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# The Development of Single Domain Antibodies for Diagnostic and Therapeutic Applications

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Additional information is available at the end of the chapter

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## Abstract

Monoclonal antibodies have become increasingly accepted as diagnostics and therapeutics for various human diseases due to their high affinity and specificity. However, several practical drawbacks are apparent for the reagents based on conventional IgG antibodies. With the emergence of antibody engineering, many problems were overcome when the recombinant antibody fragments such as Fabs, scFvs, diabodies and single domain antibodies (sdAbs), are developed. These fragments not only retain the specificity of the whole monoclonal antibodies, but are also easy to express and produce in prokaryotic expression systems. Rather unexpectedly, the natural sdAbs namely  $V_{HH}$ s,  $V_{NAR}$ s and variable lymphocyte receptors (VLRs) that comprise excellent biological activities were recently discovered in camelids, cartilaginous fish and lampreys, respectively. Due to their unique characteristics, including small size, high thermostability, stable folding in the nucleus and cytosol and long CDR3 regions which have access to cavities or clefts on the surface of proteins, these new binders are now investigated extensively as a substitute for conventional antibodies. This review describes the potential of sdAbs selected using *in vitro* display systems and their use in multiple applications.

**Keywords:** recombinant antibody, single domain antibody, diagnostic and therapeutic single domain antibody, scFv, IgNAR,  $V_{HH}$ , VLR,  $V_{NAR}$ s

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## 1. Introduction

In research applications, antibodies are widely used as binders due to their high specificity and high affinity. Antibodies can be classified into three different categories such as polyclonal antibodies, monoclonal antibodies and recombinant antibodies [1]. Polyclonal antibodies

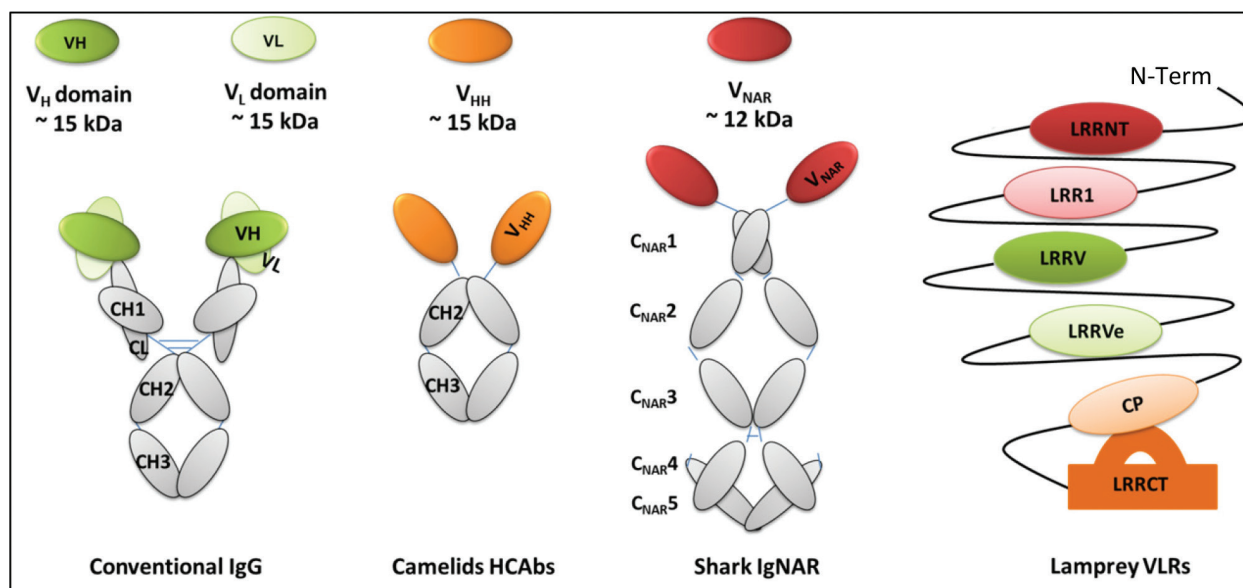
(polyclonal Abs) are heterogeneous antibody mixtures that are derived from multiple plasma cell lines. Because polyclonal antibodies comprise a mixture of different antibodies carrying numerous paratopes, they have excellent properties for recognizing antigens [2]. A monoclonal antibody (mAb) is a homogeneous antibody generated from a single B lymphocyte clone. Antibodies produced in mAb format have an extremely high specificity against a single epitope on antigens [3]. Recombinant antibodies (rAbs) are antibodies generated using molecular biology techniques. They are aimed to improve the sensitivity, selectivity, stability and immobilization properties in diagnostic applications, for example, in biosensors [4]. In making decision to use or generate polyclonal, monoclonal or recombinant antibodies, several factors should be considered, including commercial availability, possibility to raise animals, types of applications, time length of a project and costs [1]. Although a vast number of rAbs has been proposed [5–8], the natural sdAb fragments that were recently discovered from camelids ( $V_{HH}$ s), sharks ( $V_{NAR}$ s) and lampreys (VLRs) have shown to possess extraordinary features that are not found in conventional antibodies, such as a small dimension, an elevated stability and the capability of recognizing cavities and clefts on the surface of proteins that cannot be reached by conventional recombinant antibodies [9–11]. This chapter will discuss the availability of new binders derived from vertebrates and give an overview of their applications in a biomedical platform by recognizing specified targets from various diseases.

## 2. Monoclonal antibodies and their limitations

The first description of monoclonal antibody (mAbs) production was published by Nobel prize winners, Kohler and Milstein in 1984 [12]. The fusion technique developed between splenic B cells and myeloma cells is termed the hybridoma technique has revolutionized immunology and medicine. The production of mAbs is not influenced by sources of animal used, making mAbs having better homogeneity in scale-up production [13]. The mAb technology has been widely applied in biomedical research and pharmaceutical industries.

Unlike polyclonal Abs, the monospecificity of a mAb enables targeting of a single epitope. This enables a range of applications, including targeting specific members of a protein family and evaluating changes in molecular conformation and targeting protein-protein interactions. However, the specificity and sensitivity of mAbs can be reduced by small changes in the structure of the antigen determining regions, or even by minor changes in pH or salt concentration. An advantage is that, mAbs can be produced at a greater concentration and much higher purity than polyclonal Abs [13].

