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Use of a modified zirconia support in the separation of immunoproteins

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Abstract

Zirconia beads (25–38 μm in diameter) were modified with *N,N,N′,N′*-ethylenediaminetetramethylenephosphonic acid to generate a zirconia based pseudoaffinity support, further referred to as r_PEZ. The influence of pH, salt concentration and temperature on the binding of human immunoglobulin G (hIgG) to r_PEZ was studied. Temperature had no significant impact on the maximum binding capacity (Q_{max}), and the equilibrium-binding constant (K_d), whereas pH and the salt concentration had a noticeable impact on both Q_{max} and K_d. The Q_{max} value of 55 mg hIgG/ml of bead was obtained at a pH of 5.5 and found to decrease with an increase of pH. The modified zirconia support allowed the separation of immunoglobulins (IgG, IgA and IgM) from untreated human serum. Elution was possible under mild conditions with a step salt gradient. Overall protein recoveries in the range of 109–125% were obtained with human serum. Human IgG, human IgA, and human IgM yields of 29.50±6.3, 3.22±0.7, and 6.84±0.7%, respectively, were obtained at a linear velocity of 4.32 cm/min. Purity of products, obtained from a single chromatographic step was estimated to be greater than 89.0±2.6%. The utility of r_PEZ in the selective removal of immunoglobulins, as in immunoadsorption was discussed.

Keywords: Zirconia; Stationary phases, LC; Pseudoaffinity separations; Immunoglobulins; Proteins; Albumin

1. Introduction

The importance of immunoglobulins (Igs) has been well researched and documented [1]. The unique specificity that an antibody displays for an antigen makes it an invaluable tool in diagnostics [2,3] as probes for fine structural analysis [4,5], in histological examination [6], and in immunotherapy [7]. Purified immunoglobulin products have been used for medical treatment in patients with inadequate Ig levels [8]. As the discovery of new medical and diagnostic uses for immunoglobulins arise, an increased need for medical-grade Igs will continue to fuel advances in the area of Ig separation. There is a demand in the current market to design and develop methodologies for the purification of human globulin from plasma or from genetically engineered sources.

Purification schemes for Igs include precipitation with ammonium sulfate, gel filtration chromatography, ion-exchange chromatography [9], thiophilic chromatography [10], and affinity separations using immobilized protein A [4,11,12]. The development of adsorbents for the selective removal and/or immunodepletion of globulins from plasma is of great importance [13–17] and affinity type adsorbents, as an extracorporeal immunoadsorption sys-

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tem, have been investigated in experimental and clinical studies [18].

A number of affinity columns have been developed based on this principle [19] and have been successfully used to treat many disease conditions such as systemic lupus erythematosus [20], and myasthenia gravis [21]. The removal of anti-DNA antibodies by DNA fixation and removal of immune complexes and IgG by protein A immobilized on Sepharose was attempted in SLE and malignant tumors, respectively [20,22–24]. As biological ligands were used in these procedures, it was difficult to ensure an adequate supply of reagents. Additional challenge was encountered in the handling, sterilization, and preservation of these biological ligands. In addition, the leaching of ligands and the immune response resulting from the same was of legitimate concern.

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 existing supports. Hence there was an interest to develop adsorbents that operated based on physiochemical affinities or on mixed mode synthetic chemistries coupled with engineered matrices. Some examples of such applications include the immobilization of amino acids like tryptophan and phenylalanine on porous poly-(vinyl alcohol) (PVA), polyacrylamide, and chitosan beads. Using immunodiffusion methods, chitosan beads with immobilized phenyl alanine and tryptophan were used to selectively remove IgG and IgM from human plasma [25,26].

