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Review

Diagnostic and therapeutic potential of shark variable new antigen receptor (VNAR) single domain antibody

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ABSTRACT

Conventional monoclonal antibodies (mAbs) have been widely used in research and diagnostic applications due to their high affinity and specificity. However, multiple limitations, such as large size, complex structure and sensitivity to extreme ambient temperature potentially weaken the performance of mAbs in certain applications. To address this problem, the exploration of new antigen binders is extensively required in relation to improve the quality of current diagnostic platforms. In recent years, a new immunoglobulin-based protein, namely variable domain of new antigen receptor (VNAR) was discovered in sharks. Unlike conventional mAbs, several advantages of VNARs, include small size, better thermostability and peculiar paratope structure have attracted interest of researchers to further explore on it. This article aims to first present an overview of the shark VNARs and outline the characteristics as an outstanding new reagent for diagnostic and therapeutic applications.

Contents

1. Introduction

Antibodies are powerful tools in research and diagnostics due to high affinity and specificity towards target antigen. Nowadays, monoclonal antibodies (mAbs) have widely been used to detect biomarkers derived from various pathogenic diseases in various immunoassay platforms [1]. Unlike polyclonal antibodies (pAbs), the monospecificity and higher purity of mAbs have enabled them to perform in multiple applications, including evaluation of changes in molecular conformation, targeting protein-protein interactions, and identifying single members of protein families [2]. In terms of structure, antibody, also known as immunoglobulin (Ig), is a structurally complex, large hetero-tetrameric protein which is composed of two heavy chains and two light chains (Fig. 1A). The heavy chain of a conventional IgG consists of three constant domains (CH1, CH2 and CH3) and a variable domain

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(VH), whereas the light chain consists of a constant domain (CL) and a variable domain (VL).

However, the efficacy of the mAbs could be limited by their large molecular size (~150 kDa) and structural complexity, where their paratopes may not be capable to access certain antigens with cryptic epitopes, resulting to poor binding affinity [3,4]. Other than that, the degeneration of mAbs by high temperatures and humidity is the main factor affecting the sensitivity that could simply lead to false positive results in RDTs. Although many RDT devices are now kept well in sealed plastic packets to overcome humidity, the exposure to high ambient temperatures (45 °C) can always lead to the deviation of RDTs performance [5,6]. To ensure the optimum performance of RDTs in the field, the RDT devices are recommended to be stored at 4 °C or below ambient. Unfortunately, the provision of refrigerated storage system to maintain the optimum performance of RDTs has always been a problem for many low-resources regions where electricity supply is not available [6,7]. To overcome these circumstances, the exploration of new antigen binders with natural heat-stability is extensively needed for improving the current diagnostic platform.

In the early 90s, camelids and sharks were discovered to possess unconventional antibody in their immune system, known as camelid heavy-chain antibodies (HCAbs) and immunoglobulin new antigen receptors (IgNARs) respectively [8,9]. The subsequent recognition has shown the variable domains of these antibodies (camelids VHHs and sharks VNARs) contain autonomous function as single-domain antibodies. Owing to relatively small in size, high specificities for a cognate antigen, and high physiochemical stability, these naturally occurring antibody domains have been considered as promising candidates for biomedical development [10,11]. Unlike conventional antibodies which have flat or concave antigen-binding sites, a wide variety of loop lengths and structures presented in VHH and VNAR have made them capable to access more cryptic epitopes and catalytic clefts of enzymes [12–14]. While the first VHH-based drug (anti-yWF caplacizumab) has already been approved [15,16], the current progress of VNARs may substantially render new perceptions and attitudes towards these molecules in the medical and scientific communities. Hence, this article aims to review the unique characteristics of shark VNAR, and to discuss the potential uses of shark VNAR in diagnostic as well as in therapeutic platform.

