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# AbSep—An amino acid based pseudobioaffinity adsorbent for the purification of immunoglobulin G

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ABSTRACT

The present work deals with the development and characterization of a tryptophan based pseudobioaffinity adsorbent for the purification of monoclonal and polyclonal antibodies. Tryptophan as a ligand was selected based on molecular docking and experimental screening studies of the amino acids involved in IgG–Protein A interaction. The ligand was coupled to a polymethacrylate based rigid, porous SEPABEADS beaded matrix to obtain the desired affinity adsorbent, which was named AbSep. Characterization studies with regards to the effect of matrix properties (pore size, particle size, nature of matrix, spacer arm) and the medium properties (pH, conductivity, additives) were performed to elucidate the nature of IgG–AbSep interactions and to determine the optimal conditions for obtaining high binding and purity of IgG. The equilibrium binding capacity of AbSep and dissociation constant was found to be 78 mg/ml and $5.31 \times 10^{-6}$ M respectively. AbSep was able to successfully purify polyclonal human IgG from plasma and monoclonal antibody (chimeric IgG1) from CHO cell culture supernatant. Both binding and elution steps were performed at near neutral pH resulting in a purity and recovery of more than 90% and 85% respectively. Additionally, AbSep was shown to be stable to 0.5 M NaOH solutions, the preferred agent for cleaning and sanitization of chromatographic media.

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## 1. Introduction

Monoclonal and polyclonal antibodies have emerged as an important class of biopharmaceuticals for the treatment of a number of diseases such as cancer, autoimmune, immunodeficiency, infection, skin disorders, neurological disorders and other diseases. Monoclonal antibodies (MAbs) and Fc-fusion proteins account for 43% of therapeutic proteins market value [1] whereas the human polyclonal IgG is the backbone of plasma fractionation industry [2].

However, the antibody based therapies especially those involving monoclonal antibodies suffer from limitations of high cost. This high cost is due to high dosage of antibody required (>1 g per patient per year) [3] for therapy and due to high downstream processing costs. Protein A affinity chromatography which is the most widely used unit operation for the purification of MAbs is very expensive, accounting to nearly 40% of the overall downstream processing costs [4]. Moreover, being a biological ligand it suffers from limitations such as low chemical and physical stability, leachability and toxicity related issues. In case of polyclonal antibodies, Cohn fractionation process used for the purification of human polyclonal IgG, suffers from problems of low yields and low purity thus necessitating the need for additional chromatographic steps, thereby reducing the overall yield [5].

As a result, considerable work has been in progress to (a) to find alternative to Protein A affinity chromatography and (b) find alternatives to Cohn fractionation for polyclonal IgG from blood sera. In this light, attempts have been made over the last two decades for developing specific, inexpensive and robust ligands for the purification of antibodies. These ligands, at times termed as pseudobioaffinity or pseudoaffinity ligands, are coupled to suitable porous adsorbents to result in pseudobioaffinity adsorbents. Thus, adsorbents based on amines such as histidine and histamine [6–8], thiophilic derivatives [9,10], peptide/s [11,12], and those based on other low molecular weight ligands [13–16] have been reported for the purification of antibodies. However, these adsorbents have not been able to address all the problems and emerge as successful alternative to Protein A based adsorbents. It was therefore the objective of present work to attempt designing an inexpensive, physically and chemically stable, nontoxic, specific affinity adsorbent for IgG.

Work on design of affinity ligands has followed different lines that can be classified as rational, combinatorial or combined [17]. The rational approach involves retrieving structural information, such as crystallographic, NMR or homology data, about the ligand–protein, or enzyme–substrate, or protein–protein complexes from the databases. These binding complexes are

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then used as basis to design complementary pseudoaffinity, or affinity mimicking ligands, whose structure mirrors some or all of the ligand, substrate or partner protein [18]. The rapid growth in computational tools such as molecular docking software operating on powerful computers has made rational design of the ligands increasingly easy and attractive. The combinatorial approach consists of creating large libraries of molecules followed by experimental screening of the libraries for locating potential ligands. The combined method exploits both the rational and the combinatorial methods for designing of pseudoaffinity ligands.

Amino acids are some of the simplest natural molecules that have been used as affinity ligands for the purification of various biomolecules. They are stable to various physical and chemical conditions and are inexpensive than the complex biological ligands [7,14]. Different amino acids have been grafted or coupled to gel, membrane or silica supports to function as affinity media for the purification of IgG. L-histidine chromatography for IgG and other proteins has been described in the literature [6–8]. Other amino acids reported include L-tryptophan and L-phenylalanine grafted on hollow fibre membrane used for the purification of bovine IgG [19].

In the present work, an IgG affinity adsorbent – AbSep comprising of tryptophan has been developed based on the molecular docking and experimental screening studies. AbSep was characterized with regards to the effect of various parameters such as nature of base matrix, pore size, particle size and spacer arm on the binding capacity for IgG. A detailed study on the effect of pH, ionic strength and polyethylene glycol on the binding of IgG to AbSep was also carried out to elucidate the IgG–AbSep interactions. Adsorption isotherm and breakthrough studies were performed to determine the equilibrium binding capacity, dissociation constant and dynamic binding capacities. Finally, chromatographic purification studies of IgG from human plasma and monoclonal antibody from Chinese hamster ovary (CHO) cell culture supernatant using AbSep are presented. The results demonstrate the potential of this amino acid based affinity adsorbent to provide a cost effective solution for the large scale purification of antibodies.

## 2. Experimental

### 2.1. Materials

Human plasma was obtained from blood bank (KEM hospital, Mumbai, India), Human polyclonal IgG was obtained from VHB Life Sciences Inc. (Mumbai, India). Cell culture supernatant containing 200 mg/l MAb was a kind gift from Reliance Life Sciences (Mumbai, India). Bovine serum albumin (BSA) was purchased from Sisco Research Laboratories (Mumbai, India). Sepabeads® EB-EP, a polymethacrylate based rigid and porous resins having epoxy groups were kind gifts from Resindion SRL (Binasco, Italy). They are available in different particle sizes and pore sizes such as Sepabeads EBEP1 (150 μm, 300 Å), Sepabeads EBEP2 (150 μm, 600 Å), Sepabeads EBEP3 (150 μm, 1000 Å) and Sepabeads EBEP4 (75 μm, 300 Å). Profinity™ Epoxide and Macro-Prep® 50 Epoxide were gifts from Bio-Rad (CA, USA). Sepharose® CL-4B was purchased from GE Healthcare (NJ, USA). Poros® Ep 250 having epoxide groups was a kind gift from Applied Biosystems (CA, USA). HiTrap Protein G column (1 ml) and Affi-Prep Protein A resin were obtained from GE Healthcare and Bio-Rad respectively. L-tryptophan, L-tyrosine, L-phenylalanine, L-histidine, buffer ingredients and reagents were purchased from Sisco Research Laboratories (Mumbai, India). Ultra-pure water, obtained with MilliRO-MilliQ system (Millipore, USA) was used for all experiments.

### 2.2. Method of analysis

Protein and IgG concentration was determined spectrophotometrically at 280 nm using BSA and human polyclonal IgG as standard respectively with a double beam UV–VIS spectrophotometer (Shimadzu, Japan, Model UV-2550). The extinction coefficient of 1 mg/ml BSA solution and 1 mg/ml IgG solution was determined to be 0.6 and 1.4 respectively. IgG quantification during the column purification studies was done using FPLC analysis on HiTrap Protein G column. FPLC analysis was performed on automated chromatographic system (Biologic Duoflow Pathfinder, Bio-Rad). The detection was done at 280 nm and flow rate was maintained at 1 ml/min throughout the analysis. Human polyclonal IgG was used as standard for calibration. The chromatographic fractions were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) as described by Laemmli [20] using 7.5% gels on Mini-Protean II from Bio-Rad. Equal amounts of proteins were applied under non-reducing conditions. The gels were stained with method of silver staining. The developed gels were documented using gel documentation system (Gel Doc, Bio-Rad).

