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3-22-2006

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Subramanian, Anuradha; Martinez, Blanca; Holm, Jill ; Carr, Peter W. ; and McNeff, Clayton V. , "A Comparative Study of Monoclonal Antibodies (Mabs) Purified from Cell Culture Supernatant on EDTPA-Modified Zirconia Beads and Protein A-Hyper D Support" (2006). *Papers in Biotechnology.* Paper 25.
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A Comparative Study of Monoclonal Antibodies (Mabs) Purified from Cell Culture Supernatant on EDTPA-Modified Zirconia Beads and Protein A-Hyper D Support

Abstract:

Colloidal zirconia was spray dried to yield zirconia particles, which were further modified with N, N, N0, N0- Ethylenediamine tetra methylenephosphonic acid (EDTPA) to yield a support for use in bioseparations. EDTPA modified zirconia particles will be further referred to as, r_PEZ. Cell culture supernatants rich in monoclonal antibody (Mab) subtypes IgG1, IgG2a, IgG2b, and IgG3 were chromatographed on a r_PEZ column, and on a protein A-hyper D column that was purchased commercially. All Mab subtypes bound to r_PEZ and process yields in the range of 88 to 99% were obtained. The purity of the Mab products were ascertained by gel lectrophoretic analysis and were estimated to be greater than 95%. The purified Mab products obtained from r_PEZ and protein A columns were compared to the reference Mab standard in biological and enzymatic assays. The value of the dissociation constant (Kd) was found to be comparable and was in the range to that obtained with reference Mab standard (0.231+0.03 M). In addition, Mabs purified with r_PEZ had the same deglycosylation profile as the reference Mab standard. Thus, it appears that the r_PEZ purified Mab is similar in activity to Mab purified with a protein A support and in addition, the zirconia surface does not adversely impact the activity of the purified Mab.

INTRODUCTION

The importance of immunoglobulins (Ig's) 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 medi-

cal treatment in patients with inadequate Ig levels.[8] As the discovery of new medical and diagnostic uses for immunoglobulins arise, an increased need for medicalgrade Ig's 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 Ig's 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] Protein A affinity chromatography is a technique that has long been used for IgG isolation[13] and recently, protein G with a broader range of specificity has also been used in IgG isolation. The usage of both protein A and protein G in affinity chromatography is negatively impacted by the harsh elution conditions necessary for the reverse binding reaction to take place. In addition, the use of both protein A and protein G as affinity supports in chromatographic columns poses special challenges regarding regeneration and sanitation.[14] Also, a negative consequence of using this method is the leakage of immobilized protein A and protein G (whole protein and fragments) that results in the contamination of the eluted immunoglobulins.[15] This is detrimental because protein A has been linked with toxicity in both animals and humans.[16] Although protein A mimetic ligands have shown promise, ligand leakage and column regeneration are still sources of potential shortcomings. Although protein A affinity chromatography is a popular step in protein purifications, the need for less expensive, more stable alternatives to this technique has prompted many researchers to turn their attentions to the development of synthetic ligands. The techniques developed using pseudo- A. bioaffinity ligands have proven to avoid the risks associated with toxicities in elution fractions due to ligand leakage. Additionally, these ligands typically provide for a mild elution environment, thereby retaining immunological properties of the protein, have broader immunoglobulin specificity, and are more stable in chromatographic systems in comparison to ligands such as protein A and G.[17] The use of histidyl

and thiophilic ligands are limited due to their lack of specificity and selectivity. Metal chelate interaction chromatography using Cuþþ-chelate gel has been studied for the separation of Ig from blood serum and plasma.[18,19] However, concern of metal ligand leakage in the eluant presents a drawback. Additionally, the strength of bonding of the metal ion to the support, so that residual affinity for proteins remains, is often difficult to achieve.[20] 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.[28] The development of EDTPA modified zirconia as a stationary phase material is an example where both aspects may be achieved. Studies have established the usefulness of r_PEZ as a chromatographic stationary phase[21 – 27] in HPLC applications and in the efficient purification of antibodies.[29] The aim of this study is to compare the activities of antibody products purified on r_PEZ and protein A_hyper D support. The impact, if any, of the zirconiabased stationary phase on the activities of antibodies will be evaluated.

