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Study of the mechanism of interaction of antibody (IgG) on two mixed mode sorbents

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ABSTRACT

Purification of target proteins from a crude biological mixture containing proteins, peptides and other biomolecules is the chromatographic challenge. Mixed mode chromatography offers additional selectivities to improve the overall productivity of commercial bioprocesses with novel chromatographic sorbents being introduced to overcome the problem. HEA HyperCel™ (n-hexyl amine) and PPA HyperCel™ (phenyl propyl amine) are industry scalable mixed mode chromatography sorbents where both hydrophobic and electrostatic interactions are predominant. Our study focuses on understanding the underlying mechanism of interaction of protein with the sorbent. Parameters like buffer conditions, pH and temperature were tuned to study the adsorption and desorption conditions of the protein. Dynamic binding capacity of HEA HyperCel™ and PPA HyperCel™ sorbents was studied with human IgG as a model protein. Our study shows that, in HEA the interaction of IgG to the sorbent is predominantly hydrophobic as the binding is enhanced (50–60 mg/ml of sorbent) by presence of salt in buffer and increase in temperature. Binding capacity of PPA is 50–60 mg/ml of sorbent irrespective of temperature effect and/or the presence of salt. The chromatographic experiments show that the interaction could be hydrophobic or ionic or some charge transfer mechanism depending upon the buffer conditions.

1. Introduction

Industrial scale purification process has progressed vigorously over the last decades. Still, the purification of the desired protein from a crude sample to the desired level of purity is a bottle neck for industries. Due to the complexity in the purification process, final product becomes expensive. Using multifunctional sorbents, mixed mode chromatography can be exploited to achieve high selectivity between proteins.

HEA HyperCel™ and PPA HyperCel™ are novel industrial scalable chromatography sorbents designed for protein capture and impurity removal in a biopharmaceutical environment. HEA (n-hexyl amine) is an aliphatic ligand in which amine includes an n-hexyl substituent. The hexyl spacer predominantly favours hydrophobic interaction. PPA (phenyl propyl amine) is an aromatic ligand in which amine includes a phenyl propyl substituent [1]. These ligands may interact via a combination of aromatic, hydrophobic, ionic, and hydrogen bonding interactions. Each interaction may be to a particular epitope on the protein surface depending on the buffer conditions provided, which facilitates the differential binding of protein from the feedstock.

High purity monoclonal antibody production has a biotechnological relevance due to its application in diagnostics and therapeutics. Immunoglobulin G (IgG) is the most therapeutically important among all other immunoglobulin. Human IgG is an important serum protein produced world wide on a large scale as it is used to treat many diseases [2]. The purification of IgG is of great interest due to the increasing market of biopharmaceuticals consisting of antibodies (monoclonal and polyclonal) and the current focus is on immunoglobulin preparation from human serum. IgG could be purified using various conventional methods like ion exchange chromatography [3]. There are a few conventional bioaffinity ligands such as Protein A and Protein G used for the purification of IgG [4]. These are very specific but do lead to potential issues associated with the clean-in-place, ligand leakage and overall sorbent cost making it less desirable for industrial purpose [5].

To overcome the drawbacks of affinity methods, these new synthetic ligands could be a better alternative. Hence our work aims at understanding the adsorption and desorption parameters for selectivity and binding capacity of human IgG to the gels. The objectives of the study are (i) optimization of the dynamic binding capacity of the sorbents using human IgG (Cohn fractions II and III, Sigma) as a protein model, (ii) optimization of the flow rate for better binding

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of protein and (iii) understanding of the interaction of IgG in a protein mixture. From the parameters, as a function of temperature, salt, different buffer systems, the interaction mechanism(s) will be hypothesized.

2. Experimental

All experiments were performed using medium pressure pump from Bio-Rad Econo™ pump (Hercules, CA, USA) attached with a UV detector set at 280 nm. The column used was from Pall Life Sciences with 1 cm I.D. For experiments carried out at 37 °C double jacketed column XK 16, from Amersham Biosciences was used with a medium pressure p1 pump from GE Healthcare (Uppsala, Sweden). Beckman Coulter DU® 730 spectrophotometer (Fullerton, CA, USA) was used to determine absorbance at 280 nm and 595 nm for protein estimation. Bio-Rad Mini-PROTEAN system (Hercules, CA, USA) was used for SDS-PAGE analysis.