### 2.3. Selection of ligands and docking studies

X-ray crystallographic structure of binding complex between B domain of Protein A and Fc portion of IgG (PDB ID 1FC2) was used as the template for studying the interactions between Protein A and Fc region of IgG. The three dimensional structure of the IgG molecule was obtained from public domain (www.path.cam.ac.uk/~mrc7/pdb/index.html). This structure was processed and typed with CHARMm force field [21] and minimized using Smart Minimizer to obtain lowest energy. Binding sites were determined from the minimized structure of IgG using the 'Active site search' [22] algorithm and the most probable site with maximum number of points was selected for docking the potential ligands using LigandFit program [23] incorporated in Discovery Studio (Accelrys, CA, USA). Docking results were analyzed using Dock Score and ligand internal energy. The ligands with highest Dock Score and lowest internal energy were selected for experimental screening studies.

### 2.4. Preparation of adsorbents

Potential ligands were coupled on Sepabeads EBEP1, using epoxide coupling chemistry as described by Hermanson et al. [24]. Ten millilitres of adsorbent was washed extensively with acetone and distilled water to remove trace impurities and then suspended in an equal volume of 50 mM carbonate buffer pH 9.5 containing suitable concentrations of amino acid. Amino acids with low water solubility were solubilized with addition of 2 M NaOH. The reaction for coupling the ligand to the adsorbent was carried out by stirring the mixture at 45 °C for 48 h. The adsorbent was then washed extensively with 1 M NaCl and distilled water to remove any excess ligand. The unreacted epoxy groups were blocked by treatment with 1 M ethanolamine, pH 9.0 at 30 °C for 6 h. Finally, the adsorbent was washed extensively with 1 M NaCl and distilled water.

### 2.5. Screening of adsorbents

The above prepared adsorbents were screened for their affinity to IgG. A 0.5 ml of each adsorbent was added to 5 ml of 50 mM sodium phosphate buffer, pH 7 and equilibrated for 30 min on a rocker. The stock solution of IgG having a concentration of 50 mg/ml was diluted to 5 mg/ml with the equilibration buffer and 2.5 ml of

**Table 1**
Different adsorbents used to study the effect of the base matrix on IgG binding.

| Adsorbent | Base matrix | Manufacturer |
|---|---|---|
| Sepabeads EBEP1 | Polymethacrylate | Resindion SRL, Italy |
| Sepharose CL-4B | Agarose | GE Healthcare, USA |
| Profinity Epoxide | Polyacrylamide | Bio-Rad, USA |
| Poros Ep 250 | Polystyrene divinyl benzene | Applied Biosystems, USA |
| Macro-Prep 50 Epoxide | Polymethacrylate | Bio-Rad, USA |

this IgG solution was added to the equilibrated adsorbents. The mixture was incubated on a rocker for 4 h. Then the adsorbents were allowed to settle and supernatants were analyzed spectrophotometrically for IgG content. The amount of bound IgG was calculated by mass balance.

The adsorbent with the highest binding capacity was tested for its selectivity towards IgG by performing batch binding studies with bovine serum albumin (BSA). A 0.5 ml of the selected adsorbent was equilibrated with 50 mM sodium phosphate buffer pH 7 and contacted with 2.5 ml of BSA (5 mg/ml) solution for 4 h. For comparison, a 0.5 ml of the adsorbent was also mixed with IgG solution under similar conditions as used for BSA binding. The supernatants were analyzed for protein content to determine the binding abilities of the adsorbent for the target (IgG) and contaminant (BSA) proteins. The selected adsorbent will henceforth be referred as '**AbSep**'.

### 2.6. Effect of base matrix of AbSep on IgG binding

To study the effect of base matrix of AbSep, the above selected ligand was coupled to different epoxy activated base matrices (Table 1) according to the procedure described in Section 2.4. The ligand density was determined by nitrogen estimation using Kjeldahl method [25]. For coupling the ligand to the Sepharose CL-4B, the matrix having hydroxyl groups was first epoxy activated according to the procedure described by Hermanson et al. [24]. Ten millilitres of Sepharose CL-4B was washed with 200 ml of water, suction dried to a moist cake, and transferred to a 200 ml flask. The washed matrix was then mixed with 3.75 ml epichlorohydrin, 34.5 ml of 2 M NaOH, 0.125 g of sodium borohydride and continuously stirred. To this mixture, 34.5 ml of 2 M NaOH and 17 ml epichlorohydrin were added in small portions over a period of 1.5 h. The reaction mixture was stirred overnight at room temperature. Finally the adsorbent was washed successively with 200 ml each of water, 0.1 M acetic acid and water. The selected ligand was then coupled to the epoxy activated Sepharose Cl-4B. IgG binding studies were performed with each of the prepared adsorbents according to procedure described in Section 2.5.

To determine the contribution of the polymethacrylate base matrix (Sepabeads) of AbSep to IgG binding, studies were carried out with the plain matrix without ligand. The activated epoxy groups present on Sepabeads EPEP1 were hydrolyzed according to the procedure described in Hermanson et al. [24]. Ten millilitres of matrix was washed with water and dried on a sintered glass funnel, suspended in 30 ml of 2 N HCl and stirred for 6 h at 30 °C to produce a matrix containing hydroxyl groups.

### 2.7. Effect of pore size and particle size of AbSep on IgG binding

Different pore size Sepabeads were used to study effect of pore size of AbSep on IgG binding. The selected ligand was coupled to Sepabeads EBEP1, Sepabeads EBEP2, Sepabeads EBEP3 of pore sizes 300 Å, 600 Å and 1000 Å respectively according to procedure described in Section 2.4. Effect of particle size of AbSep on IgG binding was studied with Sepabeads EBEP1 and Sepabeads EBEP4 with particle size of 150 μm and 75 μm respectively. After coupling the selected ligand, the adsorbents were used for the IgG batch binding studies under the same conditions as described in Section 2.5.

### 2.8. Effect of spacer of AbSep on IgG binding

For the study of the effect of spacer arms the selected ligand was coupled to base matrix – Sepabeads EBEP4 via two and six carbon spacer arms. For introduction of two carbon spacer arm ethylenediamine was selected whereas for six carbon spacer arm hexamethylenediamine was chosen. These spacers were coupled to 10 ml Sepabeads EBEP4 according to procedure mentioned in Section 2.4. The resultant matrix consisted of terminal $-NH_2$ group and 2 additional carbon atoms or 6 carbon atoms as spacer in addition to the 2 carbon atoms of epoxy groups. For attaching the selected ligand to the matrices with spacer arms, 4 ml of each matrix was first epoxy activated with epichlorohydrin by suspending the matrix in a mixture of 1 ml epichlorohydrin and 5 ml of 0.02 M NaOH solution. The mixture was rotated at 40 °C for 6 h. The activated matrix was thoroughly washed with distilled water and then suspended in 15 ml of 0.1 M ligand solution. The reaction was carried out for 24 h at 60 °C. The adsorbents were finally washed with distilled water. A 0.5 ml each of these prepared adsorbents and adsorbent without spacer arm were evaluated for their IgG binding according to the procedure described in Section 2.5.

### 2.9. Effect of pH, conductivity and polyethylene glycol on IgG binding

To study the effect of pH on IgG binding to AbSep, 0.5 ml of adsorbent was taken in each of the seven centrifuge tubes and equilibrated for 1 h in 5 ml of different buffers having pH in the range 4–9. The following buffers were used: 25 mM acetate (pH 4, pH 4.5, pH 5), 25 mM sodium phosphate (pH 6, pH 6.5, pH 7) and 25 mM Tris–HCl (pH 8, pH 9). The equilibrated adsorbent in the tubes was then contacted with 2.5 ml of IgG (5 mg/ml) prepared in the respective equilibration buffers. After 4 h the adsorbent was allowed to settle and supernatants were analyzed for IgG content.

Effect of conductivity was studied in terms of NaCl concentration. A 0.5 ml of AbSep was taken in each of the eight centrifuge tubes and equilibrated for 1 h with 25 mM sodium phosphate buffer, pH 7 containing varying amounts of NaCl from 0 to 2 M. After equilibration, the adsorbent in the tubes was contacted with 2.5 ml of IgG (5 mg/ml) prepared in the respective equilibration buffers. After 4 h the supernatants were analyzed for the IgG content.

Effect of polyethylene glycol (PEG) 600 on binding of IgG to AbSep was studied to determine the role of hydrophobicity in IgG–AbSep interactions. PEG when added to the medium reduces the polarity of medium, thereby reducing the hydrophobic interactions between the protein and the adsorbent [26]. To study the effect of polyethylene glycol (PEG), 0.5 ml of adsorbent was taken in each of the six centrifuge tubes and equilibrated for 1 h with 25 mM sodium phosphate buffer, pH 7 containing 1 M NaCl and varying concentrations of PEG 600 from 0 to 20% (w/v). After equilibration the adsorbent in the tubes was contacted with 2.5 ml of IgG (5 mg/ml) prepared in the respective equilibration buffers. After 4 h of contacting, the supernatants were analyzed for IgG content.