EXPERIMENTAL

Reagents

All chemicals were of analytical grade or better. Sodium chloride was purchased from Fischer Scientific (Hanover Park, IL, USA). N, N, N0, N0- Ethylenediamine tetra methylenephosphonic acid (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 ELISA, and human serum albumin (HSA) were obtained from Sigma

Chemical Company (St. Louis, MO, USA). All proteins were used without further purification. Cell culture supernatant rich in Mab subclasses IgG1, IgG2a, IgG2b, and IgG3 was purchased from SOBIO (Southern Biotechnology Institute, Birmingham, AL). Pure Mab standards (subclasses) were also purchased from SOBIO. Rabbit anti-human Protein C (P-4680), goat anti-mouse IgG (A-4416), and calcium chloride (CaCl2) were purchased from Sigma (St. Louis, MO). Purified human protein C (hPC-239F) was purchased from American A Comparative Study of Monoclonal Antibodies 473 Diagnostica Inc. (Greenwich, CT). O-Phenylenediamine-2HCl (OPD) tablets were purchased from Abbot Laboratories (Chicago, IL). Reference Mab was provided as a generous gift by Dr. William Velander (Virginia Tech, Blacksburg, VA). PNGase F Kit was obtained from New England BioLab Inc. (Beverly, MA). NuPage 4–12% Bis-Tris gels were purchased from Invitrogen (Carlsbad, CA). Gelcode for staining was obtained from Pierce (Rockford, IL). 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 immunoglobulins conjugated to horseradish peroxidase (HRP) were purchased from Sigma Chemical Company (St. Louis, MO, USA). A GenesysTM 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. Chromatography. EDTPA modified particles were packed in 20 cm _ 1.0 cm (i.d.) cm and provided by Zirchrom, Inc. The chromatographic system used was a HPLC system using a Spectroflow 783 detector at 280 nm and an isocratic pump from Chromtech (Apple Valley, MN). For separation and isolation of IgG1,2a,2b,3 from the cell culture supernatant, a protocol similar to that described elsewhere was adopted.[27] In a separate experiment, 2.0mL protein A immobilized on hyper D support was packed into a Pharmacia column (1.0 cm _ 10.0 cm) and cell culture supernatants were chromatographed according to the manufacturer's instructions. In a typical appli-

cation, 1mL of supernatant was diluted with 1.0mL of 0.5M sodium citrate buffer at pH 8.4 (LB1). Feed was filtered using a Millipore 0.45 microns membrane filter and loaded on to the column at a linear velocity of 1.25 cm/min. Loosely bound proteins were washed with LB1. The elution of the bound Mab was effected with 0.5% acetic acid. The pH of the elution fraction was immediately raised to 7.0 with 1M Tris-base. All chromatographic fractions were saved and analyzed for total protein content at OD 280 nm, Mab content by specific ELISA assays and electrophoretic analysis. SDS-PAGE Analysis and Western Blot SDS-PAGE analysis under non-reducing and reducing conditions were performed using Novex electrophoresis system. Gels were stained with Gelcode to visualize the protein bands.[30] Western blots were performed with nitrocellulose membranes using standard procedures and DAB substrate was used to visualize the bands. Enzymatic Deglycosyation of Mab Standard and purified Mab of 100 mL from r_PEZ column and protein A-hyper D at 50mg/mL were denatured with 5mL of 10X denaturing buffer for 5 min at 958C. Upon cooling to room temperature, 6mL of 10X G7 buffer and 6 mL of 10% NP-40 was added. Of the enzyme, 3mL were added to each reaction tube and incubated for 1 hr at 378C. The deglycosylated proteins were analyzed by western blotting. Estimation of Kd Costar microtiter plates were coated with 100 mL per well of 0.5 mg/mL of pure hPC for 24 hr at 48C. Plates were washed three to four times with TBS/0.2% Tween-20 buffer and blocked with 200 uL of TBS/0.1% BSA buffer for 60 min at room temperature. Mabs (Mab purified with r-PEZ, Mab purified with protein A, Mab provided by Dr. Subramanian) at 40 and 20 ng/mL were incubated with pure hPC at various Ag/Ab ratios (40 to 0.078 mol/mol) in TBS/0.1% BSA-25mM EDTA buffer for 24 hr at 48C. Triplicate application of 100 mL of Mab:hPC dilutions were pipetted into the coated plates. The plates were incubated for 8 minutes at RT. After washing the plates three times with TBS/Tween buffer, the complex was detected by addition of 1:1000th diluted goat anti-mouse HRP conjugate (A-4416) and in-

cubated for 1 hr at 378C. The bound chromophorei was detected with OPD, and the reaction was stopped with 3N H2SO4. Absorbance was measured at 490 nm.

