Single-domain antibody fragments derived from heavy-chain antibodies: a review

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ABSTRACT: Single-domain antibody (sdAb) fragments derived from heavy-chain antibodies of camelids and cartilaginous fish represent a new generation of therapeutic agents and immunoreagents. Due to their unique characteristics, such as low molecular weight, high physical-chemical stability, good water solubility, and the ability to bind antigens inaccessible to conventional antibodies, they could potentially act as a substitute for conventional therapeutic drugs in the treatment of serious human diseases, and, moreover, could be broadly used in analyses and diagnostics. In this review article, an analysis of 826 publications oriented to heavy-chain antibodies and their sdAb fragments indexed in the Web of Science® database since 1993 has been carried out. Attention has predominantly been paid to papers published from 2010 to June 2012. Key publications are presented in tables and are characterised by descriptive words, abstracts and references. The presented publications have been sorted according to seven basic criteria: review articles and monographs, heavy-chain antibodies of camelids and sharks, production of sdAb fragments using recombinant technology, characteristic properties of sdAb fragments, application of sdAb fragments in therapy, application of sdAb fragments in diagnostic and immunoanalytical methods and other prospective uses of sdAb fragments. This review article should highlight the typical properties of heavy-chain antibodies and sdAb fragments which differentiate them from conventional antibodies and other available recombinant fragments, and also emphasize their extremely broad application potential, mainly in human disease therapy. At the same time it allows an easy and rapid orientation in numerous publications written on this subject, and facilitates the search for the required data.

Keywords: single-domain antibody fragment; heavy-chain antibody; antigen-binding site; camelid; shark; therapy; recombinant technology

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1. Introduction

A surprising discovery was made in the 1990s: in addition to conventional antibodies, mammals of *Camelidae* family and also some cartilaginous fish have evolved a distinctive type of antibody molecule composed entirely of two identical heavy chains (Hamerscasterman et al. 1993; Greenberg et al. 1995). Because these antibodies are devoid of light chains, they are called heavy-chain antibodies. The antigen-binding site of heavy-chain antibodies is confined to one single domain referred to as the VHH (Variable domain of the Heavy chain of the Heavy-chain antibody) in *Camelidae* and VNAR (Variable domain of the shark New Antigen Receptor) in cartilaginous fish. These variable domains can be easily expressed in bacteria, yeasts or in other hosts as recombinant single-domain antibodies (sdAb), which are the smallest available intact antigen-binding fragments. SdAb fragments derived from llama and camel heavy-chain antibodies are often referred to as nanoantibodies or nanobodies®. Due to their biophysical and pharmaceutical properties, which are conferred by their single-domain nature and small molecule size, sdAb fragments offer a broad application potential, especially as new immunotherapeutic drugs and also as efficient reagents in immunoanalytical and diagnostic methods. The aim of this review is to highlight the unique features of heavy-chain antibodies and sdAb fragments and show their numerous novel feasible applications in research and in clinical development. Publications, which contain key information concerning heavy-chain antibodies and their sdAb fragments, are in this new designed review presented in tables and are characterized with a few descriptive words, full or shortened abstracts and source references. The text in the tables contains several format imperfections, which exist in the Web of Science® database and are caused by transmission and copying of data between various information sources. The missing format of lower and upper indexes (e.g. Ig(2) instead of Ig_2 or 10(–6) instead of 10^{-6}) can be given as a typical example. Special characters, such as α, Å or °C were transcribed as alpha, angstrom or degree C, respectively. The attention is predominantly focused on papers published in the last three years, although some older key papers had been also included. The presented publications have been classified according to seven basic criteria: review articles and monographs, heavy-chain antibodies of camelids and sharks, production of sdAb fragments using recombinant technology, characteristic properties of sdAb fragments, application of sdAb fragments in therapy, application of sdAb fragments in diagnostic and immunoanalytical methods and other prospective uses of sdAb fragments. This review article should facilitate orientation in the above mentioned subjects and enable rapid and easy searching for particular data and information.

2. Review

2.1. Methods of searching and publication analysis

The publications were retrieved from the Web of Science® database using the following search profile: Topic = (nanobod* OR nanoantibod* OR "single-domain antibod*" OR "heavy-chain antibod*" OR "heavy chain-only antibod*" OR VHH OR IgNAR OR "llama* antibod*" OR "camel* antibod*" OR "new antigen receptor"), Timespan = all years or 2010–2012, Citation databases = SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH, BKCI-S and BKCI-SSH, Chemical Databases = CCR-EXPANDED and IC.

Using the above mentioned search profile, a total of 826 publications were retrieved. The first paper describing the occurrence of heavy-chain antibodies was published in 1993 by a Belgium research group. Since 2003, publication activity on the topic has sharply increased (Figure 1). In the last three years, 297 papers were published. In 2012, sixty-four papers have already been published at the time of our manuscript preparation. The older publications are predominantly oriented to the elucidation of the structure and characteristics of heavy-chain antibodies and their antigen-binding sites. The latest papers describe the possibilities of practical use of sdAb fragments, especially in medicine and therapy.

The Web of Science® utilities have been employed for search results analysis. Of all the published papers, original research articles prevail (78.2%). Our analysis shows that 262 institutions from 47 countries are concerned with the subject of sdAb fragments derived from heavy-chain antibodies. Authors, institutions, countries and scientific journals which show the highest publication activities (Top 5) are listed in Table 1.

2.2. Review articles and monographs

Eighteen review articles are listed in Table 2 and characterized by key words (left column), abstracts (in the middle), and references (right column). These papers offer an overall review regarding all important topics, especially about the structure, properties, generation and evolution of heavy-chain antibodies in camelids and sharks (Conrath et al. 2003; Tillib 2011), engineering of sdAb fragments (Deffar et al. 2009), their production, structure and properties (Muyldermans et al. 2001; Harmsen and de Haard 2007), use of sdAb fragments as building blocks for the construction of multivalent and multispecific conjugates (Saerens et al. 2008), application of sdAb fragments in therapy (Van Bockstaele et al. 2009; Wesolowski et al. 2009) and in immunoanalytical and diagnostic methods (Huang et al. 2010; de Marco 2011). All these topics are discussed in detail also in original papers, which are presented in Tables 3 to 8. Recently, the new monograph "Single Domain Antibodies" (Saerens and Muyldermans 2012) has been launched with a complete methodology and key protocols for the construction of sdAb libraries and for the selec-

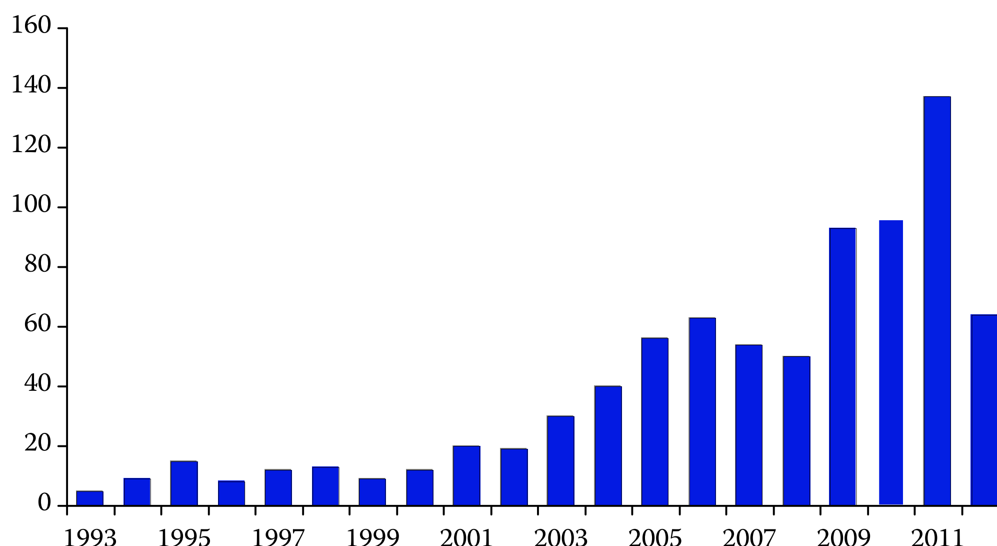

Figure 1. The number of publications on heavy-chain antibodies and single-domain antibody fragments during the period from 1993 to June 2012

tion and expression of sdAb fragments and their advanced derivatives. This publication hihlights the broad application potential of sdAb fragments and can be used as a practical guide to sdAb recombinant technologies.

2.3. Heavy-chain antibodies of camelids and sharks

Heavy-chain antibodies are homodimers of disulphide-linked heavy chains (Figure 2) that occur naturally in the serum of camelids and cartilaginous fish. The total amount of heavy-chain antibodies of all serum immunoglobulins has been determined to be about 45% in llama species, while in camels it is about 75% (Hamerscasterman et al. 1993). The function of heavy-chain antibodies in the immune system has not yet been completely explained. Due to the lack of light chains, the antigen-binding site of heavy-chain antibodies is formed by only three complementary determining regions (CDRs), compared to six CDRs in conventional antibodies. The CDR3 region of the heavy-chain antibody, which usually plays a crucial role in an antibody-antigen interaction, can be formed by a long finger-like polypeptide loop. Therefore, the antigen-binding site of the heavy-chain antibodies often exhibits a convex shape and differs sharply from the concave, groove-shaped antigen-binding site of conventional antibodies. With regard to the shape of the antigen-binding site, heavy-chain antibodies recognise preferably epitopes buried in clefts on protein surfaces, e.g. in enzyme active sites, which are usually less antigenic to conventional antibodies (Lauwereys et al. 1998; Decanniere et al. 1999; De Genst et al. 2006). The convex shaped antigen-binding site of heavy-chain antibodies is not very suitable for the binding of small molecules (haptens). Therefore, only a few papers focused on anti-hapten heavy-chain antibodies have been published to this date (Spinelli et al. 2001; Ladenson et al. 2006; Alvarez-Rueda et al. 2007). Other structural features of heavy-chain antibodies, which differentiate them from conventional antibodies, include a higher proportion of hydrophilic amino acids in variable domains (VHH/VNAR), the presence of disulfide bonds stabilizing the antigen-binding site, and an unusually high number of mutational hot spots responsible for structural variability of heavy-chain antibodies (Harmsen and de Haard 2007). In addition to structural studies, recent research has been focused also on the evolution of heavy-chain antibodies (Nguyen et al. 2002; Flajnik et al. 2011), their possible function in the immune system (Ferrari et al. 2007; Saccodossi et al. 2012), and molecular mechanisms of germline gene segment rearrangement (Nguyen et al. 2000). Some investigators have also used heavy-chain antibodies as a suitable alternative to conventional polyclonal antibodies, mainly for diagnostic purposes (Anderson and Goldman 2008; Torigoe et al. 2012). Twenty-three papers (eleven of them published from 2010 to 2012) are presented in Table 3.

2.4. Production of sdAb fragments using recombinant technology

Due to their simple modular structure and a single-gene nature, sdAb fragments are easily produced *in vitro* as recombinant proteins. The production of sdAb fragments is based on the cloning of VHH or VNAR gene segments into phage display vectors, construction of large phage libraries and selection of high-affinity binders using a biopanning process. SdAb fragments are effectively expressed in different microbial hosts, of which *Escherichia coli, Saccharomyces cerevisiae* and *Pichia pastoris* are the most commonly used (Makvandi-Nejad et al. 2011; Ezzine et al. 2012; Gorlani et al. 2012). Bacterial species enable high-yield expression of sdAb fragments in the cytosol or in periplasm, whereas yeast cells utilize secretory pathways resulting in efficient disulfide bond formation, N-glycosylation and secretion of the recombinant product into the growth medium. Recent papers also describe several alternative strategies for sdAb fragment engineering and production, such as yeast or ribosome display systems (Yau et al. 2003; Ryckaert et al. 2010), expression of recombinant fragments in filamentous fungi (Joosten et al. 2005), in mammalian cells (Bazl et al. 2007) and in transgenic mice (Zou et al. 2005). Mutagenesis and recombination of CDR regions to improve the affinity and specificity of sdAb fragments have also been discussed (Swain et al. 2010; Fanning and Horn 2011). Current studies are focused on the development of new cloning and expression strategies to produce humanised fragments, multivalent constructs and fusions of antibody fragments to other proteins (Saerens et al. 2005; Vincke et al. 2009; Bell et al. 2010; Pollithy et al. 2011). Selected articles are presented in Table 4.

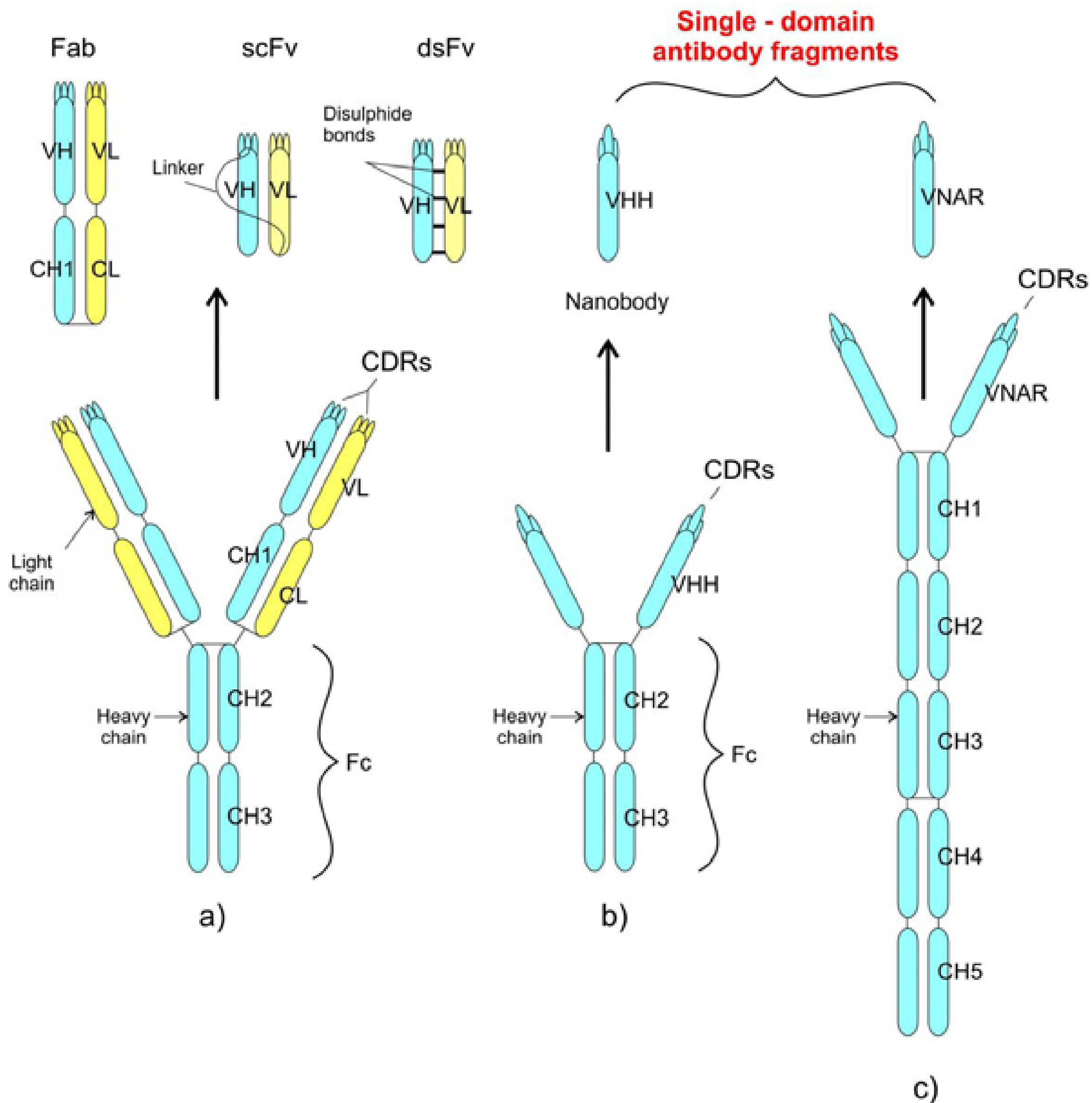

Figure 2. Structure of antibodies. (**a**) Molecule of conventional antibody (down), recombinant fragments (up); (**b**) molecule of camelid heavy-chain antibody (down), single-domain antibody fragment (up); (**c**) molecule of shark heavy-chain antibody (down), single-domain antibody fragment (up)

2.5. Characteristic properties of sdAb fragments

Recombinant sdAb fragments are formed from only one single polypeptide chain containing about 120 amino acids. Due to their size (4 × 2.5 nm) and molecular weight (12 kDa to 15 kDa), sdAb fragments are the smallest available recombinant antibodies. Their small molecular size enables sdAb to penetrate easily into tissues and intercellular spaces, but is, however, the cause of their rapid clearance from the blood by renal filtration (Cortez-Retamozo et al. 2002). The rapid clearance of sdAb fragments from the blood by kidneys can negatively affect their therapeutic activity. Therefore, several strategies to prolong the half-life of sdAb fragments in serum have been described, e.g. conjugation of sdAb to another recombinant fragment binding specifically to serum albumins or immunoglobulins (Harmsen et al. 2005). SdAb fragments that bind to and are internalised by cerebromicrovascular endothelial cells are able to cross the blood-brain barrier and transmigrate into tissues of the central nerve system (Abulrob et al. 2005). Due to their simple monomeric structure, sdAb fragments are conformationally very stable (Dumoulin et al. 2002) and refold easily after heating to achieve their original native structure (Dolk et al. 2005b; Walper et

al. 2012). They show an increased resistance to high pressure and to low pH (Dumoulin et al. 2002) and maintain their biological activity even in an environment with a high concentration of proteases and detergents (Dolk et al. 2005a; Hussack et al. 2011a). Owing to a higher content of hydrophilic amino acids, sdAb fragments are characterised by good solubility in water and limited agglutination ability (Conrath et al. 2005). Another characteristic feature particularly concerns the affinity of sdAb fragments, which remains usually equivalent to that of the native heavy-chain antibodies. This is a substantial difference from other recombinant fragments, that show often lower affinity compared with their polyclonal or monoclonal counterparts. Eight papers published from 2010 to 2012 describing typical properties of sdAb fragments are presented in Table 5. Eight older articles should also be considered.

2.6. Application of sdAb fragments in therapy

The unique physicochemical and pharmacological properties of camelid and shark sdAb fragments give them a prospective use as new-generation therapeutic agents for the treatment of serious human diseases. At present, some sdAb-based drugs are in the stage of preclinical testing on animal models or *in vitro*. Some other drugs have advanced to phase I or II of clinical testing in volunteers. The therapeutic effect of sdAb fragments is particularly based on the following principles: Firstly, a remarkable preference of sdAb fragments for binding into clefts and cavities on protein surfaces offers the possibility to develop selective therapeutics for efficient inhibition of enzymes (Paalanen et al. 2011), neutralization of proteolytic toxins (Hussack et al. 2011b) and activity modulation of cell surface proteins, such as receptors, ion channels and leukocyte ecto-enzymes (Wei et al. 2011; Altintas et al. 2012) involved in cancer and inflammatory diseases. Moreover, sdAb fragments recognize also cryptic epitopes hidden deeply in the clefts of virus capsids and in surface envelopes of parasites (Stijlemans et al. 2004; Henderson et al. 2007; Forsman et al. 2008). Secondly, intracellular expression of sdAb fragments as "intrabodies" is a potential strategy to target intracellular antigens, e.g. anti-apoptotic proteins, oncogenes or several viral proteins (Vercruysse et al. 2010). Thirdly, the high structural stability of sdAb fragments in harsh conditions makes them ideally suited for the immunotherapy of gastrointestinal disorders using oral administration (Harmsen et al. 2006; Vandenbroucke et al. 2010). Fourthly, the ability of sdAb fragments to transmigrate across the blood-brain barrier and to prevent amyloid plaque formation could be utilized in the diagnostics and therapy of neurodegenerative diseases (De Genst et al. 2010; Rutgers et al. 2011). Fifthly, a modular nature of sdAb fragments allows an easy engineering of multivalent or multispecific formats that show an increased therapeutic potency compared with monovalent sdAb fragments. Recent research is focused also on the generation of bifunctional constructs by coupling of sdAb fragments with enzymes and toxic substances for specific drug delivery to bacterial or tumour cells (Zhang et al. 2004; Stone et al. 2007). Finally, the non-human origin of camelid and shark sdAb fragments could elicit their neutralisation by the human immune response and a decrease in their therapeutic effect. Therefore, a general strategy to produce humanised sdAb fragments has been described as a promising way for lowering the potential risk of immunogenicity of therapeutic sdAb fragments (Vincke et al. 2009). More than half of the 826 analysed papers discuss possible therapeutic applications of sdAb fragments. Key publications are presented in Tables 6A to H.

2.7. Application of sdAb fragments in diagnostic and immunoanalytical methods

Their effective penetration into tissues makes sdAb fragments good candidates for the construction of imaging probes used for *in vivo* monitoring of tumours, metastatic lesions, amyloid fibrils, etc. Such immuno-imaging probes are prepared by the labelling of sdAb fragments with short-lived isotopes, mainly with ^{99m}Tc. In contrast to radiolabelled monoclonal antibodies, small sdAb fragments show rapid antigen targeting and fast clearance from blood resulting in a contrast-enhanced imaging signal, reduced accumulation of labelled fragments in liver and lower radiation burden. The main limitation of sdAb-based imaging probes, however, is their high non-specific uptake in kidney and bladder (Vaneycken et al. 2010, 2011b). An innovative approach based on sdAb fragments fused to fluorescent proteins and expressed in living cells as "chromobodies" offers the possibility to trace intracellular antigens and to modulate protein function in living cells (Schmidthals et al. 2010). Nanoantibodies can also be used in immunoassays and in biosensors

for the quantitative analysis of contaminants and toxins in food and environmental samples (Conway et al. 2010; Dona et al. 2010; Kim et al. 2012) and also for diagnostics of serious human diseases (De Marni et al. 2012). Their small size and lower aggregation propensity enable immobilisation of extremely high numbers of nanoantibody molecules on the surface of the well of microtitre plate or biosensor transducer, which leads to a substantial increase in the sensitivity of the immunodetection system. Moreover, highly stable sdAb fragments are resistant to denaturing conditions used during biochip regeneration. For details of new publications see Table 7.

2.8. Other prospective uses of sdAb fragments

Besides possible therapeutic, diagnostic and immunoanalytic applications, sdAb fragments derived from camelid and shark heavy-chain antibodies can also be employed as crystallography chaperones to stabilise the conformation of proteins during crystallisation trials (Abskharon et al. 2011). Immunoaffinity chromatography represents another prospective field for sdAb fragment application. SdAb fragments are highly resistant to the effects of organic solvents used for the elution of the captured ligands and for regeneration of immunoaffinity columns (Franco et al. 2010). Recent applications, such as the development of transport systems across epithelia (Iqbal et al. 2011), inactivation of phages infecting dairy bacterial cultures (Hultberg et al. 2007) and mimotope selection strategies (Simmons et al. 2008) show the extremely broad application potential of sdAb fragments. Ten recent publications discussing some alternative applications of sdAb fragments are listed in Table 8. This table also includes two older papers which make the review complete.

Table 1. Authors, countries, institutions and journals (Top 5) showing the highest publication activities concerning heavy-chain antibodies and single-domain antibody fragments (Web of Science®, 826 papers from 1993 to June 2012)

Item	Number of publications
Authors (685 in total)	
Muyldermans S	96
Wyns L	32
Rahbarizadeh F	28
Conrath K	25
Tanha J	23
Verrips CT	23
Institutions (262 in total)	
Free University of Brussels/Flanders Institute for Biotechnology	188
Utrecht University	61
National Research Council Canada	47
Ablynx NV	32
Ghent University	23
Countries (47 in total)	
USA	200
Belgium	193
Netherlands	115
England	78
France	63
Journals (140 in total)	
Journal of Biological Chemistry	30
Journal of Molecular Biology	25
Molecular Immunology	24
Journal of Immunological Methods	22
PLoS ONE	19

Table 2. Important review articles and monographs concerning heavy-chain antibodies and single-domain antibody fragments

Camelid immunoglobulin **IgG concentration** **Single radial immunodiffusion** **Failure of passive transfer**	Camelid immunoglobulins differ from all other known antibodies and contradict all common theories on antibody diversity. It was demonstrated that up to 75% of all serum proteins are immunoglobulin G (IgG) molecules lacking light chains. IgG(2) and IgG(3), which only consist of heavy chains, have a low molecular weight which improves their biodistribution and allows a better tissue penetration. Of special importance is the long complementary determining region (CDR) loop which inserts deep into the active site of an enzyme. This binding property was only observed in experiments to gain structural data and to point out the extraordinary value of heavy chain antibodies as biochemical and pharmacological tools. The acquisition and absorption of adequate amounts of colostral immunoglobulins are essential to the health of the neonate, Pre-colostrum serum IgG levels in camelids are low, with concentrations of 0.26 ± 0.23 mg/ml. Maximum IgG levels are reached after 24 h and kept at a plateau with concentrations of 24.52 ± 8.8 mg/dl. IgG concentrations above 10 mg/ml. indicate a successful passive transfer. IgG levels decline after 2–5 weeks and a marked increase is observed between one and two months, indicating that the immune system of the neonate has started to mature. A number of different tests are available for the assessment of IgG serum levels. Single radial immunodiffusion (SRID) is the only method that specifically measures serum IgG concentrations. It is a reliable assay to test failure of passive transfer (FPT). FPT is a major factor in neonatal mortality in camelids, but very little has been published so far. Therapeutic administration of colostrum will provide passive protection against infectious diseases for a 2–3-week period of risk, and the intravenous administration of 20–40 ml of camelid plasma helps to combat FPT.	Wernery 2001
Evolution **Jawed-vertebrates** **Sharks** **Immunoglobulin genes**	Antibodies of jawed-vertebrates are composed of paired heavy (H) and light (L) polypeptide chains. Surprisingly, the sera of camelids, nurse shark and wobbegong shark, and possibly ratfish contain antibodies that lack L-chains. In camelids, these Heavy-chain antibodies (HCAbs) are gamma-isotypes, and are functional in antigen binding. In this review we focus on the dedicated immunoglobulin (Ig) genes that encode the HCAb in Camelidae (camels, dromedaries and llamas), about their origin, and how these camel immumoglobulins evolved and acquire a large and diverse repertoire of antigen binding sites in absence of the H-L combinatorial diversity.	Conrath et al. 2003
Heavy-chain antibody **Nanobody**	It is well established that all camelids have unique antibodies circulating in their blood. Unlike antibodies from other species, these special antibodies are devoid of light chains and are composed of a heavy-chain homodimer.These so-called heavy-chain antibodies (HCAbs) are expressed after a V-D-J rearrangement and require dedicated constant gamma-genes. An immune response is raised in these so-called heavy-chain antibodies following classical immunization protocols. These HCAbs are easily purified from serum, and the antigen-binding fragment interacts with parts of the target that are less antigenic to conventional antibodies. Since the antigen-binding site of the dromedary HCAb is comprised in one single domain referred to as variable domain of heavy chain of HCAb (VHH) or nanobody (Nb), we designed a strategy to clone the Nb repertoire of an immunized dromedary and to select the Nbs with specificity for our target antigens. The monoclonal Nbs are well produced in bacteria, are very stable and highly soluble, and bind their cognate antigen with high affinity and specificity. We have successfully developed recombinant Nbs for research purposes, as probe in biosensors, to diagnose infections, and to treat diseases like cancer or trypanosomosis.	Muyldermans et al. 2009

