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Chromatographic Purification of MAbs with Non-Affinity Supports

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New protein-based therapeutics such as monoclonal antibodies (MAbs), MAb-based vaccines, growth factors and plasma proteins implies the need to study, characterize, and purify. The separation step is likely to be a bottleneck and cost-effective technology will be needed to rectify it.

Currently prevalent matrices for chromatographic separation of immunoglobulins (Igs) are based on Protein A or its recombinant versions (Protein G). They display excellent selectivity and specificity, but are expensive. A Protein A matrix costs $8,000 to 12,000 per L-resin. The typical production column volume is 100 L. The million-dollar matrix is far more expensive than the production hardware.

Affinity chromatography using Protein A/G is specific, both Protein A and Protein G are macro-molecular and fragile, expensive to obtain from bacterial or tissue-culture sources, and are difficult to im-mobilize without losing activity. The conditions used to elute IgG or an antibody bound to protein-A and protein-G sorbents are harsh and require a pH near 3.0. This can impact the biological activity of some antibody products. Hence, the product is immediately neutralized and dialyzed against a pH 7.0 buffer. To overcome this negative effect, recombinant versions of protein-A and protein-G have been developed that elute at milder conditions in the 6.0 to 7.0 pH range.

More over, The use of both proteins as affinity supports in chromatographic columns poses special challenges regarding regeneration and sanitation.[1] Some of these drawbacks preclude the use of biological ligands in practical applications and has prompted many researchers to turn their attention to the develop-ment of synthetic ligands. Smaller molecules like dyes, amino acids, metal ions, and chemical moieties show comparable affinities, and specificity of such a molecule can be increased or decreased, either at ad-sorption or desorption, to attain resolutions and degrees of purification comparable to those of immunoab-sorption.[]2–6]

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Research with human and humanized antibodies and the development of novel types of hybrid monoclonals will require the development of separation methods not based on protein A/G, because protein A/G does not recognize IgG from all species. Although Protein A chromatography is the leading choice for antibody purification, it is not the unanimous choice. The most frequently mentioned alternatives are ion exchange, hydrophobic interaction chromatography, and even relatively crude methods like ammonium sulfate and caprylic acid precipitation. Purification schemes for antibodies from the serum include precipitation,[7,8,9] ion-exchange chromatography,[10,11] thiophilic chromatography,[12,13] metal chelate interaction chromatography,[14,15] affinity separations using immobilized Protein-A/G,[1,16] hydrophobic interaction chromatography,[17,18] hydroxyapatite chromatography,[19,20] dye affinity, and ion-exchange techniques.[21–24]

Properties of Desirable Supports:

Efficiency,economics, and practicality are some of the constraints centered around the search for novel chromatographic supports and methodologies. The preparation of alternative stationary phase supports is an important area. The goal of our research is to develop support materials that offer novel selectivities, or develop new protocols that are amenable to scaleup without presenting excessive operational complexities. We focused on developing adsorbents that have a narrow range of physiochemical affinities and on mixed-mode synthetic chemistries coupled with engineered matrices.

We at the University of Nebraska explore and examine recent progress in the development of pseudoaffinity methods and the synthesis of engineered matrices. We discuss its use in the purification of antibodies from biological sources. In the first case study, the development of ligand-modified chitosan as a stationary phase material will be an example of a methodology based on pseudoaffinity and as so can be

utilized to separate and purify MAbs from cell culture supernatant. In the second case study, the development of zirconia as a stationary-phase material is an example where engineered matrices in combination with mixed-mode chemistries can be utilized to separate humanized MAbs from cell culture supernatant. The named products were purchased for use in an academic setting.

Anionic Pseudo-Bioaffinity Chromatographic Process

Some reports have been published by us on the effectiveness of a novel pseudo-bioaffinity chromatography support for the separation of Igs from complex biological fluids.[25-27] The support was synthesized by post-derivatization of bald chitosan beads with a carboxyethyl-group that contains an anionic ligand, and it can be bought from Ligochem Inc. as Ligosep Alpha. It is a new type of bead architecture. The large-diameter (600 to 800 μm), low-density chitosan (3.5% solids) permits homogeneous ligand utilization throughout the bead's interior (Figure 1). We have demonstrated that the transport of biomolecules in matrices with open architecture is a combination of convective and diffusive fluxes.[27]

It is hypothesized that the chitosan hydrogel bead resembles a network of polymer chains as opposed to conventional supports characterized by distinct pore sizes and pore geometry. The high surface area beads, in conjunction with the open architecture, create homogeneous microenvironments of low binding energy, enabling ligand specificity, which is characteristic of moderately high affinity separations. Mass transfer fluxes should be efficient under a wide range of operating conditions.

