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Purification of monoclonal antibodies from cell culture supernatants using a modified zirconia based cation-exchange support

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Abstract

A method suitable for the isolation of monoclonal antibodies (Mabs) is described. The protocol utilizes a zirconia based column modified with ethylenediamine-*N*,*N*′-tetra(methylenephosphonic) acid to create a novel cation-exchange chromatographic support. Initial experiments using a linear salt gradient demonstrate the ability of this support to efficiently separate Mab from transferrin and bovine serum albumin in a model matrix. Results of the purification of Mab from an actual cell culture supernatant over a range in protein concentrations are also shown. Analyses by enzyme-linked immunosorbent assay and gel electrophoresis demonstrate that Mabs can be recovered from a cell culture supernatant at high yield (92–98%) and high purity (>95%) in a single chromatographic step.

Keywords: Zirconia; Monoclonal antibodies

1. Introduction

1.1. Monoclonal antibody purification

Monoclonal antibodies (Mabs) which are uniform in structure and function exhibit unique specificity and selectivity for the antigen which elicited its synthesis [1,2]. The unique specificity that a monoclonal antibody displays for an antigen makes it an invaluable tool in diagnostics [3,4], as probes for fine structural analysis [5,6], in histological examinations [7], in immunoaffinity chromatography [8,9], and in immunotherapy [10]. Some of these applications require Mabs in ample quantities and in a highly purified form. Traditionally, Mabs have been produced in ascites fluid [11], but recent advances in hybridoma technology have enabled the large scale production of Mabs in mammalian bioreactor systems [8,11].

Purification schemes for Mabs from the cell culture supernatant matrix include precipitation with ammonium sulfate, ion-exchange chromatography [12], thiophilic chromatography [13], and affinity separations using immobilized protein-A [5,14,15]. Ammonium sulfate is one of the oldest and most widely used methods on the laboratory scale to preferentially precipitate Mabs from serum proteins. The method is gentle, effective, and simple, yet the product yields are low. In addition, this method is not amenable to scale-up or continuous operation, and Mabs cannot be purified to homogeneity (>95% purity).

Another commonly used method of Mab purification is an affinity technique using immobilized protein-A. This method usually results in a product with high purity and in good recovery, but the varying avidity of protein-A for IgG from different

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species and the possible contamination of the Mab product with leached protein-A makes it less attractive for preparative applications. Moreover, the low pH (pH ~3.0) often used to elute the bound Mabs from a protein-A column can induce denaturation and loss of biological activity. In addition, selection of operational parameters that yield minimal bacterial contamination and removal of non-specifically adsorbed proteins without destroying the biological activity of immobilized protein-A are often tedious.

Ion-exchange chromatography has been used as a pre-purification step prior to protein-A chromatography or a cascade of ion-exchange steps and gel filtration or a combination thereof have been also used to purify Mabs.

The presence of high concentrations of serum albumin in cell culture supernatants presents additional challenges as the contaminating proteins tend to co-purify with Mabs. For example, Mab purified using a hydrophobic mechanism (i.e. hydroxylapatite, octylsepharose) contained a substantial amount of albumin and transferrin as contaminants. This was also observed with procedures involving thiophilic adsorption [13] or DEAE-Affi Gel Blue chromatography [16]. It appears that the high ratio of albumin to Mab in cell culture supernatants when compared to ascites fluid poses additional challenges in Mab purification.

Thus there is a need for the development of new matrix technologies or purification protocols which are amenable to scale up without presenting excessive operational complexities. A novel ion-exchange matrix based on microparticulate zirconia has been developed and characterized as a sorbent for use in bioseparations. 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.

