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Camelid-derived single chain antibodies in hemostasis: mechanistic, diagnostic and therapeutic applications

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Essentials

- Single-domain antibodies (sdAbs) derived from camelids are increasingly used as research tools
- SdAbs against coagulation proteins have helped solve biochemical research issues
- SdAbs can be used as diagnostic tools and also for therapeutics in thrombosis and hemostasis
- Protein engineering increases the versatility of sdAbs and their potential applications

Abstract

Hemostasis is a complex process involving the concerted action of molecular and vascular components. Its basic understanding as well as diagnostic and therapeutic aspects have greatly benefited from the use of monoclonal antibodies. Interestingly, camelid-derived single-domain antibodies (sdAbs), also known as V_HH or nanobodies, have become available during the previous two decades as alternative tools in this regard. Compared to classic antibodies, sdAbs are easier to produce and their small size facilitates their engineering and functionalization. It is not surprising therefore, that sdAbs are increasingly used in hemostasis-related research. In addition, they have the capacity to recognize unique epitopes unavailable to full monoclonal antibodies. This property can be used to develop novel diagnostic tests identifying conformational variants of hemostatic proteins. Examples include sdAbs that bind active but not globular von Willebrand factor or free factor VIIa but not tissue factor-bound factor VIIa. Finally, sdAbs have a high therapeutic potential, exemplified by caplacizumab, a homodimeric sdAb targeting von Willebrand factor that is approved for the treatment of thrombotic thrombocytopenic purpura. In this review, the various applications of sdAbs in thrombosis and hemostasis-related research, diagnostics and therapeutic strategies will be discussed.

Introduction

Ever since the discovery of hybridoma technology by Kohler and Milstein in 1975,[1] monoclonal antibodies have represented very powerful tools to explore biological processes. Hemostasis, a complex biological process in which a cascade of events involving the concerted action of cellular and molecular players allows for a quick response to vessel injury, is no exception in this regard. Antibodies have been invaluable biological determinants not only to better understand hemostasis but also for diagnostics of hemostasis-related pathologies or more recently for their treatment.[2] Immunotherapy in the hemostasis field started with the use of Abciximab, a humanized Fab fragment against platelet glycoprotein $\alpha_{2b}\beta_3$, widely used as anti-platelet therapy in patients undergoing percutaneous coronary intervention in order to avoid ischemic complications.[3] Abciximab has the capacity to block binding of $\alpha_{2b}\beta_3$ to its ligands, fibrinogen and von Willebrand factor, thereby preventing platelet aggregation.[4] Following Abciximab, it has taken a number of years before new antibody-based treatments could become available for hemorrhagic or thrombotic diseases. However, at least in hemophilia, therapeutic antibodies are now revolutionizing the treatment of patients. [5, 6] In particular, emicizumab, a bispecific humanized IgG4 directed against coagulation factors IX and X and marketed under the name of Hemlibra has proven efficient in treating hemophilia A patients with or without inhibitors.[7] Emicizumab mimics factor VIII cofactor activity by binding simultaneously to the enzyme factor IXa and its substrate, factor X.[7] On the thrombotic side, several antibody-based treatments are currently under development, such as ACT017, a humanized Fab fragment, directed against platelet receptor GPVI and intended for stroke treatment.[8] ACT017 (Glenzocimab) blocks GPVI binding sites, leading to an inhibition of collagen-induced platelet aggregation. This antibody has now demonstrated its safety profile in a clinical phase I trial.[9] Antibodies targeting coagulation factors XI/XIa and XII/XIIa also hold the promise of becoming safe antithrombotic drugs since they are not expected to increase the bleeding risk.[10, 11]

Although classical monoclonal antibodies and their Fab fragment or single-chain variable fragments (scFv) derivatives remain most widely used, camelid-derived single-domain antibodies (sdAbs) have emerged as a credible next-generation of antibodies offering several advantages over classic antibodies owing to their peculiar properties, including their small size, robust behavior, high affinity and specificity and deep tissue penetration.[12-14] It is also important to mention that besides camelids, sdAbs can also be found in certain sharks (vNARs).[15] However, this review will focus exclusively on camelid-derived sdAbs.

