/min. The immobilization protocol varied slightly for kinetic and binding capacity studies. In binding capacity experiments, antigens were immobilized at maximum surface loading capacity, which ensured that antibody removal was not limited by the amount of immobilized antigens on the surface. Glycoconjugate solution (5 µg/ml in HBS-N buffer) was injected over the surface at a flow rate of 2 µl/min until a plateau in the binding

curve was observed, signifying that equilibrium had been reached. This typically happened within five minutes of sample injection. Repeated injections of the glycoconjugate solution after reaching equilibrium did not increase the surface loading density. For kinetic studies, a low level of antigen loading was desired to minimize the effect of diffusion limitation on the kinetic analysis.³² Immobilization was performed by injecting 5 μ l of the glycoconjugate solution (1 μ g/ml in HBS-N buffer) over the specified flow cells at a flow rate of 2 μ l/min. The amount of glycoconjugates immobilized on each surface was quantified by subtracting the baseline response level of the glycoconjugate solution before the injection from the baseline level after injection. Thus variations of synthetic A antigens (Atri-PAA¹⁰⁰⁰-biotin or Atri-PAA³⁰-biotin) were immobilized on test sensor chips. Control surfaces for measurement of non-specific binding levels included immobilized synthetic B antigens (Btri-PAA¹⁰⁰⁰-biotin or Btri-PAA³⁰-biotin) or immobilized spacers (PAA¹⁰⁰⁰-biotin).

Measurement of Anti-A Antibody Removal Capacity of Immobilized Antigens

Samples of different clones of IgM monoclonal anti-A antibodies (A16, Anti-A series1 and F98 7C6-4) were diluted to one-half their original stock concentrations in HBS-EP buffer. Each sample was then injected at a flow rate of 20 μ l/min over the surface of a sensor chip specifically prepared for binding capacity studies. Each injection was continued until a plateau in the response sensogram indicated that equilibrium was reached. After injections, the surfaces were regenerated by a single 30 second injection of 50mM NaOH solution and consequently, monoclonal antibody samples diluted to 75% of their original concentrations were injected over regenerated surfaces. Similar binding levels of monoclonal antibodies diluted to 50% and 75% their original concentrations ensured that the reported binding levels represented the maximum surface removal capacity for a given antibody. Similarly, polyclonal anti-A antibodies in plasma type B were injected over sensor chips. Unlike monoclonal antibodies, we did not dilute plasma samples because initial measurement of anti-A antibody titer in plasma samples indicated several folds lower antibody concentrations in plasma samples compared to the stock solution of monoclonal antibodies. Anti-A antibody removal capacity of immobilized antigens in each case was considered to be the equilibrium binding level in resonance units (RU). All surfaces were regenerated, by a single 30 second injection of 50mM NaOH solution at a flow rate of 90 μ l/min to remove bound monoclonal or plasma antibodies. This regeneration protocol resulted in complete removal of bound antibodies from surface. Repeated injections of monoclonal antibodies or plasma samples over each regenerated surface resulted in reproducible antibody binding levels on the surface which indicated that immobilized glycoconjugates were stable.

Statistical Analysis

The significance of the difference between the binding levels on different surfaces were evaluated using a two-tailed student's t-test with $p < 0.05$ indicating a significant difference. The difference between binding levels of different plasma samples on different surfaces were compared using one-way analysis of variance (ANOVA).

Measurement of the Kinetics of Antibody-Antigen Interactions

Monoclonal antibodies were all diluted in the dilution buffer (HBS-EP) to the following final concentrations: 0.01, 0.02, 0.04, 0.08, 0.15, 0.30, 0.61 and 1.22 nM for A-16

