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An Effective and Rapid Method for Functional Characterization of Immunoadsorbents Using POROS Beads and Flow Cytometry

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To facilitate the construction, functional characterization, and use of immunoadsorbents, we have developed a flow cytometry method that allows rapid assessment of large numbers of particle-bound antibodies. Protein G derivitized POROS beads were used to bind affinity-purified antibodies specific for synthetic peptides designed from human plasma proteins. The antibodies were covalently coupled to the beads and used to capture and release synthetic peptides that had been labeled at the C-terminus with the fluorochrome Alexa Fluor 488. Antibody coupling and specificity of antigen binding and release were measured by analysis of the POROS affinity beads by flow cytometry. The affinity-capture matrixes were also used through several antigen-binding and release cycles without loss of peptide binding efficiency. The ability to produce and characterize extremely small amounts of POROS affinity matrices will facilitate their use in protein microchemical procedures such as protein chip technology, monoclonal antibody screening and mass spectrometry, applications where analytes are limiting or present in low abundance in complex mixtures.

Keywords: POROS • perfusion chromatography • affinity capture • antibodies • flow cytometry • nanobore • mass spectrometry

1. Introduction

Antibodies have long been used as tools for identification, isolation, and quantitation of molecules in both basic and applied research. Their utility primarily depends on their remarkable specificity and their ability to bind with appropriate affinity to antigens that are often present in low amounts in complex mixtures. The demand for antibodies for many applications is growing exponentially, due in large part to the power and success of protein/peptide mass spectrometry and the post-genomic focus on proteins. Many thousands of new antibodies, both polyclonal and monoclonal, will be produced for use in a variety of methods each requiring different antibody characteristics. Unfortunately, those who work with antibodies know only too well that they behave idiosyncratically and that finding the "correct" antibody for a particular use is a challenge. In particular, assessment of antibodies for use in immunoadsorbents is labor intensive and slow, and the difficulties encountered often preclude screening of more than a few antibodies. This is made more complex by the fact that many of the relevant antigens have not been purified or are available in limited quantities, making antibody selection and functional characterization more difficult. Here, we describe a method for production and functional characterization of immunoadsorbents using only small amounts of materials. The method takes advantage of several desireable qualities of POROS beads, including their high binding capacity and flow characteristics and their stability and size, which allow their direct analysis by flow cytometry. The method is particularly useful for characterizing the effectiveness of specific immunoadsorbents prior to construction of nanoscale affinity columns used for on-line antigen separation upstream of mass spectrometry.

2. Materials and Methods

2.1 Antibodies. Polyclonal antibodies specific for synthetic peptides (designed from sequences of human plasma proteins; see the following paper by Anderson et al.) were prepared by immunizing rabbits. The proteins from which the sequences were derived and the sequences of the peptides are shown in Table 1. For production of antisera, the tryptic peptide sequences were synthesized with a C-terminal extension (Gly-Ser-Gly-Cys) in order to provide a cysteine thiol spaced away from the immunizing sequence by a relatively nonantigenic linker. This thiol was used to covalently attach the peptides to cationized bovine serum albumin carrier for immunization, for coupling to plates for enzyme-linked immunosorbent assay (ELISA), and for linking to a fluorescent chromophore for characterization of immobilized antibody supports by flow cytometry. These synthetic peptides were designated IMM 2−5 (for immunizing peptides 2−5). One additional peptide (IMM 6 from tumor necrosis factor-α; TNF-α) was also synthesized with an N-terminal extension (Cys-Gly-Ser-Gly) in order to

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Table 1. Peptides Used as Haptens for Immunization of Rabbits and for Labeling with Alexa Fluor 488

protein source	peptide	identification code: rabbit antibody	identification code: AlexaFluor 488-peptide conjugates
interleukin-6 (IL-6)	EALAENNLNLPK*GSGC*	Ab 2	ALX 2
hemopexin (Hx)	NFPSPVDAAFR*GSGC*	Ab 3	ALX 3
α_1-antichymotrypsin (AAC)	EIGELYLPK*GSGC*	Ab 4	ALX 4
tumor necrosis factor-α (TNF-α)	DLSLISPLAQAVR*GSGC*	Ab 5	ALX 5
tumor necrosis factor-α (TNF-α)	*CGSG*DLSLISPLAQAVR	Ab 6	ALX 6

explore possible specificity differences related to orientation of the peptide. An integrated commercial procedure (Express Antibody Protocol; Biosource International, Hopkinton, MA) was used to generate peptides, peptide-carrier conjugates for immunization, antisera and affinity purified antibodies. Two rabbits (for each peptide) were immunized with 4 injections of peptide-carrier conjugate and two bleeds from the animal yielding the higher titer in ELISA were pooled as the source of polyclonal antiserum. Antibodies were affinity purified on peptide-agarose conjugates and were eluted with pH 2.5 glycine-HCl followed by immediate neutralization with Tris buffer pH 9.5. Antibody concentrations were determined spectrophotometrically using an OD_{280} value of 1.43 = 1.0 mg IgG/mL. Affinity purified antibodies were assessed for purity by 1-D gel electrophoresis and staining by colloidal Coomassie Brilliant Blue G-250.[1] A portion of each antibody preparation was biotinylated, following the manufacturer's instructions, using sulfo-NHS-LC-biotin (Pierce, Rockford, IL) for use on streptavidin-coupled POROS beads (see below).

