CHAPTER 6

Immune Technology

SUMMARY

Antibodies are Y-shaped structures that function as part of the immune system. They recognize and bind to foreign molecules called antigens. Antigens are molecules that elicit an immune response. They are not recognized as "self" and are usually protein substances made by invading viruses or bacteria. Glycoproteins and lipoproteins elicit the strongest immune responses. Antibodies are ever-present within the organism. They wait for a foreign substance to appear, act on that substance, and then present it to immune system cells for memory and production of many more antibodies that are highly specific to the antigen.

Each antibody has four subunits of proteins: two light chains and two heavy chains. Each of the two types of chains has a constant region and a variable region. The chains are held together by disulfide bridges. The variable region is the part of the antibody responsible for binding portions of antigens called epitopes. The "hinge" region is the place where the heavy chains bend, creating three pieces: two identical Fab fragments and one Fc fragment. The Fab fragments house the antigen-binding site. The Fc fragment is the lower portion containing the two heavy chains that is recognized and bound by some immune system cells. Since there are so many different types of epitopes on many different pathogens, antibodies must be extremely diverse. This diversity is accomplished by shuffling around a few genes to generate new combinations of proteins at the variable regions, as opposed to the presence of separate genes for each antibody.

There are two sides to the immune system: specific immunity and acquired immunity. Acquired immunity is further divided into humoral immunity and cell-mediated immunity. Humoral immunity relies on antibodies, also called immunoglobulins, which are produced by B cells. Antigen-specific T cells moderate cell-mediated immunity by recognizing class I or II cell surface receptors called major histocompatibility complexes (MHC I or MHC II). These receptors are present on cells that are infected by a pathogen.

Antibodies can be further divided into classes based on their heavy chains. Immunoglobulin G (IgG) is the most abundant class of antibodies in blood serum and is also able to cross the placental barrier in pregnancy. IgA is another type of antibody often found in serum, colostrum, and mucosal secretions. IgA is particularly important in helping to prevent gastrointestinal infections in infants. IgM is a pentamer that acts to sequester entire pathogens that can then be targeted as a whole by immune system cells. IgD exists in small numbers, but the function is unclear. IgE is an antibody attached to certain cells that release histamines in response to allergic reactions.

Monoclonal antibodies are pure antibodies made from a single line of cells. They are more useful for biotechnology than polyclonal antibodies, which are a mixture of antibodies made from several lines of cells that all recognize the same antigen. The potential uses of monoclonal antibodies include cancer treatments. These antibodies could target very specific epitopes on the cancer cells. Antibodies are often engineered in mice, which poses a problem for humans because the human immune system recognizes the mouse antibodies as foreign and destroys them. Genetic engineering to make humanized monoclonal antibodies is one possibility to circumvent this issue. Humanizing monoclonals involves swapping out the constant region of the antibody from mice with a human constant region to create a hybrid molecule: human constant region and mouse variable region. This hybrid does not provoke a strong reaction from the immune system. Additionally, portions of the variable region that are not actively involved in binding antigen can be engineered to be more human-like. Currently, several humanized monoclonal antibodies are being used in treatments, such as for breast cancer. Trastuzumab (Herceptin) was approved by the FDA in 1998. This drug binds to a cell surface receptor (HER2), which is overproduced in chemotherapeutic-resistant breast cancers. Blocking HER2 receptor prevents cell division and induces the immune system to attack the cell. Infliximab (Remicade) treats rheumatoid arthritis by targeting TNFα in the joints of people with RA. TNFα is involved in

inflammation and immune system function. Blocking TNFα further blocks the release of proinflammatory cytokines, which reduces inflammation.

Engineered antibodies have a number of potential roles in biotechnology. Antibodies could be engineered to specifically bind an antigen on one side, and then have an active portion on the other side, which would be bound to a toxin or enzyme. The recombinant antibody would be highly specific and could be delivered straight to the source. Camels and other related animals have interesting antibody structures. The antibodies of these animals consist of only heavy chains. No light chains are involved. These newly discovered antibody structures are potentially useful in therapeutics. For example, a small molecule containing only the antigen-binding domain is more easily engineered because it is smaller, lacks disulfide bonds yet is still stable, is not easily influenced by denaturing forces, has a high affinity for antigen, and has a flexible shape. These antibodies can even recognize dimples or indentations as epitopes. The smaller molecule containing only the variable domain is called a nanobody. These nanobodies are small enough to pass through the blood-brain barrier. Diabodies are also engineered antibodies that have two antigen-binding sites pointing in opposite directions. These recombinant antibodies could potentially bind two different targets simultaneously.