1.2. Surface modification of porous microparticulate zirconia

Surface modification of zirconia prior to use as a chromatographic stationary support is necessary to create a phase which exhibits a useful selectivity for the desired separation or purification [17–23]. It is also essential to block direct chemical interactions between solutes and the native surface [22,24–27]. It is well known that zirconia's surface is populated by Lewis acid sites at a density of 1–4 $\mu mol/m^2$ [28–30]. These Lewis acid sites are the result of a zirconium(IV) atom exposed at a surface. When the coordination bonds of the zirconium(IV) atom have not been fully satisfied, it is co-ordinately unsaturated making it a strong electrophile. This is the cause of the Lewis acidity encountered with zirconia particles. These Lewis acid sites are termed 'hard' according to Pearson's scale [31].

The zirconium(IV) atom that exhibits Lewis acidity on the particle surface can form strong nonlabile bonds with electron donors that have a high charge to mass ratio or unpolarizable electrons [24,32]. Such species, termed hard Lewis bases, include carboxylates. Therefore, carboxylates (as well as any hard Lewis base moiety) present on a protein's surface can interact strongly with Lewis acid sites on unmodified zirconia. This protein–surface interaction leads to very tailed elution bands or irreversible adsorption of proteins on zirconia based columns. Both effects are detrimental in supports used for protein purifications [26].

While carboxylate species can interact with native zirconia through ligand-exchange interactions, other types of hard Lewis bases can also form strong bonds to the Lewis acid sites [32–34]. These interactions are the basis of the modification employed to create the cation-exchange phase used in this study. Aggressive modification of the zirconia particles with a phosphonate analog of EDTA, ethylenediamine-*N*,*N*′-tetra(methylenephosphonic) acid (EDTPA), is a very effective method of blocking ligand-exchange interactions between solutes and Lewis acid sites.

Here we seek to investigate the use of this new porous zirconia based adsorbent for the efficient purification of monoclonal antibodies from cell culture supernatants. The aim of this study is to show the utility of this phase in the separation and purification of antibodies from a cell culture supernatant. A single chromatographic step is sufficient to obtain highly purified antibodies with high re-

coveries. The process yields and purity of the Mab will be demonstrated.

2. Methods

2.1. Reagents

All chemicals were of analytical-grade or better. EDTPA was obtained from TCI America (Portland, OR, USA), 2-(*N*-morpholino)ethanesulfonic acid (MES) (4432-31-9) from Sigma (St. Louis, MO, USA), and 50% (w/w) sodium hydroxide solution from Fisher Scientific (Fair Lawn, NJ, USA). Sodium chloride (7647-14-5) was purchased from EM Science (Gibbstown, NJ, USA). HPLC grade 2-propanol (67-63-0) was obtained from Mallinckrodt (Paris, KT, USA). Bovine serum albumin (BSA) and transferrin where obtained from Sigma. All proteins were used without further purification. Purified murine antibody and cell culture supernatant containing Mab were generously provided by Dr. W.H. Velander (Virginia Polytechnic Institute and State University, Blacksburg, VA, USA).

Immulon II microtiter plates were purchased from Fisher Scientific (Pittsburgh, PA, USA). 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). Pre-cast 8–16% gradient polyacrlyamide gels were purchased from Novex (San Diego, CA, USA).

The water used in these studies was purified using a Barnsted Nanopure deionizing system with an organic free cartridge and a 0.2-μm final filter. All water was boiled and cooled prior to use in order to remove dissolved carbon dioxide.

2.2. Matrix preparation

Microparticulate zirconia batch COAC-15 was synthesized according to a process devised by Iler [35] and modified by Sun et al. [36]. All particles were chemically washed according to a procedure described in reference [37] prior to further surface modification to ensure removal of particle synthesis residuals. The particles are highly spherical (6-μm average diameter by scanning electron microscopy, SEM) with an average pore diameter of 220 Å (4 V/A estimation) and a BET surface area of 29 m^2/g as measured by the nitrogen adsorption isotherm at 77 K using a Micromeritics ASAP 2000 sorptometer.