2.2 Preparation of POROS Affinity Beads. In pilot experiments, biotinylated, affinity-purified antibodies were exposed to streptavidin-coupled BA20−005 Self-Pack POROS beads (Code 2−3111−00; Applied Biosystems, Foster City, CA) in suspension, washed and used without covalent cross-linking. Subsequently, to achieve a more oriented binding, the affinity-purified IgGs were bound noncovalently to Self-Pack POROS 20G Protein G beads (Code 1−5128−02; Applied Biosystems, Foster City, CA) in suspension, and then covalently cross-linked. Both types of beads were stored prior to antibody addition as a 10% (v/v) slurry at 4 °C in 20% (v/v) ethanol as supplied by the manufacturer. To prepare the beads for incubation with antibodies, 2.5 mL of the stock 10% bead slurry were centrifuged at 1000 × *g* for 5 min, and the pelleted beads were washed twice with 10 mL of loading buffer (10 mM phosphate pH 7.2/0.15 M NaCl) at room temperature (RT). The washed beads were suspended in loading buffer to give a 10% slurry and 500 μL were dispensed into each of five 1.5 mL Eppendorf microcentrifuge tubes. Different peptide-specific, affinity-purified antibodies (0.5 mg in 750 μL of loading buffer) were added to each tube (to give a total volume of 1.25 mL), the contents mixed and antibodies allowed to bind at RT for 30 min with constant shaking. The beads were pelleted by centrifugation at 1000 × *g* for 3 min and the supernatant was saved for antibody quantitation by measurement at OD_{280} nm. With the Protein G beads, after 2 more washes with loading buffer, the beads were pelleted and resuspended in freshly prepared cross-linker (30 mM dimethyl pimelimidate; Sigma Chemical Company, St. Louis, MO, in 200 mM triethanolamine, pH 8.5). After 30 min incubation at RT, the beads were centrifuged, the cross-linker was removed and the pelleted beads were resuspended in 400 μL of quenching solution (150 mM monoethanolamine, pH 9.0) for 30 min. Following this, the beads were washed twice with loading buffer, pelleted, and resuspended in 500 μL of loading buffer/0.1% sodium azide and stored at 4 °C. The method for covalent cross-linking of antibodies to POROS Protein G beads was modified from those previously described for antibody coupling to agarose[2] and to POROS Protein A and G.[3]

2.3 Preparation of Fluorescent Peptides. Peptides were conjugated at their C-terminal cysteine residues by incubation with Alexa Fluor 488 C_5 maleimide (Molecular Probes, Eugene, Oregon) using a procedure based on a previously described method [4] and on instructions from the manufacturer. Briefly, peptides were dissolved in dimethyl sulfoxide (2.0 mg/mL) and 50 μL (100 μg) were incubated for 3.5 h at 20 °C with 25 μL of the Alexa Fluor 488 reagent (10 mM in dimethyl sulfoxide). The reaction was quenched by addition of 38 μL of a 1:1000 dilution of β-mercaptoethanol in PBS (a 2-fold excess of mercaptoethanol over the Alexa Fluor 488 reagent). Conjugated peptides were stored as stock solutions at 4 °C prior to dilution and used in immunoaffinity capture experiments without further purification.

2.4 Analysis of POROS Beads by Flow Cytometry. We chose POROS 20 Protein G beads for this work because their diameter is 20 μm, an ideal size for use in the FACSCalibur flow cytometer with a 71 μm diameter sample injection port. To examine whether the beads were suitable for analysis by flow cytometry, we first tested unlabeled beads and beads that had been incubated with a 1:10 dilution of a fluorescein-conjugated murine IgG_1 monoclonal antibody (Cat No. CLP001F; Cedarlane Laboratories, Hornby, ON). This mAb is specific for surface glycoprotein antigens of procyclic forms of African trypanosomes, (the EP forms of procyclin)[5] and is able to bind to Protein G. The directly fluoresceinated mAb was only used to test antibody binding to the POROS Protein G beads. All incubations and washing steps were performed in 0.3 mL PP Micro Tubes for Micro Sedimentation (Cat No. 72.702, Sarstedt Inc., Montreal, Quebec). POROS Protein G Beads (10 μL of the stock 10% slurry) were dispensed into PP Micro Tubes and washed once with 200 μL of loading buffer containing 0.1% (w/v) octyl β-D-thioglucopyranoside (OTGP). Inclusion of the detergent was required to obtain tight pelleting of the beads. The beads were resuspended in 50 μL of loading buffer (unlabeled control) or 50 μL of a 1/10 dilution of the FITC-labeled anti-procyclin mAb. The tube contents were mixed and antibodies were allowed to bind to the beads by incubation at RT for 30 min with constant rotation on a Labquake Shaker (Labindustries, Berkeley, CA). The beads were pelleted by centrifugation at 1000 × *g* for 3 min and washed twice with 200 μL of PBS containing 0.1% OTGP before analysis by flow cytometry. Forward scatter, side scatter, and fluorescence were determined using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA). For both unlabeled and labeled beads 5000 events were analyzed. The beads were also examined by fluorescence microscopy using a Zeiss standard binocular microscope fitted with an epifluorescence attachment and a Zeiss NeoFluor 63/1.25 oil immersion objective.