2. Immunoglobulin new antigen receptor (IgNAR)

A class of natural heavy-chain only antibody known as immunoglobulin new antigen receptor (IgNAR) was discovered in cartilaginous fish in 1995 by Flajnik and co-workers [8]. IgNAR is a unique and unconventional antibody which has been identified in several different types of shark, including nurse sharks (*Ginglymostoma cirratum*), wobbegong sharks (*Orectolobus maculatus*), smooth dogfish (*Mustelus canis*), banded houndsharks (*Triakiss cyllium*) and horn sharks (*Heterodontus francisci*) [17–21]. Studies have also shown IgNAR plays a pivotal role in adaptive immune system of cartilaginous animals. The immunomicroscopic technique has exhibited IgNAR shares its structural homology with T-cell receptor variable regions, antibody VL chains, and cell adhesion molecules [8,22]. Hence, the ability of demonstrating a possible common ancestral receptor molecule in IgNAR was postulated in that it has evolved and been recruited by immune system to function as antibody-like structures [23–25]. As shown in Fig. 1B, IgNAR exists in a homodimer that is naturally devoid of light chain. Each chain in IgNAR consists of five constant domains followed by one variable domain.

The variable domain of IgNAR, or referred to as VNAR, contains only two complementarity-determining regions (CDRs), as known as CDR1 and CDR3. Unlike conventional IgG, shark VNAR has been undergone evolution by compensating CDR2. Therefore, it allows the diversity of VNAR to be achieved by long variable protruding CDR3 with additional diversity evident in CDR1 and connected through two hypervariable regions (HV), which are HV2 and HV4. Moreover, the peculiar structure of CDR3 combined with supporting intermolecular disulphide bridging has conferred VNARs to form special paratopes that can access confined epitopes [13,26–28]. Owing to the absence of CDR2, VNARs are thus far the smallest naturally occurring immunoglobulin-based protein scaffolds, with a molecular mass of about 12 kDa in animal kingdom [13]. With the presence of cysteine residues, the simple single

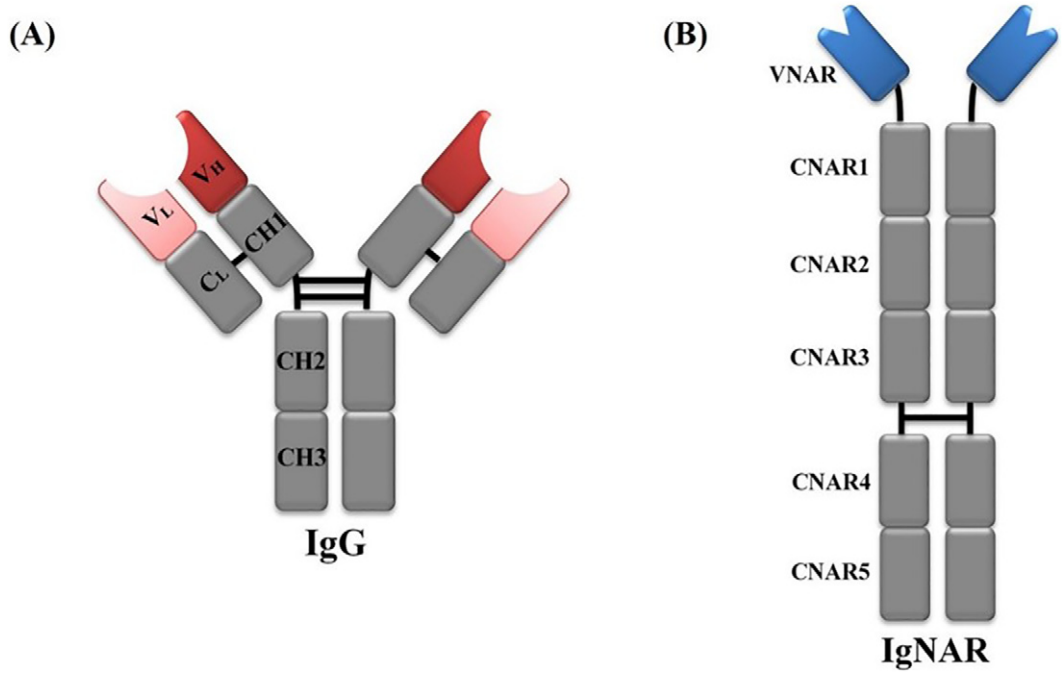

Fig. 1. The schematic representation of (A) IgG and (B) IgNAR. The coloured regions indicate variable domains of each antibody, whereas grey coloured region in each antibody indicate the constant domains.

chain structure of VNAR affords remarkable refolding properties after exposure to temperatures of up to 95 °C, making them preferable in diagnostic applications where heating may temporally occur [29].

