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Characterization and optimization of a chromatographic process based on ethylenediamine-N,N,N',N'-tetra(methylphosphonic) acid-modified zirconia particles

Sabyasachi Sarkar
University of Nebraska-Lincoln

Peter W. Carr
Department of Chemistry, University of Minnesota.

Clayton V. McNeff
ZirChrom Separations Inc. 617 Pierce Street Anoka, MN 55303, USA

Anuradha Subramanian
Department of chemical Engineering,University of Nebraska Lincoln., asubramanian2@unl.edu

Production of human and humanized antibodies as well as novel hybrid monoclonals will require the development of alternate separation strategies other than those utilizing protein A or G, as neither recognize IgG from different species. In addition separation methodologies based on protein A are prone to fouling [3–5], less amenable to scale-up and are expensive (US$8000–US$12 000 per liter of resin).

Colloidal zirconia was spray dried as outlined elsewhere [6] to generate zirconia particles, which were further classified, sintered and modified with ethylenediamine-*N,N,N',N'*-tetra(methylphosphonic) acid (EDTPA) to yield a pseudo-biospecific support for use in biochromatography. Current methods used in the preparative purification of antibodies use a multistep procedure, which includes a cation-exchange column, anion-exchange column and a protein-A based affinity column [7]. The pseudo-biospecific matrices being developed in this research study may offer a cost-effective alternative to the three-column approach.

The preparation of alternative stationary phase supports is an important area that aims to develop new support materials that offer novel selectivities or overcome the shortcomings of silica and polymeric supports [8]. In addition, there is a need to develop new matrix technologies or purification protocols that are amenable to scale up without presenting excessive operational complexities. Hence there was an interest to develop adsorbents that operated based on physiochemical affinities or on mixed mode synthetic chemistries coupled with engineered matrices. The development of zirconia as a stationary phase material is an example where both aspects may be achieved [9–12]. Its high density and excellent thermal and chemical stability provide several advantages over traditional silica or polymeric supports. In particular, the thermal and chemical stability of zirconia allows the usage of harsh cleaning agents, depyrogenation procedures, viral inactivation by detergents, decontamination by heat treatment, or combinations thereof which are routinely performed in the pharmaceutical industry. Hence the objective of this research undertaking is to gain a systematic understanding of the factors impacting the scale-up and design of a chromatographic process based on the r_PEZ solid-phase support. Specifically we seek to address: (1) the structure-function of EDTPA and understand the basis of affinity for immunoglobulins; (2) alternate methodologies to Protein-A based separations; and (3) an optimized criteria for scale-up. The aim of this study is to show the utility of this phase in the separation and purification of antibodies from human serum and cell-culture supernatants and to further characterize and optimize the chromatographic parameters.

2. Materials and methods

2.1. Reagents

All chemicals were of analytical-grade or better. Sodium chloride was purchased from Fischer Scientific (Hanover Park, IL, USA). EDTPA was purchased from TCI America (Portland, OR, USA). Bovine serum albumin (BSA), pure human immunoglobulin G (hIgG), all horseradish peroxidase conjugated anti-iinmunoglobulins used for enzyme-linked immunosorbent assay (ELISA) were obtained from Sigma (St. Louis, MO, USA). All proteins were used without further purification. Immulon II microtiter plates were purchased from Fisher Scientific (Hanover Park, IL, USA). Affinity purified goat anti-mouse (whole molecule) immunoglobulins and goat anti-mouse imrnunoglobulins conjugated to horseradish peroxidase (HRP) were purchased from Sigma. Cell culture supernatant rich in monoclonal antibodies (mAbs) was provided as a generous gift by Dr W.H. Velander (Blacksburg, VA, USA). o-Phenylenediamine·2HCl (OPD) tablets were purchased from Abbott Labs. (Chicago. IL, USA). Pre-cast NuPage 4-12% Bis-Tris gels were purchased from Invitrogen (Carlsbad, CA, USA). A Genesys 5 model from Spectronic Instruments UV–visible spectrophotometer (Rochester, NY, USA) was used to record the adsorption measurements. A bench top microcentrifuge (Eppendorf Centrifuge 5415C) was used to sediment the r_PEZ particles for batch experiments.