The conventional mAb predominantly produced as IgG after an immune response, is represented in **Figure 1**. As determined by their structural and biological properties, IgG molecules have specific features, namely their large size compared to recombinant antibody fragments, higher synthesis rate and longer half-life. IgGs are the most widely used immunoglobulins for antibody-based diagnostic and therapeutic development. Generally, conventional IgGs are characterized by having a high affinity ( $K_a$ ) ranging from  $10^{-2}$  to  $10^1$  nM, and excellent specificity for its cognate target epitope [14]. The high degree in variations of antibody specificities is



**Figure 1.** Schematic representation of conventional antibodies and natural single domain antibodies. The conventional IgGs derive from mammals while the natural single domain antibodies derive from camelids, sharks and lampreys, respectively. Single V domains are presented as colored ovals; C domains are shown gray colored. The domains in lamprey variable lymphocyte receptor (VLR) are demonstrated on the right. VLRs consist of an N-terminal cap (LRRNT), the first LRR (LRR1), multiple (usually up to seven) 24-residue variable LRRs (LRRVs), a terminal or end LRRV (LRRVe), a connecting peptide (CP) and a C-terminal cap (LRRCT), followed by an invariant 3'-terminal region.

conferred by the variable amino acid sequences in the variable regions of the heavy and light chain (V<sub>H</sub> and V<sub>L</sub>). Each variable domain is comprised of three hypervariable (HV) regions, separated by four framework regions (FR). The HV regions are known as complementarity-determining regions (CDRs), and are responsible for the identification of the specific epitope of the cognate antigen. The FR regions are major components of the backbone structure for V<sub>H</sub> and V<sub>L</sub> regions in antibodies and can potentially influence the conformation of the antigenic binding loops [15].

However, several practical drawbacks are apparent for diagnostic reagents based on conventional IgG antibodies. The complex architecture and large molecular size (~150 kDa) may result in weak binding when small size protein antigens are not easily recognized by the concave surfaces of CDR loops [16]. Initial attempts to generate single domain antibody fragments by separating expression of individual human V<sub>H</sub> or V<sub>L</sub> units was reported to result in solubility problems in aqueous solvents, higher cost and more time consuming process and the requirements for sophisticated protein engineering approaches [17]. Moreover, the failure of recognition of selected mAbs on conserved epitopes of specific antigens due to unbound reactivities mediated by the Fc region may hinder their utility for diagnostic applications [18, 19].

With the emergence of DNA engineering, surface display has been widely used to discover new antibody fragments as a means for diagnostic and therapeutic applications. An overview of principles in phage display technology, including antibody library construction, biopanning, types of bacteriophages used and antibody fragments applications are further discussed in the following sessions.

### 3. Phage display technology for new biomarker binder discovery

Screening phage display libraries are a powerful tool for identifying specific binders from libraries containing a large diversity of phage surface expressed molecules [20, 21]. Libraries construction are achieved by fusing a repertoire of genes (genotype) encoding the peptides/proteins to a gene encoding a capsid structural protein. The “displayed” peptides/proteins (phenotypes) are included in the capsid layer on the phage surface. Ideally, these proteins should not be interfered with the phage structure [22].

The display technologies have enabled exploration of new antibodies from humans or animals, including shark, camel, llama and lamprey [23–26] that may not otherwise be discovered.

#### 3.1. Antibody phage display library

Antibody phage display libraries have been extensively used for isolation of specific high affinity binders against unique antigens from different targets [27–31]. Three types of antibody library are typically constructed: naïve, synthetic and immunized libraries [32]. A naïve antibody library refers to the repertoire of antibody genes derived from non-immunized donors. Synthetic antibody libraries are constructed using synthesized mutated CDRs and synthetic frameworks whereas immunized libraries are based on a host immunized with a target antigen of disease [33].

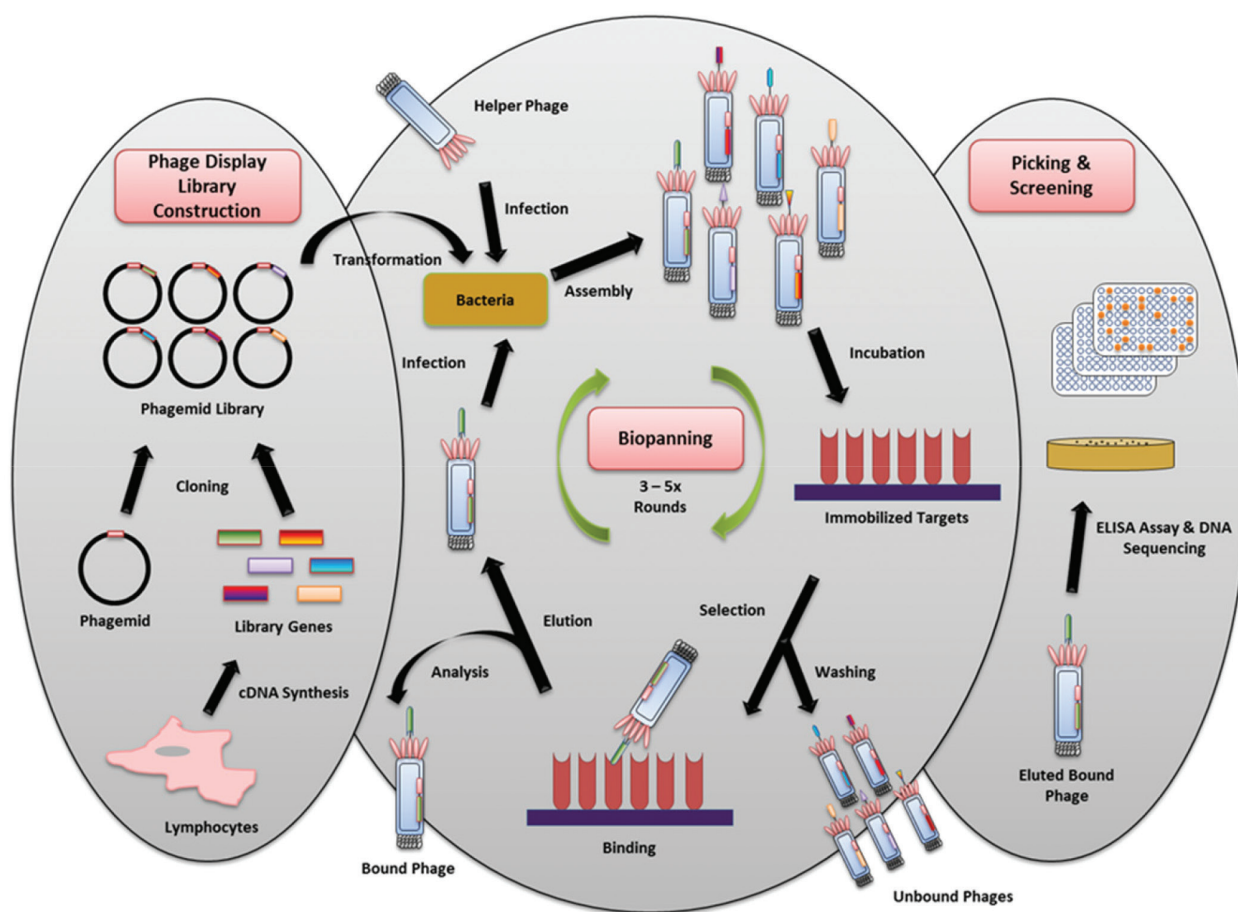
The function of the phagemid vector is akin to that of a plasmid whereby the genes of interest can be cloned directly into the multiple cloning sites upstream of the capsid structural phage protein after digestion by appropriate restriction enzymes. Phage display technology has facilitated the selection of different antibody fragments using genetic engineering approaches [34]. Many antibody fragments created (Fab, scFv and diabody) were used to overcome the limitations of conventional IgG antibodies derived from higher organism [19]. Furthermore, the presentation of single domain antibodies (sdAb) of heavy chains derived from different animals are being widely investigated, including camelids  $V_{HH}$  or Nanobodies<sup>®</sup>, sharks  $V_{NAR}$  region of IgNAR [35] and the antibody of variable-like lymphocytes (VLRs) from lamprey fish [36].

