

Structural insights and biomedical potential of IgNAR scaffolds from sharks

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Abbreviations: CDR, complementarity-determining region; 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

In addition to antibodies with the classical composition of heavy and light chains, the adaptive immune repertoire of sharks also includes a heavy-chain only isotype, where antigen binding is mediated exclusively by a small and highly stable domain, referred to as vNAR. In recent years, due to their high affinity and specificity combined with their small size, high physicochemical stability and low-cost of production, vNAR fragments have evolved as promising target-binding scaffolds that can be tailor-made for applications in medicine and biotechnology. This review highlights the structural features of vNAR molecules, addresses aspects of their generation using immunization or in vitro high throughput screening methods and provides examples of therapeutic, diagnostic and other biotechnological applications.

Introduction

Today, biological entities are one of the main drivers of the pharmaceutical industry as exemplified by their current and predicted market growth rates, which substantially exceed those of the overall sector. Within this group of biologic drugs, monoclonal antibodies (mAbs) are the highest selling class, followed by hormones, growth factors and then fusion proteins.^{1,2} The high specificity for a cognate antigen combined with Fc-mediated immune effector functions have underpinned the success of antibodies as effective tools for medical applications. With ~40 antibodies

marketed and hundreds of mAbs in clinical development, the therapeutic and economic value of mAbs is evident.^{1,3-5} Antibodies are structurally complex, large hetero-tetrameric proteins that consist of 2 heavy chains and 2 light chains (Fig. 1A). The 2 identical antigen-binding sites, i.e., paratopes, are composed of one variable domain of the light chain and one variable domain of the heavy chain, respectively. However, under certain circumstances the therapeutic and diagnostic efficacy of antibodies might be limited due to inherent attributes, e.g., structural complexity, large size. The paratope of conventional antibodies can be restricted in its ability to access certain epitopes, e.g., recessed cryptic epitopes, active sites of enzymes.⁶⁻⁸ Furthermore, the mobility, i.e., tissue penetration, of classical antibody molecules is constrained by their large size.⁹ For in vivo tumor imaging purposes, the slow blood clearance of conventional antibodies poses a problem due to their extended plasma half-life.¹⁰ Slow tumor penetration as well as nonspecific uptake by healthy tissues may represent further drawbacks of conventional antibodies in molecular imaging.¹¹⁻¹⁴ To address these issues and to increase the overall therapeutic efficacy, next-generation-antibodies, antibody fragments and non-immunoglobulin based protein scaffolds have been engineered and developed, as extensively described elsewhere.¹⁵⁻²⁴

Camelids and the cartilaginous fish possess natural antibodies composed only of heavy chains (Fig. 1B and 1C).^{25,26} The antigen binding site is formed by only one single domain, referred to as VHH and vNAR, respectively. Due to an increased frequency for polar and charged amino acids at the solvent-exposed regions corresponding to the hydrophobic VH-VL interface of conventional antibodies, vNAR and VHH domains are highly soluble.²⁷ Antigen-binding domains of heavy chain only antibodies (HCAs) combine most of the beneficial features of non-immunoglobulin-based protein scaffolds, e.g., small size, high stability, coupled with the advantageous characteristics of classical antibody molecules, most strikingly the feasibility to generate highly specific and high-affinity binders through immunization.^{6,28}

Even more interestingly, HCAs naturally complement the conventional repertoire of the aforementioned species. Whereas

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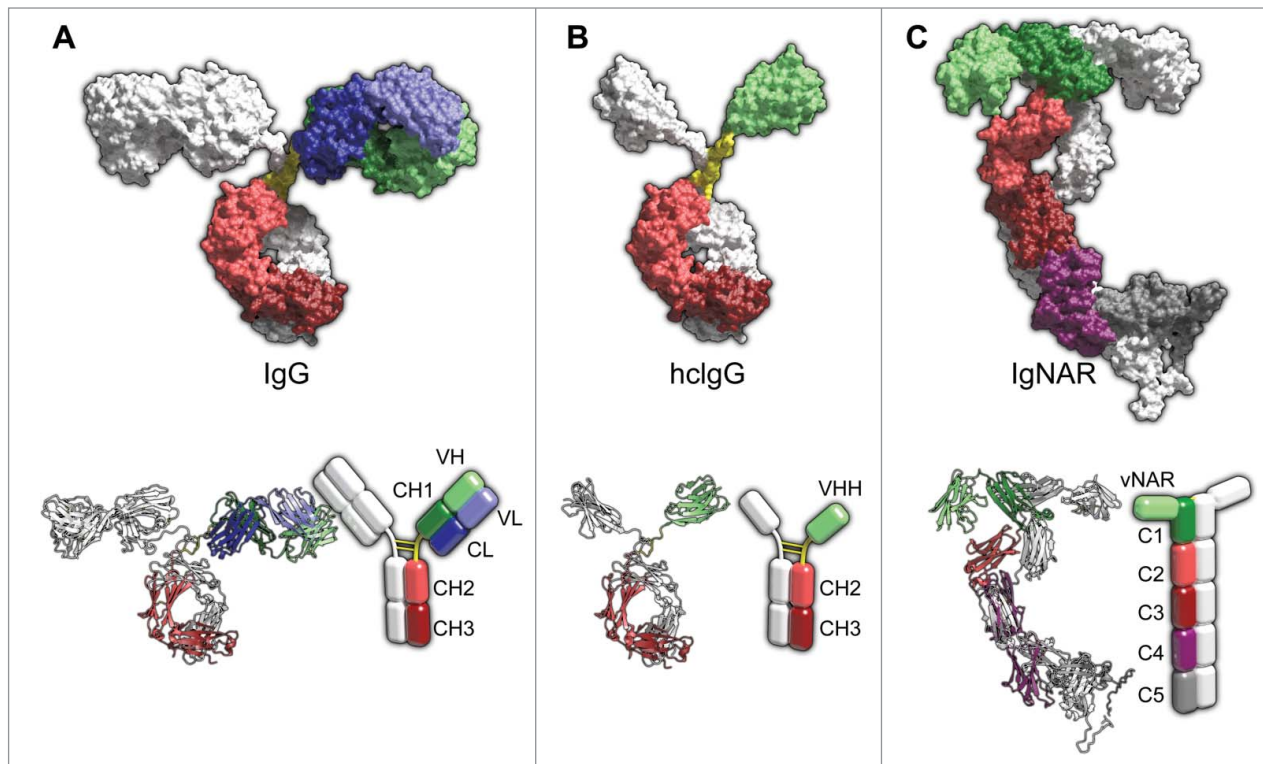


