

# Affinity Chromatography Based Upon Unique Single Chain Antibodies

*Frank Detmers, Pim Hermans, and Mark ten Haaft, BAC*

A unique kind of antibody found only in *Camelidae* offers a novel medium for affinity chromatography. The variable domain of this antibody displays characteristics that make it suitable as a ligand for the purification of a number of small molecules including peptides, proteins and antibodies. This article introduces the camelid antibody and discusses the design of ligands for affinity chromatography in a broad range of applications.

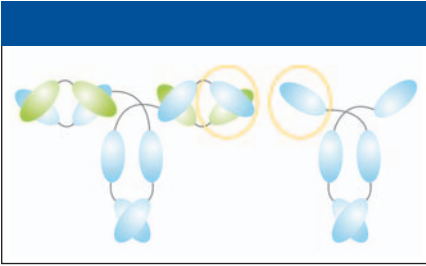
**T**he inherent chemical stability, tunable affinity, and high selectivity of affinity ligands derived from camelid single heavy chain antibody domains makes them suitable for almost any purification challenge, whether at commercial or laboratory scale. These novel ligands enable improved and streamlined purification of a broad range of molecules and complexes, meeting the increasing demand for flexible, highly selective, robust, and efficient affinity purification systems.

## Single Heavy Chain Antibodies

The camelid antibody lacks the light chains found in all classical antibodies (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 (12 kDa) derived from a fully functional immunoglobulin. Consequently, it offers improved affinity, stability, and solubility when compared with conventional antibody fragments. Their small size and unique three-dimensional (3-D) structure enables VHH domains to recognize novel epitopes that are inaccessible to classical heavy chain-light chain (VH-VL) pairs.



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

In addition to being easily modified, both at the gene and protein level, VHH domains have been cloned successfully and expressed in microbial systems, enabling safe and cost-effective production of ligands for affinity chromatography. Expression of the ligands in *Saccharomyces cerevisiae* and selection of highly versatile characteristics allows the affinity products to be customized for almost any biotherapeutic purification challenge.

### **Applications at Industrial and Laboratory Scale**

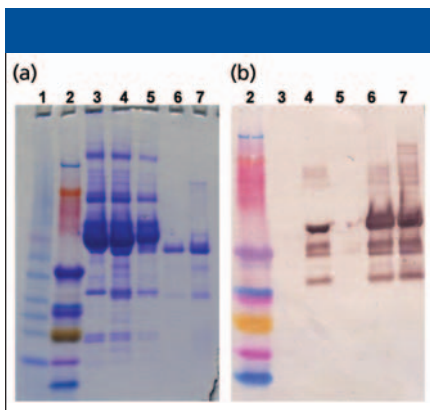
The development of VHH affinity ligands is based upon the variability, specificity, and flexibility of the mammalian immune system, enabling the production of chromatography media with affinity for a wide variety of target molecules. Ligands have been developed successfully for complex antigens such 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 also can be selected for their specificity for the format, idiotype, glycoform, or isomer of the target molecule.

### **Purification of Immunoglobulins**

VHH ligands can be selected for specificity to defined antibody regions, enabling the separation of distinct antibody populations, with a number of advantages over the traditional affinity products Protein A, G, and L. For example, VHH ligands with specificity for a unique region on the Fc domain of human IgG antibodies make it possible to purify all subclasses of IgG, including IgG3. In contrast, Protein A, which also recognizes a region of the Fc portion of IgG antibodies, does not have affinity for IgG3 and also has mild affinity for nonhuman antibodies. VHH ligands with affinity for human Fc can be used to purify both monoclonal and polyclonal human IgG populations from transgenic expression systems, and also can be used to purify polyclonal intravenous immunoglobulin (IVIg) from plasma and serum in a single step.

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 immunized 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



**Figure 2:** Purification of human Fab kappa fragments analyzed by SDS-PAGE, (a) stained with Coomassie brilliant blue and (b) western blot with a mouse anti human kappa antibody. 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.

demonstrated by purification from a sample of Fab fragments derived from polyclonal human IgG (Figure 2). As can be seen in this figure, a high-purity product is obtained in a single purification step using the VHH ligands.

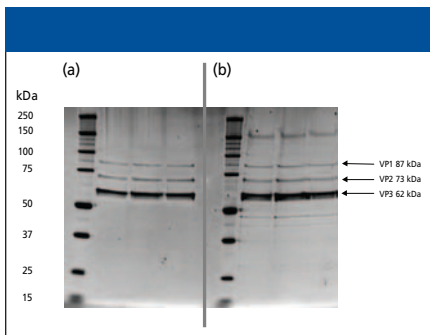
There is currently no standard affinity ligand that can purify lambda Ig light chains and so the availability of a simple affinity matrix with such specificity could have great significance for researchers in a number of areas, including the study of light chain-associated disorders. The specificity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

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. VHH ligands that display high affinity binding at a highly conserved region of the Fc region of the IgG heavy chain can be selected to create an affinity matrix for the purification of IgG 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.

## Purification of Viruses

The versatility of this novel technology has been demonstrated through a project to isolate 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, VHH ligands provide a one-step process with high selectivity to AAV subtypes 1, 2, 3, and 5, ensuring that the ligand can be used as a platform technology in the purification of different AAV subtypes (Figure 3) with improved purity compared with the conventional process. The elimination of a number of



**Figure 3:** The sypro ruby stained SDS page gel shows the difference between the purity of the purified material using (a) the VHH ligand or (b) 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 VHH process was  $1.7 \times 10^{12}$  GC/mL matrix. Recovery of the column step was 60–70%.

purification steps also will minimize product inactivation, and will therefore maximize product yield.

## Production of Custom Affinity Ligands

The production of affinity ligands from immunization 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 immunized 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. 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 analyze target binding and surface plasmon resonance to assess ligand stability. The specificity of the ligands also can be selected for at this stage. Ligands displaying suitable chromatographic characteristics are subsequently cloned as 12-kDa fragments into a *S. cerevisiae* production strain, facilitating production of the affinity ligands at any scale. Expression in *S. cerevisiae* enables high-quality, high-titer expression within a system that is totally animal component-free.

**Small scale chromatography testing:** The final stage of the development process involves the immobilization 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 immobilized 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 *Saccharomyces 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.

**Industrial scale ligand manufacturing:**

As well as making exceptional laboratory

research tools, VHH ligands are ideal for industrial-scale processes. The production of the ligands can be scaled up easily for commercial use. Production of ligands to a scale of 15 m<sup>3</sup> is possible, yielding kilograms of ligand per batch.

**VHH Affinity Advantages**

The flexibility and broad applicability of VHH ligands offers the scientific community new tools for the purification of monoclonal antibodies, Fab fragments, viruses, blood plasma proteins, and other small molecules. The development of affinity matrices based upon the variability, specificity, and flexibility of the mammalian immune system offers greater choice and significant advantage over traditional affinity chromatography systems. ■