2.3. Surface modification procedure

Surface modification of zirconia with EDTPA was developed based on a method described in an earlier paper and presented elsewhere [38]. Briefly, the procedure involves refluxing particles in a 0.1 *M* solution of EDTPA for 4 h, collecting the particles by filtration, washing, and drying prior to packing in a chromatographic column.

2.4. Chromatography

EDTPA modified particles were packed in 316 stainless-steel column blanks (Alltech, Deerfield, IL, USA), 5 cm×4.6 I.D. mm with 2-μm stainless-steel screens (Chrom Tech, Apple Valley, MN, USA) instead of stainless steel frits to minimize protein loss due to adsorption to the end fittings. The particles were slurried in 2-propanol and thoroughly degassed by sonication under vacuum for 10 min. Particles were packed by the stirred upward slurry method at 5000 p.s.i. (1 p.s.i.=6894.76 Pa). The chromatographic system used was an HP 1090L liquid chromatograph with diode array detection and Chemstation data handling system. Photometric detection was performed at 280 nm with a diode array detector controlled by the Chemstation software. Two gradient conditions were used. The first was used on a model cell culture supernatant which was produced by mixing solutions of commercially purchased proteins. This first gradient allowed us to assess the best and most efficient gradient for the isolation of Mab from a cell culture supernatant. The buffer system used for chromatography of a model cell culture supernatant consisted of 4 m*M* EDTPA, 20 m*M* MES, adjusted to pH 5.5 (with 50% solution of sodium hydroxide). A linear gradient from 0 to 1 *M* sodium chloride in 30 min was employed. The column was regenerated with the initial buffer for fifteen min prior to the next injection.

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. A step gradient to 0.5 *M* NaCl at 8 min was used to affect elution of bound Mab. Finally, a step to 1 *M* NaCl at 14 min was used to ensure elution of proteins bound by nonspecific interactions. The column was regenerated with loading buffer for 15 min prior to the next injection (flow-rate 1 ml/min).

All buffer solutions were filtered using Millipore (type HA) 0.45-μm membrane filters prior to use. All proteins including lyophilized cell culture supernatant samples were dissolved in the mobile phase.

2.5. Determination of Mab

Immulon II microtiter plates were coated with 100 μl per well of 1:200 diluted anti-mouse whole molecule in 0.1 *M* sodium hydrogencarbonate (pH 9.3) for 24 h at 4°C. Wells were washed with 0.05 *M* Tris–0.1 *M* sodium chloride–0.05% Tween (TBS-Tween) and the residual reactive sites were blocked with TBS–0.1% bovine serum albumin (BSA) for 20 min at room temperature. Various dilutions of standard and samples in TBS–0.1% BSA were added to the wells, 100 μl in each well and incubated for 20 min at 37°C. Upon incubation wells were washed four times and 1:1000 diluted HRP conjugated goat anti-mouse IgG was added to the wells and incubated for 20 min at 37°C. Wells were washed four times and 100 μl of OPD substrate was added to each well. The colorometric reaction was stopped after 3 min by the addition of 100 μl of 1.5 *M* sulfuric acid to each well. Bound chromophore was detected at 490 nm using an EL800 Bio-Tek Microplate reader. The amount of antibody immobilized on the support matrix was evaluated by subtracting the amount of Mab in the coupling step supernatant, blocking step supernatant, and 0.5 *M* sodium chloride wash step from the total Mab input.

2.6. Gel electrophoresis

The purity of the recovered Mab was analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under native and denaturing conditions. In brief, protein samples (pure Mab and fractions) were dialyzed using 10 000 molecular mass cut off Amicon spin filters and resuspended in TE buffer (10 m*M* Tris, 25 m*M* sodium chloride, 2 m*M* EDTA, pH 7.0) to a protein concentration of 0.2 mg/ml. Samples were mixed with reducing and non-reducing buffers at a ratio of 1:1 and were heated to 95°C for 5 min in a water bath. Proteins were analyzed on 8–16% gradient gel and visualized by silver staining [39]. Stained gels were further analyzed by digital image processing to assess the purity.

