



Kavanagh, Owen ORCID logoORCID: <https://orcid.org/0000-0002-2599-8511>, Elliott, Christopher T. and Campbell, Katrina (2015) Progress in the development of immunoanalytical methods incorporating recombinant antibodies to small molecular weight biotoxins. *Analytical and Bioanalytical Chemistry*, 407 (10). pp. 2749-2770.

Downloaded from: <https://ray.yorks.ac.uk/id/eprint/1134/>

The version presented here may differ from the published version or version of record. If you intend to cite from the work you are advised to consult the publisher's version:
<http://dx.doi.org/10.1007/s00216-015-8502-z>

Research at York St John (RaY) is an institutional repository. It supports the principles of open access by making the research outputs of the University available in digital form. Copyright of the items stored in RaY reside with the authors and/or other copyright owners. Users may access full text items free of charge, and may download a copy for private study or non-commercial research. For further reuse terms, see licence terms governing individual outputs. [Institutional Repositories Policy Statement](#)

RaY

Research at the University of York St John

For more information please contact RaY at
ray@yorks.ac.uk

Progress in the development of immunoanalytical methods incorporating recombinant antibodies to small molecular weight biotoxins

O Kavanagh, CT Elliott, K Campbell

Institute for Global Food Security (IGFS), School of Biological Sciences, Queen's University Belfast, 18-30

Malone Road, Belfast BT9 5BN, UK.

Abstract

Rapid immunoanalytical screening of food and environmental samples for small molecular weight (hapten) biotoxin contaminations requires the production of antibody reagents that possess the requisite sensitivity and specificity. To date animal-derived polyclonal (pAb) and monoclonal (mAb) antibodies have provided the binding element of the majority of these assays but recombinant antibodies (rAb) isolated from *in vitro* combinatorial phage display libraries are an exciting alternative due to (1) circumventing the need for experimental animals, (2) speed of production in commonly used *in vitro* expression systems and (3) subsequent molecular enhancement of binder performance. Short chain variable fragments (scFv) have been the most commonly employed rAb reagents for hapten biotoxin detection over the last two decades but antibody binding fragments (Fab) and single domain antibodies (sdAb) are increasing in popularity due to increased expression efficiency of functional binders and superior resistance to solvents. rAb-based immunochromatographic assays and surface plasmon resonance (SPR) biosensors have been reported to detect sub-regulatory levels of fungal (mycotoxins), marine (phycotoxins) and aquatic biotoxins in a wide range of food and environmental matrices however this technology has yet to surpass the performances of the equivalent mAb- and pAb-based formats. As such the full potential of rAb technology in hapten biotoxin detection has yet to be achieved but in time the inherent advantages of engineered rAb are set to provide the next generation of ultra-high performing binder reagents for the rapid and specific detection of hapten biotoxins.

Introduction

Biotoxins, a structurally diverse group of toxic compounds, are secondary metabolites produced from natural origins (e.g. animals, plants, fungi and bacteria) that detrimentally impact on human and animal health.

Intoxication occurs through consumption of contaminated foods, feeds or from environmental exposure (e.g. drinking water) and consequently the detection and quantification of biotoxins is imperative to guarantee the safety of our food and environment. An array of chemically-based analytical methods to detect biotoxins have

been described [1–4] however these methods are often laborious, expensive and are limited in some cases by the availability of toxin reference standards. Other methods of detection include functional assays and mouse bioassays but these assays have limitations due to specificity as well as ethical concerns with the latter being phased out of food safety monitoring [5, 6].

Immunoassays incorporating sensitive and highly specific biotoxin-specific antibodies offer a rapid, inexpensive alternative to the above methods. Immunoassays require antibodies generated by repeated immunisations of animals with an antigen, however the success of this approach has been somewhat limited by the fact that many biotoxins are haptens i.e. small molecular weight molecules of ≤ 1000 Da that fail to stimulate the immune system to produce an antibody response. In some cases even when they are conjugated to a larger carrier molecule the complex can fail to elicit an antibody response but can remain highly toxic. Furthermore, administration of animals with biotoxins is considered unethical as it may lead to severe adverse effects to the animal's wellbeing and in some cases the limited availability of purified toxin precludes *in vivo* generation of antibodies. Even if successful, the batch-to-batch reproducibility of polyclonal antibodies (pAb) can be poor and monoclonal antibody (mAb) development requires highly specialised equipment and labour intensive procedures and adequate storage facilities to maintain the hybridoma cell lines.

Generation of soluble functional recombinant antibodies (rAb) to haptens from combinatorial *in vitro* antibody libraries has emerged as a promising alternative to *in vivo* antibody production [7–10]. Advantages of rAbs over conventional pAb and mAb include low cost production of functional antibody in commonly used expression systems (e.g. bacteria, plants & yeast), high soluble functional protein yields in excess of 10 g/L [11] and enhancement of antibody performance by *in vitro* affinity maturation processes [7]. By mimicking the mammalian immune system large repertoires of rAb can be produced to highly toxic haptens *in vitro* thus alleviating the need for animal work. The focus of this review is to (1) analyse recent progress with the generation of hapten biotoxin rAb in the context of rapid screening immunoassays (2) examine the ability of rAb to detect sub-regulatory levels of biotoxins in food and environmental samples and (3) highlight future potentialities for this field.

Recombinant antibody combinatorial display

Advances in the field of *in vitro* combinatorial display technologies have resulted in the generation of a plethora of binding molecules to hapten contaminants composed of antibody fragments, nucleic acid aptamers, peptides and alternative protein scaffolds [7–10]. A search of the ISI Web of Science for the last 20 years, up to

and including 2014, for the topic “recombinant antibody” and “hapten”, excluding review and non-relevant articles, returns 343 research articles (Fig. 1) [12]. A further refinement of the search to include the term “phage display” yields 64 articles, or yeast-display (9 articles), ribosomal display (5 articles) and 1 article for bacterial display. What becomes apparent from Fig. 1 is that the number of articles published in this topic peaked in 1996 and the lowest number of publications and citations occurred in recent years. However an exhaustive search of publications containing the terms “phage display”, “recombinant antibody” and “hapten biotoxin” compiled from Web of Science, PubMed, Science Direct and Google Scholar indicates that the field peaked in 2008 (5 publications) but still remains active in 2013-2014 (4 publications in each year) and consequently publications in this field will be reviewed herein.

Phage display-derived recombinant antibodies

Phage display technology is an *in vitro* screening technique for identifying ligands for proteins and other macromolecules through the ability to express peptide or protein sequences as fusions to the coat proteins of a bacteriophage (viruses that infect bacteria). The ability to insert foreign proteins onto capsid proteins without affecting infectivity [13] has seen phage display develop into the most popular *in vitro* combinatorial approach in designing new drugs, vaccines and diagnostics. Phage rAb display has in many cases superseded mAb hybridoma technology through creation of highly diversified natural and synthetic *in vitro* repertoires of antigen binders. Antigen-specific rAb-displaying phage are isolated and enriched through a process of affinity selection also known as biopanning (Fig. 2A) and the clones of interest are then amplified in specialised *E. Coli* host cells and enriched by further rounds of biopanning yielding a population of antigen-specific monoclonal rAb.

Entire antibody immunoglobulin molecules are in most cases too large to be expressed on phage surfaces without negatively impacting on infectivity but smaller antibody fragments such as short-chain variable fragments (scFv) and the antigen-binding fragment (Fab) (Fig. 2B) can be efficiently displayed on virion particles [8, 14–17]. The variable heavy (H) and light (L) chains of these antibody fragments, like the parent antibody, are comprised of three areas of high sequence diversity called complementarity determination regions (H and L CDR1-3) and the CDRs on each chain are connected by stretches of relatively conserved sequence which acts as a molecular scaffold called the antibody framework [17]. Of these CDR, the H CDR3 is the most variable in terms of both length and amino acid diversity and is the major determinant of antigen

binding specificity [18]. Despite the widespread use of scFv, a major limiting factor of this technology is the low functional yields when expressed *in vitro* principally due to intracellular protein aggregation [19].

