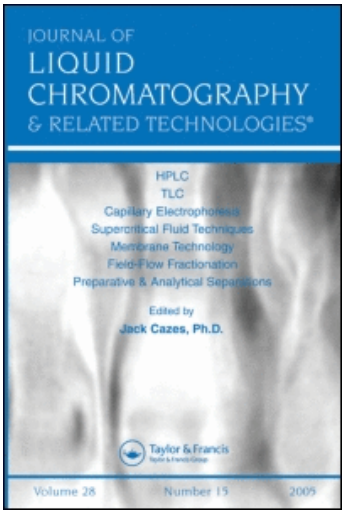

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Interaction of Immunoglobulin G with Modified Chitosan

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Interaction of Immunoglobulin G with Modified Chitosan

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ABSTRACT

Chitosan beads were modified to include a spacer arm and end-capped with a caboxyethyl-group containing anionic ligand to generate a pseudo-bioaffinity support, further referred to as Ligosep Alpha® or LMCB. To better understand the force of interaction between the immunoglobulin G (IgG) and the chitosan-based pseudo-affinity support, the equilibrium dissociation constant (K_d) was determined by static binding isotherms, as a function of temperature, and by frontal analysis at different linear velocities. The maximum static binding capacity (Q_{max}) was found to be in the range of 60–70 mg IgG per mL of gel, and unaffected by temperature. The adsorption rate constant (k_a) was determined by a split-peak approach to be

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between 46 and 404 L mol^{-1} sec^{-1} depending on the linear velocity. A governing empirical equation relating k_a to the linear velocity (u) was deduced to be $k_a = 44.4(u) + 9.3$. The kinetic parameters of the chromatographic system indicated a good capacity but a low adsorption rate constant. LMCB was found to preferentially interact with the F_{ab} and the $F_{(ab)2}$ region of the HIgG molecule. This gives the further potential of adjusting the process for purifying specific paratopes under gentle chromatographic conditions.

Key Words: Immunoglobulin G; Chitosan.

INTRODUCTION

Economics, efficiency, and practicality are some of the constraints dictating the search for novel chromatographic supports used in the industrial or large-scale purification of proteins. Affinity chromatography, ion-exchange chromatography, and other forms of electrostatic chromatography that exploit differences in biological specificity or surface charge anisotropy of proteins, have the greatest potential of impacting future trends in scalable protein separation methodologies. Research-based prediction of mass transport, biological activity behavior, kinetic and thermodynamic parameters that impact protein retention, and separation, is therefore, essential to integrate these chromatographic-based unit-operations into the purification scheme.

Affinity chromatography uses biological ligands like protein A or biotin–avidin to achieve exquisite specificity and separation. However, many of these biological ligands are macromolecular and fragile, expensive to obtain from bacterial or tissue-culture sources, and 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 that pose special challenges regarding regeneration and sanitation.[1] Some of these drawbacks preclude the use of biological ligands in practical and commercial applications, and have prompted researchers to turn their attention to the development of synthetic ligands.[2] In contrast, smaller molecules such as dyes, amino acids, metal ions, and chemical moieties show comparable affinities. Their specificity can be increased or decreased either at adsorption or desorption to attain resolutions and degrees of purification comparable with those of immunoadsorption.[3–13]

The effectiveness of a novel pseudo-bioaffinity chromatography support that has been synthesized by post-derivatization of bald chitosan beads with carboxyl group containing anionic ligand, and termed Ligosep Alpha®, for the separation of immunoglobulins (Igs) from complex biological fluids has

been demonstrated elsewhere.[14] Although the elution of retained IgG from both serum and cell culture supernatants was affected by mild desorption buffer, not much is known about the underlying mechanism of the selective recognition of IgG. While the ionic properties of both the immobilized ligand and the target protein may play an important role, the fact that selective adsorption takes place at or around the isoelectric pH of IgG is of particular interest. The influence of pH, salt concentration, and the effect of chaotropic salts have been studied. Additionally, a study of kinetic and thermodynamic factors, such as affinity constants and adsorption rate constants, of this adsorption was also undertaken.

