Strategies and Innovations: Antibody Purification

Frank Detmers and Pim Hermans at BAC look at traditional methods and novel tools for affinity purification of therapeutic antibodies

Antibodies have been a major focus in biopharmaceutical research and development for the last two decades, ever since the first development of a process for the isolation of monoclonal antibodies (mAbs) in the 1970s. Following the FDA approval of the first therapeutic mAb in 1986 (1), and as a result of significant technological advances in the 1990s, mAbs and antibody fragments are now an increasingly important class of drugs. Reports from 2008 indicate that there are currently over 200 mAb candidates in clinical development (2), with 24 mAbs having been approved for therapeutic use (1).

Therapeutic antibodies in clinical use today cover a broad range of indications, including cancer, chronic inflammatory diseases, transplant rejection, infectious diseases and cardiovascular disease. The majority of antibody drugs are full-sized IgG forms, although there is increased interest in the use of antibody fragments, with three fragment-based drugs being approved for therapeutic use in the US.

ANTIBODY CLASSES, STRUCTURE AND FUNCTION

Human antibodies are grouped into five different isotypes (IgG, IgE, IgD, IgM and IgA) and then split further into sub-classes (IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) (see Figure 1). The different isotypes have distinct functions within the body.

Antibodies are relatively large globular proteins, consisting of two heavy chains (which define the isotype and subclass) and two light chains (see Figure 2, page 46). The heavy and light chains both contain a constant region and a variable region (the antigen binding domain). Within the variable region, antigen binding complementarity determining regions (CDRs) – short hypervariable amino acid sequences – provide antibodies with a high degree of diversity, resulting in their unique antigen affinities. The antibody molecule can be further split into different regions: Fv, scFv, Fab and Fc (see Figure 2).

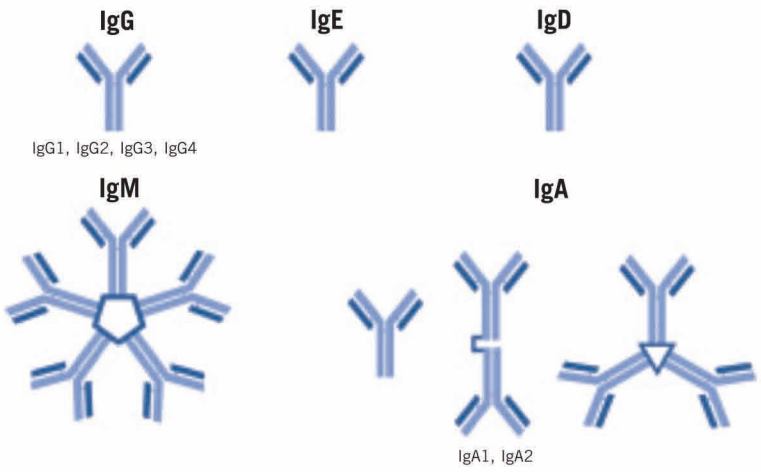
The antibody isotype most commonly focused on in drug development is IgG, and within this class only IgG1, IgG2 and IgG4 have been developed as drugs, as IgG3 has been excluded for several considerations (3). Alternative antibody classes are starting to attract attention, with a number of drugs in early stage development. Several IgM products are in preclinical and Phase I development (Pain Therapeutics, US and Argenes, Japan), whilst research and preclinical work is underway on IgA antibodies with a view to producing therapeutic molecules.

