Single domain antibodies for biomedical applications

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Abbreviations

CDR, complementarity-determining region; dAbs, domain antibodies; HV, hypervariable region; IgNAR, immunoglobulin new antigen receptor; IgNAR V domain, variable domain of IgNAR; mAbs, monoclonal antibodies; scFv, single chain variable fragment; VL, variable

domain of the light chain; VH, variable domain of the heavy chain; VHH, variable domain of camelid heavy chain antibodies; vNAR, variable domain of IgNAR

Abstract

Single domain antibodies are the smallest antigen-binding units of antibodies, consisting either only of one variable domain or one engineered constant domain that solely facilitates target binding. This class of antibody derivatives comprises naturally occurring variable domains derived from camelids and sharks as well as engineered human variable or constant antibody domains of the heavy or light chain. Because of their high affinity and specificity as well as stability, small size and benefit of multiple re-formatting opportunities, those molecules emerged as promising candidates for biomedical applications and some of these entities have already proven to be successful in clinical development.

Introduction

Monoclonal antibodies (mAbs) are indispensable molecules for therapeutic, diagnostic as well as for biotechnological applications. As one of the main drivers of the pharmaceutical industry, mAbs are the highest selling class of biological entities with current and predicted market growth rates, substantially exceeding those of the overall sector (1, 2). Their outstanding clinical efficacy in conjunction with several dozen marketed antibodies and hundreds of mAbs in clinical development is corroborating their paramount therapeutic potential (2-6). This is exemplified by the fact that five recombinant antibody therapeutics were granted first marketing approvals in 2014, as of October 2014. In addition, seven entities are currently undergoing a first regulatory review in the US or Europe (7). However, for specific applications, such as *in vivo* imaging, the efficacy of classical antibody molecules might be impaired by reason of their large hetero-tetrameric structure (Fig. 1, left). Slow blood clearance, restricted tumor penetration as well as non-specific uptake by healthy tissues

might pose problems for distinct applications (8-12) To address these issues and consequently to substantially enlarge the repertoire of biologic therapeutics and diagnostics in order to ultimately select the right molecule for an individual application, next-generation antibodies, such as antibody-drug conjugates (13, 14) or immunocytokines (15), antibody fragments (16) and scaffold proteins (17-19) were generated. Related to this, antigen-binding sites were introduced into the Fc-fragment of human IgG, significantly reducing the complex nature of a full-length antibody, as reviewed by Dimitrov and colleagues (20). In a very elegant approach, described by Rüker and co-workers, antigen-binding sites were engineered into the *C*-terminal tip of CH3, while retaining functionality of the Fc-part, e.g. long serum half-life and antibody-dependent cellular cytotoxicity (21).

In addition to engineered antibody-derivatives, the immune systems of camelids and sharks comprises non-conventional heavy chain only antibody molecules (22, 23), where antigen binding is mediated solely by one single domain, referred to as VHH and vNAR, respectively. In general, those domains exhibit high specificities for a cognate antigen, high physico-chemical stability as well as a small size and are thus considered as promising candidates for biomedical development. The transfer of key elements of VHHs onto human VH domains led to the development of single human variable heavy chain domains, referred to as camelized human VH domains (24). Nowadays, the generation of fully human single variable domain antibodies (domain antibodies, dAbs), either composed of the variable domain of an antibody heavy chain or light chain, is amenable (25). Besides, it has been shown that also monomeric human IgG derived constant domains, i.e. CH2 and CH3 can be engineered as scaffolds mediating antigen- and/or functional FcRn-binding (26).

In general, single domain antibodies exhibit high specificities for a cognate antigen, high physico-chemical stability as well as a small size allowing for enhanced tissue penetration compared to conventional antibodies. The ease of engineering as well as their capability for

modular formatting into multispecific and multivalent constructs emerge the possibility for enhanced biomedical potential.

The purpose of this review is to summarize unique features of single domain antibodies, placing the emphasis on diagnostic and therapeutic applications.

VHH domains derived from camelids

Immunoglobulin repertoires of llamas and other camelids comprise so called heavy chain only antibodies (HCAbs). Hamers-Casterman and coworkers discovered this isotype, which is lacking light chains and CH1 in the serum of camels (22). In these molecules the antigen-binding-domain is directly linked to the Fc-portion *via* a hinge region and was named VHH (variable fragments of heavy chain antibodies) (Fig. 1, middle). Although VHHs with a molecular weight of 12-15 kDa are even smaller than engineered antibody formats like Fab-fragments or scFvs, they exhibit specificities and affinities comparable to that of monoclonal antibodies (27).