#### 3.2. Biopanning of phage display

The selection of high binding clones from antibody libraries using phage display can be undertaken *in vitro* via a process called biopanning. In this process, the antibody fragments displayed on the surface of phages are incubated with an antigen of interest that is immobilized on a surface [37, 38]. Generally, immunoabsorbent ELISA microplates, uncoated cell culture dishes and immunotubes are commonly used for ligand immobilization [39]. Non-specific or unbound phages are removed by washing, whereas phage that binds specifically to the target is eluted by changing the binding conditions, depending on types of bacteriophages used in the experiment. For instance, acidic solutions of HCl or glycine buffer are used for M13 bacteriophage [40]. Other methods include use of basic solutions of triethylamine [41], enzymatic cleavage of protease site incorporated in the recombinant coat protein [42], competition with excess antigen [38] and direct bacterial elution [43] have been reported for the elution of M13 bacteriophage. For T7 phage display system, the elution buffer is 1% SDS [44].

The amplification of eluted phage is carried out by infecting the exponential growth phase of *Escherichia coli*. To assemble and produce the recombinant phage a helper phage is added [45], whereas T7 phages can be directly released from the host by cell lysis [46]. Successive rounds of biopanning varied by types of library and target antigen used. In practice, the enrichment of phages of interest can be obtained within three to six rounds of biopanning. Further rounds of selection may potentially lead to bias by the enrichment of non-specific background phages [47, 48].

Phage display is a powerful technology for the generation of antibodies for medical applications. Nowadays, approximately 30 monoclonal antibodies have been approved by FDA for use in clinical practice with many more currently being tested in clinical trials. [49, 50]. The principle of the phage display is represented in **Figure 2**, indicating the workflows of library construction, biopanning and clone screening prior to purification for functional assays.



**Figure 2.** Principle of filamentous bacteriophage M13 phage display using a phagemid vector. Antibody genes encoding for millions of variants of libraries are cloned into a phagemid vector carrying the gene encoding for one of five phage coat proteins (pIII). Large phage libraries can be obtained by transforming *E. coli* with phagemids and rescue of phages with helperphage. Hence, phages displaying the specific antibodies against immobilized targets can be selected and isolated by several rounds of biopanning. These steps involve binding, washing, elution, infection and amplification. The eluted bound phages are subsequently screened by ELISA assay and followed by DNA sequencing prior to their protein expression and purification.


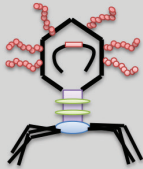
### 3.3. Types of bacteriophage utilized in phage display system

In phage display systems, different bacteriophages have been used to display a range of proteins on surface. M13 filamentous bacteriophage [51, 52] and T7 lytic phage are the most commonly used for displaying and production of antibody fragments [53, 54]. A comparison between M13 bacteriophage and T7 lytic phage are discussed in the following section and summarized in **Table 1**.

#### 3.3.1. Filamentous bacteriophage M13 system

The filamentous phage M13 is the most extensively used phage for antibody phage display [55]. Other classes of filamentous phages that have been studied include F1 and Ff phages [56, 57]. In the mature virus particle, filamentous phage M13 have a cylindrical-shaped structure, about 930 nm in length and phage proteins are encoded by a circular single-stranded DNA genome. Foreign peptides are typically displayed on the N-terminal of the minor p3 coat protein or on the major p8 coat protein with the copy numbers from 5 to more than 2000 depending on type of vectors used. However, type 3 is the most widely used display format [56, 58]. Generally, this leads to expression of 1–3 copies of the recombinant fusion protein on the phage surface.