2.5 Measurement of Antibody Binding to POROS Beads. An estimate of the amount of antibodies bound by the POROS Protein G beads was determined by measurement of the OD_{280} of the supernatants (containing unbound antibodies) after

initial incubation of the antibodies with the beads, prior to washing and covalent cross-linking.

Detection of antibodies that were covalently coupled to the beads was performed by flow cytometry. In pilot experiments, 10 μL of a 10% slurry of the antibody-coupled or sham-coupled beads were incubated with 10 μL of a 1:100 dilution of either FITC-conjugated goat anti-rabbit Ig (CL6100F; Cedarlane Laboratories Limited, Hornby, ON) or FITC-conjugated goat $F(Ab)_2$ anti-rabbit IgG (H+L) (Caltag Laboratories, Burlingame CA). Incubations were performed for 30 min at 20 °C, followed by 2 washes with 200 μL of phosphate-buffered saline containing 0.1% OTGP. Detection of bound antibodies was also performed using 10 μL of a 1:100 dilution of biotinylated Protein L (No. 29997; Pierce, Rockford IL) followed by 50 μL of a 1:100 dilution of FITC-conjugated streptavidin (CLCSA1001; Cedarlane). For flow cytometric analysis, between 1200 and 5000 events were analyzed for each sample, depending on the individual experiment.

2.6 Measurement of Antigen Binding and Release. Binding of peptide antigens to the POROS immunoaffinity beads was also measured by flow cytometry. Alexa Fluor 488-conjugated peptides (1 μL of a 1/10 dilution of stock) were incubated in Sarstedt PP Micro Tubes for 10 min at room temperature with 10 μL of a 10% suspension of bead conjugates and 10 μL of PBS/0.1% OTGP with end-over end mixing. Beads were washed twice with 200 μL of 0.1% OTGP in PBS and then analyzed by flow cytometry. Elution of antigen peptides was performed by incubating the beads with 1% or 5% (v/v) acetic acid or trifluoroacetic acid (TFA) for varying intervals (5−30 min), followed by 2−5 × 200 μL washes with loading buffer/OTGP. Beads were also examined by fluorescence microscopy after both binding and elution of peptides. Peptide binding and elution were also tested through five cycles in order to test the stability of the immunoadsorbents.

2.7 Preparation of Nanobore Columns for Mass Spectrometry. The characterized POROS immunoaffinity bead matrixes were used to prepare nanobore columns for in-line use with Q-TRAP mass spectrometry (see the following paper by Anderson et al. for methods). Mass spectrometry was used to determine the specificity of peptide binding and this could be compared with the results we obtained by analyzing the same POROS affinity matrixes using flow cytometry. In addition, the degree of peptide enrichment by the different immunoaffinity matrixes could be quantitated by the mass spectrometric technique (see the following paper by Anderson et al.).

Results

3.1 Testing of POROS Beads by Flow Cytometry. We first tested whether the 20 μm diameter beads (both streptavidin-conjugated or Protein G-conjugated) were suitable for analysis by flow cytometry, as this size is near the optimum for the sample injection port of the FACSCalibur instrument. The results from a typical experiment are shown in Figure 1. Panel A shows the forward scatter plot of unlabeled beads. The uniform, symmetrical peak reveals that the beads were uniform in size and that only a minor fraction of the population (small peak at the left) existed in a smaller, probably degraded form. This was confirmed by microscopic examination that showed mainly uniform spheres with only a few small bead fragments. Panel B shows the side scatter profile of the unlabeled beads and indicates that the beads had extremely uniform granularity or structure. Indeed, under the microscope, the POROS beads looked like small round, raspberries of uniform size. Panel C shows the background fluorescence profile (detector set for fluorescein) of unlabeled beads. Fluorescence microscopy showed that there was no visible fluorescence. Panel D shows the fluorescence profile of both unlabeled beads (grey profile) and beads incubated with a fluorescein-labeled murine mAb (anti-procyclin) known to bind to Protein G (green profile). The shift in mean fluorescence intensity after antibody binding was more than 100-fold and shows that fluorescent ligands bound to the POROS were easily detected by flow cytometry.