RESULTS

Expression Level and Purification Yields Experiments to characterize the performance of r_PEZ were performed with commercially available, pure, Mab subclasses. In order to identify the elution and retention profiles pure Mab were chromatographed on the EDTPA modified zirconia column. As judged by the chromatographic trace at 280 nm, most of the Mab was retained on the matrix and was eluted at five column volumes. A model solution, which mimics the target stream from which Mabs would be isolated (i.e., a cell culture supernatant), was produced by mixing BSA and Mab to gauge the ability of the modified zirconia phase to efficiently separate the Mab from other contaminating proteins present in the cell culture supernatant. No retention of either BSA on the matrix was observed, as judged by a large peak eluting at the dead volume of the column (data not shown). The Mab was well retained on the EDTPA modified zirconia column and eluted at 5 column volumes. A Comparative Study of Monoclonal Antibodies. Chromatographic profiles with similar characteristics to one described elsewhere[27] were obtained for the isolation of Mab from the cell culture supernatant and a representative profile is shown in Figure 1, panel B. A representative chromatographic profile for the isolation of Mab from cell culture supernatant on commercially purchased protein-A column is shown in Figure 1, panel A. The profile of the elution peak on a r_PEZ column was sharp and was approximately between 4 to 6 column volumes. In our experience, the elution peak on an immobilized protein-A column was asymmetric with shoulders and tailing. No visible peaks were observed in the regeneration step in either of the columns tested. Table 1 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 assays. 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 1 had Mab yields (based on ELISA) of in the range of 60% to 93%, while the protein A-hyper D column runs had Mab yields of in the range of 73% to 100%, respectively, with little or no detectable Mab in column fall through and wash fractions.

