HHS Public Access

Author manuscript

*Anal Bioanal Chem*. Author manuscript; available in PMC 2017 September 01.

Published in final edited form as:

*Anal Bioanal Chem*. 2016 September ; 408(22): 5985–6002. doi:10.1007/s00216-016-9585-x.

# VHH antibodies: Emerging reagents for the analysis of environmental chemicals

**Candace S. Bever**[1], **Jie-Xian Dong**[1], **Natalia Vasylieva**[1], **Bogdan Barnych**[1], **Yongliang Cui**[1,2], **Zhen-Lin Xu**[1], **Bruce D. Hammock**[1], and **Shirley J. Gee**[1,*]

[1]Department of Entomology and Nematology and UCD Comprehensive Cancer Center, University of California Davis, Davis, CA 95616

[2]Department of Oral Biology, School of Dental Medicine, University of Buffalo, State University of New York, Buffalo, NY, 14214

## Abstract

A VHH antibody (or nanobody) is the antigen binding fragment of heavy chain only antibodies. Discovered nearly 25 years ago, they have been investigated for their use in clinical therapeutics and immunodiagnostics, and more recently for environmental monitoring applications. A new and valuable immunoreagent for the analysis of small molecular weight environmental chemicals, VHH will overcome many pitfalls encountered with conventional reagents. In the work so far, VHH antibodies often perform comparably to conventional antibodies for small molecule analysis, are amenable to numerous genetic engineering techniques, and show ease of adaption to other immunodiagnostic platforms for use in environmental monitoring. Recent reviews cover the structure and production of VHH antibodies as well as their use in clinical settings. However, no report focuses on the use of these VHH antibodies to small environmental chemicals (MW <1,500 Da). This review article summarizes the efforts made to produce VHHs to various environmental targets, compares the VHH-based assays with conventional antibody assays, and discusses the advantages and limitations in developing these new antibody reagents particularly to small molecule targets.

### Keywords

VHH antibodies; environmental chemicals; sensitivity; genetic modifications; production; biosensors

## Introduction

Single variable domain on a heavy chain (VHH) antibodies, also referred to as Nanobodies®, were discovered nearly 25 years ago. Heavy chain only antibodies (HcAb) are naturally produced by camelids and sharks. The antigen binding portion of the HcAb is comprised of

*Corresponding author: Shirley J. Gee, sjgee@ucdavis.edu.

**Compliance with Ethical Standards**

The authors declare that they have no conflicts of interest. Research involving animals was reviewed and approved by the University of California Davis Institutional Animal Care and Use Committee.

the VHH fragment (Figure 1 adapted from Vincke and Muyldermans [1]). Overall, we feel the VHH technology will surpass many of the conventional antibody reagents. It utilizes key features of conventional antibody production, such as affinity maturation, however offers greatly improved screening and isolation techniques. An overview of the process for obtaining VHH is briefly diagramed in Figure 2. In addition, no animal sacrifice is needed. VHH technology also utilizes the abundant advancements in genetic engineering, such that genes are routinely spliced together and rearranged to provide a tool with superior binding that is easily purified while also containing labeling tags. Our assessment is that the VHH technology is among the most exciting developments in the antibody field in decades. For many applications in immunodiagnostics, VHH technology will be faster, cheaper and better than earlier procedures. Maybe of greater importance, VHH technology allows and will allow us to accomplish previously impossible goals.

Recent reviews have described the material [2], as well as their use in clinical and therapeutic applications [3–5], however none of these reviews focused on the application of VHH for small molecule analysis. In this paper, we describe all of the studies, to date, that have developed VHHs to small molecules (MW <1,500 Da) wherein the target is most likely relevant for environmental monitoring. We identify the methods used and critically evaluate features of each investigation and how those studies contribute to the broader understanding of the utility of nanobodies for environmental monitoring. Whenever possible, we compare the VHH-based assay to conventional antibody-based assays.

Furthermore, we addressed a variety of advantages and disadvantages of producing and using VHHs compared to poly- and monoclonal antibodies as well as other recombinant techniques for obtaining antibody fragments. The most notable advantage is that VHHs can be produced economically in unlimited amounts, are more stable when exposed to heat and solvents, and are amenable to genetic manipulations for a myriad of uses, including scaffolding, labeling, and altering specific amino acids. VHHs are 1/10th the size of conventional antibodies. Thus far, VHHs have proven to be adaptable to commonly used platforms that use conventional antibodies, such as microtiter plates, electrochemical biosensors, and lateral flow devices. With their smaller size, we surmise that the higher density of binding domains will provide an outstanding advantage in terms of increased signal and therefore higher sensitivities. A summary of characteristics associated with conventional (polyclonal, pAb and monoclonal, mAb) antibodies, as well as with VHH and another source of recombinant antibodies (scFvs, single-chain variable fragment) is provided to demonstrate that the disadvantages of using larger animals and requiring the biohazardous bacteriophage for selection is substantially outweighed by the ability to rapidly and reliably screen for, produce, and manipulate the resulting VHHs (Table 1). Ultimately, this review serves to explain what has been done and what is needed to advance the field in developing new immunoreagents and particularly VHH for environmental monitoring.

## Summary of VHH assays to environmental chemicals

Each summary provided in this section highlights a small molecular weight environmental chemical to which VHHs have been produced. They are presented in chronological order with regard to publication date. Each summary begins with a brief description of the

chemical and where it is expected to be found. For the production of VHHs, panning and screening details are identified, with particular focus on elution conditions, homo- or heterogeneity of the screening antigen, and assay buffer conditions. Key characterization information, such as cross-reactivity, assay sensitivity or limit of detection, and thermal stability of the reagent is provided, where applicable. Table 2 provides VHH assay sensitivities and, when possible, provides polyclonal antibody (pAb) or monoclonal antibody (mAb)-based assay sensitivities to the equivalent target. Table 3 summarizes other parameters beyond assay sensitivity, such as species of animal, method of elution, and other unique observations of each study.

The **reactive red azo dyes**, RR-6 and RR-120 are among a group of reactive dyes that are used to color fabric. As such, they are found in the wastewater effluent of textile factories. RR-120 has been found to be harmful to duck weed and rainbow trout and toxic to the water flea [6]. Wastewater treatment processes to degrade these chemicals such as filtration [7], UV light [8] or bioremediation are under development [9]. The body of work describing the VHH derived from llamas against the azo dyes RR-6, andRR-120 was the first demonstration of the development of a VHH for a small molecule or hapten. The compounds were coupled to bovine serum albumin (BSA) through the triazinyl group of the dyes and used as immunogens. A response to antigen was found for all three immunoglobulin (IgG) isotypes (i.e., IgG1, IgG2, and IgG3). A Ficoll discontinuous gradient was used to isolate peripheral blood cells from whole blood. The libraries were constructed in phagemid vectors for expression in either *E. coli* or yeast. The libraries were not biopanned to enrich for specific antigen binders. Instead, *E. coli* or yeast were infected and allowed to grow. At least 200 random colonies were picked and were grown up. The supernatants were then tested for binding to the haptens covalently linked to a polystyrene plate. Selected VHH had affinities between 18 and 85 nM, similar to the 8.4 nM affinity of the earlier developed mAb. No cross reactivity for RR-120 by VHH selected on RR-6 and vice versa was found [10]. In addition, their work was the first to describe a high yield of VHH from a yeast expression system where anti-hapten VHH were from 0.1 to 3.0 mg/L and anti-protein VHH were from 1.9 to 9.3 mg/L [11]. VHH had superior temperature stability and could bind antigen well at elevated temperature and higher concentrations of the chaotrope ammonium thiocyanate compared to mAbs. Both mAbs and VHHs showed no change in binding in the presence of up to 50% ethanol [10]. Further examination of the crystal structure of a VHH revealed that although VHH lack the hydrophobic cleft typically formed by VL and VH chains, the CDRs of VHH are adaptable and binding may occur in any number of ways. Both pi stacking and charge-charge interactions occur with molecules that have both aromatic and hydrophilic properties [12, 13]. Interestingly, unlike VHH previously reported for protein antigens, the VHH against reactive red dyes did not have the additional disulfide bond between CDR1 and CDR3 that has been attributed to high thermal stability. The authors proposed then that the stability could be attributed to successful refolding of the protein after heat challenge [14, 10]. To improve production, affinity and temperature stability DNA shuffling was also used with success [15].

**Picloram** is an auxinic herbicide that mimics the plant-growth hormone indole-3-acetic acid (IAA) and is widely used to control woody species and broadleaf weeds in non-crop areas. It

is the most persistent member of its family of herbicides with a half-life of 90 days. Picloram is mainly degraded by microbes that are present in soil or water. In the paper [16], the authors hypothesize "that VHHs specific for small molecules (i.e. MW <500 Da) can be isolated and affinity-matured efficiently by ribosome display technology and envisage that these unique VHHs will be valuable diagnostics in agriculture and the food industry." Previously, panning of a rather rich VHH-phage library (of size $5.4\times10^8$), constructed from a non-immunized llama, failed to give any picloram selective binders. In the present work, the authors reported successful selection of picloram selective VHH binders out of the same VHH mRNA pool they used for construction of the phage library. In comparison to the phage-display, the ribosome-display procedure did not involve any cloning and transformation steps, and could handle a VHH DNA library of a much larger size. The panning procedure was performed using a subtractive format and the hapten carrier proteins were switched at each round (Pic-OVA for rounds 1,3,5; and Pic-BSA for rounds 2,4,6). After six rounds of panning, two clones were obtained with $K_d$ values of 3 and 256 μM as determined by surface plasmon resonance (SPR) analysis. Interestingly, the CDR3 region of both VHHs consists of only five amino acids. Sequence analysis of the selected clones revealed four unique residues Pro50, Gly66, Leu87 and Met105 that are not commonly found in llama VHH antibodies. The authors speculated that separate point mutations which may have been introduced during PCR amplification steps of the ribosome display procedure could be the origin of these residues.

**Indole-3-acetic acid (IAA)**, also called auxin, is the most potent and abundant member of auxins, a class of plant hormones involved in regulation of many growth processes. Exogenous application of high concentrations of auxin leads to the symptoms of "auxin overdose" and eventually plant death due to growth abnormalities. This effect lies at the basis of the mode of action of synthetic auxins or auxinic herbicides. Several proteins have been found to bind auxin amongst which is the relatively poorly studied Auxin Binding Protein (ABP). Authors hypothesized "that antibodies that bind to IAA may be used as surrogate ABPs to study the structure-activity relationships of IAA and the auxinic herbicides binding to their putative receptor." In this work [17], authors isolated five different sdAbs from a naïve llama library with relatively low affinity for the IAA-BSA conjugate ($K_d$ ≥5 μM) as determined by SPR (surface plasmon resonance). Inhibition experiments with sdAb CSF2A ($K_d$ = 5 μM) revealed broad cross-reactivity with all auxinic herbicides with $IC_{50}$ values > 200 μM for herbicides and 800 μM for IAA. Pentamerized VHH, obtained by VHH fusion to the verotoxin B subunit, was used for stable immobilization in an oriented manner on the SPR sensor chip, which allowed for direct SPR study of VHH-hapten interactions and determining $K_d$ values. Interestingly SPR measurements of the $K_d$ values of pentamerized sdAb CSF2A for free IAA was 20 μM whereas the $K_d$ value for synthetic auxins was one-two orders of magnitude higher. CDR shuffling of all five sdAbs by StEP PCR resulted in a sdAb library with 46, 35, 10 and 10 % of the clones derived from one, two, or three parents or having point mutations respectively. Although the shuffled library did not result in sdAbs with improved affinities, affinity measurement of individual clones suggested that CDR2 of sdAb CSF2A was involved in auxin binding and CDR3 was responsible for the broad cross-reactivity for auxinic herbicides. The authors speculated that sdAb CSF2A that can discriminate between non-

active and active auxin analogues could be used for high throughput screening for new synthetic auxins.