1. Single-domain antibodies: the next-generation antibodies

1.1 General features of single-domain antibodies

The first description of functional sdAbs in camelids was reported in 1993.[16] Unlike conventional antibodies, and despite their ability to bind to their target antigen with strong affinity, a fraction of antibodies derived from camelids was found to be completely devoid of light chain. From a structural point of view, sdAbs contain the CH2 and CH3 domains but lack the first classic immunoglobins (Ig)G constant domain, CH1 (Fig. 1). The lack of CH1 domain is a necessity for their secretion in the absence of a light chain.[17] Consequently, their antigen-binding regions consist of one single VH domain termed V_HH. V_HHs were reported to be the smallest intact antigen-binding single polypeptide chains found in any natural antibody.[18] Recombinant expression of V_HHs allows for the generation of a soluble single-domain antigen-binding protein with a molecular weight of about 15kDa. Due to their small size, these sdAbs are sometimes referred to as “nanobodies”. SdAbs have functional properties similar to that of conventional antibodies in terms of affinity and specificity,[19] with a binding affinity within the pico-molar to nano-molar range.[20] However, distinctions also exist between sdAbs and the antigen-binding region of IgG, which is composed of two entities in two different polypeptide chains, VL and VH. Indeed, certain residues in the VH framework regions that are part of the interface with the VL are altered at the corresponding positions in sdAbs. This endows sdAbs with a more hydrophilic character, increasing their solubility.[21, 22] In addition, the eventual presence of an extra disulfide bond between the first and third antigen-binding loops, H1 and H3, may rigidify the sdAb structure, contributing to an increased stability and activity at high temperatures and in high concentrations of denaturants, compared to classical IgGs.[23] The sdAb paratope is defined by three highly variable loops H1, H2, and H3, which form an extended structural interface at one side of the protein (Fig. 1).[24] Despite the smaller surface of this paratope compared to the VH-VL paratope of IgG, sdAbs can create a higher diversity by exhibiting a greater structural variation in their H1, H2 and H3 loops and by increasing the length of the H3 loop.[24-28] Noteworthy, the conserved framework regions of sdAbs show a high sequence and structural homology with human VH domains of family III (VH3) [29]. This high homology not only results in a comparable immunogenicity as human VH, but also increases the chances of successful humanization of these sdAbs (see also section 4 of this review).[22] Furthermore due to their single-domain nature and lack of glycosylation, they can be produced in large amounts using standard microbial or yeast expression systems.[30-32] On the other hand, sdAbs miss some of the characteristics found in regular IgGs. For example, the absence of the Fc portion prevents these sdAbs to directly activate the complement system and immune cells, and they lack therefore cytotoxic activity.[33, 34] Furthermore, without the Fc portion, sdAbs are incapable to enter the FcRn-dependent recycling pathway. In combination with their small size, which is below the glomerular filtration capacity, this contributes to the short *in vivo* half-life of the sdAbs.[34, 35] Finally, with the antigen-binding

paratope being restricted in size compared to classic IgG, this implies that the number of residues within this paratope that could be used to improve antigen binding are limited as well.[36]

1.2 Libraries and screening options to generate sdAbs

Three kinds of libraries, immune libraries, naïve libraries and synthetic libraries can be used for the selection of sdAbs. Classic library engineering and sdAb screening strategies are presented in Fig. 2 and extensively reviewed by Hoogenboom.[37]

Traditionally, immune libraries of sdAbs are obtained upon immunization of an animal (llama, alpaca or dromedary) with the target antigen. The immunization process takes up to 6 weeks and requires weekly injections of variable amount of the target antigen supplemented with an adjuvant to boost its immunogenicity. Animals can be injected with multiple antigens (5-10), improving the economics of producing sdAbs and reducing the number of animals. Total mRNA from peripheral blood monocyctic cells is then isolated, and the V-gene repertoire is amplified to generate cDNAs encoding sdAbs which will make up the library.

sdAbs libraries can also be generated from non-immunized animals, and are therefore called naïve libraries. They come with the advantage of not being selective for a specific antigen and represent the natural diversity of the immune repertoire of the animal. This can be useful when the structure that needs to be targeted by the sdAb is poorly antigenic. It should be noted that naïve libraries often lead to generation of low affinity sdAbs which then require subsequent maturation steps.[37]

Synthetic libraries are increasingly used and consist of *in vitro* randomized libraries.[38] Starting from a unique sdAb scaffold, random primers are used to generate various CDRs lengths and sequences by PCR, creating a novel library that does not rely on the immune system of an animal. This allows the generation of unique CDR3 sequences that may have never existed *in vivo*, leading to the identification of antigen binders that can target poorly antigenic structures. One pitfall could be that such unnatural CDR3 sequences may increase immunogenicity, a potential issue if the sdAb is selected for therapeutic purposes.

The immune libraries are generally superior due to the higher affinities of the recovered sdAbs. This is likely due to the deletions/insertions seen in the V_HH genes, which occurs as clones are selected by the immune response. Such recombination events do not occur in the naïve libraries and are not naturally selected for using synthetic libraries.

One important characteristic of using cDNA-based libraries (whether they may be immune, naïve or synthetic), is that they allow for a reproducible expression of the sdAbs during each round of the selection process. This is an important advantage over the use of hybridomas, in which loss of IgG expression may occur during selection.

Independent of the approach underlying library engineering, numerous selection techniques including phage display, yeast display, bacterial display, mRNA/cDNA display, intracellular two-hybrid system, and ribosome display can be applied to facilitate screening and panning for a given antigen-specific sdAb.[39] The generation of a sdAbs library is advantageous in the sense that it can be screened multiple times, allowing for the use of several setups for antigen presentation. This is of particular interest when the target antigen presents with complicated features, as is often the case with coagulation proteins. Indeed, coagulation factors are found in a zymogen state in the circulation and needs to be activated to exert their functions.[40] Hence, activated coagulation factors are often extremely flexible in their structure, making it sometimes difficult to identify specific binders.

Following the selection protocol, sequence optimization may also be necessary to improve sdAbs production efficiency, solubility or any other desired property.

