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Single domain camel antibodies: current status

Serge Muyldermans*

Vrije Universiteit Brussel, Vlaams Interuniversitair Instituut voor Biotechnologie, Paardenstraat 65, B-1640 Sint Genesius Rode, Belgium

Abstract

The antigen-binding capacity of the paired variable domains of an antibody is well established. The observation that the isolated heavy chains of anti-hapten antibodies retain some antigen-binding capacity in the absence of light chains led to attempts to obtain an even smaller antigen-binding unit in a VH format. Unfortunately, the poor solubility, the reduced affinity for the antigen and the irreproducible outcome showed that additional protein engineering would be required to successfully generate single-domain antibody fragments. By serendipity, it was discovered that this engineering is already performed continuously in nature. Part of the humoral immune response of camels and llamas is based largely on heavy-chain antibodies where the light chain is totally absent. These unique antibody isotypes interact with the antigen by virtue of only one single variable domain, referred to as VHH. Despite the absence of the VH–VL combinatorial diversity, these heavy-chain antibodies exhibit a broad antigen-binding repertoire by enlarging their hypervariable regions. Methods are described to tap the VHH repertoire of an immunised dromedary or llama. These VHH libraries contain a high titre of intact antigen-specific binders that were matured in vivo. Synthetic libraries of a 'camelised' human VH, a mouse VH or a camelid VHH scaffold with a randomised CDR3 could constitute a valid alternative to immune libraries to retrieve useful single-domain antigen binders. The recombinant VHH that are selected from such libraries are well expressed, highly soluble in aqueous environments and very robust. Some in vivo matured VHH were also shown to be potent enzyme inhibitors, and the low complexity of the antigen-binding site is an asset in the design of peptide mimetics. Because of their smaller size and the above properties, the VHH clearly offer added-value over conventional antibody fragments. They are expected to open perspectives as enzyme inhibitors and intrabodies, as modular building units for multivalent or multifunctional constructs, or as immuno-adsorbents and detection units in biosensors.

Keywords: Camels; Heavy-chain antibodies; Llama; Molecular recognition units; Single-domain antibody; Heavy chain variable domain of conventional antibodies, VH

Abbreviations: cAb, camel single-domain antibody; CD, circular dichroism; CDR, complementarity determining region; Fv, variable fragments of conventional antibodies; HCAb, heavy-chain antibodies; scFv, single chain variable fragments; VH, heavy chain variable domain of conventional antibodies; VHH, variable domain of heavy chain of heavy-chain antibody; VL, variable domain of light chain

* Corresponding author. Fax: +32-2359-0289.

E-mail address: svmuylde@vub.ac.be (S. Muyldermans).

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1. Introduction

Köhler and Milstein (1975) introduced the monoclonal antibody technology to immortalise mouse cell lines that secrete a single type of antibody with a unique antigen specificity. This so-called hybridoma technology allows the isolation and production of monoclonal antibodies to protein, carbohydrate, nucleic acids and hapten antigens. Besides the importance as research tools, the availability of murine monoclonal antibodies opened the way to the development of diagnostics and human therapeutics. However, the application of monoclonal antibodies on a wide scale suffered from a number of technical limitations and drawbacks such as: expensive production methods based on mammalian expression systems; inability to optimise the antibody using genetic engineering techniques; and the potential immunogenicity of the mouse antibody when administered to humans (Kuus-Reichel et al., 1994).

To circumvent the latter limitation, methods were developed to generate:

1. chimeric antibodies that combine murine variable regions with the human constant regions (Boulianne et al., 1984);
2. humanised antibodies that are fully human antibody scaffolds except for their murine antigen-binding CDRs (Riechmann et al., 1988);
3. or veneered antibodies that are non-human antibody fragments in which the exposed residues in the framework regions are replaced to match the human residues at those positions (Padlan, 1991).

Of the human therapeutic monoclonal antibody products on the market, one is completely rodent (Orthoclone-OKT3, Johnson and Johnson), four are chimeric (rodent VL and VH, human constant domains: ReoPro, Centocor; Ritunax, IDEC; Simulect, Novartis; Remicade, Centocor) and three are humanised (all constant and framework regions are human, only the CDR-regions are from the original rodent antibody: Zenapax, Protein Design Labs; Synagis, MedImmune; Herceptin, Genentech; Mylotarg, Wyeth). Evidently, these examples are testimony to the therapeutic potential of these murine monoclonal antibodies and their derivatives. The generation of true human hybridomas appeared to be more difficult than first anticipated. Nevertheless, the generation of transgenic mice carrying large parts of the human immunoglobulin loci seems to generate promising human monoclonal antibodies (Green et al., 1994; Harding and Lonberg, 1995; Brüggemann and Neuberger, 1996), although they are still matured in a mouse environment and could, therefore, still contain human T-cell epitopes.

The expression of the antibody genes in bacteria should lower the production cost and facilitate the genetic engineering to optimise the antibody's performance. However, the first trials to produce antibodies in bacteria yielded disappointing low levels of functional molecules (Boss et al., 1984; Cabilly et al., 1984). More recently, gene technology has succeeded in the cloning and engineering of smaller fragments of antibody genes (Winter and Milstein, 1991; Winter et al., 1994). Certain antibody fragments such as Fab and Fv (non-covalently associated heterodimers of VH and VL domains) have been successfully expressed in bacteria (Skerra and Plückthun, 1988; Better et al., 1988), yeast, or fungi (Frenken et al., 1998). In addition, protein engineering can tailor the functionality and physico-chemical properties of the fragments leading to improved stability, reduced immunogenicity, better tissue distribution and faster blood clearance when used in vivo (Wu et al., 1996).

Despite this progress, major technical hurdles remained in implementing the technology on an industrial scale. For instance, stable Fv fragments have to be engineered by connecting the domains with a hydrophilic and flexible peptide linker to create a single-chain Fv fragment (scFv, Fig. 1, Huston et al., 1988; Bird et al., 1989), because the native Fv fragment dissociates upon dilution. Unfortunately, these scFv molecules have a reduced affinity compared to the parent antibody (Borrebaeck et al., 1992; Mallender et al., 1996; Glockshuber et al., 1990), and the linker sequences

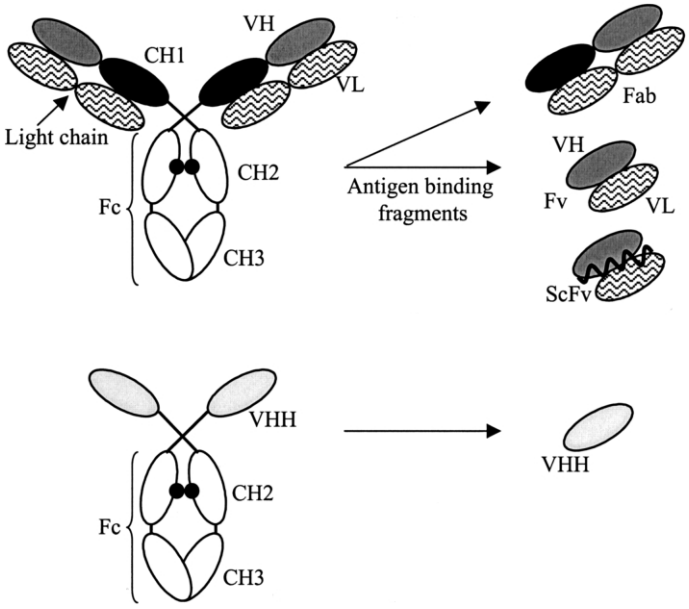

Fig. 1. Schematic illustration of the conventional (top) and heavy-chain IgG antibodies (bottom) present in camelid serum. The entire light chain (curved lines) and CH1 domain (black) are absent in HCAb. The antigen-binding domains of conventional antibodies obtained after proteolysis (Fab) or after cloning, and expression of the gene VH and VL fragments are shown. A synthetic linker introduced between the VH and VL stabilises the VH–VL dimer and forms the scFv. The recombinant VHH, the variable domain of a heavy chain of HCAb is obtained after cloning and expression. The VHH is the minimal intact antigen-binding fragment that can be generated.

frequently lead to aggregation of scFv and are easily degraded by proteolysis (Whitlow et al., 1993).

Antigen-binding fragments comprising the single variable domain (VH) of the conventional heavy chains have also been generated in the past (Ward et al., 1989). In contrast to the VL domains, such VH domains often retain the antigen-specificity of the parental antibody since their CDR3 is the major contributor to antigen binding. However, removing the VL domain from a Fv exposes a large hydrophobic surface of the VH to the solvent (i.e. the former interaction site with the VL) so that the isolated VH molecules become 'sticky' and are, therefore, difficult to produce in a soluble form. Moreover, affinity drops by one to three orders of magnitude compared to a scFv (Borrebaeck et al., 1992). Consequently, it has not been possible to develop single-domain VH antibodies as a valid alternative of monoclonal antibodies.

We noticed that serum of camels, dromedaries and llamas contains a unique type of antibodies devoid of light chains (Fig. 1, Hamers-Casterman et al., 1993). The heavy chains of these so-called heavy-chain antibodies (HCAb) have a lower MW than their counterparts in conventional antibodies due to the absence of the first constant domain, the CH1. Since the light chain is missing, the heavy-chain antibodies should bind their antigen by one single domain, the variable domain of the heavy immunoglobulin chain, referred to as VHH, to distinguish it from classic VH (Muyldermans et al., 1994). As such, the single-domain VHH is the smallest available intact antigen-binding fragment (15 kDa) derived from a functional immunoglobulin.

The cloning of the VHH in phage display vectors, selection of antigen binders by panning and expression of selected VHH in bacteria offer an attractive alternative to obtain small molecular recognition units. The VHH obtained from an immunised dromedary or llama have a number of advantages compared to the Fab, Fv or scFv de-

rived from other mammals, because only one domain has to be cloned and expressed to generate an intact in vivo matured antigen-binding fragment, and because of the intrinsic characteristics of the VHH.

