Winds of Change for Rapid Antibody Purification

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Background

There is an increasing demand for standardized and optimized affinity methods to purify a large array of proteins which can act as drugs or drug targets. It is now recognised that understanding protein structure and function is an essential key to understanding disease mechanisms. Progress in antibody-based therapeutics is growing rapidly and there are believed to be more than 400 therapeutic monoclonal antibodies currently in clinical drug development programmes, of which 26 are in clinical phase III and 76 drugs are in phase II development. One of the first monoclonal antibodies to be approved for therapeutic application was Rituxan® from IDEC Pharmaceuticals Corporation and Genentech Inc. for use in the treatment of B-cell non-Hodgkin's lymphoma in 1997. Other drugs include the humanized monoclonal antibody Herceptin® for metastatic breast cancer, humanized anti-IgE monoclonal antibody Xolair® for allergic rhinitis, chimeric monoclonal antibody Remicade® for Crohn's disease and rheumatoid arthritis, humanized monoclonal antibody, Campath® for B-cell lymphocytic leukemia and human monoclonal antibody, Humira® for rheumatoid arthritis. There are currently 16 approved therapeutic monoclonal antibodies in the market and these antibodies range from unconjugated antibodies such as anti-CD 20 chimeric murine/human IgG1 antibody Rituxan® to radio-isotope or toxin-conjugated antibodies such as yttrium 90-conjugated murine antibody, Zevalin™, approved in 2002 to treat non-Hodgkin's lymphoma.

Berthold *et al* (1) described the purification of Rituxan in their 2001 paper from harvested cell culture fluid. Rituxan® is purified using Protein A Sepharose FF chromatography, concentrated by ultrafiltration and polished by anion exchange chromatography. All industrial-scaled processes are optimized in the research laboratory. The vast majority of researchers use agarose matrices in their lab-scale chromatographic supports. Pro-Chem has pioneered new technology for pre-packing ready-to-use Protein A or G agarose resin cartridges in a wet state (Fig. 1). These resin spin columns are bundled into antibody purification kits with buffers, concentration/de-salting columns and unambiguous protocols. These packed rapid purification kits attract a substantial unconverted market that wants to recover reproducibly high levels of pure antibodies from a large amount of contaminants using familiar recombinant Protein A and G agarose resins with considerable time and cost savings compared to traditional, lengthy home-brew gravity-fed columns and expensive chromatography procedures such as HPLC. Blood plasma is believed to contain over 30,000 proteins of which albumin represents over 50 % of the total protein mass of

human serum. Purification of IgG from serum therefore, represents a considerable challenge to scientists.

Although Protein G binds has a wider reactivity profile than Protein A, the binding of antibodies to Protein G is often stronger, making elution and complete antibody recovery more difficult. Interestingly, due to the lower cost of Protein A compared to Protein G, researchers tend to experiment first with Protein A. In order to cover the broad specificity of antibody species and subclasses towards different antibody binding proteins, Pro-Chem introduced immobilized Protein A and Protein G agarose in their Protein antibody purification kits. The kits enable simple, rapid antibody purification from serum, ascites and tissue culture supernatant such as those derived from static cultures and bioreactors for a wide range of laboratory procedures such as 1-D or 2-D polyacrylamide gel electrophoresis, Western blotting, ELISA. The antibodies are sufficiently pure for radiolabeling, conjugations to chromophores or preparations of immuno-affinity columns. All modes of chromatography can be used for the separation of antibodies. Immobilized Protein A and Protein G chromatography is a common method for antibody purification and these affinity-based matrices lend themselves to the bind, wash and elute mode of operation of Proteus spin columns, partly due to the fact that they have sufficiently rapid association kinetics. The market will need new high-yielding tools to meet the significant growth in antibody purification and expression screening for the pharmaceutical industry, diagnostic companies, biotechnology laboratories, antibody manufacturers, universities and public sector research labs.