1.3 Versatility of sdAbs

The combined properties of sdAbs (*e.g.* selection of conformation-specific binders, ease of engineering, production via routine recombinant techniques, etc...), has led to a plethora of applications for sdAbs as research tools. For instance, sdAbs have been used as crystallization chaperones for the structural investigation of diverse conformational states of flexible proteins.[39, 41] Green Fluorescent Protein-targeting sdAbs have been applied to optimize super-resolution microscopy.[42] Since sdAbs are easily expressed in a variety of cell types, they also have been used to study intra-cellular processes (for which they are referred to as intrabodies). In this respect, they can be used to specifically inhibit target-proteins, signal the appearance of a specific conformational state, or direct the target protein to the intracellular degradation pathway.[38, 43, 44] In addition, numerous fusion-variants of sdAbs have been described. Apart from the classic fusion candidates (fluorescent proteins or IgG Fc-domain), these also include horseradish-peroxidase and the phospholipid-binding C1C2 domains of lactadherin.[45, 46] Besides these general applications showing (in a non-exhaustive fashion) how powerful the sdAbs technology can be, more specific use of sdAbs in the thrombosis and hemostasis field will be the focus of the next few paragraphs.

2. Single-domain antibodies as research tools in hemostasis

In the context of hemostasis, a certain number of sdAbs have now been developed in the last 15-20 years and many of those have been extremely useful, especially for biochemical studies. Indeed, many proteins relevant to maintain the hemostatic balance, can exist in different forms. Best described are enzymes and cofactors that require proteolytic activation. sdAbs have the

potential to differentiate small conformational differences and have therefore contributed to answer important research questions. Table 1 and Fig. 4 summarize sdAbs targeting hemostasis proteins.

2.1 sdAbs against factor VIIa

One example of sdAb that allowed to resolve a scientific issue relates to factor VII (FVII) which can exist in a precursor (FVII) and an activated (FVIIa) state.[47] In the 1990s, FVIIa has become available for the treatment of hemophilia inhibitor patients. Its efficacy is based on the capacity of FVIIa to activate coagulation factors IX and X, thereby leading to thrombin activation. One unanswered question remained about the mechanism of action of FVIIa, based on the apparent discrepancy between the high therapeutic doses needed compared to the low physiological concentration of FVIIa. One hypothesis was that FVIIa had to compete with endogenous FVII for binding to its cofactor Tissue Factor (TF).[48] To assess this hypothesis, our group has recently developed a sdAb (KB-FVIIa-004) capable of selectively detecting and inhibiting proteolytic activities of FVIIa but not TF-bound FVIIa.[49] Using this unique sdAb, we have been able to demonstrate the TF-independent mode of action of FVIIa *in vivo* in hemophilia A mice.

2.2 sdAbs against factor XIIa

sdAbs have also helped decipher the sequential activation steps of factor XII (FXII), a key protein of the contact system which has recently gained a lot of attention for its potential as an anti-thrombotic target.[50, 51] Upon surface binding, small amounts of FXII are converted into an activated derivative, α -FXIIa. α -FXIIa is able to convert plasma-prekallikrein (PPK) into plasma-kallikrein (PK), and in turn PK will further amplify α -FXIIa formation. However, PK will also generate β -FXIIa, a smaller derivative of α -FXIIa that lacks the surface binding heavy chain. α -FXIIa and β -FXIIa differ in that only α -FXIIa is able to activate FXI into FXIa. In contrast, both variants are able to convert PPK into PK. Since PK is responsible for the release of bradykinin, a small peptide that evokes vascular leakage and tissue swelling, it is thus important to know how much α -FXIIa and β -FXIIa is present under various physiological and pathological conditions. To improve the analysis of α -FXIIa and β -FXIIa formation, de Maat *et al* have developed sdAbs that are able to distinguish between both forms.[52] One sdAb (V_HH-B7) recognizes both variants, whereas V_HH-A10 selectively binds to α -FXIIa. These sdAbs thus represent an essential biochemical research tool to capture these molecules at different time points of the contact system and reveal previously unidentified agonist-dependent FXII activation pathways, which might or might not lead to activation of the coagulation cascade.[52]

2.3 sdAbs against Thrombin Activatable Fibrinolysis Inhibitor (TAFI)

Another hemostatic protein which has significantly benefited from sdAbs technology for its structural and mechanistic studies is TAFI, known for its modulatory function of the fibrinolytic process. TAFI circulates as an inactive carboxypeptidase-precursor, which becomes active via proteolytic activation predominantly by the thrombin/thrombomodulin complex. This proteolytic step releases the N-terminal activation peptide and opens the active site for its substrates. The main function of TAFIa is to down-regulate fibrinolysis by removing C-terminal lysines from fibrin.[53] Its physiological relevance is illustrated by the notion that elevated TAFI levels are associated with an increased thrombotic risk.

Both activation and inactivation pathways of TAFI are complex and involve multiple steps. Particularly, inactivation of TAFIa is auto-regulated by a self-destruction mechanism that involves sequential conformational changes within the protein. To better understand this process, the laboratory of Dr. Declerck has developed numerous sdAbs specific to the different conformations of the TAFI zymogen and its activated derivatives.[54-57] These sdAbs have been used in activity assays as well as in structural studies to obtain specific crystal structures, which together led to the discovery of previously unidentified conformations of this intriguing enzyme. In addition, they were useful to identify the activation peptide as an anchor for the thrombin/thrombomodulin complex, necessary to achieve physiological relevant activation rates. Together, these studies provide an excellent example of how the unique properties of sdAbs can generate novel structural insights with regard to the highly flexible structures of hemostatic proteins.