2. Results and discussion

2.1. Occurrence and composition of heavy chain antibodies

The concentration of IgG in dromedary and llama serum is approximately 5–10 mg/ml. In dromedaries, approximately half of the natural serum IgG repertoire consists of heavy-chain antibodies (Hamers-Casterman et al., 1993), llamas having a lower proportion of HCAb (25–45%) (van der Linden et al., 2000), and the remaining being conventional antibodies composed of two heavy and two light chains. From cDNA analysis, we infer the presence of at least two conventional IgG isotypes and three or four heavy-chain IgG isotypes in dromedary and llama, respectively (Nguyen, Vu and Harmsen et al., 2000).

The presence of functional HCAb in serum has not been found in any other mammals besides camelids, although non-functional heavy-chain antibodies, lacking the light chain and undefined parts of the VH and CH1 are reported as a human pathological disorder named heavy-chain disease (Seligmann et al., 1979).

The heavy chains of the dromedary or llama HCAb are composed of a variable domain (VHH) immediately followed by a hinge, the CH2 and the CH3 domain (Fig. 1). The cDNA sequence analysis illustrated that the equivalent of the CH1 domain between the VH and the hinge is clearly missing. Recently, the molecular basis for the absence of the CH1 in the HCAb was assessed by determining the entire dromedary γ2a constant gene and two llama IgG isotypes obtained from genomic libraries. In all three cases it was found that the donor splice site flanking the CH1 exon is mutated (Nguyen et al., 1999; Woolven et al., 1999). It therefore seems plausible that these point mutations at the 3′ end of the CH1 exon are responsible for the selective removal of the CH1 coding sequences during the splicing of the mRNA of the dromedary and llama HCAb. The fact that both llamas and dromedaries utilise a common mechanism to eliminate the CH1 domain indicates that the HCAb emerged in a common ancestor before these species diverged.

2.2. Differences between VH and VHH

2.2.1. Sequence difference

The dromedary and llama VHH sequences (obtained from cDNA libraries and RT-PCR clones) belong to a single gene family, namely family three. It is unlikely that dromedaries possess other VHH gene families, as a representative database of germline VHH sequences revealed the presence of some 40 different VHH genes that all evolved within the VH subgroup III (Nguyen et al., 2000).

The comparison of the human VH3 gene family with the camelid VHH shows a high degree of homology as expected for genes of the same family. However, shortly after the initial observation of the presence of heavy-chain antibodies in camelids it was noted that the VHH carry a number of remarkable amino acid substitutions in the framework-two region (Fig. 2). The VH residues in this region normally interact with the VL domain and are well conserved throughout evolution (Kabat et al., 1991). Four amino acid substitutions were reported in the framework-two region in a comparison of VH and VHH (Muyldermans et al., 1994; Vu et al., 1997). Overall, the V37F or V37Y, the G44E, L45R and W47G substitutions (in going from VH to VHH) render this side of the domain more hydrophilic, presumably lowering VHH's propensity to form a heterodimer with a VL domain. Riechmann's group (Davies and Riechmann, 1994) analysed the effect of three of these mutations (G44E, L45R, W47G) on a human VH3 expressed as an isolated domain in *E. coli*. Whereas the original isolated human VH aggregated at protein concentrations above 1 mg/ml, the aggregation of the camelised domain was significantly reduced. It is therefore possible to create a human single VH domain that behaves well in solution by mutating

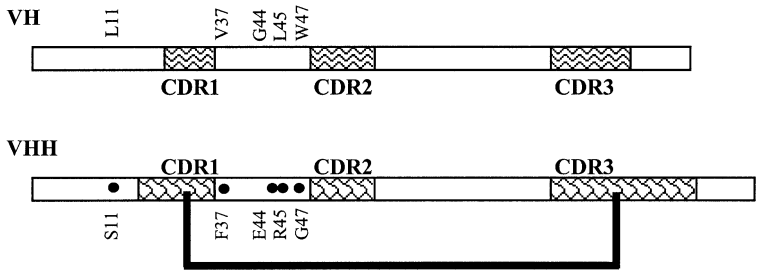

Fig. 2. Schematic representation of the differences between VH and VHH based on the sequence comparison of cDNA clones. The position of the CDR in between the framework regions is indicated. The CDR1 and CDR3 of a VHH is larger than in VH genes, and they are often connected by a disulfide bond (thick line). The hallmark amino acid substitutions in framework-one and -two are given. The numbering refers to the position of the amino acid along the sequence according to the Kabat numbering (Kabat et al., 1991).

a very limited number of residues involved in the 'former' VL interaction to mimic those residues present in dromedary VHH.

Additional, conserved substitutions occur in the scaffold or framework (FR) of camelid VHH, e.g. P14A and A83P. The amino acid substitution of the conserved L11 into S11, originally reported to occur in dromedary VHH, is less well maintained in llama VHH sequences (Vu et al., 1997). A number of VHH germline sequences also do not carry this substitution (Nguyen et al., 2000). In a VH, these residues (L11, P14, A83) are located at the antipode of the antigen-binding site and, therefore, can contact the CH1 domain (Padlan, 1994). The L11 is part of the ball-and-socket joint between the VH and CH1 (Lesk and Chothia, 1988). The absence of a CH1 domain in a HCAb suggest that the amino acids located in this part of the VHH will be solvent exposed in the intact antibody molecule. Consequently, it is logical to propose that the increase in hydrophilicity provoked by the L11S mutation would help in keeping the VHH domain soluble (Muyldermans et al., 1994; Vu et al., 1997; Nieba et al., 1997).

Besides the VH/VHH hallmark substitutions in the framework regions, the hypervariable regions of the camelid VHH seem to differ from the VH in two respects (Fig. 2). First, the hypervariable regions of VHH are on average longer than those of VH. The average CDR3 length in human and mouse VH is 12 and nine amino acids, respectively (Wu et al., 1993), whereas in dromedary VHH a length of 16–18 amino acids is most frequently observed, although in the llama a considerable fraction of the VHH seems to have a much shorter CDR of approximately six amino acids (Vu et al., 1997; Harmsen et al., 2000). In addition, the first hypervariable region that normally involves residues 31–35 in all VH (Kabat et al., 1991) is enlarged to encompass residues 27–35 in dromedary and llama VHH (Fig. 4). The VHH-specific, hypervariable residues 27–30 form the loop connecting the two β-sheets of the immunoglobulin fold, and are solvent exposed (Padlan, 1994, 1996). Their hypervariability suggests that these residues are contacting the antigen and that the somatic mutations in this area will be selected during the affinity maturation. The presence of hotspots for somatic mutation in the codons 27 and 29 in the germline VHH sequences, but not in the VH sequences, of dromedary supports the importance of this region of a VHH in antigen binding (Nguyen et al., 2000). Furthermore, the long CDR3 is most often connected by a disulfide bond to the CDR1 (Fig. 2). Sometimes, in the dromedary VHH, the disulfide bond is made between the CDR3 and a Cys45 of framework-two, whereas in llama VHH, we observed the possibility to form an interloop disulfide bond between CDR3 and a Cys50 within the CDR2 (Vu et al., 1997).

It is tentatively hypothesised that the larger hypervariable regions of the VHH will enlarge the actual surface of the antigen binding site and that this might compensate for the absence of the antigen-binding surface area provided by the VL

domain in Fv. The presence of the interloop disulfide bond will restrict the conformational flexibility of the long CDR3 loop in the antigen-free form so that immobilising the loop upon binding antigen will minimise the entropic penalty.

2.2.2. Structural differences

The crystallographic structure of four VHH (Desmyter et al., 1996; Decanniere et al., 1999; Spinelli et al., 1996, 2000), and the NMR structure of the camelised human VH (Riechmann, 1996) have been reported. A fifth crystal structure is available in the pdb under accession number 1bzq; (Desmyter et al., submitted). See Fig. 3 for three of these VHH-antigen complexes. All these structures confirm that the scaffold architecture of the VH and the VHH are identical. Nine β-strands are folded in two sheets that pack against each other and are stabilised by a conserved disulfide bond. The root mean square deviation of the framework-Cα atoms between a VHH and a human VH domain is approximately 0.75 Å (Decanniere et al., 2000, Desmyter et al., submitted). However, the surface formed by the framework-two residues of the VHH is reshaped into a much more hydrophilic region as expected from the nature of the amino acid replacements in this part of the molecule (Spinelli et al., 1996). This effect is even amplified by the rotation of the adjacent hydrophobic side-chains to expose their most hydrophilic parts to the solvent (Desmyter et al., 1996).

Unexpectedly from the sequence, the largest structural difference between a VH and VHH is to be found at the hypervariable regions. In human and mouse VH, these loops are folded in a limited number of structures, known as canonical structures (Chothia et al., 1989, 1992; Chothia and Lesk, 1987). In contrast, the antigen-binding loop structures within the VHH possess apparently many deviations on these canonical structures that could not have been easily predicted from their sequence. This does not imply that the first hypervariable loop of the VHH is unable to adopt a canonical type-1 structure. A genuine type-1 loop is formed at the first hypervariable loop of an anti-carbonic anhydrase VHH, both in the free antibody fragment, as in its complex with antigen (Decanniere et al., 2000; Desmyter et al., submitted). In contrast, none of the four published VHH structures solved by crystallography have a first hypervariable loop adopting the pre-

Fig. 3. Ribbon representation of the X-ray structure of cAb-Lys3 (left), cAb-RN05 (middle) and cAb-CA05 (right) in complex with their respective antigens, lysozyme, RNase A, or carbonic anhydrase. The VHH is shown in the lower part of the complex and the antigen is on top.

dicted canonical structure. A new canonical structure named type-4 was even proposed because the first hypervariable loop of two VHH shared the same Cα conformation (Decanniere et al., 1999, 2000).