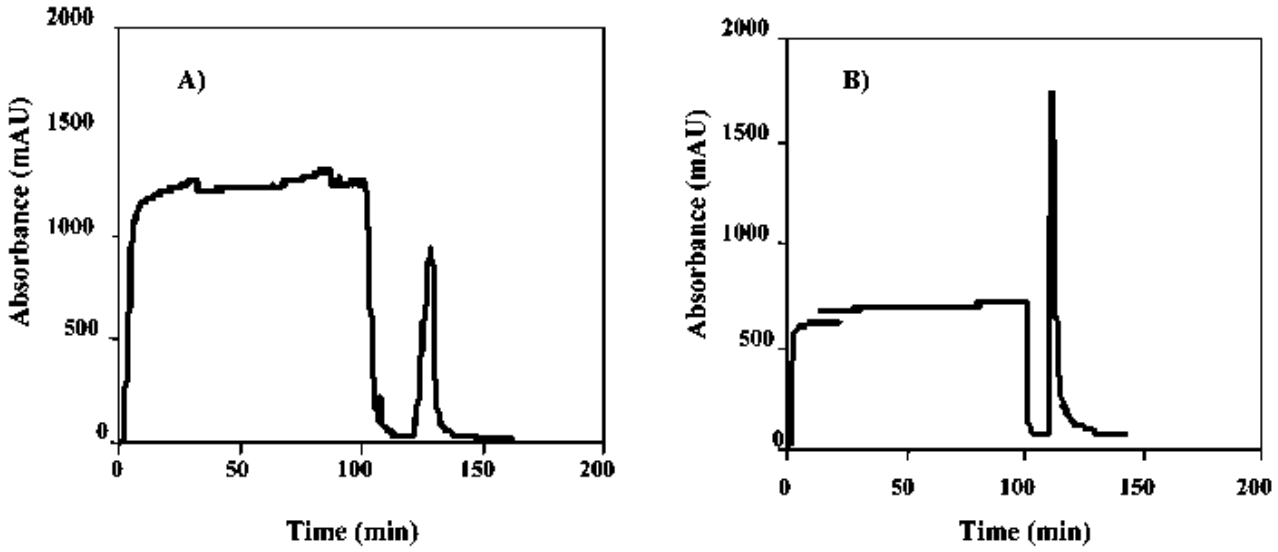

Figure 1. Separation of Mab on various chromatography columns. (A) Protein A on hyper-D and (B) EDTPA modified zirconia. EDTPA modified particles were packed in 15 cm _ 1.0 cm (i.d.) cm and provided by Zirchrom, Inc. The chromatographic system used was a HPLC system using a spectroflow 783 detector at 280 nm and an isocratic pump from Chromtech (Apple Valley, MN). Diluted Mab-rich supernatant was loaded dynamically, loosely bound proteins were washed, and bound Mab was eluted with 1M NaCl. All chromatographic operations were preformed at a linear flow rate of 6.37mL/min. Protein A immobilized on hyper D support was packed into a Pharmacia according to the manufacturer's instructions. Loosely bound proteins were washed with loading buffer and the bound Mab was eluted with 0.5% acetic acid. All chromatographic fractions were saved and analyzed for total protein content at OD 280 nm, Mab content by specific ELISA assays and electrophoretic analysis.

Table 1. Summary of chromatographic runs

r_PEZ column		Based on OD 280 nm		Based on ELISA	
Sample		% TR	% yield	% TR	% yield
Sup'nt-IgG1	Average of 17 runs	92.1	15.6	72.2	60.1
Sup'nt-IgG2a	Run 1	88.3	16.7	88.3	88.3
	Run 2	100.3	17.0	93.2	93.2
Sup'nt-IgG2b	Run 1	92.5	11.3	79.5	79.5
	Run 2	95.5	10.6	65.9	65.9
Sup'nt-IgG3	Run 1	93.3	8.3	72.7	72.7
	Run 2	98.7	12.0	81.8	81.8
Protein A-hyper D		Based on OD 280 nm		Based on ELISA	
Sup'nt-IgG1 Average of 7-runs		93.9%	21.7%	80.1%	72.9%
Sup'nt-IgG2a		84.1	18.3	90.2	90.2
Sup'nt-IgG2b		83.0	15.7	104.0	104.0
Sup'nt-IgG3		89.8	15.9	89.7	89.7

% Yield The % yield of the Mab was determined as a ratio of the total Mab in the eluate fraction (fraction 2) to the total Mab present in the feed.

% TR The % total recovery of the Mab was determined as a ratio of the total Mab present in chromatographic fractions (fall-through, wash and elution) to the total Mab present in the feed.

Figure 2 shows SDS-PAGE gel of the purified fractions from a typical chromatographic separation. Chromatographic fractions from runs in Table 1 were selected for electrophoretic analysis. Lane 1 shows a molecular weight ladder. Lanes 2 and 3 show applications of pure Mab and BSA at a total protein level of 2 mgs, respectively. Lanes 4 show a representative sample the feed cell culture supernatant. Lanes 5 and 6 show an application of IgG1-Mab purified with r_PEZ column, at a total protein level of 3 mgs. Lanes 7 and 8 show an application of Mab purified with protein-A column, at a total protein level of 3mgs. The eluate fraction from both protein A hyper D column and r_PEZ gave a band around 150 kDa similar to the pure Mab in Lane 3. In addition to the major Mab band at 150 kDa, a minor band at 56 kDa accounting for less than 2% of the area obtained by digital image processing was observed. Lane 9 and 10 show an application of column fall through at a total protein level of 3 mgs. The purity of the Mab in the eluate fraction (Lanes 4 and 5) is estimated to be greater than 98%, respec-

tively, by digital image processing. Similar electrophoretic patterns were obtained with the fractions from other runs listed in Table 1 (data not shown).