The enzyme-linked immunosorbent assay (ELISA) is used for the detection and estimation of protein concentrations in a sample. Specific antibodies are made against the target protein (antigen). The antibody also has a detection system linked to it, usually an enzyme (e.g., alkaline phosphatase) that converts a colorless substrate to a colored product. The antigens are immobilized in the bottoms of a dish, and then the antibodies linked to the detection system are added. The antibodies bind to the antigen and then are assayed for production of the colored product. The concentration of the antigen can be estimated by the intensity of the color produced during the detection process. ELISA is often used to detect diseases in animals, including humans, and plants. These assays are efficient and can be used at home or in the field. A home pregnancy test is basically an ELISA to detect for the presence of human chorionic gonadotropin (hCG), which is produced by the placenta during pregnancy.

Within cells, the location of specific proteins can be detected using either immunocytochemistry (in cell cultures) or immunohistochemistry (in prepared tissues). A primary antibody is added to permeabilized cells and targets a specific protein. A secondary antibody with a linked detection system recognizes the primary antibody bound to the antigen inside the cell. Detection of the secondary antibody also corresponds with location of the primary antibody and antigen. Fluorescent labels are the most common detection system on secondary antibodies. Detection involves excitation of the labels with UV light and subsequent digital capture of the emitted wavelengths.

Different T cells have different types of cell surface receptors called cluster of differentiation (CD), either CD4 or CD8, depending on the cell type. These surface receptors are used to physically sort the cell types present in a mixture into separate tubes using a technique called fluorescence-activated cell sorting (FACS). Antibodies labeled with two different fluorescent labels recognize either CD4 or CD8. Single droplets containing only one cell are subjected to an electrical charge. Depending on the fluorescence, the charge moves the cell to the right or left into different tubes. A third tube gathers cells that emit no fluorescence. One tube contains cells with only CD4 receptors; the other tube contains cells with only CD8 receptors. A third tube gathers cells that do not fluoresce. Flow cytometry utilizes the same principle as FACS except the cells are not sorted and retained, but rather the information is retained and plotted on a graph.

Vaccines are based on the principle that usually after a human is exposed to a pathogen, the immune system "remembers" those antigens. Upon subsequent exposure to the same pathogen, the immune system's memory takes care of the pathogen before it causes disease. Immune system cells called B cells are responsible for memory. Several types of vaccines are produced to help prevent disease. In heat-killed or denatured vaccines, the live disease agent

is killed but still maintains antigens that are recognized by the body's immune system. Some vaccines contain live disease agents that are modified so that they no longer produce the protein or toxin to cause disease. These are called attenuated vaccines. In subunit vaccines, just a portion of the protein from a pathogen is used to elicit an immune response. Subunit vaccines are developed using recombinant DNA technology. Vector vaccines are produced through genetic engineering. A gene from a pathogen is expressed in a nonpathogenic bacteria or virus. Immunity is induced against the protein from the gene and also the nonpathogenic host of the vector.

New candidate antigens are discovered using techniques such as reverse vaccinology. In this process, the genome of a pathogen that has already been entirely sequenced is scanned to identify potential candidate antigens for a new vaccine. New antigens are also discovered using the process of differential florescence induction (DFI) or *in vivo* induced antigen technology (IVIAT). Both of these approaches utilize the differential expression of genes in the pathogen upon infection within a host. In DFI, gene fusions between a library of genes from the pathogen and green fluorescent protein (GFP) are transformed into bacterial cells. The bacteria that express the gene fusion under conditions that mimic entry into a host are sorted and selected for by FACS. In IVIAT, serum from a previously infected individual is mixed with the pathogen, which sequesters only the antibodies that are specific to that pathogen, and presumably, expressed only after infection. The proteins corresponding to these antigens are identified by construction of expression libraries and subsequent analysis using the leftover antibodies.

DNA vaccines consist of only a gene for a protein that can elicit an immune response. Once the gene enters the cell, it gets expressed into protein, which is recognized by the immune system and immunity is built against it. The problem with DNA vaccines is that they have the potential to cause an autoimmune reaction if the immune system identifies specific sequence on the DNA as foreign and elicits an immune response. This reaction could spill over onto "self" DNA and cause autoimmunity. Edible vaccines provide an advantage over traditional vaccines because there is not necessarily a need for refrigerators, syringes and needles, or qualified staff to administer the vaccine. However, the vaccines must be able to withstand the acidic environment of the stomach, along with the digestive enzymes present in the stomach and intestines. Another drawback is the possibility that an attenuated agent could revert back to its virulent form and cause disease. Research into using vegetables as vaccine-delivering devices is also underway. Potatoes have been used to deliver antigens of hepatitis B to individuals in the hopes that their immune systems could be boosted without the use of more traditional vaccines. Other plants in addition to crop plants are being investigated as possible sources of oral vaccines.