2.1. Chemicals and proteins

HEA HyperCel™ and PPA HyperCel™ were supplied as a slurry in 1 M NaCl containing 20% (v/v) ethanol from Pall Life Sciences, Cergy, France. Sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride, potassium chloride, sodium acetate, Tris (hydroxymethyl) aminomethane were purchased from SRL Fine Chemicals, India. Bisacrylamide, ammonium per sulphate (APS), TEMED were from Biogene, USA. Coomassie brilliant blue-G, silver nitrate, acrylamide, human immunoglobulin G (prepared from Cohn fractions II and III), bovine serum albumin were purchased from Sigma (St Louis, MO, USA). Water used for all the experiments was treated with Milli-Q® system from Millipore (Bedford, MA, USA). All buffers were degassed prior to use.

2.2. Column preparation

The slurry of sorbents obtained was stirred to obtain a homogenous suspension and transferred to measuring cylinder. 1 ml of settled gel was slowly poured into the column without introduction of air bubbles. The column dimensions were 1 cm I.D. × 1 cm and the bed volume was 0.78 ml. The suspension was allowed to settle for 4–5 min and the column was closed with the upper plunger without any air traps. A packing flow rate of 500 cm/h was maintained. The column was thoroughly washed to remove ethanol. Then it was equilibrated with the suitable buffer before use.

In case of experiments performed at 37 °C double jacketed column was used. The gel was packed the same way as described above. Bed volume was maintained the same. Warm water was continuously recirculated in the outer jacket to maintain the temperature at 37 °C inside the column. The temperature of outlet water was periodically checked and maintained. Equilibration was done with the respective buffers at 37 °C prior to experiments. Buffers used for the study were both phosphate buffer saline (PBS) (50 mM phosphate buffer with 140 mM NaCl and 30 mM KCl, pH 7.4) and 50 mM phosphate buffer, pH 7.4.

2.3. Determination of dynamic binding capacity and its dependence on ionic strength and temperature

Determination of dynamic binding capacity (DBC) was conducted using human polyclonal IgG (Sigma). Test feedstock was applied to the column at 0.25 ml/min. During studies to assess influence of ionic strength on DBC, test feedstock contained 5 mg IgG/ml was prepared using the appropriate binding buffers. Protein was injected until a break through curve was achieved. The bound proteins were eluted using 50 mM sodium acetate buffer. After each run the column was regenerated with 1 M NaOH followed by water wash.

To assess the effect of temperature on DBC, the studies were carried out at three different temperatures (4 °C, 24 °C, and 37 °C). DBC studies at 4 °C were carried out inside a cold room which was maintained at 4 °C. For 37 °C, double jacketed column was used as mentioned in Section 2.2. At different temperatures, two different buffer systems, phosphate buffer saline (PBS) (50 mM phosphate buffer with 140 mM NaCl and 30 mM KCl, pH 7.4) were tried to find the role of buffer in the binding capacity of gel. Absorbance at 280 nm was measured for all fractions. All these experiments were done for both HEA HyperCel™ and PPA HyperCel™ sorbents.

2.4. Determination of optimum flow rate

To determine the optimum working flow rate of HEA HyperCel™ and PPA HyperCel™ sorbents, 30 mg IgG was injected from feedstock (5 mg/ml) at different flow rates (0.2, 0.4, 0.6, 0.8 and 1 ml/min). All experiments were performed at 24 °C using 50 mM PBS, pH 7.4 and 50 mM sodium acetate, pH 4 as binding and eluting buffer respectively.

2.5. Chromatographic experiments

Interaction of IgG and its separation from a mixture of protein on the HEA HyperCel™ and PPA HyperCel™ sorbents were studied. Attempts have been made to study the mode of interaction and differential binding of IgG to the sorbents in presence of BSA. For this purpose a protein mixture of pre-purified human IgG and bovine serum albumin was mixed in 1:2 proportions. The feedstock was injected to the HEA and PPA column at a flow rate of 0.6 and 0.4 ml/min (which was found to be the optimum flow rate). All chromatographic procedures were carried out at 24 °C. The chromatographic run was continuously monitored at 280 nm. Protein concentration was measured using Bradford assay [6].

2.6. Protein determination

IgG concentration was determined by measuring the absorbance at 280 nm, with the extinction coefficient of human IgG being 1.4.

Total protein concentration was determined by Bradford assay using IgG as reference protein [6].

2.7. SDS-PAGE analysis

The chromatographic fractions were analysed by 8% SDS-PAGE electrophoresis under non-reducing conditions using a Mini-PROTEAN system (Bio-Rad, USA) according to Laemmli [7]. The gels were stained with silver nitrate [8].

3. Results and discussion

3.1. Determination of dynamic binding capacity

The dynamic binding capacity of a sorbent is the amount of target protein the sorbent will bind under actual flow conditions depending on the microenvironment provided. 5 mg/ml IgG solution were prepared and injected into the column at a flow rate of 0.25 ml/min until breakthrough was achieved. Both HEA HyperCel™ and PPA HyperCel™ work under physiological conditions [1]. The buffer systems in which the DBC studies were carried out were 50 mM phosphate buffer saline, pH 7.4 and 50 mM phosphate buffer, pH 7.4. To study the effects of temperature,

Table 1
Comparative table showing the binding capacity of HEA HyperCel™ in PBS and phosphate buffers and at 4 °C, 24 °C and 37 °C.