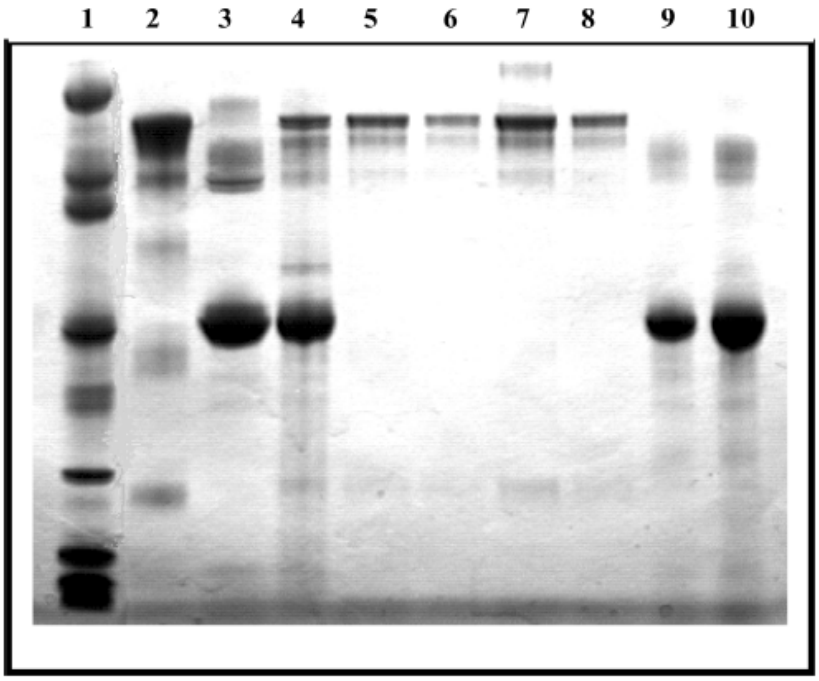

Figure 2. Sodium dodecylsulfate (0.1%)-polyacrylamide gel (4–12% gradient) electrophoresis of Mab's purified using r-PEZ and protein A-HD. Lane 1 shows a molecular weight ladder. Lanes 2 and 3 show applications of pure Mab and BSA. Lanes 4 shows a representative sample of the feed cell culture supernatant. Lanes 5 and 6 show an application of IgG1-Mab purified with r_PEZ column. Lanes 7 and 8 show an application of Mab purified with protein-A column. Lane 9 and 10 show an application of column fall through.

Figure 3 shows a SDS-PAGE gel of the starting cell culture supernatant (feed) and the purified fractions from a typical chromatographic separation on a r_PEZ column, under denaturing (reduced) conditions. Chromatographic fractions from Table 1 were selected for electrophoretic analysis. Lane 1 shows a molecular weight ladder. Lanes 2 and 3 show application of pure BSA and Mab at a total protein level of 5mgs, respectively. Lanes 4 to 7 show an application of the elution fraction from cell culture supernatants rich in IgG1, IgG2a, IgG2b, and IgG3, respectively, at a total protein level of 7mgs. Lane 9 shows the fall through fraction from a typical run at a total protein level of 10mgs. The fall through fraction gave a band around 56kDa similar to the pure BSA in Lane 2. Lane 10 shows the feed fraction to the column for a typical

run, at a total protein level of 10mgs. The eluate fraction gave two bands around 55 and 24kDa, representative of the heavy and the light chain of Mab.

