

# Novel Affinity Ligands for Bioprocessing

The discovery of a unique type of antibody, found only in the *Camelidae* family (camels and llamas), has enabled the development of novel ligands for the purification of monoclonal antibodies, Fab fragments, viruses, blood plasma proteins and other small molecules.

## By Frank Detmers, Pim Hermans and Mark ten Haaft at BAC BV

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

After receiving a Bachelor degree in biochemistry, Pim Hermans joined the R&D department at Holland Biotechnology. In this group he carried out research on 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. Pim joined the Bio Affinity Company 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.

After receiving a Bachelor degree in applied science, Mark ten Haaft joined the Protein Chemistry Group at Unilever-Bestfoods. Mark extrapolated his peptide and protein characterization and purification work to the application of enzymes and peptides as food ingredients, and leveraged this foundation in various Product Development positions at Unilever. Mark joined BAC in 1998, heading the Biochemistry and Analytical Departments. As Director of the Ligand Application Department, Mark is responsible for technical sales and new application development, and is BAC's primary interface with collaborators in the technical evaluation of BAC's affinity ligands and their applications in process and analytical affinity chromatography.



Camelid single chain antibody domains form the basis of a type of unique affinity ligand that enables improved and streamlined purification of a broad range of molecules and complexes. These ligands stand to make a significant impact on industrial bioprocessing technologies, where there is an increasing demand for flexible, highly sensitive, robust and efficient affinity purification systems.

The widespread use of affinity purification in large-scale downstream processing has been hampered by the absence of affinity ligands that fulfil the needs of large-scale affinity chromatography, including chemical stability, tunable affinity, high selectivity, short development times and cost of use. The development of safe and cost-efficient ligands that are effective for commercial-scale bioprocessing is critical for the economic viability and product authenticity of future biotherapeutics.

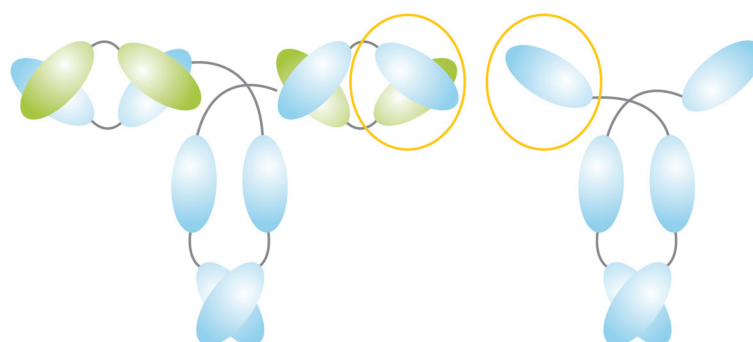
## SINGLE HEAVY CHAIN ANTIBODIES AS AFFINITY LIGANDS

The discovery of a unique type of antibody, found only in the *Camelidae* family (camels and llamas), has enabled the development of novel ligands for bioprocessing. The

camelid antibody lacks the light chains found in all classical antibodies (see Figure 1) and, as such, has only one single variable domain (VHH) by which antigens are bound, and two constant domains (CH2 and CH3).

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 3-dimensional structure enables VHH domains to recognise novel epitopes that are inaccessible to classical heavy chain-light chain (VH-VL)

**Figure 1:** The difference between classical antibodies (left) and heavy chain antibodies (right). The binding domain is circled in yellow.



pairs. In addition to being able to be modified 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 biotechnological processes.

At BAC (the Bio Affinity Company), we have developed a proprietary technology (CaptureSelect®) based on camelid VHH domains. The technology offers new opportunities in the field of custom-designed ligands for commercial-scale bioprocessing of therapeutic products 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.

were selected and produced using BAC's *S. cerevisiae* production system. The ligands were coupled to NHS Sepharose 4B Fast Flow, and specificity for IgG kappa was demonstrated by purification from a sample of Fab fragments derived from polyclonal human IgG (see Figure 2). As can be seen in this figure, a high purity product is obtained in a single purification step using the VHH ligands.

The versatility of this novel technology is demonstrated in a simple powerful solution for the purification of the typically difficult-to-purify Adeno Associated Virus (AAV). Low recoveries are common in the purification of this virus due to a five- or six-step process, which uses a combination of gradient centrifugation, ion exchange chromatography and heparin chromatography. In contrast, the CaptureSelect AAV ligands provide a one-step process with high selectivity for AAV subtypes 1, 2, 3 and 5, ensuring that the ligand can be used as a platform technology for the purification of different AAV subtypes (see Figure 3) with improved purity compared with the conventional process. The elimination of a number of purification steps also minimises product inactivation, thereby maximising product yield.

