Nanobodies and their potential applications

Nanobodies are recombinant, antigen-specific, single-domain, variable fragments of camelid heavy chain-only antibodies. The innate supremacy of nanobodies as a renewable source of affinity reagents, together with their high production yield in a broad variety of expression systems, minimal size, great stability, reversible refolding and outstanding solubility in aqueous solutions, and ability to specifically recognize unique epitopes with subnanomolar affinity, have combined to make them a useful class of biomolecules for research and various medical diagnostic and therapeutic applications. This article speculates on a number of technological innovations that might be introduced in the nanobody identification platform to streamline the generation of more potent nanobodies and to expand their application range.

**KEYWORDS**: *Camelidae* crystallization chaperone deep sequencing enzyme inhibitor heavy chain-only antibody immune repertoire intracellular delivery nanobody single-domain antibody

From heavy chain-only antibodies to nanobodies

The natural immune system receives much interest because of its importance in various fundamental and applied sciences. The soluble IgG glycoproteins circulating in the blood play an essential role in the humoral immune system of mammals. These antibodies, produced by B lymphocytes, recognize with high specificity the foreign biological or chemical substance against which they are affinity matured, and might alter the characteristics (e.g., function) of this cognate antigen. The basic structure of an IgG molecule comprising two identical heavy (H) polypeptide chains and two identical light (L) polypeptide chains is highly conserved among mammals. The molecular weight of an IgG is approximately 160,000 Da, and it is a complex molecule in which H and L chains fold into four and two domains, respectively (Figure 1). Compared with smaller antigen-binding fragments, such as Fab (molecular weight: 50,000 Da) or scFv (molecular weight: 30,000 Da), only low yields of functional recombinant IgG are obtained from microorganisms. The scFv is a man-made product composed of an N-terminal, variable domain of the H chain (VH) and an N-terminal, variable domain of the L chain (VL), joined by a short synthetic peptide linker (Figure 1). The scFv is the smallest intact antigen-binding fragment that can be derived from a conventional IgG molecule. Unfortunately, bacterial expression of scFvs cloned from previously identified hybridomas often suffers from low yields. Therefore, antigen-specific scFvs are preferably obtained in a two-step procedure: first, a large single-pot repertoire of naive or synthetic VH and VL genes are cloned in an scFv format; second, the antigen-specific scFvs are retrieved from this repertoire after phage display. It is well established that phage display panning selects for binders with the highest specificity and affinity to the target, in addition to the better producers and more stable constructs. With the objective of making even smaller antigen binders, similar single-pot libraries have been constructed based on soluble, stable, VHs with good expression, where codons for amino acids within antigen-binding loops were randomized. While such synthetic VH libraries seem to be a good source to identify single-domain antibody fragments, they can be substituted equally well by other immunoglobulin folds such as VL or fibronectin domains, as well as by engineered nonimmunoglobulin scaffolds, including ankyrin repeat proteins (DARPins), lipocalins (anticalins) or protein A (affibodies), to name just a few [1]. Each of these scaffolds has its benefits and probably limitations as well.

At the time that the first successes were experienced in retrieving antigen-specific scFvs or Fabs from large synthetic or naive libraries displayed on a phage, it was discovered serendipitously that dromedaries incorporate a unique class of antibodies devoid of L chains in their sera, in

*Gholamreza Hassanzadeh-Ghassabeh*, *Nick Devoogdt*, *Pieter De Pauw*, *Cécile Vincke*, *& Serge Muyldermans*  

1*Vrije Universiteit Brussel, Research group Cellular & Molecular Immunology, Pleinlaan 2, 1050 Brussels, Belgium*  
2*VIB, Department of Structural Biology, Vrije Universiteit Brussel, Brussels, Belgium*  
3*Vrije Universiteit Brussel, Research group ICMIC, Laarbeeklaan 109, 1090 Jette, Belgium*  
4*VIB, Nanobody Service Facility, Vrije Universiteit Brussel, Brussels, Belgium*  
5*Author for correspondence: Tel.: +32 2 629 19 69 Fax: +32 2 629 19 81 svmuylde@vub.ac.be

---

10.2217/NNM.13.86 © Serge Muyldermans

*Nanomedicine* (2013) 8(6), 1013–1026

ISSN 1743-5889
addition to the conventional H2L2-type IgGs [2]. Thus, these homodimeric H2-type antibodies were called H chain-only antibodies (HCAbs) (Figure 1). It was also noticed that the H chain of HCAbs is folded into three domains: an N-terminal domain that is variable in sequence (VHH), followed by a hinge region and two constant domains. Therefore, the equivalent of the first constant domain of the H chain of a conventional antibody is missing in H chains of H2 antibodies. The occurrence of such functional HCAbs within mammals as a component of their humoral immune system seems to be limited to Camelidae species (Camelus dromarius, Camelus bactrianus, Lama glama, Lama pacos, Lama guanicoe and Lama vicugna). However, similar functional antibodies devoid of L chains are also present in an assortment of non-mammals, such as sharks (Orectolobus maculates and Ginglymostoma cirratum) and ratfish. In these cartilaginous fish, these antibodies, commonly known as Ig-NARs, have an architecture fairly different from that of camelid HCAbs: an Ig-NAR is a homodimeric H chain, each chain consisting of one single variable antigen-binding domain (V-NAR) and five constant domains (Figure 1) [3].