The VNAR family can be categorized into four types (Fig. 2) based on the position and the number of non-canonical cysteine residues [13,14,30,31]. Type I VNAR possesses two paired cysteine residues in CDR3, and extra cysteine residues in framework 2 and 4. Based on the crystal structure analysis, the non-canonical cysteine residues which occur in framework 2 and 4 of Type I VNAR form disulphide bonds with the cysteine residues in CDR3, causing the loop to be held tightly towards HV2 region [13,32]. So far, Type I VNAR has been found only in nurse shark (*Ginglymostoma cirratum*) and may be unique to this species [25].

In Type II VNAR, a cysteine residue was identified in CDR1 and CDR3 respectively, which form disulphide bonds that brings both loops in close proximity [33,34]. Nonetheless, it lacks both cysteine motifs that anchor CDR3 to the framework region, causing CDR3 to form a protrusive structure which make it capable of targeting grooves, for example, the active site of enzymes [14]. Due to a strong selective pressure for two or more cysteine residues in CDR3 of Type I VNAR to associate with their framework 2 and 4 counterparts, this type of VNAR has a longer CDR3 compared to Type II VNAR, with an average of 21 and 15 residues, respectively [33]. However, Type II VNAR has commonly been identified in most of the shark species till recently.

Type III VNAR is predominantly expressed in new-born sharks [33]. In view of amino acid content, Type III VNAR is very similar to Type II VNAR, with the addition of conserved tryptophan residue in CDR1. However, Type III VNAR possesses shorter CDR3 which are lacking in sequence diversity. This may partly be due to the D regions of shark genes being fused with V-gene in Type III [33]. It had been hypothesized that Type III VNAR is evolved to protect new-born sharks from early stage exposure to common pathogens or may involve in the development of immune system in adult sharks. This isotype is then quickly overtaken by the more mature class of VNAR [33]. Lastly, the Type IV VNAR or sometimes referred as Type IIb lacks non-canonical disulphide bonds that are found in other types of VNAR. Thus, the topology of the antigen-binding site of Type IV VNAR is more flexible [19,25]. Moreover, Type IV VNAR with an invariant tryptophan residue in CDR1 have been identified and is referred to as Type IIIb [35].

3. Diversification of the IgNAR repertoire

In nature, the scaffold of IgNARs does not have VH-VL combinatorial diversification as they are devoid of light chains. However, the diversification process that generally occurs in conventional antibodies has been replaced by the uncommon diversification process that is in the CDR3 region. In most of the higher mammals, the antibody genes are generally arranged in translocon-organization, whereas sharks were found to have cluster-organization [3,13,31,36]. In this cluster arrangement, each cluster consists of one V segment, one or more D segments, one J segment and one C segment. The single set of C segments are arranged exclusively by the activity of RAG recombinase within individual clusters [3]. The heterogeneity of the shark IgNAR repertoire is further expanded by the extensive junctional diversification through N-region addition, P-nucleotide addition, trimming and D-region rearrangement [3,13,36,37].

Shark does not contain the conventional germinal centres as found in mammalian immunoglobulin. Nonetheless, the initial combinatorial diversity, which is primarily limited to CDR3, is further extended by the somatic hypermutation in an antigen-driven manner, with mutations clustering to the CDRs [38]. The mutational pattern and frequencies of this process are similar to that of mammalian antibodies, with a bias for transitions over transversions. This mechanism favours the serine codon AGC/T as a hotspot for mutations and majority of the changes are base replacements, which usually occur in tandem, especially in mutational hotspots and palindromic repeats [39]. Flajnik and colleagues reported somatic hypermutations that promote a gradual increase in affinity can occur after immunization, resulting in affinity maturation *in vivo* [10,13,18,31,40]. They showed that HV4 is prone to somatic mutations and may involve in antigen binding [31]. Mutations are also favoured in HV2 for Type I and in CDR1 for Type II [13,41].Therefore, it is postulated that these mutations could either directly contribute to antigen binding, or they change and stabilize the conformation of the CDR3 for antigen binding [31].

