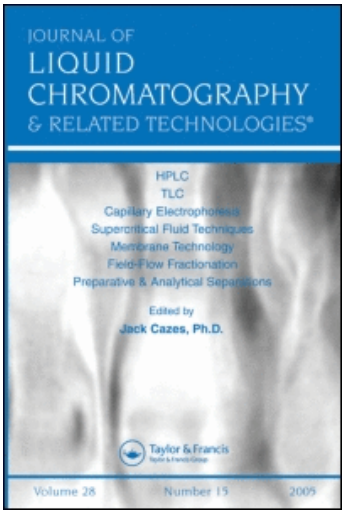

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Use of Modified Chitosan Macrospheres in the Selective Removal of Immunoglobulins

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ABSTRACT

A solution of chitosan in acetic acid was atomized into sodium hydroxide solution to yield chitosan beads that were 400–600 μm in diameter and with a solids content of 3.5%. Chitosan beads were modified to include a spacer arm and end-capped with a carboxyethyl-group containing anionic ligand to generate a support for use in bioseparations. The ligand modified chitosan beads will be further referred to as LMCB. The influence of pH, salt concentration, and chaotropic salts on the binding of immunoglobulins (Igs) to LMCB has been studied. LMCB

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supports allowed the separation of monoclonal antibodies (Mab) from cell culture supernatants. In addition, different Mab sub-species (IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3) were also retained from cell culture supernatants rich in the same and further eluted to yield purified Mab. Elution was possible under mild conditions with a step salt gradient. Overall protein recoveries in the range of 90–95% were obtained with cell culture supernatant. Purity of products obtained form a single chromatographic step was estimated to be greater than 98%.

Key Words: Pseudo-bioaffinity; Antibodies; Bioseparations.

INTRODUCTION

The importance of immunoglobulins (Igs) has been well researched and documented.[1] Purified Ig products have been used for medical treatments in-patients with inadequate Ig levels.[2] As the discovery of new medical and diagnostic uses for Igs arise, an increased need for medical-grade Igs will continue to fuel advances in the area of Ig separation. This may necessitate the design and development of efficient separation and purification processes that yield ample amounts of pure and pathogen free Ig products.

Purification schemes for antibodies from the serum include precipitation,[3–5] ion-exchange chromatography,[6,7] thiophilic chromatography,[8,9] metal chelate interaction chromatography,[10,11] affinity separations using immobilized protein A/G,[12,13] hydrophobic interaction chromatography,[14,15] hydroxyapatite chromatography,[16,17] dyne affinity, and ion-exchange techniques.[18–21] While affinity chromatography that uses protein A/G is specific, both protein A and protein G are macromolecular and fragile, expensive to obtain from bacterial or tissue-culture sources, and are difficult to immobilize without losing activity. The use of protein A and protein G in affinity chromatography is also negatively impacted by the harsh elution conditions. In addition, the use of both protein A and protein G as affinity supports in chromatographic columns poses special challenges regarding regeneration and sanitation.[13]

Some of these drawbacks preclude the use of biological ligands in practical and commercial applications and has prompted many researchers to turn their attentions to the development of synthetic ligands.[22] In contrast, smaller molecules like dyes, amino acids, metal ions, and chemical moieties show comparable affinities, and its specificity can be increased or decreased, either at adsorption or desorption, to attain resolutions and degrees of purification comparable with those of immunoadsorption. Several groups of

researchers have demonstrated the advantages of using such ligands in the separation of human and mouse Igs.[23,24] The use of immobilized amino acids, such as phenyl alanine, tryptophan, and histidine for pseudo-biospecific purification of Igs, has been demonstrated as a promising method on numerous chromatographic supports.[25–27]

The need for increased process efficiency in the production of safe protein based therapeutics continues to fuel research efforts in the field of liquid chromatography. These efforts include the design of more effective and efficient chromatography supports, new separation technologies, and cost-effective techniques to accurately characterize the systems for the purpose of scale-up design. Its abundance in nature and its similarity to cellulose, a widely used chromatography support, have resulted in the use of chitin and chitosan as a support in bioseparations and biomedical applications. The use of chitosan as a chromatography support for wastewater purification and recovery of trace metal ions has been demonstrated.[28,29] Other types of chromatographic applications include the use of highly porous chitosan as an organic acid adsorbent[30] in thin-layer chromatography to separate water-soluble food dyes,[31] amino acids,[32,33] and nucleic acids,[34,35] and in the isolation of carcinogenic heterocyclic amines using an immobilized phase.[36] Despite these applications of chitosan as an adsorbent, in comparison with other polysaccharides such as cellulose, agarose, and dextran, it has attracted relatively little, albeit increasing, attention in the application of chromatography with regard to protein recovery. Studies have shown that modified chitosan matrices are useful adsorbents for selective binding of immunoproteins, and can potentially be an effective sorbent for hemoperfusion to lower the levels of toxic immunoproteins.[27] Researchers have also found an application for the chitosan support in affinity chromatography using an immobilized protein, chicken ovomucoid.[37] Strongly basic chitosan beads, which are reported to be hard and highly porous ion exchangers, are used for the adsorption of serum albumin.[38] Chitosan has also been used in membrane chromatography as an affinity support, by coupling Cibacron Blue® to a macroporous chitosan membrane for the purification of serum albumin,[39] and as an anion-exchange membrane for protein.[33] A method for depositing and cross-linking the chitosan film on the surface of a microporous hollow fiber membrane to isolate IgG has also been investigated.[40]

The aim of the work presented in this paper is to investigate the effectiveness of a novel pseudo-bioaffinity chromatography support that has been synthesized by post-derivatization of bald chitosan beads with a carboxyethyl-group a containing anionic ligand,[41] and termed LMCB or Ligosep Alpha®, for the separations of Igs from complex biological fluids. Support parameters, which impact binding, were determined in order to

gain an understanding of the interaction between the ligand and the protein complex.