Figure 1. Structural features of intact IgG (A), camelid hclgG (B), and shark IgNAR (C) antibody formats shown as surface representation (top) as well as ribbon and schematic representations (bottom). Individual domains are colored as indicated in the schematic representation; hinge regions are colored yellow; glycans not shown. (A) IgG model is based on pdb entry 1IGT.⁹⁵ (B) Model of hclgG was generated by molecular replacement based on pdb entries 1IGT (for Fc region) and 1IEH (for VHH domain).^{95,96} 1IEH was aligned to the CH1 domain of 1IGT with YASARA structure.⁹⁷ After deletion of absent domains (VL, CL, VH, and CH1), the VHH section was connected to the Fc region via a short camelid hinge sequence (see Hamers-Casterman et al.).²⁵ Then, a 2-step energy minimization using YASARA2 force field was conducted to yield the depicted structure. (C) Coordinates of intact IgNAR including the hypothetical structure of IgNAR C5 domain were generously provided by Prof. Dr. Michael Sattler and Dr. Janosch Hennig (see Feige et al.).³⁹ Picture rendered with POV-Ray (www.povray.org/).

classical antibodies usually have planar or concave antigen binding sites, vNAR- and VHH-domains possess a wide variety of additional (in the case of vNARs) and different loop structures. This leads to a drastically expanded repertoire of available paratopes capable of accessing and binding to more cryptic epitopes and catalytic clefts of enzymes that are intractable to classical antibodies.²⁹⁻³¹ Hence, vNAR and VHH domains could add considerable value to the therapeutic development pipeline by broadening the range of druggable targets.

While camelid VHH domains have proven to be successful in early phase clinical trials,³² the engineering of vNAR domains for biomedical applications is at an earlier stage. However, significant progress demonstrating the therapeutic utility of these domains has been made in the last years. The purpose of this review is to summarize the research conducted to date of IgNARs, placing the emphasis on recent progress of the development of vNAR domains for therapeutic, diagnostic and other biotechnological applications.

The New Antigen Receptor

The cartilaginous fish (sharks, rays, skates and chimaeras) express 3 different isotypes of antibodies, IgM, IgNAR and

IgW.^{33,34} Recent analysis of the genome of African coelacanth, *Latimeria chalumnae* indicated that IgM was absent in this species, while IgW was present, supporting the notion that IgW is a primordial immunoglobulin class.^{35,36} IgNAR was first identified in the serum of the nurse shark (*Ginglymostoma cirratum*) in 1995 by Flajnik and co-workers.²⁶ It is a homodimer of heavy chains devoid of light chains. Each chain of the secretory form consists of one variable domain followed by 5 constant domains, the last 4 being homologous to IgW constant domains.³⁷ Serum IgNAR levels range from ~0.1 mg/ml to 1 mg/ml.³⁸

Based on atomic resolution structural data as well as small-angle X-ray scattering, Buchner and coworkers were able to develop a structural model of the complete IgNAR molecule (Fig. 1C).³⁹ Within the molecule, domains C1 and C3 of each chain cause dimerization of IgNAR. Despite the lack of a canonical hinge region, the variable domains are spaced sufficiently wide for binding multiple epitopes, facilitated by the wide angle of the C1 dimerization interface. A small angle between both C3 domains induces the formation of a narrow stalk for the IgNAR molecule. However, the flexibility of the stalk is induced by a disulfide-bridged linker that connects domains C3 and C4. The heavy chain-only molecule is kinked approximately in the middle of the molecule, at the location

of the flexible linker, causing its characteristic shape. Whether any effector functions are mediated by the constant region of IgNAR is currently unresolved.⁶ Finally, it is important to note that the structure of C5 shown in **Figure 1C** is completely hypothetical. This is due to a lack of structural data on this domain, which does not exhibit a fold, neither as isolated recombinant protein nor within a C4-C5 construct.³⁹

Absence of a Light Chain Partner

The homodimer IgNAR displays several unique features that are responsible for the inhibition of a potential light chain pairing. At the typical VH-VL interaction site, there is poor conservation of residues that mediate this association in mammals.⁴⁰ Instead these typically hydrophobic amino acids are frequently replaced by polar or charged residues.²⁷ For classical antibodies, a special mechanism ensures the formation of heavy- and light-chain pairing. In the endoplasmic reticulum, the heavy chain is trapped by an Ig-binding protein (BiP) *via* interaction with the CH1 domain. For the release, a light chain must displace BiP, and, consequently, only heavy- and light-chain paired antibodies are secreted.^{27,41} Flajnik and coworkers hypothesized that during evolution, a vNAR-D-J cluster recombined with an IgW cluster in a way that the IgW cluster lost its V-D-J segments and the first C exon.⁴² Indeed, the C1 domain of IgNAR is somewhat similar to the CH2 domain of IgW and may be derived from this domain.⁴³ BiP- and L-chain-interactions sites are consistently

missing in the C1 domain of IgNAR, as elegantly reviewed by Flajnik and colleagues.²⁷

The Variable Domain of IgNAR – Structural Features

The variable domain of the New Antigen Receptor shows homology to the T-cell receptor (TCR) V_{α} and also is found as a variable domain in the NAR-TCR δ .⁴⁴ It also displays sequence homology to immunoglobulin V_{κ} domains, whereas structurally it is related to V_{α} , V_{λ} , and VH domains.³⁰ The evolutionary relationship of IgNAR and TCR and their therapeutic potential was recently reviewed.⁴⁵ Moreover, since vNAR domains share structural features of cell adhesion molecules, it was suggested that IgNAR evolved from a cell-surface receptor, clearly distinguishing it from VHH, which evidently arose from an IgG lineage.^{27,46} vNAR belongs to the Ig superfamily, and accordingly it has a β -sandwich fold. However, compared to mammalian V domains, this fold only consists of 8 instead of 10 β -strands due to the deletion in the framework2-CDR2-region (**Fig. 2**).