The development of *N*,*N*,*N*′,*N*′-ethylenediaminetetramethylenephosphonic acid (EDTPA)-modified zirconia as a stationary phase material is an example where both aspects may be achieved. Studies have established the usefulness of zirconia as a chromatographic stationary phase [27–31]. The research on the use of zirconia ceramics as biomaterials was initiated 20 years ago, and now zirconia (Y-YZP) is in clinical use in total hip replacement (THR) as zirconia balls heads, but developments are in progress for its application in other medical devices. Biocompatability of zirconia was evaluated using in vitro and in vivo tests [32–39]. Here we seek to investigate the use of a new porous zirconia based adsorbent for the efficient purification of antibodies from serum fractions. The aim of this study is to show the utility of this phase in the separation and purification of antibodies from human serum. A single chromatographic step is sufficient to obtain purified antibodies. The process yields and purity of the Igs will be demonstrated.

2. Material 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-immunoglobulins used for enzyme-linked immunosorbent assay (ELISA) and human serum albumin (HSA) were obtained from Sigma (St. Louis, MO, USA). All proteins were used without further purification. Human immunoglobulin A (hIgA) and human immunoglobulin M (hIgM) were purchased from Jackson Immunoresearch (West Grove, PA, USA).

Immulon II microtiter plates were purchased from Fisher Scientific. Affinity purified goat anti-mouse (whole molecule) immunoglobulins and goat anti-mouse immunoglobulins conjugated to horseradish peroxidase (HRP) were purchased from Sigma. *o*-Phenylenediamine·2HCl (OPD) tablets were purchased from Abbott Labs. (Chicago, IL, USA). Precast NuPage 4–12% Bis–Tris gels were purchased from Invitrogen (Carlsbad, CA, USA).

A Genesys 5 Model UV–visible spectrophotometer from Spectronic Instruments (Rochester, NY, USA) was used to record the adsorption measurements. A bench top microcentrifuge (Eppendorf Centrifuge 5415C) was used to sediment the zirconia-based pseudoaffinity support (r_PEZ) particles for batch experiments.

2.2. Support matrix preparation

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

elsewhere [40]. Briefly, zirconia particles were suspended in 0.1 *M* H_4EDTPA and sonicated to ensure infiltration of the pores. The solution was then brought to a boil and maintained for 3–4 h. At the end of the reaction period, the solution was removed from the heat sources, particles allowed to settle, and the EDTPA solution was decanted. The particles were subsequently washed with multiple cycles of water. EDTPA-modified zirconia particles will be referred to as r_PEZ in this manuscript. r_PEZ particles were packed into a 5.0 cm×0.46 cm I.D. analytical column, supplied by ZirChrom (Anoka, MN, USA).

2.3. Ligand binding isotherms

Small-scale batch experiments were conducted to determine static equilibrium binding capacity of r_PEZ beads. A 200-μl volume of a 50% (v/v) slurry of r_PEZ beads was transferred to 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 9000 *g*. A 400-μl volume 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 9000 *g* for 5 min. The supernatant was subsequently pipetted off and its protein concentration was measured by 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.

In a separate experiment, isotherms were constructed with porcine IgG, and bovine IgG using a procedure similar to the one listed above.

2.4. Chromatography

The chromatographic system consisted of a Chrom Tech (Apple Valley, MN, USA) Iso-2000 isocratic pump in conjunction with an online Model 783 Spectroflow spectrophotometer (Ramsey, NJ, USA), which was used mainly as an indicator, and an SRI (Torrance, CA, USA) PeakSimple Model 203, single-channel serial port online data acquiring system. The absorbance of the fractions was then measured using the spectrophotometer.

For separation and isolation of immunoglobulins from the human crude serum solution the loading buffer consisted of 4 m*M* EDPTA, 20 m*M* MES and 50 m*M* NaCl at pH 5.5. A step gradient to 0.1 *M* NaCl around 8 min from the start of loading was used to affect elution of weakly bound non-specific proteins. Finally, a step to 1 *M* NaCl at 70 min was used to ensure elution of proteins bound by specific interactions.

All buffer solutions were filtered through a Chrom Tech metal-free solvent (type A-427) 10 μm UHMWPE (ultra-high molecular mass polyethylene) membrane filter at the time of use.