The diversity of M13 phage display libraries typically ranges from  $10^5$  to  $10^{12}$ , and is greatly dependent on the transformation efficiency of the host *E. coli*. As the proteins are secreted through periplasmic layers, the M13 phage display system represents a suitable tool to display the

Parameters	M13 filamentous phage	T7 phage
Structure diagram		
Virus behavior	Temperate phage • Colonies • Host cell = male (F-pilus)	Virulent phage • Plagues • Host cell = female
Rate of growing	Slow (~ 16 hours)	Fast (1.5 – 3 hours)
Stability range of pH	pH 1.5 – 10	pH 4 – 10
Bias of displayed peptide / protein	Physical limitation (periplasmic transport required)	Less bias than M13 phage (secreted by cell lysis)
Displaying platform	pIII or pIV protein	Capsid protein

**Table 1.** Comparison of M13 filamentous phage with T7 phage.

appropriately folded proteins containing disulfide bonds. Hence, many functional antibody fragments, enzymes and inhibitors have been displayed and selected using this system [28, 59, 60]. However, it also has the minor limitation of poor display of cytoplasmic proteins on the membrane [61]. Moreover, the removal of stop codons in the DNA library can facilitate correct display of the foreign proteins on the coat protein at the N-terminus of M13 bacteriophage [56].

### 3.3.2. T7 bacteriophage system

The bacteriophage display and cloning system using T7, T4 and  $\lambda$  phage was introduced in 1990s, and has several advantageous features over other phage display systems [62–64]. As a lytic phage, the T7 phage contains a linear double-stranded DNA genome. It has a diameter of 55 nm, with the capsid shaped in an icosahedron structure. The Novagen's T7Select® is the commercially available phage display system that takes advantage of the properties of bacteriophage T7. There are three types of vectors available in this system: for peptide display with up to 50 amino acids in high-copy number (415 per phage); 1200 amino acids display in mid-copy number (5–15 phage) and 1200 amino acids display in low-copy number (0.1–1 per phage) [64].

Fusion proteins are displayed at the C-terminal end of the T7 capsid protein (gene 10); the removal of the stop codon from foreign genes is not necessary, resulting in ease construction of a library. The diversity of T7 phage display is often dependent on the packaging efficiency into the capsid. Nevertheless, a successfully constructed library could encode a library of the size  $10^7$ – $10^8$  clones [64]. In contrast to bacteriophage M13, the secretion of library proteins through the periplasmic layer of the host cell does not occur in the T7 phage display system. This may lead to the reduction of physiochemical restriction and less bias in the library peptide diversity [65]. In addition, the T7 phage system has the advantages of being able to display a cytoplasmic protein, a major limitation of the M13 filamentous phage [61, 66].

However, folding of cytoplasmic proteins with disulfide bonds in T7 bacterial phage system do not occur quite well. This problem can be resolved by using the complementing hosts such as BLT5615 or BLT 5403 *E. coli* strain included in the T7Select® kit [65, 67, 68]. In term of general features, T7 phage grows much faster than M13. After infection, clear plaques (lawns) of T7 phages can usually be observed within 2–3 h on an LB plate at 37°C. Furthermore, the purification process of T7 phage for ELISA and DNA sequencing is also simple to perform, with only PEG/NaCl precipitation required to recover the purified phage [47, 65].

## 4. Engineered sdAb fragments from vertebrates

With the advent of recombinant DNA technology, antibody genes can be selected and amplified using phage display, yeast display, bacterial display, ribosome display, mRNA display, DNA display or mammalian cell surface display [69–73] and see chapter in this book: “Display technologies for the selection of monoclonal antibodies for clinical use” by Tsuruta et al. A range of mammalian V-gene libraries have been used to undertake *in vitro* recombinant antibodies screening projects using phage display. These include mouse [74], rabbit [75], sheep [76] and human [77]. Unlike hybridoma technology, the direct link between the geno-



type and the phenotype of displayed antibodies during selection (biopanning) can facilitate the identification of binding antibodies and corresponding antibody genes. Further, the gene encoding the desired antibody can be manipulated to improve affinity, specificity and expression or fusion to a carrier protein can be performed [38, 48, 78].

An advantage of sdAb fragments is their ease of genetic manipulation due to their smaller size, in addition ease of expression in bacterial system, low lot-to-lot variation and easy scaled-up production [79, 80]. Moreover, sdAb production is not influenced by species-specific cell fusion partner incompatibilities. Nowadays, the desired sdAb repertoire can be developed from shark, camels and humans with an appropriate set of specific primers [81]. However, an additional step of point mutations in framework regions and CDR randomization is required to construct human  $V_H$  and  $V_L$  sdAbs [81]. Regardless, the generation of sdAbs by bacterial fermentation is significantly cheaper, simpler and quicker than conventional polyclonal Abs or mAbs production [80, 82–84]. The general features of some natural sdAb fragments are described in the following section.

#### 4.1. $V_{HH}$ heavy-chain domain in camelids

Conventional immunoglobulins comprise two major parts such as the antigen-binding fragment (Fab region) and fragment crystallisable region (Fc region), with a typical molecular weight of 150 kDa. The Fab domain is responsible for antigen binding and therefore its specificity. This domain is divided into heavy (H) and light (L) chains with the molecular weights of 25 kDa each [85]. The stability of the molecular complex of an immunoglobulin is conferred by four inter-domain disulfide bonds in the hinge regions. The heavy chain can be subdivided into one variable ( $V_H$ ) region and three constant (C) regions (CH1, CH2 and CH3) while the light chain contains one variable region ( $V_L$ ) and only one constant region (CL). Lacking direct antigen-binding functions, the main role of the Fc domain is to provide effector functions such as binding to cellular receptors on macrophages and complement activation, and determination of the half-life of an antibody [86].

In addition to conventional heterotetrameric antibodies, the sera of Camelidae were discovered to possess special IgG antibodies known as heavy-chain antibodies (HCAs). Although HCAs contain both a constant (Fc) and variable domain, these antibodies are slightly different from conventional IgG by devoid of the L chain polypeptide and the first constant domain (CH1) (**Figure 1**). Therefore, the isolated variable domain region of camelids HCAs is known as  $V_{HH}$  (variable domain of the heavy chain of HCAs) or Nanobody® (Nb; Ablynx) [87].  $V_{HH}$  constitutes a binding surface to interact with the target antigen. The molecular weight of  $V_{HH}$  is 15 kDa, 10 times lower than that of a conventional antibody. It was thereby considered the smallest possible antibody fragment and has attracted the interests of many scientists [88, 89]. Moreover, the capability of camelid antibodies to retain the reversibility and binding activity after heat denaturation has enabled new applications where transient heating may occur [90].