Single domain antibodies (sdAb) or nanobodies such as the *Camelidae* VHH and shark IgNAR (Fig. 2B) possess superior physicochemical stability and high refolding capacity compared to their scFv counterparts [20–22]. Consequently sdAb are produced in soluble functional form in higher quantities from heterologous *in vitro* expression systems and are more resistant to denaturation and solvent-induced conformational unfolding [22]. The exceptional stability and solubility is due to (1) the small size of sdAb (11-15 kDa) compared to scFv (27 kDa), (2) the single domain nature of sdAb and (3) the evolutionary replacement of non-polar residues where the VL interface would be in a scFv with more hydrophilic residues. The small size and lack of VL of sdAb is compensated for by paratopes with greater sequence and topological diversity than scFv/Fab which serve to enhance the binding interface and sequence heterogeneity in the CDR regions involved in antigen-binding [23]. For example sdAb paratopes have the ability to produce much longer H CDR3 regions than for other mammalian antibodies and these finger-like antigen-binding sites are conformationally constrained by additional inter-CDR disulfide bonds [24–26]. These enlarged CDR3 regions can form convex protrusions in addition to the planar and concave (cavity) topologies of conventional antibodies [25, 27]. Hapten-scFv interaction occurs within a cavity formed between the VH and VL domains of an antibody and as sdAb lack these domains they might not be expected to bind strongly to haptens [22]. However, analysis of the crystal structure of a nanomolar affinity llama VHH interacting with its hapten antigen (azo dye Reactive Red; 728 Da) demonstrated that the hapten was indeed buried in a cavity composed of residues of the three CDR and in particular an elongated CDR3 [28, 29]. Moderate affinity hapten-specific VHH have since been isolated from immunised (nanomolar) [24] and naïve (micromolar) [30] phage VHH repertoires.

Detection of haptens using recombinant antibodies

Aspects of hapten-binding recombinant antibodies has been extensively reviewed in recent years [7–9, 22] and as a consequence where possible we will focus on more recent case studies..

Antibody engineering

In vivo-derived antibody affinity for haptens is generally limited (estimated to be 10^{-10} M or below [31]) due to fewer paratope-binding interactions provided by small molecules [32]. Phage displayed-rAb

libraries offer more promise as a platform to generate high affinity hapten-recognition molecules than conventional antibodies because the phenotype (rAb) is directly linked to the genotype (gene encoding rAb), and as such, a rAb affinity can be rapidly enhanced *in vitro* by antibody engineering processes that mimic *in vivo* affinity maturation [7, 33]. The maximum affinity generated *in vivo* is believed to be in the sub-nanomolar range ($K_D > 0.1$ nM) whereas *in vitro* antibody engineering processes can result in affinities in the picomolar-femtomolar range [34]. Another way genetic engineering processes can enhance rAb performance is by genetically fusing the detector label (e.g. alkaline phosphatase) directly to the rAb [35–37] or indirectly via affinity tags or other site-specific moieties [38, 39]. Furthermore, monomeric rAb can be genetically engineered into multimeric high avidity components or domains with differing antigen specificities that can be combined to create oligo-specific reagents that retain the sensitivity and specificity of the monomer [16, 26, 40]. Fusing several copies of a single sdAb to a pentamerisation domain such as *E. Coli* verotoxin B subunit has been reported to improve avidities several orders of magnitude greater than their monomeric counterparts [41–43].

Naïve synthetic recombinant antibody libraries

Phage displayed rAb libraries can be categorised as either immunised or un-immunised (naïve) repertoires and the latter are further classified as natural, semi- or fully synthetic. Immune libraries are commonly created from the antibody genes (e.g. VH, VL or VHH) cloned from hybridomas or lymphocytes of animals previously immunised with the antigen of interest [14, 51]. Use of immune libraries to generate rAb to haptens has been extensively reviewed elsewhere [8] and the merits of this approach are outlined in Table 1.

In general the larger and more diverse the antibody repertoire (number of B cells *in vivo* or library rAb diversity *in vitro*), the greater the probability of generating a high affinity antibody to a particular target [52]. *In vivo* antibody diversity is estimated to be in the 10^8 (mice) to 10^{12-15} (humans) range [53–55]. Larger and more diverse antibody repertoires may give rise to higher affinity antibody fragments to a wider array of target molecules. Strategies used to synthetically construct naïve (antibody repertoire constructed without prior immunisation with target antigen) libraries with enhanced antibody diversity are reviewed in more detail elsewhere [33] but include recombination of V-genes or CDR from different antibody germ lines, modification of antigen-interacting (in particular CDR3 H) and antibody framework residues on conserved antibody scaffolds [17]. This creates semi-synthetic (elongation of naturally occurring VH and VL genes only) [55] or fully synthetic (entire antigen binding region is synthetically produced and modified by mutations) libraries of

enhanced rAb diversity and size compared to the limited natural repertoire (Table1). Currently rAb synthetic library antibody diversity is limited to the 10^{10-11} range with the principal bottlenecks being transfection efficiency of the host and loss of rAb functionality due to frame shift mutations [33].

The limitations of synthetic naive libraries can be overcome by using synthetic rAb repertoires derived from known hapten-binding paratopes. A hapten-binding paratope is shaped as a pocket or cavity with contact residues located centrally whereas binding sites for larger molecules are more planar or concave with additional contact residues located on the periphery [56]. So-called hapten-“biased” or –“focused” scFv phage libraries have been constructed by engineering diversity into antigen-interacting as well as some peripheral or buried residues of an existing hapten-binding scFv resulting in isolation of scFv possessing nanomolar affinity to unrelated haptens (Table 2) [57, 58]. Another advantage to these hapten-biased libraries is that the antigen-interacting residues are optimised for binding to soluble haptens so are expected not to interact with large carrier protein molecules frequently used for affinity selection [57]. The efficacy of this approach is highlighted by the observation that hapten-biased libraries with a relatively low rAb diversity (e.g. 10^7 library size) generated between 50-100% scFv that recognise soluble haptens compared to a conventional synthetic repertoire which generated no such clones [57]. It must be noted that many of the commercially available rAb libraries are expensive and often subject to licensing agreements and some companies only offer in-house custom rAb production. Table 2 gives an overview of a selection of (semi-) synthetic rAb-based phage display libraries that have been reported to generate hapten-specific rAb.

Progress in the detection of hapten biotoxins using recombinant antibody-based assays.

Enzyme-Linked Immunosorbent Assay (ELISA)

rAb-based immunoassays to microcystins [77], aflatoxins [78] and other biotoxin haptens [79, 80] have been reviewed previously and so are not discussed in this section but are summarised in Table 4. In order to determine the true affinity of an antibody for hapten binding, direct competitive (free hapten to be measured competes in solution with hapten labelled with an appropriate detector label (e.g. horse radish peroxidase (HRP) for immobilised antibody), or, indirect competitive assay (hapten to be measured inhibits primary antibody binding to immobilised hapten and level of primary antibody binding is measured with a secondary anti-species antibody coupled to a detector label) are the most commonly employed approaches. In both cases the amount of free hapten in a sample is inversely proportional to the signal emitted by the detector label.