Monitoring of nutrition and limiting consumption of unwanted components in food is of growing interest within the general population. As a potent central nervous system stimulator, **caffeine** is frequently avoided or is desired and the development of tools that would allow caffeine monitoring in food and particularly in hot beverages is desired. Caffeine-selective VHHs were panned from libraries based on immunizing 2 camels and 3 llamas, using triethylamine for elution of VHH-phage fusions bound to immobilized caffeine/BSA antigen [18]. Four out of five camelids gave positive clones after two rounds of panning. Interestingly, one llama library produced no caffeine-selective clones, even though the pre-panning showed positive signal to the target. The most successful clone demonstrated a cross-reactivity pattern comparable to that of mAbs. The VHH proved to be thermally stable recovering more than 90% of its activity after incubation at temperatures up to 90 °C, while the mAb lost binding activity after incubation at 70 °C. Using the developed VHH, authors demonstrated the feasibility of the enzyme linked immunosorbent assay (ELISA) at elevated temperatures. The $IC_{50}$ remained unchanged when competition was performed at 70°C, indicating that binding was not affected dramatically by temperature. The accuracy of the assay was demonstrated by detection of caffeine in the range of beverages available on the market, where caffeine was not intentionally spiked, and the values obtained had good correlation with data from high performance liquid chromatography (HPLC) and data from the literature. A patent was issued on these thermostable caffeine-selective VHHs and their application to a disposable lateral flow device for caffeine monitoring in beverages at home and in restaurants [19]. In addition, the CDRs of these anti-caffeine VHHs were grafted onto an anti-RNase A antibody scaffold to produce a solid phase for immunoaffinity chromatographic separation and detection of caffeine [20].

**Methotrexate** (MTX, 4-amino-10-methylpteroylglutamic acid) is a folic acid analog that is used to treat cancer. By inhibiting dihydrofolate reductase, an enzyme responsible for the formation of one of the major cofactors in the synthesis of purines and thymidine necessary for DNA replication, it slows the growth of cancer cells [21]. A VHH against methotrexate (MTX) was generated and used as a model hapten to study how the antigen-binding mechanisms for low molecular weight antigens could result in high affinity binding considering the absence of a variable light chain (VL) [22]. Methotrexate was coupled to a commercial carrier protein, blue carrier immunogenic protein™ (Pierce Chemicals, Rockford, IL), via carbodiimide coupling. In this case, either carboxylic acid residue of MTX may have reacted to link to the carrier protein. IgG1 and IgG2/3 fractions of serum from the immunized llama were compared for binding to MTX-BSA, a homologous antigen. Both fractions had titers to MTX-BSA, with the titer of IgG1 somewhat higher. The VHH were screened from a library using three rounds of panning. Phage particles bound to the solid phase coated with MTX-BSA, were eluted with 0.1 M triethylamine, pH 11.0. Phages from the third round were then tested on MTX-BSA coated plates. Positive clones were subject to large scale production of soluble VHH that were subsequently tested for affinity using SPR. Selected VHH displayed nM binding affinities for the MTX-BSA antigen as well

as for free MTX using SPR. $IC_{50}$ data were not reported, but interpolation of the inhibition graph indicates an $IC_{50}$ of about 10 μM. Analysis of structural features revealed that like other VHHs, the CDR3 loop is longer than that found in IgG1. The CDR1–3 loops are typically credited with the high affinity binding of VHH. Using a CDR 'grafting' technique, MTX was found to 'tunnel' under the CDR1 and come in contact with a cryptic binding site in a nonhypervariable loop (CDR4). This resulted in a 1000× increase in binding of MTX. It is not known whether this tunnel effect will be universal for binding of small molecular weight compounds but it points to strategies for creating synthetic libraries or attempting *in vitro* affinity maturation [23].

**15-Acetyl-deoxynivalenol** (15-AcDON) is an acetylated metabolite of the trichothecene mycotoxin deoxynivalenol (DON). DON and its metabolites are ubiquitously found in food and feed [24]. Besides inhibiting protein synthesis, DON and 15-AcDON may also cause toxicity through dysregulation of cell signaling and changes to gene expression. Thus, their presence in the food supply is strictly regulated. The titer of the serum of a llama immunized with DON conjugated to BSA at the 15 position (15-DON-BSA) was excellent [25]. A competitive fluorescence polarization assay was developed using a homologous 15-DON-fluorescein conjugate. The resulting $IC_{50}$ for DON was 1.42 μM. The IgG in the serum was fractionated and the authors report that most binding was by what they term complete IgG. Heavy chain only antibodies apparently had significant binding but the data were not shown. VHH were selected with a panning strategy that used decreasing coating antigen (15-DON-OVA) concentration (20 to 10 μg/well), switching blocking agents for rounds 3 and 4, increasing the number of washes and eluting bound phage with triethylamine. The DNA of selected positive clones was sequenced and one containing the dominant sequence was further expressed as a pentamer by gene fusion to verotoxin B subunit. Signal for the pentamer was always higher than monomer given equivalent conditions. Thus, the authors conclude that this increased avidity is a result of panning with phage that displays VHH multi-valently and to obtain monomeric VHH that bind with high avidity may require a different display system. In competitive inhibition studies using fluorescence polarization, both the monomer and pentamer were selective for 15-AcDON in contrast to the serum antibodies that cross reacted nearly equally with DON, 3-AcDON and 15-AcDON. The $IC_{50}$s for 15-AcDON were similar, 1.24 vs 0.5 μM, for the monomer and pentamer, respectively. Surprisingly, when 15-DON-OVA was used in a competitive immunoassay on a microtiter plate the authors noted consistent inhibition by 15-AcDON, but could not obtain a complete binding curve at concentrations above 10 μM. They postulate this was due to a fast off rate of the VHH and subsequent loss of VHH during wash steps. There was no inhibition by free DON or 3-AcDON. Binding kinetics were determined by SPR. The $K_d$ for the monomer was reported at 5 μM for binding to 15-DON-HRP immobilized on the sensor chip. No binding was found for free DON. 15-AcDON and 3-AcDON were not tested for affinity binding due to solubility issues and the incompatibility of organic solvents and the sensor chip surface. Analysis of the amino acid sequence of the VHH showed no cysteines in CDR1 or CDR3 indicating that an interloop cysteine bridge, earlier postulated to be important for binding of large molecular weight antigens may not be important for small molecular weight antigens [25].

**Ochratoxin A (OTA)**, a secondary metabolite of several Aspergillus and Penicillium fungal species, is a common food contaminant that can produce nephrotoxic, teratogenic, carcinogenic, neurotoxic, and immunosuppressive activity. OTA contamination occurs worldwide, which seriously threatens public health. The OTA VHH antibodies were first cloned from an immunized alpaca by van Houwelingen's group [26]. In this case, a homologous hapten was used for both immunizing and panning. The selection of VHH antibodies was performed by panning twice against an OTA-OVA conjugate and once against an OTA-KLH conjugate. It is worth noting that the performance of one VHH in food matrices (white wine, red wine and instant coffee) was comparable to the performance in buffer. In this study, a flow-through membrane-based enzyme immunoassay was also developed. In the assay, they did not directly spot the VHH on the membrane, but instead used secondary antibodies to capture the VHH that may have enhanced the binding capability of the VHH by leaving the binding pocket exposed. Subsequently, Liu's group obtained four VHHs against OTA, and used OTA as a model to study the feasibility of phage display-mediated immuno-polymerase chain reaction (PD-IPCR) in the detection of toxic small molecular weight compounds [27]. The VHH phages were selected using gradually decreasing concentrations of OTA−OVA conjugate. The bound VHH phages were eluted with 0.2 M glycine HCl (pH 2.2) in first and second round, and OTA in the third and fourth round of panning. The detection limit of the developed VHH phage-based PD-IPCR was 3.7 pg/L, with a linear range of 0.01−1000 pg/mL. This is the most sensitive assay reported to date for the detection of OTA. In the solvent effect study, although 2.5% methanol exhibited the widest linear range and lowest LOD, there were slight differences among 5%, 10% and 20% methanol treatment groups. In Liu's second paper, the VHH was genetically fused to alkaline phosphatase to serve as the probe, and a direct competitive fluorescence enzyme immunoassay (dc-FEIA) for OTA was developed [28]. The $IC_{50}$ and the detection limit of the dc-FEIA were 0.13 and 0.04 ng/mL, respectively, with a linear range of 0.06−0.43 ng/mL. Along with speeding up the assay procedure and reducing variability by removing a step, the VHH fusion technique with the alkaline phosphatase dimer increases the avidity and thus the sensitivity of the procedure. In the methanol, ionic strength and pH effect study, the sensitivity of the dc-FEIA was affected by methanol and ionic strength. The dc-FEIA was more likely to be influenced at low pH ( ≤6.0), which may be due to the denaturation of the fusion protein caused by protein protonation at low pH. In the matrix effect study, the fusion protein was resistant to the matrix effects. In the validation study, cereal samples both spiked and naturally contaminated with OTA were analyzed. The results obtained from dc-FEIA and LC-MS/MS showed good agreement.

**Triclocarban** (3,4,4'-trichlorocarbanilide, TCC) is a broad-spectrum bactericide that is widely used in soaps, disinfectants, and other household products. TCC may cause adverse biological and toxicological effects on humans and the environment. In this work [29], TCC was used as a model hapten to study the practical aspects of producing VHH against small molecules. The titer and overall affinity of the IgG1 (conventional) subclass was relatively higher ($IC_{50}$ = 51 ng/mL) than that of IgG2 and IgG3 (monodomain). The authors demonstrated that affinity of the IgG1 to TCC was 10 times higher compared to IgG2 and IgG3. Interestingly, the panning experiments from the TCC-VHH library using classical acidic elution did not allow the selection of positive clones that could be inhibited with free

TCC. Thus, a new panning strategy was used where decreasing concentration of the free analyte was used to elute phage in the successive rounds of panning. This approach successfully gave five positive clones with the best $IC_{50}$ for TCC of 3.5 ng/mL. This was 100-fold sensitivity improvement compared to $IC_{50}$ values of other reported anti-hapten VHHs of the time. Here, only a homologous format of competitive ELISA was studied. The technique of SPR was used to measure the $K_d$ of the VHH with coating antigen and free analyte giving values of 1 and 11 ng/mL, respectively. Interestingly, the $K_d$ of the VHH-free analyte interaction was similar to $IC_{50}$ of the competitive ELISA. The VHH from selected clones showed somewhat higher reactivity to compounds structurally similar to TCC compared to the pAb [30]. The VHH demonstrated an outstanding thermostability compared to the conventional antibody. It is worth noting that not all VHHs withstand heat in the same way with some being inactivated at 85 °C and others remained active even after 1 h at 100 °C. Interestingly, opposite to what could be expected, the VHH that had additional disulfide bonds was not the most thermally stable.

Pyrethroids are a class of broad spectrum insecticides with extensive agricultural, forestry, horticulture, public health, and residential uses. Though these compounds are selectively toxic to insects and have relatively low toxicity to mammals there is still potential risk of human exposure, notably paresthesia from dermal exposure or irritation of mucous membranes. **3-Phenoxybenzoic acid** (3-PBA) is a common urinary metabolite of most pyrethroid insecticides that could be used as a biomarker of human or environmental exposure to these pesticides. In this work [31], 3-PBA was used as a model system to study the practical aspects of producing VHH against small molecules. The VHHs were selected using gradually decreasing concentrations of 3-PBA-BSA conjugate and free 3-PBA and more stringent washing. The homologous assay with the best VHH gave 100-fold higher sensitivity compared to the homologous pAb-based ELISA, and similar sensitivity to the heterologous pAb-based ELISA. Interestingly, this VHH did not recognize the heterologous hapten used in the ELISA with the pAb. The assay sensitivity achieved using a VHH against this small molecule (1.4 ng/mL) was better than those reported from most other camelid VHH-based ELISAs. Additionally, the assay sensitivity was improved 10-fold when the VHH was attached to the phage particle. This study was also the first to report the high tolerance of VHHs to organic solvents (methanol and dimethyl sulfoxide) compared to the corresponding pAb. In an effort to demonstrate the usefulness for human monitoring, this VHH assay was performed in a urine matrix with recovery values of spiked 3-PBA in the range of 80–100% and good correlation with GC-MS data.

**Brominated flame retardant** (BFR) chemicals are incorporated into plastics, electronics, and furniture to make them less flammable. One class of BFRs called polybrominated diphenyl ethers (PBDEs) has been taken off the market due to their persistence in the environment and concerns about toxicity to human health. While PBDEs have been banned from use, many of the products already manufactured that contain them remain in use. Often, the most prevalent congener of the PBDEs found in the environment is 2,2',4,4'-tetrabrominated diphenyl ether (BDE-47) and was the target for VHH production [32]. In this work, the VHH antibodies were selected using increasingly stringent phage-displayed VHH elution conditions: the antigen and analyte concentrations were decreased each round

and the free analyte was used for competitive elution. In this case, a homologous hapten was used for both immunizing and panning. The VHH antibody performed comparably to the previously described rabbit pAb for the same target [33]. The resulting detection limit was in the ppb level and cross-reactivity profiles are similar for both antibodies. The most sensitive VHH assay obtained ($IC_{50}$ = 1.4 ng/mL) utilized a heterogeneous hapten, although the homologous hapten assay was also very sensitive ($IC_{50}$ = 2.7 ng/mL). It is interesting to note that the VHH assay sensitivity improved ~100-fold when the VHH was isolated (i.e., not used while expressed on the phage particle). While not discussed in detail, the VHH assay was performed in a buffer containing 40% dimethyl sulfoxide (DMSO), indicating the ability of reagents to function in the presence of high concentrations of organic solvents. To test the thermostability of the VHH compared to the pAb, both were assessed for activity after being heated to 95 °C. The VHH retained more than 25% of its activity after being heated for 60 min, while the pAb retained none. As a demonstration that VHH can be incorporated into formats where conventional IgGs have been used, the VHH was incorporated into two biosensor formats. The VHH was coated on the surface of an electrode for electrochemical impedance detection [32] and coated onto a PDMS membrane for a lab-on-a-chip sensor [34]. In the latter, the VHH was directly labeled with horseradish peroxidase to reduce the need for additional reagents used for detection, which also provides evidence that the VHH reagent did not lose activity/functionality after being chemically labeled.