The major advantage of a  $V_{HH}$  antibody is their greater solubility compared to classical  $V_H$  [17]. This is due to the hydrophilic amino acid substitution present in the framework 2 region. Meanwhile, the single coding exon of less than 450 base pairs facilitates genetic engineering of  $V_{HH}$  fragments [91]. In addition, on account of its smaller antigen binding surface area, the

unique CDR3 region enables the heavy domain of camelids to penetrate into antigen cleft regions that are not easily recognized by conventional antibodies [92, 93]. From a phylogenetic prospect, since camelids are related to the primate lineage [94] it is possible to produce humanized  $V_{HH'}$ , a process that may be easier to perform than the complicated manipulation required to “humanize” murine or other more distant species to reduce an alloresponse, such as the human anti-mouse antibody (HAMA) response [95].

Furthermore, due to their high intrinsic domain stability, camelids  $V_{HH}$  are now under investigation as probes for diagnostics [18, 96]. The diagnostic potential of camelids  $V_{HH}$  as probes in immunodetection systems offers the possibilities of improving the diagnosis of infection [97], cancers [98] and food contaminants [99]. Although  $V_{HH}$ s do not originate from humans, the humanizations strategies of  $V_{HH}$ s have successfully been undertaken by designing a humanized scaffold region onto the antigen-binding loops (CDRs) of specific  $V_{HH}$ s can be grafted [100]. In addition, non-humanized and humanized  $V_{HH}$ s with therapeutic potential have been applied in multiple areas, including hematology [101], inflammatory diseases [102], infectious diseases [103], *in vivo* imaging [104], neurological disorders [105] and oncology [106, 107].

#### 4.2. $V_{NAR}$ heavy-chain domain in sharks

A class of naturally occurring antibodies comprising a variable domain of a heavy chain ( $V_{NAR}$ ) without a variable light chain domain was discovered in the serum of elasmobranch cartilaginous fish during early of 1990s [108–110]. These natural functional antibody repertoires were termed as immunoglobulin new antigen receptors (IgNARs). IgNARs are an unconventional and unique class of proteins found in sharks, including nurse sharks (*Ginglymostoma cirratum*) [111], wobbegong sharks (*Orectolobus maculatus*) [112], smooth dogfish (*Mustelus canis*) [113], banded hound sharks (*Triakis scyllium*) [68] and horn shark (*Heterodontus francisci*) [114]. Investigations have revealed that IgNARs function as antibody and immune response mediators in sharks. However, until now it is not clear if the IgNARs as single domain antibodies arise from TCR domains/L chains or primitive cell surface molecules [109, 115].

Several desirable biological properties of IgNAR V domains have been identified, and their potential as alternative antigen binders explored [112, 113, 116]. The natural habitat of sharks has resulted in evolving extraordinary stable antibodies such that the functionality of antibodies can be retained in a harsh environment [117]. Electron microscopic studies have indicated that the intact IgNAR exists as a disulfide-bonded homodimer that consists of a polyprotein with one variable domain ( $V_{NAR}$ ) and five constant domains ( $C_{NAR}$ ) (**Figure 1**) [118].

Similar to the camelid  $V_{HH'}$  the  $V_{NAR}$  has only a heavy-chain domain. However, the cross-species conservation of the amino acid sequence with a human VH is extremely low in a  $V_{NAR}$  domain (~25%), whereas it is more than 80% homologous to  $V_{HH}$  scaffolds in camelids  $V_{HH}$  [110, 119]. It is hypothesized that IgNARs lack many residues that exist in conventional antibodies. These are replaced by other hydrophilic residues. The greatly truncated CDR2 region, herein defined as HV2 region, has created a signature hallmark for shark  $V_{NAR}$ . Due to this unusual structure, the single variable heavy domain proteins of shark IgNARs are currently the smallest antibody fragments observed in animal kingdoms, having a size of only 12 kDa.

Yet, in combination with the peculiar feature of a long CDR3 region, these  $V_{\text{NAR}}$  domains tend to more readily penetrate cleft regions of antigens, thereby increasing the opportunity to target small target epitopes that may not be accessible to conventional IgG [120].

In terms of heat stability,  $V_{\text{NAR}}$  also possess refolding properties as found in camelids  $V_{\text{HH}}$ . The ability of retaining fully functional antigen-binding activity after exposure up to 95°C may make  $V_{\text{NAR}}$  ideally suited to protein array and diagnostic applications where transient heating may occur as part of the protein immobilization process [9, 113]. It is partly due to the presence of cysteine residues in these single domain antibodies, resulting in an extraordinary conformation [35].

$V_{\text{NAR}}$  domains are more easily produced as recombinant proteins compared to conventional antibodies. Additionally, due to hydrophilic residues present within  $V_{\text{NAR}}$  surfaces, high yields of expressed proteins associated with high solubility, are achievable and thus they can be easily produced in prokaryotic systems [112]. Therefore, the potential utility of  $V_{\text{NARs}}$  as alternative binders for clinical applications is now being investigated in a variety of areas.

### 4.3. VLRs immunoglobulin-like domains in lamprey

Lamprey and hagfish are the only surviving groups of jawless fish, having appeared since the Cambrian period. The adaptive immune system of jawless vertebrates was recognized as unique due to the rearrangement of antigen receptors which is completely different from that used by jawed vertebrates [121]. The somatic rearrangement of the variable (V) gene segments, diversity (D) segments, joining (J) segments and constant (C) segments is commonly observed in conventional Ig-based Ag receptors. However, the immune system in jawless vertebrates is predominantly regulated by recombination activating gene (RAG)-independent combinatorial assembly to generate leucine-rich repeats (LRR) cassettes for Ag recognition. Owing to these differences, antibodies in jawless fish were termed as variable lymphocyte receptors (VLRs) rather than Ig superfamily (**Figure 1**) [122].

In comparison to CDR loops used by Ig-based antibodies and T-cell receptors in many animals, the antigen-binding regions of VLRs have evolved into variable  $\beta$ -strands and C-terminal loop structural motifs, resulting in a crescent-shaped protein conformation [123, 124]. Due to the prevalence of this unusual pattern, VLRs tend to be more useful for microbial recognition [36]. Thus far, two VLR genes have been identified in lamprey and hagfish, namely VLRA and VLRB. However, the VLRB gene in lamprey shows more complexity in terms of coding sequence analysis [125].

