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Single domain antibodies for biomedical applications

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16

17 **Keywords**

18 Therapeutic antibodies, antibody engineering, single chain binding domain, IgNAR, heavy
19 chain antibody, variable domain, VHH, heavy chain antibodies, dAbs, VH, VL, antibody
20 fragment, single domain antibody, CH2, CH3

21

22 **Abbreviations**

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

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

28

29 **Abstract**

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

38

39 **Introduction**

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

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

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

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

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

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

81

82 **VHH domains derived from camelids**

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

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

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

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

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

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

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

137 Another construct is the trimeric bispecific nanobody Ozoralizumab (ATN-103) for the
138 therapy of rheumatoid arthritis. Ozoralizumab consists of two VHHs directed against TNF
139 and one albumin-binding VHH, for prolonged serum half-life *in vivo*. Possible advantages
140 over current therapeutics like Adalimumab[®], Enbrel[®] and Remicade[®] are a better efficacy and
141 reduced manufacturing costs (40). Phase II studies of Ozoralizumab as well as one open label
142 extension study have been carried out, evaluating long term safety, tolerability, and
143 pharmacokinetic properties of multiple ascending doses (clinicaltrials.gov). Other promising
144 nanobodies in clinical development target IL6 (ALX-0061), IL17 (ALX-0761), RSV (ALX-
145 0171) and RANKL (ALX-0141). In addition, VHHs are attractive for addressing difficult
146 targets, for instance G protein-coupled receptors (GPCRs). In this respect the cryptic epitopes
147 of GPCRs are located within transmembrane regions and are poorly accessible with
148 monoclonal antibodies and therefore small molecules are mostly used for GPCR targeting
149 (41). Studies highlighting this targeting approach using nanobodies were recently described
150 for two GPCRs, CXCR4 (42) and CXCR7 (43), respectively.

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

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

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

164

165 **vNAR domains derived from sharks**

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

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

185 There are several different types of vNAR molecules, categorized based on the number and
186 pattern of non-canonical disulfide bonds that are not found in classical antibody domains (49,
187 52-55). Owing to this, the different types of vNAR domains form a very diverse set of
188 additional disulfide bridges. Consequently, antigen-specific clones can be selected from a
189 unprecedented repertoire of different loop structures (56). Moreover, the architecture of the
190 paratope of those shark antibody domains seems to be predisposed to target clefts of the
191 antigen, whereas such recessed epitopes are usually not antigenic for conventional antibodies
192 (55, 57-59). Indeed, it has been shown that the active site of enzymes and clefts can be
193 targeted by vNAR domains (49, 54, 60). A more comprehensive review, addressing the
194 different types of IgNAR V domains as well as the generation of the tremendous diversity
195 found at the sequence level of the IgNAR V domain was recently published by our group
196 (47).

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

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

208 However, in contrast to camelid VHHs, which have proven to be successful in early stage
209 clinical trials (76), progress of the development of vNAR domains for clinical applications is
210 at a much earlier stage. This might be at least partially explained by the low homology of
211 shark vNAR domains with mammalian variable domains, and potential immunogenicity
212 arising thereby. Sequence identity of the IgNAR V domain with mammalian VH regions falls
213 as low as 25 % (77). Even after humanization of a shark vNAR domain a significant amount
214 of non-CDR residues still remained non-human, as described by Kovalenko *et al* (53). In
215 contrast to this, sequence homology of VHHs to human VHs is high, differing only in about
216 ten amino acids and it has been shown that VHHs can be easily humanized by loop grafting
217 (78, 79).

218 Notwithstanding, it is known that even humanized and fully human antibodies such as
219 Adalimumab[®] are able to significantly induce immunogenicity, essentially demonstrating that
220 it needs to be scrutinized for each individual therapeutic candidate how these proteins will
221 behave with regard to adverse events when injected to patients in the scope of clinical trials
222 (80-84).

223

224 **Human antibody variable domains**

225 Human domain antibodies are the smallest known fragments from variable domains of
226 conventional antibodies with retained antigen-binding function and sizes ranging between 11-
227 15 kDa. This antibody type includes antibody heavy (VH dAb) or light chain (VL dAb)
228 variable fragments and comprises three CDRs that mediate specific antigen-interaction (25).
229 In their structure, human dAbs closely resemble the architecture of paired variable fragments
230 in conventional antibodies (Fig. 1 top) (25, 85). In contrast to the naturally evolved equivalent
231 in camelids and sharks, early studies revealed that single human VH domains are prone to

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

234 The unfavorable properties were tackled in a variety of mutational approaches and initial
235 attempts aimed at the transfer of VHH key elements that attributed aggregation-resistance and
236 good solubility to camelid VHHs (85). A process was established, named “camelization”, in
237 which residues of the VL-interface were substituted with hydrophilic residues that are
238 conserved in VHHs (G44E/L45R/W47G) (86). Furthermore, it also became clear that
239 composition and length of the CDRs are determinants of aggregation-propensity and a
240 positive effect on solubility of VH dAbs was shown by the extension of the CDR3 length (25,
241 86, 87). All these studies pointed out residues of the dAb framework and the CDRs as critical
242 constituents for good biophysical properties and provided guidance for the engineering of
243 scaffolds for library applications.

244 Nowadays, convenient generation of human dAbs with desired properties and specificities has
245 been achieved using molecular evolution approaches (e.g. phage display) and repertoires of
246 naïve or synthetic human VH or VL dAbs (85, 88, 89) However, most of the human dAb
247 affinity reagents have been isolated from synthetic libraries that are usually constructed with
248 engineered single scaffolds and CDR-based diversities (90-96). For library construction, it has
249 been shown that several variants of the human germline families possess superior biophysical
250 characteristics (e.g. HV3-23) and therefore are considered as preferable scaffolds (86, 96).