Buffers	Temperature (°C)	Protein loaded (mg)	Flow through (mg)	Elution (mg)	NaOH wash (mg)	Recovery %	Binding capacity (mg)
50 mM PBS, pH 7.4	4	20	17.2	2.6	0.2	92.9	2.5 ± 0.7
	24	60	20.0	40.0	1.0	100.0	39.8 ± 2.7
	37	125	64.0	55.0	6.3	90.2	56.8 ± 1.5
50 mM phosphate buffer	4	20	18.5	1.25	0.1	83.3	1.01 ± 0.2
	24	60	20.1	37.6	0.3	94.2	32.6 ± 4.6
	37	75	33.2	32.0	3.0	76.6	32.45 ± 2

Fig. 1. HEA ligand.

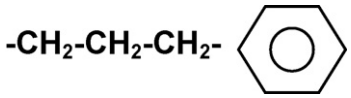

Fig. 2. PPA ligand with a phenyl residue.

experiments with these two buffers systems were performed at different temperatures.

3.1.1. Dynamic binding capacity of HEA HyperCel™

The results of DBC of HEA HyperCel™ at different buffers as a function of temperature are given in Table 1.

The presence of salt in the buffer facilitates better binding at 37 °C (55–60) compared to 24 °C (35–45 mg) and 4 °C (3 mg). An increase of ~30–40 mg/ml of sorbent and ~50–60 mg/ml of sorbent was observed at 24 °C and 4 °C respectively. On the contrary, in absence of salt in the buffer, temperature does not play a significant role in the binding capacity.

Porath et al. [9] showed that the hydrophobic adsorption of protein can be enhanced by high concentration of salt on a hydrophobic matrix. Adsorption of phosphorylase *b* to NCF-alkyl sepharose was favoured by the presence of 1 M ammonium sulphate in the binding buffer [10]. In our study, it was observed that, the binding of IgG to HEA HyperCel™ was favoured by the presence of salt (140 mM NaCl and 30 mM KCl) in the buffer. Protein in contact with aqueous solution causes ordering of water molecules along the surface of its hydrophobic patches. Addition of water structuring salts favours solvating and displacement of highly ordered water molecules surrounding the hydrophobic moiety, which enhances binding. The basis of this principle is that the Van der waals attraction forces between protein and ligand increase as the ordered structure of water increases in the presence of salt [19]. The major advantage of using these mixed mode sorbents is that it requires low concentration of salt unlike conventional hydrophobic interaction chromatography where high concentration has to be used [21,22].

The other two critical parameters which affect hydrophobic binding are the temperature and the chain length of ligand [11]. Erel *in* 1972 found that an increase in the chain length by $-CH_2$ units concomitantly increased the strength of protein binding from retardation to reversible binding to very tight binding ("irreversible" binding). His studies with phosphorylase proved that Sepharose C_4–Sepharose C_6 chain facilitated better adsorption [12]. The HEA ligand includes n-hexyl group (shown in Fig. 1). Possibly the chain length favours hydrophobic interaction of IgG to the ligand.

Hjerten proposed that the binding of proteins to hydrophobic interaction chromatography adsorbents is entropy driven, which implies that the interaction increases with an increase in temperature [13]. It may be argued that an increase in temperature favoured the change in structural conformation of IgG which enhances binding. In case of PBS buffer, in both 24 °C and 37 °C, as the protein load increases it contributes very tight binding consequently more protein is observed in NaOH wash. The lower in yield was observed when there was an increase in temperature which might be due to the loading of excess protein to the column. Increase in temperature would have also promoted very strong irreversible binding which might be one of the reasons for lower yields. Further Jennisen [14] had proved the interdependence of ionic strength, ligand chain length and temperature for the adsorption of protein to the hydrophobic sorbents. Thus the binding capacity of HEA HyperCel™ was favoured by increase in salt and temperature and possibly by the chain length of ligand.

3.1.2. Dynamic binding capacity of PPA HyperCel™

The results of DBC of PPA HyperCel™ at different buffers as a function of temperature are given in Table 2.

Dynamic binding capacity of PPA HyperCel™ was 50–60 mg/ml of sorbent at 24 °C and 37 °C in absence and presence of salt in buffer. At 4 °C, the DBC was found to be 4–5 mg/ml of sorbent. The proteins are generally adsorbed to hydrophobic stationary phase at high salt concentration, with the driving force for adsorption being a displacement of ordered water molecules around the proteins and the ligands, which leads to an increase in entropy. Common hydrophobic ligands include butyl, octyl and phenyl groups [15]. The PPA ligand the amine includes a phenyl substituent (shown in Fig. 2).