The architecture of the second hypervariable loop of VHH also appears to be more variable than in human or mouse VH (Decanniere et al., 2000). The high incidence of amino acid insertions or deletions in, or adjacent to, the first and second antigen-binding loops of VHH will undoubtedly diversify, even further, the possible antigen-binding loop conformations (Nguyen et al., 2000).

The prediction of the third hypervariable loop structure of VH has been a bottleneck for a long time, although much progress was made in recent years (Al-Lazikani et al., 1997; Morea et al., 1998; Shirai et al., 1996, 1999). Unfortunately, the new algorithms to predict the structure of the third hypervariable loop of VH cannot be applied to predict the CDR3 structure of VHH. With the exception of the llama VHH against human chorionic gonadotropin hormone, that has an unusually short CDR3 loop, the VHH with known structure have a tendency to turn their CDR3 loop outwards from the β-barrel, towards the region that would interact with the VL in a Fv structure. Therefore, the location of the long CDR3 within the VHH is incompatible with the association of a VL.

In summary, the structural analyses provide evidence that the antigen-binding loops of VHH exhibit a much larger structural repertoire than observed for VH. It is possible that the absence of the VL generates the additional freedom that makes these changes possible.

2.3. Generation of HCAb in camelids

As discussed above, a satisfactory explanation for the removal of the CH1 domain in HCAb was recently proposed (see Section 1; Nguyen et al., 1999; Woolven et al., 1999), but many other aspects of the HCAb generation remain in a state of uncertainty.

We do know that the VHH gene segments (and the VH as well) including a functional promoter sequence and recombination signal sequences, are embedded in the dromedary germline genome (Nguyen et al., 1998). Thus, the framework-two specific hallmarks of a VHH are imprinted in the dromedary genome, and are not introduced in a VH gene by a directed somatic hypermutation mechanism. Moreover, none of the VH germline segments encode a Cys in their CDR regions. In contrast, the VHH germline gene segments have the Cys in the CDR1 or at position 45, available to form, after a productive DNA recombination, a disulfide bond with a Cys in the CDR3. The longer CDR3 in VHH compared to VH is puzzling, especially since they both seem to employ the same D and J segments (Nguyen et al., 2000). It might be that the longer CDR3 in VHH is introduced by joining two D segments, or by an increased activity of terminal deoxynucleotidyl transferase during the VHH–D–J recombination. Moreover, the CDR3 of VHH often possesses a Cys, a feature that is absent in dromedary VH. The introduction of a Cys in the CDR3 of the VHH could, therefore, be due to the selection of an alternative reading frame of the D minigene or the use of alternative VHH–D–J junctions, or by the P or N nucleotide addition during the DNA recombination event.

High proportions ($\sim 30\%$) of the cDNA clones encoding VHH sequences have aberrant sizes for their CDR1 or CDR2 region. Germline VHH segments with off-sized lengths were not retrieved from the dromedary genome. Therefore, these VHH result probably from a gene conversion mechanism (Reynaud et al., 1989), although a somatic mutation mechanism (Wilson et al., 1998) cannot be formally excluded because the insertion/deletions are nearly always found adjacent to particular DNA signal sequences such as palindromes, or truncated recombination signal sequences (Nguyen et al., 2000). In any case, the off-sized loop regions of the VHH will increase the potential structural diversity of the VHH domains.

Remarkably, the codons 27 and 29, immediately upstream of the CDR1 region (CDR1 as defined by Kabat et al. (1991)), are mutated in the VHH. In principle, these germline mutations leading to the F27Y and F29Y amino acid substi-

tution are expected to be silent at the structural level (Al-Lazikani et al., 1997; Chothia et al., 1992). However, in contrast to the TTY codon for Phe, the TAY nucleotide triplet coding for Tyr is known as a hotspot for somatic hypermutation (Milstein et al., 1998). In line with this, is the observation that the amino acids at position 27 and 29 become much more variable in VHH compared to the corresponding amino acids in VH domains (Fig. 4). Hence, the CDR1 region of a VH that encompasses the amino acids 31–35 (Kabat et al., 1991) is extended in VHH to include amino acids 27–35. The whole region has a solvent exposed location and the variability in this region is expected to have two consequences. First, the mutations are most likely involved in the reshaping of the loop structure, and secondly, some of the amino acid mutations might provoke subtle surface modification that might improve the VHH–antigen fit. The crystal structures of the VHH in complex with lysozyme and RNase A confirm these expectations. The loop conformation in the CDR1 region adopts one of several possible folds (Decanniere et al., 2000), and the amino acids 27 and 29 participate actively in the recognition process of RNase A and lysozyme, respectively (Desmyter et al., 1996; Decanniere et al., 1999).

In summary, it is proposed that this frequent occurrence of 'off-sized' CDR1 and CDR2 loops and the somatic hypermutations in the CDR1 region, leading to an extended hypervariable loop region, will add to the diversity of the VHH domains. This might explain how the VHH single-domains can exhibit such a broad antigen-binding repertoire in the absence of the VH–VL combinatorial diversity.

The productive recombination product of VHH–D–J minigenes is expected to be expressed as part of a μ-chain (Tonegawa, 1983; Rajewsky, 1993). However, the co-expression of VHH with the μ constant gene is difficult to prove. At the gene level, it is difficult to amplify the VHH-μ sequences using RT-PCR, so that the few clones that are obtained might result from an artefactual PCR event (e.g. PCR cross-over). At the protein level, the production in dromedaries or llamas of IgM-like molecules containing a VHH domain

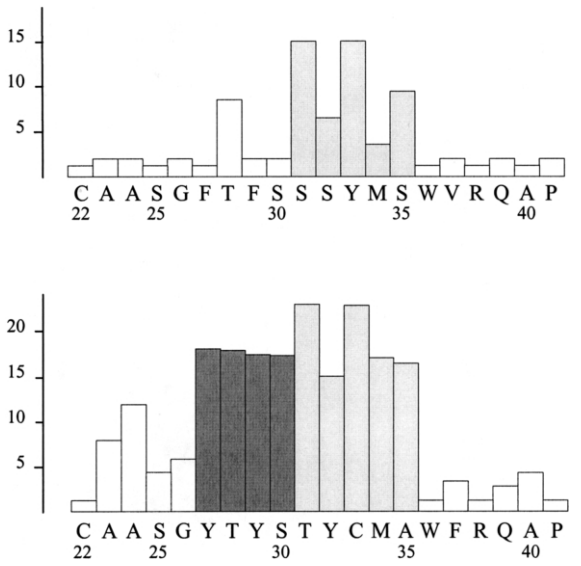

Fig. 4. Variability plot of dromedary VH (top) and VHH (bottom) from amino acid 22–41. The white bars are framework residues. The CDR1 as defined by Kabat et al. (1991) are shown in grey, while the extended hypervariable region outside the conventional CDR1, and unique for camelid VHH, is in dark grey. The variability at each position is calculated as the number of different amino acids occurring at that position divided by the frequency of occurrence of the most frequent amino acid at that position. Both databases were obtained from shotgun cloning of VH and VHH cDNA sequences, and some 20 clones of each were taken into account. The most frequent amino acid at each position for this database is given.

but without light chains remains elusive. It is difficult to envisage that VHH-μ chains containing a CH1 domain could be secreted since chaperone proteins recognising the CH1 domain need to be replaced by the light chain before secretion from the endoplasmic reticulum can proceed. Under the assumption that the constant domain of the light chain replaces the BiP chaperone and associates with the CH1 domain of the μ polypeptide (Henderschot, 1987), then the VL domain still cannot associate with a VHH domain due to the reshaped framework-two region and interference with the long CDR3 of the VHH. In contrast, there is no indication for the generation of μ polypeptide chains without CH1 domains, although such IgM-like molecules with a VHH, devoid of CH1 and light chains, could theoretically be secreted from the endoplasmic reticulum.

Since the occurrence of IgM-like molecules with a VHH in dromedaries remains uncertain, it is evident that the location and time of the antigen-binding site maturation of the VHH and concomitant $\mu-\gamma$ switch to produce the IgG-type HCAb remains purely speculative.

2.4. Immunising dromedaries or llamas

Dromedaries and llamas are easily immunised following standard procedures (Murphy et al., 1989). Immunisation with as low as 50 μg immunogen in complete and incomplete Freunds adjuvant generates antigen-specific HCAb of high affinity after 5–6 weekly boosts (Lauwereys et al., 1998). It is feasible to immunise an animal simultaneously with a mixture of several antigens and to raise an immune response to each antigen separately, making the whole procedure more cost effective (Lauwereys et al., 1998; van der Linden et al., 2000).

The HCAb repertoire of an immunised dromedary or llama displays a recognition pattern different to that of conventional IgGs. The antigen-specific serum titres obtained for proteinaceous antigens are consistently higher than those obtained for haptens. Surprisingly, the immunisation of a llama with haptens coupled to a BSA carrier lead to a higher relative proportion of HCAb against the hapten than to the carrier (van der Linden et al., 2000). The same authors also found that the immunisation of a llama with a *S. mutants* lysate resulted in a recognition pattern that differed profoundly between the HCAb and the conventional antibodies. The proteins with a MW between 60 000 and 80 000 were bound preferentially by HCAb, although overall the HCAb recognised a smaller number of proteins than the conventional antibodies.