The overall organization of the VH domain of a classical antibody and the VHH domain of an HCAb is noticeably similar. Both consist of three complementary determining regions that are located in loops connecting two beta sheets of the immunoglobulin domain and a canonical disulfide bond connecting framework 1 and framework 3 (23). But there are also distinct structural differences. The VHH-domain forms a convex shape in some cases that allows for penetration of concave epitopes and clefts such as the active site of enzymes, which are often not accessible with flat paratopes of conventional antibodies. This convex shape originates from an extraordinarily long CDR3 which can be stabilized by an additional disulfide bond either connecting CDR3 with the beginning of CDR2 in llamas, or the end of CDR1 in camels (28). Furthermore VHHs exhibit remarkable solubility, which is attributed to four conserved hydrophilic residues in framework 2. In conventional antibody derived VH domains,

homologous residues in framework 2 are hydrophobic and contribute to heavy and light chain pairing (29).

Recombinant production of VHHs emerged as a seemingly inexpensive alternative to the production of monoclonal antibodies. While the expression of conventional antibodies demands a complex folding machinery and appropriate posttranslational modifications and therefore a higher eukaryotic expression host, VHHs are easily produced in prokaryotic hosts like *E. coli* in a two to three digit milligram scale per liter (30). However, it needs to be mentioned that classical IgG antibodies can be produced in the gram per liter scale in mammalian production hosts (31) and related to the cost-of-goods of downstream processing the 'overall monetary benefit' might be questioned.

The absence of posttranslational modifications, the small size and the stable single domain structure of VHHs allow convenient protein engineering with the most common platform technologies like phage (32), yeast (33), ribosomal (34) or bacterial display (35). A widely used strategy to obtain high affinity binding molecules is the immunization of camelids with the respective antigen followed by a subsequent library generation, e.g. from blood lymphocytes followed by selection and screening (32). However, binders against several targets could also be obtained using naïve (36) and synthetic VHH libraries (37). With respect to the biomedical use of VHHs, a low immunogenicity can be expected due to the high sequence similarity to the human heavy chain variable domain, which differs in about 10 amino acids. Further attempts to reduce the risk of immunogenicity led to a general humanization strategy. Therefore, a stable humanized VHH scaffold was generated that allows grafting of antigen binding loops from other VHHs while retaining antigen affinities and specificities (38).

A variety of potential biomedical applications for VHHs are described in the literature, however we emphasize in the following section several distinct therapeutic and diagnostic applications. Although no therapeutics based on VHHs have found their way to the market

yet, six have entered clinical trials phase I or II. The popular term “Nanobody” serves as trade name for VHHs belonging to the commercial biopharmaceutical company Ablynx (www.ablynx.com), which is currently the main driver of development of VHHs for biomedical applications. A phase II study of the humanized bivalent nanobody-Fc-fusion Caplacizumab (ALX-0681) for the treatment of the rare but life-threatening disease thrombotic thrombocytopenic purpura (TTP) has recently been completed. Caplacizumab neutralizes the exposed A1 domain of ultra large von Willebrand Factor (ulvWF), thereby preventing the interaction of ulvWF with the GpIb-IX-V receptor on platelets. In TTP patients the ulvWF/GpIb-IX-V interaction is responsible for excessive platelet aggregation (39).

Another construct is the trimeric bispecific nanobody Ozoralizumab (ATN-103) for the therapy of rheumatoid arthritis. Ozoralizumab consists of two VHHs directed against TNF and one albumin-binding VHH, for prolonged serum half-life *in vivo*. Possible advantages over current therapeutics like Adalimumab®, Enbrel® and Remicade® are a better efficacy and reduced manufacturing costs (40). Phase II studies of Ozoralizumab as well as one open label extension study have been carried out, evaluating long term safety, tolerability, and pharmacokinetic properties of multiple ascending doses (clinicaltrials.gov). Other promising nanobodies in clinical development target IL6 (ALX-0061), IL17 (ALX-0761), RSV (ALX-0171) and RANKL (ALX-0141). In addition, VHHs are attractive for addressing difficult targets, for instance G protein-coupled receptors (GPCRs). In this respect the cryptic epitopes of GPCRs are located within transmembrane regions and are poorly accessible with monoclonal antibodies and therefore small molecules are mostly used for GPCR targeting (41). Studies highlighting this targeting approach using nanobodies were recently described for two GPCRs, CXCR4 (42) and CXCR7 (43), respectively.