Nanobody technology	This short review provides an introduction to the rapidly developing field of generation and utilization of "camel nanoantibodies" (or "nanobodies"). The term "nanoantibody"or "nanobody" was given to single-domain variable fragments of special type of antibodies that naturally exist (in addition to classical types of antibodies) in blood of Camelidae family animals and in some chondrichthyan fishes. The existence of very efficient technology of nanobody generation and some very useful characteristic features promise a big potential for their use in immunobiotechnology and medicine.	Tillib 2011
Nanobodies **Propeties** **Production** **Applications**	Nanobodies are antibody-derived therapeutic proteins that contain the unique structural and functional properties of naturally occurring heavy-chain antibodies. The Nanobody technology was originally developed following the discovery that camelidae (camels and llamas) possess fully functional antibodies that lack light chains. These heavy-chain antibodies contain a single variable domain (VHH) and two constant domains (CH(2) and CH(3)). Importantly, the cloned and isolated VHH domain is a perfectly stable polypeptide harboring the full antigen-binding capacity of the original heavy-chain antibody. These newly discovered VHH domains with their unique structural and functional properties form the basis of a new generation of therapeutic antibodies which were named Nanobodies. The aim of this paper is to show the properties of Nanobodies, their production and expression, applications and their clinical status.	Deffar et al. 2009
Biotechnological applications **Selection systems**	Camelids produce functional antibodies devoid of light chains of which the single N-terminal domain is fully capable of antigen binding. These single-domain antibody fragments (VHHs or Nanobodies) have several advantages for biotechnological applications. They are well expressed in microorganisms and have a high stability and solubility. Furthermore, they are well suited for construction of larger molecules and selection systems such as phage, yeast, or ribosome display. This minireview offers an overview of their properties as compared to conventional antibodies, their production in microorganisms, with a focus on yeasts, and their therapeutic applications.	Harmsen and de Haard 2007
Antigen-binding site **Mutations** **VH-VL pair** **Novel epitopes**	The antigen-binding site of antibodies from vertebrates is formed by combining the variable domains of a heavy chain (VH) and a light chain (VL). However, antibodies from camels and Ilamas are an important exception to this in that their sera contain, in addition, a unique kind of antibody that is formed by heavy chains only The antigen-binding site of these antibodies consists of one single domain, referred to as VHH. This article reviews the mutations and structural adaptations th at have ta ken place to reshape a VH of a VH-VL pair into a single-domain VHH with retention of a sufficient variability, The VHH has a potent antigen-binding capacity and provides the advantage of interacting with novel epitopes that are inaccessible to conventional VH-VL pairs.	Muyldermans et al. 2001
Cancer therapeutics	Over the years, many antibodies have been successfully generated to treat patients with life-threatening diseases, most notably cancer. While the first generation of antibodies, originating from mice, caused severe side effects and were relatively inefficient, technological advances have made it possible to obtain fully human antibodies for therapeutic use. 'Heavy-chain only' antibodies have recently been discovered in the blood of camelids. Because of their size, the antigen-binding units of these antibodies comprising only a single Ig fold are called Nanobodies. These antibody fragments have several remarkable features that make them ideal candidates as next-generation cancer therapeutics. Particularly appealing is their ability to simultaneously inhibit various crucial growth factor receptors or their ligands with a single molecule. In addition, they are easy to clone and express on the tip of filamentous phage, which opens the possibility to select for Nanobodies inducing particular biological effects. Nanobodies have potential to become important cancer therapeutics in the near future, displaying unequalled and unprecedented efficacies in treatment.	Roovers et al. 2007
Structural properties **Therapeutic application** **Clinical trials** **ALX-0081**	Nanobodies are therapeutic proteins derived from the heavy-chain variable (V(H)H) domains that occur naturally in heavy-chain-only Ig molecules in camelidae. These V(H)H domains are the smallest known antigen-binding antibody fragments. Nanobodies can be easily produced in prokaryotic or eukaryotic host organisms, and their unique biophysical and pharmacological characteristics render these molecules ideal candidates for drug development. This review describes the structural properties of nanobodies and focuses on their unique features, which distinguishes these molecules from other antibody formats and small-molecule drugs. Possible therapeutic applications of nanobodies are discussed and data from phase I clinical trials of thenovel 'first-in-class' anti-thrombotic agent ALX-0081 (Ablynx NV) are presented.	Van Bockstaele et al. 2009

Therapeutic applications **Viruses**	In 1989, a new type of antibody was identified, first in the sera of dromedaries and later also in all other species of the Camelidae family. These antibodies do not contain a light chain and also lack the first constant heavy domain. Today it is still unclear what the evolutionary advantage of such heavy chain-only antibodies could be. In sharp contrast, the broad applicability of the isolated variable antigen-binding domains (VHH) was rapidly recognized, especially for the development of therapeutic proteins, called Nanobodies. Here we summarize first some of the unique characteristics and features of VHHs. These will next be described in the context of different experimental therapeutic applications of Nanobodies against different viruses: HIV, Hepatitis B virus, influenza virus, Respiratory Syncytial virus, Rabies virus, FMDV, Poliovirus, Rotavirus, and PERVs. Next, the diagnostic application of VHHs (Vaccinia virus, Marburg virus and plant Tulip virus X), as well as an industrial application (lytic lactococcal 936 phage) will be described. In addition, the described data show that monovalent Nanobodies can possess unique characteristics not observed with conventional antibodies. The straightforward formatting into bivalent, multivalent, and/or multispecific Nanobodies allowed tailoring molecules for potency and cross-reactivity against viral targets with high sequence diversity.	Vanlandschoot et al. 2011
VHH **VNAR** **Immune function modulating** **Toxin/microbe targeting**	Antibodies are important tools for experimental research and medical applications. Most antibodies are composed of two heavy and two light chains. Both chains contribute to the antigen-binding site which is usually flat or concave. In addition to these conventional antibodies, llamas, other camelids, and sharks also produce antibodies composed only of heavy chains. The antigen-binding site of these unusual heavy chain antibodies (hcAbs) is formed only by a single domain, designated VHH in camelid hcAbs and VNAR in shark hcAbs. VHH and VNAR are easily produced as recombinant proteins, designated single domain antibodies (sdAbs) or nanobodies. The CDR3 region of these sdAbs possesses the extraordinary capacity to form long fingerlike extensions that can extend into cavities on antigens, e.g., the active site crevice of enzymes. Other advantageous features of nanobodies include their small size, high solubility, thermal stability, refolding capacity, and good tissue penetration in vivo. Here we review the results of several recent proof-of-principle studies that open the exciting perspective of using sdAbs for modulating immune functions and for targeting toxins and microbes.	Wesolowski et al. 2009
Immunosensor **Immunoaffinity chromatography** **Immuno-imaging**	With the advent of new antibody engineering technologies, conventional antibodies have been minimized into smaller antibody formats. Small size is an important advantage for current and future diagnostic development. Nanobodies (Ablynx) are among the smallest known antigen-binding antibody fragments, and are derived from the heavy-chain only antibodies that occur naturally in the serum of Camelidae. Endowed by natural evolution, these Nanobodies inherently exhibit unique biophysical, biochemical and pharmacological characteristics. In addition to their excellent potential as molecules in drug development, Nanobodies possess very attractive functional properties that aid in their development for diagnostic tools. Here we present several examples of currently available applications of Nanobodies to the field of immunosensor for cancer, immunoaffinity chromatography, in vivo and intracellular imaging.	Huang et al. 2010
Biotechnological applications	The discovery of the single-domain antibody's potentials has stimulated their use in an increasing variety of fields. The rapid accumulation of articles describing new applications and further developments of established approaches has made it, therefore, necessary to update the previous reviews with a new and more complete summary of the topic. Conclusions: Beside the necessary task of updating, this work analyses in detail some applicative aspects of the single-domain antibodies that have been overseen in the past, such as their efficacy in affinity chromatography, as co-crystallization chaperones, protein aggregation controllers, enzyme activity tuners, and the specificities of the unconventional single-domain fragments.	de Marco 2011

Immuno-imaging	Immuno-imaging is a developing technology that aims at studying disease in patients using imaging techniques such as positron emission tomography in combination with radiolabeled immunoglobulin derived targeting probes. Nanobodies are the smallest antigen-binding antibody-fragments and show fast and specific targeting in vivo. These probes are currently under investigation as therapeutics but preclinical studies indicate that nanobodies could also become the next generation of magic bullets for immuno-imaging. Initial data show that imaging can be performed as early as one hour post-injection enabling the use of short-lived radio-isotopes. These unique properties should enable patient friendly and safe imaging protocols. This review focuses on the current status of radiolabeled nanobodies as targeting probes for immuno-imaging.	Vaneycken et al. 2011a
Man-made conjugate	Antibodies are large and complex molecules, with two identical parts that bind independently of each other onto the antigen and the third part of the molecule that dictates the effector function(s). To improve the therapeutic value of antibodies, protein-engineering endeavors reduced the size of the antigen-binding moiety to a single-domain unit. Occasionally, it was demonstrated that the single-domain antigen-binding derivatives of antibodies can have – on their own – an agonistic (or antagonistic) effect on their target. The small size and strict monomeric behavior, in combination with other biochemical properties such as high solubility and high specificity and affinity for the cognate antigen, make single-domain antibodies ideal to design novel man-made conjugates harnessed with innovative effector functions outside the reach of classical antibodies.	Saerens et al. 2008
Smaller recombinant antibody fragments **Multivalent/multispecific reagent** **Single antibody domains** **Enhanced therapeutic efficacy**	With 18 monoclonal antibody (mAb) products currently on the market and more than 100 in clinical trials, it is clear that engineered antibodies have come of age as biopharmaceuticals. In fact, by 2008, engineered antibodies are predicted to account for > 30% of all revenues in the biotechnology market. Smaller recombinant antibody fragments (for example, classic monovalent antibody fragments (Fab, scFv)) and engineered variants (diabodies, triabodies, minibodies and single-domain antibodies) are now emerging as credible alternatives. These fragments retain the targeting specificity of whole mAbs but can be produced more economically and possess other unique and superior properties for a range of diagnostic and therapeutic applications. Antibody fragments have been forged into multivalent and multispecific reagents, linked to therapeutic payloads (such as radionuclides, toxins, enzymes, liposomes and viruses) and engineered for enhanced therapeutic efficacy. Recently, single antibody domains have been engineered and selected as targeting reagents against hitherto immunosilent cavities in enzymes, receptors and infectious agents. Single-domain antibodies are anticipated to significantly expand the repertoire of antibody-based reagents against the vast range of novel biomarkers being discovered through proteomics. As this review aims to show, there is tremendous potential for all antibody fragments either as robust diagnostic reagents (for example in biosensors), or as nonimmunogenic in vivo biopharmaceuticals with superior biodistribution and blood clearance properties.	Holliger and Hudson 2005
Molecular imaging **Target-oriented therapies** **Antibody derivative** **Alternative protein**	The rapid and ongoing discovery of new disease related biomarkers leads to a dramatic paradigm change in human healthcare and constitutes the basis for a truly personalized medicine. Molecular imaging enables early detection and classification of human diseases and provides valuable data for optimized, target-oriented therapies. By now, the biochemical and physiological properties of antibody derivatives or alternative protein scaffolds can be engineered for the detection of a wide range of target structures. The successful application of these reagents in animals, xenograft models and cells in preclinical research clearly demonstrate their utility for molecular imaging. Despite these promising perspectives, only a few antibodies and recombinant proteins are used yet for molecular imaging in human medicine. Especially the high safety demands and the need to eliminate off target effects in humans require extensive research and development efforts.	Romer et al. 2011

X-ray crystallography Crystallization chaperone Conformational heterogeneity	The preparation of diffraction quality crystals remains the major bottleneck in macromolecular X-ray crystallography. A crystallization chaperone is an auxiliary protein, such as fragments of monoclonal antibodies, that binds to and increases the crystallization probability of a target molecule of interest. Such chaperones reduce conformational heterogeneity, mask counterproductive surfaces while extending surfaces predisposed to forming crystal contacts, and provide phasing information. Crystallization chaperones generated using recombinant technologies have emerged as superior alternatives that increase the throughput and eliminate inherent limitations associated with antibody production by animal immunization and the hybridoma technology.	Koide 2009
Recently published monograph Single Domain Antibodies	http://www.springer.com/biomed/immunology/book/978-1-61779-967-9 The development of the hybridoma technology created the possibility to obtain unlimited amounts of monoclonal antibodies (mAb) with high specificity and affinity for any target and to introduce mAbs in a wide range of applications. Examples of antibody-based drugs in therapeutic settings and antibody-based probes in diagnostics are infinite. However, the bulky size of mAbs, costly production, and cumbersome engineering retarded or hampered regularly their streamlined development in some applications. Consequently, mAbs became the focus of many attempts to minimize the size and complexity of their antigen-binding fragments. Eventually, these efforts led to the recombinant production of smaller antigenbinding fragments such as Fab or scFv (where a synthetic linker connects the variable domains of heavy and light chain, i.e., VH and VL), and even sdAbs (single domain antibodies derived mostly of the VH). Although the first set of sdAbs offered significant advantages, they also suffered from multiple shortcomings, all of which have been remediated by elegant engineering. Interestingly, while scientists were designing, engineering, and shaping the ideal sdAb, a serendipitous discovery showed that a similar engineering occurred already in nature in the camelids, and later on, it was found that cartilaginous fish antibodies performed the exercise even earlier on in evolution. These animals have in their blood functional antibody isotype composed of heavy chains – only that lack light chains, in addition to the classical antibodies containing two heavy and two light chains. These heavy-chain antibodies (HCAbs) recognize the antigen via a single variable domain, referred to as VHH or V-NAR. The VHH or V-NAR is the smallest intact antigen-binding fragment that can be produced recombinantly at low cost. The valuable properties of man-made sdAbs, VHHs, and V-NARs including solubility and stability, high affinity and specificity for their cognate antigen, small size and strict monomeric behavior offer many opportunities. As a result, several spin-off companies have been founded in Australia, Belgium, England, Germany, Netherlands, and Scotland that introduced these proteins successfully in a wide range of applications to cover a special need in research or even to produce next-generation therapeutics in the clinic (Preface by the editors). **Titles of the contributions:** PART I OVERVIEW OF SINGLE DOMAIN ANTIBODIES From Whole Monoclonal Antibodies to Single Domain Antibodies: Think Small Introduction to Heavy Chain Antibodies and Derived Nanobodies Overview and Discovery of IgNARs and Generation of VNARs PART II SINGLE DOMAIN ANTIBODY LIBRARY CONSTRUCTION Creation of the Large and Highly Functional Synthetic Repertoire of Human VH and Vκ Domain Antibodies Preparation of a Naïve Library of Camelid Single Domain Antibodies PART III SELECTION OF SINGLE DOMAIN ANTIBODIES Selection by Phage Display of Single Domain Antibodies Specific to Antigens in Their Native Conformation Semiautomated Panning of Naive Camelidae Libraries and Selection of Single-Domain Antibodies Against Peptide Antigens Pichia Surface Display: A Tool for Screening Single Domain Antibodies Bacterial Two Hybrid: A Versatile One-Step Intracellular Selection Method Intracellular Antibody Capture (IAC) Methods or Single Domain Antibodies Selection of Functional Single Domain Antibody Fragments for Interfering with Protein–Protein Interactions Inside Cells: A "One Plasmid" Mammalian Two-Hybrid System	Saerens and Muyldermans 2012

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Table 3. Camelid and shark heavy-chain antibodies

Heavy-chain dimer **Camelus dromedarius** **Antigen-binding repertoire** **CH1 domain** **Antibody engineering**	Random association of VL and VH repertoires contributes considerably to antibody diversity. The diversity and the affinity are then increased by hypermutation in B cells located in germinal centres. Except in the case of 'heavy chain' disease, naturally occurring heavy-chain antibodies have not been described, although antigen binding has been demonstrated for separated heavy chains or cloned VH domains. Here we investigate the presence of considerable amounts of IgG-like material of M(r) 100K in the serum of the camel (Camelus dromedarius). These molecules are composed of heavy-chain dimers and are devoid of light chains, but nevertheless have an extensive antigen-binding repertoire, a finding that calls into question the role of light chains in the camel. Camel heavy-chain IgGs lack CH1, which in one IgG class might be structurally replaced by an extended hinge. Heavy-chain IgGs are a feature of all camelids. These findings open new perspectives in the engineering of antibodies.	Hamerscasterman et al. 1993
Nurse shark **New antigen receptor** **Sequence analysis** **Rearrangement** **Somatic diversification**	Immunoglobulin and T-cell receptor (TCR) molecules are central to the adaptive immune system, Sequence conservation, similarities in domain structure, and usage of similar recombination signal sequences and recombination machinery indicate that there was probably a time during evolution when an ancestral receptor diverged to the modern-day immunoglobulin and TCR(1–3). Other molecules that undergo rearrangement have not been described in vertebrates, nor have intermediates been identified that have features of both these gene families, We report here the isolation of a new member of the immunoglobulin superfamily from the nurse shark, Ginglymostoma cirratum, which contains one variable and five constant domains and is found as a dimer in serum. Analyses of complementary DNA clones show extensive sequence diversity within variable domains, which is generated by both rearrangement and somatic diversification mechanisms. Our results suggest that rearranging loci distinct from immunoglobulin and TCR have arisen during evolution.	Greenberg et al. 1995
Amino acid substitution **Disulphide bond** **Cystein**	The antigen-binding fragment of functional heavy chain antibodies (HCAbs) in camelids comprises a single domain, named the variable domain of heavy chain of HCAbs (VHH). The VHH harbors remarkable amino acid substitutions in the framework region-2 to generate an antigen-binding domain that functions in the absence of a light chain partner. The substitutions provide a more hydrophilic, hence more soluble, character to the VHH but decrease the intrinsic stability of the domain. Here we investigate the functional role of an additional hallmark of dromedary VHHs, i.e. the extra disulfide bond between the first and third antigen-binding loops. After substituting the cysteines forming this interloop cystine by all 20 amino acids, we selected and characterized several VHHs that retain antigen binding capacity. Although VHH domains can function in the absence of an interloop disulfide bond, we demonstrate that its presence constitutes a net advantage. First, the disulfide bond stabilizes the domain and counteracts the destabilization by the framework region-2 hallmark amino acids. Second, the disulfide bond rigidifies the long third antigen-binding loop, leading to a stronger antigen interaction. This dual beneficial effect explains the in vivo antibody maturation process favoring VHH domains with an interloop disulfide bond.	Govaert et al. 2012

Amino acid alignment **CDR identification** **Conformation study**	Camelids have a special type of Ab, known as heavy chain Abs, which are devoid of classical Ab light chains. Relative to classical Abs, camelid heavy chain Abs (cAbs) have comparable immunogenicity, Ag recognition diversity and binding affinities, higher stability and solubility, and better manufacturability, making them promising candidates for alternate therapeutic scaffolds. Rational engineering of cAbs to improve therapeutic function requires knowledgeof the differences of sequence and structural features between cAbs and classical Abs. In this study, amino acid sequences of 27 cAb variable regions (V(H)H) were aligned with the respective regions of 54 classical Abs to detect amino acid differences, enabling automatic identification of cAb V(H)H CDRs. CDR analysis revealed that the H1 often (and sometimes the H2) adopts diverse conformations not classifiable by established canonical rules. Also, although the cAb H3 is much longer than classical H3 loops, it often contains common structural motifs and sometimes a disulfide bond to the H1. Leveraging these observations, we created a Monte Carlo-based cAb V(H)H structural modeling tool, where the CDR H1 and H2 loops exhibited a median root-mean-square deviation to natives of 3.1 and 1.5 angstrom, respectively. The protocol generated 8–12, 14–16, and 16–24 residue H3 loops with a median root-mean-square deviation to natives of 5.7, 4.5, and 6.8 angstrom, respectively. The large deviation of the predicted loops underscores the challenge in modeling such long loops. cAb V(H)H homology models can provide structural insights into interaction mechanisms to enable development of novel Abs for therapeutic and biotechnological use.	Sircar et al. 2011
Primary structure **MALDI-TOF/TOF** **LC-MS/MS** **Sequencing**	The primary structure of a 13.6 kDa single heavy chain camelid antibody (V(H)H) was determined by matrix-assisted laser desorption ionization-time-of-flight/time-of-flight (MALDI-TOF/TOF) top-down sequence analysis. The majority of the sequence was obtained by mass spectrometric de novo sequencing, with the N-terminal 14 amino acid residues being determined using T(3)-sequencing and database interrogation. The determined sequence was confirmed by liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of a tryptic digest, which also provided high-energy collisionally induced dissociation (CID) data permitting the clear assignment of three of the 14 isobaric Leu/Ile residues. Five of the 11 Leu/Ile ambiguities could be resolved by homology comparisons with known V(H)H sequences. The monoisotopic molecular weight of the was determined by ultrahigh-resolution orthogonal electrospray (ESI)-TOF analysis and found to be 13 610.6066 Da, in excellent agreement with the established sequence. To our knowledge, this is the first time that the entire primary structure of a protein with a molecular weight > 13 kDa has been established by mass spectrometric top-down sequencing.	Resemann et al. 2010
VHH/hapten interactions **Azo-dye** **X-ray structure**	Camelids, camels and llamas, have a unique immune system able to produce heavy-chain only antibodies. Their VH domains (VHHs) are the smallest binding units produced by immune systems, and therefore suitable for biotechnological applications through heterologous expression. The recognition of protein antigens by these VHHs is rather well documented, while less is known about the VHH/hapten interactions. The recently reported X-ray structure of a VHH in complex with a copper-containing azo-dye settled the ability of VHH to recognize haptens by forming a cavity between the three complementarity-determining regions (CDR). Here we report the structures of a VHH (VHH A52) free or complexed with an azo-dye, RR1, without metal ion. The structure of the complex illustrates the involvement of CDR2, CDR3 and a framework residue in a lateral interaction with the hapten. Such a lateral combining site is comparable to that found in classical antibodies, although in the absence of the VL.	Spinelli et al. 2001

Keywords	Abstract	Reference
Caffeine **Methylxanthines** **Heat-stability** **ELISA**	We have isolated and characterized a caffeine-specific, heavy-chain-only antibody fragment (V-HH) from llama that is capable of being utilized to analyze caffeine in hot and cold beverages. Camelid species (llama and camel) were selected for immunization because of their potential to make heat-stable, heavy-chain-only antibodies. Llamas and camels were immunized with caffeine covalently linked to keyhole limpet hemocyanin, and recombinant antibody techniques were used to create phage displayed libraries of variable region fragments of the heavy-chain antibodies. Caffeine-specific VHH fragments were selected by their ability to bind to caffeine/bovine serum albumin (BSA) and confirmed by a positive reaction in a caffeine enzyme-linked immunosorbent assay (caffeine ELISA). One of these VHH fragments (VSA2) was expressed as a soluble protein and shown to recover its reactivity after exposure to temperatures up to 90 degrees C. In addition, VSA2 was able to bind caffeine at 70 degrees C. A competition caffeine ELISA was developed for the measurement of caffeine in beverages, and concentrations of caffeine obtained for coffee, Coca-Cola Classic, and Diet Coke agreed well with high performance liquid chromatography (HPLC) determination and literature values. VSA2 showed minimal cross reactivity with structurally related methylxanthines.	Ladenson et al. 2006
Methotrexate **Immune-llama library** **Anti-MTX VHH genes** **Hydrosoluble hapten**	Single-domain antibodies specific to methotrexate (MTX) were obtained after immunization of one llama (Llama glama). Specific VHH domains (V-D-J-region) were selected by panning from an immune-llama library using phage display technology. The antibody fragments specific to MTX were purified from Escherichia coli (C41 strain) periplasm by immobilized metal affinity chromatography with an expression level of around 10 mg/l. A single band around 16 000 Da corresponding to VHH fragments was found after analysis by SDS-PAGE and Western blotting, while competition ELISA demonstrated selective binding to soluble MTX. Surface plasmon resonance (SPR) analysis showed that anti-MTX VHH domains had affinities in the nanomolar range (29–515 nM) to MTX-serum albumin conjugates. The genes encoding anti-MTX VHH were found by IMGT/V-QUEST to be similar to the previously reported llama and human IGHV germline genes. The V-D and D-J junction rearrangements in the seven anti-MTX CDR3 sequences indicate that they were originated from three distinct progenitor B cells. Our results demonstrate that camelid single-domain antibodies are capable of high affinity binding to low molecular weight hydrosoluble haptens. Furthermore, these anti-MTX VHH give new insights on how the antigen binding repertoire of llama single-domain antibody can provide combining sites to haptens in the absence of a VL. This type of single-domain antibodies offers advantages compared to murine recombinant antibodies in terms of production rate and sequence similarity to the human IGHV3 subgroup genes.	Alvarez-Rueda et al. 2007
C(H)1 domain absence **Sequence analysis** **Splicing**	The molecular basis for the absence of the C(H)1 domain in naturally occurring heavy-chain antibodies of the camelids was assessed by determining the entire Camelus dromedarius gamma 2a heavy-chain constant gene. The organization of the camel gamma 2a constant heavy-chain gene obtained from a liver genomic library appears to be typical of all other mammalian gamma genes sequenced to date. It contains the switch, C(H)1, hinge, C(H)2, C(H)3, M1 and M2 exons. In contrast to the case in mouse and human heavy chain diseases, the camel gamma 2a gene shows no major structural defect, and its equivalent C(H)1 exon is intact. However, sequence analysis has revealed that the splicing site, immediately after the C(H)1 exon, is defective due to point mutations, especially the G(+1) to A(+1) transversion seems to be detrimental. It is concluded that the loss of the splice consensus signal is responsible for the removal of the entire C(H)1 domain in camel gamma 2a heavy-chain immunoglobulins. Additionally, a closer analysis of the hinge exon suggests the possible involvement of transposons in the genetic variation of mammalian C gamma hinges.	Nguyen et al. 1999

Inhibitory antibodies **Enzyme's active site** **Carbonic anhydrase** **Alpha-amylase** **Recombinant protein**	Evidence is provided that dromedary heavy-chain antibodies, in vivo-matured in the absence of light chains, are a unique source of inhibitory antibodies. After immunization of a dromedary with bovine erythrocyte carbonic anhydrase and porcine pancreatic alpha-amylase, it was demonstrated that a considerable amount of heavy-chain antibodies, acting as true competitive inhibitors, circulate in the bloodstream. In contrast, the conventional antibodies apparently do not interact with the enzyme's active site. Next we illustrated that peripheral blood lymphocytes are suitable for one-step cloning of the variable domain fragments in a phage-display vector. By bio-panning, several antigen-specific single-domain fragments are readily isolated for both enzymes. In addition we show that among those isolated fragments active site binders are well represented. When produced as recombinant protein in Escherichia coli, these active site binders appear to be potent enzyme inhibitors when tested in chromogenic assays. The low complexity of the antigen-binding site of these single-domain antibodies composed of only three loops could be valuable for designing smaller synthetic inhibitors.	Lauwereys et al. 1998
VHH **Antigen-binding loops** **RNase A** **Enzyme inhibitor** **Canonical structures**	Background: Camelid serum contains a large fraction of functional heavy chain antibodies – homodimers of heavy chains without light chains. The variable domains of these heavy-chain antibodies (VHH) have a long complementarity determining region 3 (CDR3) loop that compensates for the absence of the antigen-binding loops of the variable light chains (VL). In the case of the VHH fragment cAb-Lys3, part of the 24 amino acid long CDR3 loop protrudes from the antigen-binding surface and inserts into the active-site cleft of its antigen, rendering cAb-Lys3 a competitive enzyme inhibitor. Results: A dromedary VHH with specificity for bovine RNase A, cAb-RN05, has a short CDR3 loop of 12 amino acids and is not a competitive enzyme inhibitor. The structure of the cAb-RN05-RNase A complex has been solved at 2.8 angstrom. The VHH scaffold architecture is close to that of a human VH (variable heavy chain). The structure of the antigen-binding hypervariable 1 loop (H1) of both cAb-RN05 and cAb-Lys3 differ from the known canonical structures; in addition these H1 loops resemble each other. The CDR3 provides an antigen-binding surface and shields the face of the domain that interacts with VL in conventional antibodies. Conclusions: VHHs adopt the common immunoglobulin fold of variable domains, but the antigen-binding loops deviate from the predicted canonical structure. We define a new canonical structure for the H1 loop of immunoglobulins, with cAb-RN05 and cAb-Lys3 as reference structures. This new loop structure might also occur in human or mouse VH domains. Surprisingly, only two loops are involved in antigen recognition; the CDR2 does not participate. Nevertheless, the antigen binding occurs with nanomolar affinities because of a preferential usage of mainchain atoms for antigen interaction.	Decanniere et al. 1999
Clefts on protein surfaces **Convex paratope** **Antigen-combining site** **Lysozyme inhibitor** **Crystal structure**	Clefts on protein surfaces are avoided by antigen-combining sites of conventional antibodies, in contrast to heavy-chain antibodies (HCAbs) of camelids that seem to be attracted by enzymes' substrate pockets. The explanation for this pronounced preference of HCAbs was investigated. Eight single domain antigen-binding fragments of HCAbs (VHH) with nanomolar affinities for lysozyme were isolated from three immunized dromedaries. Six of eight VHHs compete with small lysozyme inhibitors. This ratio of active site binders is also found within the VHH pool derived from polyclonal HCAbs purified from the serum of the immunized dromedary. The crystal structures of six VHHs in complex with lysozyme and their interaction surfaces were compared to those of conventional antibodies with the same antigen. The interface sizes of VHH and conventional antibodies to lysozyme are very similar as well as the number and chemical nature of the contacts. The main difference comes from the compact prolate shape of VHH that presents a large convex paratope, predominantly formed by the H3 loop and interacting, although with different structures, into the concave lysozyme substrate-binding pocket. Therefore, a single domain antigen-combining site has a clear structural advantage over a conventional dimeric format for targeting clefts on antigenic surfaces.	De Genst et al. 2006