A direct competitive ELISA (c-ELISA) incorporating the QY1.5 scFv produced from an anti-zearalenone (ZEN) mAb [81] was immobilised to the plate surface via an affinity tag (E tag) capture antibody and free ZEN competed with a ZEN-HRP conjugate for scFv binding. QY1.5 scFv was previously found to possess similar ZEN-binding affinities to the parent mAb in buffer (Table 4) but exhibited enhanced relative cross reactivity (between 2- to 7-fold change) to the hydroxylated ZEN analogues [82] (Table 5) however it was inactivated when solvent employed for ZEN extraction exceeding 20% v/v. The ability of QY1.5 c-ELISA and ic-ELISA [82] to detect ZEN in spiked corn samples (500-3000 ng ZEN/g corn) was evaluated compared to the parent mAb [83]. The QY1.5 assay detection limits were 4 ng/ml (275 ng/g corn) with ZEN recovery rates of 95-113% by c-ELISA and 15 ng/ml (1000 ng/g corn) and recovery rate of 100-121% by ic-ELISA although the former format was found to be even more susceptible to solvent destabilisation than the latter. The mAb-based c-ELISA was 10-fold more sensitive to ZEN in maize than the equivalent scFv format which is most likely due to solvent destabilisation of QY1.5. The QY1.5 c-ELISA compared favourably to a commercial mAb-based ic-ELISA in detecting ZEN artificially contaminated wheat samples (97% correlation to Neogen Veratox ELISA (n=30)) and detected ZEN in naturally contaminated Chinese wheat samples [84] however no analytical method (e.g. HPLC or LC-MS) was employed to confirm the presence of ZEN or its metabolites therein. Both QY1.5 ELISA formats possessed sufficient sensitivity to detect sub-regulatory limits of ZEN in cereals in many countries as the maximum tolerated limits are set very high (e.g. 1000 µg/kg) (Table 5) however this is not the case in Europe where the maximum permitted levels of ZEN in processed maize are 350 µg/kg [85]. Extraction of ZEN from foods and cereals requires high concentrations of solvents (e.g. 70% methanol [86]) and as such the QY1.5 solvent susceptibility is a major limitation of these scFv-based immunoassays meaning samples have to be diluted pre-screening to reduce solvent concentrations resulting in a loss of assay sensitivity. This deleterious effect may be overcome by genetically engineering a more stable antibody fragment or alternatively the scFv could be replaced by the more solvent-resistant sdAb [22, 87]. A bi-specific binding molecule was produced by cloning of the genes encoding QY1.5 and an anti-deoxynivalenol (DON) scFv [88] onto a single expression plasmid. The subsequent divalent scFv maintained the affinity of the monovalent forms for their respective toxin and was able to detect DON or ZEN as equally well as the monomers in naturally contaminated wheat samples [89].

An aflatoxin B1 (AFB1)-specific scFv (clone C4) containing an affinity tag (FLAG tag) was used to develop an ic-ELISA where detection of scFv binding to solid phase AFB1-BSA conjugate was done using an anti-FLAG mAb and a species-appropriate HRP-labelled secondary antibody [90]. The scFv assay gave a

reproducible linear range from 98-1560 ng/ml free AFB1 in PBS with a final methanol concentration of 2.5% v/v and the rAb displayed an impressively high level of cross reactivity to seven closely related aflatoxins (Table 5). However scFv stability at the high solvent concentrations (70% methanol [91]) required to extract AFB1 from foods was not examined.

The first recombinant Fab fragment to hapten biotoxins was produced to detect a lipophilic polyether ciguatoxin (CTX) neurotoxin produced by the marine dinoflagellate *Gambierdiscus toxicus*. Generation of CTX-specific antibodies has proved challenging due to its potent toxicity, poor immunogenicity and the limited availability of purified natural CTX. This latter problem has been somewhat overcome by using chemically synthesised CTX fragments as immunogens [92]. One such synthetic hapten representing the first three rings (ABC) of the left-hand side of the CTX congener CTX3C (CTX3C-ABC) was synthesised, and used to immunise mice in order to produce phage-derived recombinant Fab from murine splenic RNA [93]. An ic-ELISA in which the Fab competed with free CTX synthetic fragments (0.001-100 μ M) or natural toxin (concentrations not published) for binding to a CTX3C-ABC protein conjugate passively coated microtiter plates. The affinity of the Fab clone 1C49 ELISA possessed moderate affinity for the free ABC synthetic fragment (8.6×10^{-8} M K_D) but considerably weaker affinity to the free ABCD fragment (2.4×10^{-5} M K_D) and the natural soluble CTX3C. The authors failed to publish the affinity of Fab 1C49 for free CTX3C but interpretation of the published results suggests that the K_D was approximately 1.3×10^{-5} M. Crystal structure analysis of 1C49 interacting with CTX indicated that the ABC fragment was deeply buried within the Fab antigen-binding pocket suggesting that the ABCD fragment and the polyether CTX3C were sterically hindered from binding to the paratope [94]. Antibodies with extremely high affinity for CTX are required for rapid screening of fish samples as trace amounts (parts per trillion [95]) of CTX can elicit toxicity and recent studies by the same group have determined that a minimum pentacyclic CTX synthetic fragment immunogen is required to produce mAb-based sandwich ELISAs that recognise ppb (nM) amounts of CTX in buffer [96–98] although no rAb derived from these mAbs have been published to date. Analysis of rAb produced from these hybridomas would be of interest and possible further enhancement of sensitivity by *in vitro* affinity maturation processes to generate rAb possessing the ultra-sensitivity required to detect CTX would be an exciting option to explore in the future.

Hu *et al* produced a scFv from the RNA of a hybridoma secreting an anti-domoic acid (DA) mAb and investigated several parameters (domain orientation, linker length, accessory protein co-expression & comparison of *E. Coli* host strains) to optimise expression of soluble functional scFv [99]. Purified scFv 2H12

was compared to the parent mAb in an ic-ELISA where antibody competed with free DA (concentrations not published) for binding to a DA-ovalbumin (OVA) conjugate coated on a microtiter plate. The amount of antibody binding to the solid phase DA was detected by addition of an appropriate HRP labelled anti-species secondary (mAb) or an anti-His tag (scFv) antibody. The 2H12 scFv ELISA was approximately 4-fold more sensitive than the parent mAb (Table 4) and although the 2H12 scFv possessed sufficient sensitivity in buffer to detect sub-regulatory amounts of DA in fish samples no assessments were performed in such samples. Another group used a sheep hyper-immunised with DA-BSA conjugate to generate an immune scFv phage library from which scFv clone DA24cB7 was isolated and used to develop an anti-DA ic-ELISA [100]. A calibration curve was constructed using the mean of 19 separate experiments over a 6 week period with free DA standards (0.63-80.3 nM) and the scFv ELISA was reported to be highly reproducible (although %CV not published) with a linear working range of 0.30 ng/mL to 5.58 ng/mL (calculated using the IC₁₀ & IC₈₀ values) with an LOD of 0.5 ng/mL (IC₂₀ value). The scFv ELISA correlated well ($R^2 = 0.91$) to HPLC analysis of 50% methanol extracted DA from naturally contaminated shellfish extracts although the ELISA was found to underestimate the results. The authors rectified this by setting a cut off value (15 mg/kg) just under the regulatory limit of 20 mg/kg (Table 5) which resulted in identification of all the HPLC-positive extracts. There are two reasons why the DA24cB7 scFv assay was more sensitive than the murine 2H12 format described above or another affinity matured DA-specific scFv (clone R4Sh-7) produced from an immunised chicken [101] (Table 4) which are (1) the species chosen to produce a DA-specific immune response and, (2) the superior affinity selection strategy used to isolate DA24cB7 (successive rounds of biopanning with reduced target hapten concentration favours the isolation of high affinity scFv). However one note of caution with all three anti-DA scFv produced to date is that the cross reactivity profiles to other marine biotoxins that may occur in shellfish samples was not reported.