### 2.10. Determination of adsorption isotherm

Adsorption isotherm was determined to find the equilibrium binding capacity and dissociation constant ($K_d$) of IgG for AbSep. A 0.5 ml of adsorbent was taken in each of the six centrifuge tubes and equilibrated for 1 h with 25 mM sodium phosphate buffer, pH 6.5. After decanting the buffer, 5 ml IgG solution of varying concentrations was added to the six centrifuge tubes and incubated for 4 h on a rocker. The supernatants were analyzed for IgG con-

tent and a graph of IgG adsorbed per ml of AbSep vs. equilibrium (supernatant) concentration of IgG (mg/ml) was plotted.

### 2.11. Breakthrough studies

Breakthrough studies or frontal analysis was carried out to determine the dynamic binding capacity of AbSep for IgG at various linear velocities. Chromatographic experiments were carried out on a preparative chromatographic system (Biologic Duoflow, Bio-Rad, USA) at room temperature (30 °C). A 5 ml adsorbent was packed in a BioScale column (10 mm i.d., 6.4 cm (L), Bio-Rad) and equilibrated with 25 mM sodium phosphate buffer containing 10% polyethylene glycol 600 (PEG 600), pH 7.0. An acetone pulse (5%, v/v) was applied to the column to determine the total column void volume. IgG solution of 15 mg/ml was prepared in 25 mM sodium phosphate buffer, pH 7 and loaded on the adsorbent at linear velocities of 50 cm/h, 70 cm/h, 100 cm/h, 200 cm/h, 300 cm/h and 500 cm/h. The IgG solution was loaded until the bed was completely exhausted. Elution of bound IgG was carried out with 25 mM sodium phosphate buffer pH 7 containing 1 M NaCl and 20% PEG 600. Fractions were collected and analyzed for IgG content. Breakthrough volume was determined at the point where IgG concentration in the flow through fraction reached 5% of its feed concentration. This breakthrough volume was corrected by subtracting the void volume and based on this corrected volume the dynamic binding capacity of AbSep was determined.

### 2.12. Purification of polyclonal IgG from human plasma

Purification of IgG from human plasma was carried out on 5 ml AbSep packed in 10 mm i.d. Econo-column (Bio-Rad, USA). The adsorbent was equilibrated with 4 column volumes (CVs) of 25 mM sodium phosphate buffer pH 7 or 25 mM sodium phosphate buffer pH 7 containing 6% PEG 600 or 10% PEG 600. The human plasma was diafiltered with 25 mM sodium phosphate buffer pH 7 using Minimate™ Tangential Flow Filtration capsule (5 K Omega membrane, Pall Life Sciences) till the conductivity reached below 5 mS/cm. One millilitre of this diafiltered human plasma was loaded onto the adsorbent. Washings were performed with 5 CVs of equilibration buffer to remove the unbound plasma proteins. Elution of adsorbed protein was then performed by passing 5 CVs of equilibration buffer containing 1 M NaCl and 20% polyethylene glycol 600. Regeneration of AbSep was done by passing 5 CVs of 0.5 M NaOH. The experiment was performed on a preparative automated chromatographic system (Biologic Duoflow, Bio-Rad) at 30 °C and at flow rate of 1 ml/min. Fractions were collected and analyzed for protein determination, IgG purity and recovery.

### 2.13. Purification of monoclonal antibody from cell culture supernatant

Fifteen millilitres of cell culture supernatant containing 200 mg/l of monoclonal antibody was diafiltered with 25 mM sodium phosphate buffer pH 7 as described in Section 2.12 and loaded on 2 ml AbSep column equilibrated with 25 mM sodium phosphate buffer pH 7 containing 10% PEG 600. Washing was performed with 4 CVs of equilibration buffer and elution was carried out with 5 CVs of 25 mM sodium phosphate buffer containing 1 M NaCl and 20% PEG 600. Regeneration was performed with 4 CVs of 0.5 M NaOH. The experiment was performed on preparative automated chromatographic system at 30 °C and at flow rate of 1 ml/min. Fractions collected were analyzed by SDS PAGE under non-reducing conditions.

**Table 2**
Docking of amino acids to IgG.

| Amino acid | Dock score | Ligand internal energy |
|---|---|---|
| Tryptophan | 67.48 | −2.579 |
| Phenylalanine | 63.17 | −1.653 |
| Histidine | 61.28 | −1.137 |
| Tyrosine | 60.35 | −1.944 |
| Glutamine | 59.92 | −0.621 |
| Lysine | 55.17 | 1.459 |
| Leucine | 55.07 | −0.596 |
| Arginine | 53.48 | −0.766 |
| Isoleucine | 51.88 | 0.054 |

### 2.14. Reusability of AbSep

Reusability studies were carried out to determine the stability of AbSep to (1) Binding and elution conditions and (2) NaOH washes used during the cleaning and sanitization steps. A 0.5 ml of AbSep was taken in a centrifuge tube and equilibrated for 1 h in 5 ml of 25 mM sodium phosphate buffer pH 6.5. The equilibrated adsorbent was then contacted with 2.5 ml of IgG (5 mg/ml) solution prepared in the equilibration buffer. After 4 h the adsorbent was allowed to settle and supernatant was analyzed for IgG content. The IgG bound to the AbSep was eluted by treating the adsorbent with 5 ml of 25 mM sodium phosphate buffer pH 6.5 containing 1 M NaCl and 20% PEG 600 for 30 min. Finally, the adsorbent was regenerated by contacting with 5 ml of 0.5 M NaOH solution for 1 h. The regenerated AbSep was again used for IgG binding studies. This cycle of IgG binding followed by regeneration was repeated ten times.

Leaching of the ligand was also determined by repeating the above runs without loading the IgG solution. The supernatants were collected during equilibration, elution and NaOH wash steps and analyzed spectrophotometrically at 280 nm for ligand content.

## 3. Results and discussion

### 3.1. Rational screening of ligands

Computational studies of the binding complex of Fc portion of IgG and B domain of Protein A (Fig. 1) showed that the interaction site is dominated by the aromatic amino acids such as phenylalanine, tyrosine and tryptophan. In addition to aromatic amino acids, amino acids such as histidine, leucine, glutamine, isoleucine and lysine were also observed to participate in the binding complex. Li et al. [14] had also analyzed the binding complex and developed an IgG affinity ligand comprising of 3-aminophenol and 4-amino-1-napthol moieties substituted on the triazine scaffold.

To determine the affinity of each of these amino acids towards IgG and narrow down the ligands for experimental screening studies, molecular docking technique was adopted. The affinity of the amino acids towards IgG was evaluated in terms of Dock Score and ligand internal energy and is shown in Table 2. Dock score is a measure of strength of binding of a ligand to target molecule (IgG) therefore, higher the score the better is the fit of ligand onto the binding site of the target molecule. Ligand internal energy on the other hand gives the bumps or conflicts between the ligand and the target molecule therefore, lower the ligand internal energy better the interaction. As can be seen from Table 2, the highest Dock Score and least ligand internal energy was obtained for tryptophan. The next best docking was shown by phenylalanine followed by histidine and tyrosine. These results were consistent with above observations that aromatic amino acids of Protein A play a significant role in IgG–Protein A interaction. Based on these studies the four amino acids *viz.* tryptophan, tyrosine, pheny-