251 Compared to VH domains, VL domains generally seem to have a higher intrinsic resistance
252 towards aggregation (85, 86, 90). Another source of VH dAbs are transgenic mice with
253 human heavy chain only repertoires. However, this platform still has to prove the delivery of
254 stable high-affinity binders (crescendobiologics.com, (96)).

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

258 protein A, resulting in an enrichment of variants with reversible-folding properties (88).
259 Similar to that, Famm and colleagues established the acid-denaturation method that allowed
260 selection of thermodynamically stable and aggregation-resistant dAbs (97). For biomedical
261 applications, the most notable feature of domain antibodies with human origin is their
262 perceived lower immunogenicity compared to their equivalents from camels and sharks which
263 is of importance particularly in long-term clinical use or in context of systemic administration
264 (98, 99). While several dAbs have been explored for a therapeutic application, no dAbs have
265 been tested in clinical phase III trials or were approved for clinical use.

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

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

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

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

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

294 Several cancer-related targets have been addressed with human dAbs. Feng and colleagues
295 isolated a high affinity binding VH dAb (HN3) against a conformation-dependent epitope in
296 Glypican-3 (GPC3) (105). HN3 was tested as bivalent Fc-fusion protein on several
297 hepatocellular carcinoma (HCC) cell lines where it specifically inhibited proliferation. In
298 addition, HN3 also repressed tumor growth in a xenograft tumor model by inducing cell-cycle
299 arrest via Yes-associated signaling (105). Other cancer related targets have been addressed,
300 including the targeting of IGF-II (m630.3), SD1 or type IV collagenase for tumor targeting
301 (106-108). Recently, another CD28-targeting human VL dAb was tested in a preclinical study
302 for the treatment of autoimmune diseases. A pharmacodynamic activity of the PEGylated VL
303 dAb was shown by inhibition of a T-cell-dependent Ab response in cynomolgus monkeys
304 without mediating cytokine release or T-cell depletion (109).

305

306 **Engineered monomeric human IgG1 CH2 and CH3 as a scaffold**

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

309 remarkable feature of engineered domains is their proposed long half-life due to functional
310 FcRn-binding. However engineered CH2 domains seem to have a high aggregation tendency
311 (110, 111). The antiparallel β -strand structure of CH2 domains is similar to that of human VH
312 domains comprising three loops (BC, DE and FG) in that the BC and FG loops at the N-
313 terminal tip can be used for diversification (112). A CH2 variant was engineered (m01s) by
314 the incorporation of an additional intra-domain disulfide-bridge between A and G strands and
315 the elimination of several N-terminal residues. In contrast to the wild-type CH2, the m01s
316 variant exhibited pronounced thermal stability, good expression yield and high solubility
317 (113). *In vivo* mice studies with injected m01s revealed a longer half-life due to FcRn-
318 interaction, compared to scaffolds of similar size that do not interact with FcRn (114).
319 Furthermore, m01s was used for phage library construction by grafting human VH CDR3
320 diversities into the loop FG and mutagenesis of the BC and DE loops. One variant was
321 isolated (m2a1) that showed selective binding to sp62 from the HIV-1 membrane proximal
322 external region (MPER) and functional FcRn-binding (110).

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

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

339

340 **Conclusion**

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

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

354 In general, engineered antibody fragments retain their specific binding characteristics with
355 high affinity and additionally, owing to their small size, gain an improved tissue penetration.

356 For some diagnostic utilization as for example *in vivo* tumor imaging, fast blood clearance of
357 single domain antibodies is desired, whereas for therapeutic applications a long serum half-
358 life is favorable. Several efforts were made to prolong the circulation time of single domain
359 antibodies for instance by PEGylation, engineering albumin binding, or formatting as Fc
360 fusion for FcRn-binding.

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

365

366 **Declaration of interest**

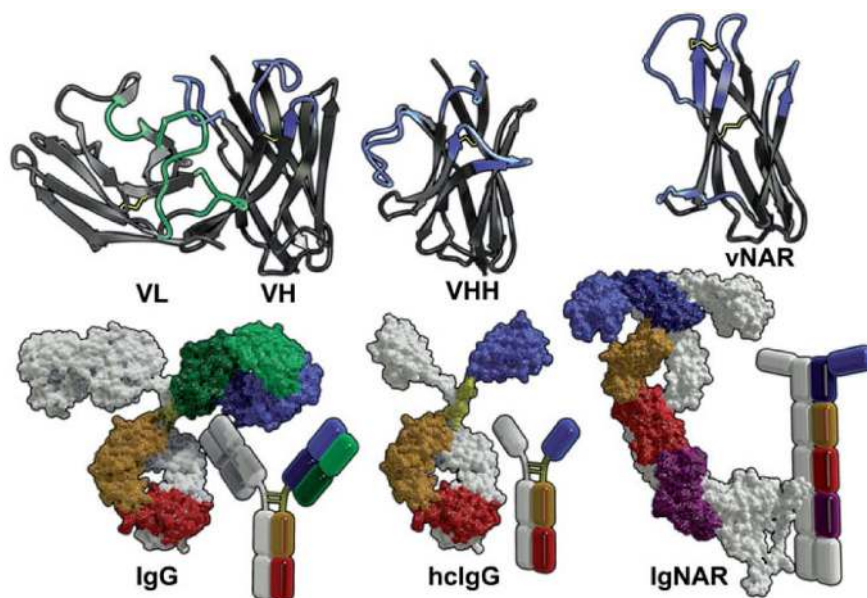
367 The authors declare no competing interests.

368

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373



374

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

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

384

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