frontiers in Immunology

PERSPECTIVE
published: 20 November 2017
doi: 10.3389/fimmu.2017.01589

Camelid Single-Domain Antibodies: Historical Perspective and Future Outlook

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Tremendous effort has been expended over the past two and a half decades to understand many aspects of camelid heavy chain antibodies, from their biology, evolution, and immunogenetics to their potential applications in various fields of research and medicine. In this article, I present a historical perspective on the development of camelid single-domain antibodies (sdAbs or V_HHs, also widely known as nanobodies) since their discovery and discuss the advantages and disadvantages of these unique molecules in various areas of research, industry, and medicine. Commercialization of camelid sdAbs exploded in 2001 with a flurry of patents issued to the Vrije Universiteit Brussel (VUB) and later taken on by the Vlaams Interuniversitair Instituut voor Biotechnologie (VIB) and, after 2002, the VIB-founded spin-off company, Ablynx. While entrepreneurial spirit has certainly catalyzed the exploration of nanobodies as marketable products, IP restrictions may be partially responsible for the relatively long time span between the discovery of these biomolecules and their entry into the pharmaceutical market. It is now anticipated that the first V_HH-based antibody drug, Caplacizumab, a bivalent anti-vWF antibody for treating rare blood clotting disorders, may be approved and commercialized in 2018 or shortly thereafter. This elusive first approval, along with the expiry of key patents, may substantially alter the scientific and biomedical landscape surrounding camelid sdAbs and pave the way for their emergence as mainstream biotherapeutics.

Keywords: camelid single-domain antibody, heavy chain antibody, V_HH, nanobody, antibody engineering, therapeutic antibody

OPEN ACCESS

Edited by:
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This is NRC publication number: 53362.

Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: *29 September 2017*
Accepted: *03 November 2017*
Published: *20 November 2017*

Citation:
Arbabi-Ghahroudi M (2017) Camelid Single-Domain Antibodies: Historical Perspective and Future Outlook. Front. Immunol. 8:1589. doi: 10.3389/fimmu.2017.01589

INTRODUCTION

The canonical view of antibodies as molecules composed of two heavy chains and two light chains was forever changed one day in 1989 following analysis of total and fractionated immunoglobulin G (IgG) molecules in the serum of a dromedary camel in the laboratory of Professor Raymond Hamers at the Vrije Universiteit Brussel (VUB). The serendipitous discovery of antibodies lacking a light chain [heavy chain-only antibodies (HCAbs)] occurred as part of a student-run project aimed at developing a serodiagnostic test for trypanosome infection in camels and water buffalos. The preliminary data showed that besides conventional IgG1 (MW ~150 kDa), two other immunoglobulin fractions (thereafter called IgG2 and IgG3; MW ~90 kDa) were present which contributed up to 75% of all serum IgGs (1–3). Comparative studies on the sera of new world camelids (*Lama glama* and *Lama pacos*) subsequently confirmed the presence of HCAbs, albeit at concentrations between

30 and 50% (1, 4–8). Following these exciting findings, it became essential to analyze the antigen-binding properties of these IgG fractions since the presence of truncated forms of heavy chain antibodies with no light chains, classically described as "heavy chain disease," had been reported in human patients (9, 10). No functional activity was reported for the pathogenic heavy chain antibodies in these patients, as these proteins were shown to bear extensive internal deletions in the variable (VH) and the first constant region (CH1) domains. By contrast, antibodies from camelids exposed to *Trypanosoma evansi* demonstrated strong binding activity in the IgG2 and IgG3 heavy chain-only fractions as shown by radio-immunoprecipitation and blotting experiments (1).