antibodies; 0.7, 1.4, 2.9, 5.8, 11.7 and 23.5 nM for Anti-A series1; and 0.1, 1.7, 3.5, 6.9, 13.9, 55.6 and 111.1 nM for clone F98 7C6-4 antibodies. The range of antibody concentrations was chosen to cover a complete range of binding levels to immobilized Atri-PAA¹⁰⁰⁰-biotin. Antibodies at each concentration were injected twice over the control and test surfaces. Injections were performed at a flow rate of 75 µl/min for three minutes in a random concentration order. Each injection was followed by a 10 minute dissociation time and a single injection of 10 mM glycine-hydrochloric acid (Glycine-HCl) (pH 1.5) at a flow rate of 90 µl/min for 30 seconds to remove the bound antibodies from the surface. A two minute stabilization time after the regeneration step followed each cycle. In all of the above experiments, a blank injection of the running buffer was included. Analysis of the kinetic data was performed as described before.³³ Briefly, the association constant (k_a) for each set of antibody-antigen interaction was obtained by globally fitting the association phase of experimental data into the following 1:1 interaction model: $dR/dt = k_a C(R_{max} - R) - k_d R$, where C represents the concentration of the antibodies in the solution, R represents the binding level at time t , and R_{max} represents the surface capacity of the immobilized antigen in resonance units. The dissociation constants (k_d) were obtained from global fitting of the dissociation phase of sensograms to the simplified form of the 1:1 interaction model: $\ln(R_o/R) = k_d t$, where R_o represents the response at the beginning of the dissociation phase. Equilibrium dissociation constants (K_D) were calculated as the ratio of k_d over k_a . For each anti-A antibody clone, k_a was reported as the average of two independent measurements and k_d was measured as the average of a series of measurements for the range of antibody concentrations studied in the experiment.

Results

Effect of the Size of PAA on Antibody Removal Capacity of Glycopolymers

Our goal was to evaluate the effect of molecular weight of polyacrylamide scaffold on the antibody removal capacity of Atri-PAA-biotin conjugates. Atri-PAA³⁰-biotin and Atri-PAA¹⁰⁰⁰-biotin (containing 20 mol% and 5 mol% of carbohydrate and biotin, respectively) were synthesized. Analogous glycoconjugates containing B antigens (Btri-PAA³⁰-biotin and Btri-PAA¹⁰⁰⁰-biotin) and the polymer without carbohydrate residues (PAA¹⁰⁰⁰-biotin) were used as controls for the SPR experiments. All glycoconjugates were immobilized on flow cells of SA sensor chips. Immobilization was performed until maximum loading on each surface was reached. The maximum loading capacity of surface was estimated 1 ng/mm² for both 30 and 1000 kDa glycoconjugates. The molar carbohydrate content was 20 mol% for all glycoconjugates, which led to equal loading of the chips with blood group A and B trisaccharides. Three clones of monoclonal anti-A antibodies and three different samples of plasma type B (polyclonal anti-A antibodies) were injected over immobilized antigens on control and test surfaces to measure maximum antibody removal capacity of each surface. The nonspecific binding level of monoclonal antibodies on the control surfaces (Btri-PAA³⁰-biotin and Btri-PAA¹⁰⁰⁰-biotin) was less than 10% of their binding level on all test surfaces. To obtain the net binding levels, the nonspecific binding levels on control surfaces were subtracted from the binding levels on test surfaces.

Figure 1 shows the net binding levels for the three clones of monoclonal antibody binding to immobilized conjugates (Atri-PAA³⁰-biotin or Atri-PAA¹⁰⁰⁰-biotin). Atri-PAA¹⁰⁰⁰-biotin showed greater capacity for monoclonal antibody removal compared to Atri-PAA³⁰-biotin. The percentage of increase in capacity was significant ($P<0.05$), but different between the three clones of antibodies (60% for A16, 30% for Anti-A series1 and 15% for F98 7C6-4). Figure 2 shows the removal capacity of different surfaces in interactions with polyclonal anti-A antibodies present in three plasma samples of type B donors. Anti-A antibody binding levels to Atri-PAA¹⁰⁰⁰-biotin increased by 150% for two of the plasma samples (#1 and #3) and by 88% for sample (#2) compared to their binding level on Atri-PAA³⁰-biotin. Average nonspecific binding levels of plasma antibodies to control surfaces were 50% and 24% of their specific binding to test surfaces containing immobilized Atri-PAA³⁰-biotin and Atri-PAA¹⁰⁰⁰-biotin, respectively. All three plasma samples showed equal binding levels to all three control surfaces (Btri-PAA¹⁰⁰⁰-biotin, Btri-PAA³⁰-biotin or PAA¹⁰⁰⁰-biotin). Thus, the increase in size of the PAA linker did not increase the nonspecific binding on surface. This confirmed that the difference between the binding levels on low and high molecular weight glycoconjugates was due to an increase in accessibility of blood group A trisaccharide conjugated to high molecular weight PAA.