**Tetrabromobisphenol-A** (TBBPA) leads sales of BFRs in the world. TBBPA and its glycidyl ether are used primarily as a reactive flame retardants covalently incorporated into epoxy and polycarbonate resins often used for electronic insulation. TBBPA is released into the environment manufacturing facilities, and when incorporated as the unreacted material, TBBPA can conceivably leach from products either during use or through means of disposing of the products. Immunoassays have been developed to detect TBBPA in soil, as well as human samples. The selection of VHH antibodies [35] was conducted using knowledge gained from prior work in isolating highly sensitive pAbs [36] and mAbs [37] to the same target. In addition, this work utilized the repertoire of previously synthesized haptens to systematically pan the phage-displayed VHH library against the homologous hapten and two heterologous haptens. Regardless of the screening hapten used for phage panning, the phage were selected and eluted by using decreasing concentrations of screening hapten and free analyte, respectively, with each successive round. The most sensitive assay identified ($IC_{50}$ = 0.4 ng/mL) resulted from panning with a heterologous hapten which varied from the immunizing hapten by having two fewer carbons in the linking arm. Due to the hydrophobicity of TBBPA, both methanol and DMSO, from 5–40%, were evaluated for their impact on sensitivity. While sensitivity was best at 10% methanol, the VHH assay was viable in all tested solvent concentrations. Additionally, pH was evaluated ranging from 4.0–11.0. The assay performed with minimal deviation between pH 7.4–11.0. These pH and solvent matrix results highlight the increased tolerance of VHH proteins to maintain function (and presumably structure) in diverse assay matrix conditions. Nonetheless, when matrix extracts from soil and fetal bovine serum were tested, they required a 100-fold and 10-fold dilution, respectively, to eliminate interference, although little explanation of how the assay was altered was provided. It is also interesting to note that a comparison was made

showing that the VHH competitive ELISA was 1000-fold more sensitive than the alpaca pAb serum assay, thus suggesting that the preliminary screening of serum may not be indicative of resulting assay performance. The VHH, as well as the pAb and mAbs, to TBBPA are highly selective for TBBPA. All antibody types exhibit negligible cross-reactivity to other compounds tested. The VHH was subjected to heating to 90 °C for 10 and 90 min, and it retained 80% and 20% of its activity, respectively. To take advantage of being able to genetically modify a protein, this VHH was genetically fused to alkaline phosphatase to serve as the reporting label [38]. Because the binding protein was directly linked to the reporting protein, the number of incubating and washing steps in the VHH-AP assay were reduced, thus speeding up the analysis time. The fusion of AP to VHH did not impact assay sensitivity or selectivity, although an unexpected finding was that the VHH-AP fusion was functional after 70 days at ambient temperature, demonstrating an advantage of the inherent stability of VHH.

The **aflatoxins** are a group of naturally occurring mycotoxins, produced mainly by *Aspergillus flavus* and *Aspergillus paraciticus*. They can occur in a wide range of products, including grains, food and feedstuff. More than 20 types of aflatoxins have been identified, among which, four aflatoxins ($B_1$, $B_2$, $G_1$ and $G_2$) occur naturally. **Aflatoxin $B_1$ ($AFB_1$)** is the most toxic, and one of the most potent carcinogens in nature. In this study [39], the VHH antibodies were selected using gradually decreasing concentrations of $AFB_1$–BSA conjugate and free $AFB_1$ and more stringent washing. Two unique clones, named Nb26 and Nb28, were selected. Both VHHs showed higher resistance to temperature and solvents than the mAb. The mAb gradually lost binding activity with increased temperatures, while the VHHs could bind to the antigen after being incubated at 95 °C for 5 min. The VHHs Nb26 and Nb28 retained about 70% and 40% of binding activity, respectively after incubation for 1 h at 85 °C. However, the binding activity of the mAb was lost after 15 min of incubation at 85 °C. Apart from temperature stability, the VHH also showed higher tolerance to methanol, acetone and acetonitrile compared with the mAb. The authors state that the temperature stability of the VHH may also be explained by their ability to properly refold after unfolding. The recovery of $AFB_1$ from spiked samples of rice, corn, peanut and feedstuff was from 80%-115%, using the VHH Nb26-based ELISA. Interestingly, the authors report that using 2% BSA in the 70% methanol in phosphate buffered saline extraction buffer aids in eliminating matrix effects, likely by providing a stabilizing effect to the antibody protein.

## Advantages of VHHs

### Large quantity production of VHHs

Once the phage-displayed VHH is selected and isolated, soluble unbound VHHs are expressed in a bacterial culture. In culture, the bacteria cells replicate on the order of every 20 min. A typical or expected yield of a 1-L culture allowed to grow for 15 h (a typical overnight expression time), is ~10 mg of protein [40]. However, the product yield is very variable and thus numerous studies of the relationship between the expression yield and expression systems, chaperones and presence of key amino acid residues in VHH framework have appeared [41]. When producing a conventional mAb from a mouse cell line, the animal must first be sacrificed to obtain the spleen cells needed for fusion with a myeloma cell line.

Once a cell line has been selected and isolated to monoclonality, there are two commonly acceptable methods to acquire antibody protein: large culture growth with media replacement and ascites production. Mammalian cells double daily, and the expected output of a 1-L culture is ~10mg, but usually requires a few days of growth [42]. Using the ascites method, a typical cell line will take ~5–7 days and yield 3–10 mL of fluid containing 10 mg/mL of protein. Unfortunately, ascites is the least humane of all of these methods and is often only used when deemed necessary. In addition, careful mammalian cell culture techniques must be employed (e.g., sterile fume hood, sterile growth chamber, use of antibiotics if necessary) to ensure no contamination, since bacterial or yeast contamination will grow faster than and kill the growth of the desired cells. On the other hand, a bacterial culture often relies on antibiotic selection and will often out-compete other bacteria that may contaminate the culture.

Ultimately, larger yields of purified immunoreagents are highly desirable as a source material for standardized regulatory tests. Even more reagent is needed when VHH are used in affinity chromatography systems. Incrementally more VHH will be needed for use in food processing. This is illustrated by their use to remove caffeine from a sample [20]. The need increases yet again for therapeutic applications. Some uses likely will involve injection into the blood stream or peritoneal cavity. Other applications may require genetic modification of the VHH to allow it to penetrate membranes to neutralize toxins. Other clinical applications could involve hemo- or peritoneal dialysis, which has been performed with conventional antibodies to paraquat (MW=257), a highly toxic herbicide [43, 44]. In the case of VHH, their lower cost and much smaller size likely will result in higher density and a far more effective reagent.

In addition, the storage of source material and the transfer of reagents among researchers are critical for the advancement of science and ensuring commercial viability of these materials. One great advantage to using VHHs may be the fact that since the genetic sequence is known and recorded, this information can be transferred between researchers and used in artificial gene synthesis. In addition, VHH source material can be stored in three physical forms: plasmid, bacteriophage, or bacterial (e.g., transformed Top 10F') cells.

## Genetic modifications

**Improve characteristics—**Ideally VHHs produced from immunized animals will have all the desired physical and antigen binding properties. Following rigorous panning and enrichment the VHH would be used as produced. In reality this is unlikely, and it is where genetic engineering may come to the aid. Although in vitro maturation approaches have been successfully used to improve stability or affinity of VHHs [45–48], little literature is available on genetic manipulations of VHHs against small molecules. Although unintentionally, in one example Yau et al. [16], selected two anti-picloram clones with three point mutations in the nucleotide sequence which most probably were introduced by intrinsic random mutagenesis during PCR steps. These mutants were selected and amplified probably because they were superior in terms of analyte binding over parent clones, but this theory was not tested by back mutation and comparison of mutated and parent VHH affinities. Authors speculated that “ribosome display technology can compensate for the

limited diversity of a VHH naive library and provide an unlimited source of affinity-matured immunoactive reagents in vitro". Another example comes from the same group, where five selected anti-auxin VHH clones were shuffled by staggered extension process (StEP). Although none of the shuffled clones had better affinity toward auxin than the best original clone, the affinity toward auxinic herbicides was significantly decreased [17]. Recently an interesting study investigating the structure – binding relationship of an anti-methotrexate (anti-MTX) VHH antibody was published by Fanning and Horn [49, 23]. CDRs 1–3 from known anti-methotrexate VHH were grafted onto an anti-RNase A VHH framework. The resulting grafted VHH had 250 times lower affinity than parental VHH. Introduction of five amino acid residues (76–80) of the anti-MTX VHH into the grafted antibody resulted in 1000-fold increase in affinity. Although this part of FR3 was not considered a hypervariable loop, it is well positioned for productive interaction with antigen as in the above example. Participation of this region in antigen binding was recently recognized and resulted in attribution of the name 'CDR4' [23]. These examples strongly suggest that for in vitro maturation techniques to be successful they should take into account the presence and participation of the CDR4 loop in antigen binding.

**Labelling applications—**An advantage of using a VHH protein is the ability to use both genetic and chemical engineering tools. Modifications could involve labeling with enzymes, fluorophores, or nanoparticles. Direct labeling of the antibody reagent often permits reducing the number of steps in the procedure. Chemical modification requires having a relatively pure protein solution. Either the protein or the label can be activated and that allows for covalent attachment. One advantage of using chemical labeling is the ability to label more than one molecule of label per target protein. In the case of colorimetric assays, increased labeling can increase the turnover of the substrate and for fluorometric assays the intensity of the signal may be increased. However, there is also the potential for the label to disrupt the function of the antibody protein thus interfering with the ability of the antibody to bind to the target. One example of a chemically labeled VHH was shown for use in a lab-on-a-chip device using the enzyme horseradish peroxidase [34]. While the ratio was not optimized in this work, it appears that a ratio of 4:1 (VHH:enzyme) worked sufficiently to achieve results comparable to using a labeled secondary reagent. An integration of chemical coupling techniques and genetic modification is illustrated by the ease with which amino acids with desired binding properties could be engineered into the VHH at a single or multiple positions. For example, the codon for tyrosine could be inserted to allow diazo linkages to form at specific locations. With the current advances in recombinant technology, there are many labels available that can be spliced in frame with the VHH nanobody gene. The genetic splicing may change the expression yield, and there is the possibility that the conjugation may inhibit the proper folding of either the label or the protein. These are common problems in expression technology that are easily solved. Already there are two examples where alkaline phosphatase (AP) has been genetically linked to a VHH [28, 38]. The resulting assays exhibited comparable to slightly improved sensitivities, while also reducing the number of steps in the analytical procedure. In addition, the use of AP allows for either a colorimetric or fluorometric product for detection, and Liu and colleagues demonstrated a 10-fold improvement in $IC_{50}$ using the fluorometric analysis [28]. Wang and colleagues also report that the fusion construct of VHH-AP is more susceptible to extreme

heat (90 °C), but the reagent retained excellent stability at ambient temperature for at least 70 days [38]. Chemical fluorophores that require chemical conjugation techniques are very different than those can be genetically fused, such as green fluorescent protein. For instance, many of the chemical fluorophore dyes have been vastly improved to resist quenching, have high quantum yield and Stokes' shift, and there are a wide range of possible conjugation methods. To date, no small molecule VHH has been labeled with a chemical fluorophore or fused to a protein fluorophore. Another future prospect is to utilize the Avi-Tag system for incorporating a biotin moiety. The Avi-Tag system has been used with VHHs to larger protein targets [50], and more recently the first demonstration for the analysis of a small molecule, methotrexate, by immunoaffinity chromatography [51]. Thus, there are a variety of molecular tags that open the door to coupling multiple affinity and detection reagents.