2.1.1. Support matrix preparation

Colloidal zirconia was spray dried to yield zirconia particles, which were further classified, modified with EDTPA and characterized as reported

elsewhere [6]. EDTPA-modified zirconia particles will be referred to as r_PEZ in this manuscript. r_PEZ particles were packed into a 50X0.46 cm I.D. analytical column, and a 20X1 cm I.D. semi-preparative column, supplied by ZirChrom (Anoka, MN, USA).

2.2. Ligand binding isotherms

Small-scale batch experiments were conducted to determine static equilibrium binding capacity of r_PEZ beads. Then, 200 μl of a 50% (v/v) slurry of r_PEZ beads were transferred into 1.5 ml microcentrifuge tubes to yield approximately 100 μl of beads. The beads were allowed to settle for at least 5 min and the liquid overlay was pipetted off after centrifuging for 5 min at 8000 rev./min. Then, 400 μl of 0.0, 1.0, 3.33, 6.67, 8.33, 10.0, 13.33, 16.67 and 20.0 mg/ml of hIgG in 4 m*M* EDTPA, 20 m*M* 2-(N-morpholino)ethanesulfonic acid (MES), 50 m*M* NaCl, pH 5.5 (LB) were added to the microcentrifuge tubes. Tubes were placed on an end-to-end rotator and allowed to rotate for 24 h at room temperature (–27"C). At the completion of the experiment the tubes were centrifuged on the bench top microcentrifuge at 8000 rev./min for 5 min. The supernatant was subsequently pipetted off and its protein concentration was measured via the absorbance at 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. Separate binding isotherm experiments were carried out as a function of temperature, salt concentration and pH of the loading buffer, keeping all other parameters constant.

2.3. Determination of dynamic bend capacity

For this study, a solution of pure hIgG in LB was used. This protein solution was added to the column, which was equilibrated with the loading buffer, at concentration of hIgG ranging at 0.5 and linear velocities of 3.01 to 48.19 cm/min. The absorbance of the effluent at 280 nm was monitored continuously. The injection was continued until the absorbance of the effluent reached 80% 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 20 m*M* MES. 4 m*M* EDTPA, 1.0 M NaCl, pH 5.5 (further referred to as EB). For each linear velocity the dynamic capacity of the column was determined as the amount of hIgG maintained per milliliter of bead.

2.4. Determination of adsorption rate constants

This experiment was done by using the split-peak approach as described elsewhere [13,14]. For this experiment a 0.83-ml column equilibrated with LB. Then, 30 ml of a 2.0 mg/ml solution of hIgG was prepared in LB. Aliquots of 1 ml were consecutively injected into the column. Unabsorbed protein was collected for each aliquot injected giving a total of 10 fractions collected for a particular linear velocity. In each peak the total amount of protein was determined by measuring absorbance at 280 nm. After all ten 1 ml aliquots were injected the retained protein was eluted and absorbance was measure at 280 nm.

2.5. Identification of the binding site

It is important to determine whether hIgG was bound to r_PEZ at the F_c or F_{ab} or $F_{(ab)2}$. Antibody fragments (F, or F,,, or $F_{(ab)2}$) were purchased commercially or prepared by enzymatic hydrolysis of HIgG with commercially available pepsin and papain kits from Pierce (Kockford, IL, USA). Antibody fragments were chromatographed separately under identical conditions, as used for IgG retention and elution protocol as described earlier. The column-wash and elution fractions were assayed by specific ELISA assays [15]. Total recovery and yield of each fragment will be assessed quantitatively.

2.6. Chromatography

EDTPA modified particles were packed in 20X1.0 cm I.D. column, which was provided by Zirchrom. The chromatographic system used was a high-performance liquid chromatography (HPLC) system using a Spectroflow 783 detector at 280 nm and an isocratic pump from Chrorntech (Apple Valley, MN, USA). For separation and isolation of mAb from the cell culture supernatant the loading buffer consisted

of 4 m*M* EDPTA, 20 m*M* MES, and 50 m*M* NaCl at pH 5.5 (LB). Typically the supernatant was diluted ten times with LB and pumped through the coluinn. Upon completion of the feed step, the column was sequentially washed with LB, followed by 4 m*M* EDTPA. 20 m*M* MES, 100 m*M* NaCl, pH 5.5 (LB1). A step gradient to 1.0 M NaCl was used to affect elution of bound IgG. The column was regenerated with 4 m*M* EDTPA, 20 m*M* MES, 2.0 M NaCl, pH 5.5 for 15 min prior to the next injection (flow-rate 5.0 ml/min). All proteins including cell culture supernatant samples were dissolved in the mobile phase. All chromatographic fractions were saved and analyzed for total protein content at 280 nm, mAb content by specific ELISA assays and electrophoretic analysis [6,15].