Effect of the Carbohydrate Content on Antibody Removal Capacity of Glycopolymers

We hypothesized that in the polyacrylamide glycoconjugates such as Atri-PAA-biotin, the space between the carbohydrate residues was not fixed. A randomly coiled flexible polyacrylamide backbone could provide multivalent binding with antibodies by adjusting the spacing between copies of A trisaccharides. Based on this hypothesis, an increase in carbohydrate content of Atri-PAA¹⁰⁰⁰-biotin from 20 mol % to 50 mol % could drastically affect the antibody removal capacity of glycopolymers by changing their flexibility. To check this hypothesis, Atri-PAA¹⁰⁰⁰-biot with 20 mol % and 50 mol % of blood group A trisaccharide content were synthesized. We did not observe a significant difference in antibody removal capacity of Atri-PAA¹⁰⁰⁰-biot with 20 or 50 mol % A trisaccharide content (Figure 3). Any further increase in the A trisaccharide content is unlikely to improve the binding capacity of surface, possibly due to an increase in the steric hindrance between the binding antibodies when PAA becomes too closely populated with conjugated A trisaccharides.

Binding Kinetics of Anti-A Antibodies to High Molecular Weight Glycopolymers

We chose Atri-PAA¹⁰⁰⁰-biot as the candidate synthetic A antigen because it showed the greatest capacity for anti-A capture. We then measured the kinetics of binding between Atri-PAA¹⁰⁰⁰-biot and monoclonal anti-A antibodies. Atri-PAA¹⁰⁰⁰-biot and Btri-PAA¹⁰⁰⁰-biot were immobilized on test and control flow cells of SA sensor chip at loading levels of 0.046 and 0.056 ng/mm², respectively. Binding curves and complete kinetic analysis for Atri-PAA¹⁰⁰⁰-biot interaction with one set of monoclonal antibodies (A16) are shown in Figures 4, 5 and 6. Figure 4 shows net binding curves obtained by subtracting response levels on control flow cells from response levels on test flow cells during double injections of A-16 antibodies (with concentrations ranging between 0.01 and 1.22 nM) over Atri-PAA¹⁰⁰⁰-biot. Repeated injection of antibodies at each concentration following the surface regeneration steps resulted in reproducible antibody binding levels indicating that immobilized Atri-

PAA¹⁰⁰⁰-biot were stable on test surfaces throughout the experiment. As shown in Figures 5.A and 5.B there was good agreement between our experimental data during the association phase and the fitting curves generated based on the 1:1 interaction model ($R^2 > 0.96$ for all fits). The dissociation phase demonstrated two stages, with faster antibody release occurring during the initial stage. This data deviated from the model in the plot of $\ln(R_o/R)$ versus time (Figure 6.A), most likely due to bivalent or multivalent binding of a fraction of the binding IgM antibody population. Due to the comparable sizes of IgM and high molecular weight glycoconjugates, multivalent binding interaction between IgM antibodies and Atri-PAA¹⁰⁰⁰-biot is expected. To minimize these effects in our analysis, we adopted the method explained by Morton et al.³⁵ and utilized only the initial stage of dissociation data in our calculations of the kinetic dissociation constants (Figure 6.B). This method excludes the population of antibodies that show smaller dissociation rate constants due to multivalent binding to immobilized A trisaccharides. Table 1 summarizes the association and dissociation rate constants obtained for all the three clones of anti-A antibodies.