Case Study Nanobody-Based Products as Research and Diagnostic Tools

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Antibodies not only are naturally made, defensive proteins, but also are exploited in multiple industries including research, diagnostics, and therapeutics. Approximately 35 monoclonal antibodies (most are humanized or chimeric) are currently approved for therapeutic use in Europe and the United States. Some of these approved antibodies are fragments, such as Fab. Research into the use of shorter scFv fragments is still ongoing, and applications have not yet been approved. Smaller antibodies have advantages in that they are faster to engineer, cheaper to make, penetrate tissue more easily, and are less immunogenic.

Camels and related animals produce heavy-chain-only antibodies. Antibody fragments derived from these are called VHH or nanobodies and have properties that make them particularly interesting for diagnostic and therapeutic processes. For example, nanobodies are smaller, have a single domain, and can associate with concave epitopes. They show great promise with regards to accessing cryptic sites or enzyme active sites.

What are chromobodies, and how do they represent a "clear breakthrough"? What role do they play in biotechnology?

Chromobodies are a VHH fused to a fluorescent protein that operates within the cytoplasm of cells and can bind specific antigens. Since they have a fluorescent tag, they can be visualized by fluorescence microscopy during localization studies without the need to genetically engineer a target protein with a fluorescent tag. The antibody would target the protein instead. They have been used to visualize native proteins, GFP-labeled proteins, and HIV virions.

GBPs are anti-GFP VHHs. What applications are using GBP-based systems?

GBPs have been fused to red fluorescent proteins (RFPs) and used to target and identify the location of GFP-fused proteins within cells. Also, linkage errors occur when full-length antibodies are coupled with organic dyes due to the larger-sized antibody and distance issues. To circumvent this issue, VHHs are smaller and would have less distance between the dye at the protein. Single molecules can also be tracked using gold nanoparticle-coupled GBP. *In vitro* protein–protein interactions have been studied using a recently developed GBP-based fluorescent-three-hybrid approach.

How else are VHHs being used in protein purification and immunoprecipitation?

VHHs have been used as ligands in protein purification techniques. VHH-based protein purification allows the columns to be regenerated more easily with an increased amount of paratopes per unit of the column support material due to the stable, monomeric nature of VHHs. VHHs with anti-human IgG, anti-human serum albumin, anti-human growth hormone, and anti-mouse IgG have been developed and commercialized. These systems outperform traditional protein purification techniques. VHHs against small, linear peptide tags have also been developed, such as the VHH purification system against KDEL, a C-terminal tag of endoplasmic reticulum proteins. In addition to protein purification applications, VHHs have been developed for immunoprecipitation roles, which have allowed the discovery of new binding sites for transcription factors.

VHHs can recognize concave sites, such as enzyme active sites. Are VHHs currently being used in applications that require recognition of concave sites? If so, give examples.

Yes. VHHs that recognize concave sites are employed to interfere with protein conformation, localization, and/or function. For example, promyelocytic leukemia protein can be relocalized to the nuclear lamina by fusing it to GFP and coexpressing GBP-lamin1. In plant cells, GBP traps nuclear GFP fusion proteins in the cytoplasm to block their activity. The catalytic activity of potato starch branching enzyme A has been inhibited using a VHH, which increases the sugar content of the tuber extracts. In animal cells, GFP fusion proteins are targeted for degradation when GBP-based fusions recruit polyubiquitination machinery. GBPs can even target different regions of GFP to increase or decrease fluorescence.

X-ray crystallography is a technique to determine the protein structure. Other than providing extra surfaces for crystallization, how have VHHs influenced crystallization techniques?

VHHs act as crystallization chaperones by either stabilizing a particular protein conformation or preventing oligomerized or misfolded structures from forming.

Because of their small size and stability, VHHs have many advantages over other detection probes in diagnostic applications. Give examples of how VHHs are used to diagnose pathogens and other medical ailments.

Monoclonal antibodies have a high degree of sensitivity for their targets, but they often lack specificity. VHHs are sensitive and specific. VHHs have been used to distinguish between *Brucella* and *Yersinia* infections in livestock and specific species of tapeworm (*Taenia*) in pigs. They have also been developed to target tumor-associated proteins and advance current imaging techniques that presently require longer exposure to radionuclides. VHHs increase the speed at which images can be obtained and, thus, decrease the radionuclide exposure time.

How are VHHs produced?