Beside their use as potential therapeutics, VHHs have also been shown to be sensitive detection probes in diagnostic applications. For the diagnosis of HIV, a VHH directed against human glycophorin A (a protein on red blood cells) was developed as a fusion protein with

p24 (HIV capsid-protein). When the fusion protein is added to the serum of HIV positive donors, rapid agglutination is induced due to cross linkage of red blood cells in the presence of anti-p24 antibodies (44).

Another approach utilizing VHHs in diagnostics is the detection of influenza H5N1, which was recently demonstrated in a sandwich ELISA format. This assay provided a lower limit of detection as compared to commercially available diagnostic kits using monoclonal antibodies (45). Furthermore radiolabeled VHHs have been used in tumor imaging. In a preclinical validation ^{99m}TC-labeled nanobodies directed against the macrophage mannose receptor, which is consistently upregulated in tumor associated macrophages (TAM), led to an efficient *in vivo* targeting and imaging and could be a novel approach for the diagnosis of cancer (46).

vNAR domains derived from sharks

Besides antibodies with the classical composition of heavy and light chains, sharks produce a heavy chain only isotype, referred to as Ig New Antigen Receptor (IgNAR) (23). IgNAR is a homo-dimer in which each chain consists of five constant domains, followed by a variable domain (vNAR, IgNAR V), solely facilitating antigen binding (**Fig 1, right**) (47). The IgNAR V domain displays several unique features, clearly distinguishing it from camelid VHH domains. Due to a deletion in the framework2-CDR2-region, the β-sandwich fold only consists of 8 instead of 10 β-strands, making the vNAR domain the smallest antigen binding antibody-like domain in the animal kingdom known to date, with a molecular mass of approximately 12 kDa (48, 49). Consequently IgNAR V domains only have two complementarity determining regions CDR1 and CDR3 (**Fig. 1, right**). However, at the CDR2 truncation site, the remaining surface exposed loop forms a ‘belt-like’ structure and it was shown that after antigen-contact, high rates of somatic mutation also occur in this loop and in a loop which corresponds to HV4 in T-cell receptors, to which vNAR domains show structural similarity. Hence, these regions have been termed HV2 and HV4, respectively (50).

Interestingly, it has been shown very recently by our group, that through randomization of HV2 in conjunction with yeast surface display, this surface exposed loop can be engineered in a way that it functions as an autonomous paratope, solely facilitating antigen binding (51). This work might pave the way for the construction of bispecific vNAR domains, which would represent the smallest bispecific antibody units known to this date.

There are several different types of vNAR molecules, categorized based on the number and pattern of non-canonical disulfide bonds that are not found in classical antibody domains (49, 52-55). Owing to this, the different types of vNAR domains form a very diverse set of additional disulfide bridges. Consequently, antigen-specific clones can be selected from a unprecedented repertoire of different loop structures (56). Moreover, the architecture of the paratope of those shark antibody domains seems to be predisposed to target clefts of the antigen, whereas such recessed epitopes are usually not antigenic for conventional antibodies (55, 57-59). Indeed, it has been shown that the active site of enzymes and clefts can be targeted by vNAR domains (49, 54, 60). A more comprehensive review, addressing the different types of IgNAR V domains as well as the generation of the tremendous diversity found at the sequence level of the IgNAR V domain was recently published by our group (47).

In addition to their aforementioned small size and paramount structural features, also the superior stability compared to conventional antibody domains and tolerance to irreversible denaturation render vNAR domains as promising candidates for clinical development (61-63). It has been successfully shown that there are multiple opportunities to re-format and functionalize the vNAR domain, including monomeric, dimeric and trimeric constructs as well as Fc-based formats, clearly demonstrating the possibility to utilize those molecules for a plethora of different applications (53, 64-68). Accordingly, target-specific vNAR molecules have been isolated against a wide range of disease-related antigens, including viral targets, toxins and cytokines as well as proteins involved in cancer and arthritis (47, 61-63, 69-74).

Above all, when higher affinities are required, several methodologies were established to optimize affinities of isolated antigen-binding molecules (63, 71, 75).

However, in contrast to camelid VHHs, which have proven to be successful in early stage clinical trials (76), progress of the development of vNAR domains for clinical applications is at a much earlier stage. This might be at least partially explained by the low homology of shark vNAR domains with mammalian variable domains, and potential immunogenicity arising thereby. Sequence identity of the IgNAR V domain with mammalian VH regions falls as low as 25 % (77). Even after humanization of a shark vNAR domain a significant amount of non-CDR residues still remained non-human, as described by Kovalenko *et* al (53). In contrast to this, sequence homology of VHHs to human VHs is high, differing only in about ten amino acids and it has been shown that VHHs can be easily humanized by loop grafting (78, 79).