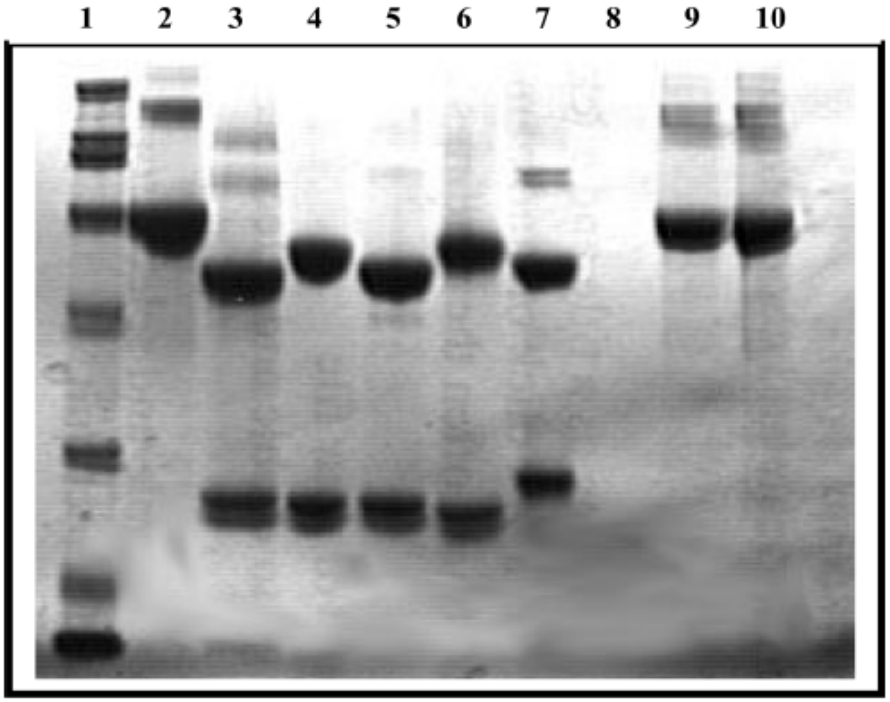

Figure 3. SDS-PAGE of purified fractions from a typical chromatographic sepa on a r_PEZ column, under denaturing (reduced) conditions. Lane 1 shows a mol weight ladder. Lanes 2 and 3 show application of pure BSA and Mab. Lanes 4 to 7 an application of the elution fraction from cell culture supernatants rich in IgG1, I IgG2b, and IgG3. Lane 9 shows the fall through fraction from a typical run. La shows the feed fraction to the column for a typical run.

As expected, the heavy chains of Mab subspecies have different mobility as evidenced by their migration patterns, and this arises from the difference in the glycosyation levels. The migration pattern was compared to that of pure Mab standards and found to be identical (data not shown). Lane 9 shows a representative fall through fraction at a total protein level of 3mgs. The fall through fraction gave a band around 56kDa similar to the pure BSA in Lane 2. Figure 4 shows the electrophoretic mobility of Mabs purified using different matrices upon treatment with N-glycosidase F. Lane 1, 3, and 5 Figure 2. Sodium dodecylsulfate (0.1%)-polyacrylamide gel (4–12% gradient) electrophoresis of Mab's purified using r-PEZ and protein A-HD. Lane 1 shows a molecular weight ladder. Lanes 2 and 3 show applications of pure Mab and BSA.

Lanes 4 shows a representative sample of the feed cell culture supernatant. Lanes 5 and 6 show an application of IgG1-Mab purified with r_PEZ column. Lanes 7 and 8 show an application of Mab purified with protein-A column. Lane 9 and 10 show an application of column fall through show a 100 ng application of Mab purified from r_PEZ, protein A-hyper D, and Mab-reference standard, respectively. Lane 2, 4, and 6 show deglycosylated Mab and the apparent faster mobility of the heavy chain due to removal of N-linked sugars is readily visible. No shift in the apparent mobility of the light chain of the Mab was observed, which is consistent with the absence of glycosylation of the light chain. Upon deglycosylation, heavy chains of all Mab samples had a similar shift in mobility.

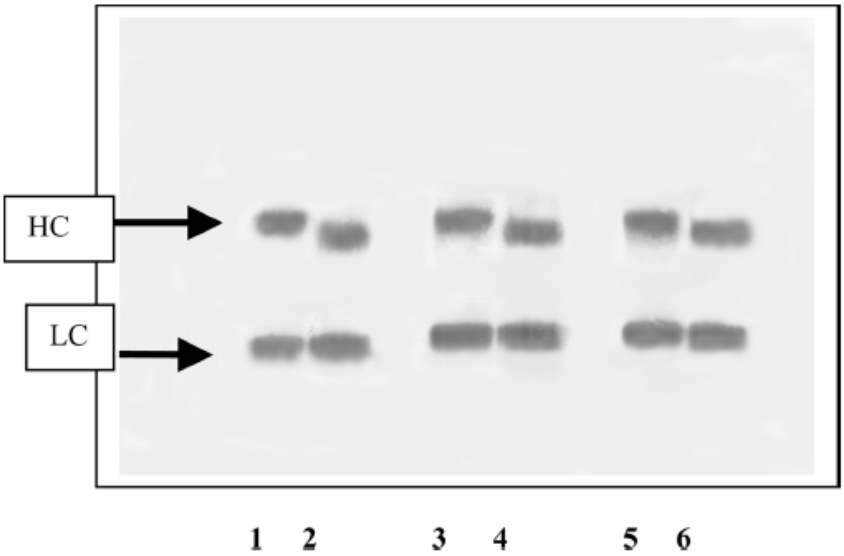

Figure 4. Electrophoretic mobility of Mabs purified using different matrices upon treatment with N-glycosidase F. Lane 1, 3, and 5 show a 100 ng application of Mab purified from r_PEZ, protein A-hyper D, and Mab-reference standard, respectively. Lane 2, 4, and 6 show deglycosylated Mab.