2.4 sdAbs against Urokinase-type Plasminogen Activator (uPA)

sdAbs have also been used for the analysis of other fibrinolytic proteins, such as Plasminogen Activator Inhibitor-1 and uPA.[58-60] uPA in particular is mainly known as a plasminogen activator, but is also involved in processes that do not require plasmin generation.[61] In search for inhibitors of uPA function, it has been observed that some of the peptide-bond based inhibitors also function as substrates. It is unclear if this dual behaviour originates (1) from conformation changes within the peptide segment of the inhibitor/substrate that inserts into the active site pocket of the enzyme, (2) from conformational changes within the enzyme structure or (3) both. To get insight into this important question, Kromann-Hansen and coworkers developed sdAbs that penetrate into the uPA active site.[58, 59] Via crystallographic analysis, they observed that the presence of such sdAbs changed the structure of uPA, favouring option 2. Interestingly, they noticed that despite the strong inhibitory effect of the sdAb, it also was slowly proteolyzed. Following mutagenesis aiming to introduce more flexibility into the rigid H3-loop, the sdAb was proteolyzed more efficiently. This suggests that also conformational changes within the peptide segment that protrudes from the active site can contribute to

whether this segment is acting as inhibitor, substrate or both. It would be interesting to investigate whether sdAbs that penetrate the active site of other serine proteases display similar features, as this may help to develop agents that display partial or transient inhibitory properties towards their targets.

2.5 sdAbs against von Willebrand factor

Although most of the above-mentioned studies have used sdAbs as biochemical tools, they can also be essential for functional studies. One such example relates to sdAbs raised against von Willebrand factor (VWF), the main molecular player in platelet adhesion to the injured vessel during primary hemostasis.[62] As many other hemostatic proteins, VWF displays important roles beyond hemostasis. In this context, our group has recently generated a sdAb binding to the VWF A1 domain (KB-VWF-006) with the unique capacity to recognize human and murine VWF.[63] This sdAb interferes with ristocetin-induced human and murine platelet aggregation by inhibiting VWF-platelet GPIIb/IIIa interaction. Because our sdAb can also recognize murine VWF, it holds the potential to be used *in vivo* in order to test the implication of the VWF-GPIIb/IIIa axis in different pathological settings. When applied to inflammatory *in vivo* models, *i.e.* immune-complex-mediated vasculitis and irritant contact dermatitis, KB-VWF-006bi (the bivalent version of KB-VWF-006) was able to reduce hemorrhages and leukocyte recruitment, supporting the previously hypothesized direct role of the VWF-GPIIb/IIIa pathway in inflammatory circumstances and during vascular permeability.[63] Similarly, in a stroke model, KB-VWF-006bi reduced thromboinflammation, highlighting the important role of the VWF A1 domain in this process.[64]

3. Single-domain antibodies as diagnostic tools in hemostasis

Although there is not any sdAb-based routine diagnostic assay available yet in clinical hematology laboratories, several sdAbs have been used for clinical research purposes, proving their usefulness in detecting specific disease-associated protein distinctions.

3.1 sdAbs against active VWF

Despite its non-enzymatic characteristic, VWF can transition from an inactive- to an active conformation in specific pathophysiologic circumstances. Indeed, pro-coagulant VWF properties largely rely on conformational changes that switch the molecule from a globular, inactive form to an open, GPIIb/IIIa-binding state, also referred as active-VWF.[65] Physical relaxation and/or mechanical activation of VWF-A1 domain in response to mechanical

supraphysiologic forces allow active VWF to interact with its partners, among which the most important are subendothelial collagen and platelets. Several years ago, a sdAb (AU/VWFA-11) was identified to selectively detect GPIIb/IIIa-binding VWF state but not globular VWF.[66] Importantly, AU/VWFA-11 has no inhibitory function. AU/VWFA-11 binding assays have been developed to directly reveal circulating active VWF and provided an alternative easy method as compared to indirect measurement of platelet aggregation in the presence of various ristocetin concentrations. Major advantage of the AU/VWFA-11 binding assay is the ability to detect active VWF even under conditions of low VWF antigen levels. It is important to mention that under physiological conditions, extremely low levels of active VWF are present in the circulation since this platelet-binding form of VWF is considered pro-thrombotic. AU/VWFA-11 assays have therefore been used to detect excessive VWF activation in several pathologic conditions.[67] Best described are a bleeding-associated rare subset of von Willebrand Disease (VWD) classified as VWD-type 2B and thrombotic thrombocytopenic purpura (TTP), which is related to disseminated thrombotic events in the microcirculation. VWD-type 2B is caused by gain-of-function genetic variants within or close to the VWF-A1 domain. These mutations destabilize VWF towards its platelet-binding conformation.[68] Using the AU/VWFA-11 binding assay, it was found that active VWF was raised two- to 15-fold.[66] Moreover, an inverse correlation was observed between the amount of active VWF and platelet count, which, in turn is associated to the severity of the bleeding phenotypes.[69]