In two subsequent reports, phage-display technology and high-resolution crystallography were utilized to (a) build a phage-display library from the lymphocytes of immunized camels and isolate monomeric antigen-specific V_HH domains in the absence of the constant regions (11) and (b) solve crystal structures of an unliganded V_HH (12) and a V_HH:lysozyme complex, reported simultaneously by the VUB team and a Dutch–French research group (13). The term V_HH was originally introduced by the VUB team in 1994 to indicate a VH domain derived from camelid heavy chain antibodies. The feasibility of isolating stable and soluble V_HH domains with nanomolar affinities against lysozyme and tetanus toxoid showed very early on the promise of these molecules as high-affinity binding moieties. Crystallography studies revealed additional salient features of an anti-lysozyme V_HH, including deep penetration of its long third complementarity-determining region (CDR3) into the active site of the enzyme; this feature had rarely been seen with conventional antibodies and required a fundamental deviation from known human canonical CDR1 structure (13). Further evidence of the unique antigen recognition behavior of V_HH domains (including enzyme inhibition) was published over the next several years (11, 14, 15), suggesting that V_HHs might probe different sets of epitopes on proteins compared with conventional antibodies. Key proof of concept for producing bivalent/bispecific V_HH modalities *via* genetic fusion (using camelid short and long hinge sequences) of anti-lyzozyme and/or anti-tetanus toxin V_HHs was also established very early on (14).

MOLECULAR ONTOGENY OF CAMELID HCAbs

Molecular biology techniques were subsequently applied to decipher the DNA sequences of HCAbs. The sequencing results showed that nature had designed HCAbs as an additional arm of the immune systems of camelid ungulates over the course of their evolutionary history. The consensus of these studies suggested camelid HCAbs possessed: (a) no CH1 domain, and therefore, a direct connection of the rearranged V_HH exon to the hinge region; (b) one of two types of long (IgG2) and short (IgG3) hinge isotypes; (c) specific conserved amino acid substitutions in framework region 2 (FR2), mainly at VH positions that make contact with the VL in classical antibodies, including Kabat positions 37, 44, 45, and 47; and (d) potentially different CDR3 amino acid composition and a broader length distribution for CDR3 compared to the heavy chains of conventional antibodies (1, 16, 17).

Later genomic studies shed light on the origin of HCAbs in dromedary camels and alpacas. It is now established that HCAbs are produced from the same *igh* locus as conventional antibodies but with distinct sets of genes for the generation of HCAbs. It is estimated that alpaca and dromedary genomes contain ~17 and ~40 V_HH genes, respectively, with an identical organization of the genes that produce conventional antibodies (18, 19). The CH1 exon is present in the genomic DNA of HCAbs but a point mutation (G to A) at the 5′ end of the CH1-hinge intron disrupts the consensus splicing site (GT) and causes omission of this region during splicing (3, 18, 20–22). A complete picture of camelid germline V gene repertoires of heavy and light chains and the classification of VH and V_HH genes is still missing. Published genomic and cDNA data have so far shown that camelid V_HH genes are highly homologous to the human VH3 family of clan III with the exception of several key amino acid substitutions in FR2, namely, Val37 → Phe/Tyr, Gly44 → Glu, Leu45 → Arg, and Trp47 → Gly (Kabat numbering), and are encoded by a distinct subset of germline V genes. Preliminary investigations of published llama V_HH sequences classified them into four subfamilies by sequence similarity, and many of the earliest-described V_HH features such as long CDR3s, additional disulfide bridges, and particular canonical structures of CDR1–3 were shown to be subfamily specific (17, 23). Subsequent studies in alpaca identified at least three V gene subgroups of the alpaca *igh* locus: IGHV1, IGHV2, and IGHV3 which are equivalent to the human IGHV families within clan I (VH families 2, 4, 6), II (VH families 1, 5, 7), and III (VH family 3), respectively, based on sequence homology. The alpaca V_HH genes clustered into six subsets by sequence similarity, but all are homologous to human IGHV3 genes (18). Furthermore, recent investigations have demonstrated the presence of genes belonging to IGHV families 1, 3, and 4 (human clan I and III) in llama and alpaca, and in addition, uncovered new camelid V genes highly homologous to the human IGHV5 and IGHV7 families (human clan II); however, no genes similar to human families 2 or 6 (within human clan I) were found (24). Interestingly, a novel promiscuous class of V genes in camelids was identified that is closely related to the human VH4 family (clan I). These VH4 homologs contribute largely to the classical antibody repertoire and lack the hallmark solubilizing V_HH residues in FR2. Nevertheless, antigen-specific VH4-family fragments with V_HH-like stability and solubility were isolated from an immune llama library (25). In the absence of a complete set of camelid germline VH and V_HH genes, most immunogenetic studies have relied on comparisons with human germline genes.