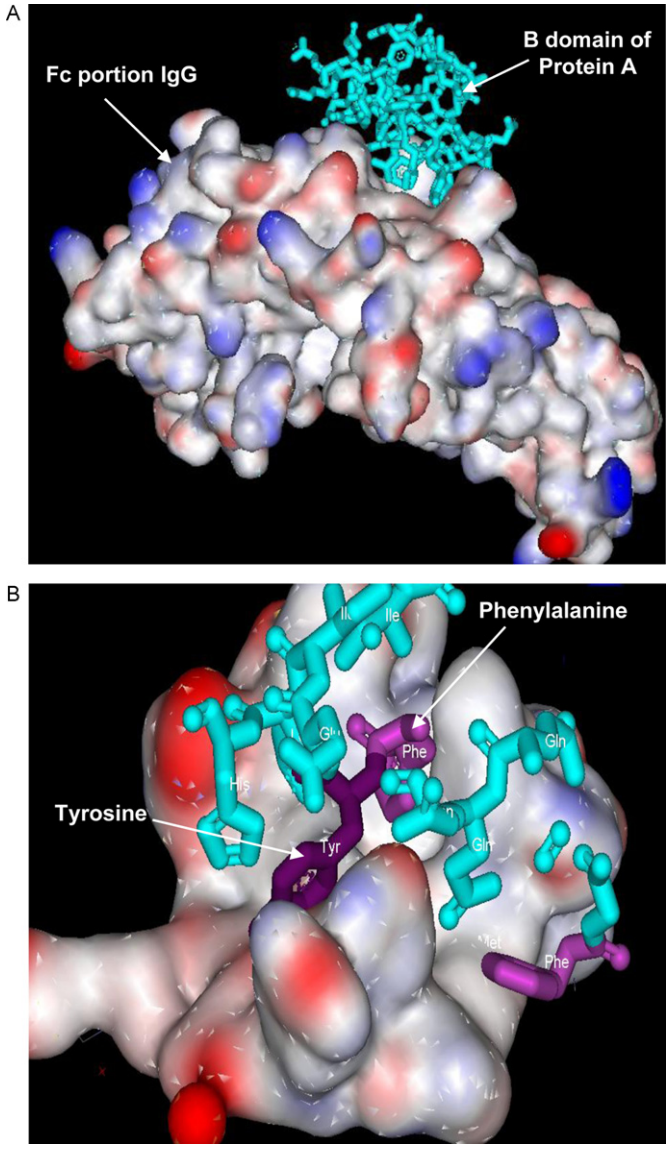


**Fig. 1.** (A) X-ray crystallographic structure of binding complex between B domain of Protein A and Fc portion of IgG (PDB file 1FC2). (B) A closer look at binding site.

lalanine and histidine were selected for experimental screening studies.

### 3.2. Screening of the adsorbents

Phenylalanine, tyrosine, tryptophan and histidine were coupled to Sepabeads EB-EP1 under identical conditions. The four adsorbents thus prepared were screened for their IgG binding and results are shown in Fig. 2. It can be seen from Fig. 2 that tryptophan showed the highest binding (19.3 mg/ml of adsorbent) as compared to phenylalanine (14.2 mg/ml of adsorbent), histidine (12.9 mg/ml of adsorbent) and tyrosine (8 mg/ml of adsorbent). It is interesting to note that this order of binding affinity of the amino acids for IgG was similar to that obtained by molecular docking studies. Sepabeads EB1-tryptophan was therefore selected for further studies.

The selectivity of Sepabeads EB1-tryptophan towards IgG was tested by carrying out batch binding studies with bovine serum albumin (BSA). For comparison, binding studies were also performed with IgG under same conditions as used for BSA binding. The adsorbent showed much lower binding for BSA (4.2 mg/ml of

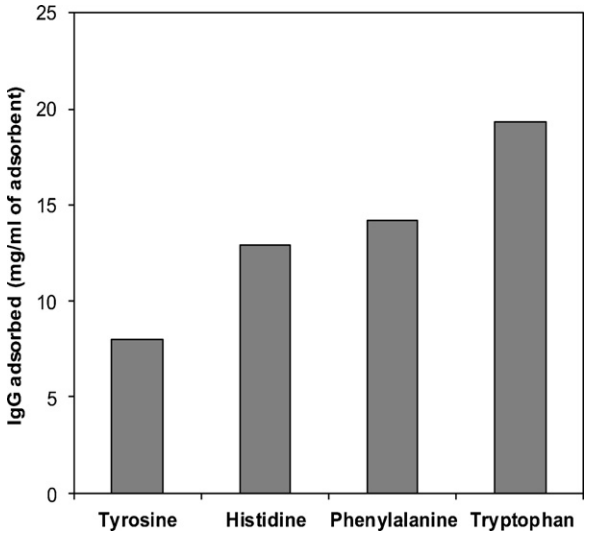


**Fig. 2.** IgG binding capacity of Sepabeads EB1-tyrosine, Sepabeads EB1-phenylalanine, Sepabeads EB1-histidine and Sepabeads EB1-tryptophan. A 0.5 ml of each adsorbent was mixed with 2.5 ml of 5 mg/ml IgG solution prepared in 50 mM sodium phosphate buffer, pH 7 for 4 h.

adsorbent) as compared to that observed for IgG (18.7 mg/ml of adsorbent), thus indicating the selectivity of adsorbent towards IgG. This adsorbent will henceforth be referred to as 'AbSep'.

### 3.3. Comparison with other base matrices

The nature of base matrix often plays a significant role in binding and selectivity exhibited by adsorbents for protein. Tryptophan was therefore coupled to different commercially used base matrices such as gel based Sepharose CL-4B (GE Healthcare, NJ, USA), Polyacrylamide based Profinity Epoxide (Bio-Rad, CA, USA) and Polystyrene-divinylbenzene based POROS EP 250 (Applied Biosystems, CA, USA). For comparison, tryptophan was also coupled to polymethacrylate base matrix Sepabeads EP (Resindion, Italy). The ligand density of Sepabeads-Trp (AbSep), Sepharose-Trp, Profinity-Trp and POROS-Trp was 75 μmol/g, 79 μmol/g, 68 μmol/g, 77 μmol/g respectively. IgG binding studies were carried out with these tryptophan coupled matrices, the results of which are presented in Fig. 3. Tryptophan bound Sepharose, Profinity and POROS adsorbents showed very low IgG binding as compared to Sepabeads-Trp (AbSep). Thus, in spite of similar ligand density the adsorbents with agarose, polyacrylamide, polystyrene-

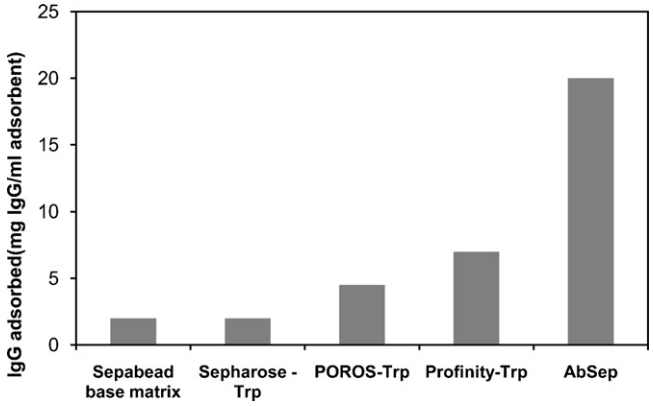


**Fig. 3.** IgG binding capacity of tryptophan coupled to different base adsorbents. A 0.5 ml of each adsorbent was mixed with 2.5 ml of 5 mg/ml IgG solution prepared in 50 mM sodium phosphate buffer, pH 7 for 4 h.

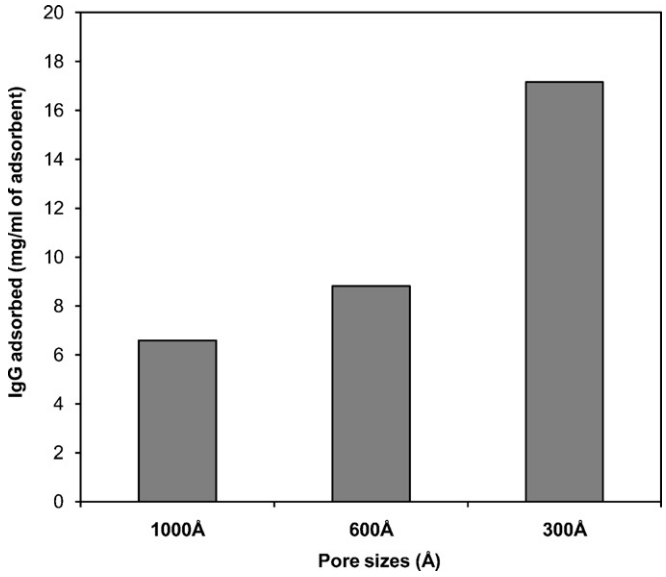


**Fig. 4.** Effect of pore size of AbSep on IgG binding. A 0.5 ml of each adsorbent was mixed with 2.5 ml of 5 mg/ml IgG solution prepared in 50 mM sodium phosphate buffer, pH 7 for 4 h.