In 2009 three more reports of AFB1-specific scFv ELISAs were published and in the first case, Wang isolated scFv with affinities of 0.4-0.7 µg/mL in buffer as determined by ELISA [102]. In the second report the authors wanted to avoid the biased immune response towards hapten derivatives and non-relevant protein carrier molecules used in immunisation protocols and instead directly coated AFB1 to the biopanning surface [103]. scFv clone H4 was isolated following affinity selection against the soluble target and incorporated into an ic-ELISA that possessed the highest affinity for free AFB1 in buffer described to that date (Table 4) and exhibited some cross reactivity to other aflatoxins (AFG1 (42%) > AFB2 (12%) > AFG2 (9%)). The bacterial expression of soluble functional clone H4 scFv was subsequently optimised to maximise the rAb yield [104]. In the third report a group in Thailand constructed a scFv-phage library (Yamo 1) from the antibody germlines cloned from

B lymphocytes of 140 non-immunised human donors [61]. A soluble AFB1-specific scFv (YM1-C3) was isolated from the Yamo-1 library which was genetically fused to an alkaline phosphate (AP) detector label (in a 1:1 ratio) [105]. Direct fusion of the AP to scFv (YM1-C3-AP) resulted in a 3-fold increase in sensitivity to free AFB1 in a single-step ic-ELISA when compared to the unlabelled scFv assay (Table 4). The relative cross-reactivity of the scFv-AP fusion products to closely related aflatoxins as determined by the ELISA was reported (AFG1>G2=B2>>M1). The authors attributed the success in generating high affinity scFv from the Yamo-1 library due to prior exposure of some of the human donors to aflatoxins.

Non-competitive assays such as the sandwich ELISA require a pair of antibodies with epitopes on different parts of the target antigen, an unlikely possibility when measuring small molecules. The development of “open sandwich-immunoassay” (OS- ELISA) has somewhat circumvented this problem by exploiting the antigen-mediated association of the VH and VL when expressed as separate entities [36]. Hara *et al* described the development and validation of a non-competitive OS-ELISA to detect the paralytic shellfish poisoning (PSP) biotoxin gonyautoxin (GTX2/3) [106]. A GTX2/3-specific mAb (GT-13a) [107] was used to produce a GTX-specific Fab from which the VH and VL domains were expressed as different soluble protein entities fused to maltose-binding protein (MBP-VH) or MBP plus affinity tag (MBP-VL-c-myc). The MBP-VH was coated onto the microtiter plate and upon the concomitant addition of free toxin and MBP-VL-c-myc an antigen-driven association of the two chains occurs. The amount of GTX-VH-VL complex present was measured using a c-myc-specific HRP-labelled secondary antibody. The Fab OS-ELISA performed similarly to previously described parent GT-13a competitive mAb-based immunoassays (albeit these assays used the parent PSP biotoxin saxitoxin (STX) as target ligand) [107–109] exhibiting the highest affinity for the immunogen used to produce the mAb (GTX2/3 0.28 ng/ml IC50) slightly less so for STX (4 ng/ml IC50) and, similar to other competitive pAb-based assays [110], minimal cross reactivity to hydroxylated analogs. The linear range of the OS-ELISA (0.05-300 ng/ml) was three orders of magnitude wider than those reported for the other GT-13a mAb-based assays and was capable of detecting four PSP toxins (GTX2/3, STX, C1/C2 and dcGTX2/3) in shellfish extracts artificially spiked at the regulatory limit of 800 µg PSP equivalents/kg shellfish meat [111, 112] (Table 5). OS-ELISA typically exhibit lower limits of detection, a broader working range and superior assay precision for hapten molecules (e.g. ZEN, estradiol (17β-E₂) and corticosteroid (11-DC)) when compared to the equivalent competitive formats [36, 113–115]. One note of caution with the OS-IA format is the high-background signal sometimes observed due to association of the V chains in the absence of antigen but this can

be reduced by site-specific mutations at the VH-VL interface [115] or prior *in vitro* combinatorial screening for compatible V chains (OS selection) [116, 117].

Huovinen *et al* modified a naïve synthetic mouse library designed for binding macromolecules (ScFvP) by introducing sequence diversity deep in the paratope and blunting CDR-H3 loops they managed to introduce a bias towards hapten binding [118]. A microcystin (MC LR)-specific scFv (clone M-MCLR1) from the resultant phage library (ScFvM) was isolated and incorporated into a competitive time-resolved fluorescence-based immunoassay (TRF-IA) consisting of free microcystin competing with a MC LR tracer labelled with the fluorescent lanthanide Europium for binding to the M-MCLR1 captured on the ELISA plate by an anti-mouse antibody. The authors acknowledged that they chose MC LR as a prototype peptide hapten target to validate their library performance and as such didn't examine the cross reactivity profile to other microcystins or validate the ELISA using environmental samples. The nanomolar affinity of the scFv assay (Table 4) was most likely as a result of the lanthanide fluorescent label employed possessing sensitivities at least 10-fold greater [119] than colorimetric or chemi-luminescent labels commonly employed in ELISAs and it would be interesting to compare the performance of M-MCLR1 scFv to a conventional Ab using the same detector label.

The first AFB1-specific Fab-based ic-ELISA was used to compare the merits of an AFB1 derivative covalently coupled to a polystyrene plate to passively adsorbed AFB1-BSA on the same surface [120]. The sensitivity of the covalent AFB1 assay was 2.7-fold greater than that of the BSA-AFB1 format in buffer (Table 4) and in both assays Fab were specific for AFB1 with some cross reactivity observed to AFG1 (Table 5) and no recognition of unrelated co-occurring mycotoxins tested (ZEN, DON and T-2 at 10 µg/ml). The covalently linked AFB1 assay permitted a shorter primary antibody incubation time, was more resistant to methanol (up to 40%) and could be re-used 4 times upon treatment with chaotropic agents (89% agreement in IC50 over successive uses) when compared to passively coated BSA-AFB1. The covalent Fab-AFB1 assay was matrix validated by measuring AFB1 in 40% methanol extracted from reference corn material containing AFB1 and AFB2 [120]. The assay was robust (<5% intra- and inter-day variation) but the authors failed to report the % recovery of AFB1 from the reference matrix or the side-by-side performance of the passively adsorbed AFB1-BSA ELISA with corn. Another interesting point would have been to compare the performance of ELISAs utilising the Fab to those incorporating a conventional antibody or the previously described scFv produced by the same group [121]. To overcome scFv inactivation at high methanol concentrations commonly used to extract aflatoxins (70-80% v/v) [91] an immune sdAb (VHH) phage display library was constructed from blood lymphocytes from an alpaca immunised repeatedly with AFB1-BSA [87]. An AFB1-specific VHH (Nb26) was

isolated by affinity selection from the library with soluble hapten elution and VHH clone Nb26 exhibited high sensitivity (0.75 ng/ml IC₅₀) to soluble AFB1 with a broad working range (0.117–5.676 µg/kg) in buffer and was specific to free AFB1 (10.1% relative cross reactivity to AFM1).