3. Results

3.1. Characterization of ion-exchange matrix

Experiments to characterize the performance of zirconia based ion-exchange matrix were performed with commercially available, pure, and well defined model proteins. In order to identify the elution and retention profiles of BSA, transferrin, and Mab, 5 μl of 3–4 mg/ml solutions of a mixture of BSA and transferrin as well as pure Mab were chromatographed on the EDTPA modified zirconia column. The resultant traces are shown in Fig. 1a and b. Fig. 1a shows the typical chromatographic profile of BSA and transferrin on the zirconia-based ion-exchange column. No retention of either BSA or transferrin on the matrix was observed as judged by a large peak eluting at the void volume of the column. Fig. 1b shows the typical well shaped chromatographic profile for pure Mab on the zirconia based ion-exchange 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 which roughly corresponds to 0.1 *M* sodium chloride in the linear gradient.

3.2. Separation of Mab in a model sample

A model matrix of a solution which mimics the target stream from which Mabs would be isolated (i.e. a cell culture supernatant) was produced by mixing transferrin, BSA, and Mab to a final concentration of 3–4 mg/ml. This model cell culture supernatant was chromatographed on the EDTPA modified column using a linear gradient of 0 to 1 *M* sodium chloride in 30 min to gauge the ability of the

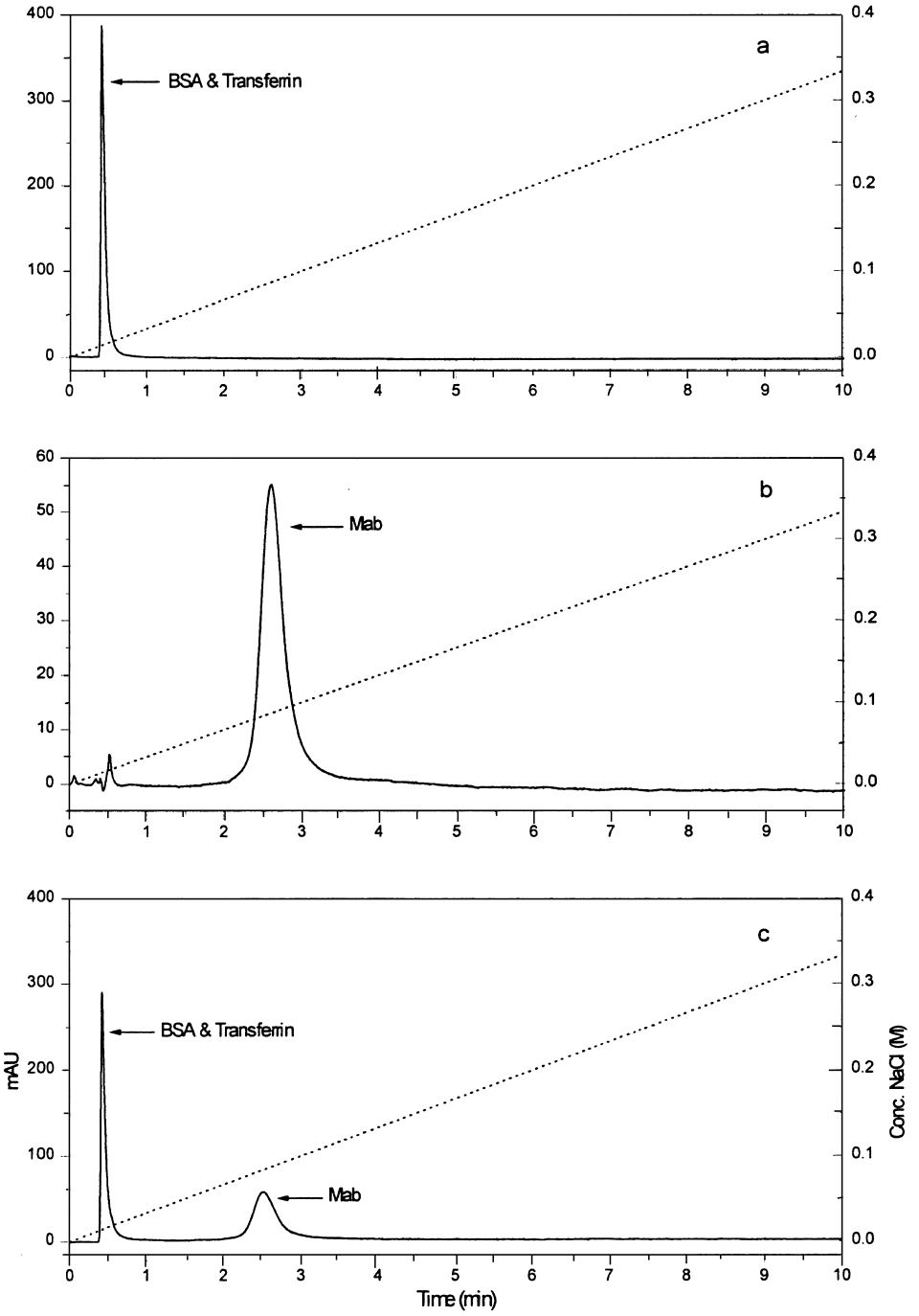

Fig. 1. Gradient elution chromatography of model cell culture supernatant components [(a) BSA and transferrin, (b) Mab] and (c) mixture of BSA, transferrin, and Mab (all concentrations 3–4 mg/ml of proteins dissolved in mobile phase) on EDTPA-modified zirconia. Particles (6 μm) were packed in 5 cm×4.6 mm I.D. columns. Mobile phase: 4 m*M* EDTPA and 20 m*M* MES adjusted to pH 5.5 (adjusted with concentrated sodium hydroxide). A linear gradient (represented by - - -) from 0 to 1 *M* sodium chloride in 30 min was employed; flow-rate 1 ml/min; temperature 30°C; detection at 280 nm (represented by —). Injection volume was 5 μl.