**Multivalent reagents to improve affinity and avidity—**It was discussed earlier that the small molecular size and single polypeptide chain of VHHs is one advantage over mAbs and other antibody fragments in terms of genetic manipulation, expression and handling. However, if bio-recognition reagents of higher molecular weight with better avidity are required VHHs can be easily expressed as multivalent reagents. Several publications have demonstrated dramatic increases in functional affinity for immobilized antigen. For example, Zhang et al. [52] reported a simple approach to produce multivalent VHHs where a sdAb is fused to the B-subunit of *Escherichia coli* verotoxin, which self-assembles to form a homopentamer and results in simultaneous sdAb polymerization giving pentabodies. Zhang et al. obtained an avidity gain of three to four orders of magnitude for pentabodies to parathyroid hormone [53]. They also mentioned that from their unpublished results pentabodies generally have observed affinities that are 1000 to 10,000-fold higher than their monomeric counterparts determined by SPR analysis [52]. Riazi et al. [54] reported a 400-fold increase in functional affinity of penta-VHHs to *Campylobacter jejuni*, a gram negative bacterium, compared to the nanobody monomer. To date, only one reference could be found on application of pentabodies for detection of small molecules. Doyle et al. [25] compared VHHs and corresponding pentabodies against 15-acetyl-deoxynivalenol in a fluorescence polarization assay. They did not observe a dramatic difference in affinities, being only 2 times different. Despite consistent assay inhibition in a direct competitive ELISA, authors were not able to precisely define the respective $IC_{50.}$ Therefore, exploring application of multivalent VHH constructs for detection of small molecules and is an interesting avenue for research.

Another interesting approach to the construction of multivalent reagents is to use multiunit proteins in conjugation with VHHs, for instance streptavidin (SC), avidin, or the recently reported rhizavidin (RZ) [55]. Unlike avidin or streptavidin, rhizavidin forms a homodimer instead of a tetramer. Authors reported that a VHH-rhizavidin bioconjugate was expressed at 20 times better yield than its streptavidin core counterparts. In addition, the VHH-RZ fusion produced a homogeneous product, while some amount of aggregated material was observed from the VHH-SC. As a plus, fusing the two proteins had no impact on their individual thermal stability. Compared to unmodified VHH, both fusions were more sensitive at detecting their corresponding analyte, but VHH-RZ appeared to have a superior dynamic range. Authors suggested that it could be related to its small size and that the fusion did not

saturate the microsphere surface as quickly as the largest SC construct. These examples demonstrate an interesting avenue for exploring multivalent VHH constructs for detection of small molecules and potential methods for improving existing assays.

## Resilience to organic solvents

It is common to use an organic solvent to extract lipophilic chemicals from environmental samples and with water miscible solvents it is often time and cost effective to add the solvent directly to the immunoassay. Also many small molecule targets are highly lipophilic, and some level of water miscible solvent can reduce binding to plastic and glass, artifacts from micelles and a host of other problems. The addition of co-solvents can also change the relative selectivity of an immunoassay. Conversely, the existence of solvent in the analytical buffer usually causes a matrix effect due to the adverse solvent effect on the conformation of the antibody. Although the matrix effect could be eliminated by dilution, this results in a higher limit of quantitation. Because VHH, like pAb and mAb, are each unique reagents that vary unpredictably, a low technology approach is to screen a series of increasing concentrations of all common co-solvents early in assay development. For reactive red dye 6, both the VHH and mAb were unaffected by concentrations of ethanol up to 50% [10]. However, many VHHs have demonstrated superior tolerance to frequently used solvents compared to conventional antibodies. In the case of 3-PBA, the VHH alone, as well as the phage-displayed VHH and pAbs were tested with a range of concentrations of methanol (MeOH) and DMSO. Both the maximal signal and the sensitivity were improved for the VHH alone and the phage-displayed VHH based-ELISA with a concentration of MeOH up to 50%, but this was not observed for the pAb-based ELISA. Similar results were observed with DMSO. The VHHs for aflatoxin described by He et al. [39] were evaluated in the presence of MeOH, DMSO, dimethylformamide (DMF), acetone and acetonitrile. The data indicated that the VHHs and mAbs were remarkably resistant to acetone. The VHH did not lose any binding ability at 80% methanol, whereas the mAb lost 50% of its activity. However, both VHHs and mAbs were adversely affected by increasing concentrations of DMSO, acetonitrile and DMF. Though not discussed in detail, the anti-BDE47 VHH worked well in the buffer containing 40% DMSO [32], and the anti-TBBPA VHH exhibited the best sensitivity with 10% MeOH [35]. There is no doubt that the ability of a VHH to perform in the presence of organic solvents is certainly favorable for detecting lipophilic analytes from real samples, which often requires solvent extraction. Another advantage in the production of VHHs is the ability to include solvents in initial panning procedures and thus select at the outset for tolerance to denaturation.

## Resilience to temperature

VHHs have been reported to be more stable than traditional pAbs, mAbs and engineered antibody fragments such as single chain fragments (scFv) and antigen biding fragments (Fab). The reason may be attributed to the small size and unique structure of VHH. To provide a sufficiently large antigen interacting surface although lacking the variable light chain, the CDR loops of VHH tend to be longer than in the VH domain of a conventional antibody. In camelid VHHs, it was originally found that the long loops were constrained with a disulfide bond to form the antigen binding site [56, 57], which was considered to contribute strongly to the stability and thermal resistance of VHHs because the removal of

this disulfide bond by site-directed mutagenesis resulted in a significant decrease in melting point and prevented refolding [58, 59]. Other researchers took a different approach and introduced a non-canonical disulfide bond into the hydrophobic core of llama VHHs between framework region 2 (FR2) and FR3, which proved to not only increase thermal stability at neutral pH, but also imparted resistance to proteolytic degradation and increased antibody stability at low pH [60, 61]. A recent thorough study of the relationship between number of disulfide bonds and the heat resistance of VHH concluded that while $T_m$ increased with the increase in number of disulfide bonds the half-life of VHH at 90 °C ($t_{1/2}^{90\ C}$) decreased [62]. The researchers also showed that the thermal loss of activity of VHH is chemical in nature and proceeds through disulfide exchange and peptide cleavage near Cys and Asn amino acid residues. Moreover they demonstrated that the replacement of Cys by appropriate amino acids will improve the heat resistance of native VHH.

For small molecules, van der Linden et al. first mentioned that even at temperatures as high as 90°C, the VHH still retained binding activity to the antigen whereas mAbs did not [10]. Most of studies focusing on VHHs for small chemicals have investigated their thermostability by testing the binding ability to antigen or $T_m$ value. These studies revealed that the VHHs have enhanced thermal stability as compared to the corresponding conventional mAbs or pAbs (Table 4). The VHH against caffeine that was reported by Ladenson et al. [18] presented far greater stability than the murine mAbs. Their VHH retained more than 90% of its activity after incubation at temperatures up to 90 °C for 20 min. By contrast, all of the binding activity of the commercial mAb for caffeine was lost after incubation at 70°C and higher. However, another VHH selective for caffeine generated by Franco et al. [20] showed weaker temperature-tolerance, because the mid-point of the unfolding transition ($T_m$) was only 56 °C. Tabares-da Rosa et al. [29] obtained five different anti-TCC VHHs. All of these VHHs were more stable than rabbit pAbs at 85 °C and 100°C. Although one VHH lost most of its reactivity at 85 °C, the other VHHs in this study retained high binding ability after 1 h incubation at 85 °C and a portion of reactivity even after 1 h at 100 °C. For the anti-BDE47 VHH, the results of thermostability indicated that the VHHs retained more than 50% of their activity after heating at 95 °C for 10 min compared to the pAbs which dropped below 6% of activity rapidly [32]. Similarly, the thermostability of VHHs for aflatoxin was better than conventional antibodies. After incubation for 1 h at 85 °C, the anti-aflatoxin VHHs still had up to 70% of binding activity, yet the binding activity of the mAb was lost after 15 min of incubation [39]. All of these results suggest that VHHs are a superior reagent compared to conventional antibodies for temperature sensitive applications, such as on-site environmental testing at elevated ambient temperatures or testing of hot samples. We anticipate that this will translate into more robust assays and longer shelf life for kits.

## Utility of VHHs in biosensor formats

As a new immunological reagent, VHHs are commonly being compared to conventional antibodies in terms of form, function and utility. One area of exploration is the utilization of VHHs in all of the same formats (e.g., plate-based assays, lateral-flow devices, electrochemical biosensors, etc.) where conventional antibodies have been used with high success. By demonstrating that formatting is not a barrier for use, VHHs will be more

rapidly accepted and used. One commonly employed biosensor technology is SPR. SPR is considered a label-free technology, wherein the signal is proportional to the change in light refraction due to binding. Because nanobodies are smaller molecular weight biorecognition molecules, the difference in size between the small molecule to be detected and the larger sensing molecule is less and thus a more distinct signal may be observed. SPR techniques were used in the study by Doyle and colleagues [25] to evaluate the binding kinetics of a VHH to the mycotoxin 15-acetyldeoxynivalenol (15-AcDON) in both a monomeric and pentameric form. VHHs against methotrexate [22], picloram [16], and auxin [17] were also evaluated by SPR technology. SPR was used to evaluate binding of VHHs against TCC towards both the coating antigen, as well as the free analyte [29]. It is reasonably anticipated that SPR will be a suitable platform for integrating VHHs for environmental monitoring. An electrochemical impedance biosensor is another label-free technology for measuring bimolecular interactions. Such a biosensor was used to evaluate the performance of both a polyclonal antibody [63] and a VHH [32] to BDE-47 and identified comparable detection limits of 1.3 ng/mL and 0.79 ng/L, respectively. Lateral flow devices are useful chemical monitoring tools relying on the movement of biorecognition molecules through a membrane support. A lateral flow device to detect caffeine has been developed and patented, which incorporates VHHs as the biorecognition molecule [19]. These avenues of biosensor research are areas of ongoing work, but so far the ability to demonstrate binding to and detection from these formats is very promising.

## Pitfalls and Limitations

### Functionally useful VHH were not obtained: Reflections

Studies that are published are often the ones that work and are noteworthy. However, recognizing that not everything works, and therefore goes unpublished, is a small reason why we felt this review would be very valuable. In our own work, hapten design is a critical aspect of assay development. Probably the greatest single reason for failure to obtain a good assay is a poor choice of a good hapten for panning and the failure to have an adequate library of competing ligands or ‘coating antigens’ for assay development. Yet, we have immunized alpacas with a few environmental targets that have not generated VHHs suitable for environmental monitoring (i.e., analyte sensitivity at environmentally relevant concentrations). One likely reason for this is the small sample size of animals immunized. When only one target is immunized into one animal and that animal fails to respond, there are no resulting antibodies to isolate. Another issue we have come across is the failure to induce the heavy chain only IgG2 or IgG3 subclass antibodies. In one example, we immunized an alpaca with a bisphenol-A immunogen conjugate that was previously used successfully to generate pAbs in rabbits (Ting Xu, China Agricultural University, Beijing, China personal communication). When the alpaca IgG1, IgG2 and IgG3 fractions were isolated, all appeared to bind to the screening antigen. However, only the binding by the IgG1 fraction could be inhibited by free bisphenol-A analyte (Figure 3). As a result, we recommend that even if screening of whole sera suggests proceeding with library production, an antibody purification should be performed to ensure that all subclasses are responding to some degree [64]. As shown in the successful experiments for generating VHH to TCC [29] and TBBPA [35], the isolated VHHs had improved sensitivity compared

to the whole sera. Another potential reason that VHH may not be successfully isolated from a responding immunized animal is that genes encoding for any of the responding IgG2 or IgG3 antibodies are not amplified (e.g., RT-PCR conditions) or isolated. This was the situation for the Goldman group, wherein responding HcAbs were isolated to 2,4,6-trinitrotoluene and used to develop immunoassays [65], but no suitable VHHs were successfully developed (personal communication). Primer bias may be a reason. Nonetheless, only a small set of primers (<10) are required for construction of diverse VHH libraries, whereas construction of mouse or human scFv libraries requires "universal primer sets" containing over a hundred different oligonucleotides [16]. Another consideration, that can occur with conventional antibodies as well, is the possibility of eliciting VHHs with a strong binding affinity for the coating antigen, but with weak or no recognition for the free analyte. These are just a few situations that have been encountered thus far and have elicited further work into understanding how to selectively induce HcAbs and ensure isolation of those genes and their corresponding VHH antibodies.

## Biohazard considerations

Development of VHHs implies use of biohazardous materials such as bacteriophages, plasmids, recombinant DNA, antibiotics, etc. These materials require careful manipulations and adequate waste disposal dedicated for hazardous materials. Biohazardous liquid waste should be decontaminated by treating with an appropriate disinfectant. Bleach added to liquid waste at 10% final concentration for 30 min is the generally preferred method. Biohazardous solid waste is autoclaved prior to routine solid waste disposal. With adequate training, dedicated equipment, and personal protective equipment, the likelihood of exposure to personnel is minimal. Good techniques are important for personal safety. In addition, phages are produced in massive numbers and lack of adequate containment can result in serious cross contamination and confusing results.