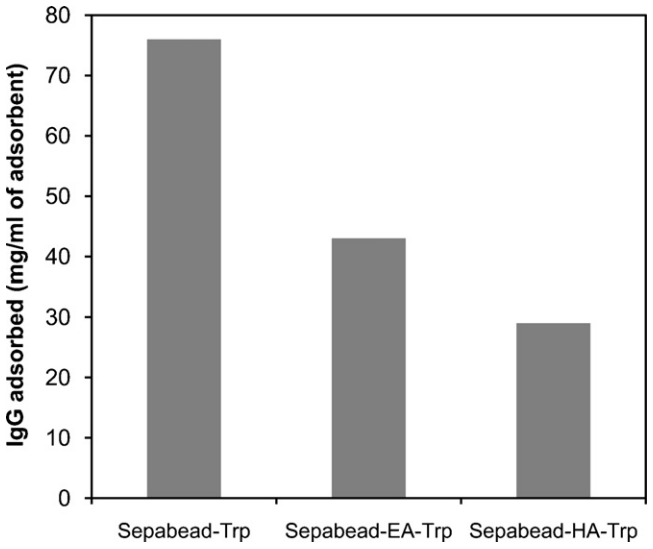


**Fig. 5.** Effect of spacer arm on IgG binding. A 0.5 ml of each adsorbent was mixed with 2.5 ml of 5 mg/ml IgG solution prepared in 50 mM sodium phosphate buffer, pH 7 for 4 h.

divinylbenzene base matrix did not show the same affinity for IgG as shown by AbSep. This meant that the polymethacrylate base matrix was playing a role in IgG binding to AbSep. To further confirm this we coupled tryptophan to another commercially available polymethacrylate base matrix Macro-Prep 50 Epoxide (Bio-Rad). The ligand density obtained on this matrix was 82 μmol/g which was close to that of Sepharose-Trp (79 μmol/g) and POROS-Trp (77 μmol/g). However MacroPrep-Trp gave a high IgG binding (40 mg/ml of adsorbent) as compared to both Sepharose-Trp (2 mg/ml of adsorbent) and POROS-Trp (4.5 mg/ml of adsorbent) thus confirming the role of polymethacrylate base matrix in IgG binding. It was also possible that the base matrix alone was responsible for binding and selectivity shown to IgG by AbSep. This aspect was investigated by carrying out IgG binding studies with only the base matrix of AbSep. From Fig. 3 it can be seen that base matrix had a very low IgG binding (2 mg/ml) as compared to ligand coupled adsorbent (19 mg/ml). This indicated that the affinity of the AbSep adsorbent towards IgG was indeed due to tryptophan. Yet it was seen that tryptophan bound to other base matrices did not show any significant binding. From these studies it was deduced that the affinity of the AbSep for IgG is due to the combination of both the ligand (tryptophan) and the polymethacrylate base matrix.

### 3.4. Effect of pore size and particle size on IgG binding

Sepabeads of different pores sizes and particle sizes were studied for their IgG binding. The effect of pore size of AbSep on IgG binding is shown in Fig. 4. It was observed that as the pore size increased, the binding of IgG to the adsorbent decreased. Adsorbent with 300 Å pore size showed maximum binding of 17.16 mg/ml of adsorbent whereas adsorbent having 1000 Å pore size gave the lowest binding (6.6 mg/ml of adsorbent). This behaviour may be due to the fact that as the pore size increases, the specific surface area of the adsorbent decreases, which in turn decreases the tryptophan density of the adsorbent. Next, the effect of particle size of AbSep was studied by using Sepabeads of pore size 300 Å and different particle sizes 150 μm (Sepabeads EB1) and 75 μm (Sepabeads EB4). AbSep of 75 μm particle size showed a very high binding capacity for IgG (76 mg/ml of adsorbent) as compared to 150 μm (17.16 mg/ml of adsorbent). This behaviour can be attributed to the increased specific surface area of 75 μm particle size AbSep which increased the ligand density to 104 μmol/g from 75 μmol/g (150 μm particle size AbSep). AbSep of pore size 300 Å and particle size 75 μm was therefore selected for further studies.

### 3.5. Effect of spacer arm on IgG binding

The interaction between the protein or macromolecule and a small ligand may be affected due to low steric availability of the ligand. The use of a spacer arm enhances the flexibility and mobility of the ligand therefore improving its access to the target's binding site. To determine whether the binding capacity of AbSep can be enhanced, adsorbents with 2 carbon spacer arm (Sepabead-EA-Trp) and 6 carbon spacer arm (Sepabead-HA-Trp) were prepared and screened for IgG binding. The introduction of 2-C spacer arm decreased the binding capacity of AbSep from 75 mg/ml of adsorbent to 43 mg/ml of adsorbent (Fig. 5). With 6-C spacer arm, the binding capacity decreased further to 29 mg/ml of adsorbent. This behaviour can be attributed to the hypothesis proposed in Section 3.3 that the IgG affinity of AbSep is due to the combination of tryptophan and polymethacrylate base matrix. The introduction of spacer arm moves the tryptophan away from the polymethacrylate base matrix thereby diminishing the contribution of polymethacrylate base matrix in IgG binding. With 6 carbon spacer arm the ligand was shifted further away from the base matrix resulting in more reduction of IgG binding capacity.

### 3.6. Effect of pH on IgG binding

The medium pH is one of the very important parameters, which determines the adsorption affinity of the protein on the chromatographic support. The adsorption medium pH affects the ionization of the functional groups on the adsorbents as well as on the protein molecules [27]. The effect of pH on IgG adsorption to AbSep was investigated between pH 4 and 9. As can be seen from Fig. 6, the amount of IgG adsorbed increased with increase in pH, reached a maximum at pH 6 and then decreased with further increase in pH. This behaviour may be explained as follows:

1. At pH < 6, IgG being a basic protein (pI ~6.5) is positively charged. The carboxyl (–COOH) group of tryptophan (ligand) which has a $pK_a$ of 2.38 is mostly converted into carboxylate ($–COO^-$) group and hence AbSep would carry negative charges. This resulted

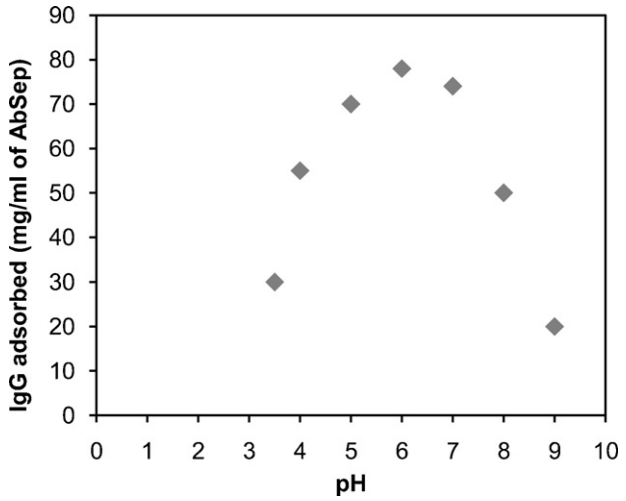


**Fig. 6.** Effect of pH on IgG binding to AbSep. Seven batches (0.5 ml) of AbSep were equilibrated with buffers of varying pH (4–9) and then mixed with 2.5 ml of 5 mg/ml IgG solution prepared in respective equilibration buffers for 4 h.

in adsorption of IgG to AbSep. As the pH was increased from 4, the dissociation of –COOH groups also increased leading to an increase in the negative charge density of AbSep. As a consequence of which, the binding of IgG to AbSep increased as shown in Fig. 6.

2. As pH was increased above 6 and as it approached the pI of IgG (~6.5) the net positive charge on IgG decreased. The electrostatic interaction between the AbSep and the IgG was minimized leading to decrease in binding. However, the binding did not decrease drastically; this may be due to hydrophobic interaction between the indole group of tryptophan and the hydrophobic groups of IgG. Therefore, some degrees of hydrophobic interaction may also play a role in AbSep–IgG interaction. As the pH was increased above pI, IgG started acquiring an overall negative charge, the –COOH group of the tryptophan also started dissociating strongly resulting in an increase of negative charges on AbSep. This repulsion led to decrease in IgG binding to AbSep.