modified zirconia phase to efficiently separate the Mab from other contaminating proteins present in the cell culture supernatant. Fig. 1c shows the chromatographic profile of the model mixture of the three representative proteins. As can be judged from Fig. 1c, BSA and transferrin co-elute at the dead volume in a narrow but somewhat tailed peak as in Fig. 1a. The Mab was well retained on the EDTPA modified zirconia column and eluted at five column volumes as in Fig. 1b.

3.3. Isolation of Mab from cell culture supernatant

Fig. 2 shows a typical chromatographic profile for the isolation of Mab from the cell culture supernatant. Unbound or very weakly retained proteins passed through the column during the first five min. The UV trace at 280 nm returns to baseline by seven min (~14 column volumes) indicating near complete elution of unbound protein.

A step change to 0.5 *M* sodium chloride was made to elute the bound Mab. A chromatographic peak at 280 nm indicates elution of bound protein which we identify as purified Mab. This peak eluted between 16–25 column volumes.

Finally, to elute any proteins bound by non-specific interactions, a step increase to 1 *M* sodium chloride at 14 min was employed. Flushing of the column continued for 6 min or approximately twelve column volumes. A small peak is observed in the UV trace during this wash step. Interestingly, the same peak is also present in blank injections and is probably a system peak from the sudden change in mobile phase composition. To restore the column for the next run, it was then flushed with loading buffer (20 m*M* MES, 4 m*M* EDTPA, 50 m*M* sodium