As high binding capacity of 50–60 mg/ml of sorbent was observed at different temperatures (24 °C and 37 °C) and in the presence/absence of salt, it may be argued that the phenyl component of the ligand promotes hydrophobic binding in presence of salt and at high temperature. However, under certain conditions, the pi electron of phenyl residues may introduce additional charge transfer interaction [16,17] which favours high binding in the absence of

Table 2
Comparative table showing the binding capacity of PPA HyperCel™ in PBS and phosphate buffers and at 4 °C, 24 °C and 37 °C.

Buffers	Temperature (°C)	Protein loaded (mg)	Flow through (mg)	Elution (mg)	NaOH wash (mg)	Recovery %	Binding capacity (mg)
50 mM PBS, pH 7.4	4	20	13.8	4.9	1.3	79.0	4.2 ± 0.6
	24	115	49.0	62.4	1.9	94.5	60.3 ± 2.2
	37	100	46.0	50	1.8	92.6	56 ± 5.2
50 mM phosphate buffer	4	20	14.6	3.5	1.2	64.8	3.7 ± 0.25
	24	100	38	58.8	1.5	94.8	56.7 ± 1.9
	37	100	49.0	50.8	2.3	99.6	54 ± 3.6

Table 3
Adsorption and recovery of IgG to HEA HyperCel™ sorbent at different flow rates.

Flow rate		Flow through (mg)	Elution (mg)	NaOH wash (mg)	Residence (s)	IgG adsorbed (mg)	IgG recovered in elution (mg)
ml/min	cm/h						
0.2	15.2	7.2	18.2	0.612	235	22.58	18.2
0.4	30.5	10.81	18.32	0.36	117	19.19	18.32
0.6	45.8	9	20.07	0.34	78	21	20.07
0.8	61.1	13.41	16.71	0.25	59	16.57	16.71
1.0	76.3	16.13	16.44	0.24	47	15.57	16.44

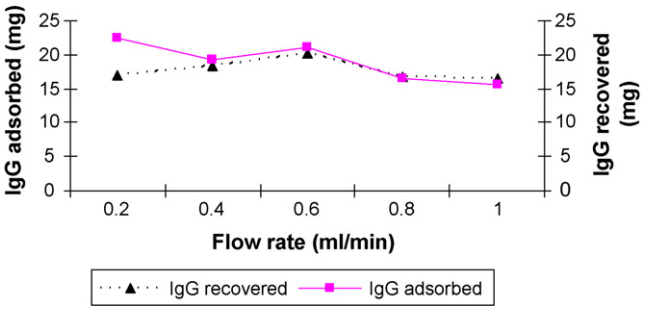

Fig. 3. Influence of flow rate in the adsorption and recovery of IgG onto HEA HyperCel™.

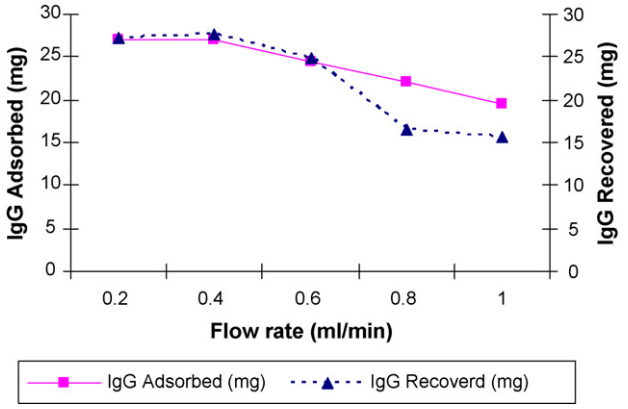

Fig. 4. Influence of flow rate in the adsorption and recovery of IgG onto PPA HyperCel™.

salt. Yield of recovery using PPA HyperCel™ is improving as there is an increase in temperature and the presence of salt. This reinforces the involvement of other interactions through charge transfer on PPA HyperCel™ which are not present on HEA HyperCel™.

DBC of these mixed mode sorbents both HEA and PPA HyperCel™ sorbents being high at physiological conditions (near neutral pH and low salt concentration) suggests the economic use of these sorbents at an industrial scale.

3.2. Determination of optimum flow rate

One of the important parameters which affect the adsorption of protein to the column is the mobile phase flow rate. The residence time of the protein depends on the flow rate. Different flow rates affected differentially the binding of IgG to HEA HyperCel™ and PPA HyperCel™. Fig. 3 shows the differential binding of protein to HEA HyperCel™ at different flow rates.