Cartilaginous fish **cDNA sequence analysis** **IgNAR** **IgW**	The cartilaginous fish (chimeras, sharks, skates and rays) are the oldest group relative to mammals in which an adaptive immune system founded upon immunoglobulins has been found. In this manuscript we characterize the immunoglobulins of the spiny dogfish *(Squalus acanthias)* at both the molecular and expressed protein levels. Despite the presence of hundreds of IgM clusters in this species the serum levels of this isotype are comparatively low. However, analysis of cDNA sequences and serum protein suggests microheterogeneity in the IgM heavy chains and supports the proposal that different clusters are preferentially used in the two forms (monomer or pentamer) of this isotype. We also found that the IgNAR isotype in this species exists in a previously unknown multimeric format in serum. Finally, we identified a new form of the IgW isotype (the shark IgD orthologue), in which the leader is spliced directly to the first constant domain, resulting in a molecule lacking an antigen-binding domain.	Smith et al. 2012
V(NAR) domain **Ribosome display** **Error-prone mutagenesis** **Mutational plasticity** **Structural modelling**	The shark antigen-binding V(NAR) domain has the potential to provide an attractive alternative to traditional biotherapeutics based on its small size, advantageous physiochemical properties, and unusual ability to target clefts in enzymes or cell surface molecules. The V(NAR) shares many of the properties of the well-characterised single-domain camelid V(H)H but is much less understood at the molecular level. We chose the hen-egg-lysozyme-specific archetypal Type I V(NAR) 5A7 and used ribosome display in combination with error-prone mutagenesis to interrogate the entire sequence space. We found a high level of mutational plasticity across the V(NAR) domain, particularly within the framework 2 and hypervariable region 2 regions. A number of residues important for affinity were identified, and a triple mutant combining AID, S61R, and G62R resulted in a K(D) of 460 pM for hen egg lysozyme, a 20-fold improvement over wild-type 5A7, and the highest K(D) yet reported for V(NAR)-antigen interactions. These findings were rationalised using structural modelling and indicate the importance of residues outside the classical complementarity determining regions in making novel antigen contacts that modulate affinity. We also located two solvent-exposed residues (G15 and G42), distant from the V(NAR) paratope, which retain function upon mutation to cysteine and have the potential to be exploited as sites for targeted covalent modification. Our findings with 5A7 were extended to all known NAR structures using an in-depth bioinformatic analysis of sequence data available in the literature and a newly generated V(NAR) database. This study allowed us to identify, for the first time, both V(NAR)-specific and V(NAR)/Ig V(L)/TCR V(alpha) overlapping hallmark residues, which are critical for the structural and functional integrity of the single domain. Intriguingly, each of our designated V(NAR)-specific hallmarks align precisely with previously defined mutational 'cold spots' in natural nurse shark cDNA sequences. These findings will aid future V(NAR) engineering and optimisation studies towards the development of V(NAR) single-domain proteins as viable biotherapeutics.	Fennell et al. 2010
IgM **IgNAR** **IgW** **Houndshark**	In this study, cDNAs encoding the secreted forms of the immunoglobulin (Ig) heavy chains of IgM, IgNAR, and IgW were cloned from the banded houndshark Triakis scyllium. Two clones for the IgM heavy chains encoded 569 and 570 amino acids, whose conserved (C) region showed 47–70% amino acid identities to those reported in other cartilaginous fish. Four clones for the IgNAR encoded 673–670 amino acids with conserved Ig-superfamily domains. The IgNAR C region showed 56–69% amino acid identities to those so far reported. High-throughput sequencing revealed that in most of the IgNAR sequences, the two variable regions (CDR1 and CDR3) each possess a cysteine residue. Three types of IgW were identified; one contained Ig-superfamily domains that are in other cartilaginous fish, one lacks the 3rd domain in the constant region, and one lacks the 3rd to 5th domains. Despite these differences, the IgW isoforms clustered with IgWs of other cartilaginous fishes and the C regions showed 47–89% amino acid identities. mRNAs for IgM and IgNAR were detected in various tissues, while IgW mRNA was mainly detected in pancreas. The banded hounded shark also has IgM, IgW and IgNAR as well as the other cartilaginous fish with unique IgW isoform.	Honda et al. 2010

New antigen receptor **Disulphide bond** **Loop stabilization** **Crystallographic studies**	The new antigen receptor (IgNAR) antibodies from sharks are disulphide bonded dimers of two protein chains, each containing one variable and five constant domains. Three types of IgNAR variable domains have been discovered, with Type 3 appearing early in shark development and being overtaken by the antigen-driven affinity-matured Type 1 and 2 response. Here, we have determined the first structure of a naturally occurring Type 2 IgNAR variable domain, and identified the disulphide bond that links and stabilizes the CDR1 and CDR3 loops. This disulphide bridge locks the CDR3 loop in an "upright" conformation in contrast to other shark antibody structures, where a more lateral configuration is observed. Further, we sought to model the Type 3 isotype based on the crystallographic structure reported here. This modelincr indicates that internal Type 3-specific residues combine to pack into a compact immunoglobulin core that supports the CDR loop regions, and that despite apparent low-sequence variability, there Is sufficient plasticity in the CDR3 loop to form a conformationally diverse antigen-binding surface.	Streltsov et al. 2005
Antibody evolution **Phylogenetic and phenotypic relationships** **Camelidae** **Sharks**	The emergence in Camelidae species of functional antibodies devoid of light chains (referred to as heavy-chain antibodies or HCAbs) is an intriguing evolutionary event. Homodimeric HCAbs have also been documented in spotted ratfish (Cos5-Abs) and nurse shark (NAR). To reveal the evolutionary history of HCAbs, we evaluated the phylogenetic and phenotypic relationships among HCAbs and conventional antibodies across taxa and confirmed the current viewpoint that different groups of HCAbs have evolved independently in the three lineages. At least, in the camelids, HCAbs are not the result of resuscitation of dormant genes. They are derived from the conventional antibodies within the Camelidae lineage, and are apparently the outcome of more recent adaptive changes occurring in the compartment of heteromeric antibodies. The shared structural properties of HCAbs across taxa are therefore explained by convergent evolution due to similar constraints related to the absence of pairing to the light chain. It appears that innovative evolutionary changes in Camelidae have led to a new level of antigen binding repertoire diversification and have allowed acquisition of novel antigen-receptor properties.	Nguyen et al. 2002
Adaptive immune response **Microbial enzyme inhibition** **P-lactamase**	In 1993, a fraction of antibodies (Abs) devoid of L chain was found naturally occurring in the Camelidae. They were found to lack L chains, as well as the first constant heavy-chain domain (CHI) and therefore they were named "heavy-chain Abs" (HCAbs). Subsequent studies focused on the functional, structural and biochemical properties of recombinant variable fragments (rVHHs) of HCAbs. It was stated that rVHHs have an augmented capacity to interact with "partially hidden" epitopes, like enzymes active sites, and have an increased stability to thermal and chemical aggression. It has been suggested that these unconventional Abs could represent an evolutionary advantage, being more efficient than conventional Abs to inhibit microbial enzymes, and thus exerting a more protective immune response against pathogens. The present work focuses on the immunobiological role of HCAbs, in their capacity to inhibit microbial enzymes. Two animal models were selected, comprising a model for common vertebrates without HCAbs (rabbits), and a model for vertebrates with both conventional and unconventional Abs (Lama glama). A recombinant bacterial P-lactamase (CTX-M-2) was selected as the microbial enzymatic antigen. After conventional immunization schedules, neither serum titers nor serum inhibitory capacity showed significant differences when rabbits and llamas were compared. These results indicate that the a priori assumption that the adaptive immune system of camelids could be better "prepared" to respond to bacterial enzymes because of the presence of HCAbs, is not always accurate. Furthermore, when the different llama antibody isotypes and subclasses were purified, it was demonstrated that the inhibitory capacity of total serum was due exclusively to IgG I. HCAbs not only failed to inhibit CTX-M-2, but instead they activated its enzymatic activity. Altogether, these results indicate that the hypotheses extrapolated from the rVHHs properties need to be revised; the real role of HCAbs in vivo remains unknown, as well as their evolutionary cause.	Ferrari et al. 2007

Immunological properties **Complement activating activity** **Anticomplementary activity** **Hemagglutination**	Heavy chain antibodies (HCAbs), devoid of the light chains and the CH1 domain, are present in the serum of camelids. IgG(2) and IgG(3) are HCAbs; whereas IgG(1) has the conventional structure. In order to study the immunological properties of llama HCAbs, from which to date little is known, llamas (Lama glama) HCAbs cDNA were cloned, sequenced and compared with other mammalian Igs. The sequence analysis showed that llama HCAbs cDNA organization is similar to other mammalian Igs and the presence of conserved binding motifs to Protein A, Protein G, Fc gamma RI, Fc gamma RIII and C1q in HCAbs were observed. In a previous work, different IgG isotypes purified by Protein A and Protein G chromatography were assayed for their ability to fix complement. Both IgG(1) and the total serum were able to fix complement, whereas IgG(2) and IgG(3) fixed complement even in the absence of antigen (anticomplementary activity). Therefore, in this work we performed the complement activating activity of the different IgG isotypes purified under physiological conditions using Sephadex G-150 and their ability to induce hemagglutination. Llamas were immunized with sheep red blood cells (RBC) stroma and the different isotypes were purified from sera. Whole serum and IgG(1) could activate complement; however, HCAbs (IgG(2) + IgG(3)) could not, despite the presence of the C1q binding motif in their primary sequence. Unlike IgG(1), the fraction corresponding to IgG(2) + IgG(3) did not display hemagglutinating activity. Our findings suggest that HCAbs cannot crosslink efficiently with different antigens and that the C1q binding site might be hindered by the proximity of the variable domains.	Saccodossi et al. 2012
Antigen-binding repertoire **Germline database** **Sequence comparison** **Evolution** **Somatic mutation**	The antigen-binding site of the camel heavy-chain antibodies devoid of light chain consists of a single variable domain (VHH) that obviously lacks the V-H-V-L combinatorial diversity, To evaluate the extent of the VHH antigen-binding repertoire, a germline database was constructed from PCR-amplified THH/V-H segments of a single specimen of Camelus dromedarius. A total of 33 VHH and 39 VH unique sequences were identified, encoded by 42 and 50 different genes, respectively. Sequence comparison indicates that the V(H)Hs evolved within the VH subgroup III, Nevertheless, the VHH germline segments are highly diverse, leading to a broad structural repertoire of the antigen-binding loops, Seven VHH subfamilies were recognized, of which five were confirmed to be expressed lit vivo. Comparison of germline and cDNA sequences demonstrates that the rearranged V(H)Hs are extensively diversified by somatic mutation processes, leading to an additional hypervariable region and a high incidence of nucleotide insertions or deletions, These diversification processes are driven by hypermutation and recombination hotspots embedded in the VHH germline genes at the regions affecting the structure of the antigen-binding loops.	Nguyen et al. 2000
Application **Human leukocyte antigen** **Cancer**	A novel monoclonal anti-pan human leukocyte antigen (HLA) class I heavy chain antibody, EMR8-5, was established. It could detect HLA-A, -B, and -C antigens in formalin-fixed paraffin embedded tissues. By immunohistochemical staining using the EMR8-5 antibody, various cancer tissues from 246 cases were examined for HLA class I expression. It was found that HLA class I expression was decreased in 20% to 42% of the cases of lung cancer, hepatocellular carcinoma, colon cancer, renal cell carcinoma, and urothelial carcinoma. In contrast, 85% of breast cancer cases had loss of or decreased HLA class I expression. Of the 35 breast cancer cases that had decreased HLA class I heavy chain expression, 33 (94%) also had decreased beta2-microglobulin expression detected by immunohistochemical staining. It was suggested that HLA class I down-regulation might be a common characteristic of breast cancer mostly caused by the down-regulation of beta2-microglobulin expression.	Torigoe et al. 2012

Application **Prion immunotherapy** **Scrapie** **PrP conformation**	Although there is currently no effective treatment for prion diseases, significant advances have been made in suppressing its progress, using antibodies that block the conversion of PrP(C) into PrP(Sc). In order to be effective in treating individuals that have prion diseases, antibodies must be capable of arresting disease in its late stages. This requires the development of antibodies with higher affinity for PrP(Sc) and systems for effective translocation of antibodies across the blood brain barrier in order to achieve high concentrations of inhibitor at the site of protein replication. An additional advantage is the ability of these antibodies to access the cytosol of affected cells. To this end, we have generated PrP-specific antibodies (known as PrioV) by immunization of camels with murine scrapie material adsorbed to immunomagnetic beads. The PrioV antibodies display a range of specificities with some recognizing the PrP (27–30) proteinase K-resistant fragment, others specific for PrP(C) and a number with dual binding specificity. Independent of their PrP conformation specificity, one of the PrioV antibodies (PrioV3) was shown to bind PrP(C) in the cytosol of neuroblastoma cells. In marked contrast, conventional anti-PrP antibodies produced in mouse against similar target antigen were unable to cross the neuronal plasma membrane and instead formed a ring around the cells. The PrioV anti-PrP antibodies could prove to be a valuable tool for the neutralization/clearance of PrP(Sc) in intracellular compartments of affected neurons and could potentially have wider applicability for the treatment of so-called protein-misfolding diseases.	Tayebi et al. 2010
Application **Competitive fluid array immunoassay** **Trinitrotoluene**	Llamas possess unique subclasses of antibodies that lack light chains, and thus are made by the pairing of two heavy chains. IgG was purified from two llamas which had been immunized with trinitrobenzene-keyhole limpet hemocyanin. Conventional IgG1 and heavy chain IgG2 and IgG3 subclasses were fractionated using affinity chromatography. The effectiveness of heavy chain antibodies for the detection of trinitrotoluene (TNT) using a competitive fluid array immunoassay was evaluated and compared to both the llama IgG1 as well as a murine monoclonal anti-TNT antibody. It was found that heavy chain antibody bound TNT with selectivity similar to conventional antibodies, yet the heavy chain antibodies possessed greater thermal stability. The titer of the heavy chain antibodies however was found to be 10-fold lower than the IgGl1; thus analytical assays were best demonstrated using the llama IgG1 conventional antibody. The TNT competitive immunoassay on the Luminex fluid analyzer had a dynamic range from similar to 100 ng/ml to 10 μg/ml. Utilizing the same two-step competitive assay format the dynamic range of the monoclonal antibody was found to have a broad range (1 ng/ml to 1 μg/ml). This method was demonstrated on TNT contaminated soil extracts using both the llama IgG1 and the mouse monoclonal validating the utility of method for analysis of held samples.	Anderson and Goldman 2008

Table 4. Production of single-domain antibody fragments using recombinant technology

E. coli **Azoxystrobin** **Metal affinity chromatography** **ELISA**	Three V(H)Hs against the model hapten, azoxystrobin (MW 403), were isolated from a hyper-immunized phage-displayed V(H)H library. This library was constructed by isolating the V(H)H-coding genes from the lymphocytes collected from a Lama glama that was immunized with azoxystrobin conjugated to bovine serum albumin (BSA). Six rounds of panning were performed against azoxystrobin conjugated to either ovalbumin (OVA) or rabbit serum albumin (RSA) to enrich clones containing V(H)Hs specific to the hapten. After screening 95 clones, three V(H)Hs (A27, A72, and A85) with different amino acid sequences were identified, expressed in soluble format in Escherichia coli HB2151, and purified using nickel-immobilized metal affinity chromatography. Competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA) showed that A27 and A85 were specific to azoxystrobin while A72 was not. The IC(50) values of A27 and A85 V(H)Hs were 7.2 and 2.0 μm, respectively. To our knowledge A85 is one of the highest affinity V(H)Hs that has yet been isolated against a hydrophobic hapten such as azoxystrobin.	Makvandi-Nejad et al. 2011
Pichia pastoris **AahI' scorpion toxin** **Alcohol oxidase promoter** **A-factor secretion signal**	Using eukaryotic hosts, antibody fragments are generally expressed by targeting to the secretory pathway. This enables not only efficient disulfide bond formation but also secretion of soluble and correctly folded product. For this goal, a recombinant vector was constructed to produce a single-domain antibody (NbAahI'22) directed against AahI'scorpion toxin using the methylotrophic yeast. The corresponding complementary DNA was cloned under control of the alcohol oxidase promoter in frame with the Saccharomyces α-factor secretion signal and then transferred to P. pastoris cell strain X-33. Using Western blot, we detected the expression of the recombinant NbAahI'22 exclusively in the culture medium. Targeting to the histidine label, the secreted nanobody was easily purified on nickelnitrilotriacetic acid resin and then tested in enzyme-linked immunosorbent assay. Interestingly, the production level of the NbAahI'22 in its new glycosylated form reached more than sixfold that obtained in E. coli. These findings give more evidence for the utilization of P. pastoris as a heterologous expression system.	Ezzine et al. 2012
Saccharomyces cerevisiae **Secretion efficiency** **J segment**	Variable domains of llama heavy-chain antibodies (VHH) are becoming a potent tool for a wide range of biotechnological and medical applications. Because of structural features typical of their single-domain nature, they are relatively easy to produce in lower eukaryotes, but it is not uncommon that some molecules have poor secretion efficiency. We therefore set out to study the production yield of VHH. We computationally identified five key residues that are crucial for folding and secretion, and we validated their importance with systematic site-directed mutations. The observation that all key residues were localised in the V segment, in proximity of the J segment of VHH, led us to study the importance of J segment in secretion efficiency. Intriguingly, we found that the use of specific J segments in VHH could strongly influence the production yield. Sequence analysis and expression experiments strongly suggested that interactions with chaperones, especially with the J segment, are a crucial aspect of the production yield of VHH.	Gorlani et al. 2012

Magnetospirillum gryphiswaldense **Magnetic nanoparticle** **Magnetosome** **Red fluorescein protein**	Numerous applications of conventional and biogenic magnetic nanoparticles (MNPs), such as in diagnostics, immunomagnetic separations, and magnetic cell labeling, require the immobilization of antibodies. This is usually accomplished by chemical conjugation, which, however, has several disadvantages, such as poor efficiency and the need for coupling chemistry. Here, we describe a novel strategy to display a functional camelid antibody fragment (nanobody) from an alpaca (Lama pacos) on the surface of bacterial biogenic magnetic nanoparticles (magnetosomes). Magnetosome-specific expression of a red fluorescent protein (RFP)-binding nanobody (RBP) in vivo was accomplished by genetic fusion of RBP to the magnetosome protein MamC in the magnetite-synthesizing bacterium Magnetospirillum gryphiswaldense. We demonstrate that isolated magnetosomes expressing MamC-RBP efficiently recognize and bind their antigen in vitro and can be used for immunoprecipitation of RFP-tagged proteins and their interaction partners from cell extracts. In addition, we show that coexpression of monomeric RFP (mRFP or its variant mCherry) and MamC-RBP results in intracellular recognition and magnetosome recruitment of RFP within living bacteria. The intracellular expression of a functional nanobody targeted to a specific bacterial compartment opens new possibilities for in vivo synthesis of MNP-immobilized nanobodies. Moreover, intracellular nanotraps can be generated to manipulate bacterial structures in live cells.	Pollithy et al. 2011
Tobacco expression system **Agrobacterium**	Several plants are the facile and economic bioreactor for large-scale production of industrial and pharmaceutical agents like proteins and antibodies. Here, we have selected tobacco as the host plant because of large scale production capability and many other advantages such as greater safety and lower production costs when compared to animal-based systems. In this study, we have subcloned VHH gene into pBI121 using phasmid pCANTAB5E. The new construct was used to transform the Agrobacterium strains C58GV3101 and LBA4404. Agrobacterium strain C58GV3101 showed a higher virulence on leaf disks of Nicotiana tabacum (NC25). Transgenic tobacco plants were then developed by introducing VHH gene under the control of CaMV 35S promoter. The presence of the VHH antibody gene in the plant genome was verified by PCR analysis and Southern hybridization experiments. Northern blot analysis showed that the genes coding for the VHH could be expressed in tobacco plants. Three lines of transgenic plant that expresses high levels of mRNA were screened in a further analysis. The expression of VHH was then observed in transgenic plants by ELISA using the specific antibody developed, the results showed three to five folds higher than non-transgenic tobaccos.	Korouzhdehy et al. 2011
Tumour necrosis factor **Fusion** **Elastin-like polypeptide** **Tobacco**	Tumour necrosis factor (TNF) is a major pro-inflammatory cytokine involved in multiple inflammatory diseases. The detrimental activity of TNF can be blocked by various antagonists, and commercial therapeutics based upon this principle have been approved for treatment of diseases including rheumatoid arthritis, Crohn's disease and psoriasis. In a search for new, improved anti-inflammatory therapeutics we have designed a single-domain monoclonal antibody (V(H)H), which recognizes TNF. The antibody component (TNF-V(H)H) is based upon an anti-human TNF Camelidae heavy-chain monoclonal antibody, which was linked to an elastin-like polypeptide (ELP). We demonstrate that ELP fusion to the TNF-V(H)H enhances accumulation of the fusion protein during biomanufacturing in transgenic tobacco plants. With this study, we show for the first time that this plant-derived anti-human TNF-V(H)H antibody was biologically active in vivo. Therefore, therapeutic application of TNF-V(H)H-ELP fusion protein was tested in humanized TNF mice and was shown to be effective in preventing death caused by septic shock. The in vivo persistence of the ELPylated antibody was similar to 24 fold longer than that of non-ELPylated TNF-V(H)H.	Conrad et al. 2011

Panning methods **Botulinum neurotoxin** **Binding kinetics**	Single-domain antibodies (sdAb) specific for botulinum neurotoxin serotype A (BoNT A) were selected from an immune llama phage display library derived from a llama that was immunized with BoNT A toxoid. The constructed phage library was panned using two methods: panning on plates coated with BoNT A toxoid (BoNT ATd) and BoNT A complex toxoid (BoNT Ac Td) and panning on microspheres coupled to BoNT A Td and BoNT A toxin (BoNT A Tx). Both panning methods selected for binders that had identical sequences, suggesting that panning on toxoided material may be as effective as panning on bead-immobilized toxin for isolating specific binders. All of the isolated binders tested were observed to recognize bead-immobilized BoNT A Tx in direct binding assays, and showed very little cross-reactivity towards other BoNT serotypes and unrelated protein. Sandwich assays that incorporated selected sdAb as capture and tracer elements demonstrated that all of the sdAb were able to recognize soluble ("live") BoNT A Tx and BoNT Ac Tx with virtually no cross-reactivity with other BoNT serotypes. The isolated sdAb did not exhibit the high degree of thermal stability often associated with these reagents; after the first heating cycle most of the binding activity was lost, but the portion of the protein that did refold and recover antigen-binding activity showed only minimal loss on subsequent heating and cooling cycles. The binding kinetics of selected binders, assessed by both an equilibrium fluid array assay as well as surface plasmon resonance (SPR) using toxoided material, gave dissociation constants (K(D)) in the range 2.2 × 10(−11) to 1.6 × 10(−10)M. These high-affinity binders may prove beneficial to the development of recombinant reagents for the rapid detection of BoNT A, particularly in field screening and monitoring applications.	Swain et al. 2010
Picloram **Ribosome display** **In vitro transcription/ translation** **Point mutation** **Binding kinetics** **Molecular evolution**	Picloram-specific variable fragments (V(HH)s) of heavy chain antibodies (HCAbs) were selected from a naive-llama library using ribosome display technology. A cDNA library of V(HH)s was constructed from lymphocytes of a non-immunized llama and engineered to allow in vitro transcription and translation. With no stop codons present on the transcripts, trimeric complexes of ribosomes, mRNAs and nascent peptides were produced for affinity selection, i.e. panning. After three cycles of panning, seven different V(HH)s all belonging to the V-HH subfamily 1 were isolated. Following another three cycles of selection, only two of the seven V(HH)s persisted. A comparison of these two sequences with known sequences in the literature suggests that point mutations may have been introduced into the DNA pool during PCR amplification steps of library construction, panning and/or cloning. Three separate point mutations causing three independent amino acid changes (nonsynonomous mutations) accumulated in the same sequence and enriched throughout the selection protocol, suggesting that these changes confer binding advantages. Surface plasmon resonance (SPR) analysis was used to determine binding kinetics of the two clones (3-1D2 and 3-1F6) representing the two different sets of isolated complementarity determining region (CDR)3s. Measured K(D)s were 3 and 254 μM, respectively. The results indicate that ribosome display technology can be used to efficiently isolate hapten-specific antibody (Ab) fragments from a naive library and concurrently introduce diversity to the selected pool thereby facilitating molecular evolution. Ribosome display technology can compensate for the limited diversity of a V-HH naive library and provide an unlimited source of affinity-matured immunoactive reagents in vitro.	Yau et al. 2003

Ribosome display **Error-prone mutagenesis** **Lysozyme** **Solvent-exposed residues**	We chose the hen-egg-lysozyme-specific archetypal Type I V(NAR) 5A7 and used ribosome display in combination with error-prone mutagenesis to interrogate the entire sequence space. We found a high level of mutational plasticity across the V(NAR) domain, particularly within the framework 2 and hypervariable region 2 regions. A number of residues important for affinity were identified, and a triple mutant combining AID, S61R, and G62R resulted in a K(D) of 460 pM for hen egg lysozyme, a 20-fold improvement over wild-type 5A7, and the highest K(D) yet reported for V(NAR)-antigen interactions. These findings were rationalised using structural modelling and indicate the importance of residues outside the classical complementarity determining regions in making novel antigen contacts that modulate affinity. We also located two solvent-exposed residues (G15 and G42), distant from the V(NAR) paratope, which retain function upon mutation to cysteine and have the potential to be exploited as sites for targeted covalent modification. Our findings with 5A7 were extended to all known NAR structures using an in-depth bioinformatic analysis of sequence data available in the literature and a newly generated V(NAR) database. This study allowed us to identify, for the first time, both V(NAR)-specific and V(NAR)/Ig V(L)/TCR V(alpha) overlapping hallmark residues, which are critical for the structural and functional integrity of the single domain. Intriguingly, each of our designated V(NAR)-specific hallmarks align precisely with previously defined mutational 'cold spots' in natural nurse shark cDNA sequences. These findings will aid future V(NAR) engineering and optimisation studies towards the development of V(NAR) single-domain proteins as viable biotherapeutics.	Fennell et al. 2010
Yeast surface display **Pichia pastoris** **Green fluorescent protein**	Yeast surface display is an efficient tool for isolating and engineering antibody fragments, both scFv and Fab. We describe the use of protein display on Pichia pastoris for the rapid selection of camelid antibodies composed only of heavy chains (nanobodies) from a library derived from a Ilama immunized with Green Fluorescent Protein. The library of nanobody-coding sequences was fused to the C-terminal part of the Saccharomyces cerevisiae alpha-agglutinin gene (SAG1) and expressed in glycoengineered P. pastoris. A high efficiency transformation protocol yielded a library of 5 × 10(7) clones. About 80% of the clones strongly expressed the nanobody fusion. Nanobody-displaying clones were rapidly enriched by fluorescence activated cell sorting (FACS), and GFP-specific nanobody-displaying clones were isolated and equilibrium dissociation constants (K(d)) determined. This technology for displaying protein libraries on P. pastoris enables the isolation and engineering of antibody-derived molecules in a robust eukaryotic expression host.	Ryckaert et al. 2010
Surface display **Expression cassettes** **Lactobacillus paracasei** **Rotavirus**	A series of expression cassettes which mediate secretion or surface display of antibody fragments was stably integrated in the chromosome of Lactobacillus paracasei. L. paracasei producing surface-anchored variable domain of llama heavy chain (VHH) (ARP1) directed against rotavirus showed efficient binding to rotavirus and protection in the mouse model of rotavirus infection.	Martin et al. 2011
Fungal expression system **Aspergillus awamori**	We report the expression and production of llama variable heavy-chain antibody fragments (V(HH)s) by Aspergillus awamori. Fragments encoding V(HH)s were cloned in a suitable Aspergillus expression vector and transformants secreting V-HH fragments were analysed for integrated gene copy-numbers, mRNA levels and protein production. Functional V(HH)s were detected in the culture medium, indicating the feasibility of producing this type of protein in a fungal expression system. Secreted V(HH)s were subjected to (extracellular) degradation, which could be partially prevented by the addition of BSA to the culture medium.	Joosten et al. 2005

Chinese hamster ovary cells **Expression vectors** **Reporter gene** **GFP-fusion protein**	There is an increasing interest in the application of nanobodies such as VHH in the field of therapy and imaging. In the present study a stable genetically engineered cell line of Chinese hamster ovary (CHO) origin transfected using two sets of expression vectors was constructed in order to permit the cytoplasmic and extracellular expression of single domain antibody along with green fluorescent protein (GFP) as reporter gene. The quality of the constructs were examined both by the restriction map as well as sequence analysis. The gene transfection and protein expression was further examined by reverse transcription-polymerase chain reaction (RT-PCR). The transfected cells were grown in 200 μg/ml hygromycin containing media and the stable cell line obtained showed fluorescent activity for more than a period of 180 days. The production of fusion protein was also detected by fluorescent microscopy, fluorescent spectroscopy as well as by enzyme-linked immunosorbent assay (ELISA) analysis. This strategy allows a rapid production of recombinant fluobodies involving VHH, which can be used in various experiments such as imaging and detection in which a primary labeled antibody is required.	Bazl et al. 2007
Expression in mouse germline **Dromedary transgene** **Gamma 2a H chain**	In mature B cells of mice and most mammals, cellular release of single H chain Abs without L chains is prevented by H chain association with Ig-specific chaperons in the endoplasmic reticiulum. In precursor B cells, however, surface expression of μ-H chain in the absence of surrogate and conventional L chain has been identified. Despite this, Ag-specific single H chain Ig repertoires, using μ-, gamma-, epsilon-, or alpha-H chains found in conventional Abs, are not produced. Moreover, removal of H chain or, separately, L chain (kappa/lambda) locus core sequences by gene targeting has prevented B cell development. In contrast, H chain-only Abs are produced abundantly in Camelidae as H2 IgG without the C(H)l domain. To test whether H chain Abs can be produced in mice, and to investigate how their expression affects B cell development, we introduced a rearranged dromedary gamma 2a H chain into the mouse germline. The dromedary transgene was expressed as a naturally occurring Ag-specific disulphide-linked homodimer, which showed that B cell development can be instigated by expression of single H chains without L chains. Lymphocyte development and B cell proliferation was accomplished despite the absence of L chain from the BCR complex. Endogenous Ig could not be detected, although V(D)J recombination and IgH/L transcription was unaltered. Furthermore, crossing the dromedary H chain mice with mice devoid of all C genes demonstrated without a doubt that a H chain-only Ab can facilitate B cell development independent of endogenous Ig expression, such as μ- or delta-H chain, at early developmental stages.	Zou et al. 2005
Universal humanized nanobody scaffold	Nanobodies, single-domain antigen-binding fragments of camelid-specific heavy-chain only antibodies offer special advantages in therapy over classic antibody fragments because of their smaller size, robustness, and preference to target unique epitopes. A Nanobody differs from a human heavy chain variable domain in about ten amino acids spread all over its surface, four hallmark Nanobody-specific amino acids in the framework-2 region (positions 42, 49, 50, and 52), and a longer third antigen-binding loop (H3) folding over this area. For therapeutic applications the camelid-specific amino acid sequences in the framework have to be mutated to their human heavy chain variable domain equivalent, i.e. humanized. We performed this humanization exercise with Nanobodies of the subfamily that represents close to 80% of all dromedary-derived Nanobodies and investigated the effects on antigen affinity, solubility, expression yield, and stability. It is demonstrated that the humanization of Nanobody-specific residues outside framework-2 are neutral to the Nanobody properties. Surprisingly, the Glu-49 -> Gly and Arg-50 -> Leu humanization of hallmark amino acids generates a single domain that is more stable though probably less soluble. The other framework-2 substitutions, Phe-42 -> Val and Gly/Ala-52 -> Trp, are detrimental for antigen affinity, due to a repositioning of the H3 loop as shown by their crystal structures. These insights were used to identify a soluble, stable, well expressed universal humanized Nanobody scaffold that allows grafts of antigen-binding loops from other Nanobodies with transfer of the antigen specificity and affinity.	Vincke et al. 2009

