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Use of spray-dried zirconia microspheres in the separation of immunoglobulins from cell culture supernatant

Anuradha Subramanian[a,*], Peter W. Carr[b], Clayton V. McNeff[c]

[a]*University of Minnesota, Department of Biosystems and Agricultural Engineering, 1390 Eckles Ave., St. Paul, MN 55108, USA*
[b]*University of Minnesota, Department of Chemistry, Smith and Kolthoff Halls, 207 Pleasant St. SE, Minneapolis, MN 55455, USA*
[c]*ZirChrom Separations Inc., 617 Pierce Street, Anoka, MN 55303, USA*

Abstract

A method suitable for the isolation of monoclonal antibodies (MAbs) on novel zirconia microspheres (20–30 μm) is described. Zirconia microspheres were generated by spray drying colloidal zirconia. Spray-dried zirconia microspheres were further classified and characterized by X-ray diffraction, BET porosimetry and scanning electron microscopy. Spray-dried zirconia microspheres were modified with ethylenediamine-*N*,*N*′-tetra(methylenephosphonic) acid (EDTPA) to create a cation-exchange chromatographic support. The chromatographic behavior of a semi-preparative column packed with EDTPA-modified zirconia microspheres was evaluated and implications for scale-up are provided. EDTPA-modified zirconia microspheres were further used to purify MAbs from cell culture supernatant. Analysis 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. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

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 ascities 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

*Corresponding author.

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with high purity and good recovery, but the varying avidity of protein A for immunoglobulin G (IgG) from different 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 has been 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, octyl-sepharose) 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 ascities 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. Matrices based on zirconia offer high density and excellent thermal and chemical stability in comparison to silica or polymeric supports. In particular, the thermal and chemical stability of zirconia allows the use 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. A novel ion-exchange matrix based on microparticulate zirconia (3.5 μm in diameter) has been developed and characterized as sorbents for use in bioseparations [17]. However, the small size of these particles precludes its use in preparative-scale chromatography.

Here we seek to investigate the use of a 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 recoveries. The process yields and purity of the MAb will be demonstrated.

2. Methods

2.1. Reagents

All chemicals were of analytical-grade or better. Ethylenediamine-*N*,*N*′-tetra(methylenephosphonic) acid (EDTPA) was obtained from TCI America (Portland, OR, USA), 2-(*N*-morpholino)ethanesulfonic acid (MES) (No. 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 (No. 7647-14-5) was purchased from EM Science (Gibbstown, NJ, USA). HPLC-grade 2-propanol (No. 67-63-0) was obtained from Mallinckrodt (Paris, KY, USA). Bovine serum albumin (BSA) and transferrin were 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 polyacrylamide gels were purchased from Novex (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. BET nitrogen

porosimetry was performed on a Micrometrics ASAP 2000 sorptometer. The X-ray diffraction spectra were taken on the Bruker AXS (Siemens) D5005 diffractometer at the Center for Interfacial Engineering, University of Minnesota, Minneapolis, MN, USA. Sintering experiments were performed in a Thermolyne 30400 furnace. Scanning electron microscopy (SEM) was performed at the Characterization Facility, University of Minnesota, Minneapolis, MN, USA.

2.2. Matrix preparation

A colloidal solution of zirconia was spray dried using a modified procedure outlined in an earlier paper and presented elsewhere [18]. The spray-dried zirconia microspheres were classified with standard mesh sieves and sintered as follows. Zirconia microspheres were heated at 400°C for 4 h in ceramic crucibles using a linear temperature ramp-up of 5°C/min. The burned particles were hardened and sintered at 750°C for 6 h and at 900°C for 3 h, using a linear temperature ramp-up of 20°C/min between each temperature. The microspheres were allowed to cool to room temperature and were further size-fractionated using a combination of one 25-μm and one 38-μm screen mesh.

2.3. Surface modification procedure

Surface modification of zirconia microspheres (25–38 μm) with EDTPA was developed based on a method described in an earlier paper and presented elsewhere [19–27].

2.4. Chromatography

EDTPA-modified particles were packed in empty 316 stainless steel columns (Alltech, Deerfield, IL, USA), 15 cm×1.0 cm I.D. semi-preparative column with 2 μm stainless steel screens (Chrom Tech, Apple Valley, MN, USA) to minimize protein loss. The particles were packed using a dry packing method. The chromatographic system used was a Hewlett-Packard 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. 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 15 min prior to the next injection.