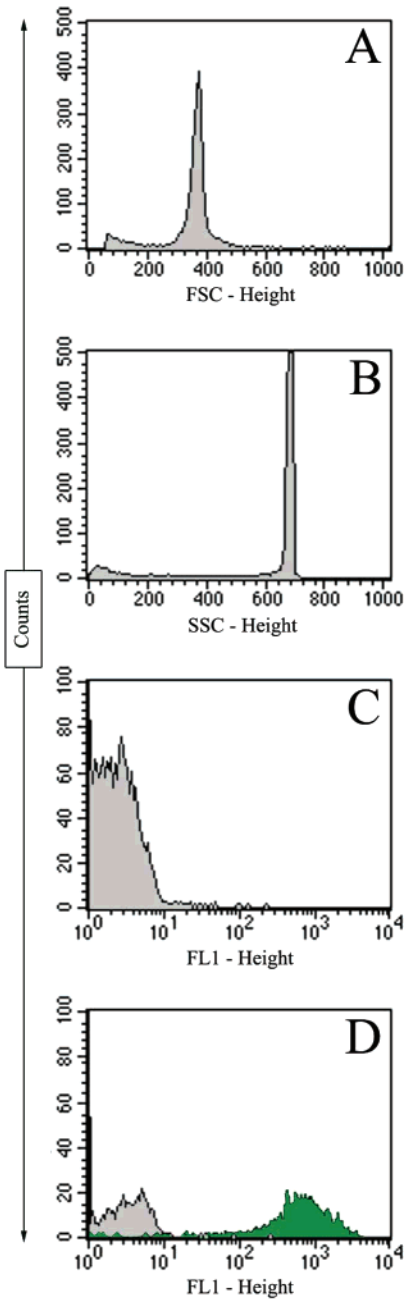

Figure 1. Determination of the suitability of POROS 20G Protein G (20 nm diameter) beads for use in flow cytometry. Panels A, B, and C show forward scatter, side scatter and fluorescence (fluorescein) respectively, of unlabeled beads. Panel D shows the fluorescence profiles of beads before (grey profile) and after (green profile) incubation with a Protein G-binding fluorescein-conjugated monoclonal antibody (mAb 247; anti-procyclin, see Materials and Methods). For each set of beads, 5000 events were analyzed.

3.2 Measurement of Antibodies Coupled to POROS Beads. Initially, we successfully used streptavidin-coupled beads to bind biotinylated affinity-purified antibodies. However, since experiments (not shown) revealed that Protein G-derivatized beads bound antibody more efficiently (and theoretically bound antibody in the correct orientation), they were subsequently used in further work. A crude estimate of antibody binding to the POROS Protein G was obtained by measuring the optical density of the antibody remaining in solution after binding of antibodies to the beads. The coupling efficiencies ranged from 27% (Ab 6) to 87% (Ab 2) suggesting that a good portion of antibodies was attached. A more direct assessment of antibody coupling was determined by flow cytometry using either FITC-labeled second antibodies (anti-rabbit IgG and $(Fab)_2$ portions of anti-rabbit IgG) or biotinylated Protein L followed by FITC-labeled streptavidin. Strong fluorescence was observed with both of the labeled second antibodies after incubation with antibody-coupled beads (not shown). However, not unexpectedly, fluorescence was also observed with both second antibodies on sham-coupled beads that contained no first antibody. As the beads were derivatized with Protein G, all antibodies would be bound directly. Therefore, in all subsequent experiments, biotinylated protein L (followed by FITC-labeled streptavidin) was used to detect bound antibodies since this reagent binds to certain kappa chain sequences of a variety of antibodies of different species, including rabbit,[6] and it does not bind to Protein G. The results of a typical experiment using indirect detection by Protein L are shown in Figure 2. As can be seen in Panel A, the control Protein G beads (no first antibody) did not bind biotinylated Protein L (black profile) whereas the positive control (streptavidin-derivatized beads; green profile) did, indicating that the biotin-Protein L/FITC-streptavidin system has the potential to work with the POROS Protein G beads. When the antibody-coupled beads were tested for binding of Protein L, strong fluorescence was seen with all of the antibodies (Panel B). Differences in fluorescence intensities could be clearly seen, allowing visual ranking of the different matrixes. The calculated median fluorescence intensities were (from the weakest to the strongest): Ab 4: 1094, Ab 2: 1700, Ab 3: 1731, Ab 6: 2128 and Ab 5: 2594. The mean fluorescence intensities were all more than 1000-fold higher than the negative control (black profile; Ab 2 with no biotinylated Protein L), demonstrating the sensitivity of the biotin-Protein L/streptavidin-FITC detection method.