VHHs are produced in bacteria, fungi, mammalian cell lines, plants, and insects, depending on desired characteristics and properties of the VHH. For example, bacteria yield high amounts of VHH in the cytoplasm or periplasm. Fungal systems secrete VHHs. Mammalian cell lines are useful for secretion of glycoproteins. Plants are similar to mammalian cell lines without the risk of pathogen contaminants and the expense associated with animal cell techniques. Production of VHHs in insects could potentially be useful for protecting inhabitants of developing countries from diarrheal diseases.

Due to their monomeric nature and small size, VHHs are more stable, easier to engineer, and less problematic in many biotechnological applications. VHHs are currently being used in basic research techniques, such as protein purification, immunoprecipitation, and X-ray crystallography, and as probes in diagnostic applications, such as ELISA, imaging, and pathogen detection. The possibilities are tremendous with regards to VHH-based systems.

Review

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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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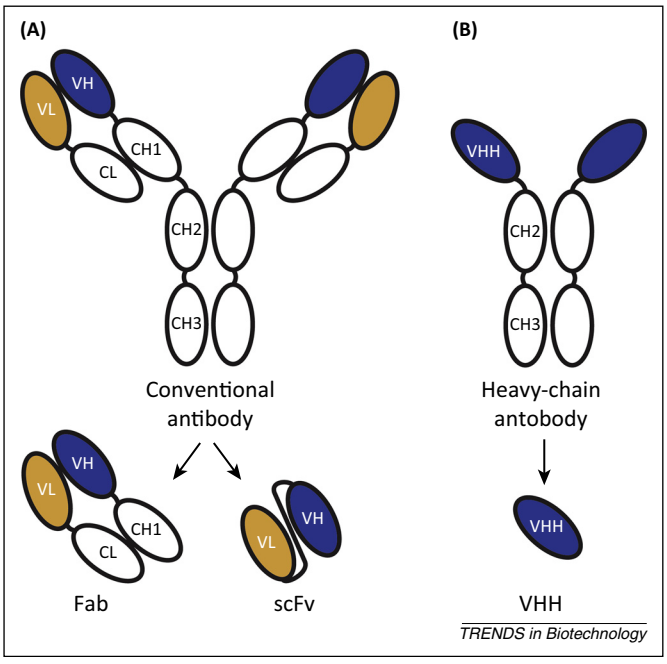

Figure 1. Antibodies and antibody-derived fragments. **(A)** The antigen-binding domains of conventional antibodies can be produced as Fab entities or scFvs, where VH and VL domains are fused by a flexible linker. **(B)** Heavy-chain antibodies lack the light chain and CH1 domain of conventional antibodies. 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

Box 2. VHHs in agriculture

Agrosavfe (http://www.agrosavfe.be/), a company established in 2013, aims to exploit VHHs as carriers for the efficient and more specific delivery of existing chemicals to crops and weeds. Their agrocapsules are engineered, depending on the application, with slow or fast release properties and covalently coupled to plant-specific VHHs [91]. In this way, VHHs can improve the chemical retention time at the target site and reduce the amount of chemicals needed. The plant-specific VHHs have been shown to bind, for example, trichomes, stomata, and the surface area of potato and grass leaves [92]. Importantly, they should also withstand harsh field conditions, such as variable pH, temperature, and salt concentrations.

The agrocapsule approach has also been applied for the VHH-based specific delivery of insecticides to insects [91,93]. To pursue good efficacy, the VHH phage selections are performed against living aphids and even whole-insect ELISAs have been developed for subsequent VHH characterisation.

translated to plant cells, where GBP is used to block the action of nuclear GFP fusion proteins by trapping them in the cytoplasm [16]. Interference with protein functioning has been demonstrated by generating an inhibitory VHH against potato starch branching enzyme A [17]. Upon targeting the VHH to potato plastids, the amylose content in tuber extracts increases due to specific VHH-based enzyme inhibition, and the increase is even higher compared to that of the antisense control lines.

Another mode of action of intrabodies is provided by deGradFP in mammalian cell lines and fruit flies [18,19]. In this approach, GFP fusion proteins are targeted for degradation *in vivo* by coexpressing the GBP fused to the F-box domain to recruit the polyubiquitination machinery (Figure 2C). Moreover, the resulting protein knockout can be restricted to certain tissues, and the extent of protein degradation can be followed over time by measuring GFP fluorescence. VHHs are also able to modify protein conformations. For example, GBPs are generated that, upon binding GFP, either increase or decrease GFP fluorescence [20]. This system has been elegantly used to monitor the translocation of the human oestrogen receptor from the cytoplasm to the nucleus in a sensitive, high-throughput manner by nuclear expression of a GFP-enhancing GBP.