In a separate experiment, 2.0-ml protein A immobilized on Hyper D support was packed into a Pharinacia column (1.0×10 cm) and cell culture supernatants were chromatographed according to the manufacturer's instructions. In a typical application, 1-ml of the cell culture supernatant 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 on to the column at a linear velocity of 1.0 cm/min. Loosely bound proteins were washed with LB_1. The elution of the bound mAb was effected with 0.570 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 280 nin and specific antibody fragment content by specific ELISA assays [6,15].

3. Results

3.1. Effect of temperature on the binding of hIgG to r_PEZ

The effect of temperature on the equilibrium binding of hIgG to r_PEZ is shown in Table 1. The temperature does not seem to have any significant effect on the binding capacities of hIgG to r_PEZ, as observed from the similar shapes of the isotherms. Furthermore, the binding isotherms follow the saturation pattern as predicted by the Langmuir model. Data was reduced by both lineweaver-busk and scatchard 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 47 to 51 mg hIgG bound per ml of beads. The K_d values were found to be in range from 4.13×10^{-6} to 5.67×10^{-6} M [moles of hIgG bound per liter of (wet) r_PEZ beads].

Table 1
Summary of binding parameters

Parameter	K_d (*M*)[a]	Q_{max} (mg/ml)
Temperature (°C)		
4	5.47×10^{-6}	51.02±5.0
15	5.27×10^{-6}	49.26±2.8
25	5.67×10^{-6}	47.85±4.8
37	4.13×10^{-6}	50.00±2.1
pH		
5.5	4.6667×10^{-6}	58±3.0
6.0	3.4667×10^{-5}	22±1.8
6.5	7.0000×10^{-5}	32±4.0
7.0	9.8667×10^{-5}	40±1.0
8.0	3.0000×10^{-4}	20±0.5
[NaCl] (M)		
0.05	4.67×10^{-6}	5521.9
0.10	8.20×10^{-6}	55±2.7
0.20	2.81×10^{-5}	30%1.3
0.40	n.a.	0

Static adsorption isotherms for the binding of hIgG to r_PEZ were carried out at different temperatures, pH and salt concentration. r_PEZ beads were contacted with different concentrations of hIgG as described in the Materials and methods section. The equilibrium data are plotted as mg of hIgG adsorbed per ml of r_PEZ beads (wet) against the concentration of HIgG in the supernatant. Experiments were done in duplicate and the standard deviation was in the range 5–15%. n.a.=not available.

[a]The standard deviation in the values of K_d reported in the table are in the range 5–8%.

In a separate experiment, r_PEZ was found to bind pure pig immunoglobulin, human IgG and bovine IgG with similar K_d and static binding capacities in the range of 25–35 mg IgG per ml of beads were obtained (data not shown). Additionally, the r_PEZ was found to interact with IgG subclasses IgG1, IgG_{2a}, IgG_{2b} and IgG_3 with similar binding affinities (data not shown).

In order 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 [14]. A cooperativity coefficient (n) of unity indicates no cooperativity. Analysis of the isotherms at 4, 10, 22 and 35 °C indicated a "n" value of 0.82, 1.10, 1.10 and 0.97, respectively, which suggests that there is no cooperativity due to protein–protein interactions.

3.2. Effect of pH on the binding of hIgG to r_PEZ

The effect of pH on the Q_{max} and K, of hIgG to r_PEZ is shown in Table 1. pH seems to have an effect on the binding of hIgG to r_PEZ. Isotherm data as a function of pH was analyzed by both Lineweaver–Burk and Scatchard analysis. similar to the aforementioned section. The static binding capacities were found to be 58, 22, 32, 40 and 20 mg hIgG bound per ml of beads at pH values of 5.5, 6.0, 6.5, 7.0 and 8.0, respectively. The K„ was found to increase from 4.60×10^{-6} to 299.710^{-6} M [moles hIgG bound per liter (wet) r_PEZ beads] with an increase in pH.