We have used the ELISA based method as described in detail elsewhere[35] to determine the binding kinetics and Kd. The method used in this study is based on the detection of the free Mab in the reaction mixture by an indirect ELISA procedure, after the Mab and the antigen reach equilibrium in solution. The underlying assumption is that the interaction between unbound Mab and the immobilized antigen (hPC in this case) does not differ significantly from the equilibrium attained between both in solution. Table 2 shows the value of the disassociation constant (Kd) obtained for Mabs purified with r-PEZ and protein A-HD, and a reference Mab sample. Previous research has documented and characterized the affinity of the Mab used in this study, and hPC the parent antigen.[31] Disassociation constant (Kd) values of 0.236+0.05 nM and 0.262+0.03nM were obtained for Mab purified using r_PEZ column and a protein-A hyper D column, respectively. A Kd value of 0.262+0.03 nM was obtained for Mab reference standard. Comparable values of Kd suggest that the Mab purified via the r_PEZ column has similar activity to Mab reference standard, and the antigenic property for the Mab was not adversely impacted either by the zirconia matrix itself or the reagents and buffer employed in the chromatographic separation.

DISCUSSION

The popularity of silica as a chromatographic support results from the cumulative impact of its mechanical strength, availability in a wide range of pore sizes, nearly ideal pore structure, and versatility of the silanization chemistry. However, the dissolution of silica in aqueous mobile phases is widely recognized and documented, and is also prone to acid-catalyzed hydrolysis of the siloxane bonds below pH 2 and dissolution above pH of 8.0 by alkali attack.[32 – 34] Thus, the preparation of alternative stationary phase supports is an important area that aims to develop new support materials that offer novel selectivities, or to overcome the shortcomings of

silica, alumina, and polymeric supports. The development of zirconia as a stationary phase material is an example where both aspects may be achieved.

Table 2. Disassociation constant (K_d) obtained for Mabs purified with r-PEZ and protein A-HD and, a reference Mab sample

Sample	K_d, nM
Mab purified on r_PEZ	0.236 ± 0.05
Mab purified on Protein A-hyper D	0.301 ± 0.01
Mab reference standard	0.262 ± 0.03

Zirconia surface has a unique surface chemistry and offers many possibilities for use in chromatography; it can be used as a bare support, it can be dynamically modified (i.e., phosphates and EDTPA), it may be coated with polymers or with inorganic layers. Additionally, its high density and excellent thermal and chemical stability provide several advantages over traditional silica or polymeric supports. Zirconia particles are extremely stable in both acidic and basic solutions.[23] The surface of bare zirconia is populated with 4 to 5 millimoles per square millimeter (mmoles/mm2) of Lewis acid sites, which are strong electrophiles and promote irreversible adsorption.[21,23] Therefore, surface modification of zirconia to titrate or neutralize the Lewis acid sites is essential before zirconia surface can be rendered useful as a support for chromatographic applications. Unless these electrophilic sites are charge titrated, zirconia particles undergo non-reversible protein adsorption and exhibit severe tailing. 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. EDTPA is a special chelator that renders zirconia Lewis acid deactivated (Figure 5). To overcome these effects, we have modified the zirconia surface with EDTPA and we have ensured that the buffers employed also contain 4mM EDTPA. Since EDTPA coating is adsorbed and not chemically

bonded, we sought to evaluate the stability of EDTPA-modified zirconia surface under a wide variety of pH and buffer conditions. Our results indicate that EDTP. A modified zirconia is stable from pH 1 to pH 10, and the EDTPA does not desorb from the surface. In these experiments, EDTPA-zirconia columns were flushed with 7000 column volumes of a pH 10 buffer and a pH 1 buffer, respectively, as separate experiments. Test solutes were periodically injected and the retention, selectivity, and efficiency were monitored. The selectivities and efficiencies of test solutes were nearly the same before and after the high pH and low pH exposure (Figure 6). Retention values of test solutes decreased by less than 3% to 4%. Thus, it is surprisingly stable.