4. Isolation of target-specific VNAR

Target-specific VNARs have been isolated from a range of shark species, including nurse shark (*Ginglymostoma cirratum*), wobbegong shark (*Orectolobus maculatus*), spiny dogfish (*Squalus acanthias*) and smooth dogfish (*Mustelus canis*) [10,19,20,42]. To isolate the target-specific

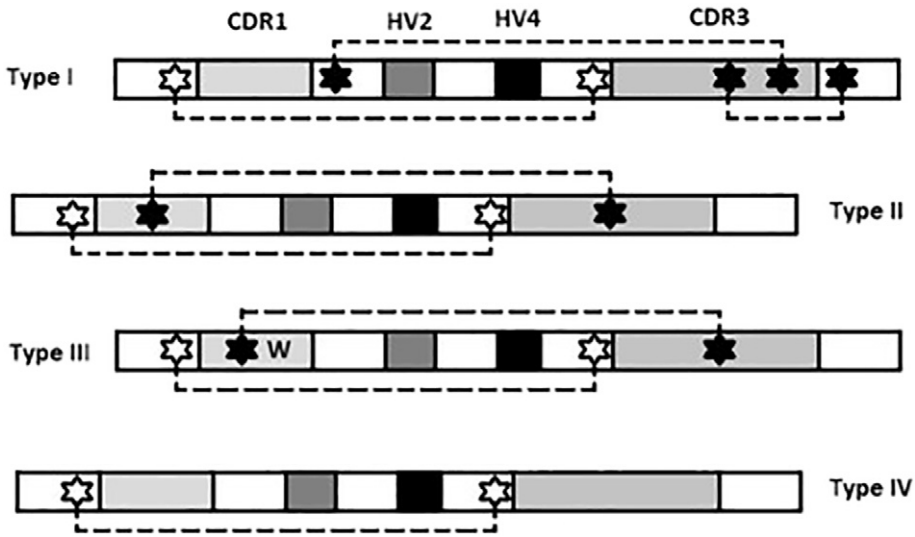

Fig. 2. The four isotypes of VNAR that shows the similarities and differences between different isotypes of VNAR. The white six-point stars indicate the canonical cysteine residues, whereas the black six-point stars indicate the non-canonical cysteine residues. The connecting dashed lines indicate the disulphide bonds. The alphabet W in Type III VNAR indicates the conserved tryptophan.

clones, several display technologies such as phage display, ribosome display and yeast display have been performed elsewhere [20,43,44]. Of these approaches, phage display technology is the most popular and widely employed for the isolation of shark VNARs against target antigen. This technology allows phage phenotype and genotype physically linked whereby the displayed peptides or proteins are expressed in fusion with phage coat protein before undergoing a screening process known as biopanning [45].In this technique, the genes encoding for antibody binding domains are fused to the bacteriophage's coat protein gene, resulting the antibodies can be displayed on the surface of bacteriophage for selection of antigen-recognizing binders [46].The principle of phage display technology is illustrated in Fig. 3.

In order to isolate target-specific antibodies using display technology, the construction of appropriate antibody library is mandatory. Thus far, several types of VNAR library have been generated, including immune library, naïve library, synthetic library or semi-synthetic library [10,19,20,47,48].Nuttall and colleagues constructed a semi-synthetic VNAR library using natural frameworks isolated from wobbegong sharks (*Orectolobus maculatus*). In the study, they employed random mutagenesis of the CDR3 region and successfully isolated recombinant VNAR that specific for Gingipain K protease from *Porphyromonas gingivalis* [20].They then extended their study by increasing the library size through the introduction of naturally occurring VNAR from naïve shark repertoire. Surprisingly, two naturally occurring VNAR proteins that were not derived from the synthetic VNAR library were isolated [26]. In 2007, Liu and co-workers successfully isolated VNARs targeting to cholera toxin, staphylococcal enterotoxin B (SEB), ricin and botulinum toxin A (BoNT/A) complex toxoid from naïve and semi-synthetic libraries made from spiny dogfish (*Squalus acanthias*) [19,49]. Target-specific VNARs with impressive affinities have also been isolated against many different targets through immunization [10,47,50]. Recently, Leow and colleagues successfully isolated several unique VNAR clones that were specific against malaria biomarkers from wobbegong shark (*Orectolobus maculatus*) through immunization [51].