Inspection of the molecular organization of the H1L2 antibodies, the camelid HCAbs and Ig-NARs, teaches us that the H2-type antibodies recognize their cognate antigen by one single domain, the VHH or the V-NAR, respectively (Figure 1). Crystal structures of VHHs reveal a prolate (rugby ball) shape of approximately 2.5 nm in diameter and 4.2 nm in length [4]. Hence, these ‘nanometer-sized antibody fragments’ are referred to as ‘nanobodies’ (Nbs) [5]. Although V-NARs and VHHs have similar sizes, the term Nb is only used for (recombinant) camelid single-domain antibody fragments.

The fascination with nanotechnology originates from altered mechanical, thermal and catalytic properties of materials as their size decreases from the micro- to nano-meter scale, enabling novel applications. Likewise, the size reduction of an HCAb into an Nb (and the concomitant reduction in valency from bivalent to monovalent) can cause a dramatic change in biological activity. Indeed, while a monoclonal HCAb with specificity to trypanosomes is harmless to the parasite (in the absence of complement), the isolation of Nbs from these HCAbs makes these antigen-binding entities highly trypanolytic by blocking a very early endocytosis step via a mechanism that has not been elucidated yet [6].

Biochemical & biophysical properties of Nbs

After vaccinating a camelid (or a cartilaginous shark) to raise an H2-type antibody response, the VHH (or V-NAR) gene fragments from lymphocytes are amplified by PCR, ligated in phage display vectors and transformed in bacteria to construct an immune VHH library. Expression of the cloned VHHs in fusion with the phage coat protein pIII, its assembly at the tip of phage particles and enrichment of phage-displayed, antigen-specific VHHs on immobilized antigens.
in two or three rounds of panning allows the identification, with a high success rate, of Nbs targeting a wide variety of antigens with nanomolar to even picomolar affinities within weeks of the first antigen injection [7]. The retrieved Nbs are successfully expressed in microorganisms, thereby providing an easy route to obtain large amounts of in vivo affinity-matured, antigen-specific single-domain antibodies. This is an important advantage over scFv or man-made scaffolds, where in vivo affinity maturation cannot be accomplished easily by immunization. In cases where vaccination is difficult (e.g., chemical and nonimmunogenic targets), it is possible to construct large single-pot synthetic VHH (or V-NAR) libraries to identify antigen binders [8]. However, the antigen-specific binders retrieved from synthetic libraries are not affinity matured and might benefit from additional sequence diversification steps and subsequent selections to generate more potent binders [9].

The crystal structures of VHHs in complex with their antigen [10] confirmed that the side of the domain (known as the framework-2 region) that contacts a VL in an H_{L2}-type antibody is resurfaced from a hydrophobic region in VH to a more hydrophilic region in a VHH. This framework-2 resurfacing by amino acid substitutions contributes to the strict monomeric behavior and the high solubility of VHH domains. Moreover, it explains the stickiness of many VHs in the absence of a VL partner. However, the reduced solubility of isolated VHs in aqueous solution can be remedied by ’camelizing’ the mouse or human VH, whereby hallmark amino acids of the framework-2 region of a camelid VHH are imprinted in the VH sequence [11]. Conversely, although Nbs are poorly immunogenic in humans, due to their high sequence identity to human VHs, it is probably good practice for prolonged therapeutic applications involving multiple administrations to first ’humanize’ the framework-2 region of Nbs to imprint an amino acid sequence closer to human VHs in that region [12]. The humanization of Nbs, like any other site-directed mutagenesis on Nbs, is straightforward as the gene is only approximately 380 base pairs.

The crystal structure of VHHs further revealed new structural organizations of the antigen-binding loops, so Nbs are more suitable than scFv paratopes for interacting with grooves on the surface of the antigen, such as the catalytic site of enzymes [10]. Indeed, it is well established that many Nbs act as enzyme inhibitors or modulate the function of the target and that the preferred antigenic sites for scFvs and VHHs are distinct. Of note, the V-NARs and VHHs seem to share this preferential binding to clefts [3].

Streamlining the Nb identification platform

Successful identification of antigen-specific Nbs is straightforward after immunizing a camelid (or shark for V-NAR) with purified, soluble, properly folded proteinaceous antigens and phage display [7]. However, immunization with more difficult targets, such as multipass membrane proteins, receptor complexes, intrinsically disordered proteins or unstructured peptides and the subsequent selections against such targets remain critical steps.

Immunization of camelids or transgenic mice

The procedures to raise HCAbs in camelids are similar to those used to elicit conventional antibodies in other animals. Various adjuvants as well as different immunization routes and boost intervals successfully induce an HCAb response in camelids. Camelid immunization simultaneously elicits conventional and HCAb responses, with the former often being dominant. Any optimized immunization protocol (e.g., a particular adjuvant and/or immunization route) favoring the HCAb response is highly desired. Taking into account that camelids are large and out-bred animals, this immunization optimization is challenging, but feasible.

Most antibody targets are membrane proteins, which are difficult to express and purify as functional recombinant proteins. The availability of such proteins in sufficient quantities for immunization and selection is, therefore, a hurdle in all antibody technology platforms. Since the vast majority of HCAbs and Nbs recognize conformational epitopes, the immunization of camelids with peptides (even coupled to larger protein carriers) is not recommended as it raises poor H_{L2}-type antibody responses.