Of the total protein injected (30 mg IgG), 0.6 ml/min flow rate favoured maximum recovery of 20 mg. Under the buffer conditions and temperature conditions provided, it is observed that slow flow rate of 0.2 ml/min (15.28 cm/h) with high residence time of 3.39 min (235 s) favours maximum adsorption of IgG, but results in lesser recovery. Higher residence time would have promoted strong hydrophobic binding, which would have resulted in lesser recovery. On the contrary, fast flow rate of 1 ml/min with residence time of 47 s resulted in less adsorption and recovery. So the optimum flow rate for the binding of IgG to HEA HyperCel™ sorbent was 46 cm/h (0.6 ml/min), the residence time was 1.31 min (78 s) (Table 3).

Fig. 4 shows the differential binding of protein to PPA HyperCel™ at different flow rates. As there is an increase in flow rate, because of decrease in residence time, the adsorption of IgG decreases. Slow flow rate of 0.4 ml/min favours maximum adsorption and recovery of 27 mg of protein.

From the results shown in Table 4, it is evident that, the increase in flow rate results in less adsorption and recovery, which is probably because of the gradual decrease in contact time between ligand and protein. So optimum flow rate for the binding of IgG to PPA HyperCel™ sorbent was 30 cm/h (0.4 ml/min), and the residence time was 1.96 min (117 s).

3.3. Separation of IgG from a protein mixture

These mixed mode ligands operate on the basis of both hydrophobic and ionic effects [1,21,22]. From the DBC studies, it has been shown that the binding IgG to the sorbents are predominantly hydrophobic. To study the binding and elution pattern of IgG from a protein mixture, binary mixture of IgG and BSA were made. Bovine serum albumin was chosen as the model protein. Experiments were carried out at both physiological conditions with PBS as a binding buffer and to explore the ionic interactions, acetate buffer at pH 5.5 was used for binding. The difference in the isoelectric point of both the proteins could be exploited to achieve separation and thereby study the ionic interactions and difference in binding to the sorbents. The isoelectric point of human IgG [Cohn

Table 4
Adsorption and recovery of IgG to PPA HyperCel™ sorbent at different flow rates.

Flow rate		Flow through (mg)	Elution (mg)	NaOH wash (mg)	Residence time (s)	IgG adsorbed (mg)	IgG recovered in elution (mg)
ml/min	cm/h						
0.2	15.2	3.1	27.29	0.47	235	26.9	27.29
0.4	30.5	2.98	27.7	0.38	117	27	27.7
0.6	45.8	5.55	24.9	0.28	78	24.45	24.9
0.8	61.1	8	16.6	0.32	59	22	16.6
1.0	76.3	10.4	15.7	0.77	47	19.6	15.7

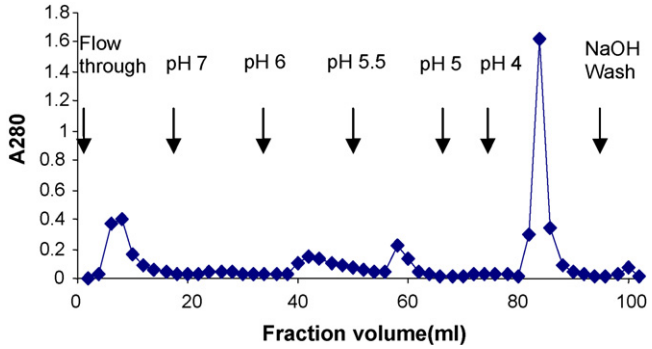

Fig. 5. Chromatogram of protein mixture (Cohn fraction human IgG and bovine serum albumin (BSA)) separation on HEA HyperCel™ at 24 °C (protein loaded, 12 mg) at a flow rate of 0.6 ml/min. Buffers used: equilibration, wash 50 mM PBS, pH 7.4 and decreasing step elution with PBS, pH 7, pH 6 and 50 mM sodium acetate buffer, pH 5.5, pH 5, pH 4. Column regenerated with 1 M NaOH.

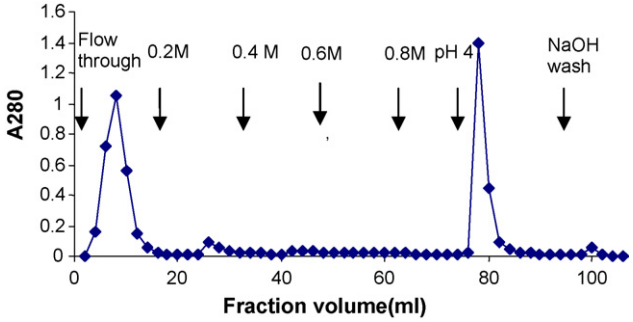

Fig. 7. Chromatogram of protein mixture (Cohn fraction human IgG and bovine serum albumin (BSA)) separation on HEA HyperCel™ at 24 °C (protein loaded, 12 mg) at a flow rate of 0.6 ml/min. Buffers used: equilibration, wash 50 mM sodium acetate, pH 5.5. Elution with step gradient using 0.2 M, 0.4 M, 0.6 M, 0.8 M and 1 M NaCl in pH 5.5 buffer and finally with sodium acetate, pH 4.0. Column regenerated with 1 M NaOH.