EXPERIMENTAL

LigoChem Inc., provided Ligosep Alpha® beads as a generous gift. We will refer to modified beads as LMCB. Lyophilized, 95% pure human IgG (hIgG), lyophilized human serum albumin (HSA), rabbit anti-hIgG, and peroxidase conjugate rabbit anti-hIgG, were purchased from Sigma Co. (St. Louis, MO). Cell culture supernatants rich in monoclonal antibodies (Mabs) (IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3) and purified Mabs were purchased Southern Biotechnology Corporation (Birmingham, AL). Protein A hyper D support was purchased from Sigma Chemical Company (St. Louis, MO). Immunoaffinity separations were performed with a Spectra/Chrom LC column (1.77 cm^2/cm), a Pharmacia C 10/20 column (1 cm^2 i.d.) (Piscataway, NJ), a Cole Parmer Materflex peristaltic pump (Niles, IL), Spectrophotometer, Spectronic® Genesys™ 5 (Rochester, NY), and a BioRad UV monitor (Hercules, CA) was used to monitor chromatography. *O*-Phenylenediamine–2HCl tablets were purchased from Abbott Laboratories (Chicago, IL). NuPage 4–12% Bis–Tris gels were purchased from Invitrogen (Carlsbad, CA) and stained with Gelcode Blue stain reagent from Pierce (Rockford, IL). Gel electrophoresis was carried out with an X-Cell II Novex unit and visualized using a Shimadzu densitometer (Columbia, MD). Immulon II microtiter plates were purchased from Fisher Scientific (Itasca, IL). Plates were read using an automated ELISA plate reader from Bio-tek (Winooski, VT).

Bead Preparation and Chemical Modification

Beads were prepared by atomizing an acidic solution (1–5%) of chitosan into a 0.1 N aqueous sodium hydroxide solution to form porous chitosan beads, which were then removed from the NaOH solution by decantation and filtration. The beads were washed multiple times with distilled water to obtain neutral pH. Chitosan beads 600–800 μm in diameter and with a solid content of 3.5% were selected for this study. The experimental conditions of the chemical modification are detailed elsewhere.[41] Briefly, the bald beads were then reacted with functional diepoxide (1,4 butanediol diglycidyl ether) to yield crosslinked chitosan units and a reactive epoxy terminal group at the distal end of a spacer arm. The reactive epoxide on the distal spacer arm was end-capped with a short chain aliphatic ligand with a carboxylic acid functionality as depicted in Fig. 1.