Similar rises in active VWF have also been measured in TTP patients, with a two- to 12-fold increase over resting, despite a very different underlying molecular mechanism. Active VWF in TTP stems from a lack of proteolytic inactivation of VWF by its specific enzyme, ADAMTS13.[70] Interestingly, higher levels of active VWF were consistently measured in patients with congenital forms of the disease, while samples from patients suffering from the acquired forms showed smaller, albeit supraphysiologic levels of active VWF. Therefore, AU/VWFA-11 is able to discriminate between congenital and acquired TTP forms which is relevant to apply therapeutic decisions.[66]

A number of pathologic conditions characterized by VWF-dependent platelet aggregates have also been associated with elevated levels of active VWF, as quantified by AU/VWFA-11-based assays.[67] This was the case in patients suffering from HELLP syndrome (*hemolysis, elevated liver enzymes, low platelets*) a severe complication of preeclampsia,[71] parasitic malaria, which is associated with thrombocytopenia and cerebral thrombotic events,[72] antiphospholipid syndrome, again characterized by low platelet counts and microthrombopathy[73] and sickle cell disease, associated with chronic hemolytic anemia and sporadic vaso-occlusion.[74]

3.2 sdAbs against FVIIa

Besides the sdAb against FVIIa developed by our group and used for mechanistic studies (see part 2.1), another group has generated a bivalent clone (α FVIIa-V_HH) which was elegantly employed in different pathologic settings. Indeed, specific levels of FVII versus FVIIa have been evaluated in cases of Systemic inflammatory response syndrome, SIRS. In this context, quantification of FVII versus FVIIa appeared useful to stratify patients with higher mortality risk. Indeed, elevated FVIIa and low FVII levels were found to associate with high mortality rate. This study highlights how constant implementation of highly selective research tools is not only beneficial for diagnosis but allows to test unexplored aspects of complex pathophysiological pathways, for example coagulation and inflammation.[75]

3.3 sdAbs against fibrinogen

Another advantage of sdAbs is that the C-terminus is opposite to the antigen-binding surface, allowing it to be used for functionalization of the antibody. This feature has recently been used for the development of a novel diagnostic assay to measure fibrinogen levels.[76, 77] Purified sdAbs targeting fibrinogen were used in an immunobased amperometric test, allowing the rapid detection of as little as 44 μ g fibrinogen/L in plasma (normal range 1.5-4.5 g/L). In follow-up studies, the same group is now developing an agglutination-based assay, that relies on the expression of sdAbs at the E- coli-surface. Incubation of plasma-samples containing fibrinogen with these sdAb-expressing bacteria results in a dose-dependent agglutination.[78] Given the high affinity and specificity, this type of approaches could be used for the detection of other hemostatic proteins as well.

4. Single-domain antibodies as therapeutic tools in thrombosis and hemostasis

Probably owing to their relatively recent discovery, sdAbs have not yet widely found their way to the clinic. There is no doubt that this situation is likely to evolve in the next few years since given their unique properties, sdAbs have a high potential in this regard. First, despite their non-human origin, sdAbs, are less immunogenic compared to murine- or rat-derived monoclonal IgG antibodies, because of their high degree of homology to the human VH3 domain.[29] In addition, humanization of sdAbs is relatively straightforward compared to the humanization of IgG molecules. Main issue is to substitute the amino acids that correspond to those in human IgG that are involved in the VH/VL interaction, which are located in framework region 2.[22] Since such substitutions may affect solubility or affinity, it is not uncommon that additional mutagenesis is performed to restore or even improve these parameters. A second advantage relates to the half-life of the sdAbs, which can be tailored dependent on its purpose. Due to its small size, a single unbound sdAb is rapidly eliminated from the circulation via renal

clearance. This could be advantageous when used for *in vivo* imaging as explained in part 5.2. Alternatively, sdAbs can form a complex with their target in the circulation, and in this complex sdAbs adopt the circulatory half-life of their target. Examples hereof are caplacizumab (discussed in part 4.1), or sdAbs targeting antithrombin, which are eliminated while bound to their respective targets VWF and antithrombin.[79, 80] Finally, sdAbs can be engineered to have a prolonged half-life, for instance via fusion to proteins with a long half-life (such as the Fc-region of IgG molecules or albumin), via fusion to a second sdAb that binds for example albumin, via multimerization to a size above the renal clearance limit, or via the formation of sdAb-containing nanoparticles.[81]

Together, these properties make sdAbs attractive candidates for therapeutical applications whether in the area of thrombosis and hemostasis or other pathologies. So far, only one sdAb has been approved for treatment but a number of other sdAbs are currently in clinical trials for treatment of rheumatoid arthritis, breast cancer or other pathologies.[13]