Notwithstanding, it is known that even humanized and fully human antibodies such as Adalimumab® are able to significantly induce immunogenicity, essentially demonstrating that it needs to be scrutinized for each individual therapeutic candidate how these proteins will behave with regard to adverse events when injected to patients in the scope of clinical trials (80-84).

Human antibody variable domains

Human domain antibodies are the smallest known fragments from variable domains of conventional antibodies with retained antigen-binding function and sizes ranging between 11-15 kDa. This antibody type includes antibody heavy (VH dAb) or light chain (VL dAb) variable fragments and comprises three CDRs that mediate specific antigen-interaction (25). In their structure, human dAbs closely resemble the architecture of paired variable fragments in conventional antibodies (Fig. 1 top) (25, 85). In contrast to the naturally evolved equivalent in camelids and sharks, early studies revealed that single human VH domains are prone to

aggregation and exhibit poor solubility, which is caused by solvent exposure of hydrophobic patches in the absence of an interacting VL domain (86).

The unfavorable properties were tackled in a variety of mutational approaches and initial attempts aimed at the transfer of VHH key elements that attributed aggregation-resistance and good solubility to camelid VHHs (85). A process was established, named "camelization", in which residues of the VL-interface were substituted with hydrophilic residues that are conserved in VHHs (G44E/L45R/W47G) (86). Furthermore, it also became clear that composition and length of the CDRs are determinants of aggregation-propensity and a positive effect on solubility of VH dAbs was shown by the extension of the CDR3 length (25, 86, 87). All these studies pointed out residues of the dAb framework and the CDRs as critical constituents for good biophysical properties and provided guidance for the engineering of scaffolds for library applications.

Nowadays, convenient generation of human dAbs with desired properties and specificities has been achieved using molecular evolution approaches (e.g. phage display) and repertoires of naïve or synthetic human VH or VL dAbs (85, 88, 89) However, most of the human dAb affinity reagents have been isolated from synthetic libraries that are usually constructed with engineered single scaffolds and CDR-based diversities (90-96). For library construction, it has been shown that several variants of the human germline families possess superior biophysical characteristics (e.g. HV3-23) and therefore are considered as preferable scaffolds (86, 96). Compared to VH domains, VL domains generally seem to have a higher intrinsic resistance towards aggregation (85, 86, 90). Another source of VH dAbs are transgenic mice with human heavy chain only repertoires. However, this platform still has to prove the delivery of stable high-affinity binders (crescendobiologics.com, (96)).

Several protocols for affinity selection by phage display have been established that consider aggregation-behavior during selection. In one study by Jespers and colleagues, aggregation-resistant dAbs were selected by applying heat-denaturing conditions in a panning against

protein A, resulting in an enrichment of variants with reversible-folding properties (88). Similar to that, Famm and colleagues established the acid-denaturation method that allowed selection of thermodynamically stable and aggregation-resistant dAbs (97). For biomedical applications, the most notable feature of domain antibodies with human origin is their perceived lower immunogenicity compared to their equivalents from camels and sharks which is of importance particularly in long-term clinical use or in context of systemic administration (98, 99). While several dAbs have been explored for a therapeutic application, no dAbs have been tested in clinical phase III trials or were approved for clinical use.

Several instances for human dAbs with therapeutic relevance are described in the following section. However this selection does not intend to provide a complete list of all early and late stage therapeutics. In contrast to a variety of studies that address therapeutic applications, diagnostic applications are underrepresented in the literature.

Research activities by GSK/Domantis led to several dAbs that have been evaluated in clinical phase I trials. Targets that have been addressed include TNF receptor-1 (TNFR1) (GSK2862277 and its predecessor GSK1995057), human albumin (GSK2374697, a genetic fusion protein of albumin binding dAbs to exendin-4) and IL-1 receptor (GSK182771), (Clinicaltrials.gov).