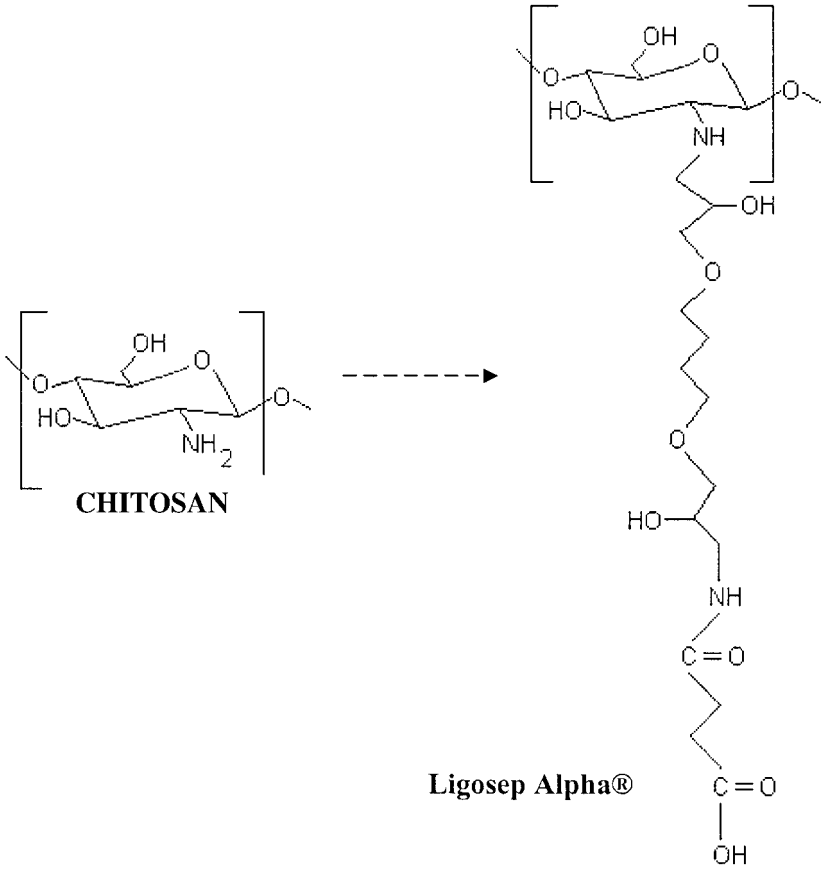

Figure 1. Schematic of the modification chemistry. The chromatographic support material was made with crosslinking chitosan with diepoxide and installation of a spacer arm between the chitosan matrix and an epoxy terminal group at the distal end of the spacer arm. The epoxide-terminated spacer-arms can then have their unreacted epoxides converted into various types of matrices. One such matrix is LMCB or Ligosep Alpha®; which is a short chain aliphatic ligand attached to the cross-linked bead with a carboxylic acid functionality (USA Pat. # 5770,712. June 23, 1998).

pH Optimization Experiment

An equilibrium binding experiment was conducted to determine optimal binding pH of hIgG and HSA on LMCB. A 50% (v/v) slurry of beads (600 μL) was transferred into 1.5 mL micro-centrifuge tubes to yield approximately

300 μL of beads, and any liquid overlay was pipetted off. A 950 μL solution of hIgG solution ranging in 3.5–4.8 mg/mL, in 10 mM KH_2PO_4, was added to the microcentrifuge tubes at the indicated pH. pH values of 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5 were used as data points in this study. Tubes were placed on an end-to-end rotator and allowed to rotate for 24 hr at room temperature (RT). At the completion of the experiment, the tubes were allowed to settle for 20 min, the supernatant was pipetted off, and protein concentration was measured spectrophotometrically at OD 280 nm. A separate but a similar experiment was used to determine the optimal binding capacity of HSA on LMCB beads. Experiments were done in duplicate.

Effects of Anti-chaotropic Salts on Binding

An equilibrium binding experiment was conducted to determine the effects of non-chaotropic sulfate salts on the binding of hIgG and HSA on LMCB. Lyophilized hIgG (7.5 mg) was re-suspended in loading buffer (10 mM KH_2PO_4, pH 6.0) containing 0.0, 0.0625, 0.125, 0.25, and 0.5 M solution of either potassium sulfate or ammonium sulfate to yield a 5 mg/mL of hIgG solution. The 5 mg/mL hIgG solution (950 μL) was placed in each microcentrifuge tube containing 300 μL beads, put on an end-to-end rotator, and allowed to rotate for 24 hr at RT. At the completion of the experiment, the tubes were allowed to settle for 20 min, the supernatant was pipetted off and protein concentration was measured spectrophotometrically at OD 280 nm.

Effects of NaCl on Binding

An equilibrium binding experiment was conducted to determine the effect of hIgG binding in the presence of varying sodium chloride (NaCl) concentrations. A 600 μL solution of a 4 mg/mL solution of hIgG in loading buffer (10 mM KH_2PO_4, pH 6.0) with NaCl molar salt concentrations of 0.0, 0.0625, 0.125, 0.25, 0.375, 0.5, and 1.0 were added to the microcentrifuge tubes containing 200 μL beads. At the completion of the experiment, the supernatant was pipetted off and protein concentration was measured spectrophotometrically at OD 280 nm.

Ligand Binding Isotherms

Small-scale experiments were conducted to obtain static equilibrium uptake capacity of LMCB. A 50% (v/v) slurry of beads (400 μL) was

transferred into 1.5 mL microcentrifuge tubes to yield approximately 200 μL of beads. hIgG in loading buffer (10 mM KH_2PO_4, pH 6.5), 500 μL of 0.0, 1.0, 3.33, 6.67, 8.33, 10.0, 13.33, 16.67, and 20.0 mg/mL were added to the microcentrifuge tubes. Experiments were carried out in duplicate. Tubes were placed on an end-to-end rotator and allowed to rotate for 24 hr at RT. In addition, identical experiments for beads were conducted with varying amounts of HSA in loading buffer. At the completion of the experiment, the supernatant was pipetted off and protein concentration was measured spectrophotometrically at OD 280 nm. In all the experiments listed above, the difference in the amount of hIgG in the feed and the amount of hIgG in the supernatant yielded the amount of hIgG bound.

Purification of Mabs

Cell culture supernatant was diluted in de-ionized water until a conductance reading of less than 2 msec was achieved. Then 10× loading buffer (100 mM KH_2PO_4, pH 6.0) was added (one tenth of the total volume of de-ionized water added) to adjust the buffer concentration and pH of the sample feed. The feed was loaded to a column (1.5 cm^2 i.d. × 13–16 cm in length) packed with Ligosep Alpha® beads at a linear velocity of 1.1 cm/min. Unretained proteins were collected, and the non-specifically bound proteins were washed with the loading buffer until the OD 280 nm returned to the baseline. The bound hIgG was eluted by making a step change to the elution buffer (10 mM KH_2PO_4, 0.5 M NaCl, pH 6.0). Upon elution, the column was washed with the elution buffer until the OD 280 nm returned to the baseline and re-equilibrated in loading buffer. The chromatographic fractions were assayed for total protein content by measuring absorbance at OD 280 nm and hIgG content by specific ELISA assays. The purity of the product was judged by electrophoretic analysis. Similar experiments were carried out with supernatants rich in different Mab sub-species.

