

VHH antibodies: emerging reagents for the analysis of environmental chemicals

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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 immunodiagnosics, 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 detect small environmental chemicals (MW < 1500 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 of 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 the VHH fragment (Fig. 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 Fig. 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 immunodiagnosics, VHH technology will be faster, cheaper, and better than earlier procedures. Perhaps 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, which have developed VHHs to small molecules (MW < 1500 Da) wherein the target is most likely relevant for environmental monitoring. We identify the

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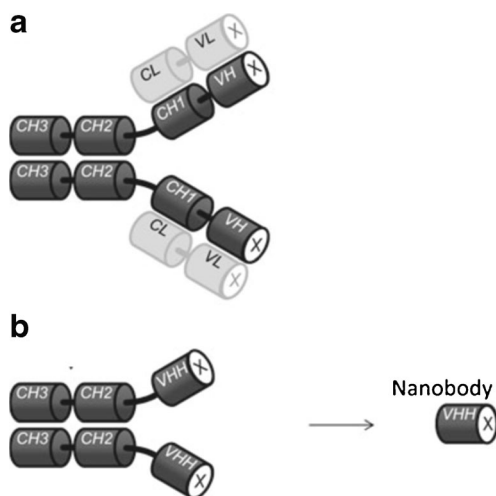


Fig. 1 Schematic representation of (a) an antibody (IgG), and (b) a camelid heavy chain antibody from which the VHH (nanobody) is derived. Adapted from Vincke and Muyldermans [1]

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 with 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 (single-chain variable fragments, scFvs) 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 VHHs 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 pAb or 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 [40]. Wastewater

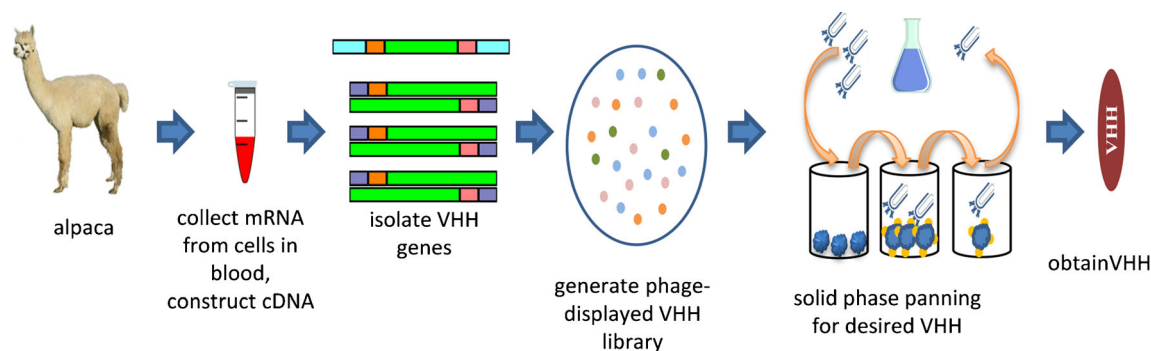


Fig. 2 Overview of the process to isolate VHH from camelids. Briefly, mRNA is collected from the animal and converted into cDNA by RT-PCR. The cDNA is amplified and digested in order to isolate the VHH

genes that are incorporated into plasmids and expressed by bacteriophage, creating a library. The library is panned for desired VHH

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

treatment processes to degrade these chemicals such as filtration [41], UV light [42], or bioremediation are under development [43]. The body of work describing the VHH derived from llamas against the azo dyes RR-6 and RR-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 triaziny 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 selective antigen binders. Instead, *E. coli* or yeast were infected and allowed to grow. At least 200 random colonies were selected and 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 [44]. In addition, their work was the first to describe a high yield of VHH from a yeast expression system where yield of 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 [6]. VHH had superior temperature stability and could bind antigen well at elevated temperature and higher concentrations of the chaotrope ammonium thiocyanate compared with mAbs. Both mAbs and VHHs showed no change in binding in the presence of up to 50 % ethanol [44]. Further examination of the crystal structure of a VHH revealed that although VHH lack the hydrophobic cleft typically formed by variable light (VL) and variable heavy (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 [45, 46]. 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 [44, 47]. To improve production, affinity, and temperature stability, DNA shuffling was also used with success [48].