The underlying rationale for this study is that a fundamental understanding of the nature of interactions between EDTPA modified zirconia and IgG, and of the impact of the chemistry and property of the base matrix on the biological properties of the purified proteins, will enable the development of robust and scalable alternate sorbents for use in preparative bioseparations. The underlying hypothesis was that zirconia being a charge-dense material Figure 5. Schematic of zirconia surface and N, N, N0, N0- Ethylenediamine tetra methylenephosphonic acid (EDTPA) may cause protein to denature upon retention. Proteins retained on immobilized metal affinity chromatographic supports (IMAC) have been shown to loose biological activity and, in some cases, have been denatured.

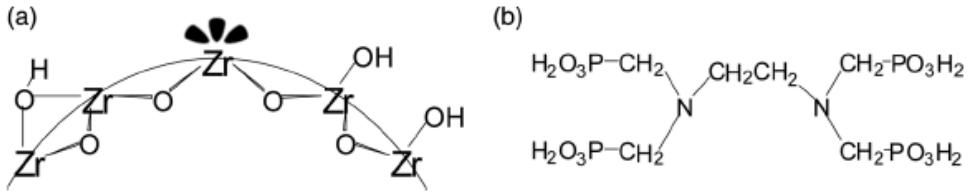

Figure 5. Schematic of zirconia surface and N, N, N′, N′- Ethylenediamine tetra methylenephosphonic acid (EDTPA).

In the absence of a spacer arm, it is conceivable that the retained Mab is in close proximity to the zirconia surface and subject to charge-induced denaturations. Hence, the activity of Mab products obtained from separation on a r_PEZ column and protein A-hyper D column were assayed for biological activity. We have used deglycosylation profile and the value of the Kd as identifiable markers. Denaturation of the Mab has shown to manifest itself in the loss of antigenicity, leading to an altered value of Kd. Our study has shown that these parameter are unaffected by either the zirconia matrix itself or the process conditions used in separation. In conclusion, our present study has shown that the zirconia surface has no negative impact on the properties of purified Mab. In conjunction with our previous studies that have been reported elsewhere, EDTPA modified zirconia columns can now be used in the preparative scale separations of immunoglobulins. In addition, other chemistries could be exploited to produce phases with different retention mechanisms. Once EDTPA has been adsorbed, it may be possible to produce phases exhibiting different selectivities. These postderivatization procedures could include cross linking with diiodoalkanes to increase both phase stability and alter the selectivity of the support.

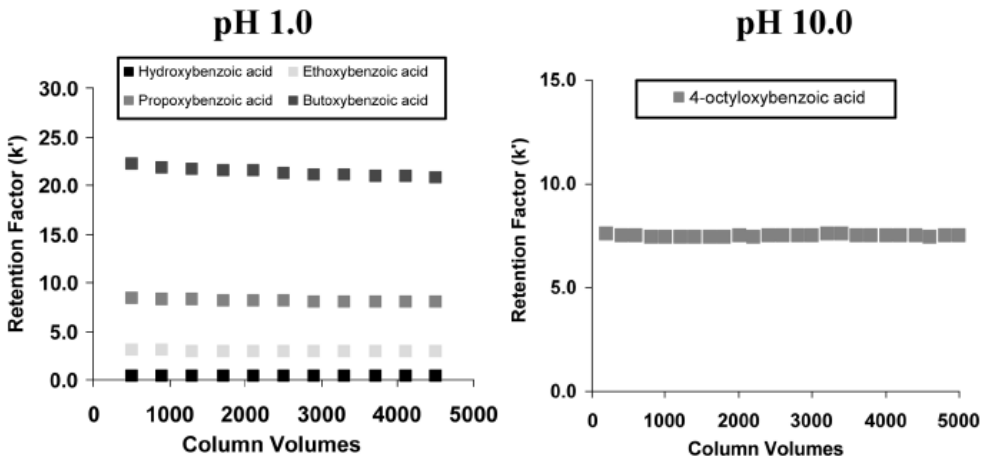

Figure 6. Exposure and evaluation conditions: Mobile phase, 15/85 acetonitrile/ 0.1 M Nitric acid, pH 1.0, or 0.1 M Ammonium hydroxide, pH 10.0; Flow rate, 1.0 mL/min.; Temperature, 30°C; Injection volume, 5 μL; Detection at 254 nm; Column, 50 mm × 4.6 mm i.d. rPEZ.

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