### 3.7. Effect of conductivity on IgG binding

The effect of conductivity on IgG binding to AbSep was studied from 0 to 2 M NaCl concentration (Fig. 7). As the NaCl concentration increased from 0 to 1 M, the binding capacity of AbSep decreased from 77 mg/ml of adsorbent to 23 mg/ml of adsorbent. This confirmed the observation made during pH study that electrostatic interactions play a major role in AbSep–IgG binding. However, as the NaCl concentration increased above 1 M the binding capacity increased from 23 mg/ml of adsorbent to 34 mg/ml of adsorbent indicating the presence of hydrophobic interactions between IgG and AbSep. Based on the these studies, it was proposed that AbSep–IgG interaction is a mixed mode comprising of electrostatic and hydrophobic interactions.

### 3.8. Effect of polyethylene glycol on IgG binding

In addition to pH and ionic strength of the medium, the binding of protein to adsorbent is also influenced by additives such as polyethylene glycol, ethylene glycol and urea. The presence of these additives decreases the polarity of solution, resulting in a reduction of hydrophobic interactions between adsorbent and protein [28].

As hydrophobic interactions were found to play an important role in binding of IgG to AbSep, it was decided to investigate the effect of polyethylene glycol (PEG) on the IgG binding. The effect of

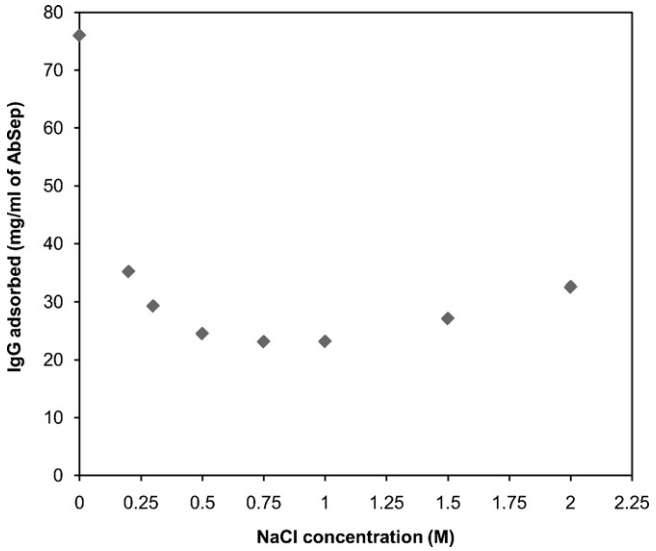


**Fig. 7.** Effect of conductivity on IgG binding to AbSep. Eight batches (0.5 ml) of AbSep were equilibrated with 25 mM sodium phosphate pH 7 buffers of varying NaCl concentrations (0–2 M). The equilibrated adsorbents were then mixed with 2.5 ml of 5 mg/ml IgG solution prepared in respective equilibration buffers for 4 h.

PEG 600 in the presence of 1 M NaCl (NaCl was added to minimize the electrostatic interactions) is shown in Fig. 8. In the absence of PEG the binding of IgG to AbSep was 27 mg/ml of adsorbent. As the concentration of PEG increased from 0 to 20% the IgG binding to AbSep decreased from 27 mg/ml of adsorbent to 7.2 mg/ml of adsorbent.

### 3.9. Adsorption isotherm

In order to evaluate the strength of binding and maximum binding of IgG to AbSep, adsorption isotherm studies were carried out. As shown in Fig. 9, the binding of IgG to AbSep followed a well defined Langmuir adsorption isotherm.

The maximum IgG binding capacity ($q_m$) of AbSep was found to be 78 mg/ml of adsorbent. This capacity is higher than the capac-

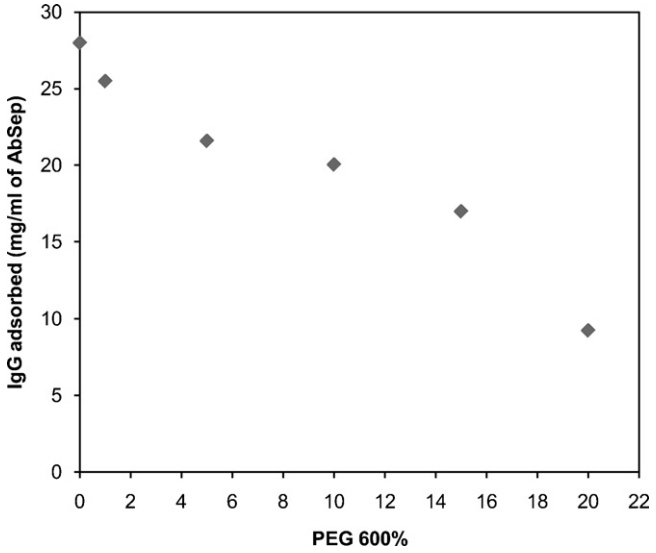


**Fig. 8.** Effect of PEG 600 on IgG binding to AbSep. Six batches (0.5 ml) of AbSep were equilibrated with 25 mM sodium phosphate pH 7 buffers containing 1 M NaCl and different concentrations of PEG (0–20%). The equilibrated adsorbent was then mixed with 2.5 ml of 5 mg/ml IgG solution prepared in respective equilibration buffers for 4 h.

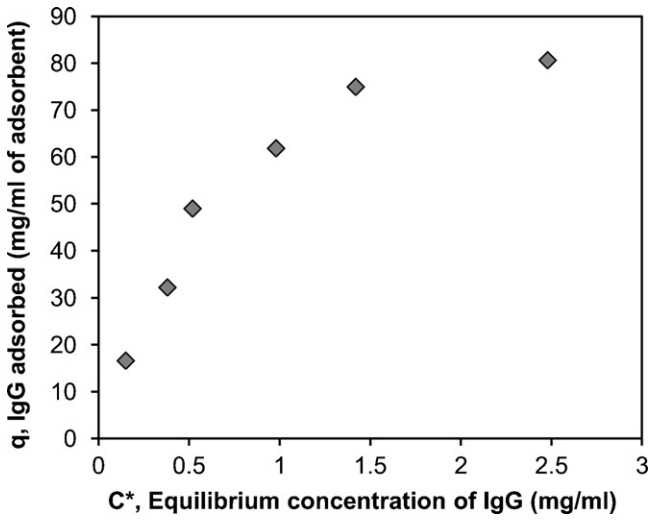


**Fig. 9.** Adsorption isotherm for IgG on AbSep. Six batches (0.5 ml) of AbSep were equilibrated with 25 mM sodium phosphate pH 6.5 buffer and then mixed with 5 ml IgG solutions of varying concentrations for 4 h.

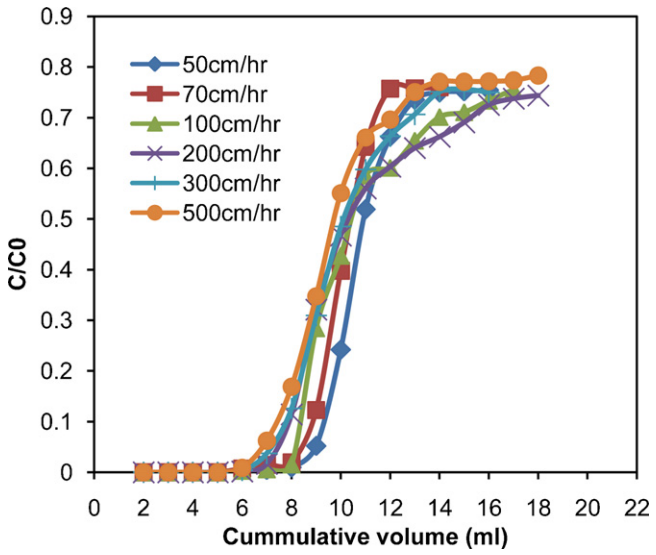


**Fig. 10.** Breakthrough curves for IgG on AbSep at different flow velocities. IgG solution (15 mg/ml) was loaded onto 5 ml AbSep column at different flow rates.

ities of pseudoaffinity adsorbents reported for IgG purification. Ngo and Khatter reported a capacity of 14 mg/ml of adsorbent for their pyridine based pseudoaffinity adsorbent [16]. Li et al. had developed a low molecular weight synthetic ligand for IgG purification. The adsorption capacity of this pseudoaffinity adsorbent for human polyclonal IgG was 20 mg/ml of adsorbent [14]. Liu et al. used a sulfamethazine ligand for IgG purification. The maximum adsorption capacity of the adsorbent for IgG was 1.95 mg/g of beads [13]. Feng et al. developed a pseudoaffinity ligand by molecular docking having a binding capacity of 17.3 mg/ml of adsorbent for human IgG [15]. The binding capacity of AbSep is also higher than the commercially available affinity ligands such as Mabsorbent A2P (ProMetic BioSciences, NJ, USA) and MEP HyperCel (Pall Corp, NY, USA). The MabSorbent A2P has a binding capacity of 50 mg/ml (Mabsorbent A2P brochure) and MEP HyperCel has a capacity of 65 mg/ml [29].