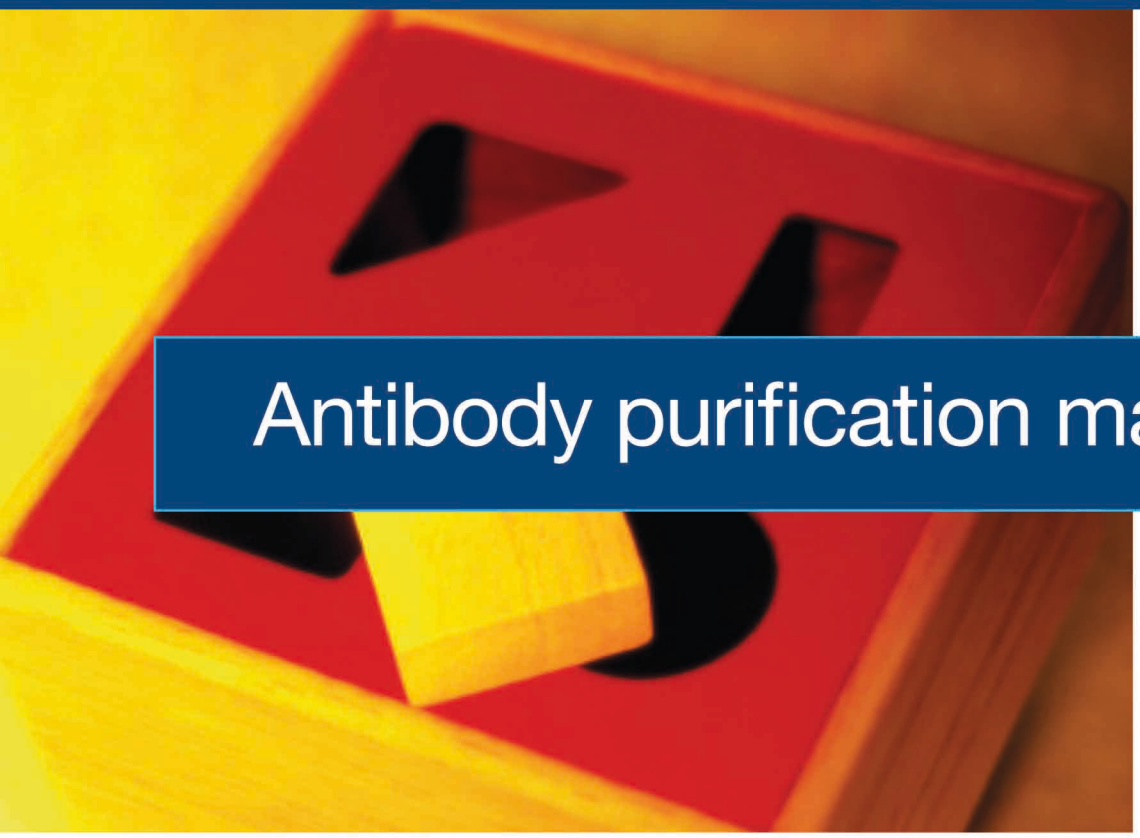
PRODUCTION OF THERAPEUTIC ANTIBODIES

Antibody drugs were originally produced in mice. It was quickly discovered, however, that murine antibodies can elicit a human anti-murine antibody (HAMA) response in the patient. To reduce this reaction, antibodies were engineered and produced as murine:human chimeric (where only the variable domains remain murine), or as humanised (where only the CDRs are murine) molecules. It is now also possible to produce fully human antibodies in transgenic mice by replacing the native murine antibody genes with human ones (see Figure 3, page 46). Over the last five years, the trend has been to opt for humanised, fully human or alternative (fragment) antibody formats.

There is a reported trend towards the study of antibody fragments as therapeutic agents (2), although whether this trend will follow through all the way to

Figure 1: Antibody isotypes and subclasses





Antibody purification made easy

It has never been simpler to select the best affinity media for the purification of your antibodies. Introducing BAC's CaptureSelect Antibody Toolbox: the world's first affinity media range designed specifically for the purification of distinct antibody populations. Now you can select an affinity matrix that is tailor-made to purify specifically the antibody format that you want – making antibody purification easier than ever before.

- Eliminate uncertainty
- Streamline protocols
- Be confident of success

CaptureSelect Human Fab kappa affinity matrix:

for all human kappa light chain containing antibodies and antibody fragments

CaptureSelect Human Fab lambda affinity matrix:

for all human lambda light chain containing antibodies and antibody fragments

CaptureSelect Human Fc affinity matrix:

for all subclasses of human IgG and IgG-Fc fusion proteins

CaptureSelect Multi Species Fc affinity matrix:

for IgG from multiple species

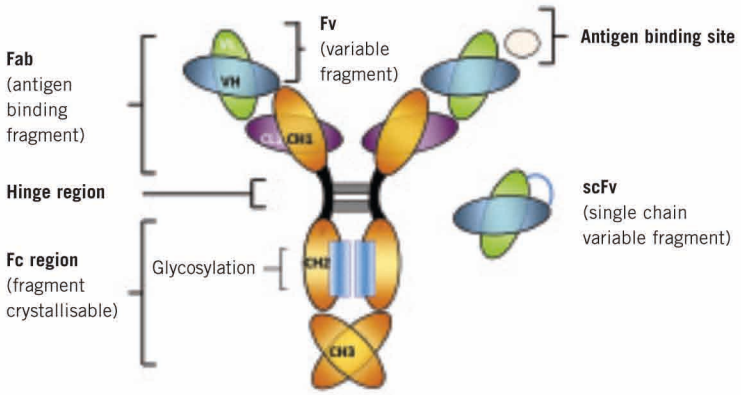
CaptureSelect Human IgA affinity matrix:

for all subclasses of human IgA

CaptureSelect IgM affinity matrix:

for all human, mouse and rat IgM antibodies and antibody fragments

Figure 2: Antibody regions represented on a schematic of a typical human IgG molecule



regulatory approval is yet to be seen. Antibody fragments, being smaller-sized molecules, have the advantage of deeper and more rapid tissue penetration, and the ability to bind otherwise inaccessible epitopes (2). A possible disadvantage of antibody fragments as therapeutics is a reported short circulatory half-life in humans. To counter this, molecules can be PEGylated or otherwise modified to increase half-life (2). There are a variety of alternative antibody formats that can be investigated as potential therapeutics (see Figure 4).

Selection of the antibody isotype and subclass during the discovery phase of drug development should be determined by the desired *in vivo* action of the drug. However, practical considerations must also be considered, such as how the antibody will be produced and purified. Expression systems for therapeutic antibodies range from transgenic animals to mammalian or bacterial cell cultures. Purification of monoclonal antibodies can be achieved using a combination of filtration, ion exchange and size exclusion chromatography steps; however, affinity chromatography provides a much quicker single-step solution.

AFFINITY PURIFICATION OF THERAPEUTIC ANTIBODIES

Protein A

Protein A is a well-established ligand for the affinity purification of antibodies. It is a surface protein originally identified in the cell wall of *Staphylococcus aureus* and has been found to bind the Fc region of IgG from many mammalian species (see Figure 5). By taking advantage of this natural affinity for IgG, a range of Protein A-based antibody purification media has been developed.

Protein A has a high affinity to human IgG1, IgG2 and IgG4 and murine IgG2a and IgG2b. It also has a moderate affinity to human IgE, IgM and IgA, and murine IgG3 and IgG1. Protein A has a low affinity for human IgG3 and does not bind IgD, or murine IgM, IgA or IgE. For alternative antibody formats, Protein A can only be useful in the purification of those fragments that retain the Fc region that Protein A recognises.

While these specificities may be ideal for certain experimental or process requirements, cross reactivity with other species' antibodies and with multiple classes and subclasses of human antibody could be problematic. A further disadvantage is imposed by the need to use a low pH to remove the bound antibody from immobilised Protein A.

Protein A strongly binds to the CH2-CH3 interface on the Fc portion of IgG antibodies. Experimental data indicate that induced fit occurs, which may explain the harsh conditions required for elution. These harsh conditions may affect the conformation of the binding sites, thereby altering the antibody effector functions of purified IgG antibodies (4). Loss of effector functions, caused by denaturation, altered folding and chemical modifications that arise during purification steps, are highly undesirable if the human or humanised antibodies are to be used for therapeutic purposes. It has also been demonstrated that the harsh elution conditions required can be linked to protein aggregation in the purified product (5).

Protein L

Protein L is an antibody binding protein found originally in *Peptostreptococcus magnus* with a binding specificity for the light chain of all antibody classes, with one limitation: Protein L will only bind a portion of antibodies that contain the variable domain of a kappa light chain (VL-κ), but will not recognise all. In humans and mice, the majority of antibodies contain a kappa light chain, while the remainder contain a lambda light chain. Protein L will bind most subtypes of human kappa light chain with the exception of VL-κII. Of the murine kappa light chains, Protein L will bind only the VL-κI subtype.