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 d. Picloram is mainly degraded by microbes that are present in soil or water. In the paper [8], the authors hypothesize “that VHHs specific for small molecules (i.e., M.W. < 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 5.4×10^8), 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 μ M as determined by surface plasmon resonance (SPR) analysis.

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 [6, 7] (RR6 733.38; RR120 1469.98)	~18-85 nM (K _d)	no	no
	Picloram (241.46)	193 µg/mL [8]	140 ng/mL [9] 10-300 ng/mL 5 ng/mL	10 ng/mL [9] 5-50 ng/mL 1 ng/mL
	Auxin (indole-3-acetic acid) (175.19)	140 µg/mL (pentabody for IAA) >43 µg/mL (herbicides) 0.5-2 mM, herbicides (K _d) 20 µM IAA (K _d)	1 pmol/assay [10] 0.1-10 0.08	5 pmol/assay [11] 0.8-10 0.5
	Caffeine (194.19)	~25 µg/mL [12] 10-50 µg/mL 4 µg/mL (homologous)	200 ng/mL [13] 116-2000 ng/mL 2 ng/mL (direct)	0.2 ng/mL [14] 0.025-1 ng/mL 0.001 ng/mL (direct)
	Methotrexate (454.44)	4.5 µg/mL [15] 1.1-13.6 µg/mL 0.9 µg/mL	(RIA) 100 ng/mL [16] 10-1000 ng/mL 1 ng/mL	(RIA) 10 ng/mL [17] 5-80 ng/mL 2 ng/mL
	15-Acetyldeoxynivalenol (338.35)	419 ng/mL (monomer) [18] 169 ng/mL (pentamer) 10-1000 ng/mL	1.9 ng/mL [19] 0.35-20 ng/mL 0.1 ng/mL (direct)	1 µg/mL [20] 0.05-20 ng/mL 0.05 ng/mL (direct)
	Ochratoxin A (403.813)	12 ng/mL [21] 8-20 ng/mL 6 ng/mL Flow-through membrane-based assay	5 ng/mL [22] 1-50 ng/mL 5 ng/mL	1.2 ng/mL 0.3-5 ng/mL 0.12 ng/mL
	Triclocarban (315.58)	3.5 ng/mL [23] 1-11 ng/mL 0.3 ng/mL (homologous)	0.69 ng/mL [24] 0.1-36 ng/mL 0.03 ng/mL	
	3-Phenoxybenzoic acid (214.22)	1.4 ng/mL [25] 0.4-8 ng/mL 0.1 ng/mL (homologous)	1.6 ng/mL [26] 0.5-5 ng/mL 0.1 ng/mL	0.6 ng/mL [27] 0.2-2.0 ng/mL 0.1 ng/mL
	BDE-47 (485.79)	1.4 ng/mL [28] 0.5-10 ng/mL 0.1 ng/mL	1.75 ng/mL [29] 0.35-8.5 ng/mL 0.2 ng/mL	22 ng/mL or in direct ELISA: 1.4 ng/mL [30] 0.3-6.5 ng/mL 0.1 ng/mL
	TBBPA (543.9)	0.4 ng/mL [31] 0.06-2.53 ng/mL 0.02 ng/mL	0.87 ng/mL [32] 0.2-6 ng/mL 0.05 ng/mL	3.87 ng/mL [33] 0.8-20.7 ng/mL 0.3 ng/mL
	Aflatoxin B ₁ (312.27)	0.754 ng/mL [34] 0.117-5.676 ng/mL 0.05 ng/mL	2 ng/mL [35] 0.4-11 ng/mL 0.3 ng/mL	0.001-6 ng/mL [36] 6-400 ng/mL 0.001 ng/mL