The finding, that VHH from the dromedary HCAb against enzymes are potent competitive inhibitors, was another unexpected observation (Lauwereys et al., 1998). This was shown by two complementary experiments. In the first approach it was demonstrated that approximately half of the HCAb against model enzymes such as α-amylase and carbonic anhydrase, can be displaced by adding a large excess of small competitive inhibitors like acarbose and dorzolamide, respectively. In contrast, conventional antibodies failed to be displaced by these drugs. In the second experimental set-up, it was illustrated that the monomeric VHH prepared from HCAb, from a dromedary immunised with α-amylase, could block the enzymatic cleavage of 2-chloro-4-nitrophenyl-maltotriose, a substrate for α-amylase. These experiments illustrated the feasibility to immunise a dromedary with enzymes to generate large quantities of HCAb that react with the enzymatic active site.

2.5. Preparing antigen-specific VHH

In principle, the antigen-specific VHH can be obtained by protease digestion of HCAb, by direct cloning of the VHH genes from B-cells, and from 'single-pot' naïve or synthetic libraries.

2.5.1. VHH from proteolysed HCAb

The availability of polyclonal VHH and VHH_2 obtained after the proteolysis of the HCAb from an immunised dromedary or llama could prove valuable to develop single-domain based immuno-reagents.

Polyclonal VHH domains, of which measurable amounts could recognise the antigen α-amylase, had previously been prepared from the IgG3 fraction of an immunised dromedary (Lauwereys et al., 1998). The HCAb isotype IgG3 was purified by protein G chromatography from the serum that contained, per ml, 0.1 mg polyclonal HCAb with specificity for α-amylase. The purified IgG3 fraction was then digested under limiting conditions with endo-Glu V8 protease that cleaves the short hinge region between the VHH and the CH2 (Hamers-Casterman, personal communication). The chromatography on Protein A of the proteolysed sample retains the Fc containing fragments (and a subfraction of the dromedary VHH), whereas the flow-through contains the majority of the VHH, of which a large proportion recognised the antigen. This method might be less applicable for llama VHH because the IgG3 titre is lower in the llama compared to the dromedary (van der Linden et al., 2000), and, in contrast to the dromedary VHH, a large fraction of the llama

VHH seems to bind protein A, so that different purification schemes should be devised.

The dromedary HCAb IgG3 isotype was also cleaved by pepsin, trypsin, or papain (Hamers-Casterman, personal communication). These proteases attack the hinge region at various positions, but mainly after the first disulfide bond that links the γ3 heavy chains. The cleavage therefore generates dimeric VHH that could be purified by passing the flow-through fraction of a protein A chromatography over a gel filtration column of Sephacryl R200.

2.5.2. Recombinant VHH

2.5.2.1. VHH repertoire cloning. Cloning the repertoire of antigen-binding fragments from an immunised animal into a phage display vector and selection of antigen-specific clones by panning became a routine method in the past decade to identify antigen-specific molecules (Winter et al., 1994; Hoogenboom et al., 1998). This powerful method is straightforward to adapt for VHH, and the single-domain nature of the VHH simplifies the effort considerably. Indeed, the repertoire cloning of conventional antibodies, e.g. in the form of scFv, involves several steps. First, two independent PCR are needed to amplify the VH and VL. In mouse (and humans as well) this PCR is complicated by the existence of multiple gene VH and VL families, where each family requires its own set of primers. Secondly, these fragments have to be joined randomly into one construct by a linker segment, either by a splicing of overlap extension or by a two-step cloning strategy. Therefore, 10^5 VH and 10^5 VL fragments amplified from 10^5 B-cells requires the cloning of 10^{10} clones to obtain all possible combinations. Admittedly, since the antigen-binding VH domains exhibit a promiscuous binding to several 'neutral' VL domains (and vice versa), there is no absolute requirement to construct the original pairs that were matured in vivo for the antigen. However, scrambling the VH and VL populations and the presence of artificial VH–VL combinations with decreased affinity implies that large libraries need to be screened in order to isolate the native combinations, and/or that many binders with sub-optimal affinity and stability will be obtained. Therefore, it seems rather counter-productive to first immunise an animal in order to optimise and mature the antibodies for the target antigen, and then to split the antigen-binding partners and to scramble them in the following step.

Tapping the antigen-binding repertoire of the HCAb of an immunised dromedary or llama is less complicated, since the intact antigen-binding fragment is encoded in a single gene fragment (the VHH). We normally start from the peripheral blood lymphocytes, isolated from an immunised dromedary or llama to prepare cDNA (Fig. 5). It was established that 20–30 ml of an immunised dromedary (containing ~20 l blood) contains sufficient B-cells that produce antigen-specific VHH.

All VHH are encoded by a single exon with homologous border sequences because they belong to one single gene family. Therefore, the complete in vivo matured VHH repertoire of an immunised animal can be amplified by one single set of primers and can be cloned immediately.

Although the amplification of the VHH repertoire is simple, the method is complicated by the requirement to avoid the cloning of camelid VH originating from the conventional antibodies. The presence of VH genes in the VHH gene library might complicate subsequent pannings due to their possible sticky behaviour (Davies and Riechmann, 1995). Two methods are in use to eliminate the VH gene fragments from the VHH pool. Both methods are based on the observation that a VH is never found within a HCAb, and on the inherent differences between the heavy chain of conventional antibodies and HCAb. The UNILEVER group (van der Linden et al., 2000) makes use of PCR primers that anneal selectively on the hinge of the HCAb. This region harbours the largest sequence differences between the different isotypes, and therefore primers can be designed that will only amplify the VHH genes (Fig. 5, procedure A). In our approach (Fig. 5, procedure B), we first employ a pan-annealing primer that binds to a conserved region of all constant γ genes in combination with a back primer that anneals at the leader signal sequence of the VH and VHH, so that all IgG isotypes will

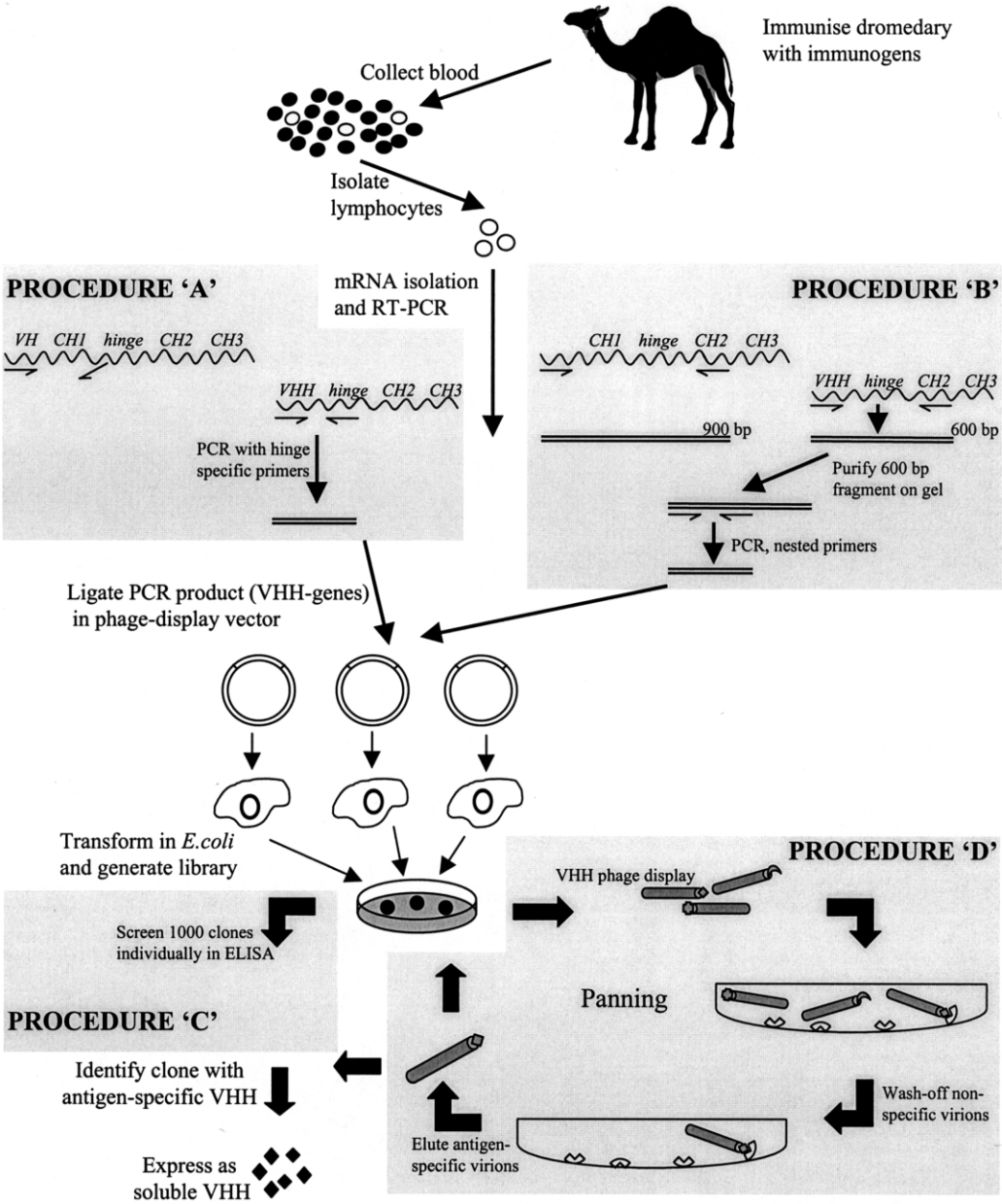

Fig. 5. Schematic overview of strategies to clone and select the VHH genes from an immunised dromedary (or llama). *See text for additional explanation.

be amplified towards their VH and VHH end. This primary PCR yields two kinds of fragments: one kind contains the CH1 exon and is derived from the heavy chain of a conventional antibody, and a second kind of fragment lacking the CH1 exon is derived from the heavy chain of the HCAb. These PCR fragments are easily separated on agarose gel and the recovery of the shorter fragment eliminates the VH sequences efficiently. A secondary PCR with nested primers

annealing at the beginning and end of the VHH is then performed to make more material and to include restriction enzyme sites for cloning purposes (Fig. 5, procedure B).