Proteus Protein A and G Antibody Purification Kits

Pro-Chem's principal task was to simplify current protein separation methods confronting biochemistry and immunology researchers with little or no chromatography knowledge who ideally want to affinity purify their antibodies easily, cost-effectively and quickly. All protocols are rigorously evaluated against a panel of protein samples and test conditions to ensure that the kits comply with all published specifications and exceed the expectations of researchers (Table 1).

Under normal circumstances, affinity separation often takes several hours to complete; in addition the researcher usually has to pack the column and prepare all the buffers, which adds considerable time to the purification step. The elaborate method of packing an empty column with loose Protein A or G resin slurry means that an experienced researcher usually prepares the column, the researcher does not know how the resin performs *in situ* and it is difficult to obtain reproducible yields of antibodies. Preparation time is substantially reduced with Proteus Protein A or G kits as all buffers and desalting/concentration columns are supplied and the spin columns are delivered pre-packed with hydrated Protein A or G agarose. Buffers to a tolerance of ± 0.1 pH

unit and manufactured under ISO 9002 accreditation are supplied with the kit to ensure that antibody capture/elution conditions are optimal.

The Proteus Mini and Midi spin columns enable batches of antibodies to be purified over many cycles of small column. The Proteus Mini and Midi spin columns are re-usable. IgG capacity of the Proteus Mini and Midi spin columns are unaffected after re-use (Fig. 2). As we have engineered re-usability into the spin columns, a regeneration protocol is included in the operating handbook.

Speed is critical when handling antibodies. The majority of purifications require fast processing times to minimize the hydrolytic actions of proteases. Many antibodies are stable in harvested cell culture fluid even at room temperature for several days. However, some murine antibodies are known to precipitate at 4 ºC and they need to be purified at the earliest opportunity. Proteus Protein A and G kits allow multiple parallel purifications to be achieved without the need to employ and queue up to use expensive PEEK tubing (protein-friendly)-based high pressure or fast protein liquid chromatography systems to achieve high yields and purities of the antibody. These LC systems are difficult to learn to use and are expensive to purchase and service. Faulty LC parts and all column accessories are often beyond the affordable budgets of many small research teams. Running samples through these LC systems is slow if separation conditions need to be optimized using different buffer formulations because these systems often need to be purged using fairly large quantities of new buffers before the purification process is initiated.

Modus Operandi

Optimal conditions for binding the target molecule to a resin are critical for successful separation of the protein. If the binding conditions are not optimal with respect to pH, salt concentration, presence or absence of metal ions etc, the interaction could be weak or non-existent.
Mouse antibodies dominate the *in vitro* diagnostic market (2). The binding capacity of mouse IgG1 to Protein A is optimal at pH 8-9, whereas the binding capacity of Protein G is high over a broader pH range. Salt concentration can significantly affect the binding of mouse antibodies to Protein A by reducing severe ionic interactions and enhancing hydrophobic interactions. For example, mouse IgG1 binds well to immobilised Protein A when the salt concentration is higher than 1 M, but binds poorly at low salt concentrations. This is the primary reason why researchers interested in capturing mouse or rat antibodies should pre-condition their samples with binding buffer. The Proteus resin plugs also incorporate a patented flow regulator which is pre-set to optimise the flow rate of the sample loading step. The flow regulator is intentionally designed to increase the residence times of the antibodies with the matrix and therefore increase the yields

and purities of the final antibody product. The sample is filtered and immediately loaded on to the spin column.