Various methods have been used to construct antibody phage display libraries. The most common method is PCR amplification of antibody genes, followed by enzymatic digestion and ligation with phagemid vector. However, such method is time consuming, and may experience a low efficiency in the ligation and transformation step. Hence, a group of researchers had developed a library construction method based on PCR extension assembly followed by self-ligation (EASeL), which significantly shortened the time needed to construct the library [52]. Using this method, a large and highly diverse VNAR library derived from six antigen-naïve nurse sharks (*Ginglymostoma cirratum*), with a library size of 10^{10} has been recently generated. From this naïve nurse shark VNAR library, a panel of VNAR binders that specific to cancer therapy-related antigens, which are glypican-3 (GPC3), human epidermal growth factor receptor 2 (HER2) and programmed cell death-1 (PD1) were isolated. Meanwhile, VNARs specific to viral antigens, such as the Middle East respiratory syndrome (MERS) and severe acute respiratory syndrome (SARS) spike proteins were also isolated recently [52]. Therefore, the findings obtained herein have convinced where VNAR clones selected from the naïve nurse shark VNAR library may potentially be developed as therapeutic antibodies after humanization process undertaken.

5. Applications of VNAR

The applications of shark VNARs have been extensively studied in recent years. In addition to immunodiagnostic applications, the potential of VNAR being developed as the reagents for immunotherapeutic applications have been well studied elsewhere. In the following section, the development of VNARs in various applications will be discussed. The

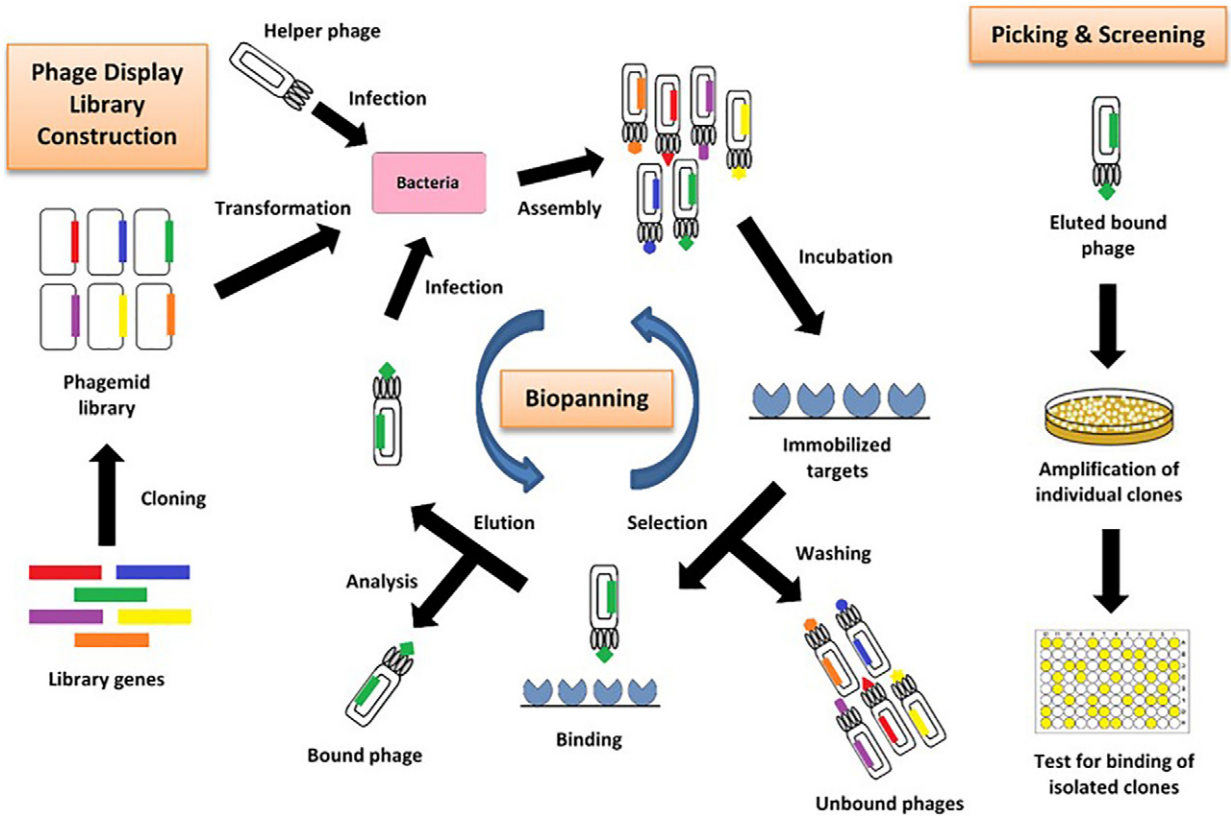

Fig. 3. The principle of phage display technology. Firstly, a library of antibody genes is cloned into the phagemid vector. The phage display library can be obtained by transforming *Escherichia coli* with recombinant phagemids, and rescue of phage with helper phage. The phage display library is then subjected to several rounds of affinity selection (biopanning) to isolate target specific binders, which involve binding, washing, elution, infection and amplification. Lastly, the isolated clones are screened by ELISA assay and followed by DNA sequencing.

application of VNAR to the biomarkers of various diseases is summarized in Table 1.

