Review

CellPress

Nanobody-based products as research and diagnostic tools

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Since the serendipitous discovery 20 years ago of *bona fide* camelid heavy-chain antibodies, their single-domain antigen-binding fragments, known as VHHs or nanobodies, have received a progressively growing interest. As a result of the beneficial properties of these stable recombinant entities, they are currently highly valued proteins for multiple applications, including fundamental research, diagnostics, and therapeutics. Today, with the original patents expiring, even more academic and industrial groups are expected to explore innovative VHH applications. Here, we provide a thorough overview of novel implementations of VHHs as research and diagnostic tools, and of the recently evaluated production platforms for several VHHs and VHH-derived antibody formats.

From conventional antibodies to antibody fragments

To date, the European Medicines Agency and US FDA have approved ~35 monoclonal antibodies (mAbs) for therapeutic applications. Most of these antibodies are chimeric or humanised full-length antibodies, whereas only a few are derived, next-generation antibody fragments, such as the 55-kDa fragment antigen-binding (Fab) (Figure 1A). Another antibody fragment, the 28-kDa single-chain variable fragment (scFv) (Figure 1A), has not yet been approved, but several are being evaluated in clinical trials [1]. This trend towards smaller antigen-binding antibody formats applies to both therapeutic and diagnostic antibodies, because antibody fragments are more amenable to faster engineering and cheaper production, and show enhanced tissue penetration and lower immunogenicity [2].

A distinct type of antibody fragment, termed VHH or nanobody, is derived from heavy-chain-only antibodies that circulate in sera of camelids, such as llamas, dromedaries, and camels (Figure 1B). This small-sized (15 kDa), autonomous VHH domain is readily produced as a highly soluble and robust entity. Its single-domain nature and strict monomeric behaviour support easy cloning, fast selection from immune or naïve VHH libraries (Box 1), and straightforward design into multivalent and pluripotent antigen-binding formats. Moreover, because VHHs prefer to associate with concave-shaped epitopes (e.g., catalytic sites of enzymes), they are able to recognise sites that are inaccessible or cryptic for conventional antibodies [3,4]. Although several VHHs have been developed as new magic bullets for therapy and are currently evaluated in phase I and II clinical trials by Ablynx (http://www.ablynx.com), VHHs have also paved the way for novel, highly valuable applications in diagnostics, protein or cell research, and even agriculture (Box 2).

VHHs in research

VHH GFP-binding protein (GBP) sets the tone

A clear breakthrough for VHHs in research was the development of chromobodies. These molecules comprise a VHH fused to a fluorescent protein and, due to the stability of the VHH, fold into functional antigen-binding entities, often even in the reducing environment of the cytoplasm within living cells. After expression and binding their specific antigen, chromobodies serve as tracers for *in vivo* intracellular target localisation studies (Figure 2A), avoiding the need for genetic modification of target proteins with fluorescent tags. As a proof-of-concept, an anti-GFP VHH, termed GBP, was fused to monomeric RFP and the resulting GFP–chromobody could specifically label cytoplasmic or nuclear localised GFP fusion proteins [5]. Other chromobodies were developed for the direct visualisation of native, endogenous proteins [6] or HIV virions [7] in living cells, and several of these nanobody-based tracers are made available by ChromoTek (http://www.chromotek.com).

The GBP is also applied in super-resolution microscopy for the visualisation of GFP fusion proteins. When full-length antibodies coupled to organic dyes are used as primary antibodies, linkage errors arise because of the distance between the organic dye and the actual localisation of the protein. Due to the smaller size of VHHs, coupling the dye to the GBP results in improved labelling with minimal linkage error [8]. Similarly, gold nanoparticle-coupled GBP is used for single-molecule tracking of GFP-tagged membrane proteins and is even internalised by electroporation to track intracellular proteins in living cells [9].

Recently, a GBP-based fluorescent-three-hybrid approach has been developed to study *in vivo* protein–protein interactions: GBP is first fixed at a particular subcellular

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Keywords: nanobody; recombinant protein production; antibody fragment; GFP-binding protein; plant production platform; yeast production platform.