DNA immunization, cell immunization and DNA prime/cell boost are potential alternatives to protein immunization. Both DNA prime followed by protein boost or cell immunization have been used to generate Nbs [13,14]. Nevertheless, the former method still requires proteins, whereas cell boost or cell immunization results in an undesired antibody response to nontarget cellular antigens, thereby complicating panning and screening steps. To optimize DNA immunization protocols without the need for protein or cell boosts, it will be necessary to systematically
analyze the target-specific Nbs identified after such immunization.

As testified by the large number of publications, immunization of camels – or better to say, the size and exotic nature of camels – has not been prohibitive in generating Nbs and in exploiting these Nbs in various applications. However, immunizing transgenic mice with the exon for the first constant domain in H chain gene(s) deleted, might streamline the Nb identification platform. The proof of principle for such mice as a source of Nbs has already been given [15,10], but the concept of transgenic mice as substitutes for camels to rapidly generate potent HCAbs and/or high-affinity Nbs still remains to be demonstrated.

**Nb selection**

Current Nb identification strategies involve enrichment of antigen-specific binders from immune or naive libraries by phage display, bacterial two-hybrid or bacterial surface display [7,8,16], and screening of individual colonies from enriched phage populations, primarily by ELISA. However, advanced approaches are in sight, and examples of such approaches are proposed below.

**Deep sequencing to avoid repertoire cloning & selection**

Recently, next-generation sequencing (NGS) was used to calculate the relative frequencies of genes encoding VH and VL in a cDNA pool of plasma cells from immunized mice. Subsequently, the most abundant VH and VL genes were synthesized and paired to obtain antigen-specific scFvs [17]. This study provides an example of bypassing tedious library construction, biopanning and ELISA screening steps. Moreover, the NGS screening identified antibodies that were lost during panning or went undetected by ELISA [18]. Interestingly, the recombinant antibodies identified by NGS, but not by standard panning and ELISA screening, were expressed in *Escherichia coli* at relatively high levels under optimized conditions.

We anticipate that implementing the NGS approach will be extremely favorable to rapidly discover antigen-specific Nb sequences. Indeed, the antigen-binding fragment of HCAbs involves only one single exon (VHH) instead of two (VH and VL) in conventional antibodies. Therefore, the most abundant VHH sequences in a pool of lymphocytes should correspond to those from HCAbs on B cells that maximally proliferated during immunization. Moreover, the single-domain Nb format immediately corresponds to the intact antigen-binding site of HCAbs, and avoids the requirement to combine all abundant VHs and VLs in all possible combinations to find the best pairs as they were affinity-matured *in vivo* during immunization.

**Selection of Nbs against proteome fractions**

It is common practice to immunize camels with a mixture of five to ten antigens and to sort by panning antigen-specific binders to each component afterwards. With this in mind, it should be possible to immunize with a crude extract of any particular cell, possibly containing a currently unknown ‘active’ protein. The camelid will raise HCAbs to immunogenic proteins and the cloned VHH library can be panned subsequently on the same proteome extract to enrich for Nbs against any component within the crude extract. After the last round of panning, individual clones can be screened by ELISA [19] or by a functional assay to identify Nbs that bind or modulate the ‘activity’ of a particular component of the mixture (see ‘Nb-based phenotypic screening’ section). Once such Nbs are isolated, they can be purified and immobilized to capture the antigen from the mixture. The captured antigen can then be identified, for example, by mass spectrometry [20].

If the biomarker is known, it is possible to use a minimal component fraction (i.e., a cell fraction enriched for the biomarker and depleted of irrelevant proteins) for immunization and subsequent phage selections. However, in cases where the biomarker of interest is unknown, the possibility to use crude, unFractionated cell extracts has an advantage over the use of minimal component fractions because the identification of the fraction containing the (undetermined) biomarker causing the phenotype of interest can be very tedious (especially when it is a multicomponent biomarker).

**Nb-based phenotypic screening**

The potential use of antibodies for high-throughput phenotypic screening to discover new targets of therapeutic value still remains unexplored. This is partly due to the inability of antibodies to access intracellular targets and the low throughput of antibody discovery platforms. Despite these shortcomings, antibodies represent an interesting class of molecules for high-throughput phenotypic screening, because of their high affinity, fine specificity and almost limitless repertoire diversity. Indeed,
the feasibility to use antibodies for the discovery of novel drugs or drug targets by phenotypic screening was recently demonstrated by using both naive and immune scFv libraries [21]. In this work, individual scFvS, from affinity selections on Pseudomonas aeruginosa whole cells, were tested by ELISA for binding to heterologous serotype strains. The scFvS exhibiting serotype-independent binding to P. aeruginosa were reconstituted into an IgG1 format and assayed for opsonophagocytic killing of P. aeruginosa. The target antigens of scFvS with such activity were then identified by assaying the binding of scFvS to P. aeruginosa mutant strains [21].

The robustness of the Nb identification technology and the possibility to generate and identify Nbs on the proteome scale [19], together with numerous data on the potent and target-specific effects of Nbs, suggest that Nbs can serve as alternatives to small chemical compounds for phenotypic screening aimed at the discovery of both novel drugs and drug targets. However, for this concept to be fully realized, future developments are vital such as incorporation of NGS into the Nb identification platform to increase its throughput and preferably the ability to target intracellular molecules in a high-throughput manner.