Sequences analysis has revealed that each VLR consists of a signal peptide (SP), hypervariable LRR regions, consisting of a 27–34 residue N-terminal LRR (LRRNT), the first 24-residue LRR (LRR1), up to nine 24-residue variable LRRs (LRRV), one 24-residue end LRRV (LRRVe), one 16-residue connecting peptide LRR (LRRCP) and a 48–63 residue C-terminal LRR (LRRCT) [126]. The assembly of VLRs entails greater recombination events in LRR modules and can efficiently generate more than  $10^{14}$  unique repertoires at a level comparable to mammalian Ig. Thus, VLRs may be a source of single-chain domains alternative to conventional Ig-based antibodies [123]. Nevertheless no single domain antibody comprising only one engineered VLR domain has been so far reported.

Having undergone evolution over millions of years, VLRs appear to have been optimized as suitable antigen receptors for humoral protection. Further analysis indicates that VLRs are extremely stable in harsh environments. Their antigen binding capability remained unchanged even after it was eluted from a column at a very basic pH (>11) [11]. In addition, the heat stability of VLRs is similar to shark IgNARs and camelids  $V_{HH}$ . For example, eluted VLRs can be stored over 1 year at 4°C, 1 month at room temperature and 36 h at 56°C. However, the degradation of Ag-binding activity occurred when the incubation period was prolonged more than 1 h at 70°C [126].

Although VLRs were discovered less than a decade ago [122], they have provided new insights into the potential of ancestral antibodies in biotechnological applications. Owing to a greater VLR library diversity as well as associated self-tolerance ability, VLRs can be efficiently used to detect antigens that may not be recognized by mammalian Ig, for example, the sensitivity of VLRB mAb targeting against *Bacillus anthracis* (BclA) was superior to that of a high affinity conventional murine IgG [11]. Furthermore, the simple modular single polypeptide structures facilitate the production of VLRs antibodies through DNA engineering. VLRs combinatorial libraries of high affinity binders can be constructed through *in vitro* random mutagenesis and loop shuffling using a surface display technology approach, for instance, yeast display system [36]. Thus, VLRs may become alternatives for the developments of new reagents in diagnostic applications to overcome the lack of Ag recognition ability of conventional monoclonal antibodies made from mammals.

## 5. Use of different recombinant antibodies for specific applications

To date, humans and mice remain the main source of complete antibodies for targeting diseases. However, with the aid of DNA technology, a number of new antibody fragments have been engineered as smaller single domain fragments to improve immunoassays, immunosensors and imaging probes in various applications. As described recently, the discovery of natural single heavy domain antibodies from camelids  $V_{HH}$  and shark  $V_{NAR}$  and in addition lamprey VLRs offers some advantages over conventional antibodies. This range of natural antibodies is expected to open various applications: to trace molecule trafficking and to inhibit protein function inside the cell as intrabody, to apply them as therapeuticum and they can be used as detection units in biosensors or immunodiagnostics. In this section, we will review the deployment of different binders in specific diagnostic applications and to what extent these binders are used.

### 5.1. Applications of camelids $V_{HH}$ domains or nanobodies®

To monitor infections, single domain antibodies naturally derived from camelids (nanobodies) may enable superior species-specific antigen detection than classical monoclonal antibodies in immunodiagnostic tests. Trypanosome infection causes African sleeping sickness and Chagas disease. Both are severe parasitic diseases caused by protozoa of the genus *Trypanosoma*. Sleeping sickness disease is mainly found in rural Africa. The antigenic variation strategy adopted by this parasite represents a major barrier to the immune system to eliminate it. Consequently, it is difficult for specific mAbs to detect genus-specific antigens [127]. By adoption of an *in vitro* selection method, novel nanobody clones were isolated that showed specificity to *T. evansi* at species level, and genus-specific reactivity against various *Trypanosoma* species [128].

Cysticercosis is a serious tissue infection caused by larval cysts of the pork tapeworm, which is prevalent in many low-income countries [129]. Monoclonal antibodies that are currently deployed in sandwich ELISAs are mainly genus-specific against *Taenia* sp., but poorly specific at a species level to identify *Taenia solium*, the major *Taenia* species threatening human health [130, 131]. To circumvent such limitations, an *in vitro* selection of nanobodies from immunized dromedaries was developed to recognize a specific marker on *T. solium*. After *in vitro* selection, the nanobodies showed no cross-reactivity against other livestock *Taenia* species, while having a very specific response to a specific 14 kDa glycoprotein (Ts14) in *T. solium*. Therefore, nanobodies showed potential as an alternative to genus-species mAb for developing unambiguous ELISA tests for human cysticercosis [97].

Apart from diagnostic reagents for infectious diseases, nanobodies have been identified as alternative binders to analyze the compositions of substances in food and beverages industries. Due to their excellent thermal stability, nanobodies showed superior behavior to classical mouse mAbs in ELISA to measure caffeine concentration in hot and cold beverages [132].

Camelid sdAbs have recently been applied in ELISA methods to detect a wide range of small molecules, including explosive materials (trinitrofluorene or TNT) [133], agents of bioterrorism (Botulinum A neurotoxin) [90], toxins (ricin, cholera and staphylococcal enterotoxin B) [134], scorpion toxin [135] and viruses (HIV, rotavirus, Vaccinia and Marburg) [136–138]. Owing to the combination of several favorable properties, camelid nanobodies have also been employed as molecules to diagnose diseases. In small molecule development, the advanced features of highly stable and unique conformational structure of nanobodies have permitted overcoming many problems faced by traditional whole antibodies and scFv fragments such as cross-reactivity and nanoparticle agglutination. The development of biosensors coupled with nanobodies (nanoconjugates system) has enabled significant improvement in the performance of a device to identify harmful bacteria (*Staphylococcus aureus*) to a nanometer scale within 10 min [139].