Recently, GBPs targeting different regions of GFP were selected. Upon coexpression of these GBPs fused to a transcriptional activation domain or a DNA-binding domain, the corresponding active transcription factor is formed specifically in GFP-expressing cells (Figure 2D) [21]. This approach can be applied to the vast collection of transgenic mouse GFP lines for the convenient generation of cell-specific transgene expression or gene knockdown by RNAi.

The VHH X factor

Determining protein structures by X-ray crystallography is difficult for 'high hanging fruits' such as membrane proteins and large protein complexes. For this reason, crystallisation chaperones in the form of antibodies and antibody

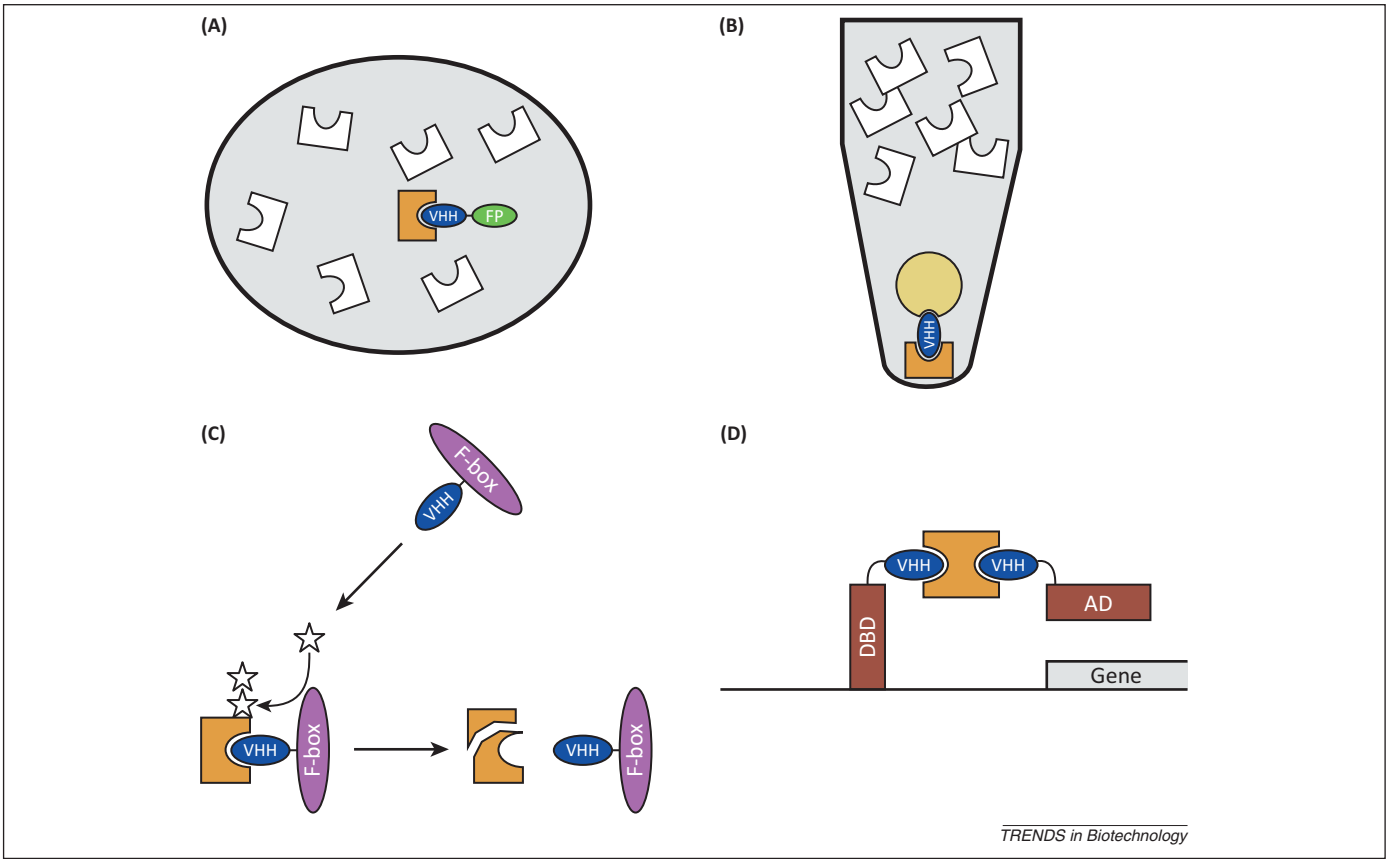

Figure 2. Innovative VHH applications in research. VHH (blue) recognises specifically its antigen (orange) within a background of contaminating proteins (white). **(A)** Chromobodies are fusions between a VHH and FP. When expressed *in vivo*, they are used for the visualisation and tracing of endogenous antigens. **(B)** VHHs are used as capturing agent for immunoprecipitation by fusing them with magnetosome particles (pale yellow). **(C)** In deGradFP, a VHH is fused to an E3 ubiquitin ligase F-box domain (pink). Upon binding its antigen *in vivo*, the antigen becomes polyubiquitinated (star) and rendered susceptible for degradation by the proteasome. **(D)** A DBD and AD are fused to complementary-binding VHHs and an active transcription factor is formed to drive gene expression in antigen-expressing cells. Abbreviations: AD, activation domain; DBD, DNAS-binding domain; FP, fluorescent protein.

fragments (in this case VHHs) have been developed. On the one hand, VHHs have facilitated crystal formation by stabilising one particular protein conformation. For example, the structure of the instable active state of the β_2 adrenergic receptor has been elucidated by growing crystals in the presence of active state-specific VHHs [22], and VHHs also provide insight into β_2-microglobulin fibril formation by binding early fibril intermediates and preventing their self-oligomerisation [23]. Similarly, a VHH that inhibits prion oligomerisation has been used to obtain structural information on the disordered N-terminal prion protein region, hence contributing to the understanding of early prion formation [24]. On the other hand, VHHs have substantially accelerated the production of well-diffracting crystals by merely providing additional contact surface to the target protein, as demonstrated for components of the bacterial type 2 secretion system [25,26]. Importantly, such chaperones are considered to maintain (one of) the natural conformations of the target instead of inducing novel architectures of the native protein [27].

VHHs as diagnostic tools

Implementation of VHHs as sensitive detection probes