fractions II and III, Sigma] is in the range of 6.85–6.95 [20] and of BSA is 4.9 [18]. Hence BSA and IgG was made as mixture in 2:1 proportion and used for the study. The purpose of this study was to possibly understand the mechanism of interaction of IgG from the protein mixture.

3.3.1. HEA ligand

The separation of IgG and BSA is shown in Fig. 5. The binding buffer used was 50 mM PBS, pH 7.4 and elution was performed with 50 mM acetate buffer using decreasing pH step gradient. The SDS-PAGE analyses of the collected chromatographic peaks are shown in Fig. 6.

The proteins were bound at physiological pH and the presence of salt in the buffer is thought to promote hydrophobic binding of proteins. The attempt was to bind the proteins at near neutral pH, pH 7.4 and elute by decrease in pH. Apart from the hydrophobic effects, the ligand carries some positive charge at near neutral pH which also favours the adsorption of acidic proteins like IgG [1]. In pH 7 and pH 6 elution, both IgG and BSA were observed. A possible explanation for this could be that a slight decrease in pH weakens the hydrophobic interaction. IgG was observed with trace albumin at pH 5.5 elution. At pH 5 elution relatively equal amount of IgG and BSA was found. So at pH below its isoelectric point, pH 5.5, IgG becomes basic which might favour the elution, where BSA still remains bound to the column. Subsequently BSA gets eluted at pH 5. pH 4 buffer elutes almost all the tightly bound proteins.

To analyse the ionic interaction of IgG to the ligand, experiment was performed with pH 5.5 buffer as the binding buffer and elution was carried out with increasing salt gradient. The result is shown in Fig. 7.

The SDS-PAGE analyses of the collected chromatographic peaks are shown in Fig. 8.

IgG was observed with a trace amount of albumin in the non-retained fraction. At 0.2 M and 0.4 M NaCl elution, little protein elutes since chloride ions competes for binding site. At higher concentration of salt (0.6 M and 0.8 M NaCl) no protein elutes instead it binds more strong to the column due to hydrophobic interaction (not shown in gel). pH 4 buffer favours elution of all the tightly bound protein. From the results obtained it is clear that pH 5.5 do not favour the adsorption of IgG because it is basic in that buffer condition.

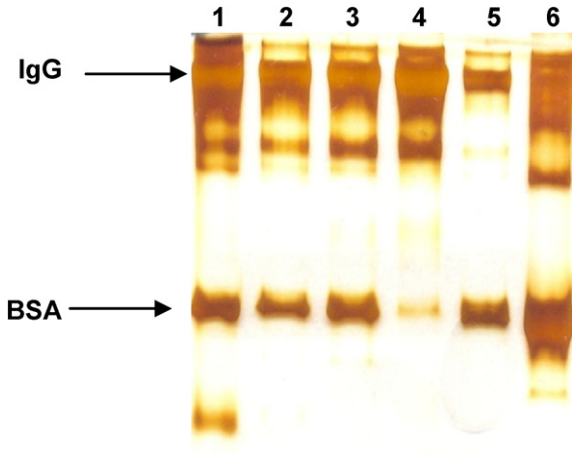

Fig. 6. 8% SDS-PAGE-non-reduced conditions. Lane 1: flow through, lane 2: pH 7 elution, lane 3: pH 6 elution, lane 4: pH 5.5 elution, lane 5: pH 5.0 and lane 6: pH 4. (*Note:* commercially available γ globulins, human and BSA from Sigma offers only 99% and 98% purity respectively, so a few contaminating proteins were also present.)