The spin columns are then washed to remove unbound non-immunoglobulin proteins. The number of wash steps for the Mini and Midi spin columns is influenced inextricably to the permitted volume of the sample chamber and resin bed volume. The wash steps ensure no carry-over of contaminating non-target proteins into the final eluate. The most common elution conditions for Protein A or G affinity and immuno-affinity separations involve a reduction in pH to between pH 2.5 and 5.5. Many monoclonal antibodies that require high pH for capture e.g. mouse IgG1 can be eluted under mild acidic conditions. For this reason, the Proteus Protein A kit contains both pH 2.5 and pH 5.5 elution buffers. It is also important to appreciate that some monoclonal antibodies are acid-labile and they can lose their activity at very low pH values. Above all, the elution conditions must preserve the integrity and activity of the target protein (3, 4). Most observed denaturation is caused by harsh elution conditions. Acidic pH is known to reduce the antibody titre, decrease immuno-reactivity and distort the antibody structure (2). It is therefore critical that the pH is restored to neutrality after elution. The Proteus spin columns have rapid elution steps, which reduces the whole purification time for the Mini and Midi columns to less than 15 min and 45 min, respectively. Negligible hold-up volume of the Protein A and G resin plug ensures high antibody recovery. In Fig. 3, Proteus Protein A spin columns have been used to purify human IgG from serum. Human IgG accounts for about 15 % of the protein mass of serum. The two dark bands, 25 kDa and 55 kDa, in the final eluate are the light and heavy chains of IgG.

Removing Bottlenecks from Antibody Purification

The Proteus Protein A and G kits are designed to purify antibodies for structural or diagnostic studies and for use as molecular probes for research and development laboratories. Proteus Protein A and G kits eliminate tedious chromatographic steps normally associated with Protein A and G chromatography. Proteus A and G spin columns reduce time-to-purity by incorporating unambiguous protocols to suit various applications, supported by a comprehensive handbook and required buffers in a convenient kit format. The beaded supports offer excellent flow properties. Large numbers of samples can be purified at the same time. The Protein A and G kits allow both inexperienced researchers and researchers with little spare time to pack columns, prepare buffers and purification schedules to safely and reproducibly purify their antibodies.

References

1. Berthold W. et al. The Rituxan Story: The Journey of a Chimeric Antibody at a conference entitled “Recovery of Biological Products 10: Learning for the Future” Cancun, Mexico, 2001.

2. Gagnon P. “Purification Tools for Monoclonal Antibodies” p. 155-198. Validated Biosystems, USA (1996).

3. Godfrey M.A.J. “Affinity Separations: A Practical Approach” (Matejtschuk, P., Ed.) p. 141-196. IRL PRESS at Oxford University Press (1997).

4. Perry M. and Kirby H.M. “Protein Purification Applications: A Practical Approach” (E.L.V. Harris and S. Angal Eds.) p. 147-166. IRL PRESS at Oxford University Press (1995).

Figure legends:

Fig. 1 showing the mode of operation of the Proteus Mini and Midi spin columns.

Fig. 2 showing that IgG capacities of Protein A and G Mini and Midi spin columns purified from rabbit serum are unaffected after re-use.

Fig. 3 is a reducing SDS-polyacrylamide gel showing human IgG purified from serum with the Proteus Protein A kit. Lane 1 represents the molecular weight markers; lane 2 represents the human serum sample; lane 3 represents pure human IgG eluted from the Proteus Protein A spin column.

Table legends:

Table 1 highlighting the specifications of the Protein A and G Mini and Midi spin columns.