0167-7799/$ – see front matter
 http://dx.doi.org/10.1016/j.tibtech.2014.03.001

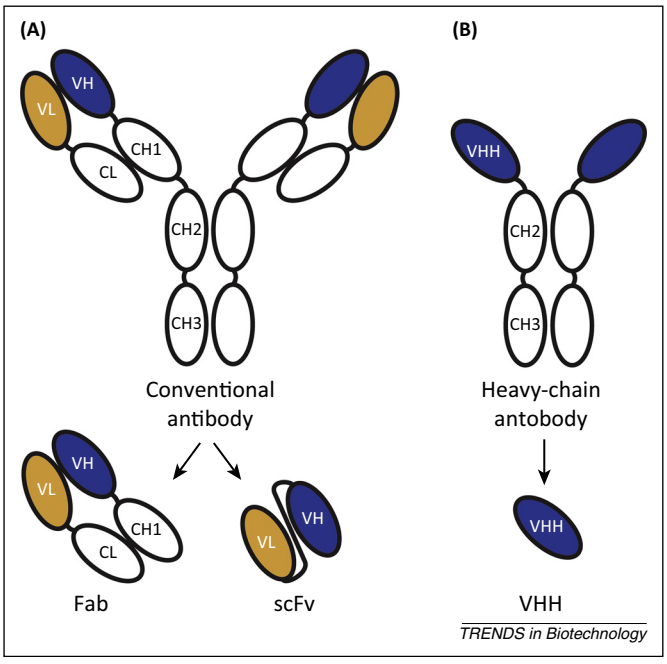

Figure 1. Antibodies and antibody-derived fragments. **(A)** The antigen-binding domains of conventional antibodies can be produced as Fab entities or scFvs, where VH and VL domains are fused by a flexible linker. **(B)** Heavy-chain antibodies lack the light chain and CH1 domain of conventional antibodies. Their antigen-binding fragment comprises an autonomous single variable domain (VHH) that is easily produced recombinantly. CH1, first constant domain of heavy immunoglobulin chain; CL, constant domain of light immunoglobulin chain; Fab, antigen-binding fragment; scFv, single-chain variable fragment; VH, variable domain of heavy immunoglobulin chain; VL, variable domain of light immunoglobulin chain.

Box 1. VHH selection by phage display

In general, following immunisation of a camelid and subsequent cloning of the VHH-only repertoire in phage display vectors, VHHs are readily selected by a few rounds of panning against an antigen, coated in wells of microtitre plates. However, because panning conditions are crucial in determining the final outcome of the VHH binding properties, alternatives exist to mimic the conditions of the intended VHH application. For example, valuable diagnostic VHHs with a preference towards denatured antigens are directly isolated by panning on polyvinylidene difluoride membranes (i.e., western panning) [79], and VHHs that were intended for formulation in shampoo, were selected in the presence of shampoo [80]. Instead of using phage display, VHHs can also be isolated by bacterial two-hybrid screening to identify VHHs for intracellular usage [81], by ribosome display [82] and by yeast [83] or bacterial [84] surface display in combination with fluorescence-activated cell sorting.

In case purified antigens are not available, one can rely on synthetic [85] or naive [86] VHH libraries. Alternatively, several reports describe the generation of VHHs by immunisation with and panning against crude protein extracts to screen for novel, unknown biomarkers or to omit the need for producing and purifying antigens [32,46,87,88]. In this approach, the lead VHHs are subsequently used as affinity ligands to identify the respective antigens by affinity purification or immunoprecipitation, followed by mass spectrometry.

In contrast to these VHH-only libraries, Kastelic and colleagues [89] constructed a mixed llama VHH–VH library and noticed a preference towards retaining VH domains after three selection rounds. Llama VHs are considered less immunogenic than VHHs, thus, they might prove useful for future therapeutic applications. Similarly, others have observed an enrichment of single-domain antibody binders with VH hallmarks after panning a naive VHH library and have argued that this bias originates from a more efficient display of such antibody fragments using the Sec-dependent periplasmic export pathway [90].

compartment by fusing it to an anchoring protein, whereas the two proteins of interest are fused to either GFP or RFP. Upon interaction, both proteins cluster together and a strong GFP–RFP colocalisation signal is observed at the location defined by the GBP. This approach has been successfully demonstrated in the nucleus and cytosol of human cells, and is used to analyse peptide inhibitors of protein–protein interactions [10].

VHHs for protein purification and immunoprecipitation

VHHs are used as protein purification ligands because of their stability, monomeric nature, and easy directional immobilisation to solid supports. The stability ensures a high column regeneration capacity, whereas the monomeric nature allows for an increased amount of paratopes per gram of support material, and sometimes for milder elution conditions as compared to full-length antibody formats. VHHs with an anti-human IgG specificity were developed for depletion of IgGs from blood, outperforming conventional protein-A-based purification [11]. These VHHs, together with VHHs against human serum albumin, human growth hormone and mouse IgG, were commercialised by BAC BV/Life Technologies (http://www.lifetechnologies.com). They also developed the CaptureSelect C-tag, a new protein affinity tag of only four amino acids (EPEA), and its corresponding anti-CaptureSelect C-tag VHH for efficient protein purification, showing that, in contrast to what one would expect based on the VHH preference for concave epitopes on properly folded proteins, VHHs can be generated against small (linear) peptide tags. This has also been confirmed by isolating VHHs against KDEL, a C-terminal signature tag of endoplasmic reticulum-resident proteins [12]. Interestingly, the easy cloning of the single-domain VHH allows the generation of a bivalent anti-KDEL VHH with enhanced functional affinity in ELISA [12].

VHHs have also been successfully evaluated for immunoprecipitation purposes [13] and for chromatin immunoprecipitation with DNA microarrays (ChIP-on-chip), leading to the discovery of novel transcription factor binding sites [14]. An inventive approach for VHH-based immunoprecipitation was developed by Pollithy and colleagues [15] (Figure 2B). Magnetosomes, which are membranous organelles present in magnetotactic bacteria, contain magnetite particles enabling orientation in a magnetic field. By expressing VHHs in-frame with a magnetosome membrane protein, VHH–coated magnetosomes were produced that were used as capturing agents for coimmunoprecipitation by magnetically separating the VHH particle–antigen complexes from nonbound protein contaminants. Such VHH particles could potentially also be used for elucidating bacterial protein complexes *in vivo*.

Targeted functional inactivation of proteins with VHHs

VHHs are stable molecules that often bind catalytic sites [3], thus, they are also exploited as *in vivo* immunomodulators (intrabodies) to interfere with protein conformation, localisation, or functioning. For example, promyelocytic leukaemia protein that is normally dispersed throughout the nucleus, is specifically redirected to the nuclear lamina by fusing it to GFP and coexpressing GBP–lamin1 as a nuclear lamina anchor [13]. The approach has also been