For separation and isolation of MAbs 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 the 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

The amounts of MAb in the chromatographic fractions were determined by the enzyme-linked immunosorbent assay (ELISA) procedure detailed elsewhere [28]. In brief, microtiter plates coated with anti-mouse IgG (whole molecule) were used to immunocapture MAb from various chromatographic fractions and the bound MAb was detected with rabbit anti-mouse-HRP conjugate. The bound chromophore was detected at 490 nm using an EL800 Bio-Tek Microplate reader.

2.6. Gel electrophoresis

The purity of the recovered MAb was analyzed by sodium dodecyl sulfate–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 dialysis membranes, lyophilized, 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 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 [29]. Stained gels were further analyzed by Shimadzu flying-spot scanning densitometry to assess the purity.

3. Results

3.1. Matrix preparation and characterization

One to five gallons of colloidal zirconia were spray-dried using a gas-fired Bowen spray drier to generate zirconia microspheres that were polydisperse (data not shown) (1 gallon=3.785 l). In order to obtain zirconia microspheres in the desired size range (25–38 μm in diameter), the spray-dried particles were sintered and classified using standard mesh sieves. The particle size was determined using a MicroTrac particle size analyzer and a mean particle size of 21.75 microns was obtained. The spray-dried particles were modified with EDTPA and further used in chromatography.

3.1.1. X-Ray diffraction

All measured peaks in the diffraction pattern on the preparative zirconia sample correspond closely both in position and relative intensity to the peaks predicted for a well-formed monoclinic crystal of zirconia. Thus it was concluded that spray-dried zirconia microspheres are pure, monoclinic crystals and similar to the reference spectra on file.

3.1.2. BET nitrogen porosimetry

The spray-dried zirconia microspheres had an average pore diameter of 260 Å, a BET surface area of 33.2 m^2/g as measured by the nitrogen adsorption isotherm, and a pore volume of 0.106 ml/g. The particles made by polymer-induced colloidal aggregation (PICA) had an average pore diameter of 220 Å and a BET surface area of 29 m^2/g as measured by the nitrogen adsorption isotherm [17].

3.1.3. Scanning electron microscopy characterization

SEM analysis of EDTPA-modified, re-screened zirconia particles suggested that spray-dried zirconia microspheres are relatively spherical with few doughnuts present (micrograph not shown).

3.1.4. Characterization of column behavior

A flow study was undertaken to measure the dependence of column efficiency on mobile phase flow-rate of a 15×1 cm semi-preparative column packed with spray-dried zirconia microspheres. 4-Dimethylaminopyridine, which has an average retention factor of 0.97 was used as a probe in this flow study. The data was analyzed in light of the Knox equation; $h=A\nu^{1/3}+B/\nu+C\nu$ and the Knox coefficients were evaluated using multivariate linear regression. An *A*-term of 1.2, *B*-term of 3.9, and *C*-term of 0.024 was obtained from the regression analysis.

3.2. Separation of MAb in a model sample

The loadability of the semi-preparative column used in this study was first evaluated by sequential injections of pure MAb (>95%) sample, where the sample size or the total MAb challenge was varied from injection to injection. The MAb was quantitatively retained on the column and eluted in 5–8 column volumes (CVs). In addition, the peak shape of the eluted MAb did not degrade over a five-fold injection of MAb sample (data not shown).

A model matrix of a solution, which mimics the target stream from which MAbs would be isolated, was produced by mixing BSA and MAb to a final concentration of 7 mg/ml. This model mixture was chromatographed on the semi-preparative column to gauge the ability of the modified zirconia phase to efficiently separate the MAb from BSA. Fig. 1a shows the chromatographic profile of the model mixture. As can be judged from Fig. 1a, BSA elutes at the dead volume in a narrow peak as in Fig. 1a. The MAb was well retained on the EDTPA-modified zirconia column and eluted between 5 and 8 CVs as shown in Fig. 1a. Additional injections of the model mixture were run and profiles similar to Fig. 1a were obtained. The ratioed area (peak area/injection volume) of the BSA and MAb peaks were analyzed for the multiple runs and a standard deviation of less than 1% was obtained.