EXPERIMENTAL

Ligochem Inc., provided Ligosep Alpha® as a generous gift. Lyophilized, 95% pure human IgG, human serum albumin were purchased from Sigma Co. (St. Louis, MO). Protein A-hyper D beads were purchased from Sigma Co. (St. Louis, MO). Potassium phosphate monobasic and sodium chloride for the buffer solutions were obtained from Columbus Chemical Industries (Columbus, WI). Immunoaffinity separations were performed with a Pharmacia HR 5/5 column (0.5 cm i.d.), a Pharmacia C 10/20 column (1 cm i.d.) (Piscataway, NJ), and a Cole Parmer Materflex peristaltic pump (Niles, IL). A Spectrophotometer, Spectronic® Genesys™ 5 (Rochester, NY), and a BioRad UV monitor (Hercules, CA) were used to monitor protein concentration and for chromatography.

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) were 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 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. The difference in the amount of HIgG in the feed and the amount of HIgG in the supernatant yielded the amount of HIgG bound.

Identification of the Binding Site

It is important to determine whether HIgG was bound to modified chitosan at the F_c or F_{ab} or $F_{(ab)2}$. Antibody fragments (F_c or F_{ab} or $F_{(ab)2}$) were purchased commercially, or prepared by enzymatic hydrolysis of HIgG, with commercially available pepsin and papain kits from Pierce Chemical Company. Antibody fragments were chromatographed separately under the same conditions as used for IgG retention and elution.[14] The column-wash and elution fractions were assayed by specific ELISA assays. Total recovery and yield of each fragment will be assessed quantitatively.

In a separate experiment, 2.0-mL protein A immobilized on hyper D support was packed into a Pharmacia column and antibody fragments were chromatographed according to the manufacturer's instructions. In a typical application, 1 mL of antibody fragment 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 microns membrane filter and loaded onto 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 fragment content by specific ELISA assays.

Detection of IgG Fragments by ELISA Assays

Immulon II microtiter plates were incubated with 100 μL/well of 5 μg/mL rabbit anti-human F_{ab} specific antisera coating buffer (0.1 M $NaHCO_3$, 0.1 M NaCl, pH 9.3), for 24 hr at 4°C. Wells were washed with washing buffer (50 mM NaCl, 20 mM Tris–HCl, 0.05% Tween, pH 7.2) and residual sites were blocked with blocking/dilution buffer (50 mM NaCl, 20 mM Tris–HCl, 0.5% casein, pH 7.2), for 30 min at RT. Diluted standard (100 μL) and samples in blocking/dilution buffer were added to each well and incubated for 30 min at 37°C. After incubation, wells were washed three times with washing buffer and 100 μL/well of 1 : 2500 diluted horse radish peroxidase (HRP), conjugated rabbit anti-human IgG was added to each well and incubated at 37°C for 30 min. Wells were washed three times and 100 μL/well of OPD substrate was added. The colorimetric reaction was stopped after approximately 3 min by the addition of 100 μL/well of 3 N sulfuric acid. Bound chromophore was detected using a Bio-Tek microplate ELISA reader at 490 nm.

A similar ELISA procedure was used for the determination of $F_{(ab)2}$ and F_c with the following changes. Plates were coated with 100 μL/well of

5 μg/mL goat anti-human $F_{(ab)2}$ and F_c specific antisera, for $F_{(ab)2}$ and F_c determination, respectively.

Kinetic Binding Experiment

Approximately 1 mL of 50% (v/v) slurry LMCB was transferred into 5 mL plastic test tubes, to yield approximately 500 μL of beads. The beads were allowed to settle for at least five minutes and the liquid overlay was then pipetted off. A sample solution of 1 : 4 HIgG to HSA was produced by mixing 25 mg of HIgG and 100 mg of HSA in 5 mL of binding buffer. Of this solution, 3.0 mL was added to each tube. Tubes were placed on an end-to-end rotator and allowed to rotate for 24 hr at RT. Aliquots were taken at times equal to 30 sec, 1, 5, 10, 20, 30, 45, 60, 120, 240 min, and 24 hr. HIgG concentration was estimated by the HIgG ELISA protocol described in the following section.

Determination of Dissociation Constant, K_d, and Dynamic Bead Capacity

For this study, a Pharmacia C 10/20 column (1 cm i.d.) was filled with 1.0 mL of beads and the length of the packed column was 1.3 cm. A solution of pure HIgG in loading buffer (10 mM KH_2PO_4, pH 6.0) was used. This protein solution was added to the column, which was equilibrated with the loading buffer at concentrations ranging from 0.5 to 25 mg/mL of HIgG and linear velocities of 1.13, 3.0, 5.66, and 9.05 cm/min. The absorbance of the effluent at 280 nm was monitored continuously. The injection was continued until the absorbance of the effluent reached 0.8–0.97 of the inlet concentration. The column was then washed with loading buffer until the absorbance at 280 nm reached the baseline. The adsorbed HIgG was then eluted with the elution buffer (10 mM KH_2PO_4, 1 M NaCl, pH 6.0). For each concentration, the dynamic capacity of the column was determined as the amount of HIgG maintained per milliliter of bead.