With a molecular mass of ~ 12 kDa, the vNAR domain is the smallest antibody-like antigen binding domain in the animal kingdom known to date.^{6,30} As a consequence, contrary to mammalian variable domains, vNAR domains have only 2 complementarity determining regions CDR1 and CDR3 (**Figs. 2, 3**). The diversity of the primary vNAR repertoire is predominantly found in CDR3. High rates of somatic mutation after antigen contact are observed in CDR1, at the CDR2 truncation site, where the remaining loop

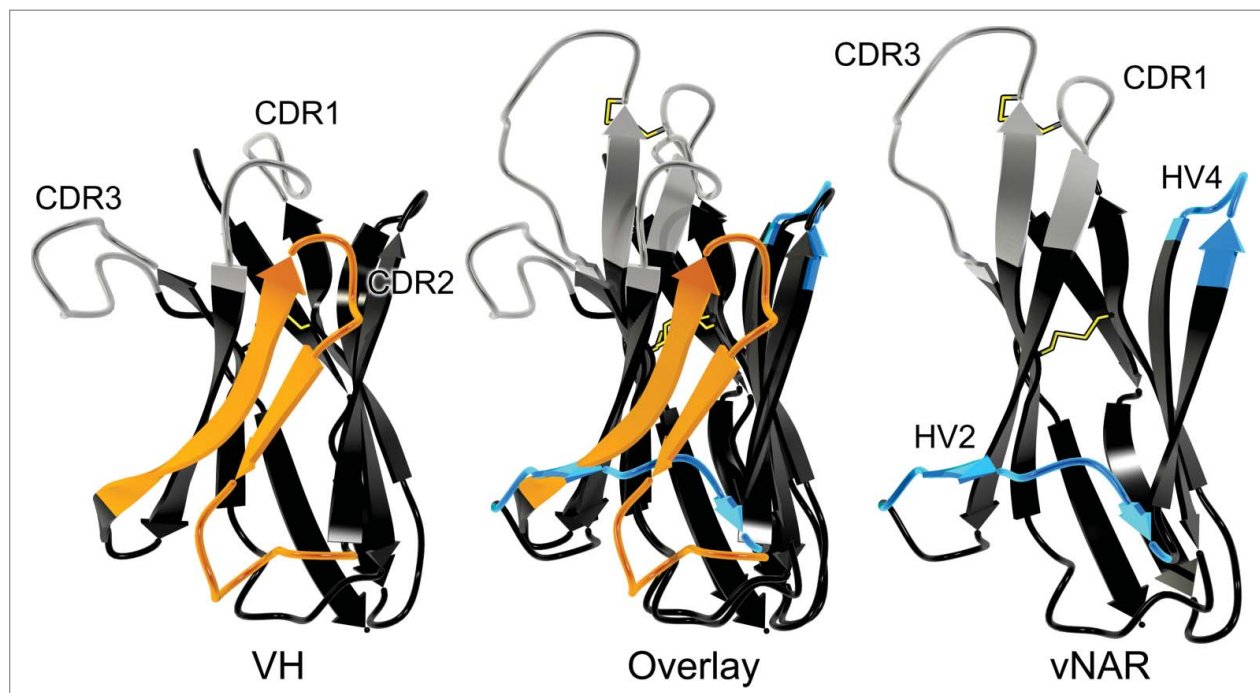


Figure 2. Comparison of VH (left; from pdb entry 1IGT) and vNAR (right, from pdb entry 2COQ) binding domains depicted as ribbon representation as well as an overlay of both structures (middle).^{31,95} CDR1 and CDR3 are shown in gray. Two β strands and CDR2 of the VH domain are highlighted in orange. These structural elements are absent in the vNAR domain which possesses HV2 and HV4 (both highlighted in blue), instead. Disulfide bonds are shown as yellow sticks. Picture rendered with POV-Ray (www.povray.org/).