Humanized nanobodies **Loop-grafting** **In vivo imaging** **Scaffold** **Carcinoembryonic antigen**	Nanobodies are a novel type of immunoglobulinlike, antigen-binding protein with beneficial pharmacologic and pharmacokinetic properties that are ideally suited to targeting cellular antigens for molecular imaging or therapeutic purposes. However, because of their camelid, nonhuman origin, the possible immunogenicity of Nanobodies when used in the clinic is a concern. Here we present a new strategy to quickly generate humanized Nanobodies for molecular imaging purposes. Methods: We genetically grafted the antigen-binding loops of NbCEA5, a Nanobody with specificity for the colon carcinoma marker carcinoembryonic antigen (CEA), onto the framework of a humanized Nanobody scaffold. This scaffold has been previously characterized in our laboratory as a stable Nanobody that can serve as a universal loop acceptor for antigen-binding loops from donor Nanobodies and has been additionally mutated at about 10 crucial surface-exposed sites to resemble the sequence of human variable immunoglobulin domains. The 3 recombinant Nanobodies (NbCEA5, humanized scaffold, and humanized CEA5 graft) were produced in bacteria and purified. Unlabeled and (99m)Tc-labeled Nanobodies were biochemically characterized in vitro and tested as probes for SPECT/CT of xenografted tumors. Results: The success of loop-grafting was confirmed by comparing these Nanobodies for their capacity to recognize soluble CEA protein in enzyme-linked immunosorbent assay and by surface plasmon resonance and to bind to CEA-positive LS174T colon carcinoma cells and CEA-transfected but not untransfected Chinese hamster ovary cells in flow cytometry. Specificity of binding was confirmed by competition studies. All Nanobodies were heat-stable, could be efficiently labeled with (99m)Tc, and recognized both soluble and membrane-bound CEA protein in binding studies. Finally, biodistribution experiments were performed with intravenously injected (99m)Tc-labeled Nanobodies in LS174T tumor-bearing mice using pinhole SPECT/micro-CT. These in vivo experiments revealed specificity of tumor targeting and rapid renal clearance for all Nanobodies, with low signals in all organs besides the kidneys. Conclusion: This study shows the potency of antigen-binding loop-grafting to efficiently generate humanized Nanobodies that retain their targeting capacities for noninvasive in vivo imaging of tumors.	Vaneycken et al. 2010
Grafting **Hapten** **Methotrexate** **Crystal structure analysis**	While several anti-hapten VHHs have been generated, little is known regarding the underlying structural and thermodynamic basis for hapten recognition. Here, an anti-methotrexate VHH (anti-MTX VHH) was generated using grafting methods whereby the three complementarity determining regions (CDRs) were inserted onto an existing VHH framework. Thermodynamic analysis of the anti-MTX VHH CDR1–3 Graft revealed a micromolar binding affinity, while the crystal structure of the complex revealed a somewhat surprising noncanonical binding site which involved MTX tunneling under the CDR1 loop. Due to the close proximity of MTX to CDR4, a nonhypervariable loop, the CDR4 loop sequence was subsequently introduced into the CDR1–3 graft, which resulted in a dramatic 1000-fold increase in the binding affinity. Crystal structure analysis of both the free and complex anti-MTX CDR1–4 graft revealed CDR4 plays a significant role in both intermolecular contacts and binding site conformation that appear to contribute toward high affinity binding. Additionally, the anti-MTX VHH possessed relatively high specificity for MTX over closely related compounds aminopterin and folate, demonstrating that VHH domains are capable of binding low-molecular weight ligands with high affinity and specificity, despite their reduced interface.	Fanning and Horn 2011

Grafting **Tobacco** **Lysozyme** **'Universal' nanobody framework**	Here we describe high-level expression, in Nicotiana benthamiana, of three versions of an anti-hen egg white lysozyme (HEWL) nanobody which include the original VHH from an immunized library (cAbLys3), a codon-optimized derivative, and a codon-optimized hybrid nanobody comprising the CDRs of cAbLys3 grafted onto an alternative 'universal' nanobody framework. His6- and StrepII-tagged derivatives of each nanobody were targeted for accumulation in the cytoplasm, chloroplast and apoplast using different pre-sequences. When targeted to the apoplast, intact functional nanobodies accumulated at an exceptionally high level (up to 30% total leaf protein), demonstrating the great potential of plants as a nanobody production system.	Teh and Kava-nagh 2010
Epidermal growth factor receptor **Pentabody** **Long serum half life** **Fc fragment**	In the search for better antibody formats for in vivo imaging and/or therapy of cancer, three types of sdAb-based molecules directed against epidermal growth factor receptor (EGER) were constructed, characterized and tested. Eleven sdAbs were isolated from a phage display library constructed from the sdAb repertoire of a llama immunized with a variant of EGFR. A pentameric sdAb, or pentabody, V2C-EG2 was constructed by fusing one of the sdAbs, EG2, to a pentamerization protein domain. A chimeric HCAb (cHCAb), EG2-hFc, was constructed by fusing EG2 to the fragment crystallizable (Fc) of human IgG1. Whereas EG2 and V2C-EG2 localized mainly in the kidneys after i.v. injection, EG2-hFc exhibited excellent tumor accumulation, and this was largely attributed to its long serum half life, which is comparable to that of IgGs. The moderate size (similar to 80 kDa) and intact human Fc make HCAbs a unique antibody format which may outperform whole IgGs as imaging and therapeutic reagents.	Bell et al. 2010
Universal framework **Grafting** **Chimera**	Camel single-domain antibody fragments (VHHs) are promising tools in numerous biotechnological and medical applications. However, some conditions under which antibodies are used are so demanding that they can be met by only the most robust VHHs. A universal framework offering the required properties for use in various applications (e.g. as intrabody, as probe in biosensors or on micro-arrays) is highly valuable and might be further implemented when employment of VHHs in human therapy is envisaged. We identified the VHH framework of cAbBCII10 as a potential candidate, useful for the exchange of antigen specificities by complementarity determining region (CDR) grafting. Due to the large number of CDRH loop structures present on VHHs, this grafting technique was expected to be rather unpredictable. Nonetheless, the plasticity of the cAbBCII10 framework allows successful transfer of antigen specificity from donor VHHs onto its scaffold. The cAbBCII10 was chosen essentially for its high level of stability (47 kJ/mol, good expression level (5 mg/l in E. coli) and its ability to be functional in the absence of the conserved disulfide bond. All five chimeras generated by grafting CDR-Hs, from donor VHHs belonging to subfamily 2 that encompass 75% of all antigen-specific VHHs, on the framework of cAbBCII10 were functional and generally had an increased thermodynamic stability. The grafting of CDR-H loops from VHHs belonging to other subfamilies resulted in chimeras of reduced antigen-binding capacity.	Saerens et al. 2005
VHH-SNAP construct **Erv1p sulfhydryl oxidase**	Camelidae single domain antibodies (VHHs) have structural and binding features that render them suitable alternatives to conventional IgG antibodies. VHHs are usually easier to produce as recombinant proteins than other antibody fragments. However, for some of the biotechnological applications for which they have been proposed, such as immunochromatography and assisted-crystallography, large amounts of purified antibodies are necessary, whereas some VHH-fusions with common tags such as GFP and SNAP are poorly expressed in the bacterial periplasm. Here we have shown that the co-expression of Erv1p sulfhydryl oxidase resulted in an astonishing yield increase of VHH-SNAP constructs expressed in the bacterial cytoplasm. The resulting recombinant antibodies were also more stable than the antibodies produced using the same plasmid, but in wild-type bacteria. Using this approach, it was possible to obtain tens of milligrams of purified fusion antibodies using a basic flask fermentation protocol. Therefore, the described method represents a valid solution to produce inexpensively large amounts of single domain antibodies for in vitro applications and we expect it will be suitable for the production of other antibody fragments.	Veggiani and de Marco 2011

Table 5. Characteristic properties of single-domain antibody fragments

Blood-brain barrier permeability **Trypanosoma brucei** **Surface glycoprotein VSG**	Experimental approach: We have assessed the blood-brain barrier permeability of Nb_An33, a nanobody against the Trypanosoma brucei brucei variant-specific surface glycoprotein (VSG). This analysis was performed in healthy rats and in rats that were in the encephalitic stage of African trypanosomiasis using intracerebral microdialysis, single photon emission computed tomography (SPECT) or a combination of both methodologies. This enabled the quantification of unlabelled and Tc-99m-labelled nanobodies using, respectively, a sensitive VSG-based nanobody-detection ELISA, radioactivity measurement in collected microdialysates and SPECT image analysis. Key results: The combined read-out methodologies showed that Nb_An33 was detected in the brain of healthy rats following *i.v.* injection, inflammation-induced damage to the blood-brain barrier, as in the late encephalitic stage of trypanosomiasis, significantly increased the efficiency of passage of the nanobody through this barrier. Complementing SPECT analyses with intracerebral microdialysis improved analysis of brain disposition. There is clear value in assessing penetration of the blood-brain barrier by monovalent nanobodies in models of CNS inflammation. Our data also suggest that rapid clearance from blood might hamper efficient targeting of specific nanobodies to the CNS. Conclusions and implications: Nanobodies can enter the brain parenchyma from the systemic circulation, especially in pathological conditions where the blood-brain barrier integrity is compromised.	Caljon et al. 2012
Beta-amyloid **Transmigration efficiency** **Alzheimer's disease** **Cerebral amyloid angiopathy**	Previously selected amyloid beta recognizing heavy chain antibody fragments (VHH) affinity binders derived from the Camelid heavy chain antibody repertoire were tested for their propensity to cross the blood-brain barrier (BBB) using an established in vitro BBB co-culture system. Of all tested VHH, ni3A showed highest transmigration efficiency which is, in part, facilitated by a three amino acid substitutions in its N-terminal domain. Additional studies indicated that the mechanism of transcellular passage of ni3A is by active transport. As VHH ni3A combines the ability to recognize amyloid beta and to cross the BBB, it has potential as a tool for non-invasive in vivo imaging and as efficient local drug targeting moiety in patients suffering from cerebral amyloidosis such as Alzheimer's disease (AD) and cerebral amyloid angiopathy (CAA).	Rutgers et al. 2011
Blood-brain barrier **Transcytosis** **Endocytosis** **Cerebral endothelial cells** **Putative receptor**	Antibodies against receptors that undergo transcytosis across the blood-brain barrier (BBB) have been used as vectors to target drugs or therapeutic peptides into the brain. We have recently discovered a novel single domain antibody, FC5, which transmigrates across human cerebral endothelial cells in vitro and the BBB in vivo. The purpose of this study was to characterize mechanisms of FC5 endocytosis and transcytosis across the BBB and its putative receptor on human brain endothelial cells. The transport of FC5 across human brain endothelial cells was polarized, charge independent and temperature dependent, suggesting a receptor-mediated process. FC5 taken up by human brain endothelial cells co-localized with clathrin but not with caveolin-1 by immunochemistry and was detected in clathrin-enriched subcellular fractions by western blot. The transendothelial migration of FC5 was reduced by inhibitors of clathrin-mediated endocytosis, K+ depletion and chlorpromazine, but was insensitive to caveolae inhibitors, filipin, nystatin or methyl-beta-cyclodextrin. Following internalization, FC5 was targeted to early endosomes, bypassed late endosomes/lysosomes and remained intact after transcytosis. The transcytosis process was inhibited by agents that affect actin cytoskeleton or intracellular signaling through PI3-kinase. Pretreatment of human brain endothelial cells with wheatgerm agglutinin, sialic acid, alpha(2,3)-neuraminidase or Maackia amurensis agglutinin that recognizes alpha(2,3)-, but not with Sambucus nigra agglutinin that recognizes alpha(2,6)-sialylgalactosyl residues, significantly reduced FC5 transcytosis. FC5 failed to recognize brain endothelial cells-derived lipids, suggesting that it binds luminal alpha(2,3)-sialoglycoprotein receptor which triggers clathrin-mediated endocytosis. This putative receptor may be a new target for developing brain-targeting drug delivery vectors.	Abulrob et al. 2005

Thermal stability **Resistance to trypsin and extreme pH** **Clostridium difficile** **Toxin A** **Oral administration**	The extreme pH and protease-rich environment of the upper gastrointestinal tract is a major obstacle facing orally-administered protein therapeutics, including antibodies. Through protein engineering, several Clostridium difficile toxin A-specific heavy chain antibody variable domains (V(H)Hs) were expressed with an additional disulfide bond by introducing Ala/Gly54Cys and Ile78Cys mutations. Mutant antibodies were compared to their wild-type counterparts with respect to expression yield, non-aggregation status, affinity for toxin A, circular dichroism (CD) structural signatures, thermal stability, protease resistance, and toxin A-neutralizing capacity. The mutant V(H)Hs were found to be well expressed, although with lower yields compared to wild-type counterparts, were non-aggregating monomers, retained low nM affinity for toxin A, albeit the majority showed somewhat reduced affinity compared to wild-type counterparts, and were capable of in vitro toxin A neutralization in cell-based assays. Far-UV and near-UV CD spectroscopy consistently showed shifts in peak intensity and selective peak minima for wild-type and mutant V(H)H pairs; however, the overall CD profile remained very similar. A significant increase in the thermal unfolding midpoint temperature was observed for all mutants at both neutral and acidic pH. Digestion of the V(H)Hs with the major gastrointestinal proteases, at biologically relevant concentrations, revealed a significant increase in pepsin resistance for all mutants and an increase in chymotrypsin resistance for the majority of mutants. Mutant V(H)H trypsin resistance was similar to that of wild-type V(H)Hs, although the trypsin resistance of one V(H)H mutant was significantly reduced. Therefore, the introduction of a second disulfide bond in the hydrophobic core not only increases V(H)H thermal stability at neutral pH, as previously shown, but also represents a generic strategy to increase V(H)H stability at low pH and impart protease resistance, with only minor perturbations in target binding affinities. These are all desirable characteristics for the design of protein-based oral therapeutics.	Hussack et al. 2011a
Heat denaturation **Epidermal growth factor receptor** **Pichia pastoris** **Malignancy**	It has been shown that, in contrast with conventional antibody fragments, the variable domains of these heavy-chain antibodies are functional at or after exposure to high temperatures. In the present study, the VHH (variable domain of heavy-chain antibody) camel antibody was subcloned into vector Ppiczc and expressed in Pichia pastoris. ORBI-83 VHH antibody recognizes the external domain of the mutant EGFR [EGF (epidermal growth factor) receptor], EGFR VIII. This tumour-specific antigen is ligand-independent, contains a constitutively active tyrosine kinase domain and has been shown to be present in a number of human malignancies. We report here that, although expression from P. pastoris resulted in a significantly increased level of expression of the anti-EGFR VIII VHH antibodies compared with Escherichia, this antibody selectively bound to the EGFR VIII peptide and reacted specifically with the immunoaffinity-purified antigen from non-small-cell lung cancer. Furthermore, thermal denaturation stability and CD spectra analysis of the Camelus bactrianus (Bactrian camel) VHH and heavy-chain antibodies at different temperature proved reversibility and binding activity after heat denaturation. Our results indicate that the P. pastoris expression system may be useful for the expression of camel single domain antibody and the ability of the expressed protein to reversibly melt without aggregation, allowing it to regain binding activity after heat denaturation.	Omidfar et al. 2007

Denaturing agents **Celiac patients** **Prolamin** **Gliadin** **ELISA**	Food intended for celiac patients' consumption must be analyzed for the presence of toxic prolamins using high delectability tests. Though 60% ethanol is the most commonly used solvent for prolamins extraction, 2-mercaptoethanol (2-ME) and guanidinium chloride (GuHCl) can be added to increase protein recovery. However, ethanol and denaturing agents interfere with antigen recognition when conventional antibodies are used. In the present work, a new method for gliadins quantification is shown. The method is based on the selection of llama single domain antibody fragments able to operate under denaturing conditions. Six out of 28 VHH-phages obtained retained their binding capacity in 15% ethanol. Selected clones presented a long CDR3 region containing two additional cysteines that could be responsible for the higher stability. One of the clones (named VHH26) was fully operative in the presence of 15% ethanol, 0.5% 2-ME, and 0.5M GuHCl. Capture ELISA using VHH26 was able to detect gliadins in samples shown as negatives by conventional ELISA. Therefore, this new strategy appears as an excellent platform for quantitative determination of proteins or any other immunogenic compound, in the presence of denaturing agents, when specific recognition units with high stability are required.	Dona et al. 2010
Enterotoxin B **Denatured protein refolding** **Diagnostic assay**	Background: Camelids and sharks possess a unique subclass of antibodies comprised of only heavy chains. The antigen binding fragments of these unique antibodies can be cloned and expressed as single domain antibodies (sdAbs). The ability of these small antigen-binding molecules to refold after heating to achieve their original structure, as well as their diminutive size, makes them attractive candidates for diagnostic assays. Results: Here we describe the isolation of an sdAb against Staphyloccocus aureus enterotoxin B (SEB). The clone, A3, was found to have high affinity (Kd = 75 pM) and good specificity for SEB, showing no cross reactivity to related molecules such as Staphylococcal enterotoxin A (SEA), Staphylococcal enterotoxin D (SED), and Shiga toxin. Most remarkably, this anti-SEB sdAb had an extremely high Tm of 85 degrees C and an ability to refold after heating to 95 degrees C. The sharp Tm determined by circular dichroism, was found to contrast with the gradual decrease observed in intrinsic fluorescence. We demonstrated the utility of this sdAb as a capture and detector molecule in Luminex based assays providing limits of detection (LODs) of at least 64 pg/ml. Conclusion: The anti-SEB sdAb A3 was found to have a high affinity and an extraordinarily high Tm and could still refold to recover activity after heat denaturation. This combination of heat resilience and strong, specific binding make this sdAb a good candidate for use in antibody-based toxin detection technologies.	Graef et al. 2011
Denaturing conditions **Refolding** **Thermodynamic stability**	A variety of techniques, including high-pressure unfolding monitored by Fourier transform infrared spectroscopy, fluorescence, circular dichroism, and surface plasmon resonance spectroscopy, have been used to investigate the equilibrium folding properties of six single-domain antigen binders derived from camelid heavy-chain antibodies with specificities for lysozymes, beta-lactamases, and a dye (RR6). Various denaturing conditions (guanidinium chloride, urea, temperature, and pressure) provided complementary and independent methods for characterizing the stability and unfolding properties of the antibody fragments. With all binders, complete recovery of the biological activity after renaturation demonstrates that chemical- induced unfolding is fully reversible. Furthermore, denaturation experiments followed by optical spectroscopic methods and affinity measurements indicate that the antibody fragments are unfolded cooperatively in a single transition. Thus, unfolding/refolding equilibrium proceeds via a simple two-state mechanism (Nreversible arrow U), where only the native and the denatured states are significantly populated. Thermally- induced denaturation, however, is not completely reversible, and the partial loss of binding capacity might be due, at least in part, to incorrect refolding of the long loops (CDRs), which are responsible for antigen recognition. Most interestingly, all the fragments are rather resistant to heat-induced denaturation (apparent T-m = 60–80 degrees-C), and display high conformational stabilities (DeltaG(H2O) = 30–60 kJ/mol). Such high thermodynamic stability has never been reported for any functional conventional antibody fragment, even when engineered antigen binders are considered. Hence, the reduced size, improved solubility, and higher stability of the camelid heavy-chain antibody fragments are of special interest for biotechnological and medical applications.	Dumoulin et al. 2002

Heat shock **Conformation studies** **Denaturation** **Refolding**	In a previous study we have shown that llama VHH antibody fragments are able to bind their antigen after a heat shock of 90 degrees C, in contrast to the murine monoclonal antibodies. However, the molecular mechanism by which antibody: antigen interaction occurs under these extreme conditions remains unclear. To examine in more detail the structural and thermodynamic aspects of the binding mechanism, an extensive CD, ITC, and NMR study was initiated. In this study the interaction between the llama VHH-R2 fragment and its antigen, the dye Reactive Red-6 (RR6) has been explored. The data show clearly that most of the VHH-R2 population at 80 degrees C is in an unfolded conformation. In contrast, CD spectra representing the complex between VHH-R2 and the dye remained the same up to 80 degrees C. Interestingly, addition of the dye to the denatured VHH-R2 at 80 degrees C yielded the spectrum of the native complex. These results suggest an induced refolding of denatured VHH-R2 by its antigen under these extreme conditions. This induced refolding showed some similarities with the well established "induced fit" mechanism of antibody-antigen interactions at ambient temperature. However, the main difference with the "induced fit0148" mechanism is that at the start of the addition of the antigen most of the VHH molecules are in an unfolded conformation. The refolding capability under these extreme conditions and the stable complex formation make VHHs useful in a wide variety of applications.	Dolk et al. 2005b
Bacillus anthracis **Thermal stability** **Denaturation** **Refolding**	Significant efforts to develop both laboratory and field-based detection assays for an array of potential biological threats started well before the anthrax attacks of 2001 and have continued with renewed urgency following. While numerous assays and methods have been explored that are suitable for laboratory utilization, detection in the field is often complicated by requirements for functionality in austere environments, where limited cold-chain facilities exist. In an effort to overcome these assay limitations for Bacillus anthracis, one of the most recognizable threats, a series of single domain antibodies (sdAbs) were isolated from a phage display library prepared from immunized llamas. Characterization of target specificity, affinity, and thermal stability was conducted for six sdAb families isolated from rounds of selection against the bacterial spore. The protein target for all six sdAb families was determined to be the S-layer protein EA1, which is present in both vegetative cells and bacterial spores. All of the sdAbs examined exhibited a high degree of specificity for the target bacterium and its spore, with affinities in the nanomolar range, and the ability to refold into functional antigen-binding molecules following several rounds of thermal denaturation and refolding. This research demonstrates the capabilities of these sdAbs and their potential for integration into current and developing assays and biosensors.	Walper et al. 2012
Dandruff **Malassezia furfur** **Shampoo** **Denaturing conditions** **Amino acid replacement**	As part of research exploring the feasibility of using antibody fragments to inhibit the growth of organisms implicated in dandruff, we isolated antibody fragments that bind to a cell surface protein of Malassezia furfur in the presence of shampoo. We found that phage display of llama single-domain antibody fragments (VHHs) can be extended to very harsh conditions, such as the presence of shampoo containing nonionic and anionic surfactants. We selected several VHHs that bind to the cell wall protein Malf1 of M. furfur, a fungus implicated in causing dandruff. In addition to high stability in the presence of shampoo, these VHHs are also stable under other denaturing conditions, such as high urea concentrations. Many of the stable VHHs were found to contain arginine at position 44. Replacement of the native amino acid at position 44 with arginine in the most stable VHH that lacked this arginine resulted in a dramatic further increase in the stability. The combination of the unique properties of VHHs together with applied phage display and protein engineering is a powerful method for obtaining highly stable VHHs that can be used in a wide range of applications.	Dolk et al. 2005a

Solubility **CDR3** **Hydrophilic amino acids** **Trypanosome surface glycoprotein**	Heavy chain only antibodies of camelids bind their antigens with a single domain, the VHH, which acquired adaptations relative to classical VHs to function in the absence of a VL partner. Additional CDR loop conformations, outside the canonical loop structures of VHs, broaden the repertoire of the antigen-binding site. The combined effects of part of the CDR3 that folds over the "former" VL binding site and framework-2 mutations to more hydrophilic amino acids, enhance the solubility of VHH domains and prevent VL pairing. cAbAn33, a VHH domain specific for the carbohydrate moiety of the variant surface glycoprotein of trypanosomes, has a short CDR3 loop that does not cover the former VL binding site as well as a VH-specific Trp47 instead of the VHH-specific Gly47. Resurfacing its framework-2 region (mutations Tyr37Val, Glu44Gly and Arg45Leu) to mimic that of a human VH restores the VL binding capacity. In solution, the humanised VHH behaves as a soluble, monomeric entity, albeit with reduced thermodynamic stability and affinity for its antigen. Comparison of the crystal structures of cAbAn33 and its humanised derivative reveals steric hindrance exerted by VHH-specific residues Tyr37 and Arg45 that prevent the VL domain pairing, whereas Glu44 and Arg45 are key elements to avoid insolubility of the domain.	Conrath et al. 2005
Serum half-live **Porcine immunoglobulin G** **E. coli F4 fimbriae**	The therapeutic parenteral application of llama single-domain antibody fragments (VHHs) is hampered by their small size, resulting in a fast elimination from the body. Here we describe a method to increase the serum half-life of VHHs in pigs by fusion to another VHH binding to porcine immunoglobulin G (pIgG). We isolated 19 pIgG-binding VHHs from an immunized llama using phage display. Six VHHs were genetically fused to model VHH K609 that binds to Escherichia coli F4 fimbriae. All six yeast-produced genetic fusions of two VHH domains (VHH2s) were functional in ELISA and bound to pIgG with high affinity (1–33 nM). Four pIgG-binding VHH2s were administered to pigs and showed a 100-fold extended in vivo residence times as compared to a control VHH2 that does not bind to pIgG. This could provide the basis for therapeutic application of VHHs in pigs.	Harmsen et al. 2005
Biodistribution studies **Blood circulation** **Tumour retention** **Tumour-targeting vehicle**	Two camel single-domain fragments, cAb-Lys2 and cAb-Lys3, recognizing an overlapping epitope of lysozyme with a dissociation constant of 2 nM and 6S nM, respectively, and a bivalent cAb-Lys3 were investigated for their ability to target transgenic tumors expressing lysozyme on their membrane. Biodistribution studies revealed that these non-immunogenic monomeric and bivalent camel single-domain antigen binders specifically target lysozyme-expressing tumors and metastatic lesions. The excess of antibody is rapidly eliminated from the blood circulation and no cAb retention was observed in normal organs. The tumor to organ cAb-ratios at 2 and 8 h were in the (2.1–10.8) : 1 and (6.2–23.7) : 1 range, respectively. The degree and specificity of tumor retention is independent of the affinity of the recombinant camel single-domain fragments for their antigen and from their univalent monomeric (15 kDa) or bivalent format (33 kDa). This study demonstrates the successful and specific in vivo targeting of tumors by camel single-domain fragments. It may open perspectives for their future use as tumor-targeting vehicle, due to their small size, soluble behaviour and because they are non-immunogenic and interact with epitopes that are less antigenic for conventional antibodies.	Cortez-Retamozo et al. 2002

Binding kinetics **Ricin toxoid** **ELISA** **Surface plasmon resonance**	Single domain antibodies are the recombinantly expressed binding fragments derived from heavy chain antibodies found in camels and llamas. These unique binding elements offer many desirable properties such as their small size (similar to 15 kDa) and thermal stability, which makes them attractive alternatives to conventional monoclonal antibodies. We created a phage display library from llamas immunized with ricin toxoid and selected a number of single domain antibodies. Phage selected on ricin were found to bind to either ricin A chain or the intact molecule; no ricin B chain binders were identified. By panning on B chain, we identified binders and have characterized their binding to the ricin B chain. While they have a poorer affinity than the previously described A chain binders, it was found that they performed dramatically better as capture reagents for the detection of ricin, providing a limit of detection in enzyme linked immunosorbent assay (ELISA) below 100 pg/mL and excellent specificity for ricin versus the highly related RCA 120 (1 to 10 000). We also reevaluated the previously isolated antiricin single domain antibody binding kinetics using surface plasmon resonance and found their K(d)s matched closely to those previously obtained under equilibrium binding conditions measured using the Luminex flow cytometer.	Anderson et al. 2010
Nanobody properties **Epidermal growth factor receptor** **Drug targeting**	The discovery of naturally occurring heavy chain only antibodies and their further development into small recombinant ‘nanobodies’ offers attractive applications in drug targeting. Here, we describe the properties of nanobodies that have been developed to target the epidermal growth factor receptor (EGFR) and contrast these to the characteristics of heavy chain only antibodies and conventional antibodies. EGFR is overexpressed in many tumors and is an attractive target for tumor-directed drug targeting.	Altintas et al. 2012

Table 6A. Application of single-domain antibody fragments in therapy: inhibition of enzymes, toxins and other soluble proteins