Determination of Adsorption Rate Constants

This experiment was done by using the split-peak approach as described elsewhere.[15] For this experiment, a Pharmacia C 10/20 column (1 cm i.d.) was filled with 1 mL of beads and equilibrated with loading buffer (10 mM KH_2PO_4, pH 6.0). A 3.5 mg/mL solution of HIgG (15 mL) was prepared in

the loading buffer. Aliquots of 1 mL were consecutively injected into the column. Unabsorbed protein was collected for each aliquot injected, giving a total of 15 fractions collected. In each peak, the total amount of protein was determined by measuring absorbance at OD 280 nm. Aliquots of 1 mL were then injected, the retained protein was eluted, and absorbance was measured at 280 nm.

Determination of Adsorption Rate

For this experiment, a Pharmacia C 10/20 column (1 cm i.d.) was filled with 1 mL beads and equilibrated with loading buffer (10 mM KH_2PO_4, pH 6.0). A solution of HIgG at concentrations of 0.5, 1.0, 2.5, 5.0, 10.0, and 15.0 mg/mL was prepared in the LB. This HIgG solution was fed to the column under dynamic conditions, and the absorbance of the effluent at 280 nm was monitored continuously. The injection was continued until the absorbance of the effluent reached 0.8–0.97 of the inlet concentration. The column was then washed with loading buffer until the absorbance at 280 nm reached the baseline. The adsorbed HIgG was then eluted with the elution buffer (10 mM KH_2PO_4, 1 M NaCl, pH 6.0). At each HIgG concentration, separate experiments were carried out at linear velocities of 1.13, 3.0, 5.66, and 9.05 cm/min. A similar set of experiments were repeated for all HIgG concentrations. The adsorption rate (mg per milliliter per minute) was calculated as the amount of protein retained per mL of support (mg/mL) as a function of time of adsorption (min).

RESULTS

Effects of Temperature on the Binding of HIgG

Static binding experiments were conducted to determine the effects of temperature on the binding of HIgG to LMCB. The results are shown in Fig. 1. Temperature does not appear to have any significant effect on the binding capacities of HIgG, as observed from the similar shapes of the isotherms. Furthermore, the binding isotherms follow the saturation pattern as predicted by the Langmuir model. 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). The Q_{max} was found to range from 66 to 88 mg HIgG bound per mL of beads. The K_d values were in the range of 1.12×10^{-5}–2.39×10^{-5} M (moles of HIgG bound per liter of beads).

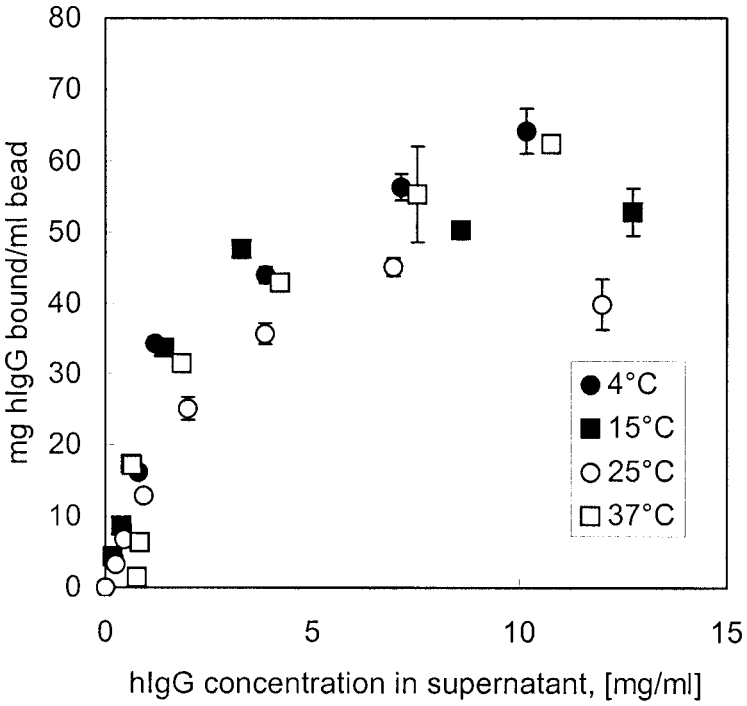

Figure 1. Static adsorption isotherms for the binding of HIgG to LMCB beads at different temperatures. LMCB beads were contacted at different concentrations of HIgG as described in the methods section. The equilibrium data are plotted as mg of HIgG adsorbed per mL of beads (wet) against the concentration 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%.