The strength of binding in terms of dissociation constant ($K_d$) of IgG to AbSep was found to be $5.31 \times 10^{-6}$ M. This $K_d$ value is within the range of $10^{-4}$–$10^{-8}$ M, which is suitable for affinity ligands [13]. Protein A based matrices have $K_d$ values in range of $10^{-6}$–$10^{-8}$ M [30].

### 3.10. Breakthrough studies

The binding capacity of an adsorbent in column mode of operation is different from the batch mode since in column mode the hydrodynamics of system greatly influences the transport and adsorption of proteins. Breakthrough studies are considered as an accurate method for quantitatively describing the characteristics of the chromatographic adsorption of biomolecules [31]. The breakthrough curves for IgG on AbSep at linear velocities from 50 cm/h to 500 cm/h are shown in Fig. 10. The dynamic binding capacity (DBC) calculated at 5% breakthrough was found to be 30 mg/ml at flow velocity of 50 cm/h (Table 3). Increasing the flow velocity from 50 cm/h to 200 cm/h decreased the DBC of AbSep from 30 mg/ml of adsorbent to 24 mg/ml of adsorbent, however with further increase of flow velocity up to 500 cm/h the DBC of AbSep remained almost constant (22 mg/ml). Additionally, there was only a linear increase in the pressure of system which is due to the rigid nature of polymethacrylate base matrix. This property of the adsorbent along with the low dependence of DBC on flow velocity makes AbSep an excellent choice for high throughput purification of antibodies.

### 3.11. Purification of IgG from human plasma

Human plasma is a complex mixture of about 100 proteins. Albumin is the major protein in the human plasma with concentration of about 35–40 mg/ml. The IgG is the second most abundant protein with concentration of 10–15 mg/ml. Purification of IgG from this complex mixture of proteins represents a major challenge to any affinity or pseudoaffinity ligands developed for IgG.

AbSep was used for the purification of IgG directly from human plasma. Though it was found from the batch studies that the maximum binding of IgG occurred at pH 6, for the purification experiment it was decided to carry out the binding at a physiological pH 7. Elution of bound IgG was carried out with buffer containing 1 M NaCl and 20% PEG 600 as the lowest IgG binding was observed at this condition during batch studies. The chromatogram and SDS PAGE are shown in Fig. 11. From SDS PAGE of the chromatographic fractions it can be seen that the major impurities such as HSA, transferrin, fibrinogen and high molecular proteins passed through the AbSep unbound. Only a slight amount of HSA and transferrin bound to the AbSep and contaminated the IgG elution fraction. This non specific binding of the impurities can be attributed to the hydrophobicity of the polymethacrylate base matrix. Therefore, further binding studies were carried out to determine the optimal conditions to minimize the non-specific binding.

#### 3.11.1. Optimization of binding conditions

As base hydrophobicity of the matrix was considered to be the primary reason for the non-specific binding, polyethylene glycol (PEG) 600 was added to the equilibration buffer. Accordingly, purification experiment was performed with equilibration buffer containing 6% PEG 600. This prevented the transferrin from bind-

**Table 3**
Dynamic binding capacities of AbSep at different flow velocities.

| Flow velocity (cm/h) | Dynamic binding capacity calculated at 5% $C_0$ (mg/ml) |
|---|---|
| 50 | 30 |
| 70 | 29 |
| 100 | 27 |
| 200 | 24 |
| 300 | 23 |
| 500 | 22 |

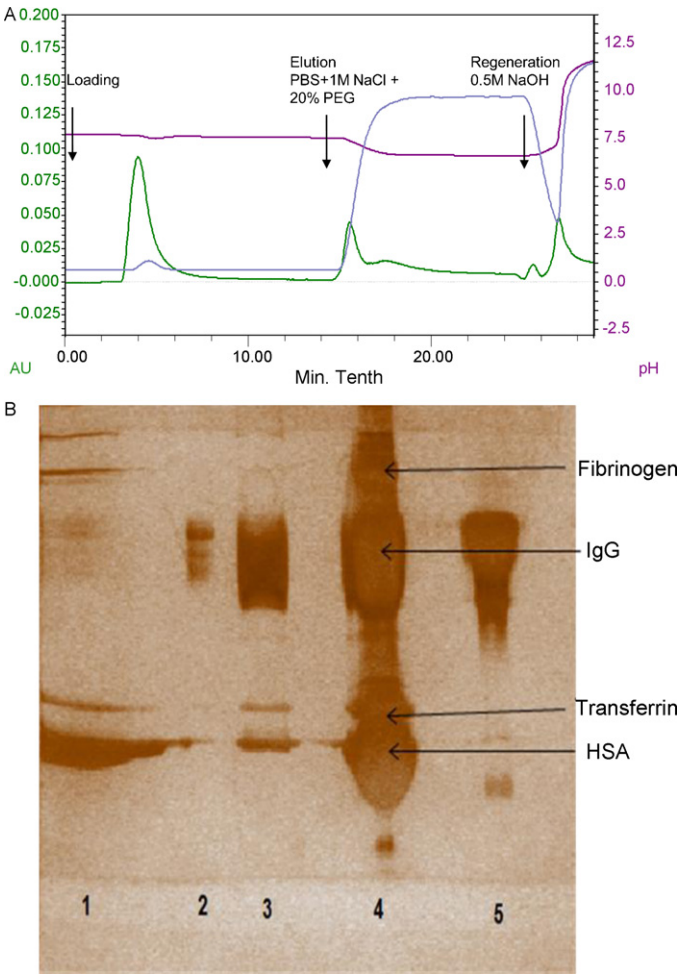


**Fig. 11.** (A) Chromatogram for the purification of IgG from human plasma using AbSep. (B) SDS PAGE analysis (non-reducing conditions) of chromatographic fractions. Lane 1: unbound fraction, Lanes 2 and 3: elution fraction, Lane 4: plasma (load) and Lane 5: IgG standard.

ing non-specifically to the adsorbent and the eluted IgG was only contaminated with trace amounts of HSA (Fig. 12, Lanes 5 and 6). Purification was then carried out with an increased PEG 600 concentration (10%) in the equilibration buffer. From the SDS PAGE

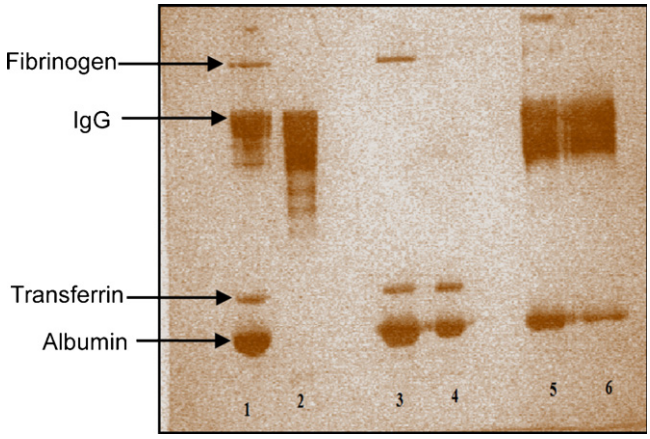


**Fig. 12.** SDS PAGE analysis (non-reducing conditions) of chromatographic fractions from purification of IgG from human plasma using 6% PEG 600 in equilibration buffer. Lane 1: plasma (load); Lane 2: IgG standard; Lanes 3 and 4: unbound; Lanes 5 and 6: elution fraction.