Protein L does not bind bovine, caprine or ovine antibodies, making Protein L a

Figure 3: Types of therapeutic antibody

Murine
100% mouse protein

Chimeric
33% mouse protein

Humanised
Approximately
10% mouse protein

Fully human
100% human proteins

Figure 4: Alternative antibody formats

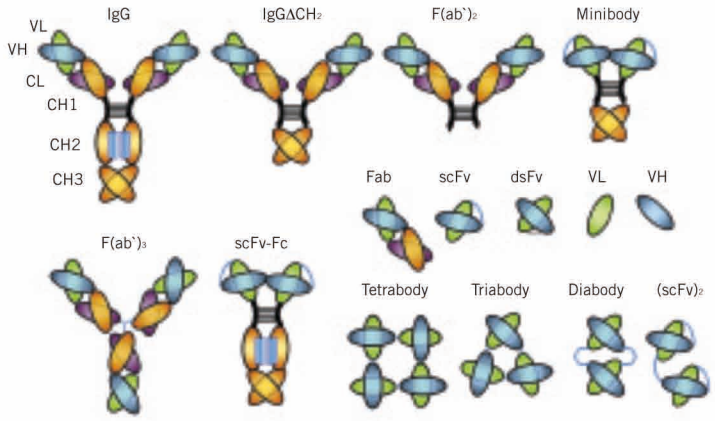
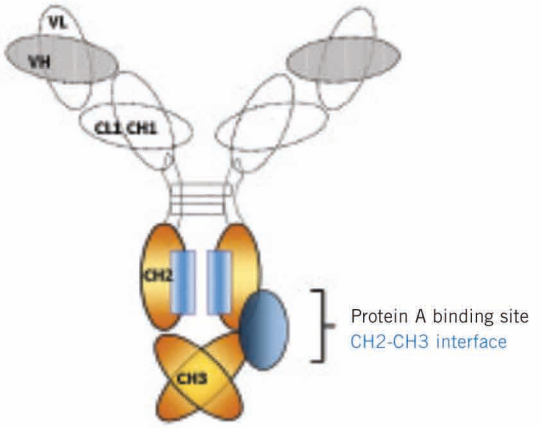


Figure 5: Protein A binding to IgG



useful affinity ligand for the purification of monoclonal antibodies, with known kappa light chain status, from cell culture that contains bovine serum. However, as with Protein A, elution of antibodies bound to Protein L resins requires a low pH. An additional drawback is that it is difficult to produce Protein L in the quantities that are needed for large-scale processes.

VHH Antibody Fragments

The use of Protein A and Protein L for the purification of antibodies can be highly effective. However, it should be remembered that these are bacterial proteins, and not designed specifically for the purposes for which we now use them. There are gaps in the functionality that they provide, and when your target protein falls into one of these gaps, the process of finding an alternative purification strategy can be extremely frustrating.

VHH antibody fragments provide a group of affinity molecules that can be designed for almost any purification target, with as narrow or as broad specificity as is required. The fragments are derived from the unique heavy chain antibodies found in *Camelidae*. Heavy chain antibodies are devoid of the entire light chain and CH1 domain found in conventional antibodies (see Figure 6). As a result of the absent light chain, antigens are bound by the variable domain of the heavy chain (VHH) only, without a loss in binding affinities compared to conventional antibodies.

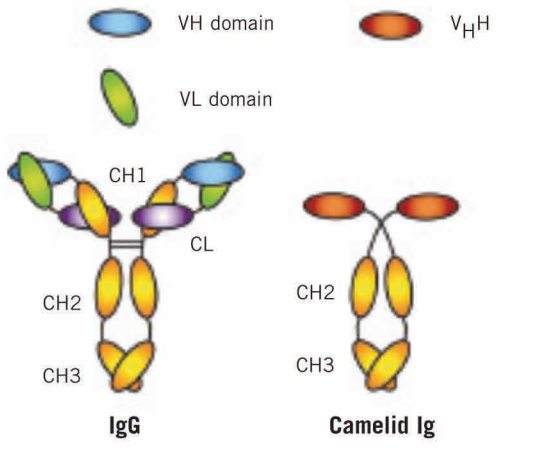
The VHH domain is the smallest known functional antigen-binding fragment that shows high affinity and stability. The equivalent fragment from conventional antibodies is the Fv domain, which consists of the heavy chain variable domain and the light chain variable domain held together by a non-covalent bond. If this bond is broken, all antigen binding functionality will be lost. scFv fragments are produced by synthetically linking the two variable domains together into a single chain, and while this retains the antigen binding functionality of the molecule, these fragments often show reduced affinity compared to the parent antibody. Because the VHH domain exists as a single polypeptide chain, it is an extremely stable and easy way to produce an antibody fragment that retains the full antigen binding activity of the parent heavy chain antibody.

VHH fragments have been used successfully as affinity ligands in chromatography processes for the purification of many types of protein, including antibodies (6,7). Ligands for a specific target molecule are identified from expression libraries that represent the heavy chain antibody repertoire of an immunised llama. Being based on the highly variable mammalian immune system, the expression libraries provide an opportunity to select VHH molecules that meet very specific requirements for specificity, affinity, elution profile and cleaning conditions. In this way, VHH affinity ligands can be created to match exactly the processes for which they are intended.

A range of VHH ligands for the purification of antibodies has been identified and developed. A VHH fragment targeting the CH2-CH3 interface of the Fc region has proven highly effective for the purification of human IgG. This ligand binds all subclasses of human IgG (IgG1, 2, 3 and 4) and is therefore a valuable tool for the production of intravenous immunoglobulin (IVIG), as well as the downstream processing of humanised or human monoclonal antibody drugs. The flexible ligand selection process has enabled desirable binding characteristics to be incorporated, including no cross-reactivity with other species' IgG and elution under mild conditions. Notably, the ligand remains effective for IgG fragments where the CH2 domain is deleted.