Although it has been proposed that dAbs have a low immunogenicity, a novel type of pre-existing anti-drug antibody was recently discovered during the development of anti-TNFR1 GSK1995057 (100). Herein, autoantibody-binding to framework sequences of the fully human VH dAbs triggered activation of TNFR1 in *in vitro* assays and in some drug-naïve subjects the release of cytokines *in vivo* (100). GSK2374697 was developed for treatment of type 2 diabetes and includes a human albumin targeting VH dAb (AlbudAb^{TM}) for half-life extension thereby prolonging the agonistic effect of the adaptor expendin-4 on the glucagon-like peptide-1 (GLP-1) receptor (101, 102).

A bivalent construct was developed by Teva/Cephalon (CEP-37247/ART621) that includes an Fc-part, a truncated CH1 region and two TNF-targeting VL dAbs (103). However, clinical research activities were stopped after a phase II clinical trial for the treatment of rheumatoid arthritis (Clinicaltrials.gov, ir.tevapharm.com).

Chen and colleagues recently published a dAb-based bispecific multivalent construct in a preclinical study (104). Herein, an engineered HIV-1 inhibitor (mD1.22) was combined with a human dAb (m36.4) that targets the coreceptor binding site of the HIV-1 envelope glycoprotein gp120. By using a human Fc-part as a scaffold, different constructs were generated with varying numbers of binding entities. The multivalent proteins exhibited improved potency in the neutralization of HIV-1 isolates, compared to the commercially available benchmark antibody VRC01 (104).

Several cancer-related targets have been addressed with human dAbs. Feng and colleagues isolated a high affinity binding VH dAb (HN3) against a conformation-dependent epitope in Glypican-3 (GPC3) (105). HN3 was tested as bivalent Fc-fusion protein on several hepatocellular carcinoma (HCC) cell lines where it specifically inhibited proliferation. In addition, HN3 also repressed tumor growth in a xenograft tumor model by inducing cell-cycle arrest via Yes-associated signaling (105). Other cancer related targets have been addressed, including the targeting of IGF-II (m630.3), SD1 or type IV collagenase for tumor targeting (106-108). Recently, another CD28-targeting human VL dAb was tested in a preclinical study for the treatment of autoimmune diseases. A pharmacodynamic activity of the PEGylated VL dAb was shown by inhibition of a T-cell-dependent Ab response in cynomolgus monkeys without mediating cytokine release or T-cell depletion (109).

Engineered monomeric human IgG1 CH2 and CH3 as a scaffold

Another class of conventional antibody derived single domains has recently been introduced for scaffold application including engineered CH2 and CH3 domains (26). The most

remarkable feature of engineered domains is their proposed long half-life due to functional FcRn-binding. However engineered CH2 domains seem to have a high aggregation tendency (110, 111). The antiparallel β-strand structure of CH2 domains is similar to that of human VH domains comprising three loops (BC, DE and FG) in that the BC and FG loops at the N-terminal tip can be used for diversification (112). A CH2 variant was engineered (m01s) by the incorporation of an additional intra-domain disulfide-bridge between A and G strands and the elimination of several N-terminal residues. In contrast to the wild-type CH2, the m01s variant exhibited pronounced thermal stability, good expression yield and high solubility (113). *In vivo* mice studies with injected m01s revealed a longer half-life due to FcRn-interaction, compared to scaffolds of similar size that do not interact with FcRn (114). Furthermore, m01s was used for phage library construction by grafting human VH CDR3 diversities into the loop FG and mutagenesis of the BC and DE loops. One variant was isolated (m2a1) that showed selective binding to sp62 from the HIV-1 membrane proximal external region (MPER) and functional FcRn-binding (110).

In addition, a monomeric soluble CH3 domain (mCH3) was developed recently (115). A structure guided mutagenesis approach led to the identification of four residues in the CH3 dimerization interface that were shown to be crucial for the generation of monomeric CH3 domains. Furthermore, the thermal stability was increased by incorporation of a disulfide bridge (from Tm= 40,6 °C to Tm=76 °C). Nevertheless, mCH3 was able to bind FcRn in a pH-dependent manner. Anyhow, the measured affinity was lower compared to wild type Fc (KD 940 nM versus 126 nM). This is attributed to the lacking CH2 domain, as residues of CH2 and CH3 in IgG1 together are responsible for FcRn interaction. In this proof of concept study the HIV-1 neutralizing dAb m36.4 was generated as a fusion protein with mCH3 (m36.4-mCH3). M36.4 targets an epitope on the HIV-1 envelope glycoprotein, which seems to be fully accessible only to small molecules. While no measurable neutralizing activity of dimeric CH3 and Fc fusion proteins was obtained, m36.4-mCH3 exhibited strong

neutralization. In this study a new scaffold was developed, which can be used as a fusion partner for small antigen binding domains that possess the advantage of good tissue penetration in combination with an expected prolonged serum half-life in *vivo,* due to FcRn mediated recycling (115).