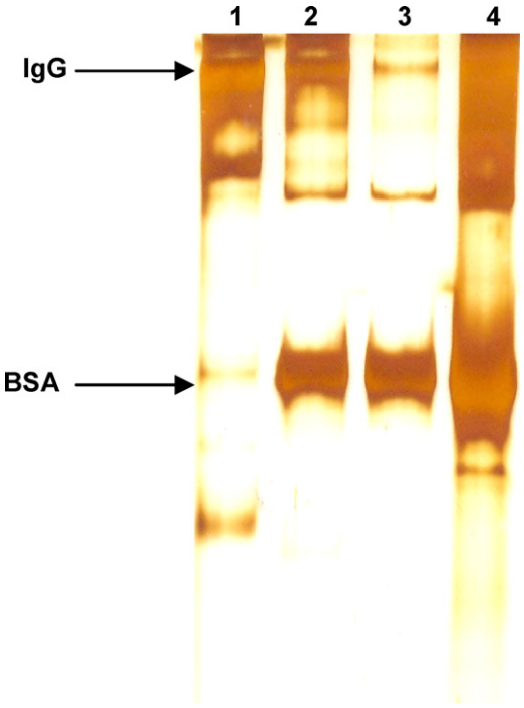

Fig. 8. 8% SDS-PAGE-non-reduced condition. Lane 1: flow through, lane 2: pH 5.5 with 0.2 M, lane 3: pH 5.5 with 0.4 M NaCl and lane 4: pH 4. (*Note*: commercially available γ globulins, human and BSA from Sigma offers only 99% and 98% purity respectively, so few contaminating proteins were also present.)

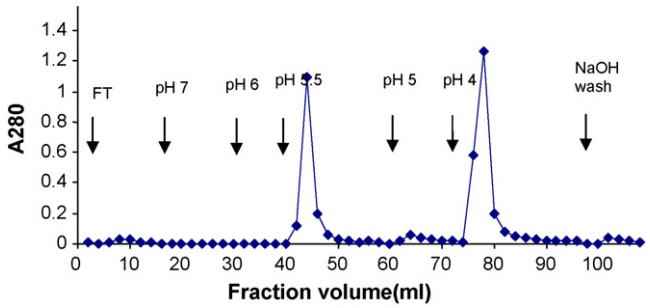

Fig. 9. Chromatogram of protein mixture (Cohn fraction human IgG and bovine serum albumin (BSA)) separation on PPA HyperCel™ at 24 °C (protein loaded, 12 mg) at a flow rate of 0.4 ml/min. Buffers used: equilibration, wash 50 mM PBS, pH 7.4 and decreasing step elution with PBS, pH 7, pH 6 and 50 mM sodium acetate buffer, pH 5.5, pH 5, pH 4. Column regenerated with 1 M NaOH.

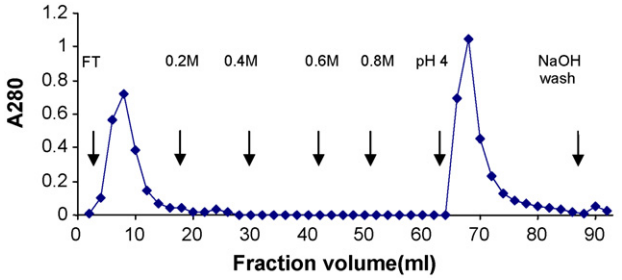

Fig. 11. Chromatogram of protein mixture (Cohn fraction human IgG and bovine serum albumin (BSA)) separation on PPA HyperCel™ at 24 °C (protein loaded, 12 mg) at a flow rate of 0.4 ml/min. Buffers used: equilibration, wash 50 mM sodium acetate, pH 5.5. Elution with step gradient using 0.2 M, 0.4 M, 0.6 M, 0.8 M and 1 M NaCl in start buffer and finally with sodium acetate, pH 4.0. Column regenerated with 1 M NaOH.

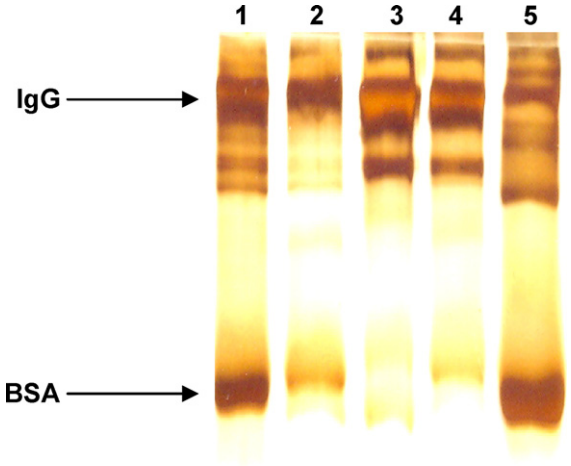

Fig. 10. 8% SDS-PAGE-non-reduced condition. Lane 1: std IgG and BSA, lane 2: flow through, lane 3: pH 5.5, lane 4: pH 5 and lane 5: pH 4. (*Note*: commercially available γ globulins, human and BSA from Sigma offers only ~99% and ~98% purity respectively, so few other contaminating proteins were also present.)