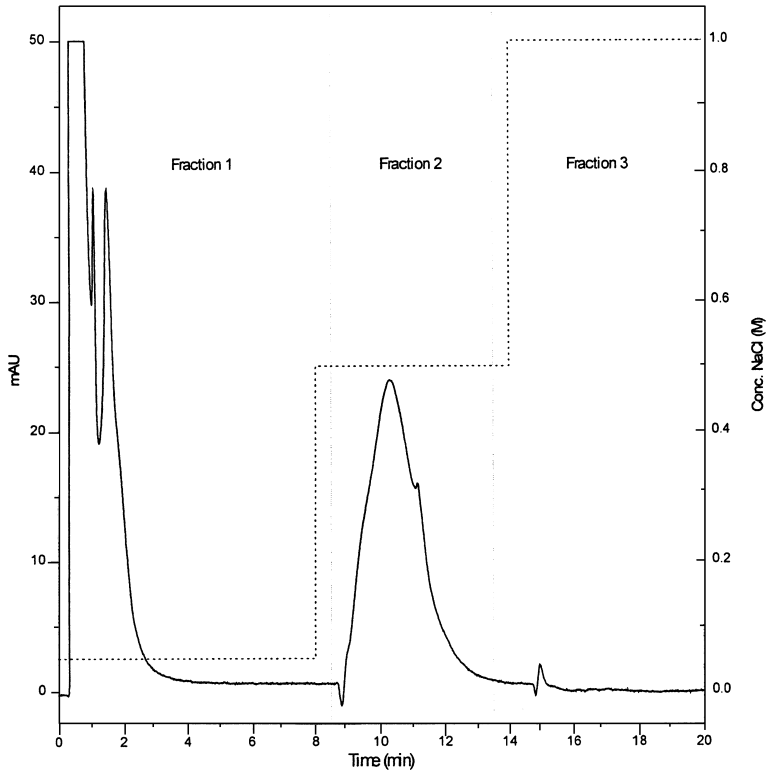

Fig. 2. Purification of Mab from a cell culture supernatant (16 mg/ml total protein by optical density estimation) on EDTPA modified zirconia. Column was the same as used in Fig. 1. Loading buffer was 4 m*M* EDTPA, 20 m*M* MES, and 50 m*M* sodium chloride at pH 5.5 (adjusted with concentrated sodium hydroxide); a step gradient (represented by - - -) 0.05 to 1 *M* NaCl in 14 min flow-rate 1 ml/min; temperature 30°C; detection at 280 nm (represented by —). Injection volume was 25 μl.

chloride at pH 5.5) for 15 min prior to the next injection.

3.4. Enzyme-linked immunosorbent assay (ELISA) of Mab from cell culture supernatant

Table 1 summarizes the Mab yield in the eluate fractions at various total protein and Mab challenges to the column. Chromatographic profiles with similar characteristics to one described earlier were obtained for all runs in Table 1. The Mab concentration in different chromatographic fractions in each individual run was estimated by the ELISA protocol described in the 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, Mab yields of 92–98% were obtained with little or no detectable Mab in column fall through (Mab which passed through with the impurities in the sample and that eluted before the main Mab peak) and wash fractions.

3.5. Gel electrophoresis

Fig. 3 shows a silver-stained, SDS–PAGE gel of the starting cell culture supernatant (feed) and the purified fractions from a typical chromatographic separation. Chromatographic fractions from runs 2 and 6 (see Table 1) were selected for electrophoretic analysis. Lanes 1 and 10 show molecular mass ladders. Lanes 2 and 3 show application of pure BSA and Mab at a total protein level of 2 μg, respectively. Lanes 4 and 7 show an application of cell culture supernatant at a total protein level of 4 μg. The cell culture supernatant has two distinct protein bands corresponding to BSA with a molecular weight of 56 000 and Mab (IgG) with a molecular mass of 150 000 with some additional minor bands. Lane 5 shows the fall through fraction from run 2 at a total protein level of 3 μg. The fall through fraction gave a band around M_r 56 000 similar to the pure BSA in lane 2. Lane 6 shows the elution fraction from run 2 at a total protein level of 3 μg. The eluate fraction gave a band around (150 similar to the pure Mab in lane 3. In addition to the major Mab band at M_r for less 150 000, a minor band at M_r 56 000 accounting for less than 2% of the area obtained by digital image processing was observed. Lane 8 shows the fall through fraction from run 6 at a total protein level of 3 μg. The fall through fraction gave a band around M_r 56 000 similar to the pure BSA in lane 2. Lane 9 shows the elution fraction from run 6 at a total protein level of 3 μg. The eluted fraction gave a band around M_r 150 000 similar to the pure Mab in lane 3. No other contaminating protein bands were observed in lane 9. The purity of the Mab in the eluate fraction (lanes 6 and 9) is estimated to be greater than 98% by digital image processing. Similar electrophoretic patterns were obtained with the fractions from other runs listed in Table 1 (data not shown).