Conclusion

In recent years, single domain antibodies have broadened the spectrum of therapeutically and diagnostically relevant proteins. New insights were gained by breaking apart the complexity of full-length antibodies of sharks, camelids and humans down to single functional fragments/units. These single domains can be remodeled, engineered and reassembled to generate new antibody-like molecules with desired properties and specificities, consisting of multiple units of the same or of different function such as for example the trimeric bispecific nanobody Ozaralizumab (ATN-103) that consists of two VHHs directed against TNF and one albumin-binding VHH (40).

Due to their small size, unique structure and nature of paratopes some characteristics of classical antibodies *e.g.* slow blood clearance, restricted penetration of solid tumors, non-specific uptake by healthy tissue and the inability to access recessed epitopes, can be overcome by single domain antibodies. Especially vNARs and VHHs are able to address targets that are not accessible by classical antibodies, such as active sites of enzymes.

In general, engineered antibody fragments retain their specific binding characteristics with high affinity and additionally, owing to their small size, gain an improved tissue penetration. For some diagnostic utilization as for example *in vivo* tumor imaging, fast blood clearance of single domain antibodies is desired, whereas for therapeutic applications a long serum half-life is favorable. Several efforts were made to prolong the circulation time of single domain antibodies for instance by PEGylation, engineering albumin binding, or formatting as Fc fusion for FcRn-binding.

In a relatively short amount of time single domain antibodies with beneficial physicochemical attributes can be generated against various therapeutically relevant targets. With candidates already being assessed in clinical phases I and II, it can be expected that single domain antibodies will find their way into the clinic in this decade.

Declaration of interest

The authors declare no competing interests.

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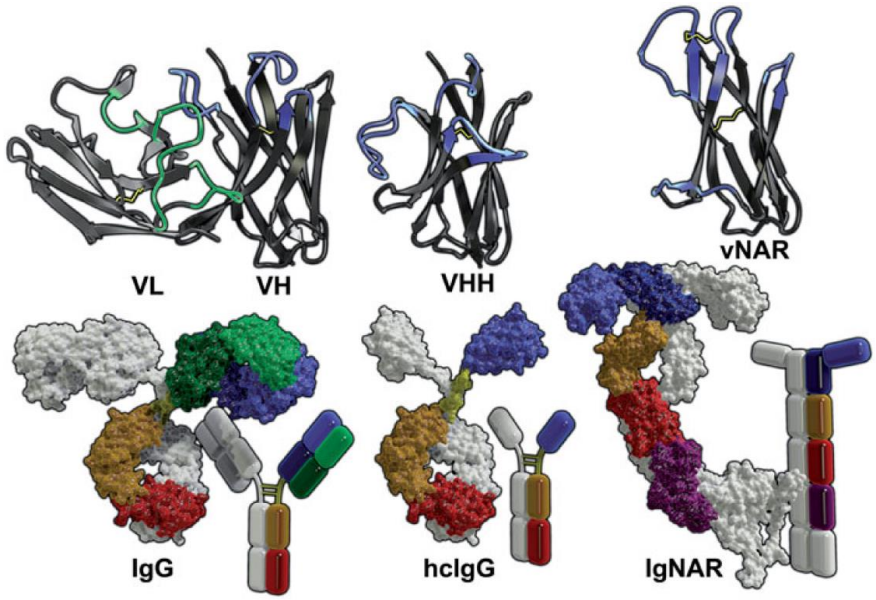

Figure 1. Structures of IgG (left; PDB-ID: 1IGT), camelid hcIgG (middle; model as described in reference (47)), and shark IgNAR (right; model was generously provided by Prof. Dr. Michael Sattler and Dr. Janosch Hennig (60)). (Top) Ribbon representations of variable

domains VL/VH, VHH, and vNAR with highlighted binding epitopes CDR1-3 (blue/green), as well as HV2 and HV4 (blue). Disulfide bonds are shown in yellow. (Bottom) Surface and schematic representations of full-length antibodies. VL: light green, CL: dark green, VH/VHH/vNAR: light blue, CH1/C1: dark blue, CH2/C2: orange, CH3/C3: red, C4: magenta, C5 (hypothetical structure) and redundant chains of homo oligomers: white, hinge regions: yellow, glycans not shown. Picture rendered with POV-Ray (http://www.povray.org/).

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