Detection probes should ideally meet most of the following characteristics: high probe accessibility, stability and selectivity towards the antigen, even in complex samples, and cost-effective large-scale production [28]. Conversely, probe accessibility is determined by probe size. When small antibody fragments are coated on adsorptive plates, the vicinity of the adsorbing surface might hinder the antigen–probe interaction [29]. However, probe accessibility is also dependent on a uniform, directional probe orientation. The accessibility of coated VHHs in ELISA can be improved by C-terminal peptide extension such as provided by a myc-His-tag or a llama long hinge region-His-tag [30], or by fusion to an Fc chain [29]. The more hydrophobic nature of the latter results in an enhanced VHH-Fc probe coating as compared to the hydrophilic VHHs for ELISA. By contrast, due to their intrinsic stability and smaller size, VHHs exhibit a better denaturation resistance during harsh probe regeneration, and a higher probe density that mediates an enhanced detection sensitivity in surface plasmon-resonance-based detection systems [28,29]. Interestingly, VHHs have also been evaluated as capturing ligands in antibody-based slide and bead arrays. More particularly, small amounts of bacterial lysates containing *in vivo* biotinylated VHHs have been applied to streptavidin beads and used for sensitive biomarker detection in patient sera [31].

For pathogen diagnosis, several VHHs have been generated in case the performance of available mAbs is unsatisfactory. Although these mAbs recognise the antigen of interest with high sensitivity, they often lack the required specificity. For example, whereas VHHs have been used successfully to distinguish between *Brucella* and *Yersinia* infections in livestock, mAbs have failed [32]. Similarly, species-specific VHHs for the detection of *Taenia solium* infection in pigs complement the existing genus-specific mAbs [33]. Diagnostic VHHs for *Trypanosoma* parasites have also been developed, without access to purified antigens: by carefully designing the panning procedure, both species-specific and genus-specific VHHs have been isolated, allowing easy species typing of the prevailing parasites [34].

Recently, a VHH-based agglutination reagent has been evaluated for HIV diagnosis [35]. The reagent comprises the fusion between an HIV antigen (i.e., HIV-1 p24) and a red blood cell-specific VHH. The presence of anti-p24 antibodies in HIV-positive serum then mediates agglutination upon addition of the p24–VHH fusion proteins. Based on this approach, diagnosing a variety of diseases could be possible when disease-specific antigens are available.

Finally, excellent VHH thermostability has been demonstrated by generating anti-caffeine VHHs for the quantification of caffeine in hot beverages. One of these VHHs could bind caffeine at 70°C and even recover its functionality after an incubation step at 90°C [36].