After cloning the amplified VHH genes in the appropriate expression vectors, a VHH library containing the repertoire of the intact in vivo matured antigen binding sites is obtained (Ghahroudi et al., 1997). In contrast to scFv libraries where relatively large libraries are required, small libraries of only 10^6–10^7 VHH individual clones have routinely resulted in the isolation of single-domain proteins with nanomolar affinity for their antigen.

2.5.2.2. Selecting the antigen-specific VHH. The VHH libraries can be screened for the presence of antigen-specific binders, either by panning or by direct colony screening. Indeed, the B-cells expressing antigen-specific VHH circulating in blood of an immunised dromedary can easily reach ~1% of the total B cell population. Therefore, the direct testing of 1000 individual clones of the VHH library is expected to yield perhaps 10 antigen binders. The colony screening with labelled antigen, as performed in the late 1980s (Huse et al., 1989), or screening of the induced cultures in ELISA is certainly feasible for the VHH libraries (Fig. 5, procedure C). However, retrieving the binders by panning is to be preferred (Fig. 5, procedure D) to the screening of individual colonies (procedure C), because the panning will also select for those binders with the highest affinities, and those that express better in bacteria. For panning, the VHH genes are cloned at the 3′ end of the minor coat gene 3 protein of filamentous phages. Phage particles can then be produced that possess the VHH–g3p fusion protein at their tip and the corresponding VHH gene encoded in the encapsulated phage or phagemid genome. VHH can therefore be selected, for several sequential rounds if needed, through panning of phage particles on immobilised antigen. Antigen bound phage particles are eluted by pH-shock and regrown through infection of bacteria. Soluble VHH can be produced after either subcloning into a soluble expression vector or through the use of bacterial non-suppressor strains and appropriate stop codons between the VHH fusion and the phage g3p (Hoogenboom et al., 1991).

The whole procedure, from the first immunisation to the cloning, selection, expression and identification of recombinant VHH antibodies is routinely performed in less than 3 months. Once again the advantage of working with small libraries should be underlined. The artefacts often observed with large libraries [over-representation of phages/virions with incomplete scFv genes, or carrying additional stopcodons or reading frame shifts (de Bruin et al., 1999)] are sufficiently suppressed in the immunised dromedary or llama VHH libraries, having a higher proportion of binders vs. non-binder clones.

In summary, the possible selection procedures to obtain antigen-specific VHH benefit from the usage of small dedicated libraries from immunised animals that contain a high ratio of binder to non-binder clones due to the in vivo proliferation of B-cells carrying antigen-specific HCAb.

2.5.3. Naïve and synthetic libraries of camel VHH domains

Despite the success to retrieve good single-domain antigen binders in a short period of time, the method still suffers from the fact that antigen is required for the immunisations. Although the antigens used for immunisation should not be of the highest purity, it is often difficult to extract the antigens in sufficient amount for immunisation. To solve this shortcoming, methods were developed to generate large ‘single-pot’ scFv (or Fab) libraries. The variability in these libraries is either introduced by grafting synthetic, randomised regions on the scaffold [i.e. synthetic libraries (Marks et al., 1991; Nissim et al., 1994; Griffiths et al., 1994; Pini et al., 1998; Knappik et al., 2000)] or by scrambling large pools of VH and VL from non-immunised individuals [i.e. naïve libraries (Vaughan et al., 1996; de Haard et al., 1999)]. Such large ‘single pot’ libraries of synthetic or naïve scFv (or Fab) proved to be a suitable source to retrieve potent antigen-binders avoiding the immunisation procedure.

Similar synthetic phage or phagemid libraries containing a randomised CDR3 on a ‘camelised’ human VH or on a mouse VH were constructed

(Davies and Riechmann, 1995; Reiter et al., 1999). Repertoires of camelised VH domains or the mouse VH were initially created by randomisation of residues within the third hypervariable loop CDR3, which at the same time was varied in length in the case of the camelised VH format. This loop contains the highest diversity in length and amino acid nature of the three VH hypervariable loops within the variable domains of heavy chains from conventional antibodies, and of dromedary and llama HCAb as well. From such a repertoire of 2×10^8 clones, camelised VH domains specific for hapten, peptide and protein antigens were selected (Davies and Riechmann, 1995; Martin et al., 1997). The dissociation constants for these camelised human VH domains in their soluble form, and their respective antigens, were in the submicromolar range (Table 1). A subsequent affinity maturation for the camelised VH hapten binders leading to a 10-fold increase in affinity could be established by phage selection of camelised VH derivatives that contained secondary mutations within the other two hypervariable loops (Davies and Riechmann, 1996a). Particular attention concerns the possible multimerisation of the selected camelised VH domains because at least in one case (Martin et al., 1997), the selected camelised VH domains dimerised in solution. Obviously, this depends on the nature of the synthetic CDR3 loop of this camelised VH binder, as it is the only portion that differs from the other, monomeric VH domains in the original library.

More recently, a mouse VH with non-conventional K44 was used as a scaffold for the generation of a single-domain phagemid library (Reiter et al., 1999). VH with this unique VL side are apparently well expressed as inclusion bodies and are easily refolded afterwards to yield monomeric VH domains. Interestingly, this mouse VH sequence belongs to the VH family I, so it indicates that the single domain antibodies can also be formed on VH scaffolds outside the family III. The repertoire of 4×10^8 individual clones was screened by panning for TNF and immunoglobulin binders. The selected binders were highly antigen-specific, correctly folded and stable monomeric molecules, with an affinity of 20 nM in case of the immunoglobulin binder.

The preliminary successes of the synthetic sin-

Table 1
Affinity of single-domain antigen binders[a]

VHH	Antigen	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_d (nM)	CDR3 (AA)
cAb-Lys2	Lysozyme	9.0×10^4	1.8×10^{-4}	2	19
cAb-Lys3	Lysozyme	4.4×10^4	2.7×10^{-3}	65	24
cAb-CA04	Carbonic anhydrase	1.3×10^5	3.9×10^{-3}	29	18
cAb-CA05	Carbonic anhydrase	6.4×10^4	5.0×10^{-3}	72	19
cAb-RN05	RNase A	2.3×10^6	8.0×10^{-2}	35	12
cAb-AMD7	α-Amylase	1.6×10^6	2.4×10^{-3}	15	16
cAb-AMD9	α-Amylase	2.4×10^5	8.0×10^{-4}	3	14
cAb-LA01	β-Lactamase	1.5×10^5	$< 1.0 \times 10^{-4}$	< 0.7	21
cAb-LA02	β-Lactamase	4.8×10^5	7.0×10^{-4}	0.6	8
cAb-LA03	β-Lactamase	2.26×10^5	2.3×10^{-3}	2	17
R2	RR6 azo-dye	1.5×10^5	3.3×10^{-3}	22	16
VH-Ox21	OxGly			146	15
VH-OS1	OxGly			25	15
VH-REVg1	Rev			220	11
VH-LS2	Lysozyme			3100	10
VH-1	IgG	2.4×10^5	4.1×10^{-3}	19	11

[a] The cAb originated from dromedary (Lauwereys et al., 1998), the R2 is from llama (Spinelli et al., 2000), the VH-Ox, REV and LS are from the synthetic camelised human VH library (Riechmann and Muyldermans, 1999), and the VH-1 is retrieved from a synthetic mouse VH library (Reiter et al., 1999).

gle domain libraries are promising, although the isolation of binders against additional antigens is required to fully assess the validity of this approach. Nevertheless, from these early successes, it is expected that a dromedary or llama VHH scaffold could also be used to generate a synthetic library by randomising its CDR3. We propose that the cAb-Lys3 (Fig. 3 left, Desmyter et al., 1996) would constitute a perfect scaffold to randomise the protruding region of the CDR3. This part of the loop is particularly accessible to solvent and should be available for antigen interaction. However, it might turn out that a number of amino acids within this part of the loop, cannot be randomised as they are required to fix the conformation of the protruding part of the loop, e.g. the tyrosines occupying an inner location within the protruding part of the CDR3 loop of cAb-Lys3.

The selection of the cAb-RN05 (Fig. 3 middle, Decanniere et al., 1999) is an alternative scaffold to construct a synthetic VHH library. This scaffold has a short CDR3 loop of only 14 amino acids, that lies over the equivalent of the 'VL side' of the domain, such that a number of amino acids seems to be essential for interacting with the framework-two residues. The structural knowledge of the VHH scaffolds enables us to randomise only those residues with an outward facing orientation, thereby minimising the generation of VHH mutants that are unable to fold properly. This limitation in exchangeable amino acids is more an advantage than a problem since the CDR3 of VHH is sufficiently long to accommodate seven to eight randomised amino acids with an outward facing side-chain, so that libraries of over 10^9 individual clones are already required to obtain a repertoire that covers all possible amino acid combinations. The exclusive randomisation of the outward facing residues within the CDR3 region should lead to libraries with a higher proportion of potentially functional binders, so that the selection of antigen binders should be more successful.

We surmise that the use of the germline VHH, as scaffold into which a synthetic CDR3 is inserted, might be even preferred, because it is expected to possess more neutral CDR1 and CDR2 regions that have not been matured to have specificity for any particular antigen. In addition, the usage of germline VHH extended with a synthetic CDR3 allows us, in subsequent steps, to generate derivatives carrying mutations in the CDR2 and especially in the extended part of the CDR1 region, in order to improve the affinity of a selected binder for a particular antigen.