3.3. Effect of salt on the binding of hIgG to r_PEZ

Ligand binding experiments were conducted under isothermal conditions with varying salt concentrations to determine the effect of salt on the binding of hIgG to r_PEZ. The data was observed to follow the Langmuir model and was reduced by Scatchard plot and Lineweaver–Busk plot analysis. The salt concentration has a significant effect on the shape of the binding profiles. The Q_{max} was found to be 55, 55 and 30 mg hIgG bound per ml of beads at NaCl concentration of 0.05, 0.1, and 0.2 M, respectively. The K, was found to vary from 4.67×10^{6} to 28.1×10^{-6} M [[moles HIgG bound per liter of (wet) r_PEZ beads] with the change in salt concentration. (Table 1).

3.4. Estimation AH,, and ΔS_o of adsorption

The thermodynamic parameters for the adsorption can be obtained by determining K_d at different temperatures. The dependence of K„ as a function of temperature was evaluated from the Langmuirian analyses of the isotherm data [14]. From the Van't Hoff reaction isotherm $\Delta G=\Delta G^{o}-RT\ln[K_d]$; when $\Delta G=0$, the value of AG" can be calculated ($\Delta G^{o}=RT\ln[K_d]$). AG" can then be calculated at a given temperature from the value of K_d. The slope of the plot of $\ln[K_d]$ vs. $1/T$ yielded AH", assuming $AH"$ is independent of temperature. A ΔH_o (adsorption value of -5.64 kJ mol^{-1} and a AS_o (adsorption value of 120 kJ K^{-1} were obtained for the interaction of IgG with r_PEZ.

3.5. Determination of the dynamic capacity and adsorption rate constant

The effects of dynamic bead capacity, Q,, on r_PEZ beads as a function of linear velocity is shown in Fig. 1. A dynamic hIgG adsorption capacity of 20.00, 13.85, and 11.53 mg/ml bead was obtained at linear velocities of 3.01, 6.02 and 12.04 cm/min, respectively. Fig. 2 shows the effect of the linear velocity on the adsorption rate constant. An association rate constant (k_a) of 982.48, 1731.65, and 3242.49 l mol^{-1} s^{-1} was obtained for linear velocities of 3.0, 6.0 and 12.0 cm/min, respectively. A best fit of the data in Fig. 2 reveals a linear trend for the data that is described by the equation $k_a=689.12\ (u)-903.39$, 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 witl1 an R^2 value of 0.994 within the range tested.

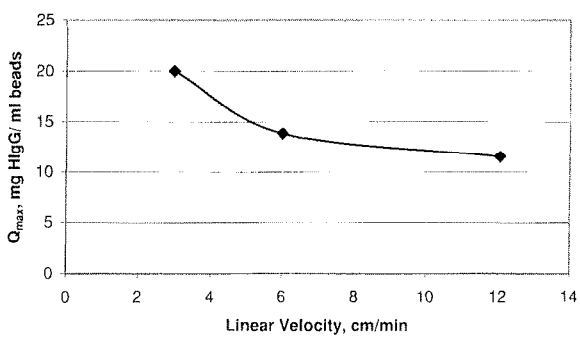

Fig. 1. Relationship between Q_{max} and linear velocity. A solution of pure hIgG at concentrations ranging from 0.5 to 25 mg/ml was continuously fed to the column until the absorbance of the effluent reached 0.8–0.97 of the inlet concentration. The column was then washed with loading buffer and the 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 3.01, 6.02 and 12.04 cm/min.

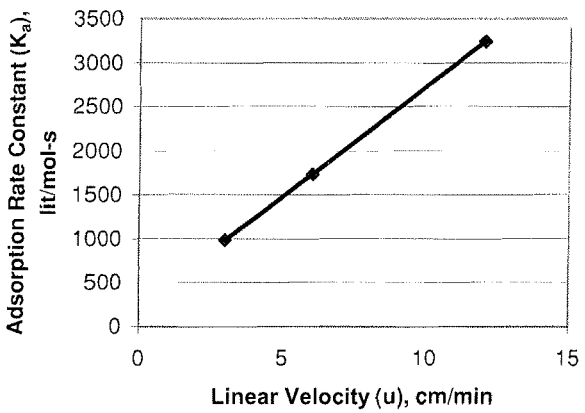

Fig. 2. Relationship between adsorption rate constant and linear velocity. At a given constant linear velocity, 15 aliquots (1-ml each aliquot) ml of a 3.5 mg/ml solution of hIgG were consecutively injected into the column. The cumulative amount of injected protein, and unadsorbed protein was calculated. The ratio of unadsorbed protein to the injected protein was expressed as f and $1-f$ gave 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 (3.01, 6.02 and 12.04 cm/min).