5.1. VNAR in immunodiagnostic applications

The VNAR isolated by Nuttall and colleagues demonstrated high affinity towards *P. falciparum* apical membrane antigen 1 (AMA1) *via* its unique CDR3 after undergoing affinity maturation [28]. AMA1 is a merozoite surface protein that importantly used for erythrocytes penetration by malaria parasites [53]. It has been reported that the binding specificity of VNAR monovalent clone towards AMA1 was comparable to a range of commercially antibody reagents [54]. Griffiths and colleagues demonstrated that the thermostability of isolated VNAR was superior to that of conventional mAbs when targeting AMA1 in various format at 45 °C. The refolding ability of VNAR was retained despite the temperature raised to 80 °C. Furthermore, VNAR was proven to have long shelf-life after incubating at ambient temperature or 37 °C for a month without losing any binding function [55]. In addition, Liu and colleagues examined the melting temperature and refolding capability of VNARs using fluorescence-based melting assay and circular dichroism. Similar to what have been observed in camelids, shark VNARs showed a wide range of melting temperature values from 42 °C to 77 °C, and capable to refold following heat denaturation [29]. Having all these excellent features, it is convincing that VNAR potentially developed as new diagnostic reagents for RDTs.

VNARs have also been demonstrated to target biomarkers derived from viral diseases such as Ebola hemorrhagic fever (EHV). EHV is a deadly viral disease caused by Ebola virus. The causative agents of EHV include *Bundibugyo ebolavirus*, *Sudan ebolavirus*, *Tai Forest ebolavirus* and *Zaire ebolavirus* [56–58]. Goodchild and co-workers successfully isolated two VNARs (DSTL096 and DSTL097) from immunized phage display libraries that specific to nucleoprotein of Ebola virus [32,50]. Further evaluation done by Anderson and co-workers confirmed that these two isolated VNARs had excellent binding affinity to nucleoprotein, but their melting temperatures were relatively low. Therefore, they employed CDR grafting technique combined with site directed mutagenesis to improve the stability of DSTL096 [32]. Using the framework of a VNAR (SP-15) which had been shown to possess good thermostability [29], two derivatives of VNAR were constructed: One where CDRs 1 and 3 along with HV2 of DSTL096 were grafted onto the framework of SP-15 (SP15-096-123), and another in which only CDRs 1 and 3 were grafted (SP15-096-13). Results from dye-melt assay showed that SP15-096-123 had excellent affinity with low melting temperature, whereas SP15-096-13 had higher melting temperature with poor affinity. However, there were only three amino acid differences in the HV2 of these two VNAR proteins. Six single- and double-point mutants that covered the intermediates between these two clones were made in order to elucidate which of the three amino acids or combinations were responsible for the affinity and stability. They found that a single change in amino acid offered a ~10 °C improvement in melting temperature while maintaining the affinity. Therefore, this study suggested that HV2 can contribute to antigen binding and stability [32].

VNARs have also been used to detect various toxins, for examples, cholera toxin, botulinum toxin A (BoNT/A) complex toxoid, ricin and staphylococcal enterotoxin B (SEB) [19,49]. Cholera is an acute, diarrheal illness caused by the infection of small intestine by *Vibrio cholera*, which secrete cholera toxin once reaching the host intestinal wall. Cholera toxin was chosen in their study because of its severe pathogenicity and commercially availability. Liu and colleagues have demonstrated the novel anti-cholera toxin VNAR binders specifically bound to soluble antigen can be isolated from naïve VNAR library. In their study, the isolated VNAR binders were able to display slow loss of antigen binding affinity over 1 h of incubation at 95 °C, while the conventional antibodies lost its antigen binding affinity in a short period of time [19]. Hence, this study has revealed VNAR is capable of binding with unacquainted antigen of interest.

Table 1
Diagnostic and therapeutic applications of VNAR on various diseases.