As in a Experiment, cell culture supernatant (an aqueous media which contains the desired antibody product, nutrients, growth factors, amino acids, vitamins, and minerals) was diluted in de-ionized water until a conductance reading of less than 2 mS was achieved. Then, a 10 times concentrated loading

buffer (100 mM KH_2PO_4, pH 6.0) was added (one tenth of the total volume of de-ionized water). A column (1.5 cm i.d. × 13–16 cm long) was packed with Ligosep Alpha beads. The feed was loaded at a linear velocity of 1.1 cm/min. Unretain-ed proteins were collected as the column fall-through and the non-specifically bound proteins were washed with the loading buffer until the OD 280 nm spectrophotometer reading returned to the baseline. The (bound) human IgG was eluted by making a step change to the elution buffer (10 mM KH_2PO_4, 0.5 M NaCl, pH 6.0). Upon elution, the column was washed with the elution buffer until the OD 280 nm reading returned to the baseline and the column re-equilibrated in loading buffer. The chromatographic fractions were assayed for total protein content by measuring absorbance at OD 280 nm, and human immunoglobulin G (hIgG) content was determined by specific Enzyme Linked Immunosorbent Assay (ELISA).

We worked with Ligosep Alpha beads and indicated that an effective isolation of MAbs from cell culture supernatant was attainable. The ability of Ligosep Alpha to bind MAbs from cell culture super-natants was evaluated in column-mode experiments. The following salient features were observed in all chromatographic traces: (1) a first large peak was obtained and was most likely due to serum proteins, non-Ig in nature. (2) the elution peak was somewhat broad and asymmetric in nature, though the Ig was well retained. This may be due to the nature of the base matrix, characterized as having conduits or a se-ries of polymeric networks comparable to large pores. A change of elution buffer by the addition of 0.5M NaCl buffer was made to elute the bound MAb.

Much of protein loaded was seen in the fall-through fraction, indicating there was little non-specific binding and the MAbs were being retained in the elution fraction. Purification experiments using column chromatography were performed in replicates with good repeatability. The percent recovery was

reported as the ratio of MAb in the elution and fall-through fractions to the total MAb in the feed solution. Values in the range of 83–100% were obtained for the linear velocities tested.

Cation Exchange Process

Botulium toxin (BoNT) has been classified by the Centers of Disease Control (CDC) as one of the six highest bioterrorism risk threats due to its potency, lethality, and ease of production.[29] Recombinant MAbs are currently being developed for the treatment of botulism.[30] One such humanized monoclonal antibody that is currently being developed at University of Nebraska is HuMab-S25. We have developed and characterized production of a MAb in a dihydrofolate reductase (dhfr)-deficient Chinese hamster ovary (CHO) cell line[31] and have analyzed a purification scheme that uses ethylenediamine-N,N'-tetra(methylphosphonic) acid (EDTPA)-modified zirconia for an initial capture and purification step, followed by a secondary purification using MEP-hypercel, a hydrophobic charge interaction chromatography (HCIC) resin. Included here is a summary of the development process.

High density and excellent thermal and chemical stability of zirconia-based supports provide several advantages over traditional 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 that are routine procedures in the pharmaceutical industry. We have successfully spray-dried colloidal zirconia to generate zirconia microspheres and then further modified with EDTPA to yield a support for bio-chromatography. The usefulness of EDTPA-modified zirconia in the separation of hIgG from cell culture supernatant and in treated serum samples has been demonstrated elsewhere.[32,33] Our previous work includes a detailed characterization of the binding constants and rate-limiting mechanisms of mass transfer in these matrices.[32-34]

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Comparison Tests of Three Supports

Culture supernatant rich in HuMab S25 was directly loaded onto chromatography columns of rPro-tein A Sepharose Fast Flow, EDTPA-modified zirconia, and MEP-hypercel (Figure 3A, 3B, 3C). The loading of S25 antibody was well below the binding capacity of the rProtein A Sepharose resin. The maximum pressure drop (3 bar) of the rProtein A Sepharose fast flow resin limits the flowrate to 90 cm/h.

Approximately 6 mg of total protein containing 1.25 mg S25 antibody was loaded onto the Fast Flow column in 26.4 mL culture supernatant. The S25 antibody was eluted off the rProtein A column with 50 mM sodium citrate (pH 3.0) in 0.6 column volumes (CVs) neutralized to pH 7.0 with 0.17 CV of 500 mM Tris base. The Protein A Sepharose Fast Flow column provided a yield of 74.8% as determined by a whole antibody ELISA. The purity of the antibody was found to be 99% based on the concentration of IgG in the elution fraction, determined by an ELISA, divided by the total protein concentration, deter-mined by a bincinchonic assay.

The EDTPA-modified zirconia column was loaded with approximately 50 mL solution consisting of a 1:1 dilution of CHO-DG44 culture supernatant and MES loading solution (40 mM MES, 8 mM EDTPA) at a pH of 5.5. This column has a smaller particle size (40 μm), which resulted in a higher initial pressure drop. However, the resin can handle pressure drops exceeding 400 bar. The S25 antibody was eluted off the column by increasing the NaCl concentration to 1M in 20 mM MES buffer containing 4 mM EDTPA. The S25 antibody eluted in 2.4 CV (Figure 3B) and neutralized to pH 7.0 with 80 μL of 500 mM Tris base. The elution fraction had a S25 antibody concentration of 542 μg/mL and a protein concentration of 1312μg/mL, which corresponds to a purity of 41.3%. The yield using the EDTPA-modified zirconia column was 89.4%.