Discussion

Polymer beads or hollow fiber membranes on which synthetic antigenic determinants of A and B blood groups are immobilized have been previously used for specific removal of anti-A or anti-B antibodies.^{19,20,23} Membranes are associated with less pressure drop, faster flow rates, and less diffusion limitations than packed beds of porous beads,³⁶ making them the more attractive option as support matrices in hemoperfusion systems. However, lower removal capacity due to limited surface area for ligand immobilization is a major obstacle in membrane adsorption techniques. In this work, we conjugated 1000 kDa PAA spacers to synthetic blood group A trisaccharide antigens and compared their anti-A removal capacity with the previously used 30 kDa glycoconjugates.²⁰ The increase in the size of PAA conjugates resulted in two to three fold increase in removal capacity per unit surface area. With this estimated increase in capacity, a hollow fiber based antibody removal device (such as the SAF device) with a surface area of about 1.5 to 2 m² would provide sufficient capacity for 100% antibody capture from blood in a single device.

Limited studies have been performed to evaluate the effect of the size of PAA on affinity and removal capacity. Shilova et al.³¹ observed no difference in the anti-B antibody binding levels on to low (30-50 kDa) or high (1000-2000 kDa) molecular weight Btri-PAA glycoconjugates physically adsorbed on ELISA plates. However, high molecular weight Btri-PAA conjugates inhibited type B erythrocyte agglutination at a 100 times lower anti-B antibody concentration than low molecular weight Btri-PAA in the solution phase reaction.³¹ We hypothesized that PAA glycoconjugates immobilized on streptavidin covered surfaces are presented in two ways. In one scenario, Atri-PAA-biotin molecules are coupled to surface by all of their biotin residues thereby adopting the “stretched” conformation (Figure 7.A). This conformation reduces mobility of the polymer backbone and interferes with interaction of the glycoconjugate with antibodies. The size of flexible loops between tethers on sensor chip surface should be equal for low and high molecular weight Atri-PAA-biotin provided that the density of biotin groups is the same for both glycopolymers. Thus, antibodies will interact equally with Atri-PAA-biotin glycopolymers regardless of their molecular weight. In the second situation, Atri-PAA-biotin polymers bind to the surface by

one or a few biotin molecules, and the glycopolymers keep their conformation close to a random coil (Figure 7.B). The diameter of Atri-PAA¹⁰⁰⁰-biotin and Atri-PAA³⁰-biotin molecules in the random coil conformation is estimated to be 500-600 Å and 90 Å respectively.³⁰ IgM antibodies (about 300-400 Å) are comparable in size to Atri-PAA¹⁰⁰⁰-biotin and are therefore capable of binding to most of the immobilized glycopolymer molecules, whereas the size of IgM antibodies does not allow binding of them to each of immobilized Atri-PAA³⁰-biotin molecules (Figure 7.B). Our results agreed well with the second scenario, i.e. high molecular weight glycoconjugates provided better accessibility of blood group A trisaccharides for antibody binding. The second scenario could also explain the earlier observed difference between antibody binding levels to glycoconjugates of different molecular weights in the solution phase.³¹

We measured the kinetics of interaction between our candidate glycoconjugates (Atri-PAA¹⁰⁰⁰-biotin) and anti-A antibodies to assess the strength of the binding interactions between synthetic antigens and anti-A antibodies. Association rate constants ranging between 10^3 and 10^7 M⁻¹s⁻¹ and dissociation rate constants ranging between 10^{-6} and 10^{-2} s⁻¹ have been previously reported for interactions between various pairs of monoclonal antibodies and their specific antigens.³⁷⁻⁴² The kinetic and affinity constants measured in this study fall within the range of antibody-antigen interaction kinetic constants, with a trend towards higher affinity interactions.

Two different levels of antigen loading on SA sensor chips were used in our studies. For our kinetic analysis, we attempted to immobilize the minimum amount of Atri-PAA¹⁰⁰⁰-biot (0.046 ng/mm²) on SA sensor chips to minimize the effect of mass transfer on the kinetic analysis.^{32,43,44,45} The low loading level of antigens also minimizes crowding of antigens on the surface and as a result, multivalent interactions between binding IgM antibodies and immobilized antigens are minimized. This behavior was ideal for quantification of the kinetics of interaction between single antibody binding sites (Fab) and immobilized Atri-PAA¹⁰⁰⁰-biot. However, to mimic the real application in SAF when measuring the binding capacity of surface, antigens needed to be immobilized on the surface at their maximum loading capacity (1 ng/mm²). This ensured that the binding reaction was not limited to the amount of available antigens on surface and that steric effects were reflected in our measurements of the surface's removal capacity.