Abbreviations used: MW, molecular weight; IC₅₀, IC₂₀₋₈₀, IC₁₀ concentrations resulting in 50, 20-80 or 10% decrease in maximum signal; pAb, polyclonal antibody; mAb, monoclonal antibody; K_d, 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; BDE-47, brominated diphenyl ether-47

*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 [6, 7] RR6 (733.38) RR120 (1469.98) Picloram [8] (241.46)	RR6-BSA (llama) RR120-BSA (llama) None (llama)	<i>E. coli</i> Yeast <i>E. coli</i> TGI (free VHH and VHH-Phage)	no panning, screened supernatants of randomly picked <i>E. coli</i> colonies EB20 [50 mM Tris-acetate (pH 7.5), 150 mM NaCl, 20 mM EDTA, 50 µg/mL Saccharomyces cerevisiae RNA] 0.1 M triethylamine 0.1 M triethylamine	4 °C to 90 °C	Crystallography Resonant mirror biosensor (K_d 18–83 nM) SPR analysis, phage ELISA	Stable in up to 4 M ammonium thiocyanate or 50 % ethanol. DNA shuffling used to alter affinity, specificity and yield in yeast Failed in phage-system, used ribosomal display. Best clones contained mutations introduced during PCR steps.
Auxin [37] (indole-3-acetic acid) (175.19) Caffeine [12] (194.19)	None (llama) caffeine carboxylate-KLH (llama and alpaca)	<i>E. coli</i> TGI (~1 mg/L) <i>E. coli</i>	0.1 M triethylamine pH 11.0	>90 % recovery after 90 °C	SPR analysis, CDR shuffling, pentamerization Patented lateral flow devices, immunoaffinity chromatographic separation and detection	SPR with immobilized pentamerized sdAbs allow direct observation of analyte-Ab interaction (K_d). Application to real samples: commercial beverages
Methotrexate [15] (454.44)	MTX-BIP (llama)	<i>E. coli</i> (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. VHH crystallized
15-Acetyldeoxymivalenol [18] (338.35) Ochratoxin A [21] (403.813)	15-DON-BSA (llama) OTA-KLH (llama)	<i>E. coli</i> (3–6 µg/L) <i>E. coli</i> BL21(DE3)pLysS	triethylamine panning 2x against OTA-OVA, 1x against OTA-KLH	Not tested Not demonstrated	fluorescence polarization, direct ELISA, SPR fused to AP	Used nested PCR, made monomer/pentamer Application to real cereal samples
Triclocarban [23] (315.58) 3-Phenoxybenzoic acid [25] (214.22) 2,2',4,4'-Tetrabromodiphenyl ether [28] (485.79)	TCC-Thyr (llama) 3-PBA-Thyr (alpaca) BDE-C1-KLH (alpaca)	<i>E. coli</i> BL21(DE3) <i>E. coli</i> TOP10F*	eluted with target analyte, decreasing concentrations eluted with target analyte, decreasing concentrations eluted with target analyte, decreasing concentrations	some inactivated at 85 °C; others remained active after 1 h at 100 °C Not demonstrated	none none	VHH that had additional disulfide bonds did not perform the best in thermal stability study. Spike-recovery validation
Tetrabromobisphenol A [31] (543.9) Aflatoxin B ₁ [34] (312.27)	T5-Thy (alpaca) AFB ₁ -BSA (alpaca)	<i>E. coli</i> TOP10F* <i>E. coli</i> TOP10F*	eluted with target analyte, decreasing concentrations panned with AFB ₁ -BSA, eluted with target analyte, decreasing concentrations	95 °C for 10, retained >50 % of its activity; 95 °C for 60, retained >25 % of its activity 90 °C heat for 10 mins 80 % activity; 90 °C heat for 90 mins 20 % activity Nb26 clone: ~70 % activity, Nb28 clone: ~70 % activity at 85 °C for 1 h	biosensors, electrochemical and chip [38] fused to AP, improved stability [39]	Spike-recovery validation