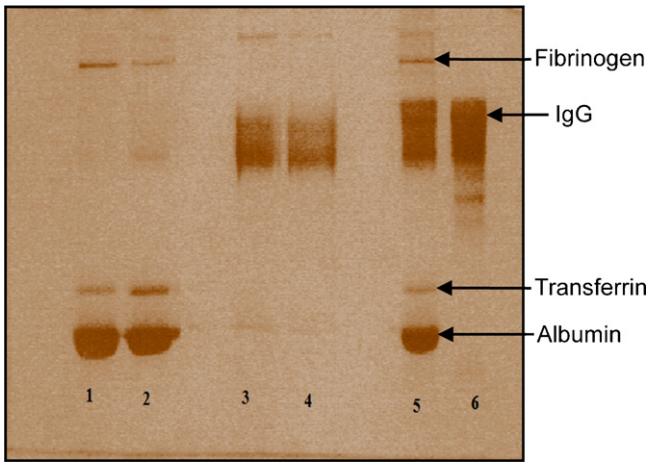


**Fig. 13.** SDS PAGE analysis (non-reducing conditions) of chromatographic fractions from purification of IgG from human plasma using 10% PEG 600 in equilibration buffer. Lanes 1 and 2: unbound fraction; Lanes 3 and 4: elution fraction; Lane 5: plasma (load); Lane 6: IgG standard.

(Fig. 13, Lanes 3 and 4) it can be seen that both HSA and transferrin passed through the column unbound and the elution fraction contained a highly pure IgG. The purity of the IgG as determined by densitometric analysis was found to be 98% and the recovery by HPLC analysis was 90%. Thus, AbSep was able to successfully capture and purify IgG from human plasma. The PEG 600 in the elution fractions was removed by diafiltration on 5 kDa membrane (5 kDa, Vivaspin, Sartorius, Germany).

### *3.12. Purification of monoclonal antibody from cell culture supernatant*

AbSep was further used for the purification of monoclonal antibody (chimeric IgG1) from cell culture supernatant. The total protein concentration in the supernatant was 0.33 mg/ml and the MAb concentration was 0.2 mg/ml. Fifteen millilitres of diafiltered cell culture supernatant was loaded onto 2 ml AbSep column. The operating conditions and buffers were same as used for IgG purifi-

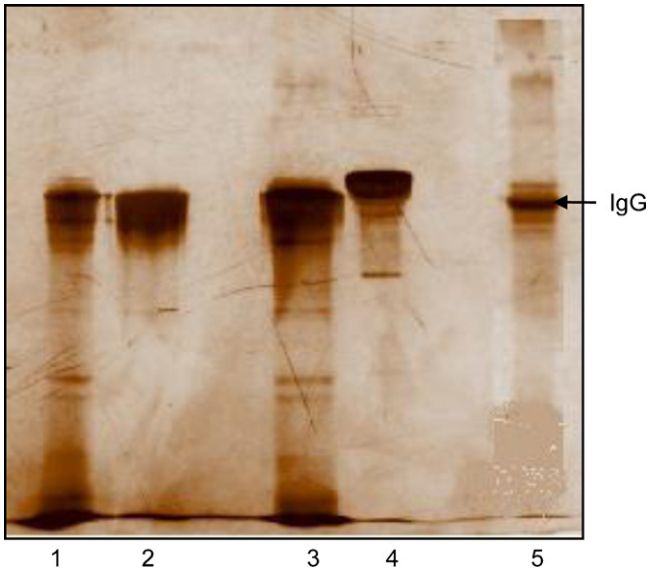


**Fig. 14.** SDS PAGE analysis (non-reducing conditions) of chromatographic fractions of MAb purification using AbSep column. Lane 1: cell culture supernatant (load); Lane 2: elution fraction; Lane 3: cell culture supernatant; Lane 4: Affi Prep Protein A elution fraction; Lane 5: pure IgG (standard).

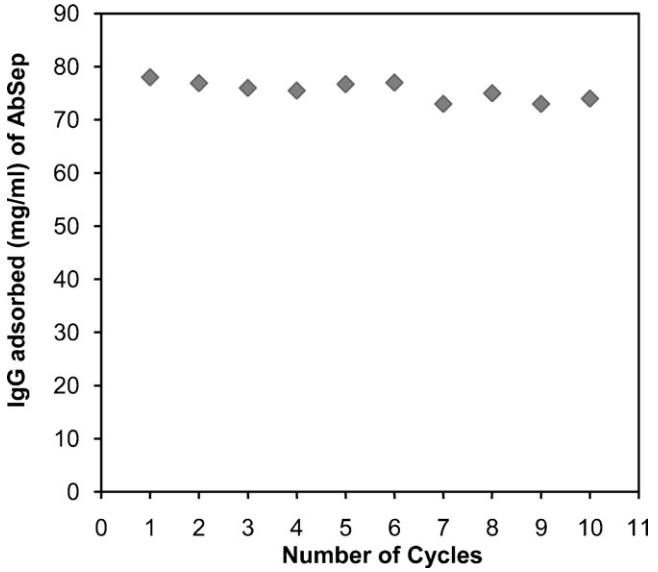


**Fig. 15.** Performance of AbSep during different cycles of IgG binding and 0.5 M NaOH regeneration.

cation from human plasma. In order to compare the performance of AbSep with Protein A, purification of MAb was carried out with Affi-Prep Protein A (Bio-Rad, USA) adsorbent using the conditions recommended by manufacturer. It can be seen from the SDS PAGE (Fig. 14, Lane 2) that elution fraction contained a highly pure and concentrated MAb free from low molecular weight impurities. The purity and recovery obtained by AbSep was 95% and 75% respectively. This performance of AbSep is comparable to that obtained with Affi-Prep Protein A which gave a purity of 96% and recovery of 81%.

### 3.13. Reusability of AbSep

Reusability of adsorbent is one of the important parameters for preparative scale operations. For the process to be economical the adsorbent should be reusable for maximum number of cycles. The reusability of the adsorbent depends upon its physical and chemical stability to process conditions and harsh chemicals used during cleaning and regeneration operations. Sodium hydroxide is the most preferred regeneration agent since it is highly effective in destroying bacteria, viruses and endotoxins. It is also essentially non-toxic yielding sodium chloride and water after neutralization. Consequently it is highly desirable if an affinity adsorbent is alkali resistant. For this to be achieved, the ligand, the coupling chemistry and the support matrix must all be equally resistant to high pH. The stability of the AbSep was tested by carrying out ten cycles of IgG binding followed by 0.5 M NaOH regeneration for 1 h after every cycle. It can be seen from Fig. 15 that binding capacity was almost same for all cycles indicating the stability of AbSep to NaOH solutions.

The stability of the AbSep was also tested by determining the leaching of the ligand-tryptophan. The above experiment was repeated without loading the IgG solution. No tryptophan was detected in the equilibration, elution and NaOH wash fractions for ten cycles thus ascertaining the robustness of AbSep.

## 4. Conclusions

A novel affinity adsorbent, named AbSep showing good specificity and binding to IgG was developed based on computational and experimental screening studies. AbSep comprises of tryptophan covalently attached to rigid polymethacrylate base matrix, Sepabeads EBEP4. The adsorption mechanism is proposed to be a mixed mode comprising of electrostatic and hydrophobic interactions. AbSep has a maximum IgG binding capacity of 78 mg/ml which is higher than that reported for commercially available pseudoaffinity adsorbents. Its affinity towards IgG determined in terms of dissociation constant ($K_d$) is $5.13 \times 10^{-6}$ M, suitable for affinity ligands. The dynamic binding capacity (DBC) of AbSep is 30 mg/ml of adsorbent which is similar to protein A based matrices. Moreover, the DBC decreases only slightly with the increase in the flow rate and the rigid nature of the matrix allows operations at high flow rates with only a linear increase in the pressure. These properties make AbSep an excellent chromatographic media for high throughput purification of antibodies.

AbSep was used to successfully purify IgG from human plasma and monoclonal antibody from cell culture supernatant. Unlike Protein A, AbSep is stable to harsh conditions such as 0.5 M NaOH required for the efficient cleaning and santization of the columns which is an important part of chromatographic purification of therapeutic proteins such as antibodies. Moreover, AbSep being an amino acid based ligand is less expensive than Protein A and also free from the immunogenic concerns associated with leakage of Protein A based matrices. In summary, the low cost, high specificity, high capacity and robustness make AbSep an excellent economic alternative for the large scale purification of monoclonal and polyclonal antibodies.

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