We previously developed a mathematical model capable of predicting the dependence of antibody removal rate on key design parameters in our proposed SAF devices.^{20, 46} This mathematical model established that maximum antibody removal rate could only be achieved in SAF devices under a diffusion limited antibody transport regime. In other words, optimum capture rate occurs only when antibody removal becomes independent of antibody-antigen reaction rates.⁴⁶ The dimensionless Damkohler number (Da) defined as

$$\left(Da = \frac{K_a C_s^i a}{D} \right)$$

was used to determine the conditions needed for antibody transport to occur in

a diffusion-limited regime. Based on this model a Da number of 10 or greater was required to achieve maximum antibody removal rate in the proposed SAF design. Both the surface binding capacity (C_s^i) and the intrinsic association rate constant (K_a) for antibody-antigen

interaction are required for accurate calculation of Da and an assessment of our design of antibody filters. The intrinsic association constants (k_a) were directly measured for three sets of monoclonal anti-A antibodies and were found to be 18, 1.2 and 0.52 cm³/nmol.s for clones A16, series 1 and F98 7C6-4. The magnitude of Da was then calculated for each set of monoclonal antibodies binding to Atri-PAA¹⁰⁰⁰-biot assuming a surface capacity (C_s^i) of 0.0017 nmol/cm² (twice the surface capacity measured previously for Atri-PAA³⁰-biot by Gautam et al.²⁰). The antibody diffusivity coefficient (D) was assumed to be 3.9×10⁻⁷ cm²/s and the fiber inner radius (a) was 0.01 cm. The value of Da for each monoclonal antibody is listed in Table 1. Values of Da for all three antibody clones were greater than 10, supporting the hypothesis that antibody removal rate becomes diffusion limited and independent of the specific affinity for antibody-antigen interaction in an Atri-PAA¹⁰⁰⁰-biotin-immobilized hollow fiber based antibody removal device.

Further experimental work is required to establish the bioactivity and biocompatibility of Atri-PAA¹⁰⁰⁰-biotin in antibody capture from whole blood in the SAF device. Rieben et al.¹⁹ previously characterized the biocompatibility of Atri-PAA³⁰ immobilized on porous particles in their interaction with plasma antibodies. Hout et al.²¹ described the feasibility of antibody capture from whole blood in an earlier-generation SAF device with Neutr-AB®, an animal based A and B glycoprotein antigen, immobilized on inner lumens of the hollow fibers. We propose that the custom-made high molecular weight glycopolymer we have presented is a promising synthetic A antigen for construction of anti-A immunoadsorption devices. A SAF device which incorporates these high molecular weight glycoconjugates as synthetic A antigens may aid in meeting the target anti-A antibody titer reduction of three to four titer steps.

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Figure 1.

Antibody removal capacity of glycopolymer immobilized surfaces. Low (Atri-PAA³⁰-biot) and high (Atri-PAA¹⁰⁰⁰-biot) molecular weight glycopolymers were deposited on streptavidin-coated sensor chips at the loading density of 1 ng/mm². Three clones of IgM anti-A antibodies (A16, Immucor and Dominion) were injected over glycopolymer immobilized surfaces until equilibrium was reached. The amount of bound antibodies per unit surface area was measured in resonance units (1000 RU~ 1 ng/mm²).

Figure 2.

Antibody removal capacity of glycopolymer immobilized surfaces (Atri-PAA³⁰-biot and Atri-PAA¹⁰⁰⁰-biot) in their interactions with polyclonal anti-A antibodies. Analogous glycoconjugates containing B antigens (Btri-PAA³⁰-biotin and Btri-PAA¹⁰⁰⁰-biotin) and the polymer without carbohydrate residues (PAA¹⁰⁰⁰-biotin) were used as controls. The loading density of glycopolymers on each surface was 1 ng/mm². Anti-A antibody titer in plasma samples #1, #2 and #3 were 32, 16 and 16, respectively. RU represents the amount of bound antibodies per unit surface area in resonance units (1000 RU~ 1 ng/mm²).