In a separate experiment, 2.0-mL protein A immobilized on hyper D support was packed into a Pharmacia column, and cell culture supernatants were chromatographed according to the manufacturer's instructions. In a typical application, 1-mL of antibody solution was diluted with 1.0 mL of 0.5 M sodium citrate buffer at pH 8.4 (LB_1). Feed was filtered using a Millipore 0.45 μm membrane filter and loaded on to the column at a linear velocity of 1.0 cm/min. Loosely bound proteins were washed with LB_1. The elution of the bound Mab was effected with 0.5% acetic acid. The pH of the elution fraction was immediately raised to 7.0 with 1 M Tris-base. All chromatographic fractions were saved and analyzed for total protein content at OD 280 nm and specific antibody content by specific ELISA assays.[45]

Gel Electrophoresis

The purity of the recovered Mab was analyzed by SDS–PAGE gel electrophoresis. In brief, all chromatographic fractions were diluted to a protein concentration of 1 mg/mL. Chromatographic samples were mixed with a non-reducing buffer at a ratio of 3 : 1 or mixed with a reducing buffer at a ratio of 1 : 1, respectively, and were heated to 95°C for 5 min in a water bath. Proteins were analyzed on NuPage 4–12% Bis–Tris gels and visualized by Gelcode Blue stain reagent or by silver staining. Stained gels were analyzed by digital image processing to assess the purity.

Determination of Mab by ELISA

The concentration of Mab in the chromatographic fractions was determined by the ELISA assays as detailed elsewhere.[46] Briefly, microtiter plates were coated goat anti-mouse IgG, and diluted standard and samples in blocking/dilution buffer were added upon washing and blocking. After sample incubation, the bound Mab was detected with horseradish peroxidase (HRP), conjugated goat anti-mouse and the bound chromophore was detected using a Bio-Tek microplate ELISA reader at 490 nm.

RESULTS

Ligand Binding Isotherm

Static binding experiments were conducted to determine the effect of temperature on the binding of IgG to LMCB. The binding isotherm follows the saturation pattern as predicted by the Langmuir model (Fig. 2). Data were reduced by Lineweaver-Burk analysis to determine the values of the static binding capacity (Q_{max}) and the equilibrium dissociation constant (K_d). A Q_{max} value of 40 mg hIgG bound per milliliter of beads and a K_d values of 1.14×10^{-5} M (moles of hIgG bound per liter of beads) were obtained.

pH Optimization

The amount of hIgG and HSA bound to LMCB as a function of pH was studied. Figure 3 shows the ratio of the amount of hIgG bound to the amount of HSA bound, as a function of pH. A binding ratio of 4.3, 5.7, 5.6, 4.2, 3.5, 1.6,

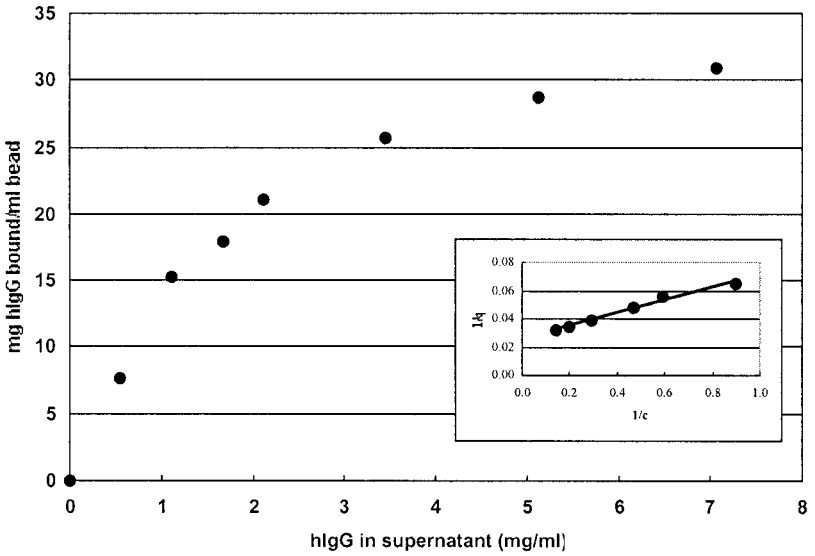

Figure 2. Static adsorption isotherms for the binding of hIgG to Ligosep Alpha® beads at RT (~30°C). Ligosep Alpha® beads were contacted at different concentrations of hIgG as described in the methods section. The equilibrium data are plotted as mg of hIgG adsorbed per mL of Ligosep Alpha® beads (wet) against the concentration of hIgG in the supernatant. The amount of hIgG bound was determined by difference. Experiments were done in duplicate and the standard deviation was in the range of 5–15%.

and 1.3 was obtained for pH values of 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0, respectively. Since the binding of hIgG is desired whereas the binding of HSA is not, for the given pH values the largest ratio gives the optimal pH. Although the value is slightly larger for a pH value of 5.5 than it is for a value of 6.0, the fact that the two values were very close and that 6.0 is closer to physiological pH prompted the use of pH 6.0 in further experiments.