2.5. Purification of immunoglobulins from serum

Human serum sample was diluted 20 times with LB and column-loaded. Typical sample applications were made with 250 μl of human serum diluted to a total volume of 5.0 ml. A linear velocity of 4.82 cm/min (0.8 ml/min) was used in this study. Upon completion of the feed loading step, the column was sequentially washed with LB, LB2 (20 m*M* MES, 4 m*M* EDTPA, 100 m*M* NaCl, pH 5.5) until the $A_{280\ nm}$ of the column effluent reached baseline. The bound Igs were eluted with a step change to the elution buffer (20 m*M* MES, 4 m*M* EDTPA, 1.0 *M* NaCl, pH 5.5). Upon completion of the elution step, the column was re-equilibrated in loading buffer. The chromatographic fractions were assayed for total protein content by measuring their absorbance at 280 nm and individual immunoprotein content by their respective specific ELISAs. The purity of the product was judged by electrophoretic analysis.

2.6. Gel electrophoresis

The purity of the recovered IgG was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). In brief, all chromatographic

fractions were diluted or concentrated to a protein concentration of 1 mg/ml. Chromatographic samples were mixed both with a non-reducing and a reducing buffer at a ratio of 1:1, respectively, and were heated to 95°C for 5 min in a water bath. Proteins were analyzed on NuPage 4–12% Bis–Tris gels and visualized by Gelcode Blue stain reagent. Stained gels were analyzed by digital image processing to assess the purity with the help of a DR-13 Shimadzu dual-wavelength densitometer (Columbia, MO, USA).

2.7. Determination of hIgG, hIgA and hIgM by ELISA

Immulon II microtiter plates were incubated with 100 μl/well of 5 μg/ml rabbit anti-human IgG in coating buffer (0.1 *M* $NaHCO_3$, 0.1 *M* NaCl, pH 9.3) for 24 h at 4°C. Wells were washed three times with washing buffer (50 m*M* NaCl, 20 m*M* Tris–HCl, 0.05% Tween, pH 7.2) and residual sites were blocked with blocking/dilution buffer (50 m*M* NaCl, 20 m*M* Tris–HCl, 0.5% casein, pH 7.2) for 30 min at room temperature. A 100-μl volume of standard and samples in blocking/dilution buffer was added to the each well and incubated for 30 min at 37°C. After incubation wells were washed three times with washing buffer and 100 μl/well of 1:2500 diluted HRP conjugated rabbit anti-human IgG was added to each well and incubated at 37°C for 30 min. Wells were washed three times with washing buffer and 100 μl/well of OPD substrate was added. The colorimetric reaction was stopped after approximately 3 min by the addition of 100 μl/well of 1.5 *M* sulfuric acid. Bound chromophore was detected using a Bio-Tek Instruments EL-800 automatic microplate ELISA reader at 490 nm (Winooski, VT, USA).

A similar ELISA procedure was used for the determination of hIgA and hIgM with the following changes. Plates were coated with 100 μl/well of 5 μg/ml goat anti-human IgA and IgM, for hIgA and hIgM determination, respectively. Volumes of 100 μl/well of 1:1000 diluted HRP conjugated goat anti-human IgA, and 1:500 diluted HRP conjugated goat anti-human IgM, were used for the hIgA and hIgM plates, respectively.

3. Results

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

Static binding experiments were conducted to determine the 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 Fig. 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 were reduced by both Lineweaver–Burk and Scatchard analysis to determine the values of the static binding capacity (Q_{max}) and the equilibrium dissociation constant (K_d). The later method proved to fit the experimental data and was subsequently used for Q_{max} and K_d determination for all the data. 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 the range of $4.13 \cdot 10^{-6}$ to $5.67 \cdot 10^{-6}$ *M* [mol of hIgG bound per liter of (wet) r_PEZ beads].