Protein kinase C **Enzyme modulator**	The 10 isozymes of the protein kinase C (PKC) family can have different roles on the same biological process, making isozyme specific analysis of function crucial. Currently, only few pharmacological compounds with moderate isozyme specific effects exist thus hampering research into individual PKC isozymes. The antigen binding regions of camelid single chain antibodies (VHHs) could provide a solution for obtaining PKC isozyme specific modulators. In the present study, we have successfully selected and characterized PKC epsilon specific VHH antibodies from two immune VHH libraries using phage display. The VHHs were shown to exclusively bind to PKC epsilon in ELISA and immunoprecipitation studies. Strikingly, five of the VHHs had an effect on PKC epsilon kinase activity in vitro. VHHs A10, C1 and D1 increased PKC epsilon kinase activity in a concentration-dependent manner (EC(50) values: 212–310 nM), whereas E6 and G8 inhibited PKC epsilon activity (IC(50) values: 103–233 nM). None of these VHHs had an effect on the activity of the other novel PKC isozymes PKC delta and PKC theta. To our knowledge, these antibodies are the first described VHH activators and inhibitors for a protein kinase. Furthermore, the development of PKC epsilon specific modulators is an important contribution to PKC research.	Paalanen et al. 2011
Anti-idiotypic **Alliinase** **Molecular mimicry** **Abzyme**	Screening of inhibitory Ab1 antibodies is a critical step for producing catalytic antibodies in the anti-idiotypic approach. However, the incompatible surface of the active site of the enzyme and the antigen-binding site of heterotetrameric conventional antibodies become the limiting step. Because camelid-derived nanobodies possess the potential to preferentially bind to the active site of enzymes due to their small size and long CDR3, we have developed a novel approach to produce antibodies with alliinase activities by exploiting the molecular mimicry of camel nanobodies. By screening the camelid-derived variable region of the heavy chain cDNA phage display library with alliinase, we obtained an inhibitory nanobody VHHA4 that recognizes the active site. Further screening with VHHA4 from the same variable domain of the heavy chain of a heavy-chain antibody library led to a higher incidence of anti-idiotypic Ab2 abzymes with alliinase activities. One of the abzymes, VHHC10, showed the highest activity that can be inhibited by Ab1VHHA4 and alliinase competitive inhibitor penicillamine and significantly suppressed the B16 tumor cell growth in the presence of alliin in vitro. The results highlight the feasibility of producing abzymes via anti-idiotypic nanobody approach.	Li et al. 2012
Allosteric effector **Dihydrofolate reductase** **Inhibition kinetics** **Crystal structure**	Although it has been known for many years that antibodies display properties characteristic of allosteric effectors, the molecular mechanisms responsible for these effects remain poorly understood. Here, we describe a single-domain antibody fragment (nanobody) that modulates protein function by constraining conformational change in the enzyme dihydrofolate reductase (DHFR). Nanobody 216 (Nb216) behaves as a potent allosteric inhibitor of DHFR, giving rise to mixed hyperbolic inhibition kinetics. The crystal structure of Nb216 in complex with DHFR reveals that the nanobody binds adjacent to the active site. Half of the epitope consists of residues from the flexible Met20 loop. This loop, which ordinarily oscillates between occluded and closed conformations during catalysis, assumes the occluded conformation in the Nb216-bound state. Using stopped flow, we show that Nb216 inhibits DHFR by stabilising the occluded Met20 loop conformation. Surprisingly, kinetic data indicate that the Met20 loop retains sufficient conformational flexibility in the Nb216-bound state to allow slow substrate turnover to occur.	Oyen et al. 2011

Sialidase **Trypanosoma cruzi**	The sialic acid present in the protective surface mucin coat of Trypanosoma cruzi is added by a membrane anchored trans-sialidase (TcTS), a modified sialidase that is expressed from a large gene family. In this work, we analyzed single domain camelid antibodies produced against trans-sialidase. Llamas were immunized with a recombinant trans-sialidase and inhibitory single-domain antibody fragments were obtained by phage display selection, taking advantage of a screening strategy using an inhibition test instead of the classic binding assay. Four single domain antibodies displaying strong trans-sialidase inhibition activity against the recombinant enzyme were identified. They share the same complementarity-determining region 3 length (17 residues) and have very similar sequences. This result indicates that they likely derived from a unique clone. Probably there is only one structural solution for tight binding inhibitory antibodies against the TcTS used for immunization. To our surprise, this single domain antibody that inhibits the recombinant TcTS, failed to inhibit the enzymatic activity present in parasite extracts. Analysis of individual recombinant trans-sialidases showed that enzymes expressed from different genes were inhibited to different extents (from 8 to 98%) by the llama antibodies. Amino acid changes at key positions are likely to be responsible for the differences in inhibition found among the recombinant enzymes. These results suggest that the presence of a large and diverse trans-sialidase family might be required to prevent the inhibitory response against this essential enzyme and might thus constitute a novel strategy of T. cruzi to evade the host immune system.	Ratier et al. 2008
Clostridium difficile **Nosocomial infection** **North America** **Exotoxin A/B** **Gastrointestinal tract**	Clostridium difficile is a leading cause of nosocomial infection in North America and a considerable challenge to healthcare professionals in hospitals and nursing homes. The Gram-positive bacterium produces two high molecular weight exotoxins, toxin A (TcdA) and toxin B (TcdB), which are the major virulence factors responsible for C. difficile-associated disease and are targets for C. difficile-associated disease therapy. Here, recombinant single-domain antibody fragments (V(H)Hs), which specifically target the cell receptor binding domains of TcdA or TcdB, were isolated from an immune llama phage display library and characterized. Four V(H)Hs (A4.2, A5.1, A20.1, and A26.8), all shown to recognize conformational epitopes, were potent neutralizers of the cytopathic effects of toxin A on fibroblast cells in an in vitro assay. The neutralizing potency was further enhanced when V(H)Hs were administered in paired or triplet combinations at the same overall V(H)H concentration, suggesting recognition of nonoverlapping TcdA epitopes. Biacore epitope mapping experiments revealed that some synergistic combinations consisted of V(H)Hs recognizing overlapping epitopes, an indication that factors other than mere epitope blocking are responsible for the increased neutralization. Further binding assays revealed TcdA-specific V(H)Hs neutralized toxin A by binding to sites other than the carbohydrate binding pocket of the toxin. With favorable characteristics such as high production yield, potent toxin neutralization, and intrinsic stability, these V(H)Hs are attractive systemic therapeutics but are more so as oral therapeutics in the destabilizing environment of the gastrointestinal tract.	Hussack et al. 2011b

Staphylococcus aureus **Hemolysin** **'Hot-cold' hemolytic activity**	Objective: To isolate and characterize Staphylococcus aureus (S. aureus) hemolysin neutralizing dAbs front phage display library of Indian desert camel. Methods: Phage display library of 5 × 10^7 dAb clones of LPS-immunized Indian desert camel constructed in our laboratory was used for selection of S. aureus exotoxin specific clones by panning technique. Enrichment of Ag-specific clones in successive rounds of panning was assessed by phage-ELISA and phage titration. Different dAb clones binding to S. aureus exotoxin Ags were expressed with C-terminal 6 X His tag in E. coli and purified by Ni-chelate chromatography. The expression was verified by SDS-PAGE and western blotting. The purified clones were tested for inhibition of 'hot-cold' hemolytic activity in vitro. Resistance to thermal inactivation of the dAb clones was studied by observing the effect of heat treatment from 50 degrees C to 99 degrees C for 30 min on the 'hot-cold' hemolytic activity in vitro. Results: Several dAb clones binding to S. aureus exotoxins were isolated and enriched by three rounds of panning. The soluble dAb clones were approximately similar to 16 kDa in size and reacted with 6 X His tag specific murine monoclonal antibody in western blot. One of the Ni-chelate affinity purified dAb.6 X His clones, inhibited S. aureus beta-hemolysin activity in vitro and resisted thermal inactivation upto 99 degrees C. Conclusions: An S. aureus beta-hemolysin neutralizing dAb clone of possible therapeutic potential has been isolated.	Pooja and Ajit 2010
Thrombin activatable fibrinolysis inhibitor **Clot lysis**	Background: Because activated thrombin activatable fibrinolysis inhibitor (TAFIa) has very powerful antifibrinolytic properties, co-administration of t-PA and a TAFIa inhibitor enhances t-PA treatment. Objective: We aimed to generate nanobodies specifically inhibiting the TAFIa activity and to test their effect on t-PA induced clot lysis. Results: Five nanobodies, raised towards an activated more stable TAFIa mutant (TAFIa A(147)-C(305)-I(325)-I(329)-Y(333)-Q(335)), are described. These nanobodies inhibit specifically TAFIa activity, resulting in an inhibition of up to 99% at a 16-fold molar excess of nanobody over TAFIa, IC(50)'s range between 0.38- and > 16-fold molar excess. In vitro clot lysis experiments in the absence of thrombomodulin (TM) demonstrate that the nanobodies exhibit profibrinolytic effects. However, in the presence of TM, one nanobody exhibits an antifibrinolytic effect whereas the other nanobodies show a slight antifibrinolytic effect at low concentrations and a pronounced profibrinolytic effect at higher concentrations. This biphasic pattern was highly dependent on TM and t-PA concentration. The nanobodies were found to bind in the active-site region of TAFIa and their time-dependent differential binding behavior during TAFIa inactivation revealed the occurrence of a yet unknown intermediate conformational transition. Conclusion: These nanobodies are very potent TAFIa inhibitors and constitute useful tools to accelerate fibrinolysis. Our data also demonstrate that the profibrinolytic effect of TAFIa inhibition may be reversed by the presence of TM. The identification of a new conformational transition provides new insights into the conformational inactivation of the unstable TAFIa.	Hendrickx et al. 2011
Tumour necrosis factor **Fusion** **Elastin-like polypeptide** **Tobacco**	Tumour necrosis factor (TNF) is a major pro-inflammatory cytokine involved in multiple inflammatory diseases. The detrimental activity of TNF can be blocked by various antagonists, and commercial therapeutics based upon this principle have been approved for treatment of diseases including rheumatoid arthritis, Crohn's disease and psoriasis. In a search for new, improved anti-inflammatory therapeutics we have designed a single-domain monoclonal antibody (V(H)H), which recognizes TNF. The antibody component (TNF-V(H)H) is based upon an anti-human TNF Camelidae heavy-chain monoclonal antibody, which was linked to an elastin-like polypeptide (ELP). We demonstrate that ELP fusion to the TNF-V(H)H enhances accumulation of the fusion protein during biomanufacturing in transgenic tobacco plants. With this study, we show for the first time that this plant-derived anti-human TNF-V(H)H antibody was biologically active in vivo. Therefore, therapeutic application of TNF-V(H)H-ELP fusion protein was tested in humanized TNF mice and was shown to be effective in preventing death caused by septic shock. The in vivo persistence of the ELPylated antibody was similar to 24 fold longer than that of non-ELPylated TNF-V(H)H.	Conrad et al. 2011

L-plastin **Malignancy** **F-actin bundling** **Filopodia**	L-plastin, a conserved modular F-actin bundling protein, is ectopically expressed in tumor cells and contributes to cell malignancy and invasion. The underlying molecular mechanisms involved remain unclear, in part, because specific inhibitors of L-plastin are lacking. We used recombinant alpaca-derived L-plastin single-domain antibodies (nanobodies) as effector of L-plastin function in cells. Key findings were compared with L-plastin down-regulation by RNAi. We show that nanobodies strongly interact with L-plastin by targeting discrete conformational epitopes with nanomolar affinity. A nanobody that selectively interacts with the tandem ABDs in L-plastin completely inhibits F-actin bundling at equimolar ratios, in contrast to a control green fluorescent protein (GFP) nanobody. This "knockout" nanobody inhibits filopodia formation, motility, and invasion when expressed in PC-3 cells. L-plastin RNA interference showed no significant effect on filopodial integrity and only marginally restrained the motile properties of cells. L-plastin nanobodies uniquely expose a fundamental role for this protein in filopodia formation and cell migration. Therefore, these molecules represent a potent instrument to ablate functions of structural proteins without manipulating gene expression. In addition, we show that they can be instrumental in uncovering new functions of proteins that remain obscured by RNAi.	Delanote et al. 2010

Table 6B. Application of single-domain antibody fragments in therapy: activity modulation of cell surface proteins

Nanobody properties **Epidermal growth factor receptor** **Drug targeting**	The discovery of naturally occurring heavy chain only antibodies and their further development into small recombinant 'nanobodies' offers attractive applications in drug targeting. Here, we describe the properties of nanobodies that have been developed to target the epidermal growth factor receptor (EGFR) and contrast these to the characteristics of heavy chain only antibodies and conventional antibodies. EGFR is overexpressed in many tumors and is an attractive target for tumor-directed drug targeting.	Altintas et al. 2012
Vascular endothelial growth factor receptor-2 **Neovascularization** **Metastasis**	Vascular endothelial growth factor receptor-2 (VEGFR2) is an important tumor-associated receptor and blockade of the VEGF receptor signaling can lead to the inhibition of neovascularization and tumor metastasis. Nanobodies are the smallest intact antigen binding fragments derived from heavy chain-only antibodies occurring in camelids. Here, we describe the identification of a VEGFR2-specific Nanobody, named 3VGR19, from dromedaries immunized with a cell line expressing high levels of VEGFR2. We demonstrate by FACS, that 3VGR19 Nanobody specifically binds VEGFR2 on the surface of 293KDR and HUVECs cells. Furthermore, the 3VGR19 Nanobody potently inhibits formation of capillary-like structures. These data show the potential of Nanobodies for the blockade of VEGFR2 signaling and provide a basis for the development of novel cancer therapeutics.	Behdani et al. 2012
Leptin receptor **Subdomain** **Neuropeptide Y** **Blood-brain barrier**	The adipocyte-derived cytokine leptin acts as a metabolic switch, connecting the body's metabolism to high-energy consuming processes such as reproduction and immune responses. Accumulating evidence suggests that leptin plays a role in human pathologies, such as autoimmune diseases and cancer, thus providing a rationale for the development of leptin antagonists. In the present study, we generated and evaluated a panel of neutralizing nanobodies targeting the LR (leptin receptor). A nanobody comprises the variable domain of the naturally occurring single-chain antibodies found in members of the Camelidae family. We identified three classes of neutralizing nanobodies targeting different LR subdomains: i.e. the CRH2 (cytokine receptor homology 2), Ig-like and FNIII (fibronectin type III) domains. Only nanobodies directed against the CRH2 domain inhibited leptin binding. We could show that a nanobody that targets the Ig-like domain potently interfered with leptin-dependent regulation of hypothalamic NPY (neuropeptide Y) expression. As a consequence, daily intraperitoneal injection increased body weight, body fat content, food intake, liver size and serum insulin levels. All of these characteristics resemble the phenotype of leptin and LR-deficient animals. The results of the present study support proposed models of the activated LR complex, and demonstrate that it is possible to block LR signalling without affecting ligand binding. These nanobodies form new tools to study the mechanisms of BBB (blood-brain barrier) leptin transport and the effect of LR inhibition in disease models.	Zabeau et al. 2012

Carcinoembryonic antigen related cell adhesion molecule **Cancer**	Carcinoembryonic antigen related cell adhesion molecule (CEACAM) 6 is over-expressed in different types of cancer cells. In addition, it has also been implicated in some infectious diseases. Targeting this molecule by an antibody might have applications in diverse tumor models. Single domain antibody (sdAb) is becoming very useful format in antibody engineering as potential tools for treating acute and chronic disease conditions such as cancer for both diagnostic as well as therapeutic application. Generally, sdAbs with good affinity are isolated from an immune library. Discovery of a new target antigen would require a new immunization with purified antigen which is not always easy. In this study, we have isolated, by phage display, an sdAb against CEACAM6 with an affinity of 5 nM from a llama immunized with cancer cells. The antibody has good biophysical properties, and it binds to the cells expressing the target antigen. Furthermore, it reduces cancer cells proliferation in vitro and shows an excellent tumor targeting in vivo. This sdAb could be useful in diagnosis as well as therapy of CEACAM6 expressing tumors. Finally, we envisage it would be feasible to isolate good sdAbs against other interesting tumor associated antigens from this library. Therefore, this immunization method could be a general strategy for isolating sdAbs against any surface antigen without immunizing the animal with the antigen of interest each time.	Baral et al. 2011
12 nanobody clones **Oligoclonal nanobodies** **Breast cancer cells** **HER2**	Modern anti-HER2 antibody therapy tends to exploit a panel of different antibodies against different epitopes on the antigen. For this aim, nanobodies are very striking targeting agents and can be easily produced against any cell-specific membrane antigen. The oligoclonal nanobodies can be used to block more than one functional epitope on a target antigen and inhibit the generation of escape variants associated with cancer therapy. In this study, 12 nanobody clones selected from an immune camel library were examined for their ability to differ between tumor markers. These oligoclonal nanobodies targeted breast cancer cells better than each individual nanobody. In epitope mapping, several nanobodies overlapped in the epitope recognized by trastuzumab and some of the non-overlapping nanobodies could affect the binding of trastuzumab to HER2. This study demonstrates that the oligoclonal nanobodies are potential therapeutic tools that can be used instead of, or in combination with trastuzumab to assess tumor viability during treatment.	Jamnani et al. 2012
Ion channel M2 **Influenza A**	Influenza A virus poses serious health threat to humans. Neutralizing antibodies against the highly conserved M2 ion channel is thought to offer broad protection against influenza A viruses. Here, we screened synthetic Camel single-domain antibody (VHH) libraries against native M2 ion channel protein. One of the isolated VHHs, M2-7A, specifically bound to M2-expressed cell membrane as well as influenza A virion, inhibited replication of both amantadine-sensitive and resistant influenza A viruses in vitro, and protected mice from a lethal influenza virus challenge. Moreover, M2-7A showed blocking activity for proton influx through M2 ion channel. These pieces of evidence collectively demonstrate for the first time that a neutralizing antibody against M2 with broad specificity is achievable, and M2-7A may have potential for cross protection against a number of variants and subtypes of influenza A viruses.	Wei et al. 2011

Table 6C. Application of single-domain antibody fragments in therapy: pathogen neutralisation

Bivalent nanobody **Influenza virus** **Intranasal administration**	Influenza A virus infections impose a recurrent and global disease burden. Current antivirals against influenza are not always effective. We assessed the protective potential of monovalent and bivalent Nanobodies (Ablynx) against challenge with this virus. These Nanobodies were derived from llamas and target H5N1 hemagglutinin. Intranasal administration of Nanobodies effectively controlled homologous influenza A virus replication. Administration of Nanobodies before challenge strongly reduced H5N1 virus replication in the lungs and protected mice from morbidity and mortality after a lethal challenge with H5N1 virus. The bivalent Nanobody was at least 60-fold more effective than the monovalent Nanobody in controlling virus replication. In addition, Nanobody therapy after challenge strongly reduced viral replication and significantly delayed time to death. Epitope mapping revealed that the VHH Nanobody binds to antigenic site B in H5 hemagglutinin. Because Nanobodies are small, stable, and simple to produce, they are a promising, novel therapeutic agent against influenza.	Ibanez et al. 2011
Multimeric/bispecific constructs **Influenza**	For efficient prevention of viral infections and cross protection, simultaneous targeting of multiple viral epitopes is a powerful strategy. Llama heavy chain antibody fragments (VHH) against the trimeric envelope proteins of Respiratory Syncytial Virus (Fusion protein), Rabies virus (Glycoprotein) and H5N1 Influenza (Hemagglutinin 5) were selected from llama derived immune libraries by phage display. Neutralizing VHH recognizing different epitopes in the receptor binding sites on the spikes with affinities in the low nanomolar range were identified for all the three viruses by viral neutralization assays. By fusion of VHH with variable linker lengths, multimeric constructs were made that improved neutralization potencies up to 4000-fold for RSV, 1500-fold for Rabies virus and 75-fold for Influenza H5N1. The potencies of the VHH constructs were similar or better than best performing monoclonal antibodies. The cross protection capacity against different viral strains was also improved for all three viruses, both by multivalent (two or three identical VHH) and biparatopic (two different VHH) constructs. By combining a VHH neutralizing RSV subtype A, but not subtype B with a poorly neutralizing VHH with high affinity for subtype B, a biparatopic construct was made with low nanomolar neutralizing potency against both subtypes. Trivalent anti-H5N1 VHH neutralized both Influenza H5N1 clade1 and 2 in a pseudotype assay and was very potent in neutralizing the NIBRG-14 Influenza H5N1 strain with IC(50) of nine picomolar. Bivalent and biparatopic constructs against Rabies virus cross neutralized both 10 different Genotype 1 strains and Genotype 5. The results show that multimerization of VHH fragments targeting multiple epitopes on a viral trimeric spike protein is a powerful tool for anti-viral therapy to achieve "best-in-class" and broader neutralization capacity.	Hultberg et al. 2011
HIV virus **CXCR4** **Epitope mapping**	The important family of G protein-coupled receptors has so far not been targeted very successfully with conventional monoclonal antibodies. Here we report the isolation and characterization of functional VHH-based immunoglobulin single variable domains (or nanobodies) against the chemokine receptor CXCR4. Two highly selective monovalent nanobodies, 238D2 and 238D4, were obtained using a time-efficient whole cell immunization, phage display, and counterselection method. The highly selective VHH-based immunoglobulin single variable domains competitively inhibited the CXCR4-mediated signaling and antagonized the chemoattractant effect of the CXCR4 ligand CXCL12. Epitope mapping showed that the two nanobodies bind to distinct but partially overlapping sites in the extracellular loops. Short peptide linkage of 238D2 with 238D4 resulted in significantly increased affinity for CXCR4 and picomolar activity in antichemotactic assays. Interestingly, the monovalent nanobodies behaved as neutral antagonists, whereas the biparatopic nanobodies acted as inverse agonists at the constitutively active CXCR4-N3.35A. The CXCR4 nanobodies displayed strong antiretroviral activity against T cell-tropic and dual-tropic HIV-1 strains. Moreover, the biparatopic nanobody effectively mobilized CD34-positive stem cells in cynomolgus monkeys. Thus, the nanobody platform may be highly effective at generating extremely potent and selective G protein-coupled receptor modulators.	Jahnichen et al. 2010

HIV virus Envelope protein CD4 receptor	Many of the neutralising antibodies, isolated to date, display limited activities against the globally most prevalent HIV-1 subtypes A and C. Therefore, those subtypes are considered to be an important target for antibody-based therapy. Variable domains of llama heavy chain antibodies (VHH) have some superior properties compared with classical antibodies. Therefore we describe the application of trimeric forms of envelope proteins (Env), derived from HIV-1 of subtype A and B/C, for a prolonged immunization of two llamas. A panel of VHH, which interfere with CD4 binding to HIV-1 Env were selected with use of panning. The results of binding and competition assays to various Env, including a variant with a stabilized CD4-binding state (gp120(Ds2)), cross-competition experiments, maturation analysis and neutralisation assays, enabled us to classify the selected VHH into three groups. The VHH of group I were efficient mainly against viruses of subtype A, C and B'/C. The VHH of group II resemble the broadly neutralising antibody (bnmAb) b12, neutralizing mainly subtype B and C viruses, however some had a broader neutralisation profile. A representative of the third group, 2E7, had an even higher neutralization breadth, neutralizing 21 out of the 26 tested strains belonging to the A, A/G, B, B/C and C subtypes. To evaluate the contribution of certain amino acids to the potency of the VHH a small set of the mutants were constructed. Surprisingly this yielded one mutant with slightly improved neutralisation potency against 92UG37.A9 (subtype A) and 96ZM651.02 (subtype C). These findings and the well-known stability of VHH indicate the potential application of these VHH as anti-HIV-1 microbicides.	Strokappe et al. 2012
Rotavirus Gastroenteritis Immunocapture electron microscopy	Rotavirus is the main cause of viral gastroenteritis in young children. Therefore, the development of inexpensive antiviral products for the prevention and/or treatment of rotavirus disease remains a priority. Previously we have shown that a recombinant monovalent antibody fragment (referred to as Anti-Rotavirus Proteins or ARP1) derived from a heavy chain antibody of a llama immunised with rotavirus was able to neutralise rotavirus infection in a mouse model system. In the present work we investigated the specificity and neutralising activity of two llama antibody fragments, ARP1 and ARP3, against 13 cell culture adapted rotavirus strains of diverse genotypes. In addition, immunocapture electron microscopy (IEM) was performed to determine binding of ARP1 to clinical isolates and cell culture adapted strains. ARP1 and ARP3 were able to neutralise a broad variety of rotavirus serotypes/genotypes in vitro, and in addition, IEM showed specific binding to a variety of cell adapted strains as well as strains from clinical specimens. These results indicated that these molecules could potentially be used as immunoprophylactic and/or immunotherapeutic products for the prevention and/ or treatment of infection of a broad range of clinically relevant rotavirus strains.	Aladin et al. 2012
Plasmodium vivax Fy blood group Duffy antigen receptor for chemokines	Fy blood group antigens are carried by the Duffy antigen receptor for chemokines (DARC), a red cells receptor for Plasmodium vivax broadly implicated in human health and diseases. Recombinant VHHs, or nanobodies, the smallest intact antigen binding fragment derivative from the heavy chain-only antibodies present in camelids, were prepared from a dromedary immunized against DARC N-terminal extracellular domain and selected for DARC binding. A described VHH, CA52, does recognize native DARC on cells. It inhibits P. vivax invasion of erythrocytes and displaces interleukin-8 bound to DARC. The targeted epitope overlaps the well-defined DARC Fy6 epitope. K(D) of CA52-DARC equilibrium is sub-nanomolar, hence ideal to develop diagnostic or therapeutic compounds. Immunocapture by immobilized CA52 yielded highly purified DARC from engineered K562 cells. This first report on a VHH with specificity for a red blood cell protein exemplifies VHHs' potentialities to target, to purify, and to modulate the function of cellular markers.	Smolarek et al. 2010

Trypanosoma brucei Variant-specific surface glycoprotein Nanobody-mediated lysis	The African trypanosome Trypanosoma brucei, which persists within the bloodstream of the mammalian host, has evolved potent mechanisms for immune evasion. Specifically, antigenic variation of the variant-specific surface glycoprotein (VSG) and a highly active endocytosis and recycling of the surface coat efficiently delay killing mediated by anti-VSG antibodies. Consequently, conventional VSG-specific intact immunoglobulins are non-trypanocidal in the absence of complement. In sharp contrast, monovalent antigen-binding fragments, including 15 kDa nanobodies (Nb) derived from camelid heavy-chain antibodies (HCAbs) recognizing variant-specific VSG epitopes, efficiently lyse trypanosomes both in vitro and in vivo. This Nb-mediated lysis is preceded by very rapid immobilisation of the parasites, massive enlargement of the flagellar pocket and major blockade of endocytosis. This is accompanied by severe metabolic perturbations reflected by reduced intracellular ATP-levels and loss of mitochondrial membrane potential, culminating in cell death. Modification of anti-VSG Nbs through site-directed mutagenesis and by reconstitution into HCAbs, combined with unveiling of trypanolytic activity from intact immunoglobulins by papain proteolysis, demonstrates that the trypanolytic activity of Nbs and Fabs requires low molecular weight, monovalency and high affinity. We propose that the generation of low molecular weight VSG-specific trypanolytic nanobodies that impede endocytosis offers a new opportunity for developing novel trypanosomiasis therapeutics. In addition, these data suggest that the antigen-binding domain of an anti-microbial antibody harbours biological functionality that is latent in the intact immunoglobulin and is revealed only upon release of the antigen-binding fragment.	Stijlemans et al. 2011
Sialidase Trypanosoma cruzi	The sialic acid present in the protective surface mucin coat of Trypanosoma cruzi is added by a membrane anchored trans-sialidase (TcTS), a modified sialidase that is expressed from a large gene family. In this work, we analyzed single domain camelid antibodies produced against trans-sialidase. Llamas were immunized with a recombinant trans-sialidase and inhibitory single-domain antibody fragments were obtained by phage display selection, taking advantage of a screening strategy using an inhibition test instead of the classic binding assay. Four single domain antibodies displaying strong trans-sialidase inhibition activity against the recombinant enzyme were identified. They share the same complementarity-determining region 3 length (17 residues) and have very similar sequences. This result indicates that they likely derived from a unique clone. Probably there is only one structural solution for tight binding inhibitory antibodies against the TcTS used for immunization. To our surprise, this single domain antibody that inhibits the recombinant TcTS, failed to inhibit the enzymatic activity present in parasite extracts. Analysis of individual recombinant trans-sialidases showed that enzymes expressed from different genes were inhibited to different extents (from 8 to 98%) by the llama antibodies. Amino acid changes at key positions are likely to be responsible for the differences in inhibition found among the recombinant enzymes. These results suggest that the presence of a large and diverse trans-sialidase family might be required to prevent the inhibitory response against this essential enzyme and might thus constitute a novel strategy of T. cruzi to evade the host immune system.	Ratier et al. 2008

Antigen variation **Variant surface glycoproteins** **Cryptic/conserved epitopes** **Trypanosomosis therapy** **Antibody: beta-lactamase fusion**	Antigen variation is a successful defense system adopted by several infectious agents to evade the host immune response. The principle of this defense strategy in the African trypanosome paradigm involves a dense packing of variant surface glycoproteins (VSG) exposing only highly variable and immuno-dominant epitopes to the immune system, whereas conserved epitopes become inaccessible for large molecules. Reducing the size of binders that target the conserved, less-immunogenic, cryptic VSG epitopes forms an obvious solution to combat these parasites. This goal was achieved by introducing dromedary Heavy-chain antibodies. We found that only these unique antibodies recognize epitopes common to multiple VSG classes. After phage display of their antigen-binding repertoire, we isolated a single domain antibody fragment with high specificity for the conserved Asn-linked carbohydrate of VSG. In sharp contrast to labeled concanavalin-A that stains only the flagellar pocket where carbohydrates are accessible because of less dense VSG packing, the single domain binder stains the entire surface of viable parasites, irrespective of the VSG type expressed. This corroborates the idea that small antibody fragments, but not larger lectins or conventional antibody fragments, are able to penetrate the dense VSG coat to target their epitope. The diagnostic potential of this fluorescently labeled binder was proven by the direct, selective, and sensitive detection of parasites in blood smears. The employment of this binder as a molecular recognition unit in immunotoxins designed for trypanosomosis therapy becomes feasible as well. This was illustrated by the specific trypanolysis induced by an antibody: beta-lactamase fusion activating a prodrug.	Stijlemans et al. 2004
Cryptic epitopes **Apical membrane antigen 1** **Plasmodium falciparurm** **Extensive polymorphism** **IgNAR**	Apical membrane antigen 1 (AMA1) is essential for invasion of erythrocytes and hepatocytes by Plasmodium parasites and is a leading malarial vaccine candidate. Although conventional antibodies to AMA1 can prevent such invasion, extensive polymorphisms within surface-exposed loops may limit the ability of these AMA1 induced antibodies to protect against all parasite genotypes. Using an AMA1-specific IgNAR single-variable-domain antibody, we performed targeted mutagenesis and selection against AMA1 from three P. falciparurm strains. We present cocrystal structures of two antibody-AMA1 complexes which reveal extended IgNAR CDR3 loops penetrating deep into a hydrophobic cleft on the antigen surface and contacting residues conserved across parasite species. Comparison of a series of affinity-enhancing mutations allowed dissection of their relative contributions to binding kinetics and correlation with inhibition of erythrocyte invasion. These findings provide insights into mechanisms of single-domain antibody binding, and may enable design of reagents targeting otherwise cryptic epitopes in pathogen antigens.	Henderson et al. 2007