The consensus of immunogenetic studies of camelid HCAbs is that repertoire diversification of these molecules may involve (a) a large number of unique V_HH gene segments recombining with DH and JH minigenes, possibly with additional non-templated nucleotide insertions leading to longer CDR3 loops; (b) somatic hypermutation, potentially of extended CDR1 regions compared with conventional antibodies; (c) acquisition of non-canonical cysteine residues in the CDRs and FR2; and

(d) involvement of FR2 residues in antigen binding and in structuring the CDR3 loop (3, 22, 26, 27). In agreement with immunogenetic analyses, several structural studies have suggested that due to the loss of VL domains, V_HH paratopes have acquired a higher structural complexity by involving more residues in antigen binding compared to classical VHs (27). As for the evolutionary origin of HCAbs, it is difficult to draw solid conclusions but several hypotheses have been proposed. A common theme among most of these has been the need for generating or expanding a new antigen-binding repertoire in *Camelidae* to address certain antigenic challenges, e.g., cryptic epitopes of commonly encountered pathogens. Phylogenetic analyses have confirmed that HCAbs diverged from conventional antibodies as a result of recent adaptive changes (22, 27–29).

HISTORY OF THE DEVELOPMENT OF CAMELID SINGLE-DOMAIN ANTIBODIES (sdAbs) AS THERAPEUTICS

Prior to the discovery of HCAbs, a single report describing the concept of sdAbs was published by Sally Ward and colleagues in 1989 (30), when they showed that VH domains from an immunized mouse, in the absence of a VL domain, could bind specifically to lysozyme and keyhole limpet hemocyanin. However, poor VH domain stability and solubility, as well as weak antigen-binding affinity compared to its fragment variable region counterpart (Fv) or to the parent antibody, were major impediments to any commercial applications (**Figure 1**).

From a historical perspective, development of camelid V_HHs as drugs has gone through three major phases. The first 10 years (1993–2003) can be classified as the exploratory phase, which coincided with the founding of Ablynx in December 2001 as a spin-off company from the Vlaams Interuniversitair Instituut voor Biotechnologie. The main developments in the first decade included: (i) the first description of V_HHs (1); (ii) sequence analyses of V_HHs with identification of V_HH germline gene segments and classification of V_HH gene subfamilies (16, 20, 23); (iii) adaptation of phage-display technology to V_HHs (11) and isolation of antigen-specific V_HHs, including several enzyme inhibitors (12, 15); (iv) solving the crystal structure of several V_HH:antigen complexes (13, 31–34); (v) development of methods for expression of V_HHs in bacteria and yeast systems and for biophysical characterization of V_HHs (35, 36); and (vi) the use of V_HHs as reagents in immunoaffinity purification and immuno-perfusion (37).

During the second phase of development (2003–2013), V_HHs began to receive more attention and publications in this area grew dramatically, surpassing 1,000 by 2013 [Ref. (38) and personal investigation on Web of Science]. Interestingly, a large and diverse group of countries and institutions (close to 50) were responsible for research on camelid V_HHs during this time, mainly for the purpose of exploring their potential applications in research, biotechnology, and medicine (38). The major hallmark of this decade was the start of preclinical and clinical studies of several nanobodies by Ablynx and others as therapeutics and imaging reagents (39, 40), including V_HHs against (i) blood glycoprotein vWF to control platelet aggregation and clot formation; (ii) viral infection (RSV); (iii) venom toxins; (iv) IL6-R for treatment of rheumatoid arthritis; and (v) the use of radiolabeled nanobodies for Her2+ tumor imaging. There were major technological advancements made in the expression of V_HHs in heterologous systems and in creating an array of bi- and multivalent V_HHs with superior efficacy during this decade.