To determine whether there were any cooperative effects due to protein–protein interaction during the adsorption process, the isotherm data (from static experiments) were analyzed by a Hill plot using the transformed Langmuir equation,[12] and the results are shown in Fig. 2. A cooperativity coefficient (n) of unity indicates no cooperativity. Analysis of the isotherms at 4°C, 15°C, and 25°C indicated n values of 0.88, 0.97, and 0.97, respectively, suggesting that there is no cooperativity due to protein–protein interactions. Analysis of the isotherms at 37°C found n values of 1.78, which suggests that there may be positive cooperativity due to protein–protein interactions.

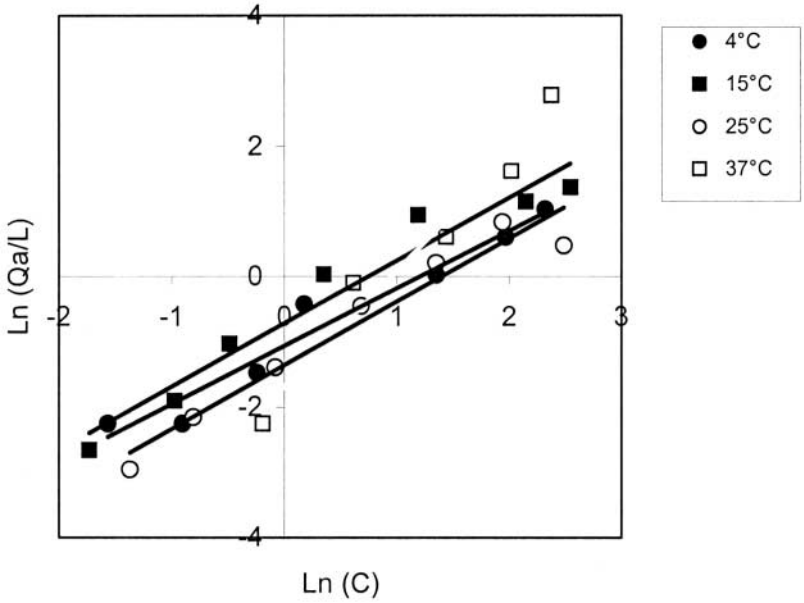

Figure 2. Determination of the protein interaction cooperativity (Hill plot of the data in Fig. 1). Data were analyzed by the transformed Langmuir equation, $\ln[Q_a/L] = \ln K_a + n \ln C$, where $L = Q_x - Q_a$ is the equilibrium free ligand concentration and n is the cooperativity coefficient. The value n was obtained from the slope of the linear plot of $\ln[Q_a/L]$ versus $\ln C$.

Identification of Binding Site

Table 1 lists the total yield and recovery obtained with each IgG fragment in separate chromatographic experiments. It appears that the F_c fragment was not retained on the LMCB under the experimental conditions, as 95% of the F_c fragment was detected in the column fall-through. The F_{ab} and the $F_{(ab)2}$ fragment were both retained on the LMCB column, albeit to varying extents. Judging from the percent yield data, it appears that a greater percentage of F_{ab} fragment (66%) was retained on the column when compared with the $F_{(ab)2}$ fragment's yield of 55%. Similar trends and percentages were obtained when the binding of the IgG fragment was tested under batch conditions (data not included).

In contrast, the F_c fragment was quantitatively retained on the protein-A column as expected (Table 1). F_{ab} and $F_{(ab)2}$ fragments were not retained on the protein A column. Similar trends and percentages were obtained when the binding of the IgG fragment to protein A-beads was tested under batch conditions (data not included).

Table 1. Percent total recovery and yield of antibody fragments on Ligosep Alpha® (LMCB) and protein-A column.