Table 6D. Application of single-domain antibody fragments in therapy: intracellular expression of single-domain antibody fragments

Apoptosis **Caspase 3** **Neurodegenerative diseases** **Cancer**	Apoptosis, or programmed cell death, is an essential process affecting homeostasis of cell growth, development, and the elimination of damaged or dangerous cells. Inappropriate cell death caused by oxidative stress has been implicated in the development of neurodegenerative diseases such as Alzheimer's, Parkinson's, and stroke. On the other hand, a defect in the cell death process leads to the development of cancer. For example, the main player of apoptosis, p53, is defective in many of the human cancers. Apoptosis is regulated by the interplay of pro-apoptotic and anti-apoptotic proteins from the Bcl-2 family and caspases. In particular, specific modulators of the activity of Caspase 3 could be very important for the development of therapies for diseases such as neurodegeneration and cancer. In this study, two V(H)Hs specific to Caspase 3 (VhhCasp31 and VhhCasp32) were isolated from a heavy chain antibody variable domain (V(H)H) phage display library and tested for their apoptosis-modulating effects. While VhhCasp31 was found to be antagonistic towards Caspase 3, VhhCasp32 was agonistic. Furthermore, when expressed as intrabodies in SHSY-5Y neuroblastoma cells, VhhCasp31 rendered cells resistant to oxidative-stress-induced apoptosis, whereas VhhCasp32 resulted in apoptosis. These V(H)H antagonist and agonist of apoptosis could have potential for the development of therapeutics for neurodegenerative diseases and cancer, respectively.	McGonigal et al. 2009
Protein kinase C **Enzyme modulator**	The 10 isozymes of the protein kinase C (PKC) family can have different roles on the same biological process, making isozyme specific analysis of function crucial. Currently, only few pharmacological compounds with moderate isozyme specific effects exist thus hampering research into individual PKC isozymes. The antigen binding regions of camelid single chain antibodies (VHHs) could provide a solution for obtaining PKC isozyme specific modulators. In the present study, we have successfully selected and characterized PKC epsilon specific VHH antibodies from two immune VHH libraries using phage display. The VHHs were shown to exclusively bind to PKC epsilon in ELISA and immunoprecipitation studies. Strikingly, five of the VHHs had an effect on PKC epsilon kinase activity in vitro. VHHs A10, C1 and D1 increased PKC epsilon kinase activity in a concentration-dependent manner (EC(50) values: 212–310 nM), whereas E6 and G8 inhibited PKC epsilon activity (IC(50) values: 103–233 nM). None of these VHHs had an effect on the activity of the other novel PKC isozymes PKC delta and PKC theta. To our knowledge, these antibodies are the first described VHH activators and inhibitors for a protein kinase. Furthermore, the development of PKC epsilon specific modulators is an important contribution to PKC research.	Paalanen et al. 2011
IRF-1 **Tumor suppressor** **Mf1 domain** **Transcriptional activity**	IRF-1 is a tumor suppressor protein that activates gene expression from a range of promoters in response to stimuli spanning viral infection to DNA damage. Studies on the post-translational regulation of IRF-1 have been hampered by a lack of suitable biochemical tools capable of targeting the endogenous protein. In this study, phage display technology was used to develop a monoclonal nanobody targeting the C-terminal Mf1 domain (residues 301–325) of IRF-1. Intracellular expression of the nanobody demonstrated that the transcriptional activity of IRF-1 is constrained by the Mf1 domain as nanobody binding gave an increase in expression from IRF-1-responsive promoters of up to 8-fold. Furthermore, Mf1-directed nanobodies have revealed an unexpected function for this domain in limiting the rate at which the IRF-1 protein is degraded. Thus, the increase in IRF-1 transcriptional activity observed on nanobody binding is accompanied by a significant reduction in the half-life of the protein. In support of the data obtained using nanobodies, a single point mutation (P325A) involving the C-terminal residue of IRF-1 has been identified, which results in greater transcriptional activity and a significant increase in the rate of degradation. The results presented here support a role for the Mf1 domain in limiting both IRF-1-dependent transcription and the rate of IRF-1 turnover. In addition, the data highlight a route for activation of downstream genes in the IRF-1 tumor suppressor pathway using biologics.	Moller et al. 2010

Hepatitis B **HBeAg** **IgNAR** **Intracellular precore protein**	The Hepatitis B virus precore protein is processed in the endoplasmic reticulum (ER) into secreted hepatitis B e antigen (HBeAg), which acts as an immune tolerogen to establish chronic infection. Downregulation of secreted HBeAg should improve clinical outcome, as patients who effectively respond to current treatments (IFN-alpha) have significantly lower serum HBeAg levels. Here, we describe a novel reagent, a single variable domain (V(NAR)) of the shark immunoglobulin new antigen receptor (IgNAR) antibodies. V(NARS) possess advantages in stability, size (similar to 14 kDa) and cryptic epitope recognition compared to conventional antibodies. The V(NAR) domain displayed biologically useful affinity for recombinant and native HBeAg, and recognised a unique conformational epitope. To assess therapeutic potential in targeting intracellular precore protein to reduce secreted HBeAg, the V(NAR) was engineered for ER-targeted in vitro delivery to function as an intracellular antibody (intrabody). In vitro data from HBV/precore hepatocyte cell lines demonstrated effective intrabody regulation of precore/HBeAg.	Walsh et al. 2011
Hepatitis B virus **HBcAg** **HBeAg**	Six single-domain antibodies (VHHs) targeting the core antigen of HBV (HBcAg) have been generated and three of these bound strongly to HBcAg of both subtype ayw and adw. These three VHHs were studied as intrabodies directed towards the nucleus or the cytoplasm of a hepatoma cell line that was co-transfected with HBV. A speckled staining of HBcAg was observed in the cytoplasm of cells transfected with nucleotropic VHH intrabodies. Moreover, an increased intracellular accumulation of hepatitis B e antigen (HBeAg) and a complete disappearance of intracellular HBcAg signal were observed with nuclear targeted HBcAg-specific VHHs. These results suggest that HBcAg-specific VHHs targeted to the nucleus affect HBcAg and HBeAg expression and trafficking in HBV-transfected hepatocytes.	Serruys et al. 2010
HIV-1 virus **Rev protein** **Replication** **Multimerization domain**	The human immunodeficiency virus, type 1 (HIV-1)-encoded Rev protein is essential for the expression of late viral mRNAs. Rev forms a large organized multimeric protein-protein complex on the Rev response element of these viral mRNA species and transports them from the nucleus to the cytoplasm, exploiting the CRM1-mediated cellular machinery. Here we report the selection of a nanobody, derived from a llama heavy-chain only antibody, that efficiently blocks the assembly of Rev multimers. The nanobody inhibits HIV-1 replication in cells and specifically suppresses the Rev-dependent expression of partially spliced and unspliced HIV-1 RNA. In HIV-susceptible cells, this nanobody thus has potential as an effective anti-HIV agent using genetic immunization strategies. Its binding site was mapped to Rev residues Lys-20 and Tyr-23 located in the N-terminal alpha-helical multimerization domain. In the presence of this nanobody, we observed an accumulation of dimeric Rev species, supporting a head-to-head/tail-to-tail molecular model for Rev assembly. The results indicate that the oligomeric assembly of Rev follows an ordered stepwise process and identify a new epitope within Rev that could guide strategies for the development of novel HIV inhibitors.	Vercruysse et al. 2010
ADP-ribosylation **Salmonella** **SpvB toxin**	ADP-ribosylation of host cell proteins is a common mode of cell intoxication by pathogenic bacterial toxins. Antibodies induced by immunization with inactivated ADP-ribosylating toxins provide efficient protection in case of some secreted toxins, e.g., diphtheria and pertussis toxins. However, other ADP-ribosylating toxins, such as Salmonella SpvB toxin, are secreted directly from the Salmonella-containing vacuole into the cytosol of target cells via the SPI-2 encoded bacterial type III secretion system, and thus are inaccessible to conventional antibodies. Small-molecule ADP-ribosylation inhibitors are fraught with potential side effects caused by inhibition of endogenous ADP-ribosyltransferases. Here, we report the development of a single-domain antibody from an immunized llama that blocks the capacity of SpvB to ADP-ribosylate actin at a molar ratio of 1 : 1. The single-domain antibody, when expressed as an intrabody, effectively protected cells from the cytotoxic activity of a translocation-competent chimeric C2IN-C/SpvB toxin. Transfected cells were also protected against cytoskeletal alterations induced by wild-type SpvB-expressing strains of Salmonella. This proof of principle paves the way for developing new antidotes against intracellular toxins.	Alzogaray et al. 2011

Botulinum neurotoxins **Protease inhibitor**	Botulinum neurotoxins (BoNTs) function by delivering a protease to neuronal cells that cleave SNARE proteins and inactivate neurotransmitter exocytosis. Small (14 kDa) binding domains specific for the protease of BoNT serotypes A or B were selected from libraries of heavy chain only antibody domains (VHHs or nanobodies) cloned from immunized alpacas. Several VHHs bind the BoNT proteases with high affinity (K(D) near 1 nM) and include potent inhibitors of BoNT/A protease activity (K(i) near 1 nM). The VHHs retain their binding specificity and inhibitory functions when expressed within mammalian neuronal cells as intrabodies. A VHH inhibitor of BoNT/A protease was able to protect neuronal cell SNAP25 protein from cleavage following intoxication with BoNT/A holotoxin. These results demonstrate that VHH domains have potential as components of therapeutic agents for reversal of botulism intoxication.	Tremblay et al. 2010
Intracellular proteins **E. coli** **Intracellular delivery** **Molecular syringe**	Intracellular proteins have a great potential as targets for therapeutic antibodies (Abs) but the plasma membrane prevents-access to these antigens. Ab fragments and IgGs are selected and engineered in E. coli and this microorganism may be also an ideal vector for their intracellular delivery. In this work we demonstrate that single-domain Ab (sdAbs) can be engineered to be injected into human cells by E. coli bacteria carrying molecular syringes assembled by a type III protein secretion system (T3SS). The injected sdAbs accumulate in the cytoplasm of HeLa cells at levels ca. 10(5)–10(6) molecules per cell and their functionality is shown by the isolation of sdAb-antigen complexes. Injection of sdAbs does not require bacterial invasion or the transfer of genetic material. These results are proof-of-principle for the capacity of E. coli bacteria to directly deliver intracellular sdAbs (intrabodies) into human cells for analytical and therapeutic purposes.	Blanco-Toribio et al. 2010

Table 6E. Application of single-domain antibody fragments in therapy: oral administration of single-domain antibody fragments

Thermal stability **Trypsin** **Extreme pH** **Clostridium difficile** **Toxin A**	The extreme pH and protease-rich environment of the upper gastrointestinal tract is a major obstacle facing orally-administered protein therapeutics, including antibodies. Through protein engineering, several Clostridium difficile toxin A-specific heavy chain antibody variable domains (V(H)Hs) were expressed with an additional disulfide bond by introducing Ala/Gly54Cys and Ile78Cys mutations. Mutant antibodies were compared to their wild-type counterparts with respect to expression yield, non-aggregation status, affinity for toxin A, circular dichroism (CD) structural signatures, thermal stability, protease resistance, and toxin A-neutralizing capacity. The mutant V(H)Hs were found to be well expressed, although with lower yields compared to wild-type counterparts, were non-aggregating monomers, retained low nM affinity for toxin A, albeit the majority showed somewhat reduced affinity compared to wild-type counterparts, and were capable of in vitro toxin A neutralization in cell-based assays. Far-UV and near-UV CD spectroscopy consistently showed shifts in peak intensity and selective peak minima for wild-type and mutant V(H)H pairs; however, the overall CD profile remained very similar. A significant increase in the thermal unfolding midpoint temperature was observed for all mutants at both neutral and acidic pH. Digestion of the V(H)Hs with the major gastrointestinal proteases, at biologically relevant concentrations, revealed a significant increase in pepsin resistance for all mutants and an increase in chymotrypsin resistance for the majority of mutants. Mutant V(H)H trypsin resistance was similar to that of wild-type V(H)Hs, although the trypsin resistance of one V(H)H mutant was significantly reduced. Therefore, the introduction of a second disulfide bond in the hydrophobic core not only increases V(H)H thermal stability at neutral pH, as previously shown, but also represents a generic strategy to increase V(H)H stability at low pH and impart protease resistance, with only minor perturbations in target binding affinities. These are all desirable characteristics for the design of protein-based oral therapeutics.	Hussack et al. 2011a

Clostridium difficile **Nosocomial infection** **North America** **Exotoxin A/B** **Cell receptor binding domain**	Clostridium difficile is a leading cause of nosocomial infection in North America and a considerable challenge to healthcare professionals in hospitals and nursing homes. The Gram-positive bacterium produces two high molecular weight exotoxins, toxin A (TcdA) and toxin B (TcdB), which are the major virulence factors responsible for C. difficile-associated disease and are targets for C. difficile-associated disease therapy. Here, recombinant single-domain antibody fragments (V(H)Hs), which specifically target the cell receptor binding domains of TcdA or TcdB, were isolated from an immune llama phage display library and characterized. Four V(H)Hs (A4.2, A5.1, A20.1, and A26.8), all shown to recognize conformational epitopes, were potent neutralizers of the cytopathic effects of toxin A on fibroblast cells in an in vitro assay. The neutralizing potency was further enhanced when V(H)Hs were administered in paired or triplet combinations at the same overall V(H)H concentration, suggesting recognition of nonoverlapping TcdA epitopes. Biacore epitope mapping experiments revealed that some synergistic combinations consisted of V(H)Hs recognizing overlapping epitopes, an indication that factors other than mere epitope blocking are responsible for the increased neutralization. Further binding assays revealed TcdA-specific V(H)Hs neutralized toxin A by binding to sites other than the carbohydrate binding pocket of the toxin. With favorable characteristics such as high production yield, potent toxin neutralization, and intrinsic stability, these V(H)Hs are attractive systemic therapeutics but are more so as oral therapeutics in the destabilizing environment of the gastrointestinal tract.	Hussack et al. 2011b
Inflammatory bowel disease **Tumor-necrosis factor** **Lactococcus lactis** **Secretion**	Inflammatory bowel disease (IBD) is a chronic inflammatory gastrointestinal disorder. Systemic treatment of IBD patients with anti-tumor necrosis factor (TNF)-alpha antibodies has proven to be a highly promising approach, but several drawbacks remain, including side effects related to systemic administration and high cost of treatment. Lactococcus lactis was engineered to secrete monovalent and bivalent murine (m) TNF-neutralizing Nanobodies as therapeutic proteins. These therapeutic proteins are derived from fragments of heavy-chain camelid antibodies and are more stable than conventional antibodies. L. lactis-secreted anti-mTNF Nanobodies neutralized mTNF in vitro. Daily oral administration of Nanobody-secreting L. lactis resulted in local delivery of anti-mTNF Nanobodies at the colon and significantly reduced inflammation in mice with dextran sulfate sodium (DSS)-induced chronic colitis. In addition, this approach was also successful in improving established enterocolitis in interleukin 10 (IL10)(–/–) mice. Finally, L. lactis-secreted anti-mTNF Nanobodies did not interfere with systemic Salmonella infection in colitic IL10(–/–) mice. In conclusion, this report details a new therapeutic approach for treatment of chronic colitis, involving in situ secretion of anti-mTNF Nanobodies by orally administered L. lactis bacteria. Therapeutic application of these engineered bacteria could eventually lead to more effective and safer management of IBD in humans.	Vanden-broucke et al. 2010
F4 fimbriae **E. coli** **Diarrhoea** **Proteolytic stability** **Gastric fluid**	We previously demonstrated that oral application of the recombinant single-domain antibody fragment (VHH) clone K609, directed against Escherichia coli F4 fimbriae, reduced E. coli-induced diarrhoea in piglets, but only at high VHH doses. We have now shown that a large portion of the orally applied K609 VHH is proteolytically degraded in the stomach. Stringent selection for proteolytic stability identified seven VHHs with 7- to 138-fold increased stability after in vitro incubation in gastric fluid. By DNA shuffling we obtained four clones with a further 1.5- to 3-fold increased in vitro stability. These VHHs differed by at most ten amino acid residues from each other and K609 that were scattered over the VHH sequence and did not overlap with predicted protease cleavage sites. The most stable clone, K922, retained 41% activity after incubation in gastric fluid and 90% in jejunal fluid. Oral application of K922 to piglets confirmed its improved proteolytic stability. In addition, K922 bound to F4 fimbriae with higher affinity and inhibited fimbrial adhesion at lower VHH concentrations. K922 is thus a promising candidate for prevention of piglet diarrhoea. Furthermore, our findings could guide selection and improvement by genetic engineering of other recombinant antibody fragments for oral use.	Harm-sen et al. 2006

Rotavirus Diarrhoea Developing countries Passive immunotherapy Transformed lactobacilli	Background. Rotavirus-induced diarrhea poses a worldwide medical problem in causing substantial morbidity and mortality among children in developing countries. We therefore developed a system for passive immunotherapy in which recombinant lactobacilli constitutively express neutralizing variable domain of llama heavy-chain (VHH) antibody fragments against rotavirus. Methods. VHH were expressed in Lactobacillus paracasei, in both secreted and cell surface-anchored forms. Electron microscopy was used to investigate the binding efficacy of VHH-expressing lactobacilli. To investigate the *in vivo* function of VHH-expressing lactobacilli, a mouse pup model of rotavirus infection was used. Results. Efficient binding of the VHH antibody fragments to rotavirus was shown by enzyme-linked immunosorbent assay and scanning electron microscopy. VHH fragments expressed by lactobacilli conferred a significant reduction in infection in cell cultures. When administered orally, lactobacilli-producing surface-expressed VHH markedly shortened disease duration, severity, and viral load in a mouse model of rotavirus-induced diarrhea when administered both fresh and in a freeze-dried form. Conclusions. Transformed lactobacilli may form the basis of a novel form of prophylactic treatment against rotavirus infections and other diarrheal diseases.	Pant et al. 2006

Table 6F. Application of single-domain antibody fragments in therapy: prevention of amyloid plaque formation and protein aggregation

Beta-amyloid transmigration efficiency Alzheimer's disease Cerebral amyloid angiopathy	Previously selected amyloid beta recognizing heavy chain antibody fragments (VHH) affinity binders derived from the Came lid heavy chain antibody repertoire were tested for their propensity to cross the blood-brain barrier (BBB) using an established in vitro BBB co-culture system. Of all tested VHH, ni3A showed highest transmigration efficiency which is, in part, facilitated by a three amino acid substitutions in its N-terminal domain. Additional studies indicated that the mechanism of transcellular passage of ni3A is by active transport. As VHH ni3A combines the ability to recognize amyloid beta and to cross the BBB, it has potential as a tool for non-invasive in vivo imaging and as efficient local drug targeting moiety in patients suffering from cerebral amyloidosis such as Alzheimer's disease (AD) and cerebral amyloid angiopathy (CAA).	Rutgers et al. 2011
Beta-amyloid Alzheimer's disease Proteolytic nanobody Aggregation	Deposition of beta-amyloid (A beta) is considered an important early event in the pathogenesis of Alzheimer's disease (AD), and reduction of A beta levels in the brain could be a viable therapeutic approach. A potentially noninflammatory route to facilitate clearance and reduce toxicity of A beta is to degrade the peptide using proteolytic nanobodies. Here we show that a proteolytic nanobody engineered to cleave A beta at its alpha-secretase site has potential therapeutic value. The Asec-1A proteolytic nanobody, derived from a parent catalytic light chain antibody, prevents aggregation of monomeric A beta, inhibits further aggregation of preformed A beta aggregates, and reduces A beta-induced cytotoxicity toward a human neuroblastoma cell line. The nanobody also reduces toxicity induced by overexpression of the human amyloid precursor protein (APP) in a Chinese hamster ovary (CHO) cell line by cleaving APP at the alpha-secretase site which precludes formation of A beta. Targeted proteolysis of APP and A beta with catalytic nanobodies represents a novel therapeutic approach for treating AD where potentially harmful side effects can he minimized.	Kasturirangan et al. 2010
Amyloidogenic lysozyme Alpha-synuclein Parkinson's disease Self-association	Nanobodies are single chain antibodies that are uniquely produced in Camelidae, e.g. camels and llamas. They have the desirable features of small sizes (Mw < 14 kDa) and high affinities against antigens (Kd similar to nM), making them ideal as structural probes for biomedically relevant motifs both in vitro and in vivo. We have previously shown that nanobody binding to amyloidogenic human lysozyme variants can effectively inhibit their aggregation, the process that is at the origin of systemic amyloid disease. Here we report the NMR assignments of a new nanobody, termed NbSyn2, which recognises the C-terminus of the intrinsically disordered protein, human alpha-synuclein (aS), whose aberrant self-association is implicated in Parkinson's disease.	Vuchelen et al. 2009

Aggregation **Alpha-synuclein** **Fibrillar amyloid structures** **Parkinson's disease**	The aggregation of the intrinsically disordered protein alpha-synuclein to form fibrillar amyloid structures is intimately associated with a variety of neurological disorders, most notably Parkinson's disease. The molecular mechanism of alpha-synuclein aggregation and toxicity is not yet understood in any detail, not least because of the paucity of structural probes through which to study the behavior of such a disordered system. Here, we describe an investigation involving a single-domain camelid antibody, NbSyn2, selected by phage display techniques to bind to alpha-synuclein, including the exploration of its effects on the in vitro aggregation of the protein under a variety of conditions. We show using isothermal calorimetric methods that NbSyn2 binds specifically to monomeric alpha-synuclein with nanomolar affinity and by means of NMR spectroscopy that it interacts with the four C-terminal residues of the protein. This latter finding is confirmed by the determination of a crystal structure of NbSyn2 bound to a peptide encompassing the nine C-terminal residues of alpha-synuclein. The NbSyn2 : alpha-synuclein interaction is mediated mainly by side-chain interactions while water molecules cross-link the main-chain atoms of alpha-synuclein to atoms of NbSyn2, a feature we believe could be important in intrinsically disordered protein interactions more generally. The aggregation behavior of alpha-synuclein at physiological pH, including the morphology of the resulting fibrillar structures, is remarkably unaffected by the presence of NbSyn2 and indeed we show that NbSyn2 binds strongly to the aggregated as well as to the soluble forms of alpha-synuclein. These results give strong support to the conjecture that the C-terminal region of the protein is not directly involved in the mechanism of aggregation and suggest that binding of NbSyn2 could be a useful probe for the identification of alpha-synuclein aggregation in vitro and possibly in vivo.	De Genst et al. 2010
Aggregation **Oculopharyngeal muscular dystrophy** **Alanine expansion** **PABPN1**	Oculopharyngeal muscular dystrophy is caused by small alanine expansions in polyadenylate binding protein nuclear 1 (PABPN1) protein resulting in its intranuclear accumulation in skeletal muscle. 3F5 llama antibody specifically interferes with the PABPN1 aggregation process in vitro and in vivo. To understand the structural basis for its epitope recognition we mapped the binding interface of 3F5 with PABPN1 and provide a structural model of the 3F5-PABPN1 complex. We show that 3F5 complementarity determining regions create a cavity in which PABPN1 alpha-helix domain resides by involving critical residues previously implicated in the aggregation process. These results may increase our understanding of the PABPN1 aggregation mechanism and the therapeutic potential of 3F5.	Impagliazzo et al. 2010

Table 6G. Application of single-domain antibody fragments in therapy: multispecific and multifunctional constructs

Pentabody **Verotoxin B-subunit** **E. coli** **Thermostability** **Protease resistance**	We describe a novel type of molecule in which single-domain antibodies (sdAbs) isolated from a naive llama single domain antibody library are linked to an oligomerization domain to generate high-avidity antigen-binding reagents. An sdAb is fused to the B-subunit of Escherichia coli verotoxin, or shiga-like toxin, which self-assembles to form a homopentamer and results in simultaneous sdAb pentamerization and introduction of avidity. Molecular modeling indicated that this fusion protein (PDB: 10JF), termed pentabody, has structural flexibility for binding to surface-presented antigen. In the instance of an sdAb specific for a peptide antigen, pentamerization resulted in a dramatic increase in functional affinity for immobilized antigen. The pentabody was expressed in high yield in E. coli in a non-aggregated state, and exhibited excellent thermostability and protease resistance. This technology provides a relatively rapid means of generating novel antigen-binding molecules that bind strongly to immobilized antigen. It is expected that pentavalent sdAbs will have general applicability in proteomics, immunochemical staining, cancer diagnosis and other applications in which antigens are presented multivalently.	Zhang et al. 2004
Biparatopic nanobody **Epidermal growth factor receptor** **Anticancer therapeutics**	The epidermal growth factor receptor (EGFR) has been shown to be a valid cancer target for antibody-based therapy. At present, several anti-EGFR monoclonal antibodies have been successfully used, such as cetuximab and matuzumab. X-ray crystallography data show that these antibodies bind to different epitopes on the ecto-domain of EGFR, providing a rationale for the combined use of these two antibody specificities. We have previously reported on the successful isolation of antagonistic anti-EGFR nanobodies. In our study, we aimed to improve the efficacy of these molecules by combining nanobodies with specificities similar to both cetuximab and matuzumab into a single biparatopic molecule. Carefully designed phage nanobody selections resulted in two sets of nanobodies that specifically blocked the binding of either matuzumab or cetuximab to EGFR and that did not compete for each others' binding. A combination of nanobodies from both epitope groups into the biparatopic nanobody CONAN-1 was shown to block EGFR activation more efficiently than monovalent or bivalent (monospecific) nanobodies. In addition, this biparatopic nanobody potently inhibited EGF-dependent cell proliferation. Importantly, in an in vivo model of athymic mice bearing A431 xenografts, CONAN-1 inhibited tumour outgrowth with an almost similar potency as the whole mAb cetuximab, despite the fact that CONAN-1 is devoid of an Fc portion that could mediate immune effector functions. Compared to therapy using bivalent, monospecific nanobodies, CONAN-1 was clearly more potent in tumour growth inhibition. These results show that the rational design of biparatopic nanobody-based anticancer therapeutics may yield potent lead molecules for further development.	Roovers et al. 2011
Bivalent nanobody **Influenza virus** **Intranasal administration**	Influenza A virus infections impose a recurrent and global disease burden. Current antivirals against influenza are not always effective. We assessed the protective potential of monovalent and bivalent Nanobodies (Ablynx) against challenge with this virus. These Nanobodies were derived from llamas and target H5N1 hemagglutinin. Intranasal administration of Nanobodies effectively controlled homologous influenza A virus replication. Administration of Nanobodies before challenge strongly reduced H5N1 virus replication in the lungs and protected mice from morbidity and mortality after a lethal challenge with H5N1 virus. The bivalent Nanobody was at least 60-fold more effective than the monovalent Nanobody in controlling virus replication. In addition, Nanobody therapy after challenge strongly reduced viral replication and significantly delayed time to death. Epitope mapping revealed that the VHH Nanobody binds to antigenic site B in H5 hemagglutinin. Because Nanobodies are small, stable, and simple to produce, they are a promising, novel therapeutic agent against influenza.	Ibanez et al. 2011

Multimeric and bispecific constructs Influenza	For efficient prevention of viral infections and cross protection, simultaneous targeting of multiple viral epitopes is a powerful strategy. Llama heavy chain antibody fragments (VHH) against the trimeric envelope proteins of Respiratory Syncytial Virus (Fusion protein), Rabies virus (Glycoprotein) and H5N1 Influenza (Hemagglutinin 5) were selected from llama derived immune libraries by phage display. Neutralizing VHH recognizing different epitopes in the receptor binding sites on the spikes with affinities in the low nanomolar range were identified for all the three viruses by viral neutralization assays. By fusion of VHH with variable linker lengths, multimeric constructs were made that improved neutralization potencies up to 4000-fold for RSV, 1500-fold for Rabies virus and 75-fold for Influenza H5N1. The potencies of the VHH constructs were similar or better than best performing monoclonal antibodies. The cross protection capacity against different viral strains was also improved for all three viruses, both by multivalent (two or three identical VHH) and biparatopic (two different VHH) constructs. By combining a VHH neutralizing RSV subtype A, but not subtype B with a poorly neutralizing VHH with high affinity for subtype B, a biparatopic construct was made with low nanomolar neutralizing potency against both subtypes. Trivalent anti-H5N1 VHH neutralized both Influenza H5N1 clade1 and 2 in a pseudotype assay and was very potent in neutralizing the NIBRG-14 Influenza H5N1 strain with IC(50) of 9 picomolar. Bivalent and biparatopic constructs against Rabies virus cross neutralized both 10 different Genotype 1 strains and Genotype 5. The results show that multimerization of VHH fragments targeting multiple epitopes on a viral trimeric spike protein is a powerful tool for anti-viral therapy to achieve "best-in-class" and broader neutralization capacity.	Hultberg et al. 2011
Biosensors Decabody Cellulose filter S. aureus	Antibody engineering has allowed for the rapid generation of binding agents against virtually any antigen of interest, predominantly for therapeutic applications. Considerably less attention has been given to the development of diagnostic reagents and biosensors using engineered antibodies. Recently, we produced a novel pentavalent bispecific antibody (i.e., decabody) by pentamerizing two single-domain antibodies (sdAbs) through the verotoxin B subunit (VTB) and found both fusion partners to be functional. Using a similar approach, we have engineered a bispecific pentameric fusion protein consisting of five sdAbs and five cellulose-binding modules (CBMs) linked via VTB. To find an optimal design format, we constructed six bispecific pentamers consisting of three different CBMs, fused to the Staphylococcus aureus-specific human sdAb HVHP428, in both orientations. One bispecific pentamer, containing an N-terminal CBM9 and C-terminal HVHP428, was soluble, non-aggregating, and did not degrade upon storage at 4 degrees C for over six months. This molecule was dually functional as it bound to cellulose-based filters as well as S. aureus cells. When impregnated in cellulose filters, the bispecific pentamer recognized S. aureus cells in a flow-through detection assay. The ability of pentamerized CBMs to bind cellulose may form the basis of an immobilization platform for multivalent display of high-avidity binding reagents on cellulosic filters for sensing of pathogens, biomarkers and environmental pollutants.	Hussack et al. 2009