Selection on cells

Purification of properly folded, multipass membrane receptors (e.g., pharmacologically important G protein-coupled receptors) or receptor complexes (e.g., integrins) for immunization and selection is a major bottleneck. For such targets, there are two solutions: either, immunization is avoided and non-affinity-matured Nbs are retrieved from naive or synthetic libraries [8], or immune libraries will be constructed after immunizing with DNA only [14] or transgenic cells that overexpress such receptors. For the latter, transgenic DUBCA cell lines might be very useful as these dromedary-derived cells are sup-
posedly poorly immunogenic in camels. During the subsequent Nb selection process, special measures need to be taken to retrieve binders targeting the (perhaps less immunodominant) receptor of interest from a vast pool of potential binders that target unwanted membrane proteins. It is imperative to eliminate the Nbs to these irrelevant antigens. This is achieved to some extent by the biopanning and rapid analysis of selective interactive ligands, the ‘BRASIL’ method [22], or by panning on two different transgenic cell lines with very different origins. Otherwise, multiple rounds of selections can be performed on a cell mixture, in which fluorescently or magnetically labeled transfected cells are diluted into unlabeled, untransfected cells and then subjected to flow cytometric or magnetic sorting. Nbs against common antigens (present on both transfected and nontransfected cells) will bind mainly to the untransfected cells due to their higher abundancy, and, therefore, those Nbs will be eliminated by sorting the labeled transfected cells.

Nbs with improved properties

In vitro affinity improvement of Nbs

In some instances, the immunization of a camelid is impossible (owing to highly toxic or pathogenic immunogens, lack of purified properly folded antigen and nonimmunogenic compounds) and, thus, alternatives to in vivo maturation might be necessary to obtain high-affinity antigen-specific binders. Generating bivalent Nb constructs might increase the functional affinity (i.e., avidity) [23], but does not change the intrinsic affinity between Nbs and antigens. In principle, improvement of the intrinsic affinity of Nbs for their antigen should be easier than that with scFvS because of Nbs’ smaller size, single-domain nature, easy folding and the presence of only three antigen-binding loops. These characteristics make the construction of second-generation libraries on a previously selected Nb scaffold less complex and allow more robust phage or yeast display selections instead of more demanding selection techniques, such as ribosome display. Libraries of Nb variants with either localized or unbiased random mutations need to be generated. The targeted options use site-specific randomization of the codons for the amino acids of the antigen-binding loops. The availability of structural information of the antigen-binding loops of the Nb, preferably in complex with its antigen, is helpful, if not critical, to pinpoint the mutagenesis hot spots. Such antigen-binding hotspots can also be identified by Ala-scanning mutagenesis [9]. Apart from these techniques based on evolution, rational approaches might also be successful. Indeed, it has been shown that mutating a limited number of amino acids, carefully selected at the periphery of the paratope, guided by parameterized quantitative descriptors (using a multivariate experimental design), and measuring the effect of these mutations on the antigen-binding kinetics could be used to construct an algorithm that predicts the kinetic binding parameters as well as the equilibrium binding constant of other possible mutations at
those positions [24]. Conversely, the unbiased, randomized Nb libraries are obtained via error-prone PCR or the use of mutator strains. These fully random approaches have the disadvantage that a vast number of dysfunctional Nbs are formed and the advantage that occasionally a mutant might emerge with an amino acid substitution outside the antigen-binding loops with indirect favorable binding properties.

Finally, the implementation of Nbs in the phage-assisted continuous evolution technology, an elegant technique in which phage fitness constitutes the driving force to select permanently novel mutants evolving spontaneously, rapidly and continuously [25], is expected to produce Nbs of greatly improved potency.

Cell-penetrating Nbs
The availability of transducing Nbs (i.e., Nbs with the capacity to penetrate the membrane of their specific target cell) that deliver functional molecules to the cytoplasm would offer an elegant solution to overcome the impermeability of cells to most macromolecules [26]. These transducing Nbs could be used to produce ‘Trojan horses’, directing proteins, DNA or siRNA specifically to diseased cells to provoke cytotoxicity, to restore a lacking function in deficient cells, or to induce production of complementary proteins. To achieve transduction, one could take advantage of the existing internalizing proteins on the target cell surface (e.g., by inducing receptor dimerization and internalization with a bivalent or bispecific Nb). Another approach would be the addition of protein transduction domains, such as the one derived from the HIV Tat protein or penetratin, or by increasing the Nbs’ arginine content, a condition that facilitates internalization [27]. Finally, a promising technology was developed whereby Nbs equipped with a short peptide are delivered into human cells, directly from E. coli via its type III protein secretion system [28]. It is surmised that expression of a cell type-specific Nb on the surface of attenuated, invasive bacterial strains might direct these engineered cells to specific organs or tissues of the host. Colonization of the target organ (e.g., solid tumors, infected or diseased cells) might then allow prolonged delivery of a protein therapy by a relevant second Nb (i.e., intrabody), injected to interfere with or reprogram the diseased cells.

Nbs in research
- Nbs as affinity capture reagents
Nbs are suitable affinity capture reagents [29] because their small size and single-domain format provide higher capacity binding surfaces and lower nonspecific background binding, as compared with larger antibody formats. Their monovalent mode of binding allows mild elution conditions, which is particularly important for sensitive molecules. Moreover, their high stability and refolding capacity permits repeated and stringent regeneration conditions.