Table 1
ELISA results at different concentrations of Mab in collected fractions

Run	Amount of Mab[a] (μg)					
	Feed[b]	Fall through (fraction 1)	Eluate (fraction 2)	Wash (fraction 3)	Yield[c] %	Overall Yield[d] %
1	21.0	0.3	19.5	0.3	92.8%	95.7%
2	26.5	0.9	25.8	0.3	97.4%	~100%
3	33.0	0.9	31	0.2	93.9%	97.3%
4	38	0.6	37	0.1	97.3%	99.2%
5	65	1.2	61	1.2	93.8%	97.5%
6	160	4.1	150	4.1	93.8%	98.9%

[a] The amount of Mab in each fraction was estimated by ELISA.

[b] Cell culture supernatant was used as feed to the columns. Sample volumes of cell culture supernatant were lyophilized and reconstituted to give the desired Mab concentrations in feed. In each feed application the ratio of BSA to Mab remained relatively constant at 3.5:1.

[c] 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.

[d] The % overall yield of the Mab was determined as a ration of the total Mab in the eluate fraction (fraction 2) to the total Mab preasent in the feed plus the fall throughout (fraction 1).

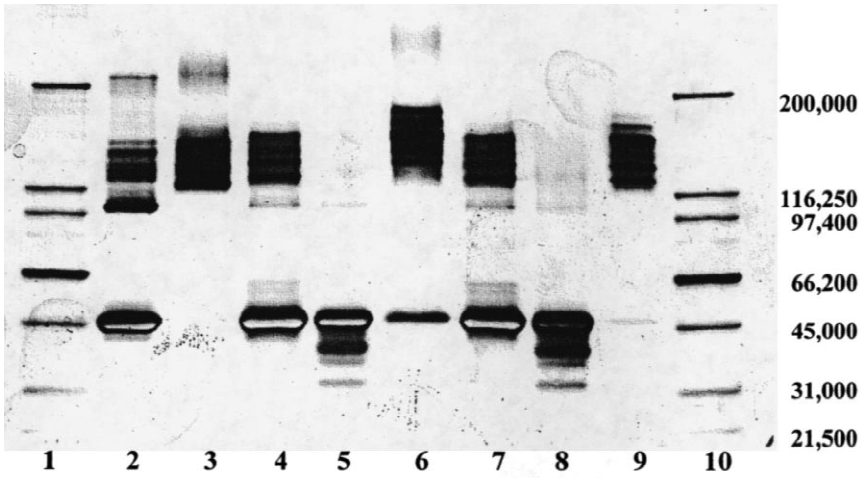

Fig. 3. Sodium dodecylsulfate (0.1%)–polyacrylamide gel (8–16% gradient) electrophoresis of Mab that was purified from cell culture supernatant using EDPTA-modified zirconia. Lanes 1 and 10 show molecular mass ladders. Lanes 2 and 3 show application of pure BSA and Mab at a total protein level of 2 μg, respectively. Lane 4 and 7 shows an application of cell culture supernatant at a total protein level of 4 μg. Lane 5 shows the fall through fraction from Run 2 at a total protein level of 3 μg. Lane 6 shows the eluted fraction from Run 2 at a total protein level of 3 μg. Lane 8 shows the fall through fraction from Run 6 at a total protein level of 3 μg. Lane 9 shows the eluted fraction from Run 6 at a total protein level of 3 μg.