Nevertheless, mAbs remain the common binding agents to identify and trace tumor-associated proteins for noninvasive *in vivo* imaging. However, limitations, particularly large size (150 kDa) and Fc regions, result in mAbs poorly penetrating into solid tumors [140]. The emergence of nanobodies offers the possibility of resolving such problems, and thereby enables nanobodies to diagnose tumor markers such as EGF receptor [141]. This will enable cancer staging predictions in blood circulation such as prostate-specific antigen [142]. More applications using camelids  $V_{HH}$  targeting antigens from various diseases are summarized in **Table 2**.

## 5.2. Applications of shark $V_{NAR}$ domains

Evidence that IgNAR is part of the shark adaptive immune response was demonstrated in a work where increasing levels of hen egg lysozyme (HEL) led to the development of specific IgNARs developed in the shark sera after 4–5 months of immunization [25]. The peculiar structure of the shark IgNAR variable domain renders it amenable to create synthetic peptide mimetics to target specific epitopes that are inaccessible to conventional antibodies [118]. Therefore,  $V_{NAR}$  may be suitable as new molecular reagents for research, diagnostic and immunotherapeutic applications.

Target antigens	Diseases	Applications	Reference
VEGF-A <sub>165</sub>	Neoangiogenesis	Diagnostic and therapeutic	[143]
HER2	Breast cancer	Diagnostic	[144, 145]
HPV-16 L1 protein	Cervical cancer	Diagnostic and therapeutic	[146, 147]
HSP-60	Brucellosis (livestock)	Diagnostic and vaccine	[23, 148]
VCAM1	Atherosclerotic lesions	Molecular imaging	[149–151]
VEGFR2	Angiogenesis	Therapeutic	[152]
TNT	Explosive	Diagnostic	[133, 153]
SEB	Toxin	Sensor and diagnostic	[134]
Ricin	Toxin	Sensor and diagnostic	[134]
BoNT/A	Toxin	Sensor and diagnostic	[90, 154, 155]
Scorpion AahII	Toxin	Neutralizing and therapeutic	[135]
EGFR	Tumors	Detection and imaging	
DARC	Malaria (by <i>P. vivax</i> )	Diagnostic or therapeutic	
LMM, ES, CSE, TSB, LLGPs, VF of <i>T. solium</i>	Neurocysticercosis	Immunodiagnosis	[97]
Heat-killed <i>B. melitensis</i> Riv1 lysates	Brucellosis	Vaccination, diagnostic, therapeutic	[141, 156–158]
Poliovirus type 1 Sabin strain particles	Poliomyelitis	Diagnostic and therapeutic	[159, 160]
CEA	Colon cancer	<i>In vivo</i> imaging	[161–163]
RSV protein F	Acute lower respiratory tract	Therapeutic	[164]
CD105	Angiogenesis related tumors	Diagnostic and therapeutic	[165, 166]
Ts14 glycoprotein	<i>T. solium</i> cysticercosis	Diagnostic	[97]
vWF	Thrombosis	Therapeutic	www.ablynx.com
TNF $\alpha$ , IL-6R, IgE	Rheumatoid arthritis	Therapeutic	www.ablynx.com
RANKL	Bone metastasis	Therapeutic	www.ablynx.com
RSV	Bronchiolitis and pneumonia	Therapeutic	www.ablynx.com
DR5	Solid tumors	Therapeutic	www.ablynx.com
Not stated	Alzheimer's disease	Therapeutic	www.ablynx.com

**Table 2.** The applications of camelids V<sub>HH</sub> against specified antigens from various diseases.

Apical membrane antigen-1 (AMA1) is a highly polymorphic 83 kDa merozoite surface protein that is essential for erythrocyte invasion in malaria parasites [143]. A V<sub>NAR</sub> isolated from a wobbegong shark showed high-binding affinity to *Plasmodium falciparum* AMA1 through its unique CDR3 region after undergoing affinity maturation [144]. The binding specificity of a monovalent V<sub>NAR</sub> clone to *P. falciparum* AMA1 was comparable with commercially available

binding reagents, derived from conventional polyclonal sera, monoclonal antibodies, small fragments (Fab and scFv) and peptides [145]. Foley and co-workers demonstrated the heat stability of purified recombinant  $V_{\text{NAR}}$  was superior to that of conventional mAbs by targeting immobilized *P. falciparum* AMA1 in various formats at 45°C, and the refolding property of  $V_{\text{NAR}}$  was retained when the temperature increased to 80°C. The excellent stability property at extreme pH and resistance to proteolytic cleavage was further evidenced by incubating  $V_{\text{NAR}}$  with homogenized murine stomach tissues under *in vivo* conditions [9]. From this point of view, it was purposed that  $V_{\text{NAR}}$  domains have potential for development as alternate binders for malaria diagnostic platforms.

Human periodontal disease is an advanced gingivitis caused by the bacterial pathogen *Porphyromonas gingivalis* [146]. Late treatments often lead to dental loss due to the accumulation of lysine gingipain (KgP). KgP is a high molecular weight polyprotease produced by *P. gingivalis* [147]. This bacterial toxin is responsible for destruction of dental tissue of host by suppressing the secretion of specific lytic enzymes from the immune system [148]. Nuttall and co-workers [149] identified two distinct clones specific to KgP from a naïve wobbegong shark  $V_{\text{NAR}}$  phage display library with synthetic CDR3 loops. The high stability and binding affinity toward *P. gingivalis* KgP indicated the potential for  $V_{\text{NAR}}$  sdAbs as a valuable source of single domain binding reagents [149].

In recent studies, shark  $V_{\text{NAR}}$  domains have been reported to detect markers from viral diseases at greater sensitivity compared to mAbs and scFvs. Ebola virus hemorrhagic fever (EVHF) is a highly lethal disease caused by Bundibugyo virus (BDBV), Sudan virus (SUDV), Tai Forest virus (TAFV) and Zaire Ebolavirus (ZEBOV) [150–152]. Shark  $V_{\text{NAR}}$  and murine scFv phage display libraries have been generated against specified markers on Zaire Ebolavirus. The results indicated that the sensitivity and thermal stability of shark  $V_{\text{NAR}}$  sdAbs against viral nucleoprotein (NP) of ZEBOV was superior in comparison to murine mAbs and scFvs. [116].