4.1 sdAbs in thrombosis

As previously mentioned, only one sdAb has been approved for therapeutic purposes, *ie* caplacizumab, a homodimeric sdAb targeting the A1-domain of VWF. Caplacizumab interferes with the interaction between VWF and GPIIb α , preventing the adhesion of platelets to VWF.[80] Originally designed to prevent thrombotic events during percutaneous coronary intervention, caplacizumab is now approved for the management of TTP. TTP is characterized by the functional deficiency of ADAMTS13, a protease important for the regulation of VWF multimer size. The absence of ADAMTS13-activity allows the secretion of ultralarge platelet-binding VWF multimers, which provokes the formation of VWF/platelet aggregates that occlude the microvasculature.[70] Caplacizumab has been evaluated in two clinical trials, the TITAN- and HERCULES-trial.[82, 83] In both trials, caplacizumab treatment was characterized by a faster resolution of thrombocytopenia, a reduction in TTP-related death, relapse and major thrombotic events as well as shorter hospitalization times. Importantly, only 3% of the patients in the HERCULES-trial developed anti-caplacizumab antibodies, with none of them affecting clinical efficacy or caplacizumab function. It is of further interest to mention that caplacizumab has been shown to reduce infarct size in an animal model for cerebral artery thrombosis, [84] while studies using a caplacizumab-mimetic (sdAb KB-VWF-006bi described in part 2.5) revealed that blocking VWF/platelet interactions reduces the inflammatory response following vascular injury.[63, 64] It would be interesting therefore to evaluate caplacizumab in other thrombotic conditions, such as ischemic stroke.

Although not yet in clinical development, there are also a number of other sdAbs that could be of interest for the management of thrombotic complications. In particular, sdAbs that target natural inhibitors of the fibrinolytic pathway could be useful in this regard. Inhibitory sdAbs

targeting TAFI (described in part 2.3) and Plasminogen activator inhibitor-1 (PAI-1), developed by the laboratory of Dr. Declerck could be used to enhance thrombolysis.[54, 57] In particular, these sdAbs could be applied to enhance thrombolysis during exaggerated pulmonary thrombosis. Interestingly, a clinical trial is under consideration to improve thrombolysis via nebulization of tissue-type plasminogen activator.[85] Since sdAbs have excellent bioavailability following nebulization,[86, 87] it would be interesting to add sdAbs targeting TAFI or PAI-1 as stand-alone or adjuvant therapy.

4.2 sdAbs in hemorrhagic disorders

Also in hemorrhagic conditions, sdAbs could be useful, and our laboratory has recently reported two potential examples in which sdAbs have been tested in pre-clinical settings. First, we used engineered sdAbs neutralizing the anticoagulant antithrombin.[79] By neutralizing antithrombin, thrombin generation in the absence of factor VIII (FVIII) or factor IX was normalized (irrespective of the presence of inhibitors). In subsequent mouse models for hemophilia A or hemophilia B with inhibitors, the presence of anti-antithrombin sdAbs normalized the bleeding tendency of these mice. Interestingly, correction of bleeding was achieved not only via a single intravenous infusion of the purified protein, but also in a gene therapy setting. Apparently, AAV-mediated expression of sdAbs is sufficiently strong to neutralize plasma proteins present in high concentrations (2.5 μ M for antithrombin). It would be of interest to also investigate sdAbs targeting other natural anticoagulants, including protease nexin-1, tissue factor pathway inhibitor, protein Z-dependent protease inhibitor, activated protein C and protein S, for their efficacy in correcting the bleeding tendency in hemophilia.

In a second approach, we replaced the B-domain of FVIII with sdAbs targeting VWF, the carrier-protein of FVIII.[88] This increased the affinity of FVIII for VWF about 25-fold (13 pM instead of 330 pM), while leaving *in vitro* and *in vivo* FVIII activity unaffected. The increased affinity resulted in a two-fold prolonged half-life. Unexpectedly, increased affinity for VWF resulted in a strongly reduced immune-response, with 7-fold less mice developing anti-FVIII antibodies compared to wild-type FVIII. Although in the early stages of development, this FVIII variant could be an attractive alternative in the treatment of patients having increased risks of inhibitor development.

5. Single-domain antibodies and challenges in hemostasis

Because sdAbs are so versatile and can be used in so many different ways, as shown in the example of the FVIII-sdAb fusion molecule, there are (almost) unlimited options in their potential applications. A few examples will be discussed below and are summarized on Fig. 4.

5.1 sdAbs and therapeutic design

An important challenge in hemostasis is to maintain the delicate balance between bleeding and thrombosis. For patients suffering from an excess or lack of coagulation, replacement therapy is often the main option but the ideal would be to direct the missing factor where it is needed, to the bleeding site or to the thrombus site. So far, such treatments are administered systemically, thereby increasing the risk of unwanted events. Due to their small size and low complexity, sdAbs are easy to combine, incorporate into fusion molecules, and can be functionalized. Therefore, the use of sdAbs for targeting active pro- or anti-coagulant molecules at the site where they are needed is an attractive option. Moreover sdAbs present a high tissue penetration capacity, which is of tremendous interest for thrombotic events.[12, 89] The field of fusion protein design with sdAbs can build on what has already been done with scFv. Such examples include the fusion protein between a scFv against the activated form of $\alpha_{2b}\beta_3$ and a FXa inhibitor peptide,[90] a scFv targeting $\alpha_{2b}\beta_3$ to a modified plasminogen,[91] or an anti-PECAM 1 scFv linked to a single-chain pro-urokinase plasminogen activator.[92] In all these examples, the fusion between the scFv and the active peptide or molecule has resulted in successful cellular targeting of the fusion protein. Compared to scFv, sdAbs will be easier to use for protein engineering due to their high solubility, stability and high yield of production.[93]