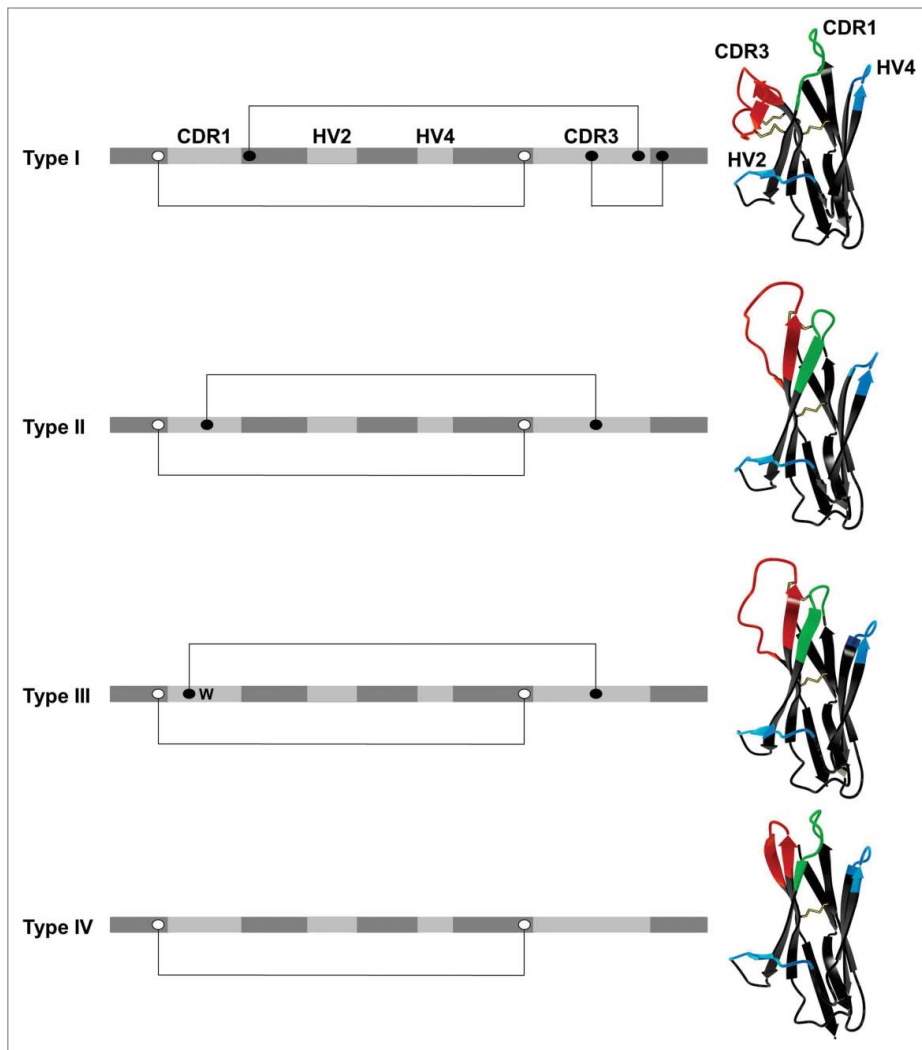


Figure 3. Different types of IgNAR V domains. Variable domains are categorized based on the presence or the absence of non-canonical cysteine residues (black dots). Canonical cysteine residues (white dots) and disulfide bonds (connecting lines), conserved tryptophan (W) as well as complementarity determining regions (CDR) and hypervariable loops (HV) are shown in their relative positions. Ribbon presentations of vNAR domains are depictions of pdb entries 1S22 (type I),³⁰ 2COQ (type II),³¹ and 4HGK (type IV)⁵² as well as a modeled type III structure based on 2COQ. The latter was generated via homology modeling using YASARA structure.⁹⁷ First, vNAR residues of 2COQ were changed to match a reported type III sequence (AAM76948 from Streltsov et al.).³¹ Then, side chain geometries were optimized followed by a 2-step energy minimization using the YASARA2 force field.⁹⁷

forms a belt-like structure at the bottom of the molecule, and in a loop that corresponds to HV4 in TCRs. Accordingly, these mutation-prone regions have been named HV2 and HV4, respectively (Fig. 2).⁴⁷ Indeed, it was shown that somatic mutations within HV4 can contribute to antigen binding.⁴⁸

Despite having a reduced number of possible antigen binding loops (4 across a single chain) compared to conventional antibodies (6 loops across 2 chains), vNAR domains bind antigens with surprisingly high affinities.^{49,50} Even from primary repertoires, where antigen binding is solely mediated by CDR3, vNAR molecules can be obtained against a given antigen with affinities in the low nanomolar range.^{48,49} The highest recorded affinities for vNAR domains, however, have been observed after

immunization with an anti-albumin binding domain, achieving picomolar levels of affinity.⁵⁰

Based on the number of non-canonical cysteine residues, which are not found in classical variable domains, vNAR molecules have been categorized into 4 types (Fig. 3).^{30,31,48,51,52} The classical Ig canonical cysteines, which stabilize the immunoglobulin fold *via* a disulfide-bond, are common to all types. Type I variable domains carry extra cysteines in framework regions 2 and 4, and, consequently, an even number of partner cysteine residues in CDR3. The determination of the crystal structure of a type I vNAR in complex with lysozyme revealed that both non-canonical framework cysteines each form disulfide-bonds with those of CDR3, causing this loop to be held tightly into the direction of HV2.³⁰ Thus far, type I variable domains of IgNAR have only been identified in the nurse shark, *Ginglymostoma cirratum*.⁶

Type II domains differ from type I by means of an additional cysteine in CDR1 and in CDR3, respectively, resulting in an intra-molecular disulfide bond that brings both loops in close vicinity. However, it lacks both cysteine motifs that anchor CDR3 to the framework in type I vNAR. As a consequence, the CDR3 region forms a protrusive ‘finger-like’ structure that is predisposed to binding into pockets or grooves, e.g., the active site of enzymes.⁵¹ According to this, it has been shown for both types that active site clefts can be penetrated by the antigen binding loops.^{30,48}

Another type, termed type III, is expressed in neonates at high frequencies.^{31,51} Akin to type II domains, this isotype is characterized by an additional

non-canonical cysteine in CDR1 and CDR3, respectively. However, in contrast to type II, type III domains comprise a restricted CDR3 diversity, highly similar in amino acid composition and length as well as a conserved tryptophan residue in CDR1 positioned adjacent to the disulfide bridge between both loops. Based on the limited CDR3 diversity it is tempting to speculate that type III vNARs evolved as a consequence of exposure to a common pathogen in early development of sharks or that it may play a role in regulatory processes during the development of the shark’s immune system.^{6,31,51}

Type IV domains differ from all described vNAR types in that they lack non-canonical disulfide bonds as found in all other vNAR types.^{50,52} Therefore, the topology of the paratope of type

IV variable domains is more flexible and not physically constrained. Type IV domains are also referred to as type IIb, according to Streltsov et al. and Liu et al.^{46,53} In addition, type IV domains with an invariant tryptophan residue in CDR1, similar to type III, have been identified.⁵⁴ Besides type III, all types of the vNAR domain give rise to high-affinity binders.^{30,48,50,55}

Diversification of the IgNAR Repertoire

In mammals, antibody diversity is generated by a process referred to as V(D)J-recombination. During B-cell development, one variable (V) segment, one diversity (D) segment and one joining (J) segment are randomly rearranged from a multitude of gene segments of the immunoglobulin heavy chain gene cluster to encode the VH domain, which is fused to a gene segment encoding a constant domain.²⁷ Similarly, for light chain generation, one V segment and one J segment are selected by chance from a pool of gene segments to produce the variable domain of the light chain that is fused to a CL gene. Diversity is further expanded in a process called junctional diversification through imprecise segment joining.⁵⁶ An additional layer of diversity is introduced by the random arrangement of the heavy chain and light chain to complete the expression of the antibody molecule.