3.3 Testing of Peptide Binding and Release. We measured the binding of each of the five Alexa Fluor 488-labeled peptides (ALX 2−6) and unconjugated Alexa Fluor 488 dye to each of the five antibody supports. An example of one of the flow cytometry profiles obtained is shown in Figure 3. In this example, the Ab 4 affinity beads were shown to bind with high specificity to the "correct" peptide ALX 4 (Panel B) when compared to the "wrong" peptide ALX 6 (Panel C) or to the negative control (Panel A). Tabulated results for all of the antibodies and peptides are presented in Table 2. Each antibody demonstrated substantial specificity with respect to peptide sequence. This is shown more clearly in Figure 4, in which the values for each antibody are normalized to the maximum signal for that antibody. Two major instances of cross-reactivity were apparent. Ab 2 bound peptide ALX 4 slightly more efficiently than it bound its homologous antigen ALX 2, indicating an unexpectedly strong cross-reaction, despite the sequence similarity between the epitopes. The related peptides ALX 5 and ALX 6 (built on the same core tryptic

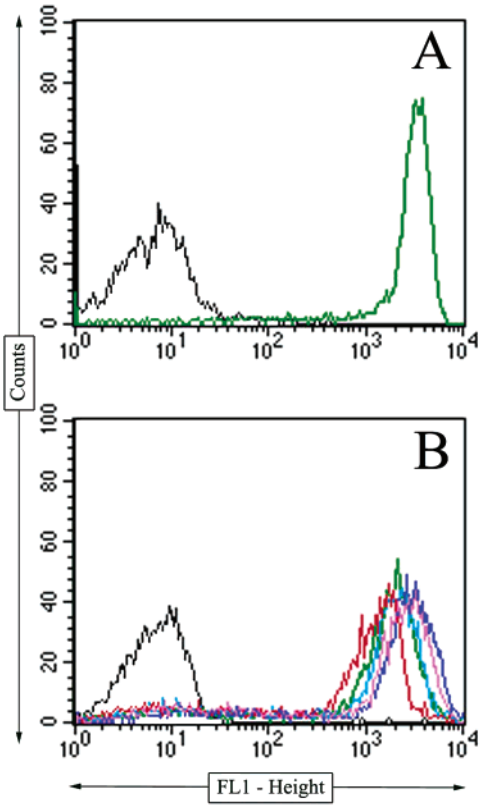

Figure 2. Flow cytometric detection of rabbit anti-peptide antibodies coupled to POROS−Protein G beads. Panel A: Pretesting of biotinylated Protein L and fluorescein-conjugated streptavidin as a detection system on POROS beads. Black profile; POROS protein G beads incubated with biotinylated protein L and fluorescein-labeled streptavidin (negative control). Green profile; POROS Streptavidin beads incubated with biotinylated Protein L and detected with fluorescein-conjugated streptavidin (positive control). Panel B: Detection of covalently coupled rabbit anti-peptide antibodies on POROS Protein G beads. Beads covalently coupled with rabbit affinity-purified Abs 2−6 (see Table 1) were incubated first with biotinylated Protein L, followed by detection with fluorescein-conjugated streptavidin. Profile designations followed by the mean fluorescence values for each affinity matrix were as follows: red, Ab 4 (1094); green, Ab 2 (1700); blue, Ab 3 (1731); pink, Ab 6 (2128) and purple, Ab 5 (2594) in increasing order of fluorescence. The black profile is a negative control (Ab 2 beads with no biotinylated protein L but with fluorescein-labeled streptavidin). For each set of beads, 2500 events were analyzed.

sequence, but extended respectively on the C- and N-termini) also exhibited cross-reaction at a level of 14−22% of the "correct" antigen. This cross-reaction is expected because of the similarity of the peptide sequences and resulting antigenic epitopes. Apart from these cross-reactions, the overall preference of each antibody for its complementary peptide (the corresponding antigen) was pronounced, with an average across the remaining Ab-peptide pairs of 110-fold more binding to correct than "incorrect" peptides (a range of 27- to 292-fold preference for the correct peptide). In absolute terms, the Ab 6 support appeared to bind much less labeled peptide than the other four supports and was not investigated further.