Nbs-based affinity capture has been used to study protein–protein interactions [30], analyze in vivo DNA–protein interactions at the genomic scale after chromatin immunoprecipitation, track protein conformations, trace bacterial infections and purify recombinant proteins. Nbs have been employed for scavenging toxins from plasma [31] and for depletion of abundant serum proteins, a step required for proteomic analysis of blood samples [32]. The use of KDEL-specific Nbs to capture endoplasmic reticulum-resident proteins [23] raises the possibility of using signal peptide-specific Nbs as a generic tool for high-throughput mapping of protein locations in physiological and pathophysiological conditions by combining Nb-based affinity capture with, for example, anti-proteome Nb arrays. Unfortunately, generating Nbs against other signal peptides (e.g., palmytoylation, sumoylation, phosphorylation, S-nitrosylation and Y-nitration) might be very challenging as these sites are less well conserved and/or located in a flexible region of the protein (i.e., poorly antigenic). Nevertheless, the KDEL and chromatin immunoprecipitation examples enforce the idea that Nb-based affinity capture, alone or in combination with other proteomics tools, might serve many purposes of modern proteomics.

- Nbs as crystallization chaperones
Nbs exhibit a good track record as chaperones to crystallize challenging proteins [33–35]. Indeed, these robust, single-domain antibody fragments often lock proteins in a particular conformation [36]. The ability of Nbs to stabilize intrinsically flexible regions or shield aggregating surfaces from contact with solvents are key features to allow the crystallization process [37]. DARPinS and anticalins appear to serve the same purpose, whereas crystallization with scFvs appears to be less successful, possibly because of the intrinsic flexibility of VH and VL interactions, or the linker. The favorable properties of Nbs allowed an impressive list of structures of highly dynamic proteins to be elucidated and identification of unknown conformations, providing insight into the mechanisms of action of their antigens. In addition, Nbs might become an essential tool to obtain valuable
structural information on partially disordered or amyloid proteins [38,39]. For example, conformation-sensitive Nb s stabilize amyloid-β protofibrils and prevent the formation of mature amyloid fibrils [40]. Structural data of such trapped amyloid intermediates might unravel the aggregation process of these tangled targets and improve our understanding of the mechanisms leading to their pathological conversion.

### Intracellular target imaging & immunomodulation

Antibodies acting inside living cells are known as ‘intrabodies’. Nbs fold surprisingly well into functional entities, even in the reducing intracellular environment. Therefore, intracellular expression of Nbs fused to fluorescent proteins produces useful chromobodies or fluobodies that can be used to trace their antigen in living cells [41,42]. This technology has been adapted to advanced super-resolution imaging techniques [43].

The intracellular robustness, together with the target-modulating activity of Nbs, makes them an ideal tool to impair antigen activity inside the cells, producing functional knockouts [41,44–46]. Apart from being a target validation tool, such Nbs might become valuable as leads to investigate molecules currently considered to be ‘undruggable’ (by definition, an undruggable target cannot be modulated appropriately by any currently known, available and safe molecule).

Nbs fused with leader signal peptides can be directed to specific subcellular compartments. The expression of Nbs, even those without intrinsic target inhibitory activity, at these sites might sequester their targets from their normal subcellular compartments, thereby inhibiting the function of the antigen. One example involves the intracellular retention of otherwise secreted proteins such as viral coat proteins [47]. Nbs, fused to a secretory signal peptide and a C-terminal KDEL peptide, sequester both the Nb and its antigen in the endoplasmic reticulum. In another setting, Nb-based intrabodies containing a PEST motif redirected the target antigen to the proteasomal degradation pathway [48]. Likewise, green fluorescent protein-tagged proteins could be depleted in vivo via the ubiquitin depletion pathway by the intracellular expression of a green fluorescent protein-specific Nb fused to the F-box domain [49]. Despite these successful examples, the broad applicability of Nb-based PEST and F-box-mediated protein knockout awaits independent confirmation.

In summary, because of their extraordinary intracellular functionality, Nbs have a clear advantage over other antibody fragments, and they will probably become a valuable research tool. However, the progression of Nb-based intrabodies towards clinical application is less certain and relies on the development of specific and efficient transduction methods. In this respect, RNAi methodologies are certainly more advanced. However, we believe that Nbs might find a niche in cases where post-translationally modified protein variants or isoforms that cannot be discriminated via RNAi need to be targeted.

### Nbs in diagnosis

#### Nbs as probes in novel biosensors

Biosensor development is a popular area of history because sensitive analyte detecting devices are required for on-site application in many major fields, such as medical diagnosis, environmental and food analysis. Many of these innovative biosensors rely on small, robust, highly specific affinity probes, where size and directional immobilization matter. Nbs are particularly suited for controlled and reproducible coupling because site-specific functional groups are easily introduced, allowing covalent and oriented binding with minimal loss of specificity and affinity [50]. The Nbs’ small size provides high binding capacity surfaces resulting in higher sensitivity, and the high stability and refolding competence of Nbs allow stringent washing and regeneration conditions.

Moreover, Nbs directionally immobilized onto solid sensor surfaces allow immunoreactions to occur very close to the sensor–solution interface, resulting in increased sensitivity. Alternatively, the analyte-capturing Nbs can be joined spontaneously to magnetic particles [51], which are suspended in the sample solution to bind analyte before being attracted to the electrode by magnetic fields. The concentration enhancement of the analyte at the surface of the electrode then leads to amplification of the signal.