## Animal husbandry considerations

As with rodents, rabbits, sheep and goats knowledge of animal husbandry specific to the species as well as the personality of the individual animal is important. A drawback of VHH technology is the management of large animals (Figure 4). While rabbits, rats and mice may be manipulated and housed in most laboratories under supervision of trained technicians, alpacas, llamas and other animals of the camelid family need special facilities for housing and a veterinarian for performing immunizations, collecting bleeds and monitoring general animal well-being. Contrary to small animals, camelids are not sacrificed at the end of the immunization round, thereby requiring their permanent housing that may not be suitable for all laboratories. Another difference to using small animals is that we routinely immunize alpacas with 2–3 immunogens at the same time. Other investigators report immunizing llamas with cocktails of 1–5 immunogens and reusing the animal after waiting at least 6 months [66]. With the increased use of camelids as pack and companion animals as well as livestock in some locations, numerous animals are available for immunization. As an alternative, commercial services are available for camelid immunization and care. cDNA and phage libraries resulting from immunization of both camelids and sharks can now be ordered.

## Concluding remarks

VHHs have many favorable properties compared to conventional and other recombinant antibody technologies, including small size, high solubility, good stability in extreme conditions (heat, pH, chaotropic media) and ability to be manipulated genetically. The assays exploiting VHHs have shown superior or comparable analytical characteristics. However, with few exceptions, current reports lack information about how well VHHs perform to detect small molecules in environmental and biological samples containing incurred residues. The possible significant advantage that VHHs could bring to the field of immunoanalysis is the possibility of their use with samples containing a high amount of matrix or high concentration of organic solvents, following sample preparation for instance.

VHH are comprised solely of the binding region with loss of the constant domain. Thus, many of the reagents designed to detect pAb or mAb conventional assays (such as goat anti-rabbit-HRP) do not work. However, the ease of manipulation of VHH facilitates adding tags, labels, and scaffolds, which can be used for detection, as well as, for purification. Scaffolds other than classical antibody regions are being developed.

To take advantage of the exquisite *in vivo* antibody affinity maturation process, animals are often immunized prior to antibody isolation. Nonetheless, non-immunized VHH libraries as well as libraries based on non-antibody scaffolds have been used successfully to isolate VHHs selective for large protein molecules. As presented in this review, this strategy has worked for two low molecular weight environmental targets (e.g., picloram and auxin).

Crystallography is a powerful tool to elucidate and study protein structure. Only a few VHH have been crystallized. The fact that the VHH usually are easy to crystallize and their small size makes solving structure relatively simple, suggest many VHH structures will soon appear. Multidimensional nuclear magnetic resonance (NMR) technology has become a robust and almost routine technique and will allow to study VHH structure, dynamics and analyte binding on the atomic level in solution [67, 68]. These emerging structures will help identify possible mechanisms and patterns of how VHHs are binding to their target, what amino acids are responsible for selective binding, and thus what amino acids can be altered in site-directed mutagenesis experiments. Knowing the structure of the binding pocket will help to tune VHHs properties in ex vivo maturation. In turn, VHHs by themselves are tools of growing interest as applied for crystallization of other proteins [66]. Acting as crystallization chaperones, VHHs help to stabilize desired conformation of highly dynamic membrane proteins, or bind conformational epitopes of functional proteins, for example.

In addition to the already noted excellent properties of VHHs, they demonstrate lower immunogenicity, better pharmacokinetics, and better ability to pass through the renal filter and blood - brain barrier and faster tissue penetration than classical antibodies [69]. For these reasons, VHHs have great potential as therapeutic agents. Indeed, conventional antibodies have been routinely used in clinical applications, for venom detoxification and sequestration in particular. However, undesirable side effects are often reported when anti-venom plasma or antibody from different species is used. In this regard, VHHs may provide an advantageous alternative for such clinical applications. A discussion of the clinical utility

of VHH is beyond the scope of this article, but many of the advantages VHH show in analytical chemistry over conventional antibodies such as small size and an ability to pan for the desired properties apply therapeutically as well. A basic difference in concept is that with classical antibodies one starts with a large protein, humanizes it and adds or removes functions that are not needed. With VHH one starts with a small binding unit and desired functions can be added. Probably the therapeutic use of VHH has been slowed by the uncertain intellectual property situation and major industries being in an intellectual well with mAb technology. This will soon change and stimulate technological advancement in the entire field. With small molecules the same hapten and panning strategies to obtain VHH for analytical chemistry can be used to obtain VHH for therapeutic applications, particularly with regard to sequestration or removal of toxins and other small molecules.

## Acknowledgments

This research was supported in part by the National Institute of Environmental Health Sciences Superfund Basic Research Program, P42 ES04699, the National Institute of Occupational Safety and Health Western Center for Agricultural Health and Safety 2U54 OH007550, and the CounterACT Program, National Institutes of Health Office of the Director, and the National Institute of Neurological Disorders and Stroke, Grant Number U54 NS079202. CB was supported by the UC Davis Environmental Health Sciences Core Center P30 ES023513. ZLX was supported by the China Scholarship Council Project 201508440069.

## References

1. Vincke C, Muyldermans S. Introduction to heavy chain antibodies and derived Nanobodies. Methods Mol Biol. 2012; 911:15–26. [PubMed: 22886243]
2. Muyldermans S. Nanobodies: natural single-domain antibodies. Ann Rev Biochem. 2013; 82:775–797. [PubMed: 23495938]
3. Roovers RC, van Dongen GA, van Bergen en Henegouwen PM. Nanobodies in therapeutic applications. Curr Opin Molec Therap. 2007; 9(4):327–335. [PubMed: 17694445]
4. Smolarek D, Bertrand O, Czerwinski M. Variable fragments of heavy chain antibodies (VHHs): a new magic bullet molecule of medicine? Postepy Hig Med Dosw (Online). 2012; 66:348–358. [PubMed: 22706121]
5. Wesolowski J, Alzogaray V, Reyelt J, Unger M, Juarez K, Urrutia M, et al. Single domain antibodies: promising experimental and therapeutic tools in infection and immunity. Med Microbiol Immunol. 2009; 198(3):157–174. [PubMed: 19529959]
6. Darsana R, Chandrasehar G, Deepa V, Gowthami Y, Chitrikha T, Ayyappan S, et al. Acute toxicity assessment of Reactive Red 120 to certain aquatic organisms. Bull Env Contam Toxicol. 2015; 95(5):582–587. [PubMed: 26350898]
7. Nejib A, Joeele D, Abdellah E, Amane J, Malika T-A. Textile dye adsorption onto raw clay: influence of clay surface properties and dyeing additives. J Colloid Sci Biotechnol. 2014; 3:98–110.
8. Sharma S, Bhunia H, Bajpai P. $TiO_2$-Assisted photocatalytic degradation of diazo dye reactive red 210: decolorization kinetics and mineralization investigations. J Adv Oxid Technol. 2013; 16:306–313.
9. Klepacz-Smolka A, Sojka-Ledakowicz J, Ledakowicz S. Biological treatment of post-nanofiltration concentrate of real textile wastewater. Fibres Text East Eur. 2015; 23:138–143.
10. van der Linden RH, Frenken LG, de Geus B, Harmsen MM, Ruuls RC, Stok W, et al. Comparison of physical chemical properties of llama VHH antibody fragments and mouse monoclonal antibodies. Biochim Biophys Acta. 1999; 1431(1):37–46. [PubMed: 10209277]
11. Frenken LG, van der Linden RH, Hermans PW, Bos JW, Ruuls RC, de Geus B, et al. Isolation of antigen specific llama VHH antibody fragments and their high level secretion by Saccharomyces cerevisiae. J Biotechnol. 2000; 78(1):11–21. [PubMed: 10702907]

12. Spinelli S, Frenken LG, Hermans P, Verrips T, Brown K, Tegoni M, et al. Camelid heavy-chain variable domains provide efficient combining sites to haptens. Biochemistry. 2000; 39(6):1217–1222. [PubMed: 10684599]
13. Spinelli S, Tegoni M, Frenken L, van Vliet C, Cambillau C. Lateral recognition of a dye hapten by a llama VHH domain. J Molec Biol. 2001; 311(1):123–129. [PubMed: 11469862]
14. Dumoulin M, Conrath K, Van Meirhaeghe A, Meersman F, Heremans K, Frenken LG, et al. Single-domain antibody fragments with high conformational stability. Protein Sci. 2002; 11(3):500–515. [PubMed: 11847273]
15. van der Linden RH, de Geus B, Frenken GJ, Peters H, Verrips CT. Improved production and function of llama heavy chain antibody fragments by molecular evolution. J Biotechnol. 2000; 80(3):261–270. [PubMed: 10949316]
16. Yau KY, Groves MA, Li S, Sheedy C, Lee H, Tanha J, et al. Selection of hapten-specific single-domain antibodies from a non-immunized llama ribosome display library. J Immunol Methods. 2003; 281(1–2):161–175. [PubMed: 14580890]
17. Sheedy C, Yau KY, Hirama T, MacKenzie CR, Hall JC. Selection, characterization, and CDR shuffling of naive llama single-domain antibodies selected against auxin and their cross-reactivity with auxinic herbicides from four chemical families. J Agric Food Chem. 2006; 54(10):3668–3678. [PubMed: 19127743]
18. Ladenson RC, Crimmins DL, Landt Y, Ladenson JH. Isolation and characterization of a thermally stable recombinant anti-caffeine heavy-chain antibody fragment. Anal Chem. 2006; 78(13):4501–4508. [PubMed: 16808459]
19. Ladenson, J.; Ladenson, R.; Landt, Y.; Crimmins, D., inventors. Washington University, assignee. Methods for determining and lowering caffeine concentration in fluids. USA patent US. 7,615,218 B2. 2009.
20. Franco EJ, Sonneson GJ, DeLegge TJ, Hofstetter H, Horn JR, Hofstetter O. Production and characterization of a genetically engineered anti-caffeine camelid antibody and its use in immunoaffinity chromatography. J Chromatog B. 2010; 878(2):177–186.
21. Zhao SS, Bukar N, Toulouse JL, Pelechacz D, Robitaille R, Pelletier JN, et al. Miniature multi-channel SPR instrument for methotrexate monitoring in clinical samples. Biosens Bioelectron. 2015; 64:664–670. [PubMed: 25441416]
22. Alvarez-Rueda N, Behar G, Ferre V, Pugniere M, Roquet F, Gastinel L, et al. Generation of llama single-domain antibodies against methotrexate, a prototypical hapten. Mol Immunol. 2007; 44(7): 1680–1690. [PubMed: 17011035]
23. Fanning SW, Horn JR. An anti-hapten camelid antibody reveals a cryptic binding site with significant energetic contributions from a nonhypervariable loop. Protein Sci. 2011; 20(7):1196–1207. [PubMed: 21557375]
24. Doyle PJ, Saeed H, Hermans A, Gleddie SC, Hussack G, Arbabi-Ghahroudi M, et al. Intracellular expression of a single domain antibody reduces cytotoxicity of 15-acetyldeoxynivalenol in yeast. J Biol Chem. 2009; 284(50):35029–35039. [PubMed: 19783651]
25. Doyle PJ, Arbabi-Ghahroudi M, Gaudette N, Furzer G, Savard ME, Gleddie S, et al. Cloning, expression, and characterization of a single-domain antibody fragment with affinity for 15-acetyl-deoxynivalenol. Mol Immunol. 2008; 45(14):3703–3713. [PubMed: 18632156]
26. van Houwelingen A, De Saeger S, Rusanova T, Waalwijk C, Beekwilder J. Generation of recombinant alpaca VHH antibody fragments for the detection of the mycotoxin ochratoxin A. World Mycotoxin J. 2008; 1:407–417.
27. Liu X, Xu Y, Xiong YH, Tu Z, Li YP, He ZY, et al. VHH phage-based competitive real-time immuno-polymerase chain reaction for ultrasensitive detection of ochratoxin A in cereal. Anal Chem. 2014; 86(15):7471–7477. [PubMed: 24992514]
28. Liu X, Xu Y, Wan DB, Xiong YH, He ZY, Wang XX, et al. Development of a nanobody-alkaline phosphatase fusion protein and its application in a highly sensitive direct competitive fluorescence enzyme immunoassay for detection of ochratoxin A in cereal. Anal Chem. 2015; 87(2):1387–1394. [PubMed: 25531426]
29. Tabares-da Rosa S, Rossotti M, Carleiza C, Carrion F, Pritsch O, Ahn KC, et al. Competitive selection from single domain antibody libraries allows isolation of high-affinity antihapten

antibodies that are not favored in the llama immune response. Anal Chem. 2011; 83(18):7213–7220. [PubMed: 21827167]