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; Thy: thyroglobulin; AP: alkaline phosphatase; 3-PBA: 3-phenoxybenzoic acid hapten; BDE-C1: brominated diphenyl ether hapten C1; AFB₁: aflatoxin B₁ hapten; BIP: blue carrier immunogenic protein; KLH: keyhole limpet hemocyanin; SPR: surface plasmon resonance

Interestingly, the CDR3 region of both VHHs consisted 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). The 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 the work [37], the authors isolated five different sdAbs from a naïve llama library with relatively low affinity for the IAA-BSA conjugate ($K_d \geq 5 \mu\text{M}$) as determined by SPR. Inhibition experiments with VHH CSF2A ($K_d = 5 \mu\text{M}$) revealed broad cross-reactivity with all auxinic herbicides with IC_{50} values $>200 \mu\text{M}$ for herbicides and $800 \mu\text{M}$ 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 VHH CSF2A for free IAA was $20 \mu\text{M}$, whereas the K_d values for synthetic auxins was one to 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 analogs 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 two camels and three llamas, using

triethylamine for elution of VHH-phage fusions bound to immobilized caffeine/BSA antigen [12]. Four out of five camels gave positive clones after two rounds of panning. Interestingly, one llama library produced no caffeine-selective clones, even though the prepanning 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 , whereas the mAb lost binding activity after incubation at 70°C . Using the developed VHH, the 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 a 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 [49]. 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 [50].

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 [51]. 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 VL [15]. Methotrexate was coupled to a commercial carrier protein, blue carrier immunogenic protein, 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

IC₅₀ of about 10 μ M. 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 \times 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 [52].

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 [53]. 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 [18]. A competitive fluorescence polarization assay was developed using a homologous 15-DON-fluorescein conjugate. The resulting IC₅₀ for DON was 1.42 μ M. The IgG in the serum was fractionated, and the authors report that most binding was by what they term complete IgG. HcAbs 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 μ g/well), switching blocking agents for rounds three and four, 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 the verotoxin B subunit. The 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 multivalently, 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 IC₅₀s for 15-AcDON were similar, 1.24 versus 0.5 μ M, 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 μ M. They postulate that 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 μ M 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 because of 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 [18].

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 [21]. 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 [54]. 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 the 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 (AP) to serve as the probe, and a direct competitive fluorescence enzyme immunoassay (dc-FEIA) for OTA was developed [55]. The IC₅₀ 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 AP

dimer apparently increased 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 the 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 [23], 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 with IgG2 and IgG3. Interestingly, the panning experiments from the TCC-VHH library using classic 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. 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 the IC_{50} of the competitive ELISA. The VHH from selected clones showed somewhat higher reactivity to compounds structurally similar to TCC compared with the pAb [24]. The VHH demonstrated an outstanding thermostability compared with 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 remaining 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 toxicity, 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

