Affinity purification for fewer bottlenecks

The single chain antibody found in the *Camelidae* family yields a variable heavy chain domain that is stable and fully functional. **Frank Detmers, Pim Hermans** and **Mark ten Haaft** of the Bio Affinity Company, outline how these properties are useful for drug discovery and bioprocessing

Camelid single chain antibody domains form the basis of a unique type of affinity ligand that enables improved and streamlined purification of a broad range of molecules and complexes. The inherent chemical stability, tunable affinity, high selectivity and short development times of Camelid antibody variable heavy chain (VHH) domains makes them suitable for commercial-scale bioprocessing; solving almost any purification challenge. They are currently employed in a wide range of bioprocess challenges by leading pharmaceutical manufacturers and form the basis of chromatography media marketed by GE Healthcare.

The unusual properties of VHH ligands also make them an ideal tool for laboratory-scale separation challenges where there is increasing demand for flexible, highly sensitive, robust and efficient affinity purification systems. The development of novel affinity ligands with these properties could play a significant role in streamlining purification processes and advancing drug discovery.

affinity ligands

The purification of immunoglobulins (Igs) is often a bottleneck for both drug discovery research and also the commercial scale production of biotherapeutics. The currently prevalent affinity matrices for purification of Igs are Protein A, Protein G and Protein L.

These affinity ligands are fragile and difficult to immobilise without losing activity and have varied affinity across Ig subclasses and species. For example, IgGs generally have a higher affinity for Protein G than Protein A and Protein G can bind IgG from a wider variety of species. Protein L has an affinity for kappa light chains from various species but does not bind well to Igs of species such as bovine, goat, sheep and horse, as they contain almost exclusively lambda chains.

The production of VHH ligands is based on the variability, specificity, and flexibility of the mammalian immune system and thereby avoids the limitations imposed by the affinity and specificity constraints of Proteins A, G and L. Now available off-the-shelf for drug discovery research, the purpose-designed VHH ligands offer significant productivity benefits over current methods due to standardised chromatography conditions and ease-of-use.

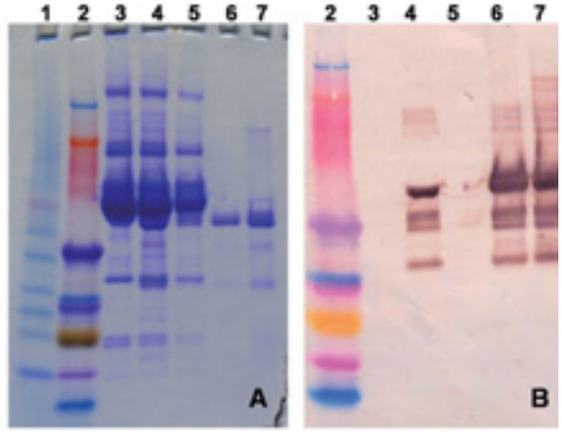


Figure 1: Purification of human Fab kappa fragments analysed by SDS PAGE, stained with coommassie brilliant blue (A) and western blot with a mouse anti human kappa antibody (B). Lane 1 and 2 MW markers; lane 3 cell culture media containing 10% FCS; lane 4 Fab spiked in cell culture media containing 10% FCS; lane 5 flowthrough fraction; lane 6 elution fraction; lane 7 Fab sample used for spiking.

VHH ligands can be selected for affinity to all human IgGs, including IgG3, and Fc fusion proteins, thereby enabling the purification of both monoclonal and polyclonal human IgG populations from transgenic expression systems. An advantage over traditional affinity matrices is the possibility of using these ligands to purify polyclonal intravenous immunoglobulin (IVIG) from plasma and serum in one step.

The inherent flexibility of the mammalian antibody production process enables VHH ligands to be selected for their very specific binding profiles, which can result in a purposefully broad-specificity affinity matrix. For example, a VHH ligand that displayed high affinity binding at a highly conserved area of the Fc region of the Ig heavy chain was selected in order to create an affinity matrix that can purify IgGs from multiple species including mouse, cow, rabbit, rat, goat, horse, human and sheep. The broad specificity of this VHH ligand has similarities with that of Protein G, and yet has the advantage of requiring only mild elution conditions and producing an improved yield of structurally intact and functional IgGs.