	Fragment F_c			Fragment $F_{(ab)2}$			Fragment F_{ab}		
	% TR	% Y	(%Y)/(%TR)	% TR	% Y	(%Y)/(%TR)	% TR	% Y	(%Y)/(%TR)
Protein A									
Run 1	88.8	85.0	0.96	91.0	0.9	0.01	97.8	0.1	0.01
Run 2	92.5	88.0	0.95	83.0	2.0	0.02	94.9	1.9	0.02
Ligosep A									
Run 1	83.0	2.8	0.03	92.5	25.0	0.27	90.8	61.5	0.68
Run 2	96.0	4.0	0.04	85.0	30.0	0.35	91.3	60.5	0.67

Notes: LMCB beads were challenged with pure antibody fragments (F_c or F_{ab} or $F_{(ab)2}$) in separate experiments. Chromatographic fractions were assayed for the specific fragment content by ELISA assays, as detailed in the material and methods section. In general, an average of three applications of three different dilutions in ELISA was used for yield calculations. Percent total recovery (TR) is defined as the ratio of the sum of the total protein in the elutate and column fall-through fractions to the total protein present in the feed. Percent yield (Y) is defined as the ratio of the IgG present in the eluate fraction to the total amount of IgG in the feed. Fragments were also chromatographed on protein A-hyper D column according to the manufacturer's instructions. The chromatographic fractions were assayed as outlined in the methods section.

Kinetic Uptake of HIgG Under Static Conditions

A small-scale experiment was conducted to determine the rate of uptake of HIgG, under batch conditions from a feed solution containing HSA and HIgG. Figure 3 shows the rate of disappearance of HIgG from solution. Since the experiment was conducted as a closed system, it is assumed that all HIgG not measured in solution is bound to the support. Maximum HIgG retention was observed at 24 hr with 91.6% disappearance of protein from solution. The largest percent of HIgG disappearance, or greatest adsorption rate, occurs within the first minute of the batch experiment, ranging from 0% adsorption at time 0 to 25% adsorption at 1 min. Fifty percent of the total equilibrium binding occurs at roughly 6 min. At 25 min, approximately

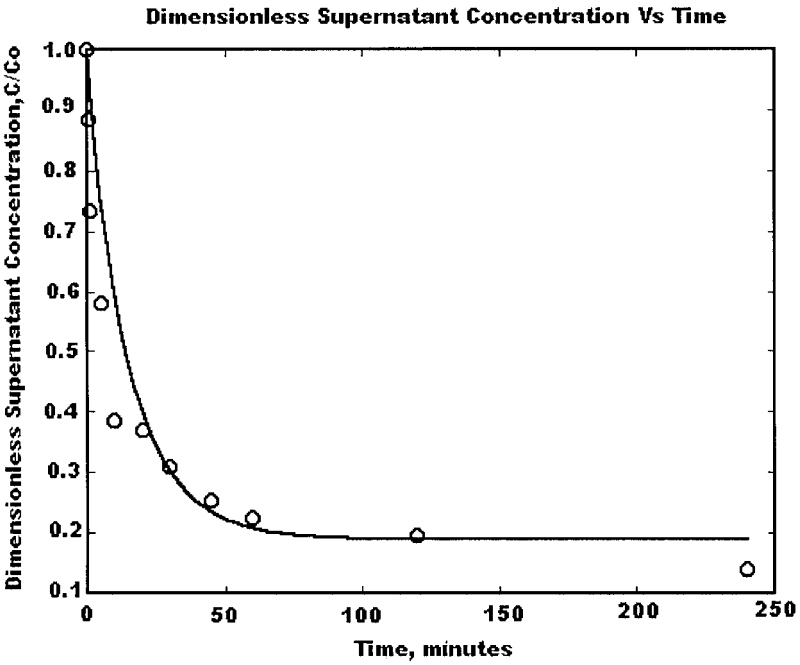

Figure 3. Batch adsorption profile for the uptake of HIgG by LMCB beads. The open circles indicate the experimental data, and the solid lines are calculated by the "kinetic rate constant model." LMCB beads were contacted with HIgG solution as described in the methods section. The supernatant was analyzed at different time intervals for the residual HIgG concentration. The data were normalized with respect to the HIgG concentration at time $t = 0$. Experiments were done in duplicate and the standard deviation was less than 8%.

78% of total binding has occurred according to our experimental data, with 90% occurring at 60 min. As shown in Fig. 3, the binding kinetics is very slow after 25 min compared with the first 25 min.