Chimeric antigen receptor **Anti-MUC1 nanobody** **Cancer immunotherapy**	The crucial role of T lymphocytes in anti-tumor immunity has led to the development of novel strategies that can target and activate T cells against tumor cells. Recombinant DNA technology has been used to generate non-MHC-restricted chimeric antigen receptors (CARs). Here, we constructed a panel of recombinant CAR that harbors the anti-MUC1 nanobody and the signaling and co-signaling moieties (CD3 zeta/CD28) with different spacer regions derived from human IgG3 with one or two repeats of the hinge sequence or the hinge region of Fc gamma RII. The PhiC31 integrase system was employed to investigate if the recombination efficiency could be recruited for high and stable expression of T cell chimeric receptor genes. The effect of nuclear localization signal (NLS) and two different promoters (CMV and CAG) on efficacy of PhiC31 integrase in human T cell lines was evaluated. The presence of integrase in combination with NLS, mediated up to 7.6 and 8.5 fold increases in CAR expression in ZCHN-attB and ZCHHN-attB cassette integrated T cells, respectively. Our results showed that highly efficient and stable transduction of the Jurkat cell line by PhiC31 integrase is a feasible modality for generating anti-cancer chimeric T cells for use in cancer immunotherapy.	Iri-Sofla et al. 2011
Nanobody-micelle conjugate **Epidermal growth factor receptor** **Lysozyme** **Drug targeting**	The aim of this study was to develop poly(ethylene glycol)-b-poly[N-(2-hydroxypropyl) methacrylamide-lactate] (mPEG-b-p(HPMAm-Lac(n)) core-crosslinked thermosensitive biodegradable polymeric micelles suitable for active tumor targeting, by coupling the anti-EGFR (epidermal growth factor receptor) EGa1 nanobody to their surface. To this end. PEG was functionalized with N-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) to yield a PDP-PEG-b-p(HPMAm-Lac(n)) block copolymer. Micelles composed of 80% mPEG-b-p(HPMAm-Lac(n)) and 20% PDP-PEG-b-p(HPMAm-Lac(n)) were prepared and lysozyme (as a model protein) was modified with N-succinimidyl-S-acetylthioacetate, deprotected with hydroxylamine hydrochloride and subsequently coupled to the micellar surface. The micellar conjugates were characterized using SDS-PAGE and gel permeation chromatography (GPC). Using the knowledge obtained with lysozyme conjugation, the EGa1 nanobody was coupled to mPEG/PDP-PEG micelles and the conjugation was successful as demonstrated by western blot and dot blot analysis. Rhodamine labeled EGa1-micelles showed substantially higher binding as well as uptake by EGFR over-expressing cancer cells (A431 and UM-SCC-14C) than untargeted rhodarnine labeled micelles. Interestingly, no binding of the nanobody micelles was observed to EGFR negative cells (3T3) as well as to14C cells in the presence of an excess of free nanobody. This demonstrates that the binding of the nanobody micelles is indeed by interaction with the EGF receptor. In conclusion, EGa1 decorated (mPEG/PDP-PEG)-b-(pHPMAm-Lac(n)) polymeric micelles are highly promising systems for active drug targeting.	Talelli et al. 2011
Nanobody-gold nanoparticle conjugates **HER2** **Photothermal therapy**	Branched gold nanoparticles are potential photothermal therapy agents because of their large absorption cross section in the near-infrared window. Upon laser irradiation they produce enough heat to destroy tumor cells. In this work, branched gold nanoparticles are blofunctionalized with nanobodies, the smallest fully functional antigen-binding fragments evolved from the variable domain, the VHH, of a camel heavy chain-only antibody. These nanobodies bind to the HER2 antigen which is highly expressed on breast and ovarian cancer cells. Flow cytometric analysis and dark field Images of HER2 positive SKOV3 cells incubated with anti-HER2 conjugated branched gold nanoparticles show specific cell targeting. Laser Irradiation studies reveal that HER2 positive SKOV3 cells exposed to the anti-HER2 targeted branched gold nanoparticles are destroyed after five minutes of laser treatment at 38 W/cm^2 using a 690 nm continuous wave laser. Starting from a nanoparticle optical density of 4, cell death is observed, whereas the control samples, nanoparticles with anti-PSA nanobodies, nanoparticles only, and laser only, do not show any cell death. These results suggest that this new type of bioconjugated branched gold nanoparticles are effective antigen-targeted photothermal therapeutic agents for cancer treatment.	Van de Broek et al. 2011

Nanobody-liposome conjugate Epidermal growth factor receptor Receptor downregulation	The epidermal growth factor receptor (EGFR) is a recognized target for tumor therapy and monoclonal antibodies (mAbs, e.g. cetuximab) have been developed to inhibit receptor activation. Besides blocking ligand (e.g. EGF) binding to the receptor, reports have shown that mAbs promote slow receptor internalization and degradation in lysosomes, i.e. downregulation. The efficacy of receptor downregulation was recently shown to depend on the size of receptor clusters formed at the cell surface. In this study, a multivalent platform is presented, consisting of nanobodies recognizing the ectodomain of EGFR (EGa1) coupled to PEG-liposomes, and the in vitro and in vivo effects of this system on EGFR internalization and downregulation were investigated. Nanobodies are the smallest functional antigen-binding immunoglobulin fragments and the EGa1 nanobody has been described as an EGFR-antagonist. EGa1-liposomes (EGa1-L) induced a more than 90% removal of EGFR from the cell surface, as a result of receptor internalization. Furthermore, this massive sequestration of EGFR mediated by EGa1-L lead to receptor degradation, while no degradation was detected with the monovalent nanobody. The downregulatory capacity here reported was found to be independent of the epitope on EGFR recognized by the grafted nanobody, and exclusive to the nanobody-liposomes, as anti-EGFR single chain variable fragments (scFv) coupled to liposomes were unable to induce this effect. Importantly, EGa1-L induced a significant inhibition of tumor cell proliferation, in vitro, an effect likely mediated by the combination of receptor downregulation and receptor antagonism. Also in vivo, EGFR downregulation was observed in tumors of mice intravenously injected with EGa1-L, indicating that this multivalent platform blocks ligand binding to the receptor and simultaneously induces the downregulation of EGFR.	Oliveira et al. 2010
Nanobody-β-lactamase conjugate Carcinoembryonic antigen Cancer therapy	We identified a nanobody with subnanomolar affinity for the human tumor-associated carcinoembryonic antigen. This nanobody was conjugated to Enterobacter cloacae beta-lactamase, and its site-selective anticancer prodrug activation capacity was evaluated. The conjugate was readily purified in high yields without aggregation or loss of functionality of the constituents. In vitro experiments showed that the nanobody-enzyme conjugate effectively activated the release of phenylenediamine mustard from the cephalosporin nitrogen mustard prodrug 7-(4-carboxybutanamido) cephalosporin mustard at the surface of carcinoembryonic antigen-expressing LS174T cancer cells. In vivo studies demonstrated that the conjugate had an excellent biodistribution profile and induced regressions and cures of established tumor xenografts. The easy generation and manufacturing yield of nanobody-based conjugates together with their potent antitumor activity make nanobodies promising vehicles for new generation cancer therapeutics.	Cortez-Retamozo et al. 2004
Nanobody-apolipoprotein L-I conjugate Trypanosomiasis therapy Immunotoxin	High systemic drug toxicity and increasing prevalence of drug resistance hampers efficient treatment of human African trypanosomiasis (HAT). Hence, development of new highly specific trypanocidal drugs is necessary. Normal human serum (NHS) contains apolipoprotein L-I (apoL-I), which lyses African trypanosomes except resistant forms such as Trypanosoma brucei rhodesiense. T. b. rhodesiense expresses the apoL-I neutralizing serum resistance-associated (SRA) protein, endowing this parasite with the ability to infect humans and cause HAT. A truncated apoL-I (Tr-apoL-I) has been engineered by deleting its SRA-interacting domain, which makes it lytic for T. b. rhodesiense. Here, we conjugated Tr-apoL-I with a single-domain antibody (nanobody) that efficiently targets conserved cryptic epitopes of the variant surface glycoprotein (VSG) of trypanosomes to generate a new manmade type of immunotoxin with potential for trypanosomiasis therapy. Treatment with this engineered conjugate resulted in clear curative and alleviating effects on acute and chronic infections of mice with both NHS-resistant and NHS-sensitive trypanosomes.	Baral et al. 2006

Nanobody-glucose oxidase conjugate **Streptococcus mutans** **Antimicrobial agents**	Enzymes such as lactoperoxidase and glucose oxidase (GOx) are used as antimicrobial agents in oral care products. Their low specificities and substantiveness can be reduced by covalent coupling of antimicrobial molecules to antibodies. Variable domains (V-HH) derived from llama heavy-chain antibodies are particularly suited for such an approach. The antibodies are composed solely of heavy-chain dimers; therefore, production of active fusion proteins by using molecular biology-based techniques is less complicated than production by use of conventional antibodies. In this study, a fusion protein consisting of V-HH and GOx was constructed and expressed by Saccharomyces cerevisiae. A llama was immunized with Streptococcus mutans strain HG982. Subsequently, B lymphocytes were isolated and cDNA fragments encoding the V-HH fragments were obtained by reverse transcription-PCR. After construction of a V-HH library in Escherichia coli and screening of the library against mutans group streptococci and Streptococcus sanguinis strains, we found two V-HH fragments with high specificities for S. mutans strains. A GOx gene was linked to the two V-HH genes and cloned into S. cerevisiae yeasts. The yeasts expressed and secreted the recombinant proteins into the growth medium. The test of binding of fusion proteins to oral bacteria through their V-HH fragments showed that S. mutans had been specifically targeted by GOx-S120, one of the fusion protein constructs. A low concentration of the fusion protein was also able to selectively kill S. mutans within 20 min in the presence of lactoperoxidase and potassium iodide. These findings demonstrate that the fusion protein GOx-V-HH is potentially valuable in the selective killing of target bacteria such as S. mutans.	Szynol et al. 2004
Bispecific antibody **Decabody** **Linker sequence** **Verotoxin B subunit**	Bispecific antibodies present unique opportunities in terms of new applications for engineered antibodies. However, designing ideal bispecific antibodies remains a challenge. Here we describe a novel bispecific antibody model in which five single domain antibodies (sdAbs) are fused via a linker sequence to the N-terminus of the verotoxin B (VTB) subunit, a pentamerization domain, and five sdAbs are fused via a linker sequence to the VTB C-terminus. Fifteen such decavalent bispecific molecules, termed decabodies, were constructed and characterized for the purpose of identifying an optimal decabody design. One of the fifteen molecules existed in a non-aggregated decavalent form. In conjunction with the isolation of sdAbs with the desired specificities from non-immune phage display libraries, the decabody strategy provides a means of generating high avidity bispecific antibody reagents, with good physical properties, relatively quickly.	Stone et al. 2007

Table 6H. Application of single-domain antibody fragments in therapy: humanised single-domain antibody fragments

VHH **CDR3** **Human scaffold protein** **Grafting** **Ubiquitin**	VHH is the binding domain of the IgG heavy chain. Some VHHs have an extremely long CDR3 that contributes to antigen binding. We studied the antigen binding ability of CDR3 by grafting a CDR3 from an antigen-binding VHH onto a nonbinding VHH. cAb-CA05-(1RI8), the CDR3-grafted VHH, had an antigen-binding ability. To find a human scaffold protein acceptable for VHH CDR3 grafting, we focused on the conserved structure of VHH, especially the N-terminal and C-terminal amino acid residues of the CDR3 loop and the Cys residue of CDR1. Human origin protein structures with the same orientation were searched in PDB and ubiquitin was selected. Ubi-(1RI8), the CDR3-grafted ubiquitin, had antigen-binding ability, though the affinity was relatively low compared to cAb-CA05-(1RI8). The thermodynamic parameters of Ubi-(1RI8) binding to HEWL were different from cAb-CA05-(1RI8). Hydrogen-deuterium exchange experiments showed decreased stability around the CDR3 grafting region of Ubi-(1RI8), which might explain the decreased antigen-binding ability and the differences in thermodynamic properties. We concluded that the orientation of the CDR3 sequence of Ubi-(1RI8) could not be reconstructed correctly.	Inoue et al. 2011

AahII scorpion toxin **Humanized nanobody**	Recently, a potent AahII scorpion toxin-neutralizing nanobody was identified. However, this NbAahII10 contains a single Cys in its first antigen-binding loop, leading to Nb dimerization upon prolonged storage. In this work, we first investigate the efficacy of NbAahII10 variants in which this Cys was substituted by Ala, Ser or Thr. Second, the NbAahII10 Cys/Ser mutant displaying the best functional properties is subsequently humanized. It is demonstrated that the maximally humanized version of NbAahII10 Cys/Ser maintains its high affinity for the antigen without conceding much on expression yield and stability. More importantly, its neutralizing capacity is preserved as all mice survive injections of seven LD(50) and 50 of mice survived nine LD(50) of the scorpion toxin. Thus, this humanized Nb is the best candidate to develop a therapy in human against the most toxic venom compound of one of the most dangerous scorpions.	Ben Abderrazek et al. 2011
Humanized nanobodies **Loop-grafting** **In vivo imaging** **Scaffold** **Carcinoembryonic antigen**	Nanobodies are a novel type of immunoglobulinlike, antigen-binding protein with beneficial pharmacologic and pharmacokinetic properties that are ideally suited to targeting cellular antigens for molecular imaging or therapeutic purposes. However, because of their camelid, nonhuman origin, the possible immunogenicity of Nanobodies when used in the clinic is a concern. Here we present a new strategy to quickly generate humanized Nanobodies for molecular imaging purposes. Methods: We genetically grafted the antigen-binding loops of NbCEA5, a Nanobody with specificity for the colon carcinoma marker carcinoembryonic antigen (CEA), onto the framework of a humanized Nanobody scaffold. This scaffold has been previously characterized in our laboratory as a stable Nanobody that can serve as a universal loop acceptor for antigen-binding loops from donor Nanobodies and has been additionally mutated at about 10 crucial surface-exposed sites to resemble the sequence of human variable immunoglobulin domains. The 3 recombinant Nanobodies (NbCEA5, humanized scaffold, and humanized CEA5 graft) were produced in bacteria and purified. Unlabeled and (99m)Tc-labeled Nanobodies were biochemically characterized in vitro and tested as probes for SPECT/CT of xenografted tumors. Results: The success of loop-grafting was confirmed by comparing these Nanobodies for their capacity to recognize soluble CEA protein in enzyme-linked immunosorbent assay and by surface plasmon resonance and to bind to CEA-positive LS174T colon carcinoma cells and CEA-transfected but not untransfected Chinese hamster ovary cells in flow cytometry. Specificity of binding was confirmed by competition studies. All Nanobodies were heat-stable, could be efficiently labeled with (99m)Tc, and recognized both soluble and membrane-bound CEA protein in binding studies. Finally, biodistribution experiments were performed with intravenously injected (99m)Tc-labeled Nanobodies in LS174T tumor-bearing mice using pinhole SPECT/micro-CT. These in vivo experiments revealed specificity of tumor targeting and rapid renal clearance for all Nanobodies, with low signals in all organs besides the kidneys. Conclusion: This study shows the potency of antigen-binding loop-grafting to efficiently generate humanized Nanobodies that retain their targeting capacities for noninvasive in vivo imaging of tumors.	Vaneycken et al. 2010
Universal humanized nanobody scaffold	Nanobodies, single-domain antigen-binding fragments of camelid-specific heavy-chain only antibodies offer special advantages in therapy over classic antibody fragments because of their smaller size, robustness, and preference to target unique epitopes. A Nanobody differs from a human heavy chain variable domain in about ten amino acids spread all over its surface, four hallmark Nanobody-specific amino acids in the framework-2 region (positions 42, 49, 50, and 52), and a longer third antigen-binding loop (H3) folding over this area. For therapeutic applications the camelid-specific amino acid sequences in the framework have to be mutated to their human heavy chain variable domain equivalent, i.e. humanized. We performed this humanization exercise with Nanobodies of the subfamily that represents close to 80% of all dromedary-derived Nanobodies and investigated the effects on antigen affinity, solubility, expression yield, and stability. It is demonstrated that the humanization of Nanobody-specific residues outside framework-2 are neutral to the Nanobody properties. Surprisingly, the Glu-49 -> Gly and Arg-50 -> Leu humanization of hallmark amino acids generates a single domain that is more stable though probably less soluble. The other framework-2 substitutions, Phe-42 -> Val and Gly/Ala-52 -> Trp, are detrimental for antigen affinity, due to a repositioning of the H3 loop as shown by their crystal structures. These insights were used to identify a soluble, stable, well expressed universal humanized Nanobody scaffold that allows grafts of antigen-binding loops from other Nanobodies with transfer of the antigen specificity and affinity.	Vincke et al. 2009

Table 7. Application of single-domain antibody fragments in diagnostic and immunoanalytic methods

Keywords	Summary	Reference
Noninvasive detection **Technetium-99m** **Atherosclerotic plaques** **Single-photon emission computed tomography**	We aimed to generate, radiolabel, and evaluate anti-VCAM1 nanobodies for noninvasive detection of atherosclerotic lesions. Methods and Results: Ten anti-VCAM1 nanobodies were generated, radiolabeled with technetium-99m, and screened in vitro on mouse and human recombinant VCAM1 proteins and endothelial cells and in vivo in apolipoprotein E-deficient (ApoE(−/−)) mice. Anontargeting control nanobody was used in all experiments to demonstrate specificity. All nanobodies displayed nanomolar affinities for murine VCAM1. Flow cytometry analyses using human human umbilical vein endothelial cells indicated murine and human VCAM1 cross-reactivity for 6 of 10 nanobodies. The lead compound cAbVCAM1-5 was cross-reactive for human VCAM1 and exhibited high lesion-to-control (4.95 ± 0.85), lesion-to-heart (8.30 ± 1.11), and lesion-to-blood ratios (4.32 ± 0.48) ($P < 0.05$ versus control C57Bl/6J mice). Aortic arch atherosclerotic lesions of ApoE(−/−) mice were successfully identified by single-photon emission computed tomography imaging. Tc-99m-cAbVCAM1-5 binding specificity was demonstrated by in vivo competition experiments. Autoradiography and immunohistochemistry further confirmed cAbVCAM1-5 uptake in VCAM1-positive lesions. Conclusions: The Tc-99m-labeled, anti-VCAM1 nanobody cAbVCAM1-5 allowed noninvasive detection of VCAM1 expression and displayed mouse and human cross-reactivity. Therefore, this study demonstrates the potential of nanobodies as a new class of radiotracers for cardiovascular applications.	Broisat et al. 2012
Tumour uptake **Tumour burden** **Epidermal growth factor receptor** **Bioluminescence imaging**	We studied the relationship between tumor uptake of the epidermal growth factor receptor (EGFR)-specific nanobody (99m) Tc-7C12 and tumor burden and evaluated the possibility of using this probe to monitor tumor response to erlotinib. The specificity and affinity of (99m)Tc-7C12 was determined on A431 cells. Cells expressing firefly luciferase were used to evaluate tumor burden using bioluminescence imaging. We evaluated the effect of erlotinib on tumor burden and (99m)Tc-7C12 uptake in vitro as well as in vivo. In vivo bioluminescence imaging was performed followed by pinhole single-photon emission computed tomography/micro-computed tomography. (99m)Tc-7C12 binds specifically to the receptor with high affinity (3.67 ± 0.59 nM). Erlotinib reduced tumor uptake and cell viability in a concentration-dependent manner. Tumor uptake of (99m)Tc-7C12 showed good correlation with tumor burden. Erlotinib treatment resulted in a progressive reduction of tumor burden and tumor uptake of (99m) Tc-7C12. (99m)Tc-7C12 binds to EGFR with high affinity and specificity. Tumor uptake is correlated with tumor burden. Quantification of (99m)Tc-7C12 uptake is promising for monitoring therapy response of EGFR-expressing tumors.	Gainkam et al. 2011
Nanoprobe **Quantum dots** **Flow cytometry** **Carcinoembryonic antigen** **Biopsy samples**	Common strategy for diagnostics with quantum dots (QDs) utilizes the specificity of monoclonal antibodies (mAbs) for targeting. However QD-mAbs conjugates are not always well-suited for this purpose because of their large size. Here, we engineered ultrasmall nanoprobes through oriented conjugation of QDs with 13-kDa single-domain antibodies (sdAbs) derived from llama IgG. Monomeric sdAbs are 12 times smaller than mAbs and demonstrate excellent capacity for refolding. sdAbs were tagged with QDs through an additional cysteine residue integrated within the C terminal of the sdAb. This approach allowed us to develop sdAbs-QD nanoprobes comprising four copies of sdAbs coupled with a QD in a highly oriented manner. sdAbs-QD conjugates specific to carcinoembryonic antigen (CEA) demonstrated excellent specificity of flow cytometry quantitative discrimination of CEA-positive and CEA-negative tumor cells. Moreover, the immunohistochemical labeling of biopsy samples was found to be comparable or even superior to the quality obtained with gold standard protocols of anatomopathology practice. sdAbs-QD-oriented conjugates as developed represent a new generation of ultrasmall diagnostic probes for applications in high-throughput diagnostic platforms.	Sukhanova et al. 2012

Chromobody **Posttranslational modifications**	The understanding of cellular processes and their pathophysiological alterations requires comprehensive data on the abundance, distribution, modification, and interaction of all cellular components. On the one hand, artificially introduced fluorescent fusion proteins provide information about their distribution and dynamics in living cells but not about endogenous factors. On the other hand, antibodies can detect endogenous proteins, posttranslational modifications, and other cellular components but mostly in fixed and permeabilized cells. Here we highlight a new technology based on the antigen-binding domain of heavy-chain antibodies (VHH) from Camelidae. These extremely stable VHH domains can be produced in bacteria, coupled to matrices, and used for affinity purification and proteome studies. Alternatively, these VHH domains can be fused with fluorescent proteins and expressed in living cells. These fluorescent antigen-binding proteins called "chromobodies" can be used to detect and trace proteins and other cellular components in vivo. Chromobodies can, in principle, detect any antigenic structure, including posttranslational modifications, and thereby dramatically expand the quality and quantity of information that can be gathered in high-content analysis. Depending on the epitope chosen, chromobodies can also be used to modulate protein function in living cells.	Schmidthals et al. 2010
deGradFP **Green fluorescent protein** **Protein knockout**	The use of genetic mutations to study protein functions in vivo is a central paradigm of modern biology. Recent advances in reverse genetics such as RNA interference and morpholinos are widely used to further apply this paradigm. Nevertheless, such systems act upstream of the proteic level, and protein depletion depends on the turnover rate of the existing target proteins. Here we present deGradFP, a genetically encoded method for direct and fast depletion of target green fluorescent protein (GFP) fusions in any eukaryotic genetic system. This method is universal because it relies on an evolutionarily highly conserved eukaryotic function, the ubiquitin pathway. It is traceable, because the GFP tag can be used to monitor the protein knockout. In many cases, it is a ready-to-use solution, as GFP protein-trap stock collections are being generated in Drosophila melanogaster and in Danio rerio.	Caussinus et al. 2012
Alzheimer's disease **Peptide conformation** **Amyloid beta** **Neurotoxicity**	Neurotoxic oligomers of amyloid beta (A beta) peptide have been incriminated in the pathogenesis of Alzheimer's disease. Further exploration of this issue has been hampered to this date by the fact that all previously described anti-A beta antibodies are unable to discriminate between the different conformations of the peptide (oligomers, protofibrils and fibrils). Here we describe the generation of novel camelid single-chain binding domains (VHHs) that recognizes specifically low molecular-weight (MW) oligomers. Three VHH specific for A beta were obtained from an immunized alpaca phage display library. Two were able to recognize selectively intraneuronal A beta oligomers: furthermore, one of them, V31-1, prevented A beta-induced neurotoxicity and inhibited fibril formation. This study confirms that VHHs may recognize non-conventional epitopes and illustrates their potential for the immunodiagnostic of diseases due to protein accumulation.	Lafaye et al. 2009

Humanized nanobodies **Loop-grafting** **In vivo imaging** **Scaffold** **Carcinoembryonic antigen**	Nanobodies are a novel type of immunoglobulinlike, antigen-binding protein with beneficial pharmacologic and pharmacokinetic properties that are ideally suited to targeting cellular antigens for molecular imaging or therapeutic purposes. However, because of their camelid, nonhuman origin, the possible immunogenicity of Nanobodies when used in the clinic is a concern. Here we present a new strategy to quickly generate humanized Nanobodies for molecular imaging purposes. Methods: We genetically grafted the antigen-binding loops of NbCEA5, a Nanobody with specificity for the colon carcinoma marker carcinoembryonic antigen (CEA), onto the framework of a humanized Nanobody scaffold. This scaffold has been previously characterized in our laboratory as a stable Nanobody that can serve as a universal loop acceptor for antigen-binding loops from donor Nanobodies and has been additionally mutated at about 10 crucial surface-exposed sites to resemble the sequence of human variable immunoglobulin domains. The 3 recombinant Nanobodies (NbCEA5, humanized scaffold, and humanized CEA5 graft) were produced in bacteria and purified. Unlabeled and (99m)Tc-labeled Nanobodies were biochemically characterized in vitro and tested as probes for SPECT/CT of xenografted tumors. Results: The success of loop-grafting was confirmed by comparing these Nanobodies for their capacity to recognize soluble CEA protein in enzyme-linked immunosorbent assay and by surface plasmon resonance and to bind to CEA-positive LS174T colon carcinoma cells and CEA-transfected but not untransfected Chinese hamster ovary cells in flow cytometry. Specificity of binding was confirmed by competition studies. All Nanobodies were heat-stable, could be efficiently labeled with (99m)Tc, and recognized both soluble and membrane-bound CEA protein in binding studies. Finally, biodistribution experiments were performed with intravenously injected (99m)Tc-labeled Nanobodies in LS174T tumor-bearing mice using pinhole SPECT/micro-CT. These in vivo experiments revealed specificity of tumor targeting and rapid renal clearance for all Nanobodies, with low signals in all organs besides the kidneys. Conclusion: This study shows the potency of antigen-binding loop-grafting to efficiently generate humanized Nanobodies that retain their targeting capacities for noninvasive in vivo imaging of tumors.	Vaneycken et al. 2010
HER-2 **Breast cancer** **Noninvasive imaging**	Accurate determination of tumor human epidermal growth factor receptor 2 (HER2)-status in breast cancer patients is possible via noninvasive imaging, provided adequate tracers are used. In this study, we describe the generation of a panel of 38 nanobodies, small HER2-binding fragments that are derived from heavy-chain-only antibodies raised in an immunized dromedary. In search of a lead compound, a subset of nanobodies was biochemically characterized in depth and preclinically tested for use as tracers for imaging of xenografted tumors. The selected compound, 2Rs15d, was found to be stable and to interact specifically with HER2 recombinant protein and HER2-expressing cells in ELISA, surface plasmon resonance, flow cytometry, and radioligand binding studies with low nanomolar affinities, and did not compete with anti-HER2 therapeutic antibodies trastuzumab and pertuzumab. Single-photon-emission computed tomography (SPECT) imaging quantification and biodistribution analyses showed that (99m)Tc-labeled 2Rs15d has a high tumor uptake in 2 $HER2^{+}$ tumor models, fast blood clearance, low accumulation in nontarget organs except kidneys, and high concomitant tumor-to-blood and tumor-to-muscle ratios at 1 h after intravenous injection. These values were dramatically lower for an irrelevant control (99m)Tc-nanobody and for (99m)Tc-2Rs15d targeting a $HER2^{-}$ tumor.	Vaneycken et al. 2011b