30. Ahn KC, Kasagami T, Tsai HJ, Schebb NH, Ogunyoku T, Gee SJ, et al. An immunoassay to evaluate human/environmental exposure to the antimicrobial triclocarban. Environ Sci Technol. 2012; 46(1):374–381. [PubMed: 22077920]
31. Kim HJ, McCoy MR, Majkova Z, Dechant JE, Gee SJ, Tabares-da Rosa S, et al. Isolation of alpaca anti-hapten heavy chain single domain antibodies for development of sensitive immunoassay. Anal Chem. 2012; 84(2):1165–1171. [PubMed: 22148739]
32. Bever CR, Majkova Z, Radhakrishnan R, Suni I, McCoy M, Wang Y, et al. Development and utilization of camelid VHH antibodies from alpaca for 2,2',4,4'-tetrabrominated diphenyl ether detection. Anal Chem. 2014; 86(15):7875–7882. [PubMed: 25005746]
33. Ahn KC, Gee SJ, Tsai HJ, Bennett D, Nishioka MG, Blum A, et al. Immunoassay for monitoring environmental and human exposure to the polybrominated diphenyl ether BDE-47. Environ Sci Technol. 2009; 43(20):7784–7790. [PubMed: 19921894]
34. Chen A, Wang R, Bever CR, Xing S, Hammock BD, Pan T. Smartphone-interfaced lab-on-a-chip devices for field-deployable enzyme-linked immunosorbent assay. Biomicrofluidics. 2014; 8(6): 064101. [PubMed: 25553178]
35. Wang J, Bever CR, Majkova Z, Dechant JE, Yang J, Gee SJ, et al. Heterologous antigen selection of camelid heavy chain single domain antibodies against tetrabromobisphenol A. Anal Chem. 2014; 86(16):8296–8302. [PubMed: 25068372]
36. Xu T, Wang J, Liu SZ, Lu C, Shelver WL, Li QX, et al. A highly sensitive and selective immunoassay for the detection of tetrabromobisphenol A in soil and sediment. Anal Chim Acta. 2012; 751:119–127. [PubMed: 23084060]
37. Xu C, Ou J, Cui Y, Wang L, Lv C, Liu K, et al. Development of a monoclonal antibody-based enzyme-linked immunosorbent assay for tetrabromobisphenol A. Monoclon Antib Immunodiagn Immunother. 2013; 32(2):113–118. [PubMed: 23607346]
38. Wang J, Majkova Z, Bever CR, Yang J, Gee SJ, Li J, et al. One-step immunoassay for tetrabromobisphenol A using a camelid single domain antibody-alkaline phosphatase fusion protein. Anal Chem. 2015; 87(9):4741–4748. [PubMed: 25849972]
39. He T, Wang Y, Li P, Zhang Q, Lei J, Zhang Z, et al. Nanobody-based enzyme immunoassay for aflatoxin in agro-products with high tolerance to cosolvent methanol. Anal Chem. 2014; 86(17): 8873–8880. [PubMed: 25079057]
40. Barbas, CF., III; Burton, DR.; Scott, JK.; Silverman, GJ. Phage Display: A Laboratory Manual. Cold Spring Harbor New York: Cold Spring Harbor Laboratory Press; 2001.
41. Gorlani A, Hulsik DL, Adams H, Vriend G, Hermans P, Verrips T. Antibody engineering reveals the important role of J segments in the production efficiency of llama single-domain antibodies in Saccharomyces cerevisiae. Prot Eng Design Selec. 2012; 25(1):39–46.
42. Harlow, E.; Lane, D. Antibodies: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Labororatory Press; 1988.
43. Azhari R, Margel S, Labes A, Haviv Y. Specific removal of paraquat by hemoperfusion through antiparaquat conjugated agarose-polyacrolein microsphere beads. J Biomed Mat Res. 1987; 21(1): 25–41.
44. Nagao M. Production and toxicological application of anti-paraquat antibodies. Jap J Leg Med. 1989; 43(2):134–147.
45. Doshi R, Chen BR, Vibat CR, Huang N, Lee CW, Chang G. In vitro nanobody discovery for integral membrane protein targets. Sci Reports. 2014; 4:6760.
46. Strokappe N, Szynol A, Aasa-Chapman M, Gorlani A, Forsman Quigley A, Hulsik DL, et al. Llama antibody fragments recognizing various epitopes of the CD4bs neutralize a broad range of HIV-1 subtypes A, B and C. Plos One. 2012; 7(3):e33298. [PubMed: 22438910]
47. Yau KY, Dubuc G, Li S, Hirama T, Mackenzie CR, Jermutus L, et al. Affinity maturation of a V(H)H by mutational hotspot randomization. J Immunol Methods. 2005; 297(1–2):213–224. [PubMed: 15777944]

48. Zielonka S, Weber N, Becker S, Doerner A, Christmann A, Christmann C, et al. Shark Attack: high affinity binding proteins derived from shark vNAR domains by stepwise in vitro affinity maturation. J Biotechnol. 2014; 191:236–245. [PubMed: 24862193]
49. Bond CJ, Wiesmann C, Marsters JC Jr, Sidhu SS. A structure-based database of antibody variable domain diversity. J Mol Biol. 2005; 348(3):699–709. [PubMed: 15826665]
50. Rossotti MA, Pirez M, Gonzalez-Techera A, Cui Y, Bever CS, Lee KS, et al. Method for sorting and pairwise selection of nanobodies for the development of highly sensitive sandwich immunoassays. Anal Chem. 2015; 87(23):11907–11914. [PubMed: 26544909]
51. Davenport KR, Smith CA, Hofstetter H, Horn JR, Hofstetter O. Site-directed immobilization of a genetically engineered anti-methotrexate antibody via an enzymatically introduced biotin label significantly increases the binding capacity of immunoaffinity columns. J Chromatog B. 2016
52. Zhang JB, Li QG, Nguyen TD, Tremblay TL, Stone E, To R, et al. A pentavalent single-domain antibody approach to tumor antigen discovery and the development of novel proteomics reagents. J Molec Biol. 2004; 341(1):161–169. [PubMed: 15312770]
53. Zhang JB, Tanha J, Hirama T, Khieu NH, To R, Hong TS, et al. Pentamerization of single-domain antibodies from phage libraries: A novel strategy for the rapid generation of high-avidity antibody reagents. J Molec Biol. 2004; 335(1):49–56. [PubMed: 14659739]
54. Riazi A, Strong PCR, Coleman R, Chen WX, Hirama T, van Faassen H, et al. Pentavalent single-domain antibodies reduce *Campylobacter jejuni* motility and colonization in chickens. Plos One. 2013; 8(12):e83928. [PubMed: 24391847]
55. Liu JL, Zabetakis D, Walper SA, Goldman ER, Anderson GP. Bioconjugates of rhizavidin with single domain antibodies as bifunctional immunoreagents. J Immunol Methods. 2014; 411:37–42. [PubMed: 24946086]
56. Govaert J, Pellis M, Deschacht N, Vincke C, Conrath K, Muyldermans S, et al. Dual beneficial effect of interloop disulfide bond for single domain antibody fragments. J Biol Chem. 2012; 287(3):1970–1979. [PubMed: 22128183]
57. Spinelli S, Frenken L, Bourgeois D, de Ron L, Bos W, Verrips T, et al. The crystal structure of a llama heavy chain variable domain. Nat Struc Biol. 1996; 3(9):752–757.
58. Saerens D, Conrath K, Govaert J, Muyldermans S. Disulfide bond introduction for general stabilization of immunoglobulin heavy-chain variable domains. J Molec Biol. 2008; 377(2):478–488. [PubMed: 18262543]
59. Turner KB, Zabetakis D, Goldman ER, Anderson GP. Enhanced stabilization of a stable single domain antibody for SEB toxin by random mutagenesis and stringent selection. Pro Eng Design Selec. 2014; 27(3):89–95.
60. Hussack G, Hirama T, Ding W, Mackenzie R, Tanha J. Engineered single-domain antibodies with high protease resistance and thermal stability. Plos One. 2011; 6(11):e28218. [PubMed: 22140551]
61. Zabetakis D, Olson MA, Anderson GP, Legler PM, Goldman ER. Evaluation of disulfide bond position to enhance the thermal stability of a highly stable single domain antibody. Plos One. 2014; 9(12):e115405. [PubMed: 25526640]
62. Akazawa-Ogawa Y, Uegaki K, Hagihara Y. The role of intra-domain disulfide bonds in heat-induced irreversible denaturation of camelid single domain VHH antibodies. J Biochem. 2016; 159(1):111–121. [PubMed: 26289739]
63. Radhakrishnan R, Suni II, Bever CS, Hammock BD. Impedance Biosensors: Applications to Sustainability and Remaining Technical Challenges. ACS Sustainable Chemistry & Engineering. 2014; 2(7):1649–1655. [PubMed: 25068095]
64. Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hamers C, Songa EB, et al. Naturally occurring antibodies devoid of light chains. Nature. 1993; 363(6428):446–448. [PubMed: 8502296]
65. Anderson GP, Goldman ER. TNT detection using llama antibodies and a two-step competitive fluid array immunoassay. J Immunol Methods. 2008; 339(1):47–54. [PubMed: 18755196]
66. Pardon E, Laeremans T, Triest S, Rasmussen SG, Wohlkonig A, Ruf A, et al. A general protocol for the generation of Nanobodies for structural biology. Nat Prot. 2014; 9(3):674–693.
67. Leung IKH. NMR spectroscopy - a simple yet powerful tool in chemical biology. Chem New Zealand. 2015; 79:42–50.

68. Renisio JG, Perez J, Czisch M, Guenneugues M, Bornet O, Frenken L, et al. Solution structure and backbone dynamics of an antigen-free heavy chain variable domain (VHH) from Llama. Proteins. 2002; 47(4):546–555. [PubMed: 12001233]
69. Rahbarizadeh F, Ahmadvand D, Sharifzadeh Z. Nanobody; an old concept and new vehicle for immunotargeting. Immunol Invest. 2011; 40(3):299–338. [PubMed: 21244216]
70. van der Linden R, de Geus B, Stok W, Bos W, van Wassenaar D, Verrips T, et al. Induction of immune responses and molecular cloning of the heavy chain antibody repertoire of Lama glama. J Immunol Methods. 2000; 240(1–2):185–195. [PubMed: 10854612]
71. Deschamps RJA, Hall JC, Mcdermott MR. Polyclonal and monoclonal enzyme immunoassays for picloram detection in water, soil, plants, and urine. J Agric Food Chem. 1990; 38(9):1881–1886.
72. Weiler EW, Jourdan PS, Conrad W. Use of immunoassay in plant-science. 21. Levels of indole-3-acetic-acid in intact and decapitated coleoptiles as determined by a specific and highly sensitive solid-phase enzyme-immunoassay. Planta. 1981; 153(6):561–571. [PubMed: 24275876]
73. Mertens R, Eberle J, Arnscheidt A, Ledebur A, Weiler EW. Monoclonal-antibodies to plant-growth regulators. 2. Indole-3-acetic-acid. Planta. 1985; 166(3):389–393. [PubMed: 24241522]
74. Fickling SA, Hampton SM, Teale D, Middleton BA, Marks V. Development of an enzyme-linked-immunosorbent-assay for caffeine. J Immunol Methods. 1990; 129(2):159–164. [PubMed: 2351834]
75. Carvalho JJ, Weller MG, Panne U, Schneider RJ. A highly sensitive caffeine immunoassay based on a monoclonal antibody. Anal Bioanal Chem. 2010; 396(7):2617–2628. [PubMed: 20155491]
76. Hendel J, Sarek LJ, Hvidberg EF. Rapid radioimmunoassay for methotrexate in biological-fluids. Clin Chem. 1976; 22(6):813–816. [PubMed: 945138]
77. Kato Y, Paterson A, Langone JJ. Monoclonal-antibodies to the chemotherapeutic agent methotrexate - Production, properties and comparison with polyclonal antibodies. J Immunol Methods. 1984; 67(2):321–336. [PubMed: 6707474]
78. Usleber E, Schneider E, Martlbauer E, Terplan G. 2 Formats of enzyme-immunoassay for 15-acetyldeoxynivalenol applied to wheat. J Agric Food Chem. 1993; 41(11):2019–2023.
79. Sinha RC, Savard ME, Lau R. Production of monoclonal-antibodies for the specific detection of deoxynivalenol and 15-acetyldeoxynivalenol by ELISA. J Agric Food Chem. 1995; 43(6):1740–1744.
80. Thirumala-Devi K, Mayo MA, Reddy G, Reddy SV, Delfosse P, Reddy DVR. Production of polyclonal antibodies against ochratoxin A and its detection in chilies by ELISA. J Agric Food Chem. 2000; 48(10):5079–5082. [PubMed: 11052781]
81. Shan G, Huang H, Stoutamire DW, Gee SJ, Leng G, Hammock BD. A sensitive class specific immunoassay for the detection of pyrethroid metabolites in human urine. Chem Res Toxicol. 2004; 17(2):218–225. [PubMed: 14967009]
82. Liu Y, Wu AH, Hu J, Lin MM, Wen MT, Zhang X, et al. Detection of 3-phenoxybenzoic acid in river water with a colloidal gold-based lateral flow immunoassay. Anal Biochem. 2015; 483:7–11. [PubMed: 25957127]
83. Wang J, Li H, Shelver WL, Wang ZH, Li QX, Li J, et al. Development of a monoclonal antibody-based, congener-specific and solvent-tolerable direct enzyme-linked immunosorbent assay for the detection of 2,2',4,4'-tetrabromodiphenyl ether in environmental samples. Anal Bioanal Chem. 2011; 401(7):2249–2258. [PubMed: 21822776]
84. Reddy SV, Mayi DK, Reddy MU, Thirumala-Devi K, Reddy DV. Aflatoxins B1 in different grades of chillies (*Capsicum annum* L.) in India as determined by indirect competitive-ELISA. Food Addit Contam. 2001; 18(6):553–558. [PubMed: 11407753]
85. Li PW, Zhang Q, Zhang W. Immunoassays for aflatoxins. Trends Analyt Chem. 2009; 28(9):1115–1126.