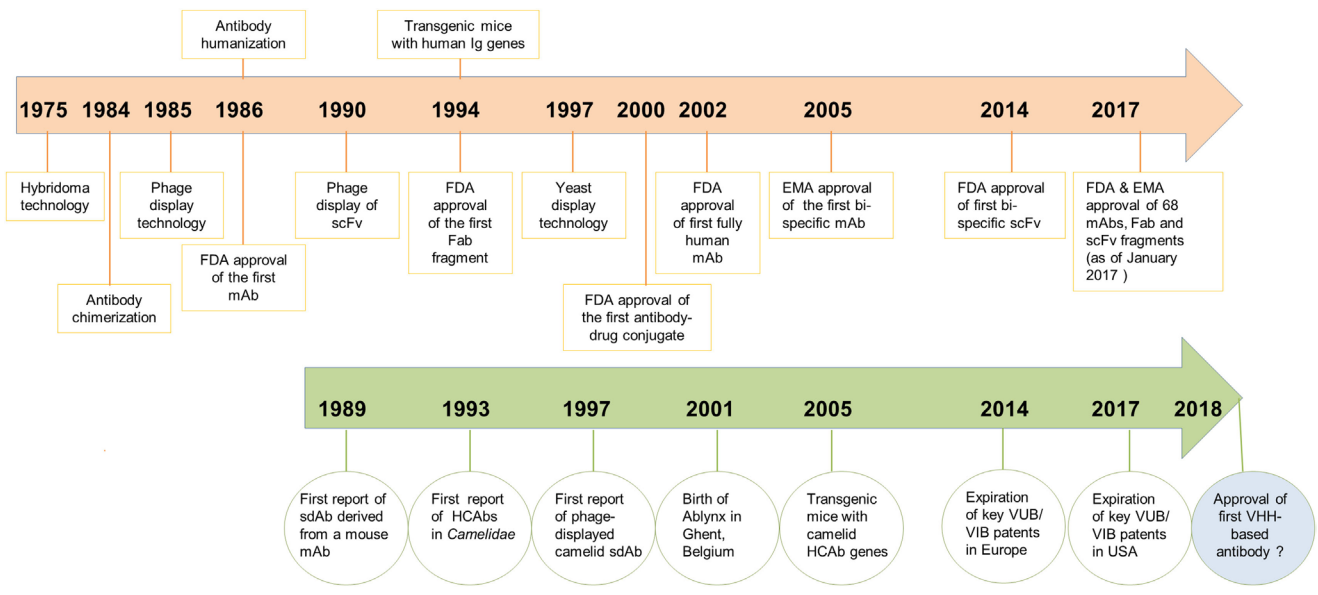

FIGURE 1 | Chronological timeline of major scientific developments in the field of antibody engineering since the discovery of monoclonal antibodies (mAbs) in 1975 leading to the regulatory approval of mAbs, antigen-binding fragments (Fabs) and scFvs as therapeutics. Developments for mAbs are shown in orange and developments of V_HHs/heavy chain-only antibodies (HCAbs) in green. Regulatory approval of the first V_HH-based antibody drug is expected in 2018.

Now in the third phase of development (2014–present), publications continue to grow and more V_HHs have entered into clinical trials or advanced closer to the market. The main patent claims on camelid antibody fragments expired in the summer of 2014 in Europe and in the summer of 2017 in America. Ablynx has expanded its collaborations with large biophama players, such as Merck, Boehringer Ingelheim, Sanofi, and so on, with more than 20 preclinical and clinical programs. It is expected that the first V_HH-based drug (Caplacizumab; bivalent anti-vWF nanobody for treating rare blood clotting disorders) will reach the market sometime in 2018 (www.ablynx.com). Meanwhile, IP limitations on the composition of matter of V_HHs are diminishing and more biotechnology companies (39) are showing interest in commercialization of these domain antibodies as therapeutics, diagnostics, and research reagents (**Figure 1**).