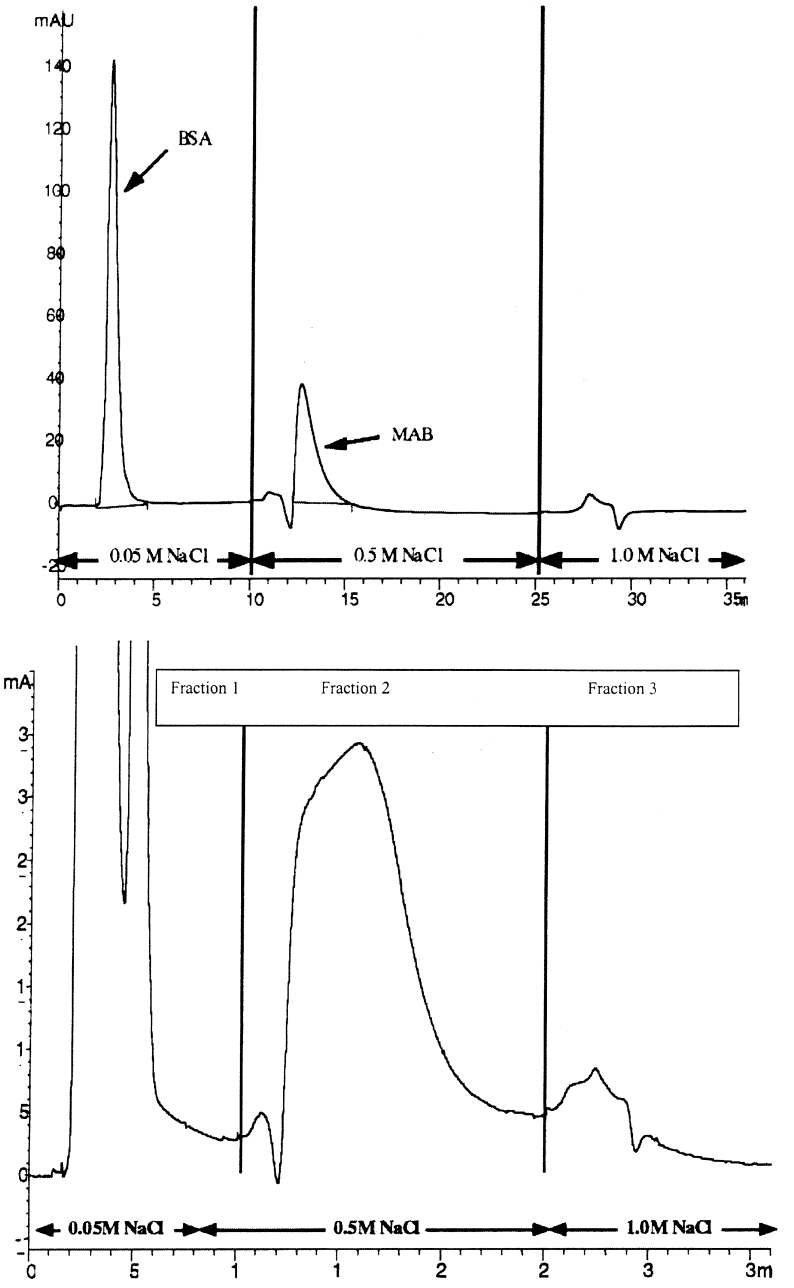

Fig. 1. (a) Gradient elution chromatography of model cell culture supernatant components (BSA and MAb) (all concentrations 3–4 mg/ml of proteins dissolved in mobile phase) on EDTPA-modified zirconia. Particles (25–38 μm) were packed in 15×1 cm I.D. columns. Mobile phase: 4 m*M* EDTPA and 20 m*M* MES adjusted to pH 5.5 (adjusted with concentrated sodium hydroxide). A step gradient from 0 to 0.5 *M* sodium chloride was employed in the elution regime and 1.0 *M* sodium chloride was used to regenerate the column. A flow-rate of 1 ml/min was maintained; temperature of operation was 30°C; and detection performed at 280 nm. Sample injection volume was 50–100 μl. (b) Purification of MAb from a cell culture supernatant on EDTPA-modified zirconia microspheres. Column was the same as used in (a). 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 0.05 to 1 *M* NaCl in 14 min; flow-rate 1 ml/min; temperature 30°C; detection at 280 nm. Sample injection volume was 100–200 μl.

3.3. Isolation of MAb from cell culture supernatant

Fig. 1b 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 5 min. The UV trace at 280 nm returns to baseline by 7 min (~14 CVs) 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 that we identify as purified MAb. This peak eluted between 16 and 25 CVs. 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 12 CVs. 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 the next run, it was then flushed with loading buffer (20 m*M* MES, 4 m*M* EDTPA, 50 m*M* sodium chloride at pH 5.5) for 15 min prior to the next injection.

3.4. 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 the 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 and wash fractions.