VHH-based immunoassay **3-phenoxybenzoic acid** **Pyrethroid insecticides**	We expressed VHHs from an immunized alpaca and developed a VHH-based immunoassay using 3-phenoxybenzoic acid (3-PBA), a major metabolite of pyrethroid insecticides as a model system. A phage VHH library was constructed, and seven VHH clones were selected by competitive binding with 3-PBA. The best immunoassay developed with one of these VHHs showed an IC50 of 1.4 ng/ml (limit of detection (LOD) = 0.1 ng/ml). These parameters were further improved by using the phage borne VHH, IC50 = 0.1 ng/ml and LOD = 0.01 ng/ml. Both assays showed a similar tolerance to methanol and dimethylsulfoxide up to 50% in assay buffer. The assay was highly specific to 3-PBA and its 4-hydroxylated derivative, 4-hydroxy 3-PBA, (150% cross reactivity) with negligible cross reactivity with other tested structural analogues, and the recovery from spiked urine sample ranged from 80 to 112%. In conclusion, a highly specific and sensitive VHH for 3-PBA was developed using sequences from immunized alpaca and phage display technology for antibody selection.	Kim et al. 2012
Botulinum neurotoxin **Toxin neutralizing capability** **High temperatures**	Background: There are currently 7 known serotypes of botulinum neurotoxin (BoNT) classified upon non-cross reactivity of neutralizing immunoglobulins. Non-neutralizing immunoglobulins, however, can exhibit cross-reactivities between 2 or more serotypes, particularly mosaic forms, which can hamper the development of highly specific immunoassays, especially if based on polyclonal antisera. Here we employ facile recombinant antibody technology to subtractively select ligands to each of the 7 BoNT serotypes, resulting in populations with very high specificity for their intended serotype. Methods and Findings: A single llama was immunized with a cocktail of 7 BoNT toxoids to generate a phage display library of single domain antibodies (sdAb, VHH or nanobodies) which were selected on live toxins. Resulting sdAb were capable of detecting both toxin and toxin complex with the best combinations able to detect 100s-10s of pg per 50 µl sample in a liquid bead array. The most sensitive sdAb were combined in a heptaplex assay to identify each of the BoNT serotypes in buffer and milk and to a lesser extent in carrot juice, orange juice and cola. Several anti-A(1) sdAb recognized A2 complex, showing that subtype cross-reactivity within a serotype was evident. Many of our sdAb could act as both captor and tracer for several toxin and toxin complexes suggesting sdAb can be used as architectural probes to indicate BoNT oligomerisation. Six of 14 anti-A clones exhibited inhibition of SNAP-25 cleavage in the neuro-2A assay indicating some sdAb had toxin neutralizing capabilities. Many sdAb were also shown to be refoldable after exposure to high temperatures in contrast to polyclonal antisera, as monitored by circular dichroism. Conclusions: Our panel of molecularly flexible antibodies should not only serve as a good starting point for ruggedizing assays and inhibitors, but enable the intricate architectures of BoNT toxins and complexes to be probed more extensively.	Conway et al. 2010
ELISA **Toxin** **SdAb-alkaline phosphatase fusion**	Naive libraries of single domain antibodies (sdAbs) enable rapid isolation of binders to nearly any target. These binders, however, lack the benefits bestowed by in vivo affinity maturation and typically have low affinity toward their targets. We expressed five low-affinity toxin binding sdAbs, previously selected from a naive library derived from variable regions of llama heavy chain-only antibodies, as fusions with a hyperactive mutant Escherichia coli alkaline phosphatase (AP) and examined the impact on apparent affinity and utility. AP spontaneously dimerizes in solution, effectively dimerizing the fused sdAbs, imparting avidity in place of the lower affinity monomeric interactions. The sdAb-AP fusion also combines the target recognition domain with a signal transduction domain, commonly used in enzyme-linked immunosorbent assays (ELISAs). The functional affinity of the sdAb-AP fusions, often increased by a factor of 10 over unfused sdAbs, and their utility as tracer reagents in ELISAs was dramatically improved, giving limits of detection of 300 ng/ml or less, whereas parental sdAbs gave no discernible signal at the toxin concentrations tested. The fusion of sdAbs to AP presents a valuable route to facilitate the implementation of sdAb-based immunoreagents rapidly selected from existing naive libraries toward new or emerging threats.	Swain et al. 2011

ELISA **Ricin** **Botulinum A toxin** **Cy-3 fluorescent dye**	Previously, we selected sdAb that were specific for both ricin and botulinum A (BoNT A) toxin complex from phage display libraries of sdAb and evaluated the solubly expressed protein. Here, phage-displayed sdAb were used as reporter reagents and compared to soluble, unfused sdAb. We found that using phage-displayed sdAb as reporter elements in immunoassay formats gave improved detection over using unfused, soluble sdAb reporters. In enzyme-linked immunosorbent assays (ELISAs), the lowest level of toxin detected for both ricin and BoNT A toxoid complex was decreased by one to two orders of magnitude using phage-displayed sdAb as reporter reagents. Use of the phage preserved the ability to discriminate ricin and RCA120 by at least a factor of 10 fold. In an effort to reduce the number of steps in the assays, we directly labeled phage displaying sdAb with a Cy-3 fluorescent dye. Signal was greatly decreased using the dye-labeled phage compared to biotinylated phage followed by streptavidin-phycoerythrin. In these assays the use of phage-displayed sdAb gives more sensitive detection than soluble sdAb alone, however directly dye labeling the phage failed to provide responses of a similar magnitude.	Goldman et al. 2010
Antibody microarrays **Surface expression** **Fibroblast growth factor receptor 1**	The preparation of effective conventional antibody microarrays depends on the availability of high quality material and on the correct accessibility of the antibody active moieties following their immobilization on the support slide. We show that spotting bacteria that expose recombinant antibodies on their external surface directly on nanostructured-TiO(2) or epoxy slides (purification-independent microarray – PIM) is a simple and reliable alternative for preparing sensitive and specific microarrays for antigen detection. Variable domains of single heavy-chain antibodies (VHHs) against fibroblast growth factor receptor 1 (FGFR1) were used to capture the antigen diluted in serum or BSA solution. The FGFR1 detection was performed by either direct antigen labeling or using a sandwich system in which FGFR1 was first bound to its antibody and successively identified using a labeled FGF. In both cases the signal distribution within each spot was uniform and spot morphology regular. The signal-to-noise ratio of the signal was extremely elevated and the specificity of the system was proved statistically. The LOD of the system for the antigen was calculated being 0.4 ng/ml and the dynamic range between 0.4 ng/ml and 10 μg/ml. The microarrays prepared with bacteria exposing antibodies remain fully functional for at least 31 clays after spotting. We finally demonstrated that the method is suitable for other antigen-antibody pairs and expect that it could be easily adapted to further applications such as the display of scFv and IgG antibodies or the autoantibody detection using protein.	De Marni et al. 2012
Competitive assay **Triclocarban**	Using triclocarban (TCC) as a model hapten, we found that conventional antibodies, IgG1 fraction, reacted with free TCC with a higher relative affinity (IC(50) 51.0 ng/ml) than did the sdAbs (IgG2 and IgG3, 497 and 370 ng/ml, respectively). A VHH library was prepared, and by elution of phage with limiting concentrations of TCC and competitive selection of binders, we were able to isolate high-affinity clones, K(D) 0.98–1.37 nM (SPR), which allowed development of a competitive assay for TCC with an IC(50) = 3.5 ng/ml (11 nM). This represents a 100-fold improvement with regard to the performance of the sdAb serum fraction, and it is 100-fold better than the IC(50) attained with other antihapten VHHs reported thus far. Despite the modest overall antihapten sdAbs response in llamas, a small subpopulation of high-affinity VHHs is generated that can be isolated by careful design of the selection process.	Tabares-da Rosa et al. 2011

Denaturing agents **Celiac patients** **Prolamin** **Gliadin** **ELISA**	Food intended for celiac patients' consumption must be analyzed for the presence of toxic prolamins using high delectability tests. Though 60% ethanol is the most commonly used solvent for prolamins extraction, 2-mercaptoethanol (2-ME) and guanidinium chloride (GuHCl) can be added to increase protein recovery. However, ethanol and denaturing agents interfere with antigen recognition when conventional antibodies are used. In the present work, a new method for gliadins quantification is shown. The method is based on the selection of llama single domain antibody fragments able to operate under denaturing conditions. Six out of 28 VHH-phages obtained retained their binding capacity in 15% ethanol. Selected clones presented a long CDR3 region containing two additional cysteines that could be responsible for the higher stability. One of the clones (named VHH26) was fully operative in the presence of 15% ethanol, 0.5% 2-ME, and 0.5 M GuHCl. Capture ELISA using VHH26 was able to detect gliadins in samples shown as negatives by conventional ELISA. Therefore, this new strategy appears as an excellent platform for quantitative determination of proteins or any other immunogenic compound, in the presence of denaturing agents, when specific recognition units with high stability are required.	Dona et al. 2010
ELISA **Taenia solium** **Cysticercosis**	Taenia solium cysticercosis is a major helminth zoonosis in developing countries. Pigs are the intermediate hosts mediating transmission of infection. Specific assays to diagnose living cysts in pigs are lacking. The monodonal-based antigen detection ELISA is genus-specific and cross-reactions with Taenia hydatigena hamper the use of this test to screen pigs. We, therefore, aimed to introduce nanobodies, camelid-derived single-domain antibodies specific for T. solium cysticercosis, to develop unambiguous tests. Nanobodies were cloned following immunization of two dromedaries with T. solium antigen and eight T. solium-specific nanobodies were selected after phage display. Their binding characteristics and potential for the diagnosis of porcine cysticercosis were investigated. The nanobodies do not cross-react with T hydatigena, Taenia saginata, Taenia crassiceps or Trichinella spiralis and were categorized into four epitope-binding groups. The target protein was identified as 14 kDa diagnostic glycoprotein (Ts14), but the nanobodies also reacted with other proteins of the same family. Nanobodies were tested in a sandwich ELISA with cyst fluid, and one particular nanobody detected its cognate serum antigens in a species-specific inhibition ELISA. Considering their beneficial production and stability properties, these highly specific nanobodies constitute a promising tool to diagnose cysticercosis after further improvement of the sensitivity and future assay validation.	Deckers et al. 2009
Biosensor **Ebolavirus** **Clinical and environmental samples** **IgNAR** **Haemorrhagic fever**	Members of the genus Ebolavirus cause fulminating outbreaks of disease in human and non-human primate populations with a mortality rate up to 90%. To facilitate rapid detection of these pathogens in clinical and environmental samples, robust reagents capable of providing sensitive and specific detection are required. In this work recombinant antibody libraries were generated from murine (single chain variable domain fragment; scFv) and nurse shark, Ginglymostoma cirratum (IgNAR V) hosts immunised with Zaire ebolavirus. This provides the first recorded IgNAR V response against a particulate antigen in the nurse shark. Both murine scFv and shark IgNAR V libraries were panned by phage display technology to identify useful antibodies for the generation of immunological detection reagents. Two murine scFv were shown to have specificity to the Zaire ebolavirus viral matrix protein VP40. Two isolated IgNAR V were shown to bind to the viral nucleoprotein (NP) and to capture viable Zaire ebolavirus with a high degree of sensitivity. Assays developed with IgNAR V cross-reacted to Reston ebolavirus, Sudan ebolavirus and Bundibugyo ebolavirus. Despite this broad reactivity, neither of IgNAR V showed reactivity to Cote divoire ebolavirus. IgNAR V was substantially more resistant to irreversible thermal denaturation than murine scFv and monoclonal IgG in a comparative test. The demonstrable robustness of the IgNAR V domains may offer enhanced utility as immunological detection reagents in fieldable biosensor applications for use in tropical or subtropical countries where outbreaks of Ebolavirus haemorrhagic fever occur.	Goodchild et al. 2011

Biosensor **Decabody** **Cellulose filters** **S. aureus**	Antibody engineering has allowed for the rapid generation of binding agents against virtually any antigen of interest, predominantly for therapeutic applications. Considerably less attention has been given to the development of diagnostic reagents and biosensors using engineered antibodies. Recently, we produced a novel pentavalent bispecific antibody (i.e., decabody) by pentamerizing two single-domain antibodies (sdAbs) through the verotoxin B subunit (VTB) and found both fusion partners to be functional. Using a similar approach, we have engineered a bispecific pentameric fusion protein consisting of five sdAbs and five cellulose-binding modules (CBMs) linked via VTB. To find an optimal design format, we constructed six bispecific pentamers consisting of three different CBMs, fused to the Staphylococcus aureus-specific human sdAb HVHP428, in both orientations. One bispecific pentamer, containing an N-terminal CBM9 and C-terminal HVHP428, was soluble, non-aggregating, and did not degrade upon storage at four degrees C for over six months. This molecule was dually functional as it bound to cellulose-based filters as well as S. aureus cells. When impregnated in cellulose filters, the bispecific pentamer recognized S. aureus cells in a flow-through detection assay. The ability of pentamerized CBMs to bind cellulose may form the basis of an immobilization platform for multivalent display of high-avidity binding reagents on cellulosic filters for sensing of pathogens, biomarkers and environmental pollutants.	Hussack et al. 2009
SRP-sensor **ELISA** **M. tuberculosis** **Phage display selection strategies** **Heat shock protein**	Background: Recombinant antibodies are powerful tools in engineering of novel diagnostics. Due to the small size and stable nature of llama antibody domains selected antibodies can serve as a detection reagent in multiplexed and sensitive assays for M. tuberculosis. Methodology/Principal Findings: Antibodies for Mycobacterium tuberculosis (M. tb) recognition were raised in Alpaca, and, by phage display, recombinant variable domains of heavy-chain antibodies (VHH) binding to M. tuberculosis antigens were isolated. Two phage display selection strategies were followed: one direct selection using semi-purified protein antigen, and a depletion strategy with lysates, aiming to avoid cross-reaction to other mycobacteria. Both panning methods selected a set of binders with widely differing complementarity determining regions. Selected recombinant VHHs were produced in E. coli and shown to bind immobilized lysate in direct Enzymelinked Immunosorbent Assay (ELISA) tests and soluble antigen by surface plasmon resonance (SPR) analysis. All tested VHHs were specific for tuberculosis-causing mycobacteria (M. tuberculosis, M. bovis) and exclusively recognized an immunodominant 16 kDa heat shock protein (hsp). The highest affinity VHH had a dissociation constant (KD) of 4 × 10(−10) M. Conclusions/Significance: A broad set of different llama antibodies specific for 16 kDa heat shock protein of M. tuberculosis is available. This protein is highly stable and abundant in M. tuberculosis. The VHH that detect this protein are applied in a robust SPR sensor for identification of tuberculosis-causing mycobacteria.	Trilling et al. 2011

Table 8. Other prospective uses of single-domain antibody fragments

Crystal structure **Editosome** **Trypanosoma brucei**	The parasite Trypanosoma brucei, the causative agent of sleeping sickness across sub-Saharan Africa, depends on a remarkable U-insertion/deletion RNA editing process in its mitochondrion. A approximately 20 S multi-protein complex, called the editosome, is an essential machinery for editing pre-mRNA molecules encoding the majority of mitochondrial proteins. Editosomes contain a common core of twelve proteins where six OB-fold interaction proteins, called A1-A6, play a crucial role. Here, we report the structure of two single-strand nucleic acid-binding OB-folds from interaction proteins A3 and A6 that surprisingly, form a heterodimer. Crystal growth required the assistance of an anti-A3 nanobody as a crystallization chaperone. Unexpectedly, this anti-A3 nanobody binds to both A3(OB) and A6, despite only similar to 40% amino acid sequence identity between the OB-folds of A3 and A6. The A3(OB)-A6 heterodimer buries 35% more surface area than the A6 homodimer. This is attributed mainly to the presence of a conserved Pro-rich loop in A3(OB). The implications of the A3(OB)-A6 heterodimer, and of a dimer of heterodimers observed in the crystals, for the architecture of the editosome are profound, resulting in a proposal of a 'five OB-fold center' in the core of the editosome.	Park et al. 2012
X-ray crystallography **Prion** **In situ proteolysis** **Microseed matrix screening**	Prion proteins (PrPs) are difficult to crystallize, probably due to their inherent flexibility. Several PrPs structures have been solved by nuclear magnetic resonance (NMR) techniques; however, only three structures were solved by X-ray crystallography. Here we combined in-situ proteolysis with automated microseed matrix screening (MMS) to crystallize two different PrP(C)-nanobody (Nb) complexes. Nanobodies are single-domain antibodies derived from heavy-chain-only antibodies of camelids. Initial crystallization screening conditions using in situ proteolysis of mouse prion (23–230) in complex with a nanobody (Nb_PrP_01) gave thin needle aggregates, which were of poor diffraction quality. Next, we used these microcrystals as nucleants for automated MMS. Good-quality crystals were obtained from mouse PrP (89–230)/Nb_PrP_01, belonged to the monoclinic space group P 1 21 1, with unit-cell parameters a = 59.13, b = 63.80, c = 69.79 angstrom, β = 101.96° and diffracted to 2.1 angstrom resolution using synchrotron radiation. Human PrP (90–231)/Nb_PrP_01 crystals belonged to the monoclinic space group C2, with unit-cell parameters a = 131.86, b = 45.78, c = 45.09 angstrom, β = 96.23° and diffracted to 1.5 angstrom resolution. This combined strategy benefits from the power of the MMS technique without suffering from the drawbacks of the in situ proteolysis. It proved to be a successful strategy to crystallize PrP-nanobodies complexes and could be exploited for the crystallization of other difficult antigenantibody complexes.	Abskharon et al. 2011
X-ray crystallography **Conformational intermediates** **Amyloidogenesis** **Beta 2-microglobulin**	Atomic-level structural investigation of the key conformational intermediates of amyloidogenesis remains a challenge. Here we demonstrate the utility of nanobodies to trap and characterize intermediates of beta 2-microglobulin (beta 2m) amyloidogenesis by X-ray crystallography. For this purpose, we selected five single domain antibodies that block the fibrillogenesis of a proteolytic amyloidogenic fragment of beta 2m (Delta N6 beta 2m). The crystal structure of Delta N6 beta 2m in complex with one of these nanobodies (Nb24) identifies domain swapping as a plausible mechanism of self-association of this amyloidogenic protein. In the swapped dimer, two extended hinge loops-corresponding to the heptapetide NHVTLSQ that forms amyloid in isolation-are unmasked and fold into a new two-stranded antiparallel beta-sheet. The beta-strands of this sheet are prone to self-associate and stack perpendicular to the direction of the strands to build large intermolecular beta-sheets that run parallel to the axis of growing oligomers, providing an elongation mechanism by self-templated growth.	Domanska et al. 2011

Keywords	Abstract	Reference
Transport systems **Immunoglobulin receptor** **Carrier system** **Transcytosis** **Epithelial monolayer**	The polymeric immunoglobulin receptor (pIgR) ensures the transport of dimeric immunoglobulin A (dIgA) and pentameric immunoglobulin M (pIgM) across epithelia to the mucosal layer of for example the intestines and the lungs via transcytosis. Per day the human pIgR mediates the excretion of 2 to 5 grams of dIgA into the mucosa of luminal organs. This system could prove useful for therapies aiming at excretion of compounds into the mucosa. Here we investigated the use of the variable domain of camelid derived heavy chain only antibodies, also known as VHHs or Nanobodies, targeting the human pIgR, as a transport system across epithelial cells. We show that VHHs directed against the human pIgR are able to bind the receptor with high affinity (similar to 1 nM) and that they compete with the natural ligand, dIgA. In a transcytosis assay both native and phage-bound VHH were only able to get across polarized MDCK cells that express the human pIgR gene in a basolateral to apical fashion. Indicating that the VHHs are able to translocate across epithelia and to take along large particles of cargo. Furthermore, by making multivalent VHHs we were able to enhance the transport of the compounds both in a MDCK-hpIgR and Caco-2 cell system, probably by inducing receptor clustering. These results show that VHHs can be used as a carrier system to exploit the human pIgR transcytotic system and that multivalent compounds are able to significantly enhance the transport across epithelial monolayers.	Emmerson et al. 2011
Transport systems **Micelle** **EGFR** **Doxorubicin** **Encapsulation efficiency**	Core-crosslinked thermosensitive and biodegradable polymeric micelles were actively targeted to EGFR-overexpressing cancer cells by conjugating an anti-EGFR nanobody on the surface of the micelles. A methacrylated doxorubicin derivative containing an acid sensitive hydrazone spacer was encapsulated and subsequently covalently attached during core-crosslinking of the micelles. Encapsulation efficiency was 60% and doxorubicin (DOX) was completely released after 24 h at pH 5, while hardly any DOX was released at pH 7.4. DOX-loaded micelles showed toxicity similar to free doxorubicin towards ovarian carcinoma cells.	Talelli et al. 2010
Drug delivery vehicle **Sodalis glossinidius** **Paratransgenesis** **Trypanosoma brucei**	Background: Sodalis glossinidius, a gram-negative bacterial endosymbiont of the tsetse fly, has been proposed as a potential in vivo drug delivery vehicle to control trypanosome parasite development in the fly, an approach known as paratransgenesis. Despite this interest of S. glossinidius as a paratransgenic platform organism in tsetse flies, few potential effector molecules have been identified so far and to date none of these molecules have been successfully expressed in this bacterium. Results: In this study, S. glossinidius was transformed to express a single domain antibody, (Nanobody) Nb_An33, that efficiently targets conserved cryptic epitopes of the variant surface glycoprotein (VSG) of the parasite Trypanosoma brucei. Next, we analyzed the capability of two predicted secretion signals to direct the extracellular delivery of significant levels of active Nb_An33. We show that the pelB leader peptide was successful in directing the export of fully functional Nb_An33 to the periplasm of S. glossinidius resulting in significant levels of extracellular release. Finally, S. glossinidius expressing pelBNb_An33 exhibited no significant reduction in terms of fitness, determined by in vitro growth kinetics, compared to the wild-type strain. Conclusions: These data are the first demonstration of the expression and extracellular release of functional trypanosome-interfering Nanobodies in S. glossinidius. Furthermore, Sodalis strains that efficiently released the effector protein were not affected in their growth, suggesting that they may be competitive with endogenous microbiota in the midgut environment of the tsetse fly. Collectively, these data reinforce the notion for the potential of S. glossinidius to be developed into a paratransgenic platform organism.	De Vooght et al. 2012

Blood-brain barrier **Drug biodistribution** **In vivo imaging** **Liposome nanocarrier**	The development of imaging and therapeutic agents against neuronal targets is hampered by the limited access of probes into the central nervous system across the blood brain barrier (BBB). The evaluation of drug penetration into the brain in experimental models often requires complex procedures, including drug radiolabeling, as well as determinations in multiple animals for each condition or time point. Prospective in vivo imaging of drug biodistribution may provide an alternative to "classical" pharmacokinetics and biodistribution studies in that a contrast-enhanced imaging signal could serve as a surrogate for the amount of drug or biologic delivered to the organ of interest. For the brain-targeting applications, it is necessary to develop formulation strategies that enable a simultaneous drug and contrast agent delivery across the BBB. In this chapter, we describe methods for encapsulating drugs into liposome nanocarriers with surface display of both the imaging contrast agent for one or multiple imaging modalities and the single-domain antibody that undergoes receptor-mediated transcytosis across the BBB. Contrast-enhanced imaging signal detected in the brain after intravenous injection of such formulation(s) is proportional to the amount of drug delivered into the brain parenchyma. This method allows for a prospective, noninvasive estimation of drug delivery, accumulation, and elimination from the brain.	Iqbal et al. 2011
Mimotope selection **IgNAR** **Plasmodium falciparum**	Mimotopes mimic the three-dimensional topology of an antigen epitope, and are frequently recognized by antibodies with affinities comparable to those obtained for the original antibody-antigen interaction. Peptides and anti-idiotypic antibodies are two classes of protein mimotopes that mimic the topology (but not necessarily the sequence) of the parental antigen. In this study, we combine these two classes by selecting mimotopes based on single domain IgNAR antibodies, which display exceptionally long CDR3 loop regions (analogous to a constrained peptide library) presented in the context of an immunoglobulin framework with adjacent and supporting CDR1 loops. By screening an in vitro phage-display library of IgNAR variable domains (V(NAR)s) against the target antigen monoclonal antibody MAb5G8, we obtained four potential mimotopes. MAb5G8 targets a linear tripeptide epitope (AYP) in the flexible signal sequence of the Plasmodium falciparum Apical Membrane Antigen-1 (AMA1), and this or similar motifs were detected in the CDR loops of all four V(NAR)s. The V(NAR)s, 1-A-2, -7, -11, and -14, were demonstrated to bind specifically to this paratope by competition studies with an artificial peptide and all showed enhanced affinities (3–46 nM) compared to the parental antigen (175 nM). Crystallographic studies of recombinant proteins 1-A-7 and 1-A-11 showed that the SYP motifs on these V(NAR)s presented at the tip of the exposed CDR3 loops, ideally positioned within bulge-like structures to make contact with the MAb5G8 antibody. These loops, in particular in 1-A-11, were further stabilized by inter- and intra- loop disulphide bridges, hydrogen bonds, electrostatic interactions, and aromatic residue packing. We rationalize the higher affinity of the V(NAR)s compared to the parental antigen by suggesting that adjacent CDR1 and framework residues contribute to binding affinity, through interactions with other CDR regions on the antibody, though of course definitive support of this hypothesis will rely on co-crystallographic studies. Alternatively, the selection of mimotopes from a large ($< 4 \times 10^8$) constrained library may have allowed selection of variants with even more favorable epitape topologies than present in the original antigenic structure, illustrating the power of in vivo selection of mimotopes from phage-displayed molecular libraries.	Simmons et al. 2008

Bacteriophage p2 **Major capsid protein** **Receptor-binding proteins** **Neutralisation assay**	Background: Bacteriophages infecting lactic acid bacteria (LAB) are widely acknowledged as the main cause of milk fermentation failures. In this study, we describe the surface-expression as well as the secretion of two functional llama heavy-chain antibody fragments, one binding to the major capsid protein (MCP) and the other to the receptor-binding proteins (RBP) of the lactococcal bacteriophage p2, by lactobacilli in order to neutralise lactococcal phages. Results: The antibody fragment VHH5 that is directed against the RBP, was fused to a c-myc tag and expressed in a secreted form by a Lactobacillus strain. The fragment VHH2 that is binding to the MCP, was fused to an E-tag and anchored on the surface of the lactobacilli. Surface expression of VHH2 was confirmed by flow cytometry using an anti-E-tag antibody. Efficient binding of both the VHH2 and the secreted VHH5 fragment to the phage antigens was shown in ELISA. Scanning electron microscopy showed that lactobacilli expressing VHH2 anchored at their surface were able to bind lactococcal phages. A neutralisation assay also confirmed that the secreted VHH5 and the anchored VHH2 fragments prevented the adsorption of lactococcal phages to their host cells. Conclusion: Lactobacilli were able to express functional VHH fragments in both a secreted and a cell surface form and reduced phage infection of lactococcal cells. Lactobacilli expressing llama heavy-chain antibody fragments represent a novel way to limit phage infection.	Hultberg et al. 2007
Protein switches **Combinatorial histidine library** **pH-dependent binding**	There is growing interest in the development of protein switches, which are proteins whose function, such as binding a target molecule, can be modulated through environmental triggers. Efforts to engineer highly pH sensitive protein-protein interactions typically rely on the rational introduction of ionizable groups in the protein interface. Such experiments are typically time intensive and often sacrifice the protein's affinity at the permissive pH. The underlying thermodynamics of proton-linkage dictate that the presence of multiple ionizable groups, which undergo a pK(a) change on protein binding, are necessary to result in highly pH-dependent binding. To test this hypothesis, a novel combinatorial histidine library was developed where every possible combination of histidine and wild-type residue is sampled throughout the interface of a model anti-RNase A single domain VHH antibody. Antibodies were coselected for high-affinity binding and pH-sensitivity using an in vitro, dual-function selection strategy. The resulting antibodies retained near wild-type affinity yet became highly sensitive to small decreases in pH, drastically decreasing their binding affinity, due to the incorporation of multiple histidine groups. Several trends were observed, such as histidine "hot-spots", which will help enhance the development of pH switch proteins as well as increase our understanding of the role of ionizable residues in protein interfaces. Overall, the combinatorial approach is rapid, general, and robust and should be capable of producing highly pH-sensitive protein affinity reagents for a number of different applications.	Murtaugh et al. 2011
Immunoaffinity chromatography **Caffeine** **Grafting**	This work demonstrates the feasibility of using a camelid single domain antibody for immunoaffinity chromatographic seperation of small molecules. An anti-caffeine VHH antibody was produced by grafting the complementanty determining sequences of a previously generated antibody onto an anti-RNase A antibody scaffold followed by expression in E. coli. Analysis of the binding properties of the antibody by ELISA and fluorescence-based thermal shift assays showed that it recognizes not only caffeine, but also theophylline, theobiomine and paraxanthine, albeit with lower affinity. Further investigation of the effect of environmental conditions, i.e., temperature, pH, and ionic strength, on the antibody using these methods provided useful information about potential elation conditions to be used in chromatographic applications. Immobilization of the VHH onto a high flow-through synthetic support material resulted In a stationary phase capable of separating caffeine and its metabolites.	Franco et al. 2010

Protein conformation Spectral properties Green fluorescent protein Protein translocation	Protein conformation is critically linked to function and often controlled by interactions with regulatory factors. Here we report the selection of camelid-derived single-domain antibodies (nanobodies) that modulate the conformation and spectral properties of the green fluorescent protein (GFP). One nanobody could reversibly reduce GFP fluorescence by a factor of 5, whereas its displacement by a second nanobody caused an increase by a factor of 10. Structural analysis of GFP-nanobody complexes revealed that the two nanobodies induce subtle opposing changes in the chromophore environment, leading to altered absorption properties. Unlike conventional antibodies, the small, stable nanobodies are functional in living cells. Nanobody-induced changes were detected by ratio imaging and used to monitor events such as the tamoxifen-induced nuclear localization of estrogen receptor. This work demonstrates that protein conformations can be manipulated and studied with nanobodies in living cells.	Kirchhofer et al. 2010

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