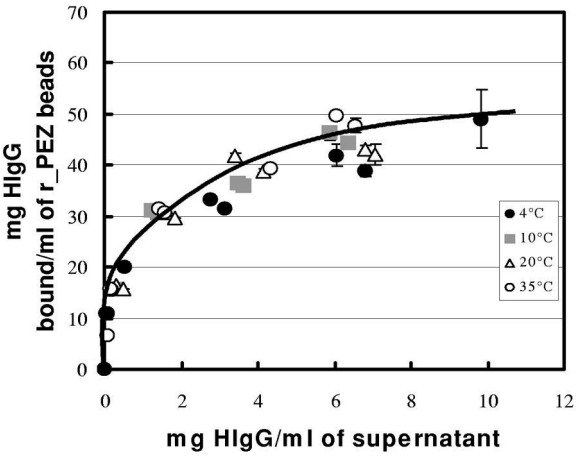

Fig. 1. Static adsorption isotherms for the binding of hIgG to r_PEZ at different temperatures. r_PEZ beads were contacted with different concentrations of hIgG as described in the Materials and methods section. Temperature of the experiment is indicated on the graph. The equilibrium data are plotted as mg of hIgG adsorbed per ml of r_PEZ beads (wet) against the concentration of hIgG in the supernatant. The amount of hIgG bound was determined by difference. Experiments were done in duplicate and the standard deviation was in the range of 5–15%.

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).

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

The effect of pH on the Q_{max} and K_d of hIgG to r_PEZ is shown in Fig. 2. pH seems to have an effect on the binding of hIgG to r_PEZ. Isotherm data as a function of pH were analyzed by both Lineweaver–Burk and Scatchard analysis, similar to the previous 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_d was found to increase from $4.60 \cdot 10^{-6}$ to $299.7 \cdot 10^{-6}$ *M* [mol hIgG bound per liter (wet) r_PEZ beads] with an increase in pH.

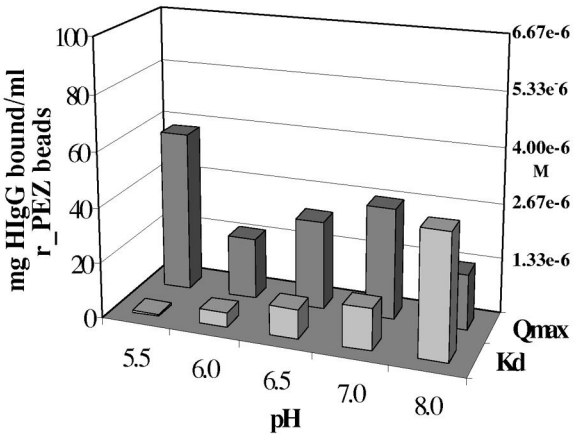

Fig. 2. Representation of Q_{max} and K_d of hIgG on r_PEZ beads at varying pH. The maximum amount of total hIgG loaded was 20 mg per ml of r_PEZ beads. The data are plotted as mg of hIgG adsorbed per ml of (wet) r_PEZ beads. Values were calculated using Scatchard plots after reducing data obtained from static adsorption isotherms at different pH values as described in the Materials and methods section. The coefficient of determination was greater than 0.87.

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 were observed to follow the Langmuir model and were reduced by Scatchard Plot and Lineweaver–Burk plot analysis. The salt concentration has a significant effect on the shape of the binding profiles. The Q_{max} values were found to be 55, 55 and 30 mg hIgG bound per ml of beads at NaCl concentrations of 0.05, 0.1, and 0.2 *M*, respectively. The K_d values were found to vary from $4.67 \cdot 10^{-6}$ to $28.1 \cdot 10^{-6}$ *M* [mol hIgG bound per liter of (wet) r_PEZ beads] with the change in salt concentration (Fig. 3).

3.4. Column chromatography

Fig. 4 shows a typical chromatographic profile for the isolation of Igs from the crude human serum. Unbound or very weakly retained proteins passed through the column during the first 20 min. To elute any proteins bound by non-specific interactions, a step increase to 0.1 *M* sodium chloride at 6 min was employed. The millivolt trace returns to baseline by 50 min (60 column volumes) indicating near complete removal of unbound proteins. A step change to 1 *M* sodium chloride was made to elute the bound Igs. A chromatographic peak at 77 min indicates elution of bound protein that we identify as Igs. This peak was eluted between 11 and 12 column volumes.