In addition to AFB1, other aflatoxins are harmful on their own and monitoring of the sum of aflatoxins (AFB1+B2+G1+G2) is also essential to limit the total aflatoxin content of food and foodstuffs [122, 123]. With this in mind Edupuganti *et al* produced AFB2-specific rAbs from a scFv-phage library produced from the splenic RNA of mice immunised with AFB2-BSA [124]. AFB2 is the dihydroxy derivative of AFB1 which albeit has reduced toxicity than AFB1 is present in trace amounts in foods and feed but has a lower susceptibility to decontamination methods than AFB1. Two clones were found to be highly specific to AFB2 when screened by ic-ELISA (Table 5) and the more sensitive clone (E9) (85 ng/ml IC₅₀ in buffer) and was incorporated into a SPR-based assay for further analysis (next section). In another study Zhang *et al* produced a series of high affinity mAbs using AFB1-BSA immunogen with a range of diverse cross-reactivity profiles that possessed high affinity to either total aflatoxins (clone 1C11; AFB1+B2+G1+G2 at 1.2-18.0 pg/ml IC₅₀) or AFM1 alone (clone 4F3; 0.27 ng/ml IC₅₀) [125]. The same group subsequently constructed a phage scFv library composed of the VH and VL of almost 20 hybridomas secreting mAbs (including clones IC11 and 4F3) with diverse specificity to aflatoxins [126]. From this immunised repertoire two scFv of interest were rapidly isolated (without biopanning) and incorporated into ic-ELISAs. The first scFv clone (2G7) was found to possess the highest reported sensitivity to free AFB1 to date (0.01 ng/ml IC₅₀ in buffer) and was highly specific to AFB1. The second clone (1A7) also exhibited excellent affinity to AFB1 (0.02 ng/ml IC₅₀ in buffer) but also strong cross reactivity to AFB2 (166%), AFG1 (100%) and to some extent to AFM1 (11%). It is worth noting that the scFv clones of interest were isolated according to their ability to recognise AFB1 and as such it may be possible to select scFv candidates exhibiting different cross-reactivity profiles by using more appropriate target ligands. Cows can metabolise AFB1 into AFM1 whose levels in dairy products are regulated for raw milk and infant formulas at 0.05 and 0.025 ppb respectively [122] and as the immune library potentially contains 4F3 antibody fragments that exhibit ppb sensitivity for AFM1 it should be possible to isolate high affinity scFv by screening with AFM1 target ligand.

Detection of the hepatotoxic microcystin (MC) congeners, other than MC-LR, is of critical importance and animal-derived pan-specific antibodies have been raised against the invariant toxic Adda amino acid ((3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-dienoic acid) common to the majority of MC and nodularins

has been described [127, 128]. 3-D structural modelling an anti-MC LR scFv 3A8 [129] (Table 4) binding to MC LR identified the Adda residue as the most likely epitope for this rAb which seemed to be confirmed somewhat by an scFv-AP conjugate dose-dependent binding to an Adda mimic by ELISA [130]. In the same report, real-time SPR analysis of the scFv-AP binding directly to immobilised MC-LR-OVA seems to have identified a possible flaw with some scFv as two binding affinities were detected ($K_{D1} = 0.22 \text{ nM}$ & $K_{D2} = 8.73 \text{ }\mu\text{M}$) suggesting the presence of a heterologous population (either mis-folded or aggregated scFv). This observation was borne out by the relatively weak affinity of the 3A8-AP for free MC-LR in a one-step ic-ELISA ((IC₅₀ = 2.97 μM & LOD (IC₁₀) of 0.25 μM in buffer). This work very elegantly highlights the need to fully characterise monoclonal scFv in order to ensure the presence of a single homogenous functional rAb population and suggests the greater potential of the more thermostable sdAb which are less prone to mis-folding.

A Chinese group recently developed a scFv-based ELISA to measure the carcinogenic mycotoxin fumonisin B1 (FB1) found in contaminated corn samples [131]. The scFv was cloned from a hybridoma expressing an anti-FB1 mAb (1D11) and ic-ELISA analysis showed that the scFv performance was very similar to the parent mAb. Both antibody ELISAs possessed similar affinity to FB1 in buffer (Table 4) and were specific to FB1 with less than 10% cross reactivity to the closely related FB2 and no recognition of other co-occurring unrelated mycotoxins (Table 5) although the scFv ELISA linear range (2.1-76.45 $\mu\text{g/L}$) was marginally higher than that of the parent 1D11 (1.67-61.74 $\mu\text{g/L}$). The scFv assay successfully validated in corn samples according to the criteria set out by the European Commission Decision 2002/657/EC [132]. The analytical limit calculated from 20 known blank corn samples ranged from 1.40 to 6.30 $\mu\text{g kg}^{-1}$ due to the crude nature of the matrix and LOD (8.32 $\mu\text{g/kg}$) were calculated from the same corn samples spiked with known concentrations of FB1 with intra- (9.72-10.26 %CV) and inter-assay (11.28-14.03 %CV). Furthermore the scFv ELISA displayed good agreement ($R^2 = 0.97$) to HPLC analysis of 30 naturally FB1-contaminated corn samples. Council Directive 2002/657/EC [132] established criteria and procedures for the validation of analytical methods to ensure the quality and comparability of analytical results generated by official laboratories and established common criteria for the interpretation of test results. One of the requirements is that the detection capability ($CC\beta$) (15 $\mu\text{g/kg}$) must be less than or equal to the regulatory limit set which worldwide ranges from 1000-4000 $\mu\text{g/kg}$ corn (Table 5).

Biosensor-based immunoassays

Immuno-biosensors enable the real-time analysis of antigen-antibody interactions which is subsequently detected by a transducer or sensing device (e.g. optical, electrochemical, acoustical, piezochemical or micromechanical [141]). Optically-based BIAcore instruments are a widely used example of biosensors which enables biospecific interaction analysis (BIA) of antigen-antibody binding with high sensitivity and speed of analysis. The BIAcore technology utilises the phenomenon of surface plasmon resonance (SPR) where one interacting molecule (ligand) is bound to the biosensor surface (flow cell on sensor chip) while the other (analyte) is delivered to the surface in a continuous flow through a microfluidic system [9, 110, 141]. This technology also offers the advantage of real time analysis of antibody-antigen interaction kinetics (e.g. association (K_{ON}) and dissociation rate (K_{OFF}) constants) and requires no labelling of biomolecules. SPR depends on a change in mass when an antigen binds to an antibody and as hapten analytes are too small to measure a significant change in mass by direct binding, competitive assay formats are usually employed [110]. This format requires the immobilisation of the biotoxin-specific antibody to the chip surface which can in many cases be problematic [142] and so the majority of SPR-based assays to measure haptens employ the competitive format. The ic-SPR assay is based on the same principle described previously for the ic-ELISA where the analyte to be measured is incubated with antibody before injecting the mixture over the immobilised target ligand (low molecular weight biotoxin either as a free derivative or coupled to a carrier macromolecule) and the amount of biotoxin present in a sample is inversely proportional to the level of antibody binding. One caveat of ic-SPR is that the competing antibody must possess moderate affinity for the immobilised hapten (in other words possesses a relatively fast dissociation rate (K_{OFF})) so as the chip can be completely regenerated with relatively weak agents thus permitting a higher number of binding-regeneration cycles.

The first reported study of incorporation of a hapten biotoxin-specific rAb into an immuno-biosensor was by Moghaddam who wanted to address the difficulties in obtaining scFv capable of distinguishing soluble hapten over those generated towards the carrier protein and/or derivatised hapten immunogen [73]. They compared the efficacy of isolating scFv specific to free AFB1 from either a naïve human pooled lymphocyte-derived repertoire [143] or a hapten-biased semi-synthetic library [58]. The latter approach yielded the most success in isolating scFv to free AFB1 but only when hapten elution of the scFv-phage during biopanning was employed. A purified AFB1-specific scFv (Afla-45) was characterised by ic-SPR using a BIAcoreX biosensor wherein Afla-45 was pre-incubated with a range of concentrations of soluble AFB1 (0.5-4000 nM corresponding to 0.25 to 100 times Afla-45 concentration) or other mycotoxins (60 nM) and subsequently injected over a carboxy-methylated dextran-based flow cell (CM5) coupled to BSA-AFB1 or BSA alone. The

BSA signal generated was subtracted from that of the AFB1-BSA and the heterogeneous ligand-binding model was used to analyse scFv binding kinetics. Afla-45 exhibited nanomolar affinity to free AFB1 (Table 4) in buffer and recognised AFG1 equally well but not M1 or M2 or the unrelated ochratoxin A. The authors used AFB1 as a model antigen to demonstrate that a hapten-biased scFv library possessing specificity for one hapten (phOx) can be modified by rationale design to generate high affinity binders to other structurally unrelated haptens and as such no validation of Afla-45 performance in food samples has been undertaken to date (personal communication).