The experimental data for the protein adsorption obtained under static conditions were compared with the "kinetic rate constant model" discussed in detail elsewhere.[16] The only unknown parameter was the forward rate constant (k_1), whereas the isotherm parameters K_d and Q_{max} determined from static binding experiments were used and the reverse rate constant (k_2) was equated to K_d and k_1. The simulation was run with a variety of values of the unknown parameter k_1, and the value that gave the best fit to the experimental data was reported. The agreement between the experimental data and simulation is shown in Fig. 3. A k_1 value of $0.0055\,min^{-1}$ and a k_2 value of $0.0102\,min^{-1}$ were obtained. The values reported are derived from an average of three independent experiments and a percent error of less than 5% was noted.

Determination of the Dynamic Capacity

The dynamic isotherm was obtained from frontal analysis at four different linear velocities. The shape of the isotherm obtained indicated a Langmuir-type of adsorption and the dynamic bead capacity, Q_x was obtained from Scatchard analyses of the isotherms.[14] The effects of dynamic bead capacity, Q_x as a function of linear velocity are shown in Fig. 4. A dynamic HIgG adsorption capacity of 18.1, 14.6, 9.7, and 10.0 mg/mL bead was obtained at linear velocities of 1.1 (t_R, 1.18 min), 3.0 (t_R, 0.43 min), 5.6 (t_R, 0.23 min), and 9.0 (t_R, 0.14 min) cm/min, respectively. The column residence times (t_R) are shown in parentheses. The dynamic bead capacity decreases, to a critical point, with increasing linear velocity or decreasing t_R. It seems that a ceiling dynamic bead capacity of about 10 mg HIgG/mL bead is achieved at linear velocities of 5.6 cm/min and higher in the range tested in our laboratory.

Determination of the Adsorption Rate Constant

The effect of total protein injected to the column on the adsorption yield of HIgG was studied at varying linear velocities. At all linear velocities, the adsorption decreased with increasing cumulative amounts of HIgG injected, and the adsorption rate constant (k_a) was determined from the linear portion of the curve. Figure 5 shows the effect of the adsorption rate constant, which varies with linear velocity. An association rate constant of 46.1,

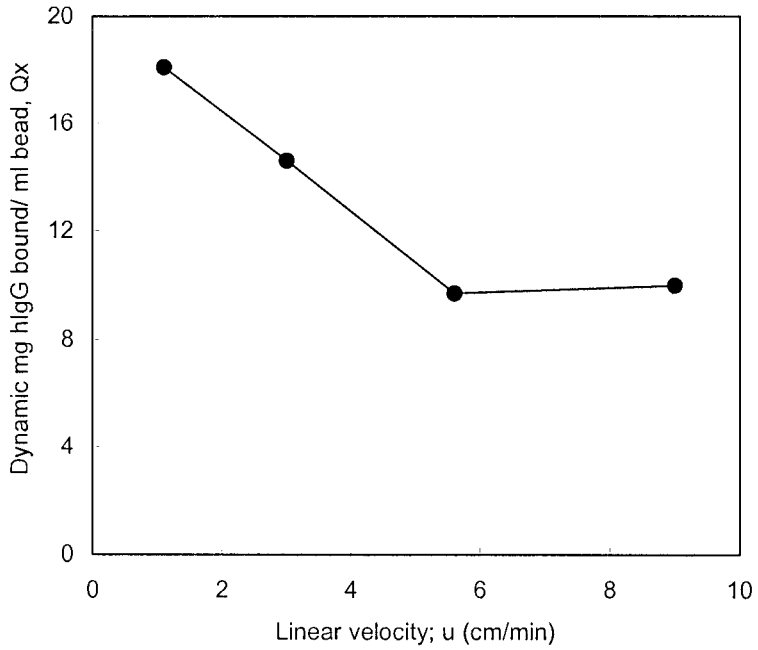

Figure 4. A solution of pure HIgG at concentrations ranging from 0.5 to 25 mg/mL was continuously fed to the column (1.3 × 1.0 cm) until the absorbance of the effluent reached 0.8–0.97 of the inlet concentration. The column was then washed with loading buffer and the adsorbed HIgG was eluted. For each concentration the dynamic capacity of the column was determined as the amount of HIgG maintained per milliliter of bead. Separate experiments were carried out at linear velocities of 1.13, 3.0, 5.66, and 9.05 cm/min.

157.6, 258.5, and 404.2 L mol^{-1} s^{-1} was obtained for linear velocities of 1.1, 3.0, 5.6, and 9.0 cm/min, respectively. A best fit of the data [Fig. 5(b)] reveals a linear trend for the data obtained. The equation $k_a = 44.4{*}u + 9.3$, where k_a is the rate constant (L mol^{-1} s^{-1}) and u is the linear velocity (cm/min), gives a good linear fit with an R^2 value of 0.994 within the range tested.