CAMELID sdAbs: PROS, CONS, AND APPLICATIONS

Immunization of *Camelidae* against targets of interest leads to the *in vivo* maturation of HCAb and conventional antibody repertoires. Construction of phage-display libraries is performed by cloning of amplified V_HH repertoires with minimal modification, thus presenting an authentic picture of *in vivo*-matured heavy chain repertoire diversity. By contrast, in both scFv libraries (requiring the artificial joining of VH and VL domains by a synthetic linker) and antigen-binding fragment (Fab) libraries derived from conventional antibody repertoires, natural VH–VL pairings are usually lost. The potential for direct cloning of V_HH repertoires from immunized camelids, the smaller library sizes required to capture the immune V_HH repertoire, the stability of the libraries, the feasibility of displaying V_HHs on a phage or alternative display formats, and the ease of sub-cloning and expression of antigen-specific V_HHs are among the major technical advantages of the camelid V_HH platform over conventional antibody platforms.

Key characteristics of V_HHs include their high affinity and specificity (equivalent to conventional antibodies), high thermostability, good solubility and strictly monomeric behavior, small size (2.5 nm in diameter and about 4 nm in length; ~15 kDa), relatively low production cost, ease of genetic engineering, format flexibility or modularity, low immunogenicity, and a higher penetration rate into tissues (3, 41–44). The short half-life of V_HHs in blood circulation is well suited to certain applications such as tumor imaging or delivery of toxin or radioisotopes to diseased tissues where rapid clearance is required. However, the pharmacokinetic behavior of V_HHs can also be improved by extending their half-lives using different formatting options, including PEGylation or fusion to serum albumin or an anti-serum albumin moiety (43, 45, 46). The immunogenicity of V_HHs domains can also be minimized by humanization (47–49). As with all antibodies of non-human species origin (and even fully human antibodies), immunogenicity and toxicity must be investigated empirically for humanized V_HHs. A complete picture of the immunogenicity of non-humanized and humanized camelid V_HHs is lacking due to insufficient data, but anti-drug immune responses may have been a major reason for the clinical failure of a humanized tetravalent Nanobody®targeting the DR5 receptor (50). As of 2016, V_HHs have been isolated against more than 120 therapeutically important targets relevant to oncology, *in vivo* imaging, hematology, infectious diseases, neurological, and inflammatory disorders, with some in advanced stages of clinical trials (39).

One of the unique characteristics of V_HHs is their ability to target antigenic epitopes at locations which are difficult to access by large molecules such as conventional monoclonal antibodies (mAbs). Examples include intracellular targets (51, 52) or epitopes concealed from mAbs in protein structures (53), G protein-coupled receptors (54, 55), and ion channels (3). V_HHs are ideally suited for such applications due to their small size, target specificity, and long CDR3 loops, bypassing many drawbacks related to small-molecule synthetic drugs such as fine specificity and off-target toxicity (56). As "intrabodies," V_HHs are also ideally suited for cytosolic expression due to their ability to fold in the reducing intracellular environment. This feature likely reflects the single disulfide linkage present in the V_HH domain, as compared to the multi-domain structure and multiple disulfide linkages of conventional antibodies, and may not be completely general to all V_HHs but appears to be quite common; intracellular expression of V_HHs has been widely and productively exploited for *in vivo* cellular imaging (5, 57) as well as to inhibit the function of viral proteins (58, 59). There have been several excellent reviews covering V_HH applications in different areas of basic and applied research and a detailed description of each application is beyond the scope of this article (3, 39, 41, 43, 57, 60–65).