2.5.4. Immunised vs. synthetic VHH libraries

The availability of synthetic VHH libraries (or camelised human VH, or mouse VH) definitely offer a solution to identify antigen binders in cases where it is impossible to immunise (lack of antigen, antigen is toxic or not immunogenic). However, we feel that the synthetic VHH libraries might be inferior to the VHH libraries of immunised dromedary or llama. The present success rate in isolating binders from synthetic VH libraries, as inferred from the literature, needs to be confirmed. Moreover, since the binders obtained from naïve or synthetic libraries are not matured against the antigen, we expect that the affinities will be low (in the μM range) so that lengthy in vitro affinity maturation steps are required to improve on their binding capacity. This involves, again, the randomisation of some residues of the selected VHH and the construction of a second library that should also be as large as possible to meet with a fair chance of success. Therefore, in the end it might be much faster to use the potential of the dromedary immune system to simultaneously proliferate the antigen-binding cells and perform the affinity maturation, and then to generate a relative small, immunised VHH library.

2.6. Unique features of VHH domains

The single domain nature of VHH gives rise to several unique features as compared to antigen-binding derivatives of conventional antibodies. Besides the advantages of easy cloning (single gene) and selection from an in vivo matured library, the recombinant VHH have other techno-

logical, functional and physico-chemical advantages such as:

- the high expression yields and ease of purification;
- a highly soluble and stable, single-domain immunoglobulin fold;
- the generation of antigen-specific, high-affinity binders;
- the recognition of unique conformational epitopes with the dominant involvement of its long CDR3; and
- the close homology to human VH fragments.

2.6.1. High expression yields and purification of recombinant VHH

The expression of the recombinant VHH produces a single domain protein of only 15 kDa. This is half the size of the smallest intact, antigen-binding fragment that can be generated from conventional antibodies (Fv or scFv) (Fig. 1). The recombinant VHH contain (depending on the expression vector) a C-terminal His_6, c-myc or an haemaglutinin tag that are routinely used for detection in Western blots, or ELISA, by the appropriate specific antibodies.

In contrast to VH domains of conventional antibodies, the VHH domains of camel heavy-chain antibodies are expressed efficiently as soluble and non-aggregating recombinant proteins due to their unique hydrophilic substitutions in framework-two: V37F or V37Y; G44E; L45R or L45C; and W47, most often substituted by G (Fig. 2). Without any optimisation of conditions, recombinant camelid single domain antibodies are routinely obtained at levels of 5–10 mg/l when expressed in *E. coli* grown in shaking culture flasks. This is on average 10 times higher than most scFv constructs (Ghahroudi et al., 1997). With other expression systems it is even possible to obtain higher yields of VHH expression. Production levels of 9.3 mg/l/OD660 or $\sim$250 mg secreted protein per litre of *Saccharomyces* yeast culture in shake flasks could be obtained (Frenken et al., 2000). This suggests that in high cell-density fermentation, with ODs of over 100 (40–80 g dry weight per litre), a production level of over 1 g/l should be achievable. Of course, variations in the expression levels of different VHH are observed frequently, but so far no specific amino acid could be identified that would explain this observed expression variability. It is postulated that the screening of the VHH for the desired antigen-binding specificity should preferably be performed directly in the final production organism, to avoid the reduced expression yields upon changing the expression host. It would therefore, in this context be an advantage if one could develop VHH screening methods in yeast, in case this organism is selected as expression host.

In preliminary tests we also cloned the VHH in tobacco plants and estimated a functional VHH production yield of 0.5 μg/g leaf, corresponding to 0.1% of the total protein extract (K.B. Vu, personal communication). Thus, transgenic plants might be an economic alternative to produce extremely large amounts of VHH (Benvenuto et al., 1991).

Recombinant VHH can be purified following various procedures. The most versatile bacterial expression systems direct the expressed VHH towards the periplasm (Skerra, 1993). Proteins in this compartment are folded and the oxidising environment stimulates the disulfide bond formation, leading to functional VHH. A simple osmotic shock (Skerra and Plückthun, 1988) extracts the majority of the periplasmic proteins that are already highly enriched in VHH. Further purification is most conveniently achieved by IMAC (Immobilised Metal Affinity Chromatography) when the recombinant VHH are cloned with a His_6 C-terminal tag. Alternatively, the llama VHH or the 'camelised' human VH can be purified by Protein A chromatography. Most Fv possessing VH of subgroup III (Sasso et al., 1991), most llama VHH (van der Linden et al., 1999), the camelised human VH (Davies and Riechmann, 1995; Riechmann and Muyldermans, 1999), and a minor fraction of dromedary VHH (Ghahroudi et al., 1997) can all be adsorbed by Protein A containing supports. This purification scheme has the additional advantages that only the properly folded molecules are retained.

Finally, the production of VHH as inclusion bodies should also facilitate the purification be-

cause the unfolded VHH are readily folded into functional entities when denaturing agents are removed by dialysis or brought under the critical concentration by dilution.

2.6.2. Solubility and stability of recombinant VHH

The VH within conventional antibodies interacts through large hydrophobic areas with the VL and CH1 domains. Consequently, the separate expression of the VH domain leads to formation of inclusion bodies, or to a folded domain containing large hydrophobic patches on its surface that renders this polypeptide sticky. In contrast, the VHH domains are naturally soluble because of the substitutions of hydrophobic by hydrophilic residues in the framework-two region. It has been experimentally shown that replacing the hydrophobic framework-two residues in human conventional VH by the camelid specific sequences results in increased solubility and lack of aggregation (Davies and Riechmann, 1996b). In addition, the absence of engineered structures, such as linkers which may lead to aggregation (e.g. as observed for scFv), promotes the solubility of VHH, even at concentrations above 10 mg/ml.

The VHH tested so far are remarkably stable: three VHH retained 100% and one VHH retained 80% of its binding activity after a 200-h (1 week) incubation at 37°C (Ghahroudi et al., 1997). Therefore, it seems that the VHH will have a very good 'shelf-life'. Interestingly, the VHH with the slightly reduced shelf-life was the only one of the VHH tested that lacked an interloop disulfide bond. In addition, purified recombinant VHH recognising lysozyme or β-lactamase retained full antigen-binding activity after a 24-h incubation in mouse serum at 37°C (K. Conrath, personal communication). It is, therefore, expected that the VHH will also perform well in vivo.

VHH also exhibits a good thermal resistance. Davies and Riechmann (1995) followed the thermal denaturation of the camelised, human VH by CD and observed that the introduction of an interloop disulfide bond significantly improves the stability of the isolated domain. Melting temperatures (T_m) as high as 72°C were observed. Two out of six llama VHH directed against azo-dyes performed even better. These were able to bind antigen specifically at temperatures as high as 90°C, whereas all mouse monoclonal antibodies against the same antigen loose their functionality at this temperature (van der Linden et al., 1999). Therefore, at least some of the camelid VHH can for example be used in solutions that are pasteurised without losing the ability to interact with the antigen.

The resistance of VHH against the denaturing effect of chaotropic reagents (urea, guanidinium hydrochloride and ammonium isothiocyanate) was tested. The fully denatured state of the purified VHH could not always be obtained with urea, and the guanidinium denaturation confirmed the stable protein fold. The observation that the fully guanidinium-denatured VHH refolded immediately upon diluting the solution into water suggests that the VHH should easily refold from inclusion bodies. More importantly, the robust property against various denaturing conditions suggests that VHH might be extremely powerful as immuno-adsorbents (Berry and Davies, 1992) or could be used as detection probes in biosensors. The efficient refolding from strongly denaturing conditions further supports the possibility to sanitise and to regenerate the VHH multiple times.

2.6.3. Recombinant VHH are specific and bind their antigen with nanomolar affinity

The VHH isolated from the immunised libraries are highly specific for the target antigen, and do not cross-react with other non-related antigens (Ghahroudi et al., 1997). For example, the cAb-Lys3 that was retrieved by hen egg-white lysozyme, recognises the hen lysozyme and the turkey lysozyme that differs by only six conserved amino acids substitution, but no reaction was observed with the more distantly related human lysozyme. VHH could also be obtained that discriminate the surface antigen of different hepatitis serotypes (M. Lauwereys, personal communication).

We routinely measure the affinity of the purified, monomeric antigen binders selected from the immunised libraries (Table 1). The determination of the kinetic rate constants k_{on} and k_{off} by resonant mirror (IAsys, Fisons) yielded values

between 10^4–10^6 M^{-1} s^{-1} and 10^{-2}–10^{-4} s^{-1}, respectively. This results in K_d in the nanomolar range (Muyldermans and Lauwereys, 1999). Affinities in the same range were also observed for the binder to the azo-dye hapten, RR6 (Spinelli et al., 2000). Thus, the affinity of the selected VHH domains for their respective antigen is similar to the affinity of monomeric scFv fragments derived from monoclonal antibodies. Although the synthetic camelised human VHH library might be much better for isolating model hapten binders such as phenyloxazolon, we see from Table 1 that the antigen affinities of the VHH from the immunised libraries are 100–1000 times better than the camelised human VHH against the same model proteinaceous antigens. Similarly, we expect that the VHH from immunised libraries will be 10–100 times better than most scFv isolated from naïve or synthetic libraries. However, these latter libraries can sometimes yield highly specific binders with affinities in the subnanomolar range (Vaughan et al., 1996).

2.6.4. Dromedary VHH recognise unique conformational epitopes

The antigen-binding surface of conventional antibodies forms a flat or concave surface, depending on the antigen, being a protein or hapten, respectively (Webster et al., 1994; Padlan, 1996). Since large exposed loops are normally not encountered in conventional antibodies, these antibodies cannot bind into large cavities on the antigen surface. However, grooves and cavities play a critical role in multiple biological activities as these often form the specific interaction site between two molecules, e.g. receptor-ligand or enzyme-substrate (Laskowski et al., 1996). Antibodies that specifically bind into such active sites therefore represent powerful tools to modulate biological activities.