The MEP-hypercel column was loaded with approximately 50 mL supernatant from the CHO-DG44 S25 cells. The column was washed with 5 CV phosphate buffer saline (PBS, pH 7.2) and eluted using 50 mM sodium citrate (pH 4.0). The S25 antibody was eluted from the MEP-hypercel column in 2.7 CV (4.5 mL), similar to the EDTPA-modified zirconia column. The elution fraction had a S25 antibody concentration of 319 μg/mL and a total protein concentration of 729 μg/mL, which corresponds to a purity of 43.7%. The yield for the MEP-hypercel column was 74.8%, similar to that achieved using the rProtein A column. The loss of S25 antibody appears to be due to irreversible binding onto the resin because there was little antibody in either the flow-through or the wash fractions.

Western Blotting Leads to Hypothesis

A non-denaturing SDS-PAGE gel was run to compare purity of the EDTPA-modified zirconia and MEP-hypercel purified samples to that purified using rProtein A Sepharose Fast Flow (Figure 4). Both rProtein A purified samples were very pure with no visible bands corresponding to non-IgG proteins. There are numerous bands that correspond to contaminating proteins from the CHO DG44 S25 culture supernatant purified using the EDTPA-modified zirconia column. All three fractions contain a high level of contaminating proteins and as a result, were combined.

Referring to Figures 3B and 3C, the S25 elution peak from the MEP-hypercel column is significantly broader than that obtained from the other columns and has a shoulder on the front. This shoulder suggests an improvement in purity could be obtained by elution at several pH steps. Comparing the EDTPA-modified zirconia and MEP-hypercel peaks, it was observed that the contaminating bands in the MEP-hypercel column were different from those occurring on the EDTPA modified zirconia column.

Chromatographic Purification of MAbs with Non-Affinity Supports

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Chromatography in Series

Analytical data were interpreted to conduct another test. The EDTPA-modified zirconia column and MEP-hypercel column were run in series to improve the purity of S25 antibody. The zirconia column was chosen as the first purification step because it can be operated at higher pressure drops and higher flowrates. In addition, the high antibody recovery makes it the preferred choice for an initial purification step. Numerous impurities that result in large bands in the S25 antibody elute taken from the EDTPA modified zirconia column were removed in the MEP-hypercel column. The S25 antibody from the zirconia column was dialyzed into PBS (pH 7.2) using an 8000 kDa molecular weight cutoff (MWCO) dialysis membrane. The dialyzed sample was loaded onto the MEP-hypercel column, followed by 5 CVs with PBS. The load into the MEP-hypercel column was much less than the amount of supernatant previously loaded onto the column. The S25 antibody was eluted with 50 mM sodium citrate (pH 4.0).

Flow-through of partly purified S25 antibody sample loaded onto the MEP-hypercel column had an absorbance of about 70 mAU (Figure 3D). This corresponds to protein that is being removed using the MEP-hypercel column. The S25 antibody was eluted at pH 4.0 and a peak height of 370 mAU was observed, which is much lower than readings observed in the other columns due to the decreased antibody load. The antibody was eluted in 2 mL volume, and 0.4 mL 500 mM Tris base was immediately added to bring the pH to 7.0. The final S25 antibody concentration was 164 µg/mL and the final total protein concentration was 229 µg/mL, resulting in a final purification of 71.6%. This is a significant improvement over the purity obtained using the EDTPA-modified zirconia column alone. The final yield for two-column purification was 71.8%, just slightly less than that obtained from a single rProtein A column.

Chromatographic Purification of MAbs with Non-Affinity Supports

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S25 antibody, purified using columns in series, was run on reducing SDS-PAGE gel, along with the samples purified using the EDTPA modified zirconia, rProtein A Sepharose Fast Flow, and the MEP-hypercel alone (Figure 5). Comparison of lanes 6, 7, and 9 shows the improvement in S25 antibody purity obtained after running columns in series. The activities of purified antibodies were analyzed by monitoring equilibrium binding kinetics using BIAcore equipment.[35] The K_d of the antibodies purified using either the protein-A column or the two step-method proposed had similar K_d values and are listed as follows: K_d was 1.96×10^{-9} M^{-1}, with a k_{on} of 6.03×10^5 $M^{-1}s^{-1}$ and a k_{off} of 1.18×10^{-3} s^{-1}. These values are also similar to ones previously determined for other antibodies.[30,35]

Conclusion

A combination of non-affinity based methods may serve as an alternative to protein-A based separations. Further exploration of biomimetic ligands is needed to find alternatives to the protein A/G chromatographic process. Process validation and process economics require a design of separation strategies that take into account the nature of the product. Quantum leaps in separation technologies will likely happen when advances in ligand installation and specificity are combined with matrices that can be operated at low to moderate pressures.

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