Besides protein fusion, another interesting possibility relies on the potential incorporation of sdAbs in the capsid of gene-delivery vectors such as adeno-associated viruses (AAV). This was recently reported by Eichhoff *et al*, who elegantly generated AAV variants whose capsid VP1 protein was fused with receptor-specific sdAbs. These AAV variants demonstrated high specificity for the cells expressing the respective sdAbs targeted receptor.[94] Future development of such strategies will help generate highly tissue-specific AAV vectors that would increase gene-therapy efficiency. This would be particularly interesting in the hemophilia field where improvement in gene-therapy efficiency could lead to lower doses of virus particles injected and therefore to less adverse effects such as liver toxicity.

Still with the aim of achieving a better targeted therapy, sdAbs can be incorporated into drug delivery systems such as liposomes, micelles or nanoparticles.[95-97]

5.2 sdAbs and bioimaging

To date, thrombus characterization suffers from the lack of precise tools for structure and composition analysis, which is crucial to make a medical decision. This is particularly true in stroke where “time is brain” but where recanalization treatment will be more or less successful according to the etiology of the thrombus.[98] Thus, markers of thrombus composition and also of thrombus maturity are critically needed to decide whether anticoagulation, pharmacological

thrombolysis, mechanical thrombolysis, or a combination of these is required.[99] The recent availability of thrombi obtained by thrombectomy has only further highlighted the differences in thrombus composition and therefore the need for more adapted and targeted therapies.[100, 101] However, the imaging technologies that are currently available such as single photon emission computed tomography (SPECT), positron emission tomography (PET), magnetic resonance imaging (MRI) and optical imaging techniques, despite their advantages, do not yet allow the precise characterization of a thrombus composition that would be needed upon patient arrival. This is why mixed imaging techniques which can combine molecular/functional/anatomical imaging are gaining increasing attention. They rely on the accuracy and precision of radiolabelled markers and sdAbs have many of the desired qualities required to constitute excellent markers for imaging. Their small molecular weight (about 15 kDa) is well below the renal elimination cut-off (~50 kDa). As a result, sdAbs size favors their tissue penetration and allows for their rapid renal clearance. This leads to a high signal-to-noise ratio and rapid imaging after administration, as demonstrated in the case of tumor imaging.[102, 103] It is therefore possible to imagine using sdAbs fused to imaging probes for detection of fibrin/red blood cells/VWF or activated platelets in order to gain knowledge in thrombus structure. Again, the feasibility of such an approach was demonstrated using scFv. A synthetic protein-based nanoparticle containing a near infrared fluorescent molecule and functionalized with a scFv specific for activated platelets was engineered by Bonnard *et al.* This construct exhibited a strong potential as biocompatible molecular imaging probe in an *in vivo* model of carotid arterial thrombosis.[104]

Conclusion

sdAbs have only begun to demonstrate their utility in biomedical research. The thrombosis and hemostasis community has rapidly seized their potential by developing very interesting biological tools against complex proteins and by using them to tackle unanswered biochemical issues. Notably, so far, no sdAbs against platelet receptors have been developed. Importantly, the first clinically approved sdAb is in the thrombosis and hemostasis field, demonstrating the swift embracement of this technology by our community. However, as a laboratory that has invested in sdAb technology for the past six years, we can testify that although they constitute great tools, not all sdAbs are equal and working with some of them can prove challenging. Affinity maturation steps to optimize sdAbs are often necessary and can be time consuming. Only future will tell whether sdAbs will be the magic bullets of next-generation immunotherapy strategies.

Author contribution

All authors have contributed to the writing of the review and approved the final manuscript.

Conflict of interest

P.J. Lenting, O.D. Christophe and C.V. Denis are co-inventors on 4 patents related to the use of single-domain antibodies for therapeutic purposes.

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Table 1: Overview of sdAbs used in Thrombosis and Hemostasis field

Target protein	Camelid species/ Synthetic library	sdAb nomenclature	Binding site	Activity	Application	References
Antithrombin	Llama	KB-AT-01 – 07 ; KB-AT-23	None defined	Neutralization of antithrombin function	Research /Therapy	[79]
Factor VIIa	Llama	α FVIIa-V _H H	FVIIa protease domain	Detection of FVIIa but not FVII	Diagnostics	[75]
Factor VIIa	Llama	KB-FVIIa-004	FVIIa protease domain	Inhibits free FVIIa but not TF-bound FVIIa activity	Research	[49]
Factor XIIa	Llama	A10	Catalytic domain	Binds selectively to α -FXIIa	Research /Diagnostics	[52]
Factor XIIa	Llama	B7	Catalytic domain	Binds both α -FXIIa and β -FXIIa	Research /Diagnostics	[52]
Fibrinogen	Dromedary	VFIB1, -2, -3	None defined	High-affinity binding to fibrinogen	Diagnostics	[76] [77]
PAI-1	Alpaca	V _H H-s-a98	Surface loops around central β -sheet A	Switches PAI-1/PA to substrate pathway	Research	[105]
PAI-1	Alpaca	V _H H-2w-64 (Nb64)	Surface loops around central β -sheet A	Switches PAI-1/PA to substrate pathway	Research /Therapy	[60] [105]