The release of peptides from the POROS affinity matrixes was also tested. Pilot experiments showed that 5% trifluoroacetic acid was more efficient at eluting peptides than 5% acetic

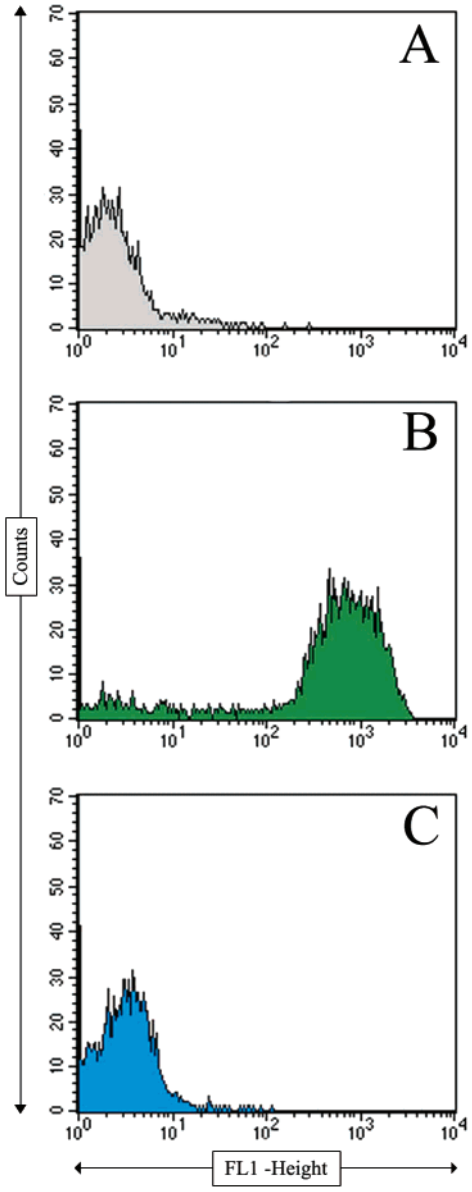

Figure 3. Detection of fluorescent peptide antigens bound to antibody-coupled POROS Protein G beads. Panel A: Ab 4 beads plus Alexa Fluor 488 (negative control). Panel B: Ab 4 beads plus ALX 4 (homologous peptide); Panel C: Ab 4 beads plus ALX 6 (heterologous peptide control). For each set of beads, 2500 events were analyzed.

Table 2. Binding of AlexaFluor 488-Labeled Peptides to POROS Antibody Matrices Measured by Flow Cytometry

	peptide-AlexaFluor 488 conjugates					
POROS-Ab matrices	ALX 2	ALX 3	ALX 4	ALX 5	ALX 6	Alexa Fluor control
Ab 2	1134	5	1275	29	11	11
Ab 3	41	2458	42	54	17	5
Ab 4	32	14	2763	19	9	4
Ab 5	31	11	26	1723	382	3
Ab 6	8	3	3	29	213	2

acid (not shown) thus subsequent experiments were performed with TFA under a variety of conditions. The results with one affinity matrix (Ab 2), are shown in Figure 5. Panel A shows that the "correct" peptide (ALX 2: green trace) was bound with

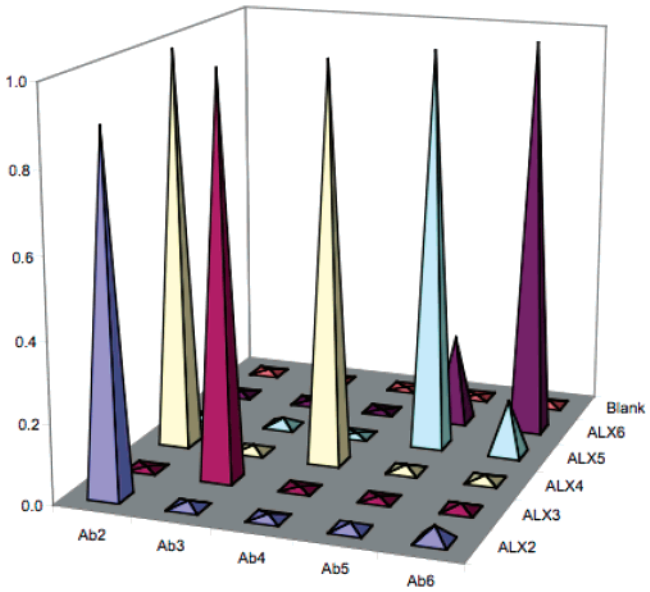

Figure 4. Relative binding of Alexa Fluor 488-labeled peptides to five affinity purified anti-peptide antibodies (Ab 2−5) immobilized on POROS determined by flow cytometry. The binding of four peptides, ALX 2−6, by POROS beads affinity matrixes containing either their homologous (specific) or heterologous (nonspecific) antibodies was analyzed. The values for each antibody are normalized to the maximum fluorescence intensity for that antibody. Each value is the median fluorescence intensity for 1200 flow cytometer events. The gray shading shows the value for the matched antibody-peptide pairs.