Effects of Adsorption Rate at Varying Linear Velocities

The effects of HIgG feed concentration on the adsorption rate of HIgG at varying linear velocities were evaluated. The adsorption rate increases with HIgG feed concentration and linear velocity. Empirical equations that relate

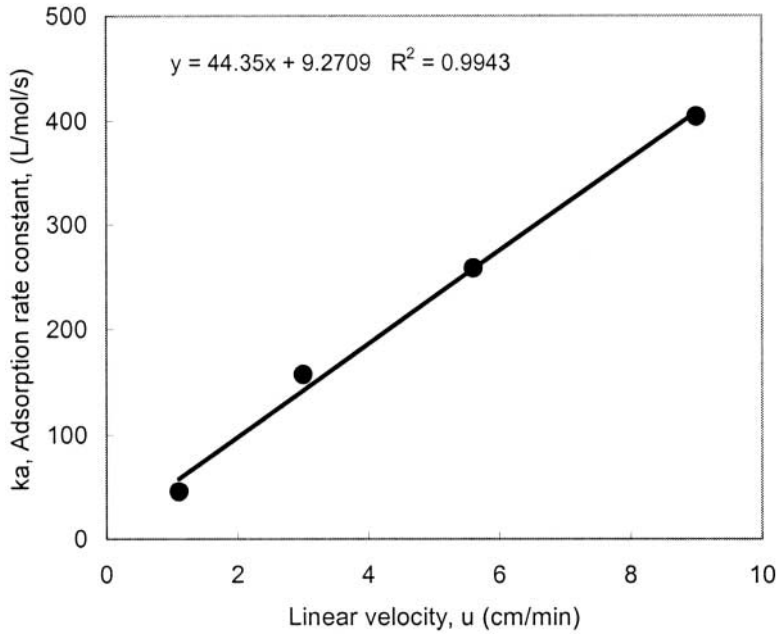

Figure 5. At a given constant linear velocity, 15 aliquots (1 mL each aliquot) mL of a 3.5 mg/mL solution of HIgG were consecutively injected into the column. The cumulative amount of injected protein and unadsorbed protein was calculated. The ratio of unadsorbed protein to the injected protein was expressed as f and $1 - f$, giving the adsorption yield. The rate constant k_a was determined from the linear region of the graph and plotted as a function of the linear velocity. Similar experiments were performed at other linear velocities studied (1.1, 3.1, 5.3, and 9.91 cm/min).

the adsorption rate changes with feed concentration at linear velocities of 1.1, 3.0, 5.6, and 9.0 cm/min are shown in Table 2. The adsorption rate is the highest at the highest experimental feed concentration of 15 mg HIgG/mL, more than 20 times than that of the lowest concentration at 0.5 mg HIgG/mL.

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

Table 2. Effects of flow rate on the rate of adsorption.

HIgG feed concentration (mg/mL)	Equation
0.5	$AR = 0.11(u)^{0.74}$
1.0	$AR = 0.24(u)^{0.64}$
2.5	$AR = 0.41(u)^{0.75}$
5.0	$AR = 0.82(u)^{0.63}$
10.0	$AR = 1.3(u)^{0.67}$
15.0	$AR = 2.3(u)^{0.59}$

Notes: The data obtained in Fig. 5 were approximated to the equation $AR = a[u]^b$, where a and b are constants, u is the linear velocity, and AR is the adsorption rate. The adsorption rate (mg per milliliter per minute) was calculated as the amount of protein retained per mL of support (mg/mL)/time of adsorption (min).

of chitosan beads (400–600 μm in diameter) with innovative matrix architecture, which differentiates chitosan beads used in this study from all other liquid chromatography bio-separation 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 high surface area beads in conjunction with the open architecture create homogeneous microenvironments of low binding energy, and this consequently enables ligand specificity characteristic of moderately high affinity separations.

In our ongoing studies, we have utilized the primary amine of the chitosan polymer backbone in a crosslinking reaction with a di-epoxide to yield a derivatized chitosan plus a spacer (or Tether) with a reactive epoxide group.[14] This latter structure was subsequently substituted with low molecular weight ligands upon nucleophilic reactions. We have used a carboxylic group containing anionic ligand to yield a modified chitosan beads. In particular, we have demonstrated the ability of modified chitosan beads to enrich and purify antibodies from cell culture supernatants and serum samples in good yields and high purity.[17] Interestingly, modified chitosan beads were found to interact specifically with IgG, IgA, and IgM over serum albumin and the binding was mediated through a differential in binding capacity.[17] In other words, LMCB beads were able to bind IgG specifically over BSA from biological mixtures with little to none of BSA binding. Thus, we hypothesize that Ligosep A maybe used as sorbents in the antibody pre-purification steps.