PAI-1	Alpaca	V _H H-s-a27	None defined	Neutralization of PAI-1 function	Research	[105]
PAI-1	Alpaca	V _H H-s-a93	Vicinity of reactive center loop	Interferes with initial PAI-1/PA complex formation	Research	[105]
PAI-1	Alpaca	V _H H-2g-42 (Nb42)	Vicinity of reactive center loop	Interferes with initial PAI-1/PA complex formation	Research /Therapy	[60] [105]
PN-1	Synthetic library	KB-PN1-A08, -B11, -F06, -H12	Reactive center loop	Neutralization of human and murine PN-1 function	Research	[106]
hTAFI	Alpaca	V _H H-TAFI-i and V _H H-TAFI-a series	None defined	Interfere with TAFI activation and/or activity	Research	[54] [55] [57]
hTAFI	Alpaca	V _H H-TAFI-a51 V _H H-TAFI-i103	None defined	Stimulate activity of TAFI zymogen		[56]
mTAFI	Alpaca	V _H H-TAFI-i49	Lys212 & Arg227	Stimulates activity of TAFI zymogen	Research	[107]
uPA	Alpaca	Nb4	Active site	Inhibits uPA activity	Research	[59]
uPA	Alpaca	Nb7	37s & 70s loops of the N-terminal β -barrel	Allosteric inhibitor of uPA activity	Research	[58]
uPA	Alpaca	Nb22	Active site	Inhibits uPA activity	Research	[58]
VWF	Llama	ALX-0081 (caplacizumab)	Human A1 domain	Inhibition of VWF/glycoprotein Ib α interaction	Therapy	[80] [82] [83]

VWF	Llama	KB-VWF-006bv	Human/murine A1 domain	Inhibition of VWF/glycoprotein Ib α interaction	Research	[63] [64]
VWF	Llama	AU-VWFa-11	Human/murine A1 domain	Specifically detects platelet-binding VWF conformation	Diagnostics	[66] [67] [69] [73] [74] [72]

PAI-1: plasminogen activator inhibitor-1; PN-1: protease nexin-1; hTAFI: Thrombin-activatable fibrinolysis inhibitor; mTAFI: murine TAFI; uPA: urokinase plasminogen activator; PA: Plasminogen activator; VWF: von Willebrand factor

Figure legends

Figure 1: Structural features of classical and camelid heavy chain antibodies

(A) In a conventional IgG, pairing of a variable heavy and variable light chains are required to assemble the paratope, while in camelid heavy chain antibody the V_HH domain does not require a light chain to bind the antigen. Inset: the secondary structure of heavy chain only antibodies showing three hyper-variable loop regions known as the loop H1, 2 and 3. **(B)** comparison of the antigen-binding surfaces of conventional IgG (left) and heavy chain only antibody (right). Adapted from Mitchel *et al.*[26]

Figure 2: Strategies for creation and selection of SdAbs libraries

SdAb diversity is obtained from B cells of naïve or immunized animal, or from synthetic DNA library. Expression of single sequences results in the presence of a unique sdAb variant at the surface of each individual host (typically phages, bacteria or yeast cells). Multiple rounds of selective pressure are applied to the host, resulting in the identification of strong antigen binders. Adapted from Hoogenboom [37]

Figure 3: Targets for sdAbs in the coagulation & fibrinolysis pathways

An overview of the major activation and inactivation reactions in the coagulation and fibrinolysis pathway are shown. Red circles indicate sdAbs and their targets that have been mentioned in this review.

APC: activated Protein C; α 2AP: α 2-antiplasmin; AT: antithrombin; F: factor; Plm: plasmin; Plg: plasminogen; PAI-1: plasminogen activator inhibitor-1; PC: protein C; PN1: Protease nexin-1; ProtS: protein S; ProtZ: protein Z; TAFI: thrombin-activatable fibrinolysis inhibitor; TM: thrombomodulin; TF: tissue factor; TFPI: tissue factor pathway inhibitor; tPA: tissue-type plasminogen activator; uPA: urokinase-type plasminogen activator; VWF: von Willebrand factor; ZPI: protein Z-dependent protease inhibitor.

Figure 4: Examples of potential applications of sdAbs in relation to thrombosis and hemostasis

The ease of manipulation of sdAbs makes them an ideal choice for the design of therapeutic and diagnostic tools. SdAbs can be incorporated in larger structure to participate in the gain-of-function of therapeutic compound, conjugated by cleavable or non-cleavable linker. Due to their small size (associated with a high tissue penetration) and fast clearance from the bloodstream, sdAbs are suitable for the design of imaging probes. This can be achieved by the direct conjugation of target-specific sdAbs with radionucleides, or by incorporating target-

specific sdAbs in nanoparticles. In the case of sdAbs with direct pro- or anti-coagulant properties, increasing their half-lives requires their fusion to other proteins such as albumin or albumin adaptors, or to the Fc domain of conventional IgGs. For example, bivalent constructs bridging a target to an albumin molecule would potentially increase the half-life of the target protein to that of albumin.