Upon immunisation of dromedaries with enzymes, significant proportions of the HCAb generated are competitive enzyme inhibitors that interact with the cavity of the active site (Lauwereys et al., 1998). In contrast, conventional monoclonal antibodies that are competitive enzyme inhibitors are, by far, more the exception than the rule. It is, therefore, clear that camelid heavy chain antibodies recognise unique epitopes that are currently out of reach for conventional antibodies.

This was corroborated by the isolation of recombinant VHH that bind specifically into cavities on the surface of enzymes (Desmyter et al., 1996; Lauwereys et al., 1998). Inhibitory VHH have been isolated against a variety of enzymes including lysozyme, carbonic anhydrase, α-amylase and β-lactamase (Lauwereys et al., 1998; K. Conrath, personal communication).

In particular, the long CDR3-loop of the in vivo matured VHH from a dromedary HCAb accounts usually for most of the binding interaction with the antigen. The crystal structure of an anti-lysozyme VHH, the cAb-Lys3 (Fig. 3 left), which was identified as an inhibitor, gave a satisfactory explanation for the efficient blocking of the enzyme. This VHH carries a long CDR3 loop of 24 amino acids, of which the first 10 amino acids form a loop that extends from the remaining antigen-binding site and inserts into the active site of the lysozyme. This was the first example of an antigen-binding site with a large protruding loop. Most antibodies reacting with large antigens form a surface that is essentially flat, eventually with some small undulations from individual side-chains that protrude (Webster et al., 1994; Padlan, 1996). The residues of the protruding loop of cAb-Lys3 interact with the catalytic residues of the enzyme and seven consecutive amino acids forming the bulge of the loop mimic the natural substrate of the enzyme (Transue et al., 1998). It was therefore hypothesised that the long CDR3 of the VHH could be a perfect lead to design new drugs against various enzymes (Sheriff and Constantine, 1996). However, the observation that the catalytic activity of some enzymes can be blocked totally by VHH with a much shorter CDR3 loop (e.g. cAb-AMD9 inhibits α-amylase and contains a CDR3 of nine residues) indicates that the insertion of a long protruding CDR3 loop into the active site cleft will not be the only mechanism to block the antigenic enzyme. The recent crystal structure of R2, an anti-hapten llama VHH, indicates that the non-canonical conformation of the CDR1 loop might also be sufficiently separated from the other

antigen-binding loops to interact with cavities on the antigen surface (Spinelli et al., 2000).

The capacity of binding in the cavities on the antigenic surface or to inhibit the enzymatic activity of enzymes might also be maintained in the VHH extracted from synthetic libraries. Support for this statement was given by Martin et al. (1997), who isolated a hepatitis C protease inhibitor from the camelised human VH library. Remarkably, the binder was selected from the library with only 11 amino acids in its CDR3. Again, this observation demonstrates that the presence of a long CDR3 in the VHH is not a prerequisite for the enzyme inhibition.

2.6.5. Homology of VHH to human VH sequences

Over the last 15 years more than 200 antibodies have made it into the clinic, illustrating the diagnostic and therapeutic value of antibodies. One major drawback is that the large majority of the present therapeutic antibodies are of murine origin, and that these can elicit an immune response in the patient (Courtenay-Luck et al., 1986). Some of the toxicity seen with mouse antibodies has been resolved by shuffling the murine and human antibody sequences to various extends to construct chimeric, humanised, or veneered antibodies (Boulianne et al., 1984; Riechmann et al., 1988; Padlan, 1991).

VHH provide opportunities to develop a new generation of minimal sized therapeutic antibodies where the unique features of VHH offer added value over conventional antibody fragments. The sequence comparison of the human VH and dromedary VHH demonstrates their high degree of identity. The 'humanisation' of camel VHH is consequently more straightforward as compared to their murine counterparts because they are smaller and more homologous to human antibodies (Vu et al., 1997). Based on the structural information of VHH, we foresee that most of the substitutions other than the key camel-specific substitutions in framework-two can be replaced by the human sequence without altering the performance of the VHH. The VHH will therefore be good candidates to humanise by veneering, as devised by Padlan (1991). Evidently, the VHH-specific residues in the framework-two should not be modified since they are either involved in the interaction with the long CDR3 or they are solvent exposed and essential for the single-domain nature and the unique physico–chemical features of the isolated VHH.

2.7. Possible applications fields for VHH

The VHH are distinguished from the scFv by their unique properties of: size; stability; solubility; ease of cloning a library and selecting highly antigen-specific binders that were matured in vivo; expression yield; and a less complex paratope. Obviously, these features should lead to a number of applications where VHH should perform better than other antibody formats. Their advantages as detection units on biosensors or usage in immuno-adsorbents, or to generate potent competitive enzyme inhibitors were already discussed. We will now focus on the possibility to develop VHH as:

- in vivo imaging agents;
- leads to derive peptide-based drugs;
- modular building blocks for manifold constructs; and
- intrabodies.

2.7.1. VHH as in vivo imaging agent

Fast and reliable in vivo diagnosis at an early stage of the disease remains one of the major challenges in infections and cancer diagnosis. The ideal cancer-imaging agent should deliver an amount of label sufficient to detect the smallest metastases against a low level of non-specific background signal. It should localise promptly by penetrating the tumour and bind to its target antigen with high affinity without staining the necrotic area. In addition, the unbound conjugate should clear rapidly from the system to reduce the time between administration and imaging, and minimise whole body exposure to the radiation.

Commercial imaging agents and those in clinical trials are derived from murine monoclonals. The complete antibodies suffer from immunogenicity, while slow clearance and poor tumour

penetration, because of size, lead to a high background. The reduced sizes of antibodies, such as scFv have improved biodistribution and faster renal clearance (Yokota et al., 1992). However, scFv tend to be unstable, have, usually, a low affinity and may accumulate into necrotic areas.

The minimal sized VHH-based imaging agents would represent a valuable alternative in this respect. The superior penetration potential of VHH due to their smaller size, combined with high affinity target binding and fast clearance from the circulation, represents an ideal basis for imaging purposes. We recently obtained evidence in a murine tumour model that tumour-specific VHH are cleared more rapidly from the circulation than a scFv, but still can specifically localise to the tumour site (V. Cortez-Retamozo and H. Revets, personal communication). A few hours after administration, a tumour to blood ratio above 10 could be demonstrated with a radiolabelled VHH.

VHH are easily amenable to an optimisation of pharmaco–kinetic properties through engineering of their size or oligomeric state. In addition, the labelling efficiency can be increased through the addition of reactive groups at the C-terminal end of the VHH, diminishing the risk to modify critical residues involved in binding.

Probably, a successful imaging agent can form the basis for the development of a second generation radio-immuno-therapeutic. However, this would require the repeated administration of high doses of VHH, and therefore, the VHH should not be immunogenic. We analysed the possible immunogenicity of our VHH in mice (H. Revets, personal communication), and failed to indicate the presence of circulating immunoglobulins directed against the VHH that was injected intravenously, even after repeated doses. In addition, we could not demonstrate a T-cell response against the dromedary VHH in the treated mice. At least from the mouse model system it seems that the VHH have the potential to generate potent in vivo therapeutics.

2.7.2. VHH-derived peptide-based drugs

In order to increase the therapeutic window of certain protein-based drugs from an acute to a chronic application, several biotech and pharmaceutical companies attempt to design and produce small-molecular peptide derivatives that retain the activity and specificity of the original protein. Peptides have a broad range of potential clinical benefits, with applications in major disease areas, such as: immune system disorders; cancer; and cardiovascular and neurological disorders (Latham, 1999).

It is hypothesised that the antigen-specific VHH might constitute better leads for a drug design approach than scFv (Sheriff and Constantine, 1996). The antigen-binding site of conventional antibodies is dispersed over six CDR-loops on two domains (Padlan, 1996). In contrast, the single domain VHH contain only three antigen binding loops, with the unusually long CDR3 loop providing the majority of binding affinity for proteinaceous antigens. In the three structures of VHH-protein complexes available so far, the VHH-CDR3 contributes between 50 and 100% to the antigen interacting surface (Table 2). This loop is furthermore constrained via an intra-molecular disulfide bond, rendering it an ideal starting point for the design of constrained peptides mimicking the CDR3-loop structure. In contrast, the only VHH-hapten structure that is available (Spinelli et al., 2000) revealed the dominant role of the first hypervariable loop in the interaction with the dye. The contribution of the CDR1 in providing the antigen-interacting surface was approximately double that of the third hypervariable loop with its 18 amino acids. However, even for this llama VHH, it is noticed that the binding energy is provided mainly by one loop. It should therefore be possible to design in a first step, a peptide that maintains much of the original antigen-binding properties. An Ala scanning experiment on the synthetic peptide might then reveal in the next step the essential residues (Laune et al., 1997), so that, ultimately, small chemical derivatives harbouring the specific binding specificity of the original VHH can be generated. It is noticed that this strategy to yield peptide mimetics can be employed even in the absence of the VHH structural knowledge.

Overlapping synthetic peptides of the cAb-Lys3 were generated to test the feasibility of this ap-

Table 2
Interaction surface area of CDR with antigen (in Å^2) and the contribution (in %) of each CDR is given for the four VHH crystal structures solved in complex with their antigen

	CDR3 (AA)	K_D (nM)	Surface (Å^3)	CDR1	CDR2	CDR3
cAb-Lys3	24	65	847	14.2%	15.4%	70.4%
cAb-RN05	12	35	570	43.9%	–	56.1%
cAb-CA05	19	72	619	8.0%	1.0%	91.0%
R2	16	22	295	43.0%	33.6%	23.4%

proach. The peptides that contained the CDR3 loop sequences retained considerable binding affinity to lysozyme (C. Granier and D. Laune, personal communication) and were shown to be competitive inhibitors. Thus it is concluded that the cavity binding propensity of the CDR3 of VHH can, therefore, form the ideal lead to design peptidomimetic-based drugs acting as enzyme inhibitors, and probably also as receptor agonists or antagonists.