Effects of Anti-chaotropic Salts on Binding Capacity

The effect of anti-chaotropic salts on the relative amount of hIgG and HSA bound on LMCB was studied. Figure 4 shows the ratio of the amount of hIgG bound to the amount of HSA bound, as a function of $(NH_4)_2SO_4$ and K_2SO_4 concentration. A hIgG to HSA binding ratio of 2.1, 2.8, 1.9, and 1.0 was obtained at $(NH_4)_2SO_4$ concentrations of 0.0625, 0.125, 0.25, and

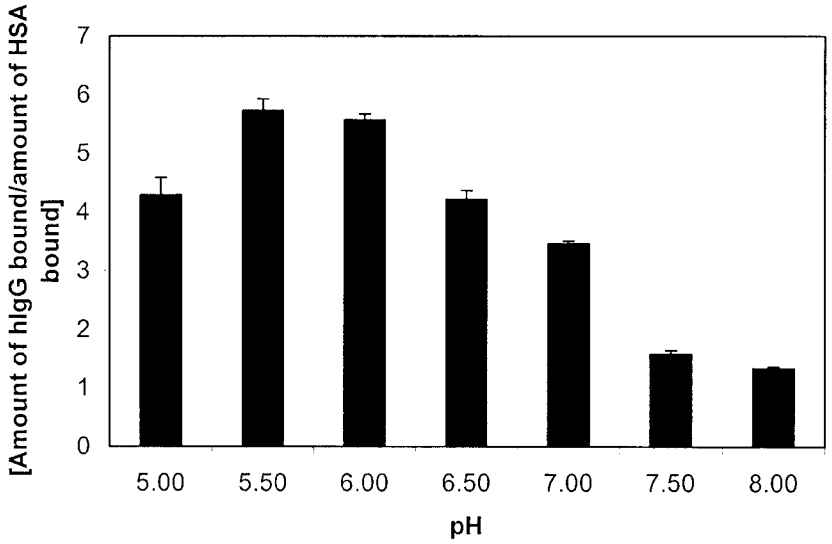

Figure 3. Ratio of the amount of hIgG bound to the amount of HSA bound to Ligosep Alpha® beads at varying values of pH. Briefly, Ligosep Alpha® beads were contacted with hIgG solution in loading buffer, at a concentration of 5.0 mg/mL as described in the methods section, as function of pH. Similar but separate experiments were carried out with HSA. Upon completion of the incubation step, the concentration of the residual protein in the supernatant was measured spectrophotometrically at OD 280 nm. The amount of hIgG or HSA bound was determined by difference. Experiments were done in duplicate and the standard deviation was in the range of 5–10%.

0.5, respectively. A ratio hIgG to HSA binding ratio of 1.5, 1.5, 1.2, and 1.1 was obtained at potassium sulfate molar concentrations of 0.0625, 0.125, 0.25, and 0.5, respectively. The binding ratio on the LMCB with the addition of 0.0625–0.5 M ammonium and potassium sulfate varies between 1.0 and 2.8. In the pH optimization experiment, at a pH value of 6.0 and the same protein target binding capacities, the binding ratio was 5.6. Thus, the addition of sulfate salts decreased the binding ratio when compared with a buffer containing no sulfate salts.

Effects of NaCl on Binding Capacity

The influence of ionic strength on the retention of hIgG on LMCB is presented in Fig. 5. The efficiency of adsorption is maximum in the absence of

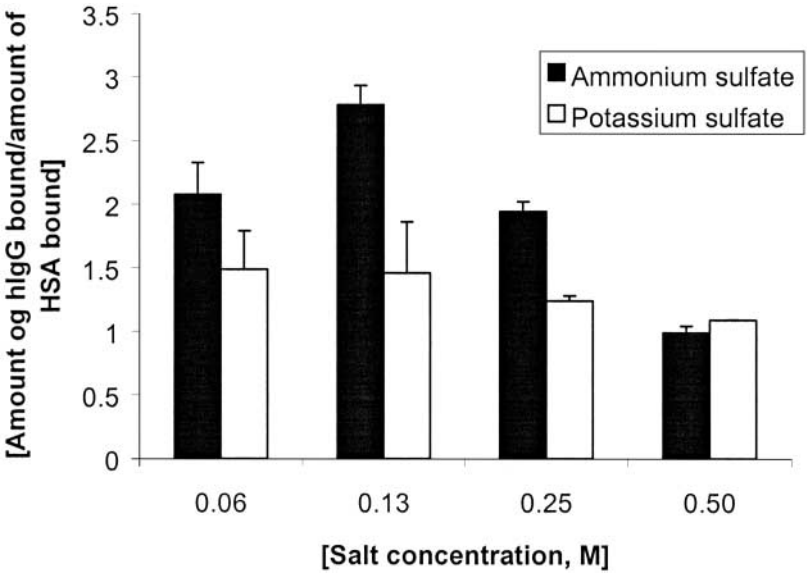

Figure 4. Ratio of the amount of hIgG bound to the amount of HSA bound to Ligosep Alpha® beads at varying ammonium sulfate and potassium sulfate concentrations. Briefly, Ligosep Alpha® beads were contacted with hIgG solution in loading buffer, at a concentration of 5.0 mg/mL as described in the methods section, at varying concentrations of chaotropic salts. Similar but separate experiments were carried out with HSA. Upon completion of the incubation step, the concentration of the residual protein in the supernatant was measured spectrophotometrically at OD 280 nm. The amount of hIgG or HSA bound was determined by difference. Experiments were done in duplicate and the standard deviation was in the range of 5–10%.