#### Nbs for noninvasive in vivo imaging

Noninvasive molecular imaging uses labeled tracers to visualize molecules and cellular biomarkers in the whole body without disturbing the host. This technology is very useful to investigate biological processes in animal models and is expected to become a major tool in nuclear medicine departments of hospitals to diagnose and stratify patients following PET and SPECT scans. An ideal (clinical) tracer should be stable in vivo, with the ability to reach all sites in the body, generate signals over a long range of biomarker concentrations, interact with the
biomarker with high affinity and specificity, easy to label with dyes or radionuclides, inexpensive, and nontoxic. In clinical imaging, the generation of contrast at the site of biomarker expression within hours of tracer administration is essential. A Nb meets most, but not all, requirements to become a generic imaging tracer [52]. Nbs are usually stable, easily labeled, have low immunogenicity and have affinities in the low nanomolar to high picomolar range. Their small size assures optimal tissue penetration and fast blood clearance, so that high contrast is attained within hours of administration. Their target specificity has been demonstrated using gene-deficient animals [53] or via coinjection of an unlabeled competitor [54]. In preclinical animal models, we have shown focal uptake of radiolabeled Nbs in tumors targeting markers of cancer cells [52] or tumor-infiltrating macrophages (Figure 2) [53], in arthritic lesions, atherosclerotic plaques [54] and immune organs [53]. A Phase I study with an anti-HER2 Nb for PET imaging of breast cancer patients is currently ongoing.

Nb-based imaging also has some limitations. First, their fast renal excretion leads to high signals in the kidneys and bladder, and imaging of specific tracer uptake at nearby sites is obstructed. Second, Nbs rarely penetrate the blood–brain barrier [55] and, therefore, might be suboptimal in the field of neuroimaging. Third, while Nb-based imaging of cell surface receptors is straightforward, tracing intracellular or soluble targets is more challenging. Finally, GMP production of Nbs is far more expensive than other useful tracers that are based on peptides or chemical compounds.

Nbs for therapy

- Serum half-life extension

Antibody fragments, including Nbs, display short serum half-life profiles. Although this is highly desirable for certain applications, such as imaging with radiolabeled tracers, many therapeutic applications require a slow drug clearance rate to avoid high doses and frequent administration. PEGylation or modification with a serum albumin-specific small chemical compound, and genetic fusion with conformationally disordered polypeptides (e.g., HAPylation and PASylation), with a protein having an inherently long serum half-life (e.g., serum albumin) or with a molecule binding to an abundant serum protein are among the many different approaches used to extend the serum half-life of therapeutics [56]. However, these modifications might have adverse effects on antibody functionality [57].

Nbs specific for abundant serum proteins, such as albumin and IgG, have a long serum half-life [58,59], and fusion of such Nbs to other therapeutic compounds provides a generic approach to extend the plasma half-life of this therapeutic. Other studies described the extension of Nbs’ serum half-life by PEGylation [60], pentamerization and fusion with IgFc [61]. The small amount of data on Nbs’ serum half-life extension makes it difficult to speculate on the preferred method for each application. However, it is evident that methods that adversely affect Nbs’ advantages should be avoided. For instance, fusion of Nbs to albumin or IgFc may complicate the expression of Nbs in microorganisms, and pentamerization (by fusion to verotoxin) may render Nbs

---

Figure 2. Maximal intensity projection of fused pinhole SPECT/micro-computed tomography image of a mouse bearing a TSA tumor implanted in the mammary fat pad. The image was taken 3 h after injection with a 99mTc radio-labeled anti-macrophage mannose receptor nanobody together with an excess of bivalent, unlabeled macrophage mannose receptor nanobody to reduce tracer uptake in organs other than the tumor. Note the accumulation of the tracer inside the tumor, visualizing mannose receptor-positive tumor-infiltrating macrophages [53].
immunogenic. It is also worth noting that fusion with IgG-specific Nb s for serum half-life extension may provoke Ig-mediated immune effector cell activation at undesired locations.

Nbs against envenoming
The preferred serotherapeutic application of Nbs over other antibody formats is illustrated in the field of envenoming caused by scorpions or snakes [62,63]. Current antivenoms are polyclonal immunoglobulin fragments purified from the blood of venom-immunized horses and sheep. However, these antivenoms are often associated with low potency, variable efficacy and severe adverse effects (e.g., serum sickness). This low potency is mainly attributed to a poor immune response of host animals because most toxic compounds within venom are small and poor immunogens. Owing to their small size and the absence of an Fc region, Nbs diffuse rapidly through the body and reach a tissue biodistribution that closely matches that of the small venom toxins. In addition, even after capturing and neutralizing the toxin, the toxin–Nb complex remains sufficiently small to be eliminated rapidly via the kidneys. As a result, a bispecific Nb against the *Androctonus australis hector* AahI and AahII toxin – the most toxic molecules in scorpion venom – was shown to possess, in mouse models, a higher neutralization capacity than the current anti-scorpion toxin immunotherapies [64]. Recently, *in vivo* echocardiography in rats demonstrated the ability of this 30-kDa construct to successfully prevent the fatal hemodynamic disturbances provoked by a lethal dose of venom [65]. Intact camelid IgG antibodies and, in particular, their Nb derivatives are equally or more potent than the conventional antivenom in neutralizing the lethal, hemorrhagic and coagulopathic effects of west African viper (*Echis ocellatus*) venom [62]. Further research to define the pathology-inducing compounds of the complex snake venoms might lead to the identification of Nbs against conserved epitopes, which may eventually result in the development of polyreactive toxin-specific antivenoms. Finally, the production of sustainable Nb-based recombinant antivenoms is expected to contribute favorably to the reproducibility, safety and treatment cost of envenomed victims.