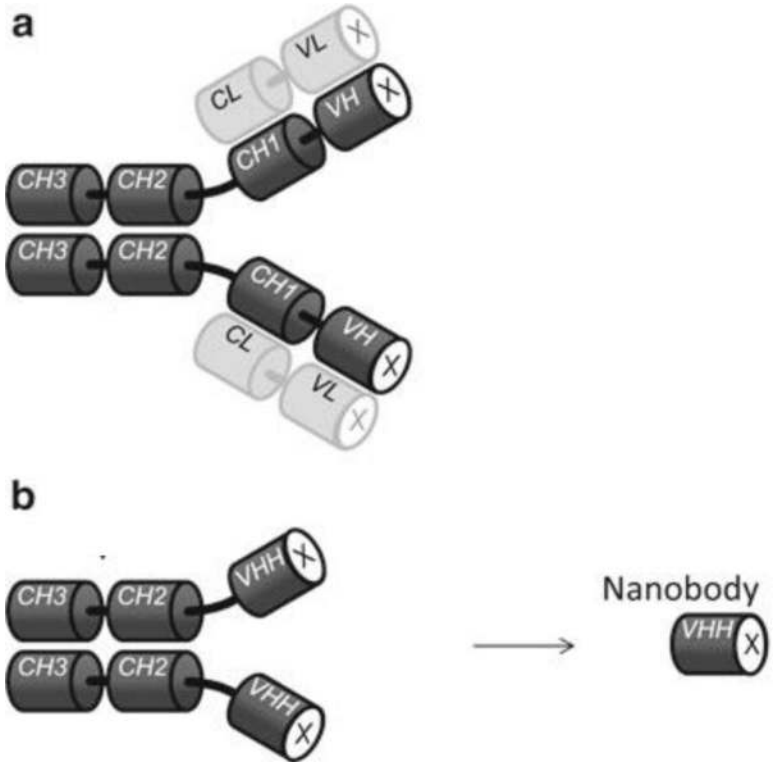



**Figure 1.**
Schematic representation of (a) an antibody (IgG) and (b) a camelid heavy chain antibody from which the VHH (nanobody) is derived. Adapted from Vincke and Muyldermans [1].

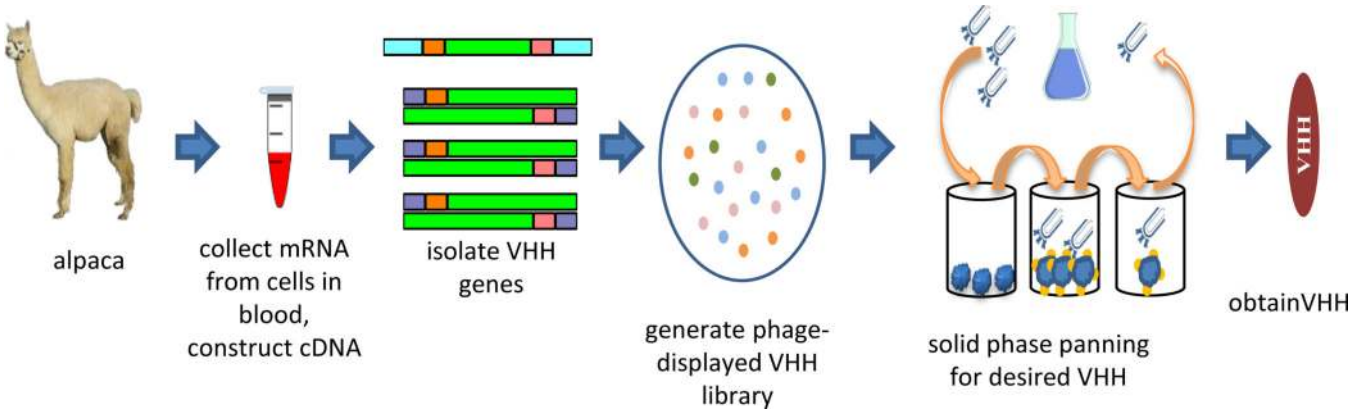



**Figure 2.**

Overview of the process to isolate VHH from camelids. Briefly, mRNA is collected from the animal and converted into cDNA by RT-PCR. The cDNA is amplified and digested in order to isolate the VHH genes that are incorporated into plasmids and expressed by bacteriophage, creating a library. The library is panned for desired VHH.

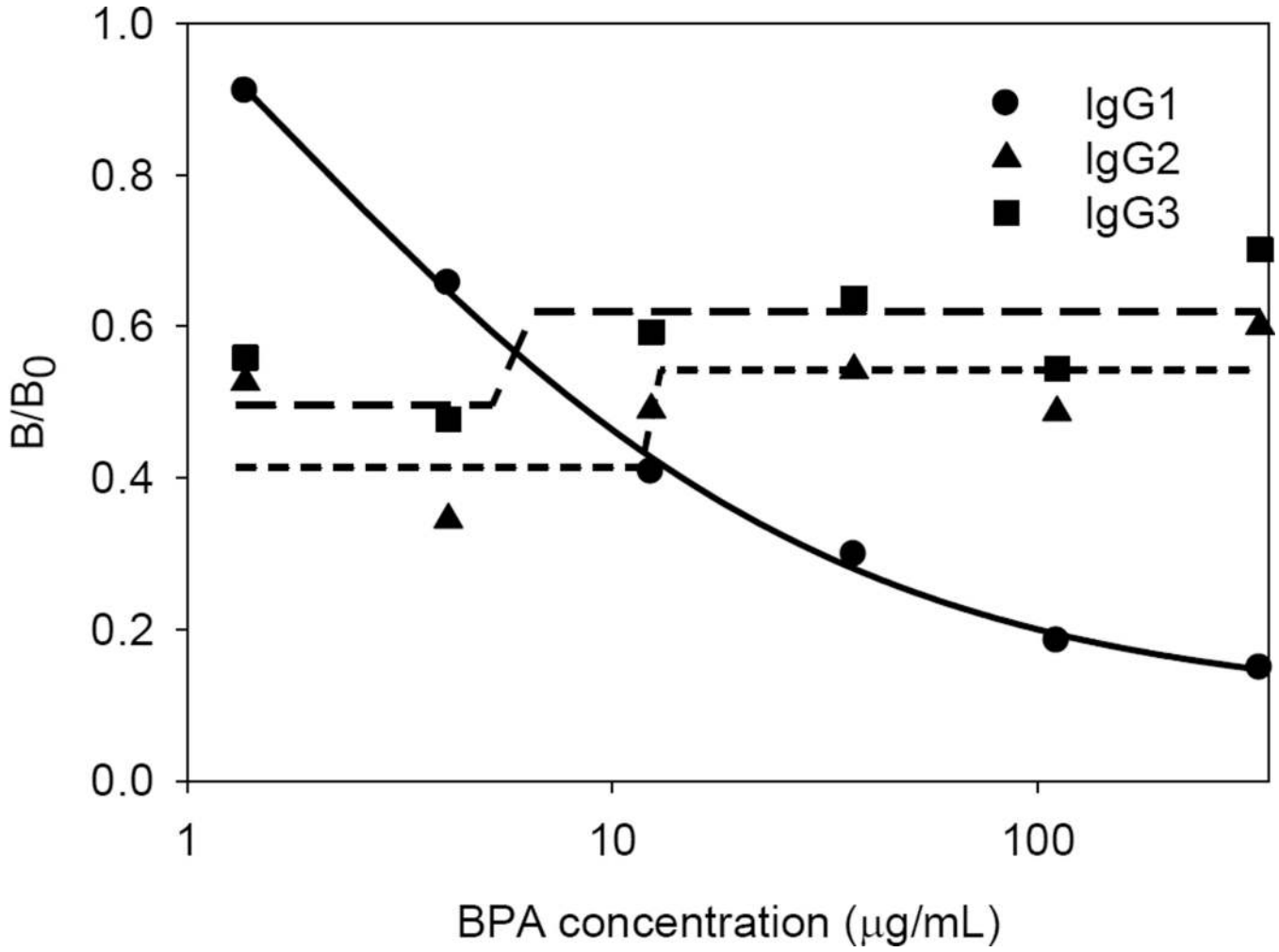



**Figure 3.**
Competitive inhibition profiles of subclass antibody fractions (IgG1, IgG2, and IgG3) from the same alpaca to bisphenol A.

**Figure 4.**
Alpaca housed at UC Davis.

**Table 1**

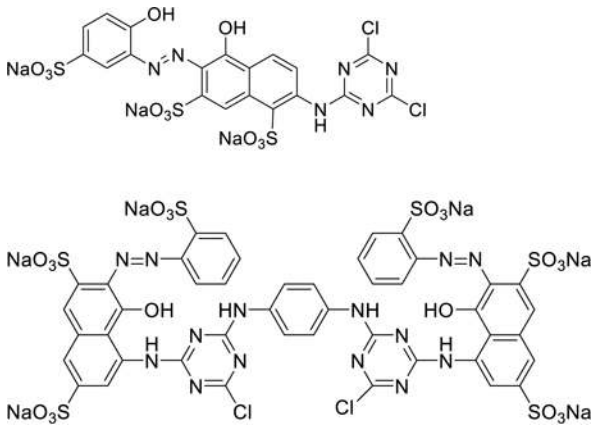
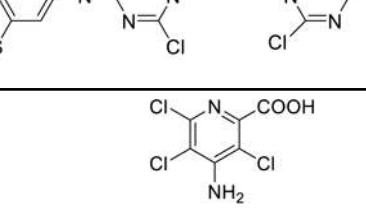
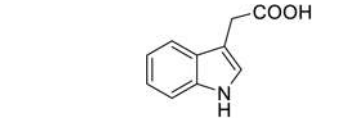
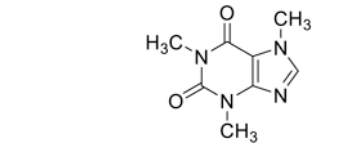
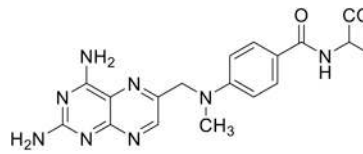
Comparison of characteristics associated with each immunoreagent technology.