VHHs in preclinical noninvasive diagnostic imaging

Antibodies targeted against tumour-associated proteins and labelled with a radionuclide are applied as tracers for noninvasive *in vivo* tumour imaging via positron emission tomography (PET) and single-photon emission computed tomography (SPECT). The tracers should exhibit a high tumour-to-background signal, high target specificity, fast clearance of unbound tracer, high stability, good solubility, and low immunogenicity. As a result of the long serum half-life of full-length antibodies, imaging of sufficient contrast can only be performed several days post-injection and hence long-lived radionuclides are required, which also increase the toxicity. In this respect, VHHs have recently gained interest as valuable alternatives because their fast extravasation, good tumour penetration, and rapid renal clearance of excess tracer allow sensitive imaging of target tissue already within a few hours post-injection [37,38]. In addition, this early imaging allows the use of short-lived radionuclides, resulting in more patient-friendly diagnoses. The disadvantage of VHHs, however, is the high accumulation of unbound VHHs in kidneys, soon after tracer injection, resulting in a higher radiation dose for kidneys and a complicated analysis of nearby tissues [37,39,40]. However, the signal can be reduced by alternative labelling reactions and by co-injection of gelofusin.

Radiolabelled VHHs, such as ^{99m}Tc-VHH, are already used in several contexts, such as to discriminate between moderate and high epidermal growth factor receptor expression for improved prognosis of cancer therapy [38], to evaluate breast cancer diagnosis in a preclinical setting [37], and to monitor the status of inflammatory responses by visualising dendritic cells [41]. Interestingly, a method has been proposed to reduce further the background level encountered with radiolabelled VHHs [40]. First, unlabelled bivalent VHHs are injected, of which the majority occupy all extratumoural sites because they are apparently too large and of too high avidity for efficient tumour penetration. A second injection with monovalent ^{99m}Tc-VHH then results in specific labelling of the tumour. It remains to be seen whether this approach becomes widely applicable. Besides these promising results with ^{99m}Tc-VHHs in SPECT, progress has also been made for *in vivo* imaging with PET [37,39].

Finally, because tracers should not induce any immune response, the effect of VHH humanisation has been investigated by grafting the antigen-binding loops onto a humanised VHH scaffold [42]. Although the VHH experiences a slightly reduced affinity *in vitro* upon humanisation, both the parental and humanised VHH have shown good tumour labelling. Alternatively, nonradioactive VHH-based probes with near-infrared fluorophores have also been developed for tumour imaging and these allow faster imaging as compared to approved mAbs targeting the same antigens [43,44].

Production of VHHs and VHH-derived formats

Evidently, large quantities of VHHs are required for some of these applications. To meet these demands, VHHs and VHH-based proteins have been expressed in bacteria, fungi, mammalian cell lines, plants, and insects.

Bacteria

The vast majority of VHHs and VHH-derived formats (among others, chromobodies and tandem-linked VHHs) has been produced in bacteria, namely in *Escherichia coli*, either periplasmically or cytoplasmically. By targeting VHHs to the periplasmic space, disulfide bridges are formed and VHHs are readily purified after preparing periplasmic extracts [45]. Periplasmic VHH production yields typically range from 1 to 10 mg/l [33,39,46], with few VHHs reaching 50–70 mg/l [47,48]. By contrast, 60–200 mg/l purified VHH is routinely obtained upon cytoplasmic protein accumulation [35,49,50]. However, sometimes the reducing environment of the cytoplasm prevents the formation of functional VHHs. To overcome this limitation, conserved cysteines have been substituted to generate disulfide-bridge-free VHHs [51], or the VHH has been fused to cytoplasm-stable proteins to provide stability [52], or the complementarity determining regions have been grafted onto a universal VHH scaffold with demonstrated stability even in a reducing environment [53], or chaperones have been coexpressed for successful disulfide bridge formation [54], whether or not in combination with mutant *E. coli* strains exhibiting a less-reducing cytoplasm (i.e., SHuffle cells) [35,50].

Fungi

VHHs produced in fungi are generally targeted for secretion by yeasts (i.e., *Saccharomyces cerevisiae* and *Pichia pastoris*), although a few reports exist on the production in filamentous fungi [55,56]. By producing 71 different VHHs in *S. cerevisiae*, 100-fold differences in production levels were observed (from <1 to >100 mg/l) and the achieved production level was strongly influenced by the secretion efficiency [57]. The latter mainly correlated with the overall hydrophobicity of the VHH [58] and with the identity of the J-encoded segment that corresponds to the C-terminal end of the VHH, and is thought to interact with yeast chaperones via several key residues for correct protein folding [57].

By producing VHHs in both *E. coli* and *P. pastoris*, cross-platform comparisons are possible: VHHs that were initially purified at 0.5–3 mg/l from *E. coli* periplasm, were secreted at 8–18 mg/l by *P. pastoris* using baffled shake flasks [59,60]. Importantly, these mg/l yields in bacteria and yeasts at laboratory scale can be translated into g/l in high cell density fermenters [58,61,62]. High accumulation levels (>1 g/l in clarified medium) combined with straightforward purification have made *P. pastoris* the preferred host for the large-scale production of VHHs in industry (Ablynx) (personal communication).