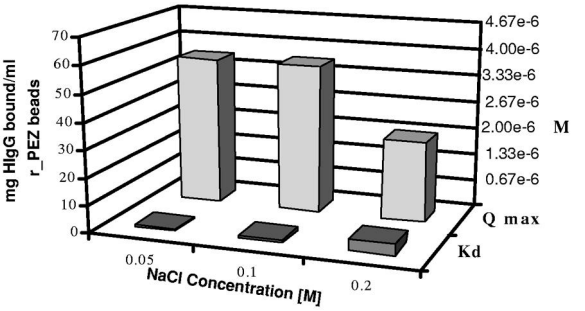

Fig. 3. Q_{max} and K_d of hIgG on r_PEZ beads at varying NaCl concentrations. The maximum amount of total hIgG loaded was 20 mg per ml of r_PEZ beads. The data are plotted as mg of hIgG adsorbed per ml of (wet) r_PEZ beads. Values were calculated using Scatchard plots after reducing data obtained from static adsorption isotherms at different salt concentrations as described in the Materials and methods section. The coefficient of determination was greater than 0.92.

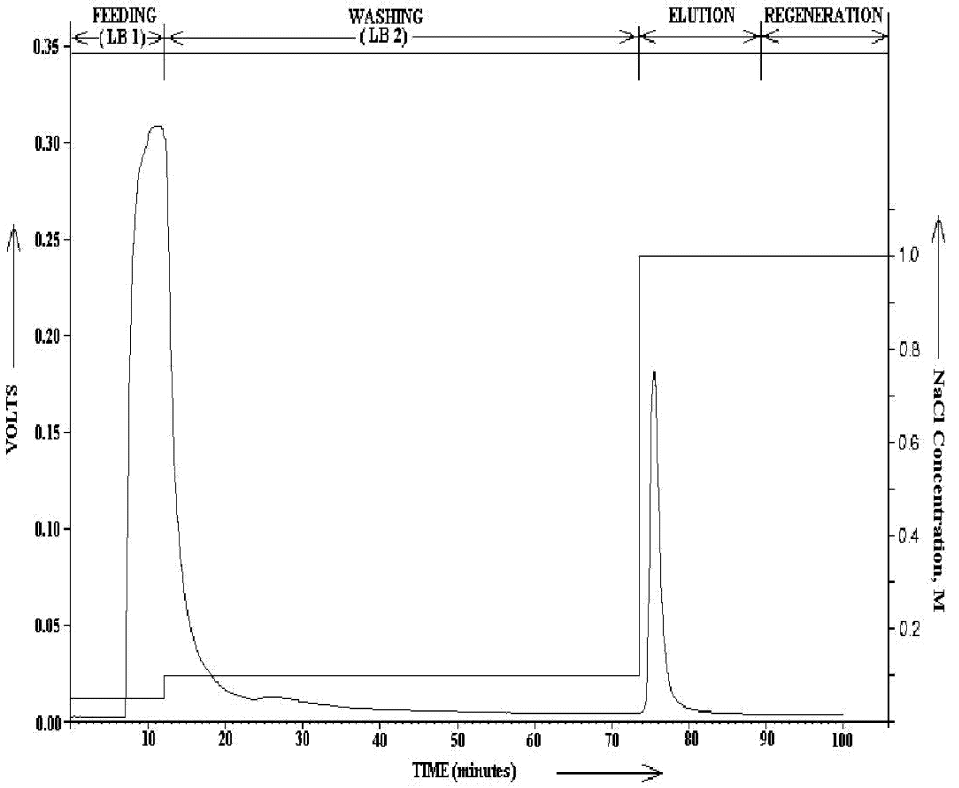

Fig. 4. Purification of Igs from diluted human serum on EDTPA modified zirconia (r_PEZ). Particles (25–38 μm) were packed in 50 mm×4.6 mm I.D. analytical columns. Loading buffer was 4 m*M* EDTPA, 20 m*M* MES, and 50 m*M* NaCl at pH 5.5 (adjusted with conc. NaOH); elution buffer was 4 m*M* EDTPA, 20 m*M* MES, and 500 m*M* NaCl at pH 5.5; linear velocity 4.82 cm/min; temperature 27°C; detection at 280 nm. Individual Ig content in the chromatographic fractions was determined by specific ELISAs.