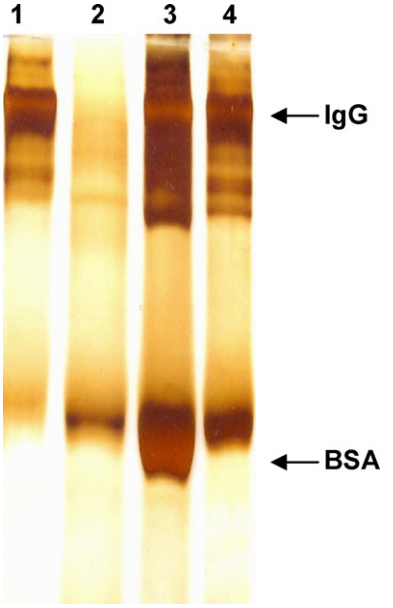

Fig. 12. 8% SDS-PAGE-non-reduced condition. Lane 1: flow through, lane 2: 0.2 M NaCl, lane 3: pH 4 and lane 4: std IgG and BSA. (*Note*: commercially available γ globulins, human and BSA from Sigma offers only 99% and 98% purity respectively, so few contaminating proteins were also present.)

3.3.2. PPA ligand

The separation of IgG and BSA is shown in Fig. 9. The binding buffer used was 50 mM PBS, pH 7.4 and elution was performed using a decreasing pH step gradient was with 50 mM acetate buffer. The SDS-PAGE analyses of the collected chromatographic peaks are shown in Fig. 10.

The proteins were bound at physiological pH and the presence of salt in the buffer is thought to promote hydrophobic binding of proteins. The attempt was to bind the proteins at near neutral pH, pH 7.4 and elute by decrease in pH. PPA HyperCel™ binds strongly both the proteins as relatively less protein was observed in flow through. As observed with HEA HyperCel™, pH 5.5 does not favour the adsorption of IgG as pure IgG with trace amount of BSA was observed. The PPA ligand is also found to carry some positive charge, so at pH 5.5 IgG becomes basic and so gets eluted. This effect was proved by using pH 5.5 as a binding buffer, which results are shown in Fig. 11. SDS-PAGE analyses of the chromatographic fractions were shown in Fig. 12.

IgG was observed in non-retained fractions with a trace of albumin at pH 5.5. In 0.2 M NaCl elution, little of protein elutes since chloride ions competes for binding site. On increasing the salt concentration to 0.4 M, 0.6 M and 0.8 M in the elution buffer, no protein elutes instead it binds more strong to the column due to hydropho-

Table 5
Summary of dynamic binding capacity of HEA and PPA HyperCel™ sorbents.

Buffers	Temperature (°C)	Binding capacity (mg/ml sorbent)	
		HEA HyperCel™	PPA HyperCel™
50 mM PBS, pH 7.4	4	2.5 ± 0.7	4.2 ± 0.6
	24	39.8 ± 2.7	60.3 ± 2.2
	37	56.8 ± 1.5	56 ± 5.2
50 mM phosphate buffer, pH 7.4 (no salt)	4	1.01 ± 0.2	3.7 ± 0.25
	24	32.6 ± 4.6	56.7 ± 1.9
	37	32.45 ± 2	54 ± 3.6

bic interaction. At pH 4 almost all the tightly bound protein elutes due to electrostatic repulsion. The presence of IgG in flow through and in elution could be justified that the multifunctional nature of the ligand would have favoured both hydrophobic and ionic interactions of IgG to the sorbent.

4. Conclusion

HEA and PPA synthetic ligands work with different modes of interaction based on the buffer conditions provided. HEA HyperCel™ and PPA HyperCel™ have a high binding capacity of 35–45 mg/ml and 55–60 mg/ml of sorbent respectively at room temperature. The hydrophobic component of the HEA ligand is sensitive to temperature as the binding capacity of the sorbent increases to 55–60 mg/ml of sorbent at 37 °C. The binding capacity of these sorbents depending on the temperature and effect of salt are summarized in Table 5.

From the chromatographic experiments, it is clear that mode of interaction of IgG to HEA HyperCel™ is predominantly hydrophobic, as the increase in temperature, presence of salt and the ligand chain length favours hydrophobic interaction. In absence of salt, some kind of ionic interaction takes place. The phenyl group of the PPA ligand favours hydrophobic as well as charge transfer interaction based on the microenvironment conditions. An added advantage to these gels is that, unlike conventional hydrophobic interaction chromatography, these gels require a minimum amount of salt (0.2 M) in buffer for an efficient binding.

In general, in case of both the sorbents, pH 5.5 does not favour the retention of IgG. At this pH IgG remains basic and is eluted. Partial separation of IgG from BSA was achieved.

The mixed mode nature of the sorbents could be exploited to separate a mixture of proteins by bidimensional separation mechanism (based of isoelectric point of the protein and relative hydrophobicity). The use of these specialized sorbents may reduce the number of process steps and could be used in the secondary polishing step in the purification of IgG from various feedstocks.

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