high efficiency whereas an "incorrect" peptide (ALX 5: orange trace) was not (approximately 100-fold less binding on the arbitrary, logarithmic fluorescence intensity scale). Some nonspecific binding was apparent with this peptide (compare the red profile with the orange profile in panel A) because beads incubated with the Alexa Fluor 488 dye alone showed much less fluorescence than those incubated with the labeled heterologous peptide. This weak nonspecific binding was also seen with the other labeled peptides (data not shown). Panel B shows the results of the TFA elution experiments. A 30 min TFA treatment eluted peptide ALX 2 from the matrix (compare the red profile with the green profile). Further recycling of the affinity matrix revealed an interesting phenomenon: after acid elution and a second loading of peptide, there was an increased binding of peptide (compare the blue and green profiles in Figure 5B). This effect was also seen in peptide binding and elution experiments using the same POROS affinity matrixes in nanoscale LC columns and using mass spectrometry to measure peptide elution (see the following paper by Anderson et al.). Finally, to test the recyclability of the matrix, five peptide load and elution cycles were performed with 0.1% TFA. The results (Figure 5, Panel C) showed that the POROS matrix could easily withstand the acid elution treatment and retained full peptide binding capacity at least throughout 5 load-elution cycles. In fact, after 5 load-elution cycles, the binding of peptide was increased when compared with the first cycle. The arbitrary fluorescence units in Figure 5C have been adjusted so that the profiles are centered thus the fluorescence intensities cannot be directly compared to data from the separate experiments shown in Figure 5, A and B). It is important to note that both forward and side scatter profiles remained the same from start to finish (not shown), indicating that the POROS bead integrity

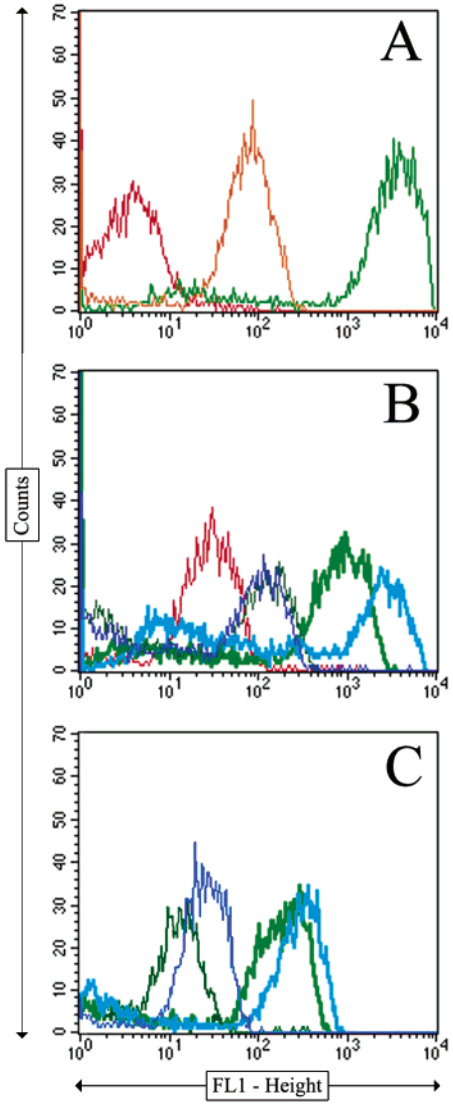

Figure 5. Flow cytometric analysis of peptide binding and release from POROS antibody beads. Panel A: Specificity testing of POROS Ab 2. Green profile: binding of homologous peptide ALX 2. Orange profile: binding of heterologous peptide ALX 5 (specificity control). Red profile: binding of Alexa Fluor 488 dye (negative control). Panel B: Binding and release of peptides from POROS immunoadsorbent Ab 2. Green profile, binding of peptide ALX 2. Thin green trace, binding of peptide ALX 2 after 30 min elution with trifluoroacetic acid (TFA). Light blue profile, repeat binding of peptide ALX 2 after first TFA elution. Dark blue profile, TFA elution after second binding of peptide ALX 2. Red profile, Binding of peptide ALX 5 (specificity control). Panel C: binding and elution of peptide ALX 2 to POROS Ab 2 immunoaffinity matrix after 1 (green) and 5 (blue) load-elution cycles. Note: the fluorescence units are not directly comparable to those in separate experiments shown in parts A and B. For each set of beads, 2500 events were analyzed.

remainedunchanged. Examination of the beads at intervals during the 5 peptide load-elution cycles showed that the beads were uniformly strongly fluorescent when peptides were bound whereas after elution no fluorescence could be seen.

4. Discussion

Those who have worked with immunoadsorbents know that it is often difficult to make them reproducibly and test their effectiveness. Antibody-coupled affinity matrixes are typically used in a column format and bound and eluted antigen is detected by polyacrylamide gel analysis. Such procedures are time-consuming and not particularly quantitative, especially if the antibody being used is not ideal and if the amount of antigen is limiting. These limitations often impede the construction and testing of sets of immunoadsorbents and certainly preclude large-scale screening for the most suitable candidates among large numbers of potentially available antibodies. They also inhibit systematic studies that require generating and characterizing multiple versions of a support, such as optimizing the protocols for cross-linking of antibodies on protein G.