Diseases	Target antigens	Applications	References
Ebola haemorrhagic fever	Nucleoprotein	Diagnostic	[32,50]
Malaria	AMA1	Diagnostic	[28,54]
Toxin	Cholera toxin	Diagnostic	[19]
Toxin	BoNT/A	Sensor and diagnostic	[49]
Toxin	Ricin	Sensor and diagnostic	[49]
Toxin	SEB	Sensor and diagnostic	[49]
Human immunosilent target processes	Tom70	Detection	[27]
Periodontal disease	Gingipain K protease	Neutralization	[26]
Arthritis, cancer or Alzheimer's disease	HTRA1	Therapeutic	[44]
Cancer	EpCAM	Therapeutic	[44]
Cancer	EphA2	Therapeutic	[44]
Cancer	GPC3	Therapeutic	[52]
Cancer	HER2	Therapeutic	[52]
Cancer	mAb idiotope	Therapeutic	[59]
Cancer	PD1	Therapeutic	[52]
Cancer (*in vitro* angiogenesis)	VEGF	Therapeutic	[60]
Hepatitis B	HBeAg	Therapeutic	[61]
MERS	MERS spike protein	Therapeutic	[52]
Neurological disease	Myostatin	Therapeutic	[62]
Pro-inflammatory cytokine	rhTNFα	Therapeutic	[17,63]
SARS	SARS spike protein	Therapeutic	[52]
Uveitis	Induced costimulatory ligand	Therapeutic	[64]

5.2. VNAR in immunotherapeutic applications

Zielonka and colleagues have recently performed the rapid isolation of target-specific VNARs with artificial CDR3 loops against three distinct disease targets, namely epithelial cell adhesion molecule (EpCAM), Ephrin type-A receptor 2 (EphA2), and human serine protease (HTRA1) from the VNAR library of bamboo shark (*Chiloscyllium plagiosum*) using yeast display techniques associated with subsequent affinity maturation of target-specific VNARs. In their study, a range of antigen binding VNARs were selected after screening the randomized VNAR-CDR3 repertoires over three antigens as stated. Due to inferiority of binding affinity, the sub-libraries were undergone affinity maturation by randomizing CDR1 region in response to rapid isolation of target-specific VNARs. As a result, the EpCAM- and HTRA1-binding VNAR clones isolated from the sub-libraries exhibited greater binding affinity compared to the clones that isolated from the initial library. From the outcomes, this work has proved where stepwise affinity optimization by first randomizing CDR3 and accumulating low affinity binders followed by diversification of CDR1 in this population of enriched variants could be a useful strategy to obtain VNARs with affinities in the low nanomolar range. To determine the thermostability, EpCAM-binding VNAR clones that were isolated from both initial library and sub-library were produced as soluble proteins and characterized through thermal shift assays. The results from the thermal shift assays showed that the EpCAM-binding VNAR clones exhibited high *Tm* values ranging from around 65 °C to over 75 °C, suggesting the target binders with high affinity can be generated *in vitro* without undermining the thermostability of the VNAR scaffold [44].

Camacho-Villegas and co-workers have utilized phage display technology to isolate targeted VNAR by immunizing a horn shark (*Heterodontus francisci*) with recombinant human tumour necrosis factor alpha (rhTNFα). TNFα is a pro-inflammatory cytokine that is predominantly involved in diseases such as rheumatoid arthritis and psoriasis. According to their results, the isolated VNAR clone had the ability to identify rhTNFα and neutralize it *in vitro*. Therefore VNAR

was suggested to be potentially developed as immunotherapeutic drug for the diseases by suppressing the over secretion of pro-inflammatory cytokine in the body [17].

On the other hand, anti-idiotypic binders have been proven as robust tools to determine pharmacokinetic studies of therapeutic antibodies and cancer vaccines development. In order to develop specific capturing ligands, Könning and colleagues isolated the anti-idiotypic VNARs that recognize therapeutic antibodies (cetuximab and matuzumab) from semi-synthetic libraries. Without applying any negative selections, a few of VNAR binders that showed high specificity towards both cetuximab and matuzumab were successfully isolated using yeast display technology. Surprisingly, each anti-idiotypic VNAR binder isolated was not cross reacting to either cetuximab or matuzumab [59]. Therefore, the combination of semi-synthetic VNAR libraries with yeast surface display would have been a promising technique for rapid isolation of anti-idiotypic VNARs. This novel approach is ideally used for immunotherapeutic platform development.