As IgNARs are devoid of light chains, they lack H-L combinatorial diversification. Correspondingly, one would expect a dramatically restricted primary repertoire. However, this lack of diversification process is at least partially compensated through the diversity achieved within the CDR3 region. Whereas mammalian antibody genes are organized in the translocon-format, shark antibody genes are exclusively arranged in the cluster-organization (Fig. 4).^{6,30,42,48} Nurse sharks comprise 4 IgNAR gene

clusters, though only 2 are expressed in adult life, one encodes type I and one encodes type II IgNARs.^{30,48} Each IgNAR cluster comprises one V segment, 3 D segments and one J segment and a single set of C segments. Rearrangement occurs solely within this cluster resulting in a VD₁D₂D₃J assembly. Hence, 4 rearrangement processes generate the complete vNAR domain. However, the order of rearrangements remains to be determined.⁴² The interfaces between the V segment, the 3 D segments and the J segment encode for CDR3. Consequently, diversity in both sequence and length of the primary repertoire is nearly entirely found in CDR3.^{51,57,58} Extensive junctional diversification through N-region addition, P-nucleotide addition, trimming and D-region rearrangement further expands the heterogeneity of the primary repertoire of IgNAR.^{6,30,42,59} The type III gene cluster represents an exception within the recombination process. As a result of the fusion of the D₁ segment and the D₂ segment in the nurse shark, only 3 rearrangement events occur, explaining the restricted diversity of this type. In contrast to this, in the spiny dogfish the type III IgNAR clusters are not partially germline-joined, indicating that germline-joining of Ig clusters might be a species-specific event.⁶⁰

Sharks do not possess conventional germinal centers. Nevertheless, the initial combinatorial diversity, which is mainly restricted to CDR3, is further expanded by extensive somatic hypermutation in an antigen-driven manner, with mutations clustering to the CDRs.⁵⁷ The mutational pattern and frequencies of this process are similar to that of mammalian immunoglobulins with a bias for transitions over transversions. The mechanism favors the serine codon AGC/T as a hotspot for mutations and most of the changes are base substitutions. Surprisingly, base changes often occur in tandem, particularly in mutational hotspots and palindromic repeats.⁵⁸ It was first shown

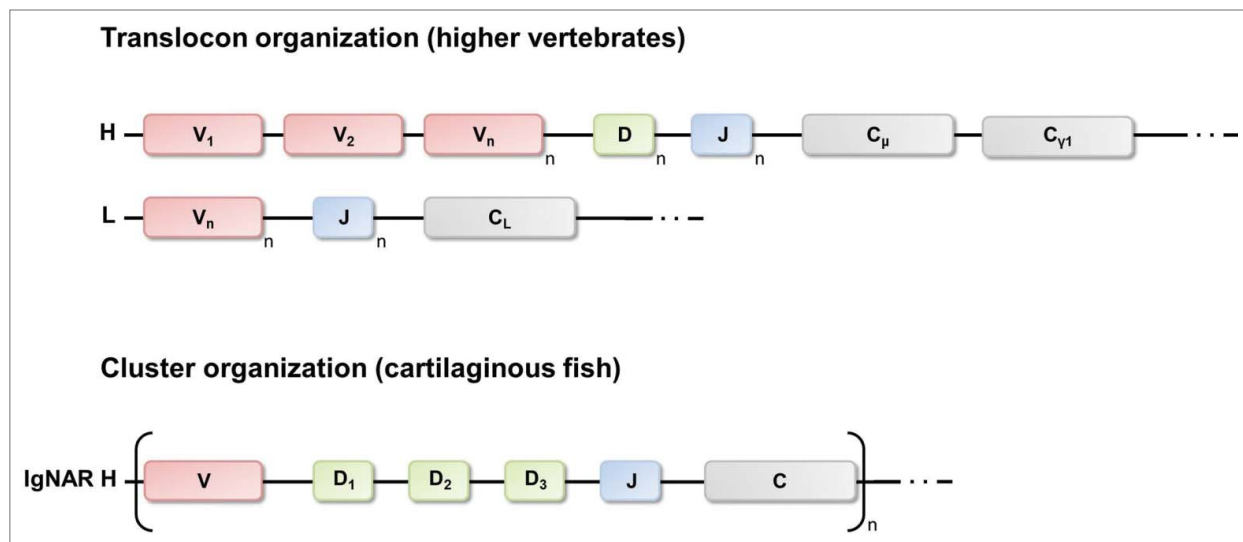


Figure 4. Translocon arrangements of immunoglobulin genes in higher vertebrates and cluster configuration of IgNAR genes of cartilaginous fish. In the translocon organization there are many variable (V) segments upstream of many diversity (D, only for heavy chains) and joining (J) segments that recombine randomly to encode the variable domain of the heavy chain or the light chain. IgNAR genes (like all Ig genes of the cartilaginous fish) are organized in the cluster configuration. Each cluster contains for the variable domain one V segment, 3 D segments and one J segment. Recombination occurs exclusively within one cluster. H, heavy chain loci; L, light chain loci, C, constant region.

by Flajnik and coworkers, that after immunization, somatic mutations promoted an incremental increase in affinity, giving clear evidence for in vivo affinity maturation in sharks.^{30,38,47-49} Furthermore, they demonstrated that HV4 is prone to somatic mutations and, even more interesting, these mutations can be involved in antigen binding.⁴⁸

Consistent with the structure of type I and type II IgNARs (Fig. 3), mutations are favored in CDR1 for type II vNARs and in HV2 for type I vNARs.^{27,30} It is hypothesized that those mutations could either directly contribute to antigen binding or they could indirectly have an effect on the paratope such that they stabilize and influence the conformation of the antigen-binding CDR3.