4. Discussion

Our experiments with the model cell culture supernatant (mixture of BSA, transferrin, and Mab) show that while the Mab is retained on the column and elutes in a symmetrical peak, the impurities (BSA and transferrin) are not retained and elute in a sharp but somewhat tailed band at the dead volume of the column. In addition, both BSA and transferrin were unretained on the support under a variety of mobile phase and gradient conditions (data not shown). These experiments indicate that an effective isolation of Mab from contaminating proteins was achieved more through a high differential in binding capacity and less through a high number of plates or efficiency. In other words, while the solid-phase is not specific for the Mab as a protein-A column would be, the modified zirconia column is much more selective for the Mab relative to the major impurities in a real sample from a cell culture.

This selectivity facilitated the use of step gradient conditions for the efficient purification of the Mab. This is of particular interest in large-scale separations where linear gradient elution has proven to be time-consuming and inconvenient. The step gradient allowed for adequate purification (>95%) with high recoveries (>95%) and will likely prove attractive in large scale purification of Mabs. Three significant features are noted in the chromatographic trace in Fig. 2. First, the peaks eluting between 0.5 and 5 min are due to impurities in the feed. The first large band is due most likely to transferrin and BSA. The two peaks that elute within the tail are due to proteins in low concentration that were not accounted for in the model cell culture supernatant used in the initial experiments (see Fig. 1).

The second significant feature in the UV trace of Fig. 2 is the elution profile of the Mab. Though the fraction is well retained, the elution peak is somewhat broad and asymmetric. In addition, a small shoulder is present. The relatively high concentration of total protein challenge to the matrix (~16 mg/ml) in an injection volume of 25 μl likely contributes to peak broadening. In addition, the shoulder on the Mab peak contributes to the asymmetry. This shoulder is probably due to some microheterogeniety within the Mab present in the cell culture supernatant.

An interesting observation from the chromatogram in Fig. 3 is the absence of a protein peak during the high-salt column wash and regeneration steps. A small peak was recorded at approximately fifteen

min and a similar peak was also present in blank injections (injections of 25 μl of loading buffer). This peak is probably the result of the step change in mobile phase and not due to the elution of proteins due to nonspecific interactions. The absence of proteins retained by nonspecific interactions is a welcome result on zirconia-based phases, both bare and polymer coated, which are plagued by these deleterious interactions. This helps support our view that the modification used to impart a cation-exchange type of retention mechanism also adequately blocks ligand-exchange interactions between protein and the support surface.

5. Conclusion

We have successfully used EDTPA modified zirconia to separate Mab from cell culture protein contaminants on a laboratory scale. Specifically, a single-step purification on the modified zirconia matrix yields purified Mab with very low levels of contaminating proteins like BSA and transferrin. In order to better visualize proteins upon gel electrophoresis we have chosen silver-staining protocol over the widely used coomassie blue method. The better sensitivity of the silver staining protocol for protein detection enables us to better assess the purity of the Mab in the eluate fraction.

Large scale applications of the separation methodology developed in this study will require scale-up with respect to particle size and the length of the column. The particles used in these experiments were 6 μm, typical for separations on an analytical scale. Optimal pressure drop and flow properties in preparative columns require particles on the order of 25 to 100 μm. Future efforts will be directed towards the synthesis and evaluation of a 35–50-μm EDTPA modified zirconia particle. We expect a decrease in efficiency with a column packed with larger particles, but this lack of efficiency should not significantly hinder the ability of the column to perform the desired separation. We believe this to be true because the ability of the modified zirconia column to purify Mab from a cell culture supernatant does not arise entirely from the number of plates delivered by the column. Rather it stems from the highly selective binding of Mab by the support relative to other constituents in the sample matrix. Therefore, a column packed with 35- to 50-μm modified zirconia particles with reasonable efficiency should be able to achieve the same degree of purity and recovery at much higher throughput.

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