any added NaCl in the buffer. The effect of NaCl on binding was also evaluated by isotherm analyses (data not shown). From isotherm experiments and analyses, static binding capacities of 31, 22, and 5 mg of hIgG bound per millileter of beads at NaCl concentrations of 0.0, 0.05, and 0.125 M NaCl were obtained, respectively.

Column Chromatography

The ability of LMCB to bind Mabs from cell culture supernatants was evaluated in column-mode experiments. Unbound or weakly retained proteins passed through the column during the first nine column volumes (CV). The ultraviolet (UV) trace at 280 nm returned to a baseline by ten CV indicating

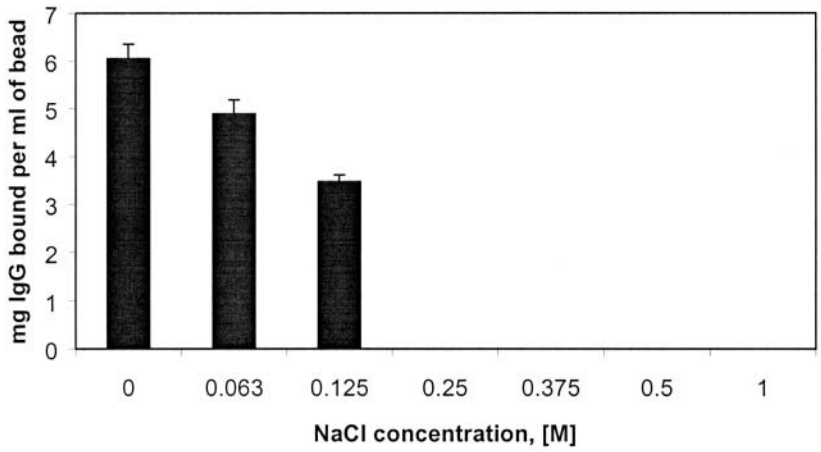

Figure 5. Amount of hIgG bound to Ligosep Alpha® beads at varying sodium chloride (NaCl) concentrations. Briefly, Ligosep Alpha® beads were contacted with hIgG solution in loading buffer, at a concentration of 4.0 mg/mL as described in the methods section, at varying NaCl concentrations. Upon completion of the incubation step, the concentration of the residual protein in the supernatant was measured spectrophotometrically at OD 280 nm. The amount of hIgG bound was determined by difference. Experiments were done in duplicate and the standard deviation was in the range of 4–7%.

a complete elution of unbound proteins. A change of elution buffer by the addition of NaCl was made to elute the bound Mab. A chromatographic peak at 280 nm indicates elution of bound proteins, which was identified to be composed mainly of Igs. This peak eluted between 3 and 5 CV. The column was then washed with the elution buffer until the UV trace reading returned to a baseline.

Yield of Mab

Total percent recovery and total percent yield of Mab on LMCB is shown in Table 1. The total protein concentration in different chromatographic fractions was obtained by measuring the absorbance at a wavelength of 280 nm. The total percent recovery was determined by taking a ratio of the total protein in all chromatographic fractions to the protein in the feed sample. Using cell culture supernatant as the feed sample, total recoveries of 83–100% were achieved for all runs. The total percent yield was determined by taking a ratio of the total protein in all elution fractions to the protein in the feed

Table 1. Summary of the total recovery and yields of Mabs on Ligosep Alpha® and protein A-hyper D.

Run	Column type	Total recovery[a] (%)	Total recovery[b] (%)	Yield[b] (%)
A1	Ligosep Alpha®	98.2	98.9	78.34
A2	Ligosep Alpha®	100.0	91.1	82.5
B1	Protein A-HD	83.5	92.3	62.73
B2	Protein A-HD	94.5	95.9	89.6

Notes: Ligosep Alpha® beads were challenged with diluted cell culture supernatant in 10 mM $KHPO_4$ at pH 5.5 at a flow rate of 1.0 mL/min. Bound Mab was eluted with in 10 mM $KHPO_4$, 1.0 M NaCl at pH 5.5. Two independent runs were performed. Protein A-hyper D column was operated according to manufacturer's instructions. The total protein concentrations in column-fractions were determined by OD 280 nm. Individual Mab concentrations in fractions were determined by ELISA assays. In general, an average of triplicate application of three different dilutions in ELISA assays was used for yield calculations. Percent total recovery is defined as the ratio of the sum of the total protein in eluate and column fall-through fractions to the total protein present in the feed. Percent yield is defined as the ratio of the Ig present in the eluate fraction to the total amount of Ig in the feed. ELISA values were used to estimate the yields.
[a]Determined by ELISA assays.
[b]Determined spectrophotometrically by OD 280 nm.

sample. The majority of protein loaded was seen in the fall through fraction indicating that there was little non-specific binding and the Mabs were being retained for the elution fraction. Purification experiments using column chromatography were performed in duplicate with good comparison.