The AFB1-specific clone C4 scFv characterised by ELISA in the previous section was purified by immobilised metal affinity chromatography (IMAC) and incorporated into an BIAcore-based ic-SPR where a series of dilutions of AFB1 (0-195 ng/ml) were incubated with the rAb before injecting the mixture over a CM5 flow cell containing AFB1-BSA [90]. Multiple regeneration steps with high pH solution (10 mM NaOH) of the freshly immobilised conjugate ensured only stably coupled AFB1-BSA remained on the chip resulting in consistent binding responses over 50 cycles of sampling and non-specific binding of the scFv to a flow cell immobilised with BSA alone was negligible. The working range in buffer was determined to be between 3-195 ng/ml free AFB1 and the assay possessed acceptable accuracy and precision (intraday CV% 0.1-16.5 & interday CV% 3.7-11.8). Most interestingly the assay was more sensitive in detecting AFB1 in spiked grain samples than in buffer exhibiting a LOD as low as 0.75 ng/ml (detection range = 0.75-48 ng/ml). The clone C4 scFv assay, unlike Afla-45 scFv, exhibited a superior cross-reactivity profile (in particular recognises both AFM1 and AFM2) and was able to detect sub-regulatory amounts of AFB1 in corn (Table 5). The same group described a BIAcore inhibition SPR-based assay incorporating either a monomeric or dimeric AFB1-scFv where immobilised AFB1 derivative as target antigen was employed instead of a protein conjugate [121]. The reason for this change in immobilised ligand was due to difficulties encountered in regenerating the BSA-AFB1 chip. Studies revealed that a slightly stronger regeneration solution (25 mM NaOH) was required for the complete removal of dimer which still enabled over 500 reproducible binding cycles to be completed. The monomeric assay had a working range of 0.375-12 ng/ml of free AFB1 in PBS containing 5% methanol and the dimer exhibited a slightly broader working range and recovery rates of around 80-110% were described reflecting a high degree of assay accuracy and reproducibility at least when screening in buffer. The scFv displayed a high specificity for AFB1 with only slight cross-reactivity to other aflatoxins (AFM1 & AFG1=12.5%). The sensitivity of the scFv-based SPR assays were comparable to a pAb-based SPR assay described by the same

group (linear range 3-98 ng/ml) [144] although the pAb exhibited superior cross-reactivity to other aflatoxins (Table 5).

Hu *et al* [99] hypothesised that a higher density of the anti-DA scFv 2H12 (27kDa) can be attached to a biosensor flow cell surface compared to the larger but lower affinity parent mAb molecule (160-180 kDa) would result in an extremely sensitive assay to detect DA in fish samples. The immobilisation characteristics of the scFv clone on various mesoporous silicate surfaces [145, 146] and the ability of 2H12 to be genetically engineered to correctly orientate the scFv for optimal antigen binding [147] were subsequently investigated but to date no 2H12-based biosensor to measure DA in fish samples or seawater has been described.

The performance of an AFB1-specific scFv (clone H4 described in previous section) produced by Yang was assessed with a BIAcore instrument by pre-incubation with free AFB1 (0-2.0 ng/ml) before injecting over an AFB1-BSA chip. The authors claim the ic-SPR assay possessed similar affinity (IC₅₀) to the ic-ELISA mentioned previously but didn't publish the value however the K_D for soluble AFB1 in buffer was determined using the Langmuir 1:1 model to be 1.2×10^{-12} M. A scFv isolated from an immunised phage library using an AFB1-specific mAb (2C12) as a template [139] was highly-specific for AFB1 and unable to recognise any other unrelated mycotoxins (OTA, FMB1, FMB2, DON and T-2 toxin) tested by ELISA. The scFv affinity for free AFB1 in buffer (Table 4) was approximately 17-fold lower than that of the parent mAb. The anti-AFB2 scFv clone E9 produced by Edupuganti [124] (reviewed in the previous section) exhibited nanomolar affinity (Table 4) for an immobilised AFB2 derivative using a BIAcore3000 instrument and exhibited marginally higher affinity to free AFB2 in buffer (8 ng/ml IC₅₀) than in an almond matrix (11 ng/ml IC₅₀) with acceptable assay precision over 5 separate days (CV% <11).

Lateral flow immunoassays

Lateral flow immunoassays (LF-IA) are a commonly used example of a micro-fluidic immunosensor where flow through of the analyte in running buffer and detector antibody (usually conjugated to colloidal gold for visual detection or fluorescent nanoparticles for fluorescent scanner detection) suitable support surface such as nitro-cellulose is driven by capillary forces [148]. Following on from the successful isolation of microcystin-specific scFv ELISAs from naïve synthetic rAb repertoires [64, 129] (Table 4) capable of detecting MC LR in water below the WHO guidelines [149, 150] the same group produced a commercially available scFv-based LF-IA dipstick (Microcystin kit ImmunoStrip® (ISK 84300) Agdia Inc., USA). The assay is based on the competitive inhibition of colloidal gold-labelled scFv (scFv-Au) detector antibody by analyte [137]. The scFv

LF-IA was validated using a range of water qualities (distilled, tap or lake water) artificially fortified with seven microcystins or the closely related nodularin which were detected at 0.5 µg/l (Table 5). Despite this the manufactures recommend a detection threshold of 10 µg/L⁻¹ which enables users to determine if the MC levels are approaching the moderate risk guideline value for recreational activity in water of 20 µg/L⁻¹ [150]. The scFv LF-IA distinguished toxic from non-toxic cyanobacterial extracts and correlated well with HPLC analysis of real life water samples contaminated with harmful algae blooms giving no false positives. The authors did note that the dipstick failed to work in brackish water samples which suggests some element of pre-screening de-salination of these samples would be required in these cases. The first LF-IA published to quantify MC LR in water used fluorescently-labelled (Alexa-647) mAb as the detector signal and a portable fluorescent scanner was designed to measure the signal [151]. This assay system possessed a higher sensitivity than the scFv-based assay (0.05 µg/l LOD in buffer) and the authors also noted a deleterious effect when monitoring low levels of microcystins in river water samples albeit at levels over an order of magnitude below the WHO guidelines (MC LR concentration < 0.1 ng/l). LF-IA technology is compatible with multi-analyte detection simply by adding several lines of immobilised antigen or antibodies with different specificities to the strip and dipsticks employing conventional Abs to measure up to six co-occurring *Fusarium* mycotoxins in cereals (ZEN, DON, T-2, HT-2, FB1, FB2 & FB3) have been described [152].

Year published	Biotoxin target	Library	Recombinant antibody form (clone ID)	Assay platform	Affinity to free biotoxin	Reference	Comments
1997	ZEN	Immunised (Hybridoma)	scFv (QY1.5)	ELISA	14 ng/ml (IC50)	[82]	Comparable affinity to parent mAb but enhanced cross reactivity
2000	MC LR	Human synthetic (Tomlinson I)	scFv (3D10)	ELISA	4×10^{-6} M (IC50)	[133]	First rAb isolated from naïve synthetic phage library
2001	ZEN	Immunised (Hybridoma)	scFv (QY1.5)	ELISA	4 ng/ml (LOD)	[83]	More sensitive than QY1.5 ic-ELISA but more susceptible to solvent destabilisation
	AFB1	Human naïve	scFv (Afla-45)	SPR	6×10^{-9} M (K_D)	[73]	Only semi-synthetic library generated high affinity scFv
2002	MC LR	Human semi-synthetic (Griffin.1)	scFv (3A8)	ELISA	4.5×10^{-9} M (IC50)	[129]	Incorporated into immunoaffinity column for concentration of MC LR from water
	MC LR	Human semi-synthetic (Griffin.1)	scFv	ELISA	13×10^{-9} M (IC50)	[64]	scFv 300-fold greater sensitivity than those isolated from synthetic scFv repertoire
	AFB1	Immunised (mouse splenocytes)	scFv	ELISA SPR	98 ng/ml 0.75 ng/ml (LOD)	[90]	scFv sensitivity higher in grain than assay buffer