V_HHs are also well suited in the generation of bi- and multi-specific antibodies. In the field of antibody therapeutics, it is now widely accepted that monotherapy of cancer and other diseases may not result in effective outcomes, in particular due to the problem of acquired resistance (66, 67). Bispecific antibodies provide a possible solution in which they could bind simultaneously to a tumor-associated antigen and another activating molecule, e.g., CD3 on T cells, leading to tumor killing/lysis through lymphocyte recruitment, or alternatively, could target two or more tumor epitopes (bi-paratopic) or antigens simultaneously. Bispecific V_HHs may be uniquely positioned for these applications given their simple design and small size relative to other antibody fragments, which may result in better solid tumor penetration rates, homogeneous production at high yield in microbial systems, and ease of fusion to a heterodimerization motif, therefore bypassing issues related to some linker peptides such as aggregation and immunogenicity (45, 66, 68, 69). Interestingly, all of the V_HH-based therapeutic candidates in clinical trials are composed of bivalent, trivalent, or higher valency formats (39). It has been shown that some V_HHs, when properly selected, are able to transmigrate through human brain endothelial cell layers spontaneously and, possibly through a receptor-mediated process (70–72); bispecific molecules incorporating these V_HHs can, thus, deliver attached cargo (e.g., therapeutics) into the brain in rodents (73).

Despite the many advantages of V_HHs, there are several drawbacks to be considered as well. The fact that the antigen-binding

paratope of camelid HCAbs has been restricted to a single domain of about 110 amino acids will automatically put more weight on each and every residue in the V_HH domain. The extended CDR1, longer CDR3, involvement of FR2 in antigen binding and shaping the CDR3 loop, the role of the "CDR4" (residues 76–80) loop in antigen binding, and extensive somatic hypermutation are some of the evolutionary mechanisms adapted to compensate repertoire diversity due to the lack of a VL domain (3). Therefore, there may be limitations on the extent of manipulation and engineering that can be tolerated by antigen-specific V_HHs. For example, complete humanization of camelid V_HHs involving the mutation of residues outside the antigen-binding loops often drastically compromises antigen-binding affinity, V_HH stability, and the expression yield (unpublished data). A survey of the literature clearly demonstrates that almost all V_HHs isolated to date have originated from direct camelid immunization, or from large naïve camelid libraries, although recently, successful isolation of V_HHs from synthetic or semi-synthetic libraries against a number of protein antigens has also been reported (74–77). All available pieces of evidence support the notion that the V_HH domain is a highly complex molecule and that each amino acid (depending on its position) may have direct and indirect effects on the molecule's stability and structural integrity, as well as on antigen-binding affinity and specificity.

Another limitation of V_HHs is their low propensity to bind small molecules, likely due to their dominant convex surface topology as compared to the flat or concave topologies found on conventional antibody fragments (e.g., scFv, Fab). In a number of llama immunization trials, we and others have been able to generate strong conventional immune responses, but rather weak HCAb responses, against several haptens and carbohydrate antigens (unpublished data). However, repeated immunization of camelids with small molecules conjugated or fused to larger proteins has led to the successful isolation of V_HHs against caffeine (78), red dye (79), and linear peptides (80, 81) with affinities ranging from micromolar to low nanomolar. The biophysicochemical properties of V_HHs suggest that they would be well suited to many immunodiagnostic platforms for detecting small molecules and environmental chemicals; however, isolation of high-affinity V_HHs suitable for such applications seems to be a difficult task, although not impossible (3, 64, 65, 78, 82, 83). Immunization of large animals and heterogeneity in immune responses among individual outbred animals is another consideration which is important when alternative immunization techniques such as DNA immunization are applied. DNA immunization has had limited success in camelid and other large animals and reproducibility is often a major issue to be tackled (84–87). To overcome this limitation, transgenic mice bearing either a rearranged dromedary γ2a chain or hybrid llama/human antibody loci have been generated that produce a form of dromedary or human heavy chain antibodies (88–90).