The Mab concentration in different chromatographic fractions was estimated by an ELISA assay specific for hIgG. These values are shown in Table 1 for LMCB. The percent recovery is reported as the ratio of Mab in the elution and fall through fractions to the total Mab in the feed solution, and values in the range of 83–100% were obtained for the linear velocities tested. Total Mab percent yield is reported as the ratio of Mab in the elution fraction to the total Mab in the feed solution. Total percent yield in the range of 78–83% and 63–90% were obtained for independent runs with LMCB and protein A-hyper D, respectively.

Gel Electrophoresis

Figure 6 shows a Gel-code™ reagent stained SDS–PAGE gel under non-reducing conditions of the cell culture supernatant (feed) and the

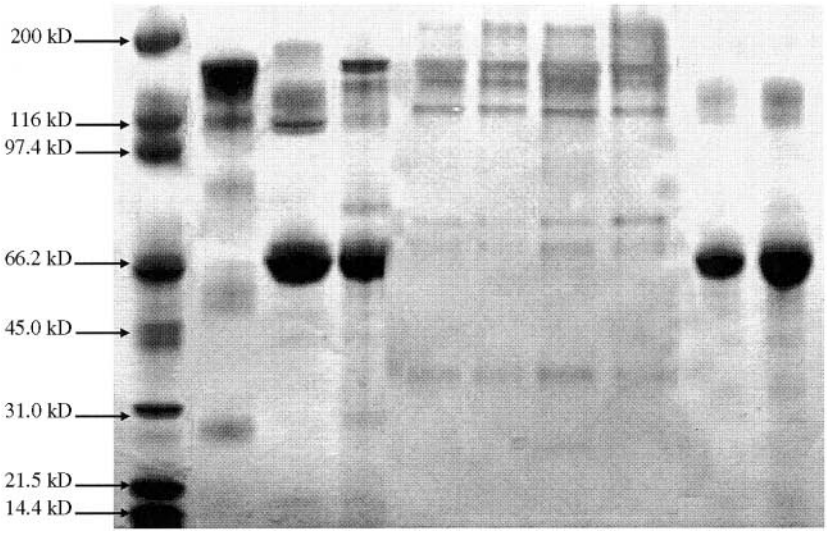

Figure 6. SDS–GAGE electrophoresis of chromatographic fractions. Sodium dodecylsulfate (0.1%)–polyacrylamide gel (4–12% gradient) electrophoresis of Mabs that were purified from cell culture supernatant using Ligosep Alpha®. Lane 1 shows a molecular weight ladder. Lane 2 shows an application of pure Mab. A total protein level of 15 μg was loaded to each lane. Lane 3 shows an application of pure BSA. Lane 4 shows an application of cell culture supernatant sample that was used as the feed. Lanes 5 and 6 show the elution fractions from two independent runs using LMCB. Lanes 7 and 8 show the elution fractions from two independent protein A–hyper D runs. Lanes 9 and 10 show representative fall through fractions obtained from independent runs.

chromatographic fractions from a typical separation run on LMCB. Lane 1 shows a molecular weight ladder. Lane 2 shows an application of pure Mab. A total protein level of 15 μg was loaded to each lane. Lane 3 shows an application of pure BSA. Lane 4 shows an application of cell culture supernatant sample that was used as the feed. Lanes 5 and 6 show the elution fractions from two independent runs using LMCB. Lanes 7 and 8 show the elution fractions from two independent protein A-hyper D runs. Lanes 9 and 10 show representative fall through fractions obtained from independent runs. The eluate fractions, shown in lanes 5–8, gave a band around 150 kDa, similar to pure Mab. The purity of hIgG in the eluate fraction was estimated to be greater than 98% by digital image processing, with BSA accounting for approximately 1% of the area in the fraction.

DISCUSSION

The long-term goals of our research are to develop methods to produce hydrogel based matrices, and to further modify hydrogel surfaces with unique and targeted chemistries to yield chemically bonded surfaces with novel selectivities. Our current research efforts have enabled the preparation of chitosan beads (400–600 μm in diameter) with novel matrix architecture, which differentiates chitosan beads used in this study from all other liquid chromatography bioseparation matrices. The matrix is a bead of large diameter, low density chitosan (3% solids), which permits homogeneous ligand utilization throughout the bead interior. We hypothesize that the chitosan hydrogel bead resembles a network of polymer chains, as opposed to conventional supports that are characterized by distinct pore sizes and pore geometry. Furthermore, the base chitosan matrix has been further derivatized to yield a pseudo-bioaffinity support for use in bioseparations (Fig. 1).[41] We seek to better understand the effect of support characteristics and support activation chemistry on the parameters impacting the binding of Igs from biological fluids. Thus, studies aimed at understanding the adsorption mechanism and the nature of interactive forces between Igs and binding site on LMCB beads were undertaken.