## PRODUCTION OF CUSTOM AFFINITY LIGANDS

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

### Library Construction

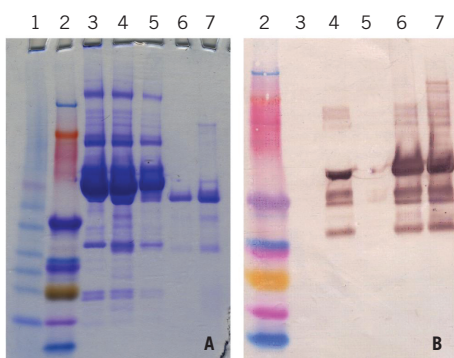
The mRNA encoding VHH fragments is isolated from the peripheral blood lymphocytes of immunised llama through amplification using polymerase chain reaction techniques. The DNA is cloned into *Saccharomyces cerevisiae* creating a VHH library, which will then be screened for ligands that bind to the target molecules. The library will contain as many as 10<sup>8</sup> potential ligands. In order 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.

## LIGAND DISCOVERY AND TUNABLE SPECIFICITY

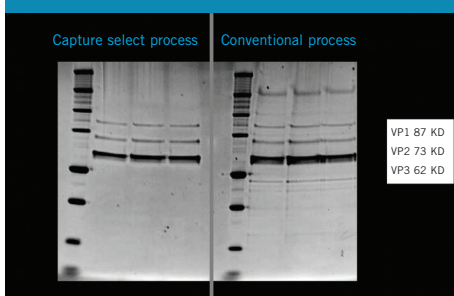
The VHH ligand discovery process is applicable to a wide variety of target molecules. Ligands have been successfully developed for complex antigens such as: bacteria and viruses; proteins, antibody fragments and carbohydrates; and even very small molecules such as haptens, dyes and peptide tags. The fact that large libraries of VHH ligands are screened during the discovery process makes it possible to 'tune' the specificity of the ligand. Ligands can also be selected for their specificity for the format, idiotype, glycoform or isomer of the target molecule.

The tunable specificity of VHH ligands is exemplified by the design of affinity ligands for the separation of human IgG kappa and lambda molecules. From the library obtained from a llama immunised with polyclonal human IgM antibodies, clones producing the VHH domain were screened against different human IgG monoclonal antibodies. Ligands that showed binding to human IgG kappa, but not to IgG lambda,

**Figure 2:** Purification of human Fab kappa fragments analysed by SDS PAGE, stained with coomassie brilliant blue (A) and western blot with a mouse anti-human kappa antibody (B): Lanes 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, flow-through fraction; lane 6, elution fraction; and lane 7, Fab sample used for spiking.



**Figure 3:** The sypro ruby-stained SDS page gel shows the difference between the purity of the purified material using the CaptureSelect ligand, and the conventional process. The samples are purified AAV batches. The picture clearly shows the viral proteins (VP1, 2 and 3) of AAV and the impurities that are in the sample of the conventional process. Capacity of the CaptureSelect process was 1.7E12 GC/ml matrix. Recovery of the column step was between 60 and 70%.



## 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 for 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 stage reduces the number of potential ligands from a library of approximately  $10^8$  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, and then 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.

## LIGAND MANUFACTURING

The production of VHH ligands can be easily scaled up for commercial use (see Figure 4). Production of ligands to a scale of  $15\text{m}^3$  is possible, yielding kilograms of ligand per batch. Ligands are produced following a fed batch protocol, and primary recovery is performed with micro- and ultra-filtration. The final recovery of the ligands is carried out through two chromatography steps. Production of ligands using *S. cerevisiae*, in which the ligand gene is integrated into the yeast genome, ensures that the ligand products are entirely free of *E.coli* or animal-derived components.



## VHH AFFINITY ADVANTAGES

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. In addition, the ease of custom-designing these ligands provides companies with tailored purification systems for various large-scale applications, such as scavenging of impurities or product isolation from complex media.

The development of affinity ligands based on the variability, specificity and flexibility of the mammalian immune system offers huge potential to the bioprocessing industry. The time taken from immunisation to proof-of-principle of ligands in small scale chromatography experiments can be as short as six to nine months, and the ease of large-scale production in well-established *S. cerevisiae* production strains makes VHH ligands ideal affinity tools – conferring benefits in terms of reduced cost of purification, higher quality products and increased flexibility in the purification process.

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**Figure 4:** Large-scale production of VHH affinity ligands.