CAMELID sdAbs VERSUS mAbs

The first therapeutic mAb, Orthoclone OKT3, a murine IgG2a for the prevention of kidney transplant rejection, hit the market little more than a decade after the discovery of hydridoma technology in 1975 (91–94). Currently, mAbs constitute about half of marketed biological products and, as of January 2017, 68 mAbs have been approved by the Food and Drug Administration (FDA) in the USA and/or by the European Medicine Agency (EMA) in Europe. The projected global sales of mAbs will be close to $100 billion in 2017 (44, 95). The lack of restrictive IP on the original technology is considered by many as a driving force that allowed researchers to develop effective research tools and diagnostic mAb-based reagents without limitation. The introduction of antibody fragments, such as Fab and scFv (the "second generation" of antibodies), combined with the power of phage-display technology in the late 1990s, opened new horizons in the world of antibodies and empowered researchers with the ability to clone the entire immunoglobulin repertoire of mammalian immune B cells and to isolate specific antibody fragments virtually against any target (96–98). This technology led to the development of the first FDA-approved fully human mAb, Humira, which was obtained from a phage-displayed human antibody library 12 years after the initial paper by McCafferty and co-workers on the construction of phage-displayed human antibody libraries (99–101). Further developments in antibody engineering have so far resulted in three FDA-approved therapeutic Fabs (95).

Overwhelming evidence in the literature suggests that camelid V_HHs, as the so-called "third generation" of antibodies, have many added features that supersede those of conventional mAbs and antibody fragments (Fab and scFv). Although V_HHs have already been commercialized for non-medical applications (63, 102), the research and medical communities eagerly await the first V_HH-based therapeutic to gain approval. If we consider the 9- to 13-year time span between the discovery of the key technology enabling conventional mAbs (hybridoma technology) and the FDA-approval of a mAb or an antibody fragment, a longer time has been required for the development of the first V_HH-based therapeutic. It is unclear if technical challenges, regulatory hurdles, or the need to define a unique niche/indication for V_HHs, have been involved in the prolonged delay of the first V_HH-based therapeutic. It is obvious that issues related to downstream processing, stability, immunogenicity, toxicity, safety, and potency of a V_HH-based therapeutic product will be doubly scrutinized by FDA and EMA since it would represent the first product of its kind to enter the market. The fact that the first potential Ablynx product is an engineered bivalent anti-vWF nanobody and is produced in a microbial system may have raised additional red flags for the approving regulatory bodies.

CONCLUDING REMARKS

Over a quarter century has passed since the first observation by Hamers and colleagues of camelid HCAbs. This finding was a significant milestone in the field of antibody engineering and opened many new opportunities and applications. It was also instrumental in reviving the concept of sdAbs, which had been originally suggested by Ward et al. a few years earlier. The unique and extraordinary features of HCAbs and their antigen-binding

domains (V_HHs) have with no doubt attracted many researchers and commercial entities to the field of antibody engineering. V_HHs are now closer than ever to approval as pharmaceutical drugs to fight a wide range of diseases, including cancer, inflammation, hematology, and respiratory diseases, with five V_HH-based drugs in various stages of clinical development. V_HHs have also been shown to be effective as therapeutics against infectious disease, particularly in viral therapy, as well as robust reagents in the field of diagnostic and imaging applications. While the commercial applications of V_HHs have been slowed by IP limitations, it is probable that demand, as well as extensive research on these antibody domains, will ultimately supersede these limitations and bring many more of these molecules into use as biopharmaceutical reagents within the next decade.

AUTHOR CONTRIBUTIONS

MA-G conceived and wrote the manuscript.

ACKNOWLEDGMENTS

The author gratefully acknowledges Greg Hussack, Roger MacKenzie, Kevin Henry, and Kristin Kemmerich for reading and providing comments on the text.

FUNDING

This work was supported by funding from the National Research Council Canada.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.