Mammalian cell lines

As a result of their ability to secrete properly folded glycoproteins and their well-established regulatory guidelines, mammalian cell lines are generally used for industrial glycoprotein production. However, as VHHs are easily produced in bacteria and yeasts, mammalian cell lines have not been evaluated for this purpose. By contrast, next-generation VHH-Fc antibodies, that are desired for increased functional affinity and prolonged serum half-life [63], require a eukaryotic protein folding machinery and have been produced at 100 mg/l in mammalian cell cultures [64,65].

Plants

Plants are regarded as a valuable alternative for the expensive production in mammalian cell lines and are free of possible contamination with human pathogens. Moreover, plants offer a range of production systems that could create additional benefits for the pursued product, such as convenient, long-term protein storage in seeds. However, low VHH accumulation levels of <1% of total soluble protein (TSP) are reported in *Arabidopsis thaliana* leaves [66] and seeds [67], and a medium VHH accumulation of 3.4% of TSP is achieved in transgenic tobacco leaves [68]. In comparison, producing the latter VHH in *P. pastoris* yields 10–15 mg of purified VHH per litre of culture [69]. In contrast to these low and medium accumulation levels, a codon-optimised, apoplast-targeted VHH constitutes 30% of TSP in agroinfiltrated *Nicotiana benthamiana* leaves [70].

Several reports describe an enhanced VHH accumulation level after fusing the VHH to a stabilising partner. For example, the level of VHHs that is barely detectable in tobacco chloroplasts, shifts to 3% of TSP when they are fused to β-glucuronidase; an enzyme known to be stable in the chloroplast stroma [71]. Similarly, fusing a VHH to elastin-like polypeptide repeats improves the accumulation level from 0.003 to 1.7% of TSP in transgenic tobacco leaves [72], and maximum levels of 0.13% and 16% of TSP are reported for VHH and dimeric VHH-Fc antibodies, respectively, in *A. thaliana* seeds [67]. The latter platform is also used as proof-of-concept for producing anti-enterotoxigenic *E. coli* (ETEC) secretory IgAs based on VHH-Fc to combat ETEC-caused post-weaning diarrhoea in piglets, and is now under evaluation for the production of VHH-Fc antibodies in crops such as soybean seeds as feed for oral passive immunisation [73]. Alternatively, anti-rotavirus VHHs are produced at high levels of 11.6% of TSP in rice seeds in combination with RNAi-based suppression of endogenous rice seed proteins [74]. These VHH-containing seeds could be administered orally without the need for purification, are easily digestible, and provide high resistance of the VHHs towards thermodenaturation upon boiling.

An innovative approach to produce a polyclonal VHH mix in plants, referred to as 'plantiserum', has been proposed recently (http://rybicki.wordpress.com/2013/06/16/polyclonal-antibodies-from-plants/). Initially, Julve and colleagues [75] examined the process of superinfection exclusion, in which a plant cell infected by a tobacco mosaic virus is resistant to superinfection with a related virus. Based on these results, a library of anti-snake venom VHHs has been cloned into a deconstructed, tobacco-mosaic-virus-based vector for subsequent agroinfiltration of *N. benthamiana* leaves. Following cell-to-cell movement of viral RNA from agroinfiltrated cells to neighbouring leaf cells, mosaic patterns of leaf cells infected with one particular strain (and hence one VHH coding sequence) are formed because of superinfection exclusion. Protein extracts from these leaves are then considered to be polyclonal VHH plantiserum.

Insects

Living insects in nonsterile conditions have been proposed for the large-scale, cheap production of anti-rotavirus VHHs in developing countries [76]. The VHHs are intended for oral administration and hence no relevant contaminating agents should be present, therefore, living insects are preferred over bacterial and mammalian production systems. VHHs accumulate to 3.6% of TSP and have been successfully evaluated in piglets, where they fully protect the host against human rotavirus strain-induced diarrhoea [77].

Concluding remarks

The peculiar properties of VHHs have stimulated their introduction in various applications: their high stability allows oral administration and *in vivo* immunomodulation; their distinct paratope enables recognition of epitopes inaccessible for full-length antibodies; and their small size make them better diagnostic detection probes. However, besides VHHs, non-immunoglobulin protein scaffolds are also considered highly potent binders for diverse applications [78]. Hence, we believe VHHs will become even more widely applicable in the future, complementary to other antibody formats and protein scaffolds.

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