Fig. 1

Fig. 2

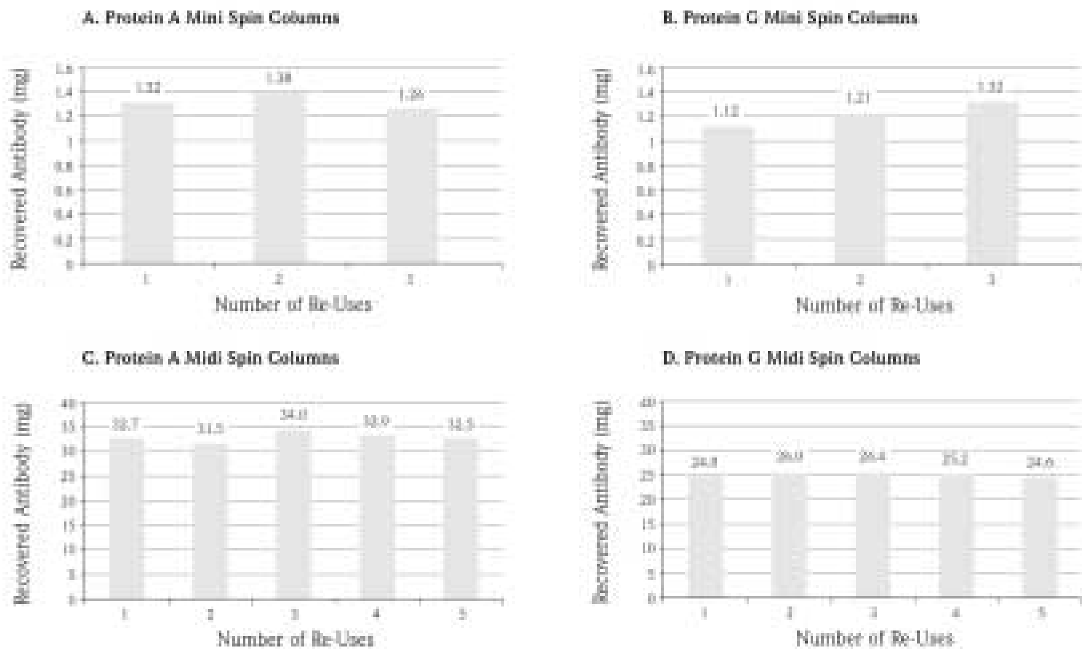

Fig. 3

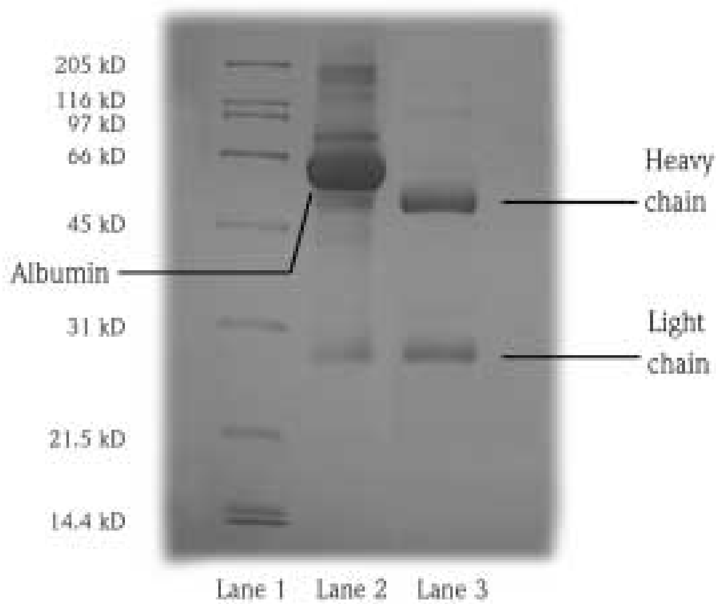

Table 1

MINI & MIDI SPECIFICATIONS

Kit	PROTEIN A	PROTEIN G
Source	Recombinant Protein A expressed in *E. coli* (N.B. No toxic bacterial contaminants normally found in native Protein A)	Recombinant Protein G expressed in *E. coli* (N.B. The recombinant Protein G lacks the albumin-binding domain found in native Protein G)
Binding capacity per use	1 mg human IgG (Mini) 20 mg human IgG (Midi)	1 mg human IgG (Mini) 20 mg human IgG (Midi)
Typical number of uses per plug	3 (Mini) - 5 (Midi)	3 (Mini) - 5 (Midi)
Supporting Proteus matrix	Covalently coupled to agarose resin	Covalently coupled to agarose resin
Resin bed volume	0.23 ml (Mini) - 1.6 ml (Midi)	0.23 ml (Mini) - 1.6 ml (Midi)
Ligand density	3.5 mg Protein A/ml resin	2 mg Protein G/ml resin
Bead size range	60-165 μm	45-165 μm
Maximum volume	0.65 ml (Mini) - 20 ml (Midi)	0.65 ml (Mini) - 20 ml (Midi)
Storage temp. for resin plugs	2-8 °C	2-8 °C
Recommended working pH	pH 2.5-9.0	pH 2.0-9.0
Plastic construction	Polypropylene	Polypropylene
Color coded end-caps	Red	Yellow