[25], 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 with 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, MeOH, and dimethyl sulfoxide, DMSO) compared with 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 its persistence in the environment and concerns about toxicity to human health. Although 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'-tetrabromodiphenyl ether (BDE-47), and was the target for VHH production [28]. 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 [29]. The resulting detection limit was in the ppb level, and cross-reactivity profiles were similar for both antibodies. The most sensitive VHH assay obtained ($IC_{50} = 1.4$ ng/mL) utilized a heterologous 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). Although not discussed in detail, the VHH assay was performed in a buffer containing 40 % 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 with 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, whereas 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 [28], and coated onto a PDMS membrane for a lab-on-a-chip sensor [38]. 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 of 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 [31] was conducted using knowledge gained from prior work in isolating highly sensitive pAbs [32] and mAbs [33] 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 ($IC_{50} = 0.4 \text{ ng/mL}$) resulted from panning with a heterologous hapten, that 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 % MeOH, 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 and 11.0. These pH and solvent matrix results highlight the increased tolerance of VHH proteins to maintain function (and presumably structure) under 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 exhibited 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 AP to serve as the reporting label [39]. 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 d 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 [34], 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 MeOH, 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 80–115 %, using the VHH Nb26-based ELISA. Interestingly, the authors reported that using 2 % BSA in the 70 % MeOH in phosphate buffered saline extraction buffer aided 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 [56]. 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 [57]. 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 ~10 mg, but usually requires a few days of growth [58]. Using the ascites method, a typical cell line will take ~5–7 d 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 [50]. 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 [59, 60]. 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 10 F') 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 [61–64], little literature is available on genetic manipulations of VHHs against small molecules. Although unintentionally, in one example Yau et al. [8] 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. The 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 immune-active 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 [37]. Recently, an interesting study investigating the structure–binding relationship of an anti-methotrexate (anti-MTX) VHH antibody was published by Fanning and Horn [52]. 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’ [65]. 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.

Labeling 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 [38]. Although 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 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 AP has been genetically linked to a VHH for a small molecule [39, 55]. 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 [55]. 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 d [39]. Chemical fluorophores that require chemical conjugation techniques are very different than those that 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 [66], and more recently the first demonstration for the analysis of a small molecule, methotrexate, by immunoaffinity chromatography [67]. 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. [68] reported a simple approach to produce multivalent VHHs where a VHH is fused to the B-subunit of *Escherichia coli* verotoxin, which self-assembles to form a homopentamer and results in simultaneous VHH polymerization giving pentabodies. Zhang et al. obtained an avidity gain of three to four orders of magnitude for pentabodies to parathyroid hormone [69]. 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 [68]. Riaz et al. [70] reported a 400-fold increase in functional affinity of penta-VHHs to *Campylobacter jejuni*, a gram negative bacterium, compared with the VHH monomer. To date, only one reference could be found on application of pentabodies for detection of small molecules. Doyle et al. [18] 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 two fold different. Despite consistent assay inhibition in a direct competitive ELISA, the authors were not able to precisely define the respective IC_{50} . Therefore, exploring the application of multivalent VHH constructs for detection of small molecules 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, avidin, or the recently reported rhizavidin (RZ) [71]. Unlike avidin or streptavidin, rhizavidin forms a homodimer instead of a tetramer. The authors reported that a VHH-rhizavidin bio-conjugate 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 (truncated recombinant core of streptavidin). 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. The 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 larger SC construct. These examples demonstrate an interesting avenue for exploring multivalent VHH constructs for the 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 % [44]. However, many VHHs have demonstrated superior tolerance to frequently used solvents compared with 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 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. [34] 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 % MeOH, 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 [28], and the anti-TBBPA VHH exhibited the best sensitivity with 10 % MeOH [31]. 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

scFvs and antigen binding 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 VL 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 [72, 73], 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 [74, 75]. Other researchers took a different approach and introduced a noncanonical disulfide bond into the hydrophobic core of llama VHHs between framework regions 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 [76, 77]. A recent thorough study of the relationship between the 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 ($t_{1/2}^{90\text{ °C}}$) decreased [78]. The researchers also showed that the thermal loss of activity of VHH was chemical in nature and proceeded 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 [44]. Most of the 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 compared with the corresponding conventional mAbs or pAbs (Table 4). The VHH against caffeine that was reported by Ladenson et al. [12] 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. [50] showed weaker temperature tolerance because the mid-point of the unfolding transition (T_m) was only 56 °C. Tabares-da Rosa et al. [23] obtained five different anti-TCC VHHs. All of these VHHs were more stable than rabbit pAbs at 85 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 studies indicated that the VHHs retained more than 50 % of their activity after heating