To restore the column for the next run, it was then flushed with LB for 30 min prior to the next injection.

3.5. Immunoprotein yield from serum

Total percent recovery and total percent yield of human serum proteins on r_PEZ is shown in Table 1. The total protein concentration in different chromatographic fractions was obtained by measuring the absorbance at a wavelength of 280 nm. The total percent recovery was determined by taking a ratio of the total protein in all chromatographic fractions to the protein in the feed sample. Total protein recoveries over 100% were achieved for all runs. The total percent yield of Igs was determined by taking a ratio of the total immunoprotein in all elution fractions to the protein in the feed sample. Total hIgG yields of 29.48±6.3% were obtained for four independent runs. Total hIgA yields of 3.22±0.7% were obtained for four independent runs. Total hIgM yields of 6.84±0.7% were obtained for four independent runs.

3.6. Gel electrophoresis

Fig. 5 shows an SDS (0.1%)–polyacrylamide gel (4–12% gradient) electrophoresis of human serum (feed) and the chromatographic fractions from a typical run on r_PEZ, under non-reducing conditions. Lane 1 shows a molecular mass ladder. Lane 2 shows an application of pure hIgG. A total protein level of 3.2 μg was loaded to each standard lane. Lanes 3 and 4 show an application of hIgA and hIgM. Lane 5 shows an application of pure HSA. Lane 6 shows an application of crude human serum

Table 1
Summary of the total recovery and yields of Igs on r_PEZ

Run No.	Feed[c]	Flow through[c] (mg)	Eluate[c] (mg)	Yield (%)				Total protein recovery[c] (%)
				Total[b]	hIgG[a]	hIgA[a]	hIgM[a]	
1	3.40	3.05	0.74	36.39	26.94	2.55	6.90	111.47
2	3.87	3.45	1.14	41.14	37.67	2.73	–	118.60
3	3.50	2.91	0.90	40.69	30.57	3.96	6.16	108.86
4	3.91	4.14	0.76	33.83	22.73	3.64	7.46	125.31

r_PEZ beads were challenged with diluted human serum in 4 m*M* EDTPA, 20 m*M* MES, and 50 m*M* NaCl at pH 5.5 at a linear velocity of 4.82 cm/min. Bound IgG was eluted with 4 m*M* EDTPA, 20 m*M* MES, and 500 m*M* NaCl at pH 5.5. Four independent runs were performed. The total protein concentrations in column fractions were determined by $A_{280\ nm}$. Individual Ig (hIgG, hIgA and hIgM) contents were determined by ELISAs as described in the Materials and methods section. In general, an average of triplicate application of three different dilutions in ELISAs was used for yield calculations. Percent total recovery is defined as the ratio of the sum of the total protein in eluate and column fall-through fractions to the total protein present in the feed. Percent yield is defined as the ratio of the Ig present in the eluate fraction to the total amount of Ig in the feed. ELISA values were used to estimate the yields.

[a] Determined by ELISAs.

[b] The total yield is defined as the sum of individual immunoprotein yields.

[c] Determined spectrophotometrically by $A_{280\ nm}$.

used as column feed. The serum had two distinct protein bands corresponding to HSA with a molecular mass of 56 000 and hIgG with a molecular mass of 150 000. Some additional minor bands were also observed. Lane 7 shows a pooled flow though and subsequent washing fraction from a typical column run at a linear velocity of 4.82 cm/min. Lanes 8, 9 and 10 show the pooled elution fractions from the column runs at room temperature (~27°C) with the same linear velocity. The flow through fractions gave a band around M_r 56 000 similar to the pure HSA in Lane 5. It is observed that very little of HSA is present in the elution fractions. The eluate fractions, shown in lanes 8–10, gave a band around M_r 150 000 similar to pure hIgG. The purity of hIgG in the eluate fractions were estimated to be greater than 89% by digital image processing with HSA accounting for approximately 11% of the area in the fraction. Similar electrophoretic patterns were obtained with the fractions from duplicate column runs. Under reducing conditions the eluate fractions show three distinct bands around M_r 150 000, 162 000 and 900 000 which correspond to hIgG, hIgA and hIgM, respectively (gel not shown).