Nbs against infections
Antibodies are natural defense molecules against bacterial, viral and parasite infections. It is well established that polyclonal antibodies are more effective at combating such infections than monoclonal antibodies. However, the possibility to screen thousands of antigen-specific Nbs from immune VH H libraries, in conjunction with their intrinsic capacity to target epitopes, which are cryptic for conventional antibodies, has provided access to a number of neutralizing Nbs against a myriad of pathogens [66,67]. A successful Phase I clinical trial with Nbs targeting respiratory syncytial virus has demonstrated the potential of Nbs to combat infections [102]. Since Nbs are devoid of any Fc region, they do not have Fc-mediated effector functions and are unable to neutralize and eliminate the pathogen. However, several Nbs have an inherent neutralizing effect that is masked in HCAbs because pathogens have evolved to escape this antibody-mediated threat [6]. Alternatively, the strict monomeric nature of Nbs facilitates their fusion with molecules with innovative effector functions, such as enzymes or toxic molecules, in order to eradicate the pathogen. Additional success with Nb therapies will come from more patient-friendly administration (topical, inhalation or oral delivery, and colonizing bacteria-releasing therapeutic Nbs) and from the variety of strategies used to obtain improved blood levels over a prolonged time.

Nbs against amyloidosis
The search for lasting treatments that target the underlying causes and alter the natural course of amyloidosis disorders (e.g., Alzheimer’s, Parkinson’s and Huntington’s diseases), linked with misfolding or aggregation of proteins into fibrillar deposits, is an extremely active area of clinical investigation. Regarding Alzheimer’s disease, active and passive immunotherapy have been suggested for more than a decade as potential strategies to retard or even reverse disease progression. The form of the amyloid-β peptide that leads to synaptic, neurotic and behavioral abnormalities has been difficult to define, although growing evidence suggests that oligomeric amyloid-β intermediates are probably more toxic than the polymeric fibrils [68]. Antibody-mediated interference must prevent the conformational transitions that lead to the assembly of oligomers, protofibrils and fibrils, which ultimately lead to plaque deposition. The clearance should, therefore, preferably be performed before the proteins cluster into the toxic oligomers, by only targeting a specific subset of amyloid-β precursors. The single-domain nature of Nbs and their availability to work with immune repertoires greatly facilitates their isolation as highly specific probes for pathological target structures with excellent affinity [69,70].
In addition, Nbs are readily available for large-scale expression, and if penetration in the CNS appears to be necessary for efficacy, some Nbs that recognize unique epitopes on epithelia have been described to transmigrate through the human blood–brain barrier in vitro and in vivo via active transport [71,72].

## Nbs against pathologies & cancer

Therapeutic antibodies often rely on activation of immune effector mechanisms for optimal efficacy. Due to the lack of Fc, which mediates immune effector cell activation, Nbs in therapeutic intervention are entirely dependent on intrinsic antagonistic antigen targeting, and possibly on agonistic signaling. Conversely, lack of Fc in Nbs may result in fewer unwanted immune-mediated side effects.

The application of Nbs to combat diseases and pathologies mainly focuses on the inhibition of ligand–receptor interactions. Examples include antagonizing anti-von Willebrand factor Nbs to block thrombosis initiation [73], anti-TNF-α Nbs to treat arthritis [74] and EGF–EGFR blocking Nbs to treat cancer [75]. Other examples include Nbs that keep antigens in an inactive receptor state [76] or provoke receptor activation (agonist action) for apoptosis induction of cancer cells. One advantage of Nbs is their small size, which means that dense tissues can be penetrated by bivalent or trivalent Nb complexes [59]. Hence, they are effective even at occluded sites. Since Nbs are usually rapidly excreted from the body, their serum half-life needs to be controlled (see ‘Serum half-life extension’ section).

Recently, Nbs have been employed as a cell (re-)targeting moiety in larger theranostic vehicles, such as lentviruses [77], microbubbles [78], branched nanogold particles [79] and albumin nanoparticles [80], bio-nanocapsules [81], and polymeric micelles [82]. The toxicological profile of these relatively large particles in clinical settings still needs to be evaluated, as well as their targeting capacity after systemic administration.

### Bispecific Nbs & Nb-based T-body

Complex diseases are often multifactorial in nature; therefore, necessitating simultaneous targeting of multiple factors or pathways for maximal therapeutic effect. This can be achieved by combination therapy (i.e., simultaneous administration of different antibodies) or, alternatively, by using multispecific antibodies. Compared with combination therapy, multispecific antibodies provide the possibility to reduce the costs of production, preclinical testing and clinical trials [83]. Bispecific antibodies hold great promise as next-generation therapeutics, especially for cancer treatment [83–85].

The interesting properties of Nbs, in particular their strict monomeric behavior, single-domain nature and minimal size, make them attractive candidates for the development of manifold constructs [86,87]. Based on the publicly available data, this notion has surprisingly largely stayed in the explorative phase. This might be, at least in part, due to the restrictions resulting from intellectual property rights associated with the use of Nbs, taking into account that the major driving force behind bispecific antibodies is the desire to finally bring them to clinics. With some of these patents coming to an end soon, we anticipate much faster progress in the field of bispecific Nbs in the near future.

Bispecific antibodies have often been used to activate immune effector cells and their rerouting to cancer cells [83]. An alternative approach to the use of bispecific antibodies for rerouting and activation of T cells is the T-body approach (i.e., generation of T cells expressing chimeric receptors consisting of an antibody directed to a tumor-specific antigen) [88,89]. The small size and high stability/folding capacity of Nbs suggest that they could offer advantages over other antibody formats for the generation of a T-body. Proof of concept for Nb-based T-bodies has been provided [90], but further studies are required to catalyze the translation of this concept into effective therapies.