The effect of hIgG feed concentration on the adsorption rate of hIgG on r_PEZ was evaluated as a function of linear velocities. The adsorption rate increases with increasing hIgG feed concentration and linear velocity. Empirical equations that relate 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 10 mg hIgG/ml, more than *20* times than that of the lowest concentration at 0.5 mg hIgG/ml.

3.6. Identification of binding site

Table 3 lists the total yield and recovery obtained with each IgG fragment, as separate chromatographic experiments. The F,, and the $F_{(ab)2}$ fragment were both retained on the r_PEZ column. It appears that the F_{ab} and $F_{(ab)2}$ fragments were retained to a similar extent or in other words r_PEZ has similar affinity for the F,,,and $F_{(ab)2}$ fragments. F, fragment was also retained but to a lesser extent. Similar trends and percentages were obtained when the binding of the IgG fragment were tested under batch conditions (data not included).

In contrast, the F_c fragment was quantitatively retained on the Protein A column as expected (Table 3). Both F„ 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).

3.7. Expression level and purification yields

Table 4 summarizes the total protein recovery and mAb yield in the eluate fractions for both r_PEZ and protein–Hyper D columns. The mAb concentration in different chromatographic fractions in each individual run was estimated by the ELISA protocol described in the Materials and methods section. The percent yield of mAb in the eluate fraction was determined as a ratio of the total mAb in the eluate fraction to the total mAb challenge. In most cases. total mAb recoveries of 80–99% were obtained. The r_PEZ column runs shown in Table 4 had mAb yields of 65.4 and 72.5%, while the protein A–Hyper D column runs had mAb yields of *77.2* and 82.6%,

Table 2
Effect of the flow-rate on the rate of adsorption

Feed concentration (mg/ml)	Emperical equation
0.5	$y=9\times10^{-8}x+2\times10^{-7}$
2	$y=3\times10^{-7}x+8\times10^{-7}$
5	$y=1\times10^{-6}x-2\times10^{-7}$
10	$y=2\times10^{-6}x-6\times10^{-8}$

The data obtained was approximated to the following equation: $AR=au+b$ where a and h are constants and "u" is the linear velocity and "AR" is the adsorption rate. The adsorption rate (mg ml^{-1} min^{-1}) was calculated as follows: Amount of protein retained per ml of support (mg/ml)/time of adsorption (min).

Table 3
Percent total recovery and yield of antibody fragment5 on ı PEZ 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	SS.8	85.0	0.96	91.0	0.9	0.01	97.8	0.1	0.01
	Run 2	97.5	88.0	0.95	83.0	2.0	0.02	94.9	1.9	0.02
r_PEZ	Run *1*	57.5	16.3	0.28	47.5	35.8	0.75	57.3	42.0	0.73
	Run 2	67.5	20.0	0.30	48.0	38.0	0.79	62.5	39.1	0.63

r_PEZ were challenged with pure antibody fragments (F_c or F_{ab} or F,;,,,,)as separate experiments. Chromatographic fractions were assayed for the specific fragment content by ELISA, as detailed in the Materials and methods section. In general, an average of triplicate application of three different dilutions in ELISAs were 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-throngl1 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 manufacturer's instructions. The chromatographic fractions were assayed as outlined in the Materials and methods section.

respectively, with little or no detectable mAb in the unretained and wash fsactions.

3.7.1. Gel electrophoresis

Fig. 3 shows the sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of the purified fractions from a typical chromatographic separation. Chromatographic fractions from Run A1 and Run B1 (see Table 4) were selected for electrophoretic analysis. Lanes 1 shows a molecular mass ladder. Lanes 2 and 3 show applications of pure BSA and mAb at a total protein level of 2 μg, respectively. Lanes 4 and 5 show an application of mAb purified with r_PEZ column, at a total protein level of 3 μg. Lanes 6 and 7 show an application of mAb purified with Protein A–Hyper D column, at a total protein level of 3 μg. The eluate fraction from both Protein A Hyper D column and r_PEZ gave a band around M, 150 000 similar to the pure mAb in lane 3. In addition to the major mAb band at M, 150 000, a minor band at M, 56 000 accounting for less than 0.5% of the area obtained by digital image processing was observed. Lane X shows an application pure mAb reference standard under denatured conditions at a total protein level of 3 μg. Lane 9 shows an application mAb purified with Protein A–Hyper D column under denatured conditions at a total protein level of 3 pg. Lane 10 shows an application mAb purified with r_PEZ column under denatured conditions at a total protein level of 3 μg. The purified mAb gave two distinct bands around M, 50 000 and 25 000 similar to the pure mAb in Lane 8. The purity of the mAb in the eluate fraction (lanes 4–7) is estimated to be greater than 98%, respectively by