Recently, Cabanillas-Bernal and co-workers successfully isolated anti-VEGF VNARs which can potentially inhibit *in vitro* angiogenesis. In their study, three synthetic VNAR libraries were constructed, using frameworks without cysteine, with one cysteine and with two cysteine residues within the CDR3.The libraries were then used for selection against six mammalian proteins, which are aquaporin 1 (AQP1), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF-2), leukemia inhibitory factor (LIF), carcinoembryonic antigen (CEA) and glycophorin A (GYPA). At least one VNAR was found for each of these antigens, and a clone from the library without cysteine in the CDR3 was selected with all the antigens. The isolated anti-VEGF VNARs were then undergone three-dimensional *in vitro* angiogenesis assay and *in silico* analysis. The results from *in vitro* angiogenesis assay suggested that VNARs are capable of inhibiting *in vitro* angiogenesis, whereas the results from *in silico* analysis showed that VNARs from synthetic libraries could rival the VNARs with affinity maturation by *in silico* modelling [60].

6. Formatting and humanization of VNAR

VNAR is a promising therapeutic candidate due to its stability, solubility and extraordinary binding loop topology. However, the inherent small size causes VNAR to have short serum half-life. This can be a major limitation when tumour-targeting is needed since it can be rapidly cleared *in vivo* by glomerular filtration [35].Therefore, Müller and co-workers developed a VNAR-based half-life extension tool which increases the serum circulating time of a fusion partner. Their study involved the *N*- and *C*-terminal fusion of a naïve VNAR with an anti-human serum albumin (HSA) VNAR isolated from an immunized spiny dogfish (*Squalus acanthias*), which known as E06 [47]. The fusion constructs were shown to retain high binding affinity to HSA. In comparison with their unconjugated parental domains, the fusion constructs exhibited significantly increased *in vivo* half-life. In addition, the fusion constructs displayed the E06 was capable to enhance the pharmacokinetics effect in mouse, rat and monkey [47].

For therapeutic applications, the humanization of non-human immunoglobulin scaffolds is required to minimize the immunogenicity, and to enhance the thermodynamic stability, folding and expression properties. The first humanized VNAR was reported by Kovalenko and colleagues where the isolated anti-HSA VNAR E06 clone was used as a model to construct a number of humanized variants known as huE06v1.10 [65]. Humanization of E06 was performed by converting more than half of the non-CDR residues to those of a human germline V_K1 sequence. The resulting humanized E06 molecules have largely retained the specificity and affinity of antigen binding of the parental VNAR. Crystal structures in complex with HSA showed the humanized variant, huE06v1.10 bound in a manner different from the E06 [65].

Steven and co-workers then extended this work by employing huE06v1.10 as template to isolate domains with enhanced biophysical properties and reduced antigenicity. The huE06v1.10was mutagenized, followed by refinement of clones *via* an off-rate selection screening on HSA. The lead clones were assessed for any tendency to dimerize, tolerance to *N*- and *C*-terminal fusions, stability, affinity and relative antigenicity in human dendritic cell assays. In addition, the functionality of the clones was verified *in vivo* through the extension of serum half-life in a typical drug format [66]. From these analyses, a clone known as BA11 showed insignificant antigenicity, high affinity and high stability for mouse, rat and HSA. When these attributes were combined with demonstrable functionality in a rat model of pharmacokinetics, the BA11 was established as their clinical candidate [66].

7. Conclusion

Conventional antibodies derived from higher mammals are widely employed as immunodiagnostic reagents due to its specificity. However, the limitations of using conventional antibodies in RDTs leading to misdiagnosis have been reported elsewhere. Therefore, it has become important to extensively investigate new binders with better stability from other biological sources using surface technology. The natural heavy-chain only antibody derived from sharks, known as VNAR, has been discovered in recent years. As highlighted in this review, shark VNAR can be superior to that of conventional antibodies in many aspects. One example is that the capability of VNAR to access cryptic and recessed epitopes which are usually not antigenic for conventional antibodies has made a breakthrough in medical research. In addition to smaller size and high solubility, the excellent thermostability is an advantageous property suggesting that this antibody can be applied in immunodiagnostics especially for the countries in tropical region. Since many new findings have been discovered in shark VNAR, the continuous efforts of research alliances may allow this heavy-chain only antibody to overcome the hindrance faced by corresponded diseases treatment in therapeutic platform.

Authors' contributions

All authors made contribution to this study and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

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