VHH fragments that target the constant domain of the light chain region (CL) have also been identified. As for Protein L, the VHH ligand against human kappa light chains will bind to all antibody isotypes that contain a kappa light chain (IgG, IgM and IgA). However, in the case of the VHH ligand, the affinity is specific for

Figure 6: Comparison of conventional IgG structure versus Camelid heavy chain antibody



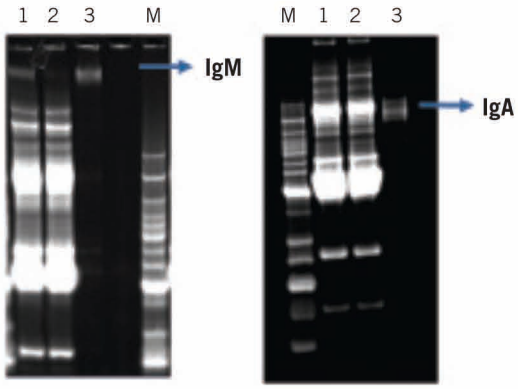
human antibodies and covers all subtypes of kappa light chain.

Similarly to the VHH ligand for kappa light chains, a lambda light chain-specific ligand has been identified. As there is presently no alternative affinity ligand that specifically binds to human lambda light chains, this VHH fragment represents an important and unique resource for both research and industrial processes for the purification of human antibodies and Fab fragments thereof.

Altogether, the Fc, kappa- and lambda-light chain specific VHH fragments represent a toolbox that comprehensively covers all that Proteins A and L provide and more. The VHH ligands offer a number of advantages over the existing purification media, the most useful being complete control of species specificity (here we have described human antibody specificity, but alternative or multiple species specificity can also be provided) and the ability to use mild elution conditions.

Further specificity is also possible with VHH fragments. For example, ligands for the purification of IgM and IgA have been identified (see Figure 7), as well as a ligand for the specific purification of IgG4. All three of these targets have been largely overlooked as therapeutics, despite having some unique and potentially valuable properties, as a result of difficulties encountered in isolating the molecules. It is to be hoped that the availability of a simple-to-use VHH ligand, which enables a one-step purification process, will open up these areas of therapeutics research.

Figure 7: Purification of IgM and IgA with VHH ligands, in a one-step process, at pH3-4. (1) Human serum, (2) Flow-through, (3) Elution, (M) Marker



VHH ligands have been successfully applied as purification media to isolate monoclonal antibody products, although the unique specificity of these ligands has been best demonstrated by the extraction of contaminating bovine IgG from a polyclonal human IgG product expressed in a transgenic cow.

CONCLUSION

The monoclonal antibody market is fast growing, and has the potential to produce some extremely important drugs for a broad range of diseases. It is important that research and development of these drugs is not limited unnecessarily by production constraints – downstream processing being a common hurdle. In particular, the growing interest in antibody fragments as therapeutics requires alternative purification strategies in order to fill in the gaps left by Protein A and Protein L. The identification and development of the VHH domain as an affinity ligand for the purification of antibodies has demonstrated unique flexibility and adaptability as a platform technology. Through selection and optimisation, it is possible to generate a ligand for specific targets, or conversely, to create a universal chromatography medium for the blanket purification of whole antibody populations.

About the authors

Dr Frank Detmers is Director of Ligand Application at BAC. In 2001, he received his PhD at the department of Molecular Microbiology at the University of Groningen (the Netherlands). He worked as a postdoctoral researcher at the Department of Cell Physiology at the Nijmegen Center of Molecular Life Sciences (NCMLS, Nijmegen, the Netherlands). He joined BAC in 2004 and the focus of his work is immobilisation of affinity ligands on solid supports and the development of applications of ligands in healthcare.

After receiving a Bachelors degree in Biochemistry, Pim Hermans joined the R&D department at Holland Biotechnology. In this group, he carried out research on the production and purification of recombinant cytokines and monoclonal antibodies for diagnostic and research purposes. In 1993, Pim joined the Bio-Immunochemistry group at Unilever-Bestfoods, where he was involved in the early development and exploration of camelid derived single domain antibodies (VHHs). Pim joined BAC in 2002. As Director of the Ligand Discovery Department, Pim is responsible for the development of VHH-based affinity ligands for applications in process, and analytical affinity chromatography.

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