Table 4
Summary of the total recovery and yields of mAbs on r_PEZ and Protein A–Hyper D

Run	Column type	Total recovery[a] (%)	Total recovery[b] (%)	Yield[b] (%)
A1	r-PEZ	95	83.9	65.4
A2	r-PEZ	90.8	99.2	72.5
B1	Protein A–Hyper D	81.9	80	77.2
B2	Protein A–Hyper D	91.8	87.1	82.6

Percent total recovery is defined as the ratio of the sum of the total protein in eluate and column fall-through fractions to the total protein present in the feed. Percent yield is defined as the ratio of the Ig present in the eluate fraction to the total amount of Ig in the feed. ELISA values were used to estimate the yields. In general, an average of triplicate application of three different dilutions in ELISA assays was used for yield calculations.

[a] Determined by ELISA assays.

[b] Determined spectrophotometrically at 280 nm.

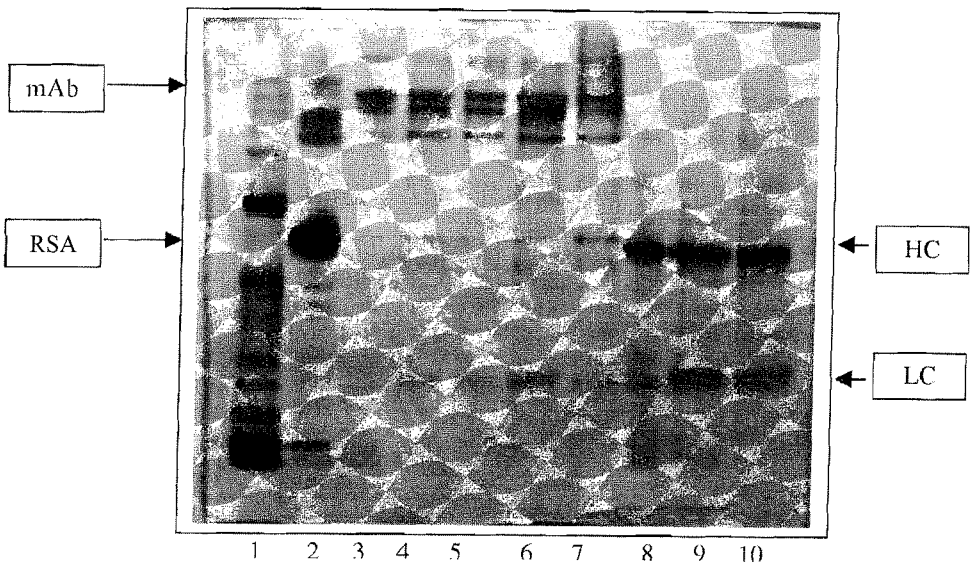

Fig. 3. Silver stained gel of reduced and non-reduced elutions from r-PEZ and Protein A–Hyper D. Sodium dodecylsulfate (0.1%)–polyacrylamide gel (4–12% gradient) electrophoresis of mAbs that were puritied from cell culture supernatant using r-PEZ. Lanes: 1, molecular mass ladder; 2 and **3,** applications of pure RSA and reference mAb standard, respectively; 4 and 5, applications of purified mAb product from r_PEZ runs; *5* and 6, applications of purified mAb product from protein A–tryper D runs; 8–10, reference mAb standard, r_PEZ puritied mAb and Protein A–Hyper D puritied mAb obtained performed under reducing conditions.

digital image processing. Similar electrophoretic patterns were obtained with the fractions from other runs listed in Table 4 (data not shown).