Selection of Antigen-Specific vNAR Domains From Shark Immune Repertoires

Antigen-specific vNAR domains have been generated from the immune repertoire of a number of different shark species, including the nurse shark,⁴⁹ the wobbegong shark,^{55,61} the spiny dogfish,^{50,53,62} the banded houndshark^{63,64} and the bamboo shark.⁶⁵ Target-specific clones are generally isolated using different display technologies, such as phage display^{49,55} or ribosome display.⁶⁶ We recently showed that antigen-specific vNAR molecules can also be selected using yeast surface display as platform technology.⁶⁵ A potential advantage of yeast surface display over the above mentioned display technologies is the amenability of single-cell online and real-time analysis, as well as subsequent characterization of individual library candidates in terms of specificity, affinity and stability.^{67,68}

There are several distinct strategies for library establishment. Binders can be selected from immunized sharks,^{49,50} from the naïve shark repertoire,⁵³ or from a synthetic vNAR library,^{55,69} where the vNAR molecule serves as a scaffold with randomized loops and from semi-synthetic repertoires. Here, additional diversity is included through the randomization of one or more antigen-binding loops.^{62,70} For the most part, immunization is the preferred route to obtain high affinity binders. An additional advantage of immunization is that sharks are evolutionary very distant to humans. This greatly reduces the likelihood of immune tolerance that would reduce the induction of target-specific responses when antigens are well conserved across species. Consequently, antigen-specific vNAR molecules have been generated with impressive affinities against a multitude of different targets *via* immunization.^{49,50,71,72} However, the process of immunization of sharks is protracted compared to standard mammalian protocols, and not every species tested has proven successful.^{50,73} For instance Dooley et al. and also our group (unpublished results) were unable to detect an antigen-specific IgNAR response after the immunization of the small spotted catshark, *Scyliorhinus canicula*.⁷⁴

Antigen-specific vNAR fragments have also been isolated from non-immunized libraries against a plethora of different targets, including viral targets, cytokines, proteins involved in cancer and arthritis and toxins.^{53,62,64,65,70,75,76} Table 1 illustrates vNARs selected against therapeutically relevant targets from immunized

Table 1. Published vNAR domains against disease related targets. Adapted from ref. 54

Target	Potential Application	Reference
VHSV	Anti-viral	64
TNF- α	Endotoxic shock	98
HSA	Half-life extension	50
HBeAg of HBV	Anti-viral	75
Ebola virus	Immunodiagnostic	72
Cholera toxin	Biosensor	53
Staphylococcal enterotoxin B	Sensors	62
Ricin	Sensor	62
Botulinum toxin	Sensor	62
AMA1	Malaria diagnosis	66,76
EpCAM	Cancer diagnosis & therapy	65
HTRA1	Arthritis therapy	65
EphA2	Cancer diagnosis & therapy	65
IL-8	Anti-inflammation	unpublished results

VHSV: Viral hemorrhagic septicaemia virus, TNF: Tumor necrosis factor, HBeAg: Hepatitis B e antigen, HBV: Hepatitis B virus, HSA: Human serum albumin, AMA1: Malarial apical membrane antigen-1, EpCAM: Epithelial cell adhesion molecule, HTRA1: Human serine protease HTRA1, IL-8: Interleukin 8.

and non-immunized origins (Table 1). Surprisingly, binders selected from such libraries often show good affinities to their target.^{61,62,75,76} Nonetheless, when higher affinities are required, vNARs can be optimized using in vitro affinity maturation. In this respect 3 different methodologies have been established. Nuttall and co-workers were able to improve the affinity for an AMA1-specific IgNAR V domain using error-prone PCR resulting in ~10-fold enhanced affinity.⁷⁶ In a more recent approach, the same group employed a mutagenesis system dependent on low fidelity RNA polymerase from Q β bacteriophage to introduce diversity into IgNAR antibody libraries for affinity maturation. With this novel strategy they were able to select mutated vNAR molecules with a more than 20-fold enhanced affinity compared to the wild type clone.⁶⁶

We recently established a stepwise in vitro affinity maturation methodology for the generation of high affinity binders derived from shark vNAR domains. To this end, binders were selected from a library in which CDR3 was totally randomized using yeast surface display. Affinities of binders were significantly improved by CDR1 diversification and sublibrary screening, resulting in enhanced molecules with affinities in the low nanomolar range.⁶⁵ Interestingly, this method resembles the natural strategy of the immune system of nurse sharks to generate high affinity antibodies.

Therapeutic and Diagnostic Attributes of vNAR Domains

The tremendous diversity found at the sequence-level of the CDR3-loop of IgNAR, as well as the multiplicity of the structural topologies formed by the antigen-binding site of the vNAR domain (Fig. 5), render IgNARs promising alternatives to