2004	DON	Immunised (hybridoma)	scFv	SPR	$3.7 \times 10^{-6} \text{ M (K}_D\text{)}$	[134]	scFv affinity weaker than parent mAb ($8.8 \times 10^{-8} \text{ M K}_D$)
	CTX	Immunised (mouse splenocytes)	Fab (1C49)	ELISA	Affinity to natural CTX not published	[93]	Fab had weak affinity (10^{-5} M) for natural CTX
2005	AFB1	Not published	scFv monomer/dimer	SPR	0.375 ng/ml (monomer) 0.19 ng/ml (dimer) (LOD)	[121]	AFB1 derivative immobilised on flow cell permits over 500 reproducible binding-regeneration cycles
	DA	Immunised (Hybridoma)	scFv (2H12)	ELISA	245 ng/ml (IC50)	[99]	More sensitive than parent mAb (1000 ng/ml IC50)
	FB1	Human synthetic (ETH-2)	scFv (BFB1-02)	SPR	$4.08 \times 10^{-7} \text{ M (K}_D\text{)}$	[68]	scFv specificity for FB1 & FB2
2006	DA	Immunised (Chicken)	scFv (R4Sh-7)	ELISA	156 ng/ml (IC50)	[101]	scFv affinity-matured to be more sensitive than parent pAb (1000 ng/ml IC50)
2007	DON	Immunised (hybridoma)	scFv	ELISA	8.2 ng/m(IC50)	[88]	Equivalent affinity but enhanced cross reactivity to DON metabolites of scFv when compared to parent mAb
	ZEN	Immunised (hybridoma)	scFv	OS-IA	20 ng/ml (LOD)	[113]	OS-IA working range (0.1-10 $\mu\text{g/ml}$) broader than that of ic-ELISA (3-0.1 $\mu\text{g/ml}$)
2008	ZEN	Immunised (Hybridoma)	scFv (QY1.5)	c-ELISA	1 $\mu\text{g/ml}$	[84]	Compared favourably to commercial ELISA when screening real life wheat samples

	ZEN/DON	Immunised (Hybridoma)	Bispecific scFv dimer (QY1.5)	ELISA	$9.6 \times 10^{-10} \text{M}$ (K_{aff})	[89]	Bispecific scFv retained affinity of monomeric scFv
	15-ac-DON	Immunised (Llama)	VHH (NAT-267 monomer or pentamer)	FPIA	0.5 μM (monomer) 1.24 μM (dimer) (IC50)	[135]	Monomer & pentamer affinity comparable to parent pAb (1.42 μM)
	OTA	Immunised (alpaca)	VHH	ELISA	12 ng/ml (IC50)	[136]	First rAb produced to OTA
	DA	Immunised (Sheep splenocytes)	ScFv (DA24cB7)	ELISA	2.6 ng/ml (IC50)	[100]	Retained sensitivity of parent pAb (2.8 ng/ml IC50)
2009	AFB1	Human synthetic (Tomlinson I)	scFv	ELISA	0.4 $\mu\text{g/ml}$	[102]	Compared four elution conditions and found hapten elution/trypsinisation was optimal
	AFB1	Human synthetic (Tomlinson I+J)	scFv	ELISA SPR	0.4 ng/ml (IC50) $1.2 \times 10^{-12} \text{M}$ (K_D)	[103]	scFv stabilised by interdomain disulfide bond
2010	MC LR	Not published	scFv	LF-IA	0.1-0.5 $\mu\text{g/l}$	[137]	Commercially available scFv-based lateral flow device for detecting microcystins and nodularin
	FB1	Immunised (Hybridoma)	scFv	ELISA	Not reported	[138]	Parent mAb possesses 12-fold greater affinity (10 ppb LOD & 220 ppb IC50)
2011	AFB1	Human naïve 140 human donors	scFv	ELISA	0.12 $\mu\text{g/ml}$ (YM1-C3)	[105]	Direct fusion to alkaline phosphatase (AP) label enhances sensitivity 3-fold

		(Yamo-1)			0.034 µg/ml (YM1-C3-AP) (IC50)		
	AFB1	Immunised (hybridoma)	scFv	SPR	1.16×10^{-7} M (K_D)	[139]	Parent mAb 17-fold better affinity (6.95×10^{-9} M) then scFv
2012	MC LR	Murine naïve synthetic	Fab	TRFIA	0.435 µg/ml (IC50)	[140]	MC-LR scFv rapidly isolated from mouse naïve library analysed by time-resolved fluorometry
2013	GTX2/3	Immunised (Hybridoma)	scFv	OS-ELISA	0.05 ng/ml (LOD)	[106]	Wider working range of OS-ELISA (0.05-300 ng/ml) compared to equivalent ic-ELISA (0.7-130 ng/ml)
	MC LR	Murine naïve synthetic (ScFvM)	scFv (M-MCLR1)	c-TRFIA	23 nM (IC50)	[118]	MC-LR scFv rapidly isolated from mouse naïve library analysed by time-resolved fluorometry
	AFB2	Immunised (mouse splenocytes)	scFv (E9)	ELISA SPR	3 ng/ml (LOD) 0.9 ng/ml (LOD)	[124]	scFv–SPR biosensor capable of detecting sub-regulatory levels of AFB2 in artificially contaminated almonds
	AFB1	Not published	Fab	ELISA	27 ng/ml (IC50)	[120]	Sensitivity of AFB1 covalent immobilised to microtiter plate was 2.7-fold greater than that of passively coated AFB1-BSA assay (77 ng/ml IC50)
2014	AFB1	Immunised (alpaca)	VHH (Nb26)	ELISA	0.75 ng/ml (IC50)	[87]	Produced a rAb resistant to high solvent concentrations

	AFB1	Immunised (Pooled hybridoma)	scFv (2G7)	ELISA	0.01 ng/ml (IC50)	[126]	Developed a scFv capable of measuring total aflatoxins
	MC LR	Human semi-synthetic (Griffin.1)	scFv-AP (3A8)	ELISA	2.97 μ M (IC50)	[130]	Moderate 3A8 affinity due to heterologous scFv population
	FB1	Immunised (Hybridoma)	scFv	ELISA	12.67 μ g/L (IC50)	[131]	Similar affinity (10.16 μ g/L IC50) and specificity to parent mAb

Abbreviations: fluorescence polarization immunoassay (FPIA), open sandwich ELISA (OS-ELISA), affinity constant (K_{aff}), time-resolved immunoassay (TRFIA), lateral flow immunoassay (LF-IA)

Table 3. Recombinant antibodies (rAb) produced to hapten biotoxins.