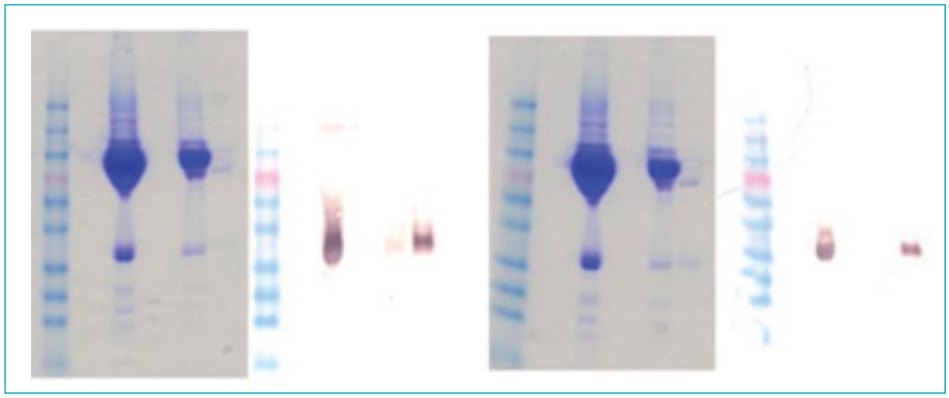
light chain purification

The tunable specificity of VHH ligands is exemplified by the design of affinity ligands for the separation of human kappa and lambda light chain molecules. From the library obtained from a llama immunised with polyclonal human IgM antibodies, it is possible to select ligands that show binding to human kappa chains, but not to lambda molecules, and vice versa. Specificity for human kappa light chain antibodies is demonstrated by purification from a sample of Fab fragments derived from polyclonal human IgG (see fig. 1). As can be seen in this figure, a high purity product is obtained in a single purification step using the VHH ligands.

There is currently no standard affinity ligand that can purify lambda Ig light chains and so the availability of a simple affinity matrix with such specificity could have great significance for researchers in a number of areas, including the study of light chain-associated disorders.

The specificity of these ligands for human immunoglobulins makes them ideal tools for healthcare related studies and applications. Moreover, use of the Fab kappa/lambda ligands together offers a unique affinity media for pan-Ig purification or depletion applications (see fig. 2).

The camelid antibody lacks the light chains found in all classical anti- ▶



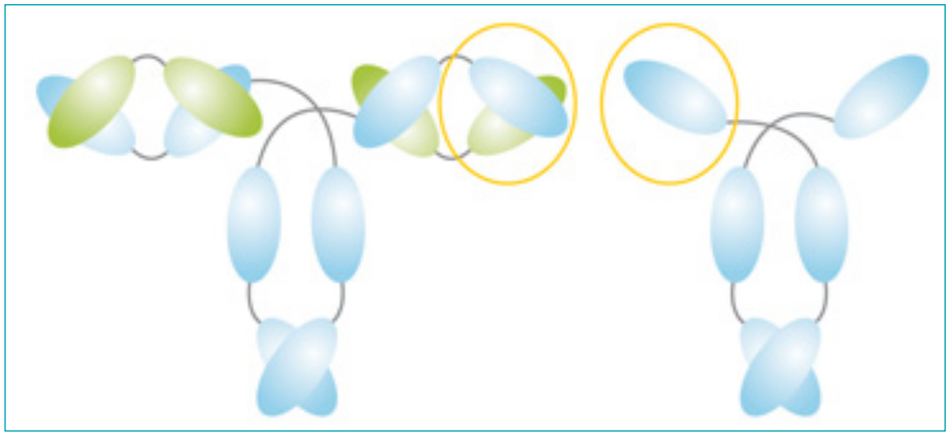


Figure 2 (top): Purification of human Ig lambda (A, B) and human Ig kappa (C, D) from human serum, analysed by SDS PAGE stained with coommassie brilliant blue (A, C) and western blot with a mouse anti human lambda antibody (B) and a mouse anti human kappa antibody (D). Lane 1: MW marker; Lane 2: Human plasma; Lane 3: flowthrough fraction; Lane 4: elution fraction

Figure 3 (above): The difference between classical antibodies (left) and heavy chain antibodies (right). The binding domain is encircled in yellow

bodies (see fig. 3) and, as such, has only one single variable domain (VHH) by which antigens are bound, and two constant domains (CH_2 and CH_3).