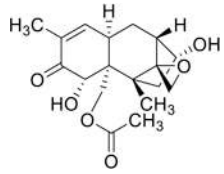
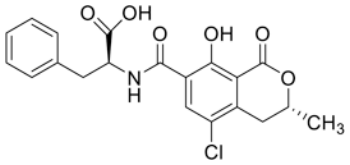
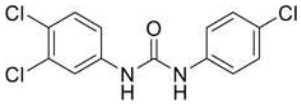
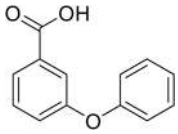
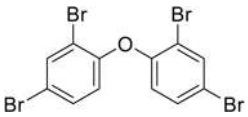
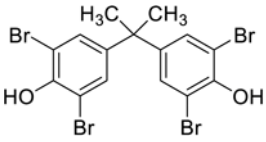
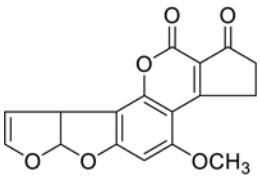
| Characteristic | VHH | mAb | pAb | | scFv |
|---|---|---|---|---|---|
| **Typical animal species used** | alpacas, llamas, camels | mouse | mouse, rabbit | goat | mouse |
| **Size of housing needed** | large | small | Small | large | small |
| **Animal sacrifice** | no | yes | usually, for largest blood collection | not common | yes |
| **Utilizes affinity maturation** | yes | yes | yes | | yes |
| **Biohazards** | some | minimal to none | minimal to none | | some |
| **Time needed for screening/isolating clones** | days | weeks | not applicable | | days |
| **Production yield** | unlimited | unlimited | finite | | unlimited |
| **Genetic manipulation** | amenable | not amenable | not amenable | | amenable |
| **Scaffolding** | amenable | not amenable | not amenable | | amenable |
| **Chemical labeling** | amenable | amenable | amenable, but requires purification | | amenable |
| **Temperature stability** | high | low | low | | low |
| **Biosensor formats** | amenable | amenable | amenable, but often requires additional purification | | amenable |

*Anal Bioanal Chem*. Author manuscript; available in PMC 2017 September 01.

## Table 2

VHH to small molecular weight compounds to date and comparison of sensitivities to polyclonal and monoclonal antibodies.

| Structure | Analyte (MW) | Assay sensitivity, $IC_{50}$ Linear range, $IC_{20–80}$ LOD, $IC_{10}$ | | |
|---|---|---|---|---|
| | | Nanobody | pAb | mAb |
|  | Azo dyes [11, 70] (RR6 733.38; RR120 1469.98) | ~18–85 nM ($K_d$) | no | no |
|  | Picloram (241.46) | 193 μg/mL [16] | 140 ng/mL [71]<br>10–300 ng/mL<br>5 ng/mL | 10 ng/mL [71]<br>5–50 ng/mL<br>1 ng/mL |
|  | Auxin (indole-3-acetic acid) (175.19) | 140 μg/mL * (pentabody for IAA)<br>>43 μg/mL * (herbicides)<br>0.5–2 mM, herbicides ($K_d$)<br>20 μM IAA ($K_d$) | 1 pmol/assay [72]<br>0.1–10<br>0.08 | 5 pmol/assay [73]<br>0.8–10<br>0.5 |
|  | Caffeine (194.19) | ~25 μg/mL [18]<br>10–50 μg/mL<br>4 μg/mL (homologous) | 200 ng/mL [74]<br>116–2000 ng/mL<br>2 ng/mL (direct) | 0.2 ng/mL [75]<br>0.025–1 ng/mL<br>0.001 ng/mL (direct) |
|  | Methotrexate (454.44) | 4.5 μg/mL [22]<br>1.1–13.6 μg/mL<br>0.9 μg/mL * | (RIA)<br>100 ng/mL [76]<br>10–1000 ng/mL<br>1 ng/mL | (RIA)<br>10 ng/mL [77]<br>5–80 ng/mL<br>2 ng/mL |

| Structure | Analyte (MW) | Assay sensitivity, $IC_{50}$ Linear range, $IC_{20–80}$ LOD, $IC_{10}$ | | |
|---|---|---|---|---|
| | | **Nanobody** | **pAb** | **mAb** |
|  | 15-Acetyldeoxynivalenol (338.35) | 419 ng/mL (monomer) [25]<br>169 ng/mL (pentamer)<br>10–1000 ng/mL | 1.9 ng/mL [78]<br>0.35–20 ng/mL<br>0.1 ng/mL (direct) | 1 μg/mL [79]<br>0.05–20 ng/mL<br>0.05 ng/mL (direct) |
|  | Ochratoxin A (403.813) | 12 ng/mL [26]<br>8–20 ng/mL<br>6 ng/mL<br>Flow-through membrane-based assay | 5 ng/mL [80]<br>1–50 ng/mL<br>5 ng/mL | 1.2 ng/mL<br>0.3–5 ng/mL<br>0.12 ng/mL |
|  | Triclocarban (315.58) | 3.5 ng/mL [29]<br>1–11 ng/mL<br>0.3 ng/mL (homologous) | 0.69 ng/mL [30]<br>0.1–36 ng/mL<br>0.03 ng/mL | |
|  | 3-Phenoxybenzoic acid (214.22) | 1.4 ng/mL [31]<br>0.4–8 ng/mL<br>0.1 ng/mL (homologous) | 1.6 ng/mL [81]<br>0.5–5 ng/mL<br>0.1 ng/mL | 0.6 ng/mL [82]<br>0.2–2.0 ng/mL<br>0.1 ng/mL |
|  | BDE-47 (485.79) | 1.4 ng/mL [32]<br>0.5–10 ng/mL<br>0.1 ng/mL | 1.75 ng/mL [33]<br>0.35–8.5 ng/mL<br>0.2 ng/mL | 22 ng/mL or in direct ELISA:<br>1.4 ng/mL [83]<br>0.3–6.5 ng/mL<br>0.1 ng/mL |
|  | TBBPA (543.9) | 0.4 ng/mL [35]<br>0.06–2.53 ng/mL<br>0.02 ng/mL | 0.87 ng/mL [36]<br>0.2–6 ng/mL<br>0.05 ng/mL | 3.87 ng/mL [37]<br>0.8–20.7 ng/mL<br>0.3 ng/mL |
|  | Aflatoxin $B_1$ (312.27) | 0.754 ng/mL [39]<br>0.117–5.676 ng/mL<br>0.05 ng/mL | 2 ng/mL [84]<br>0.4–11 ng/mL<br>0.3 ng/mL | 0.001–6 ng/mL [85]<br>6–400 ng/mL<br>0.001 ng/mL |

Abbreviations used: MW, molecular weight; $IC_{50}$, $IC_{20–80}$, $IC_{10}$ concentrations resulting in 50, 20–80 or 10% decrease in maximum signal; pAb, polyclonal antibody; mAb, monoclonal antibody; Kd, dissociation constant; IAA, indole-3-acetic acid; RIA, radioimmunoassay; IgG2, immunoglobulin G fraction 2; IgG3, immunoglobulin G fraction 3; SPR, surface plasmon resonance; ELISA, enzyme linked immunosorbent assay; TBBPA, tetrabromobisphenol A,

*units converted to ng/mL from initial publication.

**Table 3**

Characterization of VHH against small molecular weight compounds

| Analyte (MW) | Immunogen-protein (animal) | Expression system (Yield %) | Elution conditions | Temperature stability (range) | Formats and uses | Comments |
|---|---|---|---|---|---|---|
| Azo dyes [11, 70]<br>RR6<br>(733.38)<br>RR120<br>(1469.98) | RR6-BSA<br>(llama)<br>RR120-BSA | *E coli*<br>Yeast | no panning, screened supernatants of randomly picked E coli colonies | 4 °C to 90 °C | Crystallography<br>Resonant mirror biosensor ($K_d$ 18 – 83 nM) | Stable in up to 4M ammonium thiocyanate or 50% ethanol. DNA shuffling used to alter affinity, specificity and yield in yeast |
| Picloram [16]<br>(241.46) | None<br>(llama) | *E.coli*<br>TG1<br>(free VHH and VHH-Phage) | EB20 [50 mM Tris–acetate (pH 7.5), 150 mM NaCl, 20 mM EDTA, 50 μg/mL Saccharomyces cerevisiae RNA] | | SPR analysis, phage ELISA | Failed in phage-system, used ribosomal display. Best clones contained mutations introduced during PCR steps. |
| Auxin [17]<br>(indole-3-acetic acid)<br>(175.19) | None<br>(llama) | *E.coli*<br>TG1<br>(~1 mg/L) | 0.1 M triethylamine | | SPR analysis, CDR shuffling, pentamerization | SPR with immobilized pentamerized sdAbs allow direct observation of analyte-Ab interaction ($K_d$). |
| Caffeine [18]<br>(194.19) | caffeine carboxylate-KLH<br>(llama and alpaca) | *E. coli* | 0.1 M triethylamine | >90% recovery after 90°C | Patented lateral flow devices, immunoaffinity chromatographic separation and detection | Application to real samples: commercial beverages |
| Methotrexate [22]<br>(454.44) | MTX-BIP<br>(llama) | *E. coli*<br>(2 – 20 mg/L) | 0.1 M triethylamine pH 11.0 | Not tested | SPR ($K_d$ 29 – 515 nM; $K_d$ competitive assay, 80 nM) | Utilized CDR grafting to explore mechanism of binding.<br>VHH crystallized |
| 15-Acetyldeoxynivalenol [25]<br>(338.35) | 15-DON-BSA<br>(llama) | *E. coli*<br>(3–6 μg/L) | triethylamine | Not tested | fluorescence polarization, direct ELISA, SPR | Used nested PCR, made monomer/pentamer |
| Ochratoxin A [26]<br>(403.813) | OTA-KLH<br>(llama) | *E.coli*<br>BL21(DE3)plysS | panning 2× against OTA-OVA, 1× against OTA-KLH | Not demonstrated | fused to AP | Application to real cereal samples |
| Triclocarban [29]<br>(315.58) | TCC-Thyr<br>(llama) | *E.coli*<br>BL21(DE3) | eluted with target analyte, decreasing concentrations | some inactivated at 85 °C; others remained | none | VHH that had additional disulfide bonds did not perform the best in |

| Analyte (MW) | Immunogen-protein (animal) | Expression system (Yield %) | Elution conditions | Temperature stability (range) | Formats and uses | Comments |
|---|---|---|---|---|---|---|
| | | | | active after 1h at 100 °C | | thermal stability study. |
| 3-Phenoxybenzoic acid [31] (214.22) | 3-PBA-Thyr (alpaca) | *E.coli* TOP10F' | eluted with target analyte, decreasing concentrations | Not demonstrated | none | Spike-recovery validation |
| 2,2',4,4'-Tetrabromodiphenyl ether [32] (485.79) | BDE-9-KLH (BDE-C1) (alpaca) | *E.coli* TOP10F' | eluted with target analyte, decreasing concentrations | 95 °C for 10, retained >50% of its activity; 95 °C for 60, retailed >25% of its activity | biosensors, electrochemical and chip[34] | |
| Tetrabromobisphenol A [35] (543.9) | T5-Thy (alpaca) | *E.coli* TOP10F' | eluted with target analyte, decreasing concentrations | 90 °C heat for 10 mins 80% activity; 90 °C heat for 90 mins 20% activity | fused to AP, improved stability[38] | |
| Aflatoxin $B_1$ [39] (312.27) | $AFB_1$-BSA (alpaca) | *E.coli* TOP10F' | panned with $AFB_1$-BSA, eluted with target analyte, decreasing concentrations | Nb26 clone: ≈ 70% activity, Nb28 clone: ≈ 70% activity at 85 °C for 1h | | Spike-recovery validation |

Abbreviations used: BSA, bovine serum albumin; Pic, picloram hapten; CDR, complementarity determining region; OVA, ovalbumin; KLH, keyhole limpet hemocyanin; MTX, methotrexate hapten; DON, deoxynivalenol hapten; TNB, trinitrobenzene hapten; OTA, ochratoxin hapten; TCC, triclocarban hapten; Thyr, thyroglobulin; AP, alkaline phosphatase; 3-PBA; 3-phenoxybenzoic acid hapten; BDE-9, brominated diphenyl ether hapten 9; $AFB_1$, aflatoxin $B_1$ hapten; BIP, blue carrier immunogenic protein.

*Anal Bioanal Chem*. Author manuscript; available in PMC 2017 September 01.

**Table 4**

Summary of cysteine residues and locations within VHH gene sequences.

| Target analyte | Total # of cysteines | Location of cysteines | VHH more stable than IgG? |
|---|---|---|---|
| RR6 dye [14, 10] | 2 | FR1, FR3 | Yes |
| Picloram [16] | 2 | FR1, FR3 | Not tested |
| Auxin [17] | 2 | FR1, FR3 | Not tested |
| Caffeine [18] | 2 | FR1, FR3 | Yes |
| Methotrexate [22] | 2 | FR1, FR3 | Not tested |
| 15-AcDON [25] | 2 | FR1, FR3 | Not tested |
| Ochratoxin A [28] | 2 | FR1, FR3 | Yes |
| TCC [29] | 2 | FR1, FR3 | Yes |
| | 4 | FR1, FR2, FR3,CDR3 | Yes |
| 3-PBA [31] | 4 | FR1, FR2, FR3, CDR3 | Not tested |
| BDE-47 [32] | 2 | FR1, FR3 | Yes |
| TBBPA [35] | 2 | FR1, FR3 | Not tested |
| Aflatoxin $B_1$ [39] | 2 | FR1, FR3 | Yes |