The underlying rationale for this study is that a fundamental understanding of the nature of interactions between LMCB and Ig's, and of the mechanism of biomolecules transport in these matrices, will enable the development of robust and scalable alternate sorbents for use in preparative bioseparations. To gain a better understanding of the interaction between the protein and the binding site, it is often necessary to quantify important factors, such as capacity of the affinity matrix and the disassociation constant of the corresponding protein–ligand interaction. Further studies aimed at understanding the adsorption mechanism and the nature of interactive forces between IgG and binding site on LMCB beads were undertaken. A study of the kinetic and thermodynamic parameters, such as affinity constants and adsorption rate constants of this interaction, was undertaken.

It is informative 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 temperatures studied indicates a Langmuir-type isotherm. 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, as determined by the Langmuir adsorption data, was calculated to be 64–87 mg IgG per mL of beads, which is comparable with that reported for protein A-Sepharose and protein A-Ultragel. The dissociation constant, K_d, was determined to be in the range of 1.12×10^{-5}–2.4×10^{-5} M, which indicates medium affinity and is typical for a pseudo-affinity ligand.[18]

The high capacity and selectivity displayed by the matrix for IgG in equilibrium, prompted us to study the kinetic aspects of this adsorption for an eventual scale-up of IgG separation from plasma or biological fluids. The values obtained for the dynamic capacity at the experimental linear velocities tested, ranged from 9.7–18.1 mg IgG per mL of beads, which again is comparable to the values reported for protein A-Sepharose and protein A-Ultragel under similar chromatographic conditions and column residence times. It is to be noted, the dynamic capacities reported here are for very short residence times (length of column/linear velocity) in the columns. Our ongoing research has shown that when a sufficiently long column is used to provide longer column residence times, dynamic binding capacities in the range of 35–45 mg IgG/mL of beads were obtained (data not shown). The dynamic dissociation constants were in the range of 4×10^{-7}–1.6×10^{-5} M for the linear velocities tested, indicating weak to medium affinity and consistent with the easy and non-denaturing desorption of bound IgG in column-mode.

The adsorption rate constant, k_a, which was determined by the split-peak method, gave a range of values from 46.1–404.2 $L\,mol^{-1}\,s^{-1}$ for linear velocities of 1.1–9.0 cm/min, respectively. The linear increase in k_a for the range of velocities tested, indicates that the adsorption is velocity dependent

and that a change in linear velocity will influence Ig retention. This equation can be utilized in the scale-up separation of IgG by process scale chromatography with LMCB.

In our work, the adsorption rate increases with increasing linear velocity. This is in good agreement with our adsorption rate constant results, which follow the same linear trend. Additionally, for the same linear velocity, the adsorption rate increases with increasing HIgG feed concentration. This is due to the greater number of IgG molecules in solution and, consequently, more in the vicinity of the binding sites. Empirical relationships relating the adsorption rate with linear velocity, u, in the form of $AR = a(u)^b$ were derived for column-mode HIgG retention and are presented in Table 2. These equations can be used in the design and scale-up of HIgG separations.

One of the interesting findings of this study is the affinity of the LMCB matrix for the F_{ab} region over the F_c region of the antibody. In fact, our data suggest that it does not interact with the F_c fragment. We have indeed created a pseudo-bioaffinity matrix by modifying the epoxy-derivatized chitosan with an anionic ligand. This finding has a great potential in clinical immunoassay techniques. We will be now able to use the selectivity in binding to separate paratopes by adjusting elution conditions.

We postulate that the interactions are governed by a combination of ionic, electrostatic, and van der Waals interactions. We conclude that hydrophobic forces probably play a minor role.[17] It is possible to fine-tune the selectivity of Ligosep Alpha® for a special subset or subclass of IgG, by choosing the appropriate buffer. The weak affinity interactions are very advantageous for a high throughput and recovery of labile proteins such as IgG, compared with protein A immobilized gels. The high capacity and the reproducibility are attractive features in using such a system for scale-up operations. We are currently exploring spectroscopic methods to better understand the affinity of Ligosep Alpha® beads.

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