The single VHH domain is the smallest intact and functional antigen-binding fragment (12kDa) derived from a fully functional immunoglobulin. Consequently it offers improved affinity, stability and solubility compared with conventional antibody fragments. Their small size and unique 3D structure enables VHH domains to recognise novel epitopes that are inaccessible to classical heavy chain-light chain (VH-VL) pairs.

In addition to being able to modify them easily, both at gene and protein level, VHH domains have been successfully cloned and expressed in microbial systems, enabling safe and cost-effective production, making them ideal candidates for application as affinity ligands in bench research and industrial-scale bioprocessing.

BAC used the camelid VHH domain to develop proprietary CaptureSelect affinity ligands. These ligands are the world's only VHH-based affinity ligands and offer new opportunities for the purification of almost any small molecule product from complex media. Expression of the ligands in *Saccharomyces cerevisiae* and selection of highly versatile characteristics allows the affinity products to be custom made for almost any biotherapeutic purification challenge.

contact
BAC BV
Huizerstraatweg 28
1411 GP Naarden
The Netherlands
info@bac.nl
www.bac.nl

customised ligands

The production of affinity ligands from immunisation through to identification and testing of the best ligands has been streamlined into a process that can be separated into three stages:

Library construction: The mRNA encoding VHH fragments are isolated from the peripheral blood lymphocytes of immunised llama through amplification using polymerase chain reaction techniques. The DNA is cloned into *S. cerevisiae* creating a VHH library that will then be screened for ligands that bind to the target molecules. The library will contain as many as 108 potential ligands.

To select clones to put through to the library screening process a broad test is performed to check the proportion of clones that cross-react with the molecule of interest. This step is performed on a fraction of the clones in the library using ELISA. The percentage of reactive clones is measured and, if high enough, the whole library is progressed to the screening stage. If a low percentage of cross-reactivity is observed then the library is put through an enrichment step using a proprietary yeast display system. The enriched library is then ready for screening.

Library screening: During the screening process, specific requirements are incorporated that closely relate to the final chromatography process, such as elution conditions and stability of the ligand at certain pH values. These characteristics are checked using ELISA techniques to analyse target binding, and Surface Plasmon Resonance to assess ligand stability.

The specificity of the ligands can also be selected at this stage. For example, it may be desirable that the ligands bind to all human IgG but not to bovine IgG; alternatively, ligands that bind to IgG across a number of different species could be selected. Ligands displaying suitable chromatographic characteristics are subsequently cloned as 12kDa fragments into a *S. cerevisiae* production strain, facilitating production of the affinity ligands at any scale. Expression in *S. cerevisiae* enables high-quality, high-titre expression within a system that is totally animal free. This screening reduces the number of potential ligands from a library of around 108 to around 20 clones of interest.

Small scale chromatography testing: The final stage of the development process involves the immobilisation of the ligands onto a solid support for small-scale affinity chromatography testing. The VHH ligands can be coupled to matrices, membranes or magnetic beads using various coupling chemistries, allowing the most suitable solid support to be selected for each application. Small batches of several ligands are produced in shake flasks, purified and immobilised onto the chosen matrix. Product testing for chromatographic characteristics, such as binding conditions, elution conditions and dynamic capacity is carried out before the most suitable ligands are chosen for full scale production.

Production of ligands using *S. cerevisiae*,, in which the ligand gene is integrated into the yeast genome, ensures the ligand products are entirely free of *E.coli* or animal derived components.

As well as laboratory research tools, VHH ligands are ideal for industrial-scale processes. The production of the ligands can be easily scaled up for commercial use. Production of ligands to a scale of $15m^3$ is possible, yielding kilograms of ligand per batch.

Novel ligands like the CaptureSelect VHH ligands offer the biopharmaceutical industry new tools for the purification of monoclonal antibodies, Fab fragments, viruses, blood plasma proteins and other small molecules. The development of affinity matrices based on the variability, specificity and flexibility of the mammalian immune system offers greater choice and significant advantage to the biotherapeutics industry. ■