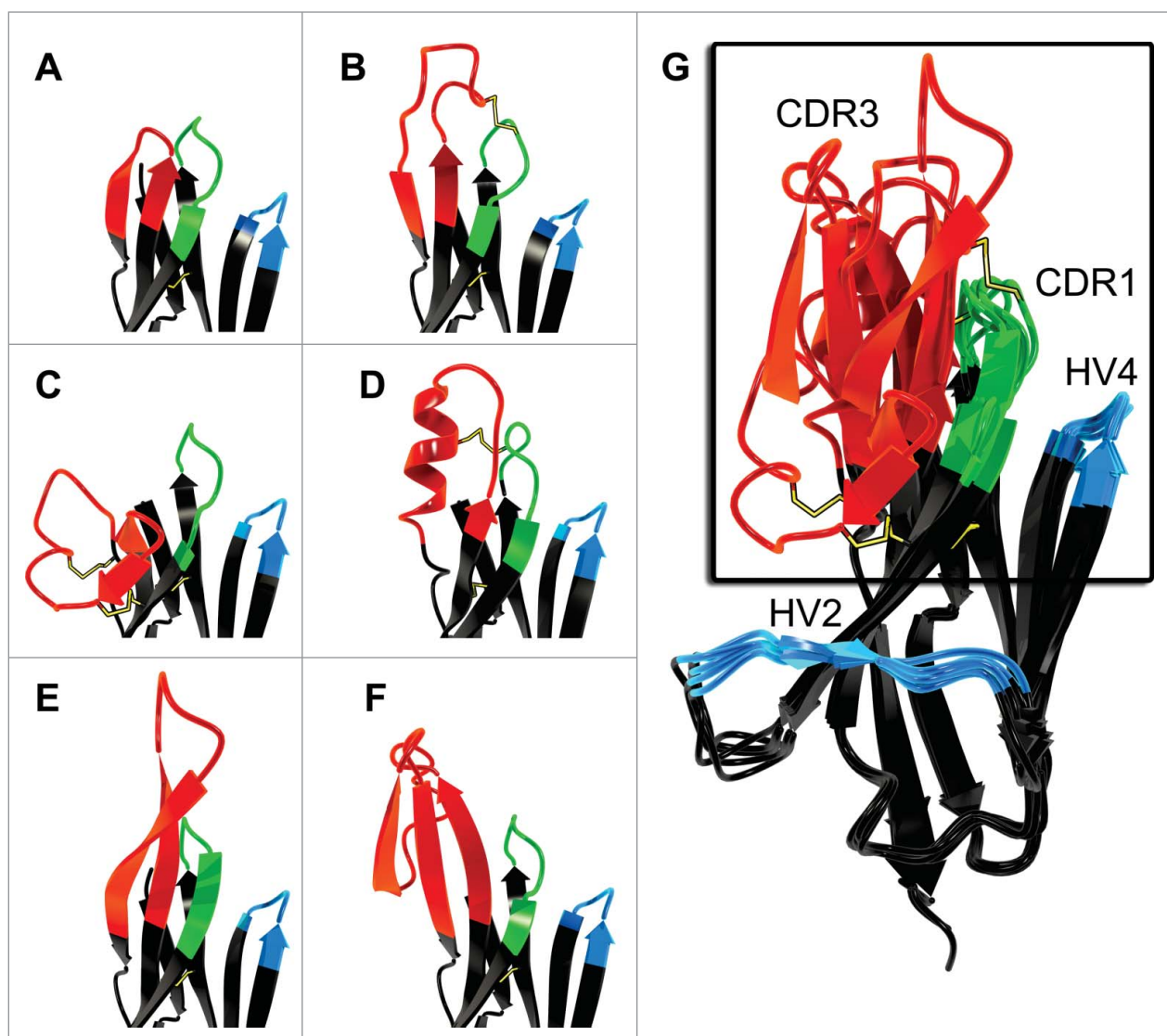


Figure 5. Examples of CDR3 variability in vNAR domains depicted in ribbon representation. **(A)** Short loop (type IV, pdb entry 4HGK).⁵² **(B)** Large loop with one disulfide constraint (type II, pdb entry 2COQ).³¹ **(C)** Highly constrained loop tethered by 2 cystine motifs (type I, pdb entry 1SQ2).³⁰ **(D)** Extended CDR3 forming an α -helical motif (type II, pdb entry 2I25).⁴⁸ **(E)** Extended CDR3 forming a 2-stranded β -sheet (type IV, pdb entry 2Z8V).⁷⁸ **(F)** Extended CDR3 incorporating an amyloid- β p3 fragment (type IV, pdb entry 3MOQ).⁹⁴ **(G)** Overlay of structures **A–F**. Disulfide bonds are shown in yellow. Picture rendered with POV-Ray (www.povray.org/).

conventional antibodies.^{30,31,48} As described above, the different types of vNAR domains form, if any, a very diverse set of disulfide-bridges. Consequently, antigen-specific clones can be selected from a very large, unprecedented repertoire of different loop structures.⁷⁷ Moreover this unique paratope-architecture of shark domains seems to be predisposed to target clefts of the antigen, whereas recessed epitopes are usually not antigenic for conventional antibodies.^{27,31,46,78} Indeed, it has been shown that the active site of enzymes and clefts can be targeted by vNAR domains.^{30,39,48}

Above all, vNARs exhibit many additional properties that render them interesting for diagnostic and therapeutic applications. It has been demonstrated that vNARs are extraordinarily stable proteins,^{49,53,62,69,72} which is probably a consequence of the

harsh physiological environment - the blood of sharks contains 350 mM urea - those molecules are exposed to.⁵⁴ The superior thermal stability and tolerance to irreversible thermal denaturation compared to scFv- and mAb-formats was elegantly demonstrated by Lonsdale and colleagues, as well as by Goldman and coworkers.^{53,79} Even type IV domains, which lack the non-canonical loop stabilizing disulfide-bond, show superior thermal stability, with T_m values that for the most part exceed 70°C.⁶⁵ In contrast to this, scFv fragments often show T_m values in the range between 50°C and 65°C.^{80,81} However, T_m may sometimes be below 40°C, requiring optimization of their thermal stability.^{82,83} Concordantly, it has been shown that the C2 and C4 domains of IgNAR are very stable. Buchner and coworkers were able to identify structural elements that contribute to their high

stability. Compared to mammalian constant domains, C2 and C4 domains of IgNAR contain an additional salt bridge and an extended hydrophobic core. The transfer of these key elements of enhanced stability to a human antibody domain improved its stability significantly.³⁹

The inherent small size of the IgNAR V domain is an additional therapeutic and diagnostic attribute. It can be hypothesized that this property leads to a greater mobility with reference to tissue penetration. Especially for *in vivo* imaging, where a high contrast to background ratio is crucial, this feature is beneficial, due to an advantageous pharmacokinetic profile, i.e., a much shorter residence time in the blood compared to classical antibodies.¹⁰ Furthermore it is assumed that the small molecular weight of the vNAR domain implicates the opportunity to target epitopes otherwise only accessible to small molecules.⁶

Re-Formatting of vNAR Domains

The simple single chain molecular architecture of vNAR domains affords the benefit of multiple re-formatting opportunities to tailor the final product for purpose. Many formats have been successfully proven, including monomeric, dimeric and trimeric (binding more than one target) in addition to Fc-based formats, with all demonstrating the inherent flexibility of these domains.^{50,52,54,84}