As in the case with camelids nanobodies, highly diversified shark  $V_{\text{NAR}}$  libraries have also been used to detect different kind of toxins, including staphylococcal enterotoxin B (SEB), ricin and botulinum toxin A (BoNT/A) complex toxoid [153] and cholera toxin (CT) [113]. In addition,  $V_{\text{NAR}}$  sdAbs have been reported to recognize immunosilent targets in human, for example, the 70 kDa translocase of outer membrane (Tom70) [154]. Owing to the findings of negligible cross-reactivity with other antigens and superior heat stability, shark  $V_{\text{NAR}}$  domains may be a potent source of sdAbs with thermal stability over conventional antibodies in diagnostic and biotherapeutic applications [155, 156]. The applications of recombinant shark  $V_{\text{NAR}}$  sdAbs against specified antigens from various diseases are summarized in **Table 3**.

### 5.3. Applications of lamprey VLRs

The variable lymphocyte receptors (VLRs) discovered from jawless fish had recently attracted interests and is leading to the development of new monoclonal antibodies for biomedical applications [11, 36, 157]. Despite possessing an unusual structure, VLRs have been shown to have excellent binding ability to specified targets (**Table 4**).

Cooper and co-workers demonstrated high specificity of recombinant VLRs for BclA, a major anthrax spore coat which could be produced from an immunized sea lamprey (*Petromyzon*

Target antigens	Diseases	Applications	Reference
Kgp protease ( <i>P. gingivalis</i> )	Periodontal disease	Neutralization	[173]
rhTNF $\alpha$	Pro-inflammatory cytokine	Therapeutic	[114, 181]
AMA1 ( <i>P. falciparum</i> )	Malaria	Diagnostic	[168, 169]
Nonfibrillar oligomer formation	Alzheimer's disease	Modeling	[182]
Zaire ebolavirus viral nucleoprotein	Ebolavirus Haemorrhagic Fever	Diagnostic	[116]
HBeAg	Hepatitis B virus	Immunolocalization and diagnostic	[183]
Cholera toxin	Toxin	Diagnostic	[113]
SEB	Toxin	Sensor and diagnostic	[177]
Ricin	Toxin	Sensor and diagnostic	[177]
BoNT/A	Toxin	Sensor and diagnostic	[177]
Tom70	Human immunosilent target processes	Detection	[178]
GPCR's ion channels		Therapeutic	<a href="http://www.adalta.com.au">www.adalta.com.au</a>
Anti-thrombotic drug targets	Cardiovascular disease	Diagnostic and therapeutic	<a href="http://www.adalta.com.au">www.adalta.com.au</a> <a href="http://www.adalta.com.au">www.adalta.com.au</a>
	Blood brain barrier	Therapeutic	<a href="http://www.ossianix.com">www.ossianix.com</a>
	Gastrointestinal tract	Therapeutic	<a href="http://www.ossianix.com">www.ossianix.com</a>
Myostatin	Neurological disease	Therapeutic	<a href="http://www.ossianix.com">www.ossianix.com</a>
	Uveitis	Therapeutic	<a href="http://www.elasmogen.com">www.elasmogen.com</a>

**Table 3.** The applications of shark V<sub>NAR</sub> against specified antigens from various diseases.

*marinus*) using hybridoma technology [11]. *Bacillus anthracis* is the causative agent for anthrax and the only pathogenic species in the genus *Bacillus* [158]. Due to their extreme dormancy and durability, anthrax spores have long been considered ideal biological weapons [159–161]. In this work, the recombinant monoclonal VLRs were shown to be capable of identifying bacteria at a genus level, by differentiating the C-terminal domain of BclA *Bacillus anthracis* from non-coated bacteria of *Bacillus cereus* [11].

Target antigens	Diseases	Applications	Reference
BclA glycoprotein	<i>B. anthracis</i> spores (anthrax)	Diagnostic	[11]
HEL, $\beta$ -gal, cholera toxin subunit B, R-phycoerythrin, and B-trisaccharide	Complex protein antigens	Affinity determination	[39, 189]
C1q and C3 proteins	Cytotoxicity for bacteria and tumor cells	Binding interaction	[190]

**Table 4.** The applications of lamprey VLRs against specified antigens from various diseases.



In another study, a large library of recombinant VLRs was constructed to target lysozyme,  $\beta$ -gal, cholera toxin subunit B, R-phycoerythrin and B-trisaccharide antigens using yeast surface display technologies [36]. This high-throughput technology platform offers the potential of rapid identification and isolation of monoclonal VLRs that specifically bind to target antigens with affinities in the micromolar to nanomolar range [36]. Using such display methods, the specificity of selected VLR antibodies can recognize the target antigen with high binding affinity up to 100-fold compared to conventional mouse mAb [36]. These data indicate that the function of VLRs is comparable or perhaps better than that of mammalian IgG antibodies. Therefore, it is speculated that VLRs may be an alternative reagent for the future development of therapeutic and diagnostic applications.

## 6. Conclusion

The fields of antibody engineering have undergone major advancements in the past few decades. New surface display technologies, in particular phage display and yeast display, are powerful tools that could facilitate the isolation of new antibodies with high specificities for a broad range of targets. Due to their affinity, which often is similar to conventional antibodies and reliable production, recombinant antibodies are becoming increasingly important in the field of diagnosis and therapy for targeting a wide range of diseases such as cancer, inflammatory, autoimmune and viral diseases. In view of natural scaffold design, previous studies showed that the sdAbs repertoires derived from animals such as camelid  $V_{HH}$ s, shark  $V_{NAR}$ s and lamprey VLRs contain several advantages over conventional antibodies. One of the unusual characteristics shared among the sdAbs is that they possess better penetration ability. This feature allows the sdAbs to effectively penetrate into antigen clefts (enzyme active sites, viral capsids and cell surface receptors) which are not easily recognized by the concave surfaces of CDR loops of complex conventional antibodies. To date, due to their ability to target both refractory antigens and immunosilent epitopes, the engineered antibody fragments coupled with latest screening technologies have extensively been used in positron emission tomography and high-sensitivity (nonradioactive, noninvasive) laser technologies for medical imaging. To sum up, it is believed that with rapid progress in antibody engineering technologies, sdAbs will become indispensable as clinical and research reagents in the next decades.

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