### Conclusion & future perspective

The possibility to rapidly obtain in vivo affinity-matured Nbs, combined with their strict monomeric behavior, and favorable biochemical and physical properties, make Nbs unique among multitarget recognition molecules that are currently available. Therefore, it is expected that Nbs will make a substantial difference in therapy, diagnostic screenings and research. Although no Nb-based product is currently approved as a therapeutic agent, the fast identification of a Nb-based lead gives Ablynx-NV a substantial advantage, as shown by the various Nb-based products that are already in the pipeline and/or in advanced clinical phases. Besides Nbs developed to combat cancer, diseases such as amyloidoses, viral infections or toxin envenoming could be treated with future Nb-based therapeutics. Evidently, the development of Nbs into therapeutics remains a high-risk, high-reward business, and commercialization of Nbs will be faster in the diagnostic field and as a research tool. For
diagnostic applications, Nbs developed for non-invasive in vivo imaging of tumors and lesions definitely have great potential. In addition, their application as a highly specific probe on µ-arrays or in novel biosensors will grow steadily in the very near future. Finally, as a research tool, Nbs are already quite useful as crystallization chaperones or when fluorescently labeled as tracers of antigen trafficking inside living cells at the highest possible resolution. The Nbs with an intrinsic antigen-modulating activity (e.g., enzyme activation or inhibition), sometimes even leading to the production of functional antigen knockouts, will be a valuable research tool for target validation.

Acknowledgements
The authors wish to thank all personnel from their laboratory and collaborators all over the world for sharing their exciting data and for their much-appreciated contributions in the past 20 years.

Financial & competing interests disclosure
The authors give special thanks to granting authorities from the EU (AFFINOMICS project 241481), NATO (Science for Peace), Belgium (Ministry of Health), Flanders (IWT and FWO), VIB (Technology funds) and OZR of Vrije Universiteit Brussel, ‘Vlaamse Liga tegen Kanker’ and ‘Stichting tegen kanker’ for their financial support. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Open Access
This work is licensed under the Creative Commons Attribution-NonCommercial 3.0 Unported License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-nd/3.0/

Executive summary

Biochemical & biophysical properties of nanobodies
- An optimized technology platform has been developed to identify nanobodies (Nbs) against soluble, properly folded, immunogenic proteins from the immune variable domain of heavy chain-only antibody phage display libraries.
- Such Nbs are naturally endowed with good biochemical and biophysical properties.

Streamlining the Nb identification platform
- Development of transgenic mice that can be immunized to elicit heavy chain-only antibodies and be used to generate Nbs may further streamline the Nb technology.
- Novel immunization protocols to maximally stimulate the heavy chain-only antibodies’ immune response need to be developed.
- Further streamlining of the Nb identification platform for difficult targets (transmembrane biomarkers) is envisaged by DNA immunization or cell immunization, and panning on cells.
- Next-generation sequencing technologies should be introduced for rapid target-specific Nb identification obviating the need to construct immune libraries and panning.
- Immune Nb libraries against (sub-)proteomes are predicted to constitute a valuable source for novel target identification, especially when linked with phenotypic screening technologies that are still in their infancy.
- Affinity maturation technologies are available, but introducing Nbs in the phage-assisted continuous evolution technology might allow generation of Nbs with novel innate effector functions.
- Tools need to be developed for obtaining cell-specific and cell-penetrating Nbs for intracellular delivery of therapeutics.

Nbs in research
- Nbs are excellent capturing agents to purify targets from complex mixtures or chromatin immunoprecipitation.
- Nbs are key molecules as crystallization chaperones.
- Nbs are useful tools for tracing and immunomodulation of intracellular antigens at the highest resolution.

Nbs in diagnosis
- Nbs have a future as minimal-sized antigen-specific probes in µ-arrays and innovative biosensors.
- Nbs are readily labeled and their fast renal clearance makes them ideal tools for noninvasive in vivo imaging.

Nbs for therapy
- The very fast blood clearance of Nbs emphasizes the need for technologies to increase the blood half-life of Nbs for therapeutic purposes.
- The small, monomeric format of Nbs makes them a perfect plug-and-play tool to generate bispecific Nbs or larger pluripotent constructs with an adaptable serum half-life, that are able to combat envenoming with small toxins, viral, bacterial or parasite infections, and various pathologies.
- Generation of Nbs that cross the blood–brain barrier, the attachment of Nbs to lentiviral particles or even on the surface of bacteria, or the creation of Nbs that are secreted by bacteria will create novel therapeutic opportunities.
References

Papers of special note have been highlighted as:
* of interest
** of considerable interest


25. Introduces an elegant and powerful technology for a phase-assisted continuous directed evolution of genes linked to protein production in Escherichia coli.


29. Injecting nanobodies (Nbs) into mammalian cells using nonpathogenic bacterial strains carrying a TSS5 is a promising technology for in vitro and in vivo intrabody applications that target host cell functions and signaling pathways.


42 Method for using any green fluorescent protein-tagged construct in single-molecule super-resolution microscopy using green fluorescent protein-specific Nbs coupled to organic dyes is described.


Websites

101 Crescendo. www.crescendo.com
102 Ablynx. www.ablynx.com

Nanomedicine (2013) 8(6)