3.5. Gel electrophoresis

Fig. 2 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 1 and 2 (see Table 1) were selected for electrophoretic analysis. Lane 1 shows a molecular mass ladder. Lane 2 shows a 3 μg application of pure BSA. Lanes 3 and 7 show application of pure BSA and MAb at a total protein level of 2 μg, respectively. Lanes 4 and 8 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 mass 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 1 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

Table 1
Summary of ELISA results

	Amount of MAb[a] (μg)					
	Feed[b]	Fall Through	Eluate	Wash	Yield[c] (%)	Total recovery[d] (%)
Run 1	588.88	18.41	564.16	5.61	95.8	99.88
Run 2	605.40	18.32	544.06	4.89	89.87	93.70

[a] The amount of MAb in each fraction was determined by ELISA. Each sample was assayed at different dilutions with triplicate application of each dilution to the ELISA plate. The average values are reported here. In all cases an assay regression value in the range of 0.95–099 was obtained.

[b] Cell culture supernatant was used as feed to the columns. 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 % total recovery of the MAb was determined as a ratio of the total MAb present in fractions 1, 2 and 3 to the total MAb present in the feed.

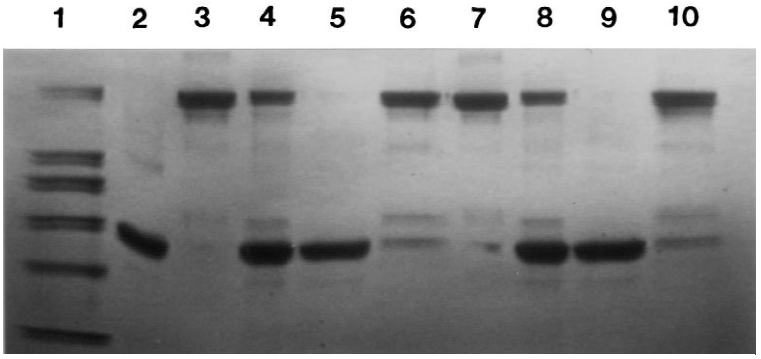

Fig. 2. Sodium dodecyl sulfate (0.1%)–polyacrylamide gel (8–16% gradient) electrophoresis of MAb that was purified from cell culture supernatant using EDPTA-modified zirconia. Lane 1 shows a molecular mass ladder. Lanes 2 and 3 show application of pure BSA and MAb at a total protein level of 2 μg, respectively.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

shows the elution fraction from run 1 at a total protein level of 3 μg. The eluate fraction gave a band around M_r 150 000 similar to the pure MAb in lane 3. In addition to the major MAb band at M_r 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 9 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 10 shows the elution fraction from run 2 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. Other contaminating protein bands were observed in lane 10. 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).

4. Discussion

Our previous work with analytical columns packed with EDTPA-modified micro-particulate zirconia (3 μm) produced by the PICA method indicated that an effective isolation of MAb from contaminating proteins present in cell culture supernatant was attainable [17]. Separation of MAb from other contaminating proteins was likely 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 EDTPA-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.

The chromatographic properties of the spray-dried zirconia microspheres were quite similar to the PICA-derived materials used in analytical-scale columns. The spray-dried particles proved to be mechanically robust, withstood dry packing, and were operable at high linear velocities. In addition, the spray-dried zirconia microspheres could be cleaned, re-coated with EDTPA in a dynamic mode and reused with no apparent loss in resolution. The value of the *A*-term (1.2) obtained from the Knox equation [30] indicates a well-packed column. The *B*-coefficient, which had a value of 3.9, indicates some channeling in the column and this can be expected

when small columns are packed with large particles. These wall effects can be reduced in larger diameter columns. The *C*-term, which had a value of 0.024, indicates that there was a weak dependence of the column efficiency on mobile phase velocity. We can possibly use this to our advantage when optimizing preparative separations and purification cycle times.

Three significant features were noted in the chromatographic trace in Fig. 1b. The first large peak is due most likely to transferrin and BSA. The second significant feature in the UV trace of Fig. 1b 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 50–100 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. 1b is the absence of a protein peak during the high-salt column wash and regeneration steps. A small peak was recorded at approximately 15 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 spray-dried colloidal zirconia to generate zirconia microspheres that can be used as a support in bio-chromatography. A semi-preparative column packed with zirconia microspheres was employed to separate MAb from cell culture protein contaminants. Specifically, a single-step purification on the modified zirconia matrix yields purified MAb with very low levels of contaminating proteins like BSA and transferrrin. Our future efforts will focus on the development of a purification strategy for human immunoglobulins from serum using zirconia microspheres.

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. We are currently focusing our efforts on the packing and evaluation of a 15×2.1 cm preparative column. Future efforts will also be directed towards the synthesis and evaluation of a 100–200 μm EDPTA-modified zirconia particle. We expect a decrease in efficiency with 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 100–200 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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