Methods that allow moderate throughput of antibodies for development of micro-immunoadsorbents and identification of bound antigens have been developed[7] but their characterization relies on gel electrophoresis and the adsorbents are not recyclable. Because there is a growing need for antibodies for a variety of proteomics applications, we sought a method that is amenable to screening of large numbers of antibodies for their utility in antigen capture techniques. For example, antibodies are required for use in immunoadsorbents for antigen discovery and identification,[7] for immunodepletion,[3] in biochips,[8] and in nanobore chromatography for antigen concentration prior to identification and/or quantitation by mass spectrometry (see the following paper by Anderson et al.). As described in the current paper, the use of POROS beads and flow cytometry facilitates the functional characterization of immunoadsorbents and theoretically can be scaled up to allow screening of large numbers of antibodies.

POROS beads offer several desireable characteristics as affinity capture matrixes: they can be derivatized with a variety of functional groups, including Protein G (used in the current paper) that allows binding of antibodies in the "correct" orientation, they have a large surface area giving them a high binding capacity and they have excellent flow characteristics, important for rapid chromatographic procedures (see the following paper by Anderson et al.). Most importantly, as shown in the work reported here, their size (20 μm), uniformity, and translucence are perfectly suited for analysis of fluorescence in a flow cytometer. Additionally, the beads are remarkably stable, retaining their physical integrity and function throughout several antigen binding and elution cycles. Thus selection of beads with particular fluorescence characteristics could be achieved by fluorescence-activated cell sorting, thus allowing fine-tuning of immunadsorbent characteristics.

Care must be taken in deciding which reagents to use for detection of antibodies and antigens bound to the POROS adsorbents. If the beads are derivatized with immunoglobulin-binding ligands such as Protein A or G, second antibodies must be avoided as they can bind directly to these ligands. We observed this in our experiments, even when we used $F(ab)_2$ portions of second antibodies to avoid binding of the Fc portions to the Protein G on the beads. Contrary to popular belief, Protein A and G can also bind to Fab portions of immunoglobulins.[9] We therefore used Protein L to specifically detect immunoglobulin on the beads. Protein L is an elongated molecule expressed on the surface of some isolates of *Peptostreptococcus magnus* and binds to the framework regions of the variable domain of κ light chains from any class of Ig.[10] As this interaction is with light chains, there is no Ig class restriction. In addition, although Protein L binds close to the antigen-binding region, it does not affect Ig-antigen interaction.[6]

In the work reported here, Alexa Fluor 488-conjugated peptides were used as antigens and their binding and release from the POROS immunoadsorbents could be directly measured by flow cytometry. For purified antigens, including peptides, proteins and glycoconjugates, this direct labeling approach (through a variety of functional groups) should work well. Detection of binding of antigens that are not easily purified presents a greater challenge. It should be possible to use indirect immunofluorescenceand flow cytometry with antibodies specific to the relevant antigen as long as suitable controls are used to account for background binding to immunoglobulin-binding ligands (if any) on the beads. This could be minimized by using Fab portions of the specific antibody followed by detection with fluorescent Protein A, G, or L.

Measuring the binding of antigen to populations of apparently homogeneous POROS beads revealed that not all beads bind the same amount of antibody or antigen. Indeed, the fluorescence histograms suggest that there are beads of similar size that bind at least 2−3 times as much antibody or antigen as other beads from the same batch. It will thus be possible in future studies to preparatively select high-binding beads from a population by fluorescence-activated bead sorting (in the same instrument used here as an analytical flow cytometer) for improved performance in microscale applications such as MS.

The ability to quantitatively and conveniently assess antigen binding capacity and specificity over successive binding/elution cycles is an important additional feature of the method. Although antibodies are not often reused in most current ELISA or immunoprecipitation protocols, there are important advantages to re-usable antibodies for online enrichment or subtraction, not the least of which is lower cost per cycle. In addition, when antibody amount is limiting, the ability to carry out multiple successive captures from a large pool of antigen allows accumulation of much more antigen than the available amount of antibody.[11] Polyclonal antibody columns have been shown to be recyclable, some hundreds of times,[12] but not all antibodies survive the harsh elution conditions. This fact makes it important to select antibodies that can withstand multiple load-elution cycles.

An ideal immunoadsorbent should have a high enough affinity for the relevant antigen to allow its rapid enrichment from complex mixtures and should be robust enough to withstand multiple binding and elution cycles. Selection of the most effective antibodies thus becomes the most important aspect of immunoadsorbent construction. Which species of animal produces the most suitable antibodies and whether polyclonal or monoclonal antibodies will prove to be the most appropriate remain to be determined. The flow cytometry method using POROS beads described in this manuscript allows a systematic approach to this antibody problem.

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