The small size of vNAR domains contributes to rapid renal clearance *in vivo* and represents a major drawback when non-imaging applications such as tumor-targeting are required. However, reduced size can be advantageous with regard to tumor penetration and ultimately the right format for the individual application needs to be determined. Fast glomerular filtration can be circumvented by multimerization of single vNAR domains, as has been shown by Müller et al.⁵⁰ Their investigations covered the *N*- as well as *C*-terminal fusion of a naïve vNAR domain with an anti-human serum albumin (HSA) vNAR originating from an immunized shark and isolated *via* phage display.⁸⁵ The fusion constructs retained high-affinity binding to HSA and exhibited significantly increased *in vivo* half-lives compared to their unconjugated parental domains. The size of such dimeric formats is in the range of 25 kDa, and, compared to other antibody fragment formats such as scFvs, achieves double the binding site capacity while attaining increased affinities toward demanding, cryptic epitopes, which is a hallmark of vNAR proteins owing to their unique topology. In addition to dimeric fusions, a trimeric construct comprising the naïve vNAR domain at both termini of the anti-HSA vNAR displayed improved pharmacokinetics in *in vivo* studies in different species.⁵⁰ In another study conducted by Nuttall and colleagues, several approaches for the generation of bivalent shark antibodies with enhanced functional affinity were investigated.⁸⁴ Best affinities were obtained through *C*-terminal covalent or domain mediated linkages.

As has been shown extensively for rodent mAbs, there is a plethora of rational as well as empirical humanization strategies available to reduce immunogenic responses caused by animal-derived immunoglobulins.⁸⁶⁻⁹⁰ Rational design and the grafting

of CDR loops of a xenogenic antibody onto a suitable human scaffold exhibiting a similar sequence has culminated in the development of several blockbuster pharmaceuticals routinely used in the clinic (e.g., trastuzumab, bevacizumab).⁹¹

The sequence identity of the IgNAR V domain with mammalian VH regions falls as low as 25%.⁴⁹ In order to minimize the immunogenic potential of vNAR domains, Kovalenko and coworkers were able to engineer the aforementioned anti-HSA shark vNAR domain⁵⁰ by converting more than half of the framework amino acids to those of the human germline VK1 sequence DPK9.⁵² This sequence bears the highest structural resemblance to the corresponding vNAR domain, and concomitantly represents one of the most stable human frameworks for downstream development. Determination of the binding constants of humanized vNAR variants yielded antigen affinities similar to those of the parental construct (14.8 nM *vs* 13.6 nM for the parental molecule). The opposite approach, i.e., starting with a structurally related human VH domain and converting that to a vNAR-like domain by clipping the CDR2 region, extending CDR3 and introducing stabilizing residues and additional disulfides, may also be a viable alternative as already shown for the camelization of human VH domains.⁹² To enhance the expression of humanized vNARs in mammalian cells, they were *C*-terminally fused to human Fc domains.⁵² This conjugation strategy can also contribute to the formation of dimers due to the interactions of 2 vNAR-Fc conjugates at the respective human constant domains. Fusions to human Fc, besides increasing the overall molecular weight and thus counteracting early renal clearance, can elicit *in vivo* immune effector functions and ultimately intensify the immune response *via* antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. According to Kovaleva et al., the humanized IgNAR V domain variant showed negligible immunogenicity in dendritic cell assays.⁵⁴ Notably, also no significant immunogenic effects were observed after subcutaneous application of the parental, non-humanized anti-HSA vNAR in rodents and non-human primates.^{50,54} However, it remains to be scrutinized more meticulously how these proteins will behave when administered to patients in the scope of clinical trials.

Besides diagnostic and therapeutic applications, vNAR domains, due to their small size, high stability and their ability to sustain repeated cycles of unfolding and folding are also promising biomolecules for biotechnological applications to serve for example as high affinity capturing agents for purification of biomolecules or as tools for diagnostic applications. It was recently shown that vNAR fragments can be coupled covalently and site-specifically onto crystalline nanocellulose that serves as a protein-capturing nanoscaffold.⁹³ This evidence, coupled with the demonstrated stability and flexibility of vNAR domains, would predict more biotechnological applications can be expected to show up in the next years. Additionally, the vNAR domain can be utilized to gain information about pathological processes that at present are not completely understood. This was exemplified by Nuttall and colleagues, who grafted parts of the A β -peptide involved in Alzheimer's disease into CDR3 of a vNAR domain, and thus solved the crystal structure of the amyloid- β p3 fragment (Fig. 5F).⁹⁴

Conclusion

Antibodies are essential molecules for biomedical and biotechnological applications. As described above, shark IgNARs differ greatly from conventional antibodies in many respects. Because of their unique structural features, these molecules have emerged as promising candidates for therapeutic, diagnostic and biotechnological applications. One striking example is that vNAR domains may complement classical antibodies in terms of drugable antigens. IgNAR V domains possess the potential to access cryptic and recessed epitopes that are usually not antigenic for conventional antibodies, e.g., hydrophobic clefts, active sites. However, this has only been shown for targeting the active site of lysozyme, as well as for targeting the hydrophobic cleft of AMA1, and therefore requires further exploration.^{30,48,78} Their small size, high stability and the feasibility to re-format those molecules are additional desirable attributes. During the last years, constant progress on shark IgNAR research has been made. Related to this, vNAR domains targeting a plethora of therapeutically relevant antigens were generated and several methodologies for affinity-maturation of target-specific vNAR domains, as well as for re-formatting, e.g., humanization, multimerization, have been established. Moreover, a structural model of the complete IgNAR molecule revealed deeper insights into the extraordi-

nary shape, stability and mode of action of this unique binding domain. Information gained from this might pave the way for the next generation of classical antibody variants showing improved stability. Fundamentally, it can be expected that this exceptional molecule, which evolved several hundred million years ago, might add value to the continuously evolving and exciting field of biologic drug development. Finally, it needs to be emphasized that, in view of future clinical applications, the immunogenic potential of vNAR domains requires further in depth investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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