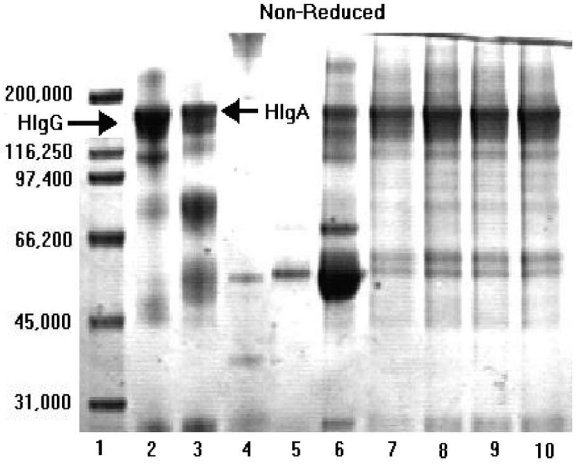

Fig. 5. Sodium dodecyl sulfate (0.1%)–polyacrylamide gel (4–12% gradient) electrophoresis of Igs that were purified from human serum using r_PEZ. Lanes: 1=molecular mass ladder; 2–4: pure samples of hIgG, hIgA and hIgM, respectively; 5=pure HSA, 6=the human serum feed; 7=the column fall through fraction; 8–10: the pooled elution fractions of different runs performed under identical conditions. Lanes 6–10 contain at least 7 to 10 μg of total protein.

4. Discussion

Zirconia spheres 28–35 μm in diameter were further end-capped with EDTPA to yield a pseudo-affinity support for use in bioseparations. 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 Waal interactions. The same forces probably play a role in pseudoaffinity systems, but their role and magnitude perhaps differ. It is desired to establish the type of interactions governing and prevalent in pseudoaffinity 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, 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.

Pseudoaffinity 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 Langmuir-type isotherm and is well fit to the simple Langmuir equation [41]. We hypothesize that at these temperatures, hIgG binds uniformly with a high affinity for the binding sites until it reaches a maximum binding energy. The static capacity of r_PEZ as determined by the Langmuir adsorption data was calculated to be 55–58 mg IgG per ml of beads, which is comparable to that reported for protein A-Sepharose and protein A-Ultragel. The dissociation constant, K_d, was determined to be $4.7\cdot 10^{-6}$ *M*, which indicates medium affinity and is typical for a pseudoaffinity ligand [41,42].

Our work with r_PEZ beads indicated that an effective isolation of immunoglobulins from human serum was attainable. This work may help develop pseudoaffinity matrices for use immunoadsorption columns. The chromatographic properties of r_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 pseudoaffinity interactions. In other words, the r_PEZ matrix is not specific for immunoglobulins as a protein A matrix would be, but the pseudoaffinity interactive forces confer a unique specificity for immunoglobulins over other serum proteins. This selectivity facilitated the use of a step gradient for the separation of Igs from a human serum sample.

5. Summary

We have successfully spray-dried colloidal zirconia to generate zirconia microspheres that can be used as a support in biochromatography. An analytical scale column packed with zirconia microspheres was employed to separate Igs from human serum. Our future efforts will focus on the optimization and scale-up of a purification strategy for human immunoglobulins from serum using zirconia microspheres. Studies are underway to estimate the parameters relevant for scale-up. In parallel studies, we are undertaking a detailed analysis of the protein content in the elution fractions. Future work will address the blood compatibility of r_PEZ. Such detailed studies are needed to achieve a sounder scientific basis for the utilization of r_PEZ in biomedical applications.

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