It is evident that the VHH that are retrieved from a synthetic library, in which the CDR3 is the only randomised region, will be even more efficient in the design of peptide mimetics, since only the CDR3 of the antigen binder is different from the non-binders in the library.

2.7.3. VHH as modular building blocks for manifold constructs

Expanding the applications of conventional antibodies through the design of antibodies with multiple functions requires extensive protein engineering skills. An abundance of applications becomes available for bispecific antibodies or other immuno-fusions.

2.7.3.1. Bispecific and multivalent antibodies. Bivalent and bispecific antibodies (Fig. 6) have many practical applications in immuno-diagnosis and therapy. Bivalency can allow antibodies to bind to antigens with great avidity (Rheinnecker et al., 1996; Terskikh et al., 1997). Bispecificity permits the cross-linking of two antigens, for example in

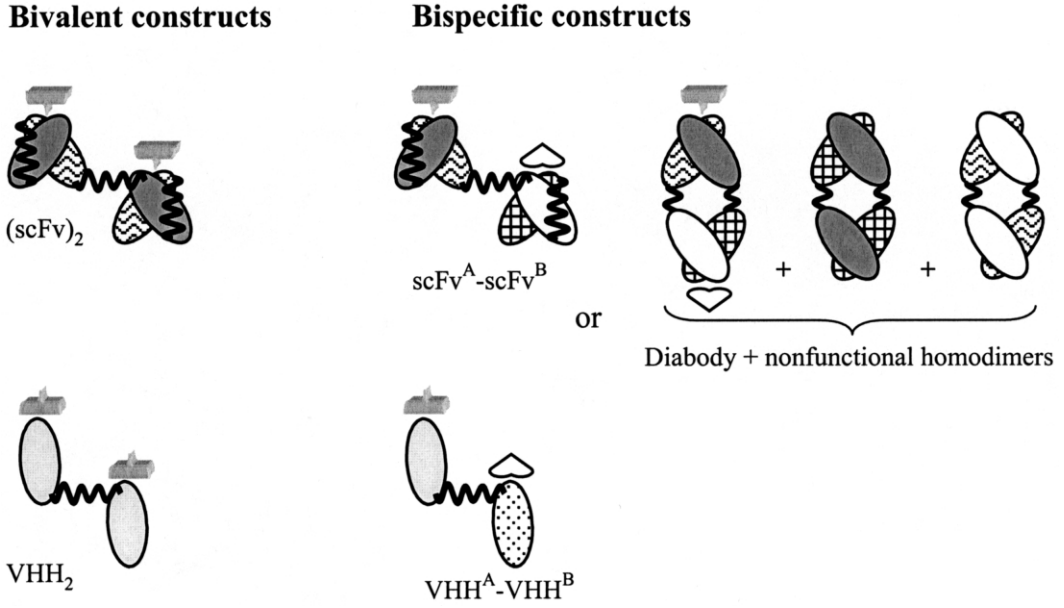

Fig. 6. Bivalent (left) and bispecific (right) antigen-binding constructs formed by expression of tandemly cloned scFv or VHH, separated by a linker. Bispecific antigen-binding constructs are generated by cloning two conventional scFv of different specificities spaced by a long linker (scFv^A–scFv^B) or by a shorter linker and scrambling the VH and VL domains (diabodies). Also, the VHH of different specificities can be cloned and expressed as one polypeptide with a linker. We selected the upper structural hinge of dromedary IgG2a in our VHH-based constructs.

recruiting cytotoxic T-cells to mediate killing of a tumour cell (Bohlen et al., 1993; Kurucz et al., 1995). The ideal characteristics of a multispecific antibody are: minimal size; absence of a linker peptide prone to aggregation or susceptible to proteolysis; high expression yields; high solubility; and high stability.

Bivalent or bispecific constructs of scFv have been obtained by tandem cloning of two scFv, each binding to the same or to a different antigen or epitope, respectively, and spaced by a linker peptide (Fig. 6). These constructs, therefore, contain three linkers and are prone to aggregation. So unfortunately, large-scale clinical development is being hampered by low expression levels and sensitivity to proteolytic degradation at the level of the three synthetic linkers. An alternative was experimentally tested by Holliger et al. (1993) by linking the VH gene of a first antibody (A) and the VL gene of a second antibody (B) with a shortened linker, and vice versa. The co-expression of these two hybrid scFv in the same cell created two polypeptide chains, VHA–VLB and VHB–VLA, that are forced to dimerise since the shortened linker does not allow pairing of the domains within one polypeptide chain. The heterodimers of these two polypeptide chains carry two antigen-binding sites in the same molecule, but homodimers are non-functional (Fig. 6). The functional expression of these, so-called diabodies, is often low because homo- and heterodimers are equally well formed. Moreover, the aberrant angle between the VH–VL pairs may be detrimental for antigen binding (Hudson, 1998).

Thus, diabodies and $scFv_2$, which are low molecular weight bivalent or bispecific antibody fragments, with a size similar to that of a Fab fragment, have run into the same problems of low recovery because of unproductive association between the VH and VL chains of two different antibodies. We realise that camelid VHH offer a clear opportunity in each application where bispecific or multivalent antibodies are needed.

The generation of bispecific and bivalent constructs with VHH is straightforward because the intact antigen-binding site is contained in one domain (Fig. 6). Moreover, VHH are soluble and remarkably robust, allowing a high versatility as building blocks for manifold constructs. We successfully expressed tandemly cloned VHH with different or an identical antigen specificity, spaced by the natural structural upper hinge of camel HCAb. The resulting bispecific and bivalent proteins were expressed in bacteria in shake flasks at yields of 0.5-mg purified constructs per litre culture (Conrath et al., 2001). These bifunctional constructs based on individual VHH with different specificities retained fully the original functionality. There was no sign of dimerisation or aggregation of the polypeptide chains. Furthermore, the choice of the natural hinge provides a good serum stability of the constructs. It also allows sufficient mobility of the individual VHH so that the original antigen recognition of both subunits is not impeded.

2.7.3.2. Immunofusions. An extension of the above approach consists of replacing one of the VHH by a toxin or enzyme resulting in functional immuno-fusions (Reiter and Pastan, 1996; Senter et al., 1988). Cancer therapy based on immunotoxins, i.e. a tumour-specific antibody chemically or genetically fused to a toxin, is a valid therapeutic option. This is the basis of the 'magic bullet' approach, whereby an antibody is used as a delivery vehicle for the toxin, thereby limiting the toxin dose resulting in less side effects (e.g. toxicity towards healthy tissue, immunogenic response to the toxin). The toxin can also be replaced by an enzyme (e.g. prodrug activation via antibody–enzyme fusions), or a radioactive ligand (e.g. radioimmunotherapy).

The first antibody therapeutics in cancer patients were directed to lymphomas because these tumours are easily accessible in the bloodstream. Antibodies that can fight solid tumours should have high affinity for a tumour-specific antigen and an optimal size to deliver the highest dose of label or toxin.

We think that VHH are excellent vehicles to deliver toxins to particular tumour targets. Given the small molecular weight of VHH, the tumour penetration potential of VHH is expected to be superior to that of conventional antibodies. The inherent solubility and stability turn VHH into the most versatile building block for manifold

constructs. All these features are beneficial to keep the size of the immunofusion minimal for optimal tissue penetration and clearance rate. Finally, VHH recognises other conformational epitopes than conventional antibodies recognise. Therefore, VHH-based immuno-toxins may target presently unexplored or unrecognised tumour antigens.

The construction of immuno-toxins with VHH is straightforward because the intact antigen binding site is contained in one single domain that can easily be fused genetically to any other protein of choice (enzymes, toxins, etc.). We have successfully constructed a bifunctional fusion between a lysozyme-specific VHH and bacterial alkaline phosphatase (K. Silence, personal communication). This construct retained the specific binding to lysozyme and the phosphatase enzymatic activity. The apparent antigen-binding affinity increased because the alkaline phosphatase is extracted from the periplasm as a dimer, and therefore we also obtained two VHH moieties per molecule.

2.7.4. VHH as intrabodies

The intracellular expression of antibodies (intrabodies) is a powerful strategy to inhibit in vivo function of selected molecules, but it is limited by the unpredictable behaviour of antibodies when intracellularly expressed (Cattaneo and Biocca, 1999). Intrabodies may hold promise for a variety of applications including plant biotechnology, human gene therapy and functional genomics. A most illustrative example of this approach was given by Tavladoraki et al. (1993) who demonstrated that the expression of antibody fragments, against the coat protein of artichoke-mottled-crinkled virus, in tobacco plants reduced the production of infective virus particles. The availability of inhibitory, antagonistic or agonistic antibodies would increase the success rate and broaden considerably the applicability of the intrabody technology. In the same vein, it is expected that the expression within one cell, of multiple intrabodies, each binding to a different antigen, might be far more effective to combat, for example, a viral infection (Rondon and Marasco, 1997). Indeed, it should be no problem to generate multicistronic constructs of multiple dromedary VHH genes each separated by an internal ribosomal entry site (IRES), and to co-express all these as intrabodies. The single domain nature of each VHH will avoid their aggregation so that each of them could interfere with the proper function of their target.

Finally, the intracellular expression of multidomain antibodies (scFv) has proven problematic due to incorrect assembly of the VH and VL chains and proteolytic degradation of the (synthetic) linkers. Although some selected scFv have been functionally expressed in mammalian cells (Visintin et al., 1999), the more versatile expression of libraries of scFv still awaits experimental proof.

The high ratio of antigen binders obtained in a small VHH library of an immunised dromedary opens, theoretically, the possibility to construct, immediately, a VHH intrabody library in mammalian cells and to screen the transformants individually for the antigen targeting and possible interference with target function.

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