In affinity chromatography, interaction between the immobilized ligand and the solute molecule is based on similarity of charge, hydrophobic, ionic, and van der Waal interactions. The same forces probably play a role in pseudo-bioaffinity systems, but their role and magnitude perhaps differ. It is desired to establish the type of interactions governing and prevalent in pseudo-bioaffinity systems in order to better optimize the processing conditions, namely pH, ionic strength, salt concentration, and temperature.

In the interaction between a chromatographic support and the molecule that is being purified, pH of the buffer plays an important role. The net charge on a protein is altered at varying pH values, which leads to varying bonding interactions between the biomolecule and the chromatographic support. We obtained an adsorption pH optimum of 6.0 for this support.

Pseudo-bioaffinity chromatography supports exploit certain structural binding features of proteins. The Ligosep Alpha® support, for example, utilizes structural characteristics of IgG or Mab to establish a protein–ligand interaction, although the mechanism of binding is yet to be determined. Because of the unknown nature of the mechanism, an experiment adding various concentrations of two salts, potassium sulfate and ammonium sulfate, to the binding buffer were conducted in order to determine if binding on the beads is salt promoted. Sulfate salts are anti-chaotropic salts and, according to the Hofmeister series, have the ability to stabilize proteins and increase hydrophobic interactions, thereby promoting protein adsorption.[42] If the

protein–ligand interaction of the Ligosep Alpha® support was based, in part, on hydrophobic interactions the addition of low concentration sulfate salts would increase the binding capacity of hIgG to the support. Poor capacities were obtained with the addition of the sulfate salts in comparison to a buffer without. Therefore, it is reasonable to deduce that the mechanism of binding is not due to any hydrophobic interactions. Additionally, binding based on hydrophobic interactions tends to increase with increasing temperature.[43] This trend was not observed in our work in which retention of Mab or IgG at both 4°C and 37°C were similar (data not included).

Moreover, the presence of high salt concentration in the binding buffer resulted in negligible binding of the hIgG in comparison with that in the absence of NaCl. This indicates the involvement of electrostatic and possibly hydrogen bond interactions between the proteins and the ligand.

As stated, it appears that charge–charge interactions play an important role in the ligand-Ig complex. At the pH value for which binding of IgG was determined to be optimal, a pH of 6.0, hIgG has a net positive charge. Therefore, at the same pH, we hypothesize that the tether bound to the support must carry a net negative charge, since the support backbone itself is non-reactive, for charge–charge interactions to occur.

When determining the factors that govern protein–ligand interactions, it becomes important to understand how the chemical and physical properties of the matrices binding sites affect protein adsorption. It is therefore, useful to study equilibrium adsorption data with the intention of determining how the support reacts to protein binding with increasing concentrations. The shape of the equilibrium adsorption curve at the temperature studied, indicates a Langmuir-type isotherm and is well fit to the simple Langmuir equation.[44] We hypothesize that at these temperatures, hIgG binds uniformly with a high affinity for the binding sites until it reaches a maximum binding energy. The static capacity of r_PEZ, as determined by the Langmuir adsorption data, was calculated to be 40 mg IgG per milliliter of beads, which is comparable with that reported for protein A–sepharose and protein A–ultragel (Technical handbook, Pierce Chemical Company). The dissociation constant, K_d, was determined to be 1.14×10^{-5} M, which indicates medium affinity and is typical for a pseudo-bioaffinity ligand.[43,44]

Our work with LMCB beads indicated that an effective isolation of Mabs from cell culture supernatant was attainable. Separation of Mabs from other proteins was likely through a differential in binding capacity mediated by pseudo-bioaffinity interactions. In other words, the LMCB or Ligosep Alpha® matrix is not specific for Igs as a protein A matrix would be, but the pseudo-bioaffinity interactive forces confer a unique specificity for Igs over other serum proteins. This selectivity facilitated the use of a step gradient for the separation of Mab from a cell culture supernatant. This is of particular

interest in large-scale separations where linear gradient has proven to be time consuming and inconvenient. Using a similar protocol, we were also able to separate and enrich sub-species of Mab (IgG_{2a}, IgG_{2b}, and IgG_3) from respective cell culture supernatants (data not included).

The chitosan beads were mechanically and chemically stable and withstood high linear velocities. The following salient features were observed in all chromatographic traces: (1) At first, large peak was obtained and was most likely due to serum proteins, non-Ig in nature. (2) The elution peak was somewhat broad and asymmetric in nature, though the Ig was well retained. This may be due to the nature of the base matrix, which is characterized as having conduits or a series of polymeric networks comparable with large pores.

CONCLUSION

We have shown that matrices based on natural biopolymers like chitosan can be designed to yield chromatographic supports for use in biochromatography. Specifically, we have demonstrated the utility of these matrices in pseudo-bioaffinity mode. We believe the true leaps in throughput and productivity in bioprocessing will result when the merits of large bead technology will be merged with new and improved activation or ligand immobilization strategies. The ability of Ligosep Alpha® to enrich Mabs from cell culture supernatants was demonstrated. We are also evaluating the performance of beads end-capped with a family of anionic ligands.

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