Figure 3.

Effect of the carbohydrate content on antibody removal capacity of glycopolymer immobilized surfaces. Atri-PAA¹⁰⁰⁰-biot molecules with 20% or 50% A trisaccharide content were deposited on streptavidin-coated sensor chips at a loading density of 1 ng/mm². Three plasma samples and a sample of monoclonal IgM anti-A antibodies (Immucor) were injected over glycopolymer immobilized surfaces until equilibrium was reached. Anti-A antibody titer in plasma samples #2 and #3 and Immucor antibodies were 16 and in plasma sample #1 the antibody titer was 32. RU represents the amount of bound antibodies per unit surface area in resonance units (1000 RU~ 1 ng/mm²).

Figure 4.

Real time plot of monoclonal anti-A antibody (A16) binding level on Atri-PAA¹⁰⁰⁰-biot immobilized surface. Atri-PAA¹⁰⁰⁰-biot molecules were deposited on streptavidin-coated sensor chips at a loading density of 0.046 ng/mm². A16 antibodies were injected over Atri-PAA¹⁰⁰⁰-biot immobilized surface at various concentrations (0.01, 0.02, 0.04, 0.08, 0.15, 0.30, 0.61 and 1.22 nM).

Figure 5.

Measurement of association constant for binding interactions between A-16 antibodies (at concentrations of 0.15, 0.30, 0.61 and 1.22 nM) and Atri-PAA¹⁰⁰⁰-biot immobilized surface.

A: First order rate constant (k_s) was measured as the slope of the plot of dR/dt versus R at each antibody concentration. R represents the antibody binding level on surface at any time point (t) in resonance units (RU). **B:** Plot of the first order rate constants (k_s) versus antibody concentrations (C). The slope of the plot corresponds to the association rate constant, k_a .

Figure 6.

Measurement of dissociation constant for binding interactions between A-16 antibodies and Atri-PAA¹⁰⁰⁰-biot immobilized surface. **A:** Plot of $\ln (R_o / R)$ versus time demonstrates two stages of dissociation phase with faster antibody release occurring during the initial stage. R and R_o represent the antibody binding level on surface at any time point (t) and at the beginning of the dissociation phase, respectively. **B:** Linear regression of $\ln (R_o / R)$ versus time for the initial dissociation stage. The slope of the plot corresponds to the dissociation constant, k_d .

Figure 7.

Two hypothetical presentations of immobilized glycopolymers on surface. **A:** Atri-PAA-biotin polymers are coupled to surface by all of their biotin residues thereby adopting the “stretched” conformation. **B:** Atri-PAA-biotin polymers bind to the surface by one or a few biotin molecules, and the glycopolymers keep their conformation close to random coil.

Table I

Kinetic and equilibrium affinity constants for binding interactions between three clones of IgM anti-A antibodies and Atri-PAA¹⁰⁰⁰-biot.

Monoclonal Antibody Clone and Source	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)	$Da = \left(\frac{k_a C_s^i a}{D} \right)$
A-16 (Hematological Center)	$(1.8 \pm 0.1) \times 10^7$	$(3.8 \pm 2.2) \times 10^{-4}$	2.1×10^{-11}	785
Series1 (Immucor)	$(1.2 \pm 0.2) \times 10^6$	$(1.4 \pm 0.7) \times 10^{-3}$	1.2×10^{-9}	52
F98 7C6-4 (Dominion)	$(5.2 \pm 1.2) \times 10^5$	$(6.4 \pm 3.7) \times 10^{-3}$	1.2×10^{-8}	23

^a k_a and k_d represent association and dissociation constants; K_D represents equilibrium dissociation constant ($K_D = k_d / k_a$).

^b Da represents the Damkohler number in a hollow fiber based antibody removal device. The magnitude of Da was calculated assuming a surface capacity (C_s^i) of 0.0017 nmol/cm², an antibody diffusivity coefficient (D) of 3.9×10^{-7} cm²/s and a fiber inner radius (a) of 0.01 cm.