4. Discussion

The concentration of Lewis acid sites on the surface of bare unmodified zirconia) is estimated to be 4–5 mmol/mm^2 [8]. The great affinity of zirconia for phosphate and the low solubility of zirconium phosphate in aqueous media made the intentional modification of zirconia with EDTPA an attractive concept [8]. EDTPA is a special chelator the renders zirconia Lewis acid deactivated. Hence, zirconia spheres 28–35 μm in diameter were further modified with EDTPA to yield a pseudo-affinity support for use in bioseparations. Additionally, we include 4 m*M* EDTPA in all our buffers to ensure the surface concentration is constant. However, our ongoing studies have demonstrated that EDTPA-modified zirconia is stable from pH 1 to 10 and the EDTPA does not desorb from the surface.

In this study, further studies aimed at understanding the adsorption mechanism and the nature of interactive forces between Igs and EDTPA modified zirconia were undertaken. In affinity chromatography, interaction between the immobilized ligand and the solute molecule is based on complimentarity of charge. hydrophobic, ionic and Van der Waals interactions. The same forces probably play a role in cation-exchange dominated pseudo-affinity systems, but their role and magnitude perhaps differ. It is desired to establish the type of interactions governing and prevalent in pseudo-biospecific systems in order to better optimize the processing conditions, namely pH, ionic strength, salt concentration and temperature.

In the interaction between a chromatographic support and the molecule that is being purified pH of the buffer plays an important role. The net charge on a protein is altered at varying pH values, which leads to varying bonding interactions between it and a chromatographic support. At the pH value for which binding of IgG to the r_PEZ support was determined to be optimal, a pH of 5.5, hIgG has a net positive charge. Therefore, at the same pH, we hypothesize that the EDTPA modified r_PEZ must carry a net

negative charge, for charge–charge interactions to occur. Additionally at this pH, human serum albumin (HSA) carries a net negative charge [isoelectric point, $pI_{[HSA]}$=4.9] which minimizes interaction with r_PEZ. This is verified by the fact that little HSA was present in the elution fractions.

Pseudo-affinity chromatography supports exploit certain structural binding features of proteins. The r_PEZ, for example, utilizes structural characteristics of hIgG to establish a protein–ligand interaction, although the mechanism of binding is yet unknown. The presence of high salt concentration in the binding buffer resulted in negligible binding of the hIgG in comparison to that in absence of NaCl. This indicates the involvement of electrostatic and possibly hydrogen bond interactions between the proteins and the ligand.

When determining the factors that govern protein–ligand interactions it becomes important to understand how the chemical and physical properties of the matrices binding sites affects protein adsorption. It is therefore useful to study equilibrium adsorption data with the intention of determining how the support reacts to protein binding with increasing concentrations. The shape of the equilibrium adsorption curve at all temperatures studied indicates a Langinuir-type isotherm and is well fit to the simple Langmuir equation [16]. We hypothesize that at these temperatures, hIgG binds uniformly with a high affinity for the binding sites until it reaches a maximum binding capacity. The static capacity of r_PEZ as determined by the Langmuir adsorption data was calculated to be 55–58 ing IgG per ml of beads, which is comparable to that reported for Protein A-Sepharose and Protein A-Ultragel [17]. The dissociation constant, K_d, was determined to be 4.7×10^{-6} M, which indicates medium affinity and is typical for a pseudo-affinity ligand [13,14].

Our work with r_PEZ beads indicated that an effective isolation of mAb from cell supernatant was attainable. This work may help develop pseudo-affinity matrices for use as immunoadsorption columns. The chromatographic properties of s-PEZ are comparable to commercially available stationary phases. The r_PEZ beads are mechanically and chemically stable and can withstand high linear velocities. Separation of human immunoglobulins from other serum proteins was likely through a differential in binding capacity mediated by pseu affinity interactions. In other words, the r_PEZ ma is not specific for immunoglobulins as a Protein matrix would be, but the pseudo-affinity interact forces confer a unique specificity for immun globulins over other serum proteins. This selectiv facilitated the use of a step gradient for the sep ration of mAb from cell culture supernatant.

5. Summary

We have successfully spray-dried colloidal zi conia to generate zirconia microspheres that can b used as a support in bio-chromatography. An sem preparative-scale column packed with zirconi microspheres was employed to separate Igs from cel culture supernatants. Our future efforts will focus o the validation of optimization and scale-up parame ters obtained in this study. Studies are underway t estimate the transport parameters relevant for scale up.

Acknowledgements

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