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 [44, 47]	2	FR1, FR3	Yes
Picloram [8]	2	FR1, FR3	Not tested
Auxin [37]	2	FR1, FR3	Not tested
Caffeine [12]	2	FR1, FR3	Yes
Methotrexate [15]	2	FR1, FR3	Not tested
15-AcDON [18]	2	FR1, FR3	Not tested
Ochratoxin A [55]	2	FR1, FR3	Yes
TCC [23]	2	FR1, FR3	Yes
	4	FR1, FR2, FR3, CDR3	Yes
3-PBA [25]	4	FR1, FR2, FR3, CDR3	Not tested
BDE-47 [28]	2	FR1, FR3	Yes
TBBPA [31]	2	FR1, FR3	Not tested
Aflatoxin B ₁ [34]	2	FR1, FR3	Yes

at 95 °C for 10 min compared with the pAbs, which dropped below 6 % of activity rapidly [28]. 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 [34]. All of these results suggest that VHHs are a superior reagent compared with 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 immunologic reagent, VHHs are commonly being compared with 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 to be a label-free technology wherein the signal is proportional to the change in light refraction due to binding. Because VHHs 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 [18] to evaluate the

binding kinetics of a VHH to the mycotoxin 15-AcDON in both a monomeric and pentameric form. VHHs against methotrexate [15], picloram [8], and auxin [37] 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 [23]. 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 pAb [79] and a VHH [28] 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 [49]. These avenues of biosensor research are areas of ongoing work, and 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 (Fig. 3). As a result, we recommend that even if screening of whole sera suggests

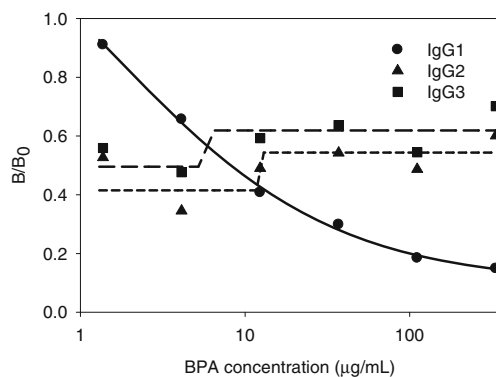


Fig. 3 Competitive inhibition profiles of subclass antibody fractions (IgG1, IgG2, and IgG3) from the same alpaca to bisphenol A

proceeding with library production, an antibody purification should be performed to ensure that all subclasses are responding to some degree [80]. As shown in the successful experiments for generating VHH to TCC [23] and TBBPA [31], the isolated VHHs had improved sensitivity compared with 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 Anderson and Goldman, wherein responding HcAbs were isolated to 2,4,6-trinitrotoluene and used to develop immunoassays [81], 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 [8]. Another consideration, which 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 to understanding how to selectively induce HcAbs and ensure isolation of those genes and their corresponding VHH antibodies.

Biohazard considerations

Development of VHHs implies the 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 (Fig. 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 wellbeing. 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 [82]. 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 with conventional and other recombinant antibody technologies, including small size, high solubility, good stability in extreme conditions (heat, pH, chaotropic media) and ability to be



Fig. 4 Alpaca housed at UC Davis

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 in 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 classic 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 structures 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 studying VHH structure, dynamics, and analyte binding on the atomic level in solution [83, 84]. 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 used for the crystallization of other proteins [82]. Acting as crystallization chaperones, VHHs help to stabilize the 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, better ability to pass through the renal filter and blood-brain barrier, and faster tissue penetration than conventional antibodies [85]. For these reasons, VHHs have great potential as therapeutic agents. Indeed, conventional antibodies have been routinely used in clinical applications, in particular for venom detoxification and sequestration. 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 conventional 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. It is probable that 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.

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Conflict of interest The authors declare that they have no conflicts of interest.

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