Regulatory Limits	Assay format	Lowest detected levels in food matrix	Matrix tested	Antibody cross-reactivity (%)	Reference
AFB1					
12-0.1 ppb (µg/kg) [122] 300-20 ppb [153] 200-1 ppb [154]	Fab ic-ELISA	12 ppb (35 ppb IC50)	grain	AFB2 2, AFG1 18, AFG2 1	[120]
	VHH ic-ELISA (clone Nb26)	3 ppb IC50	Peanut, rice, corn, feedstuff	AFB2 1, AFG2 2, AFM1 11	[87]
	pAb c-ELISA	8 ppt	Rice, beans	Not reported	[155]
	cAb-ELISA ^{N/A}	20 ppb	Corn, wheat, peanut butter & feeds	Total Aflatoxins (AFB1+B2+G1+G2) but % cross reactivity not reported	[126][102]Neo gen Corp., USA (Agri-Screen for aflatoxin)
	cAb-ELISA ^{N/A}	1.75 ppb	Cereals & feed	Total Aflatoxins AFB1 100, AFB2 48, AFG1 75, AFG2 18	Biopharm AG, Germany (RIDASCREE N® Aflatoxin Total)
	pAb c-ELISA	25 ppt 2.5 ppb	cereals & nuts animal feed	AFB2 20, AFG1 17, AFG2 4	Europroxima BV; The Netherlands (5121AFB)
	scFv ic-SPR (clone C4)	0.75 ppb	grain	AFB2 53, AFG1 76, AFG2 68, AFM1 50, AFM2 23, AFB2a 18, AFG2a 22	[90]
	mAb ic-SPR	0.2 ppb	grain	Not reported	[156]
	pAb Ic-SPR	3 ppb	No	AFB2 68, AFG1 66, AFG2 50, AFM1 35, AFM2 2, AFB2a 33, AFG2a 16	[144]
AFB2	scFv SPR (E9)	0.9 ppb	Almonds	AFB1 0, AFG1 0, AFG2 0, AFM1 0	[124]

DON					
8 -0.9 ppb (µg/kg) Commission et al. 2007) 2 –0.3 ppb [158]	scFv ELISA	8 ppb IC50	Wheat	3-Ac-DON 6, 15-Ac-DON 5, Nivalenol < 1, T-2 < 1	[88]
	mAb ELISA	8 ppb IC50	Wheat	3-Ac-DON 8, 15-Ac-DON 5, Nivalenol < 1, T-2 < 1	Neogen Corp, USA (Veratox 5/5) [88]
	cAb-ELISA ^{N/A}	3.7-18.5 ppb	Cereals, malt, feed, beer & wort	3-Ac-DON 100, 15-Ac-DON 19, Nivalenol 4, T-2 < 1	Biopharm AG, Germany (RIDASCREE N® DON)
	pAb ELISA	10-30 ppb	Cereals, food, feed & beer	3-Ac-DON 96, 15-Ac-DON < 1, Nivalenol 40, T-2 < 1	Europroxima BV; The Netherlands (5121DON)
	cAb-ELISA ^{N/A}	1 ppm	Wheat products	3-Ac-DON but % cross reactivity not reported	Neogen Corp., USA (Agri-Screen for DON)
FB1					
3 - 1 ppm (mg/kg) [158] 4 – 2 ppm [85] 2 – 4 ppm [159]	scFv ELISA	8 ppb (13 ppb IC50)	wheat	FB2 5, AFB1 < 1, DON < 1, ZEN < 1, OTA < 1	[131]
	mAb ELISA	10 ppb IC50		FB2 9, AFB1 < 1, DON < 1, ZEN < 1, OTA < 1	
	mAb ELISA	2.2 ppb IC50	maize	FB2 154, AFB1 < 1, DON < 1, OTA < 1	[160]
	pAb ELISA	0.05 ppb	maize	FB2 72, FB3 5, AFB1 < 1	[161]
	cAb-ELISA ^{N/A}	25 ppb	Corn & corn products	FB2 40, FB3 100	Biopharm AG, Germany (RIDASCREE

					N® fumonisin)
	pAb ELISA	2.3 ppb	maize	FB2 27, FB3 76	Europroxima BV; The Netherlands (5121FUM)
	cAb-ELISA ^{N/A}	5 ppb	Barley, corn, rice, soybean & wheat	Total fumonisins (FB1, B2, B3) but % cross reactivity not reported	Neogen Corp., USA (Agri-Screen for fumonisins)
OTA					
80-0.5 ppb (µg/kg) [162] 50-3 ppb [158]	VHH ic-ELISA	17-21 ppb (IC50)	Wine, coffee	OTB 2.5 , L-Phe <1, coumarin <1	[136]
	mAb ic-ELISA	2.5 ppb	Cereals	OTB 17, AFB1<8.5, FB1 9.4, DON 9.6	[163]
	pAb c-ELISA	10 ppb	Soybean, cereals, corn, coffee	OTB 0.8 , OTC 167, citrinin <1, L-Phe <1	[164]
	cAb-ELISA ^{N/A}	1.25-2.5 ppb 50 ppt	Cereals, feed Beer	OTC: 44, OTB: 14, Ochratoxin α< 1	Biopharm AG, Germany (RIDASCREE N® Ochratoxin A)
	pAb ELISA	1-0.5 ppb	Wine, beer, cereals, cacao, chocolate, green coffee & feed	OTB 9.3, ochratoxin-α <1 ,coumarin <1, D-Phe <1	Europroxima BV; The Netherlands (5121OCH)
	cAb-ELISA ^{N/A}	1 ppb	Cereals, coffee &	OTB 18	Neogen Corp., USA (Veratox for

			dried fruits		Ochratoxin)
ZEN					
1000-50 ppb (µg/kg) Cereals & maize [158] 350-100 ppb [85]	scFv c-ELISA	275 ppb	Maize	α-ZEL 82, β- ZEL 26, α- ZAL 62, β- ZAL 26	[82–84]
	scFv ic-ELISA	1000 ppb			
	mAb c-ELISA	50 ppb		α-ZEL 26,β- ZEL 11, α- ZAL 8, β- ZAL 10	
	mAb c-ELISA (AOAC official method)	800 ppb	Maize, wheat & feed	α-ZEL 107, β- ZEL 29, α- ZAL 35, β- ZAL 25	[86]
	mAb c-ELISA	150 ppm	Feed	α-ZEL 101, β- ZEL 121, α- ZAL 122, β- ZAL 152	[165]
	cAb-ELISA ^{N/A}	1.75 ppb 0.25 ppb	Cereals, feed Beer	α-ZEL 42 β- ZEL 14	Biopharm AG, Germany (RIDASCREE N® Zearalenon)
	pAb c-ELISA	15.8 ppb 58.4 ppb	Cereals Animal feed	α-ZEL 175, β- ZEL 103, α- ZAL 146, β- ZAL 194	Europroxima BV; The Netherlands (5121ZON)
	mAb c-ELISA	25 ppb	Corn, wheat, barley	α-ZEL 73, β- ZEL 23, α- ZAL 36, β- ZAL 15	Neogen Corp., USA (Veratox® for ZEN)
MC LR					
1.0 ppb (µg/L) cell-bound or free in drink water [149]	scFv ic-ELISA (3A10)	0.8 ppb	distilled water/cyano bacterial extracts	MC-RR, 2250, -LW 2, -LF 2, Nod 38	[129]

20.0 ppb recreational water [150]	pAb ic-ELISA	1.0 ppb	Fresh, brackish & marine water	MC-LW 50, -LF 93, -LY 125, -LA 125, Nod 44	[166]
	pAb c-ELISA	0.31 ppb	cyanobacterial extracts/surface water	MC-RR 100, -YR 82, Nod 66	Strategic Diagnostic Inc., USA (Enviroguard® Microcystins) [167]
	pAb ic-ELISA	0.05 ppb	Surface/drinking water	MC-RR 50, -LW 118, -LF 108, , -YR 167, Nod 100	[127]
	mAb ELISA	0.1 ppb	Surface water	MC-RR 8, -LW < 1, -LF < 1, -YR 12, Nod < 1	[168]
	cAb-ELISA ^{N/A}	0.16-2.5 ppb	Surface water	M-RR 54, -YR 35, -LA 62, Nod 68	Envirologix™, USA (QuantiPlate™ Kit for Microcystins)
	scFv LFD	0.5 ppb	Tap, distilled & raw water	MC-RR 100, -LW 500, -LF > 100 -LY > 100 -D-RR 100 -Me-LR 50 Nod 500	[137]
	scFv LFD	0.095 ppb	Surface water	Cross-reactivity to MC-RR & -YR detected but not reported as % relative cross-reactivity	[151]
GTX2/3					
800 ppm (µg STX equivalent/kg shellfish meat) [111, 112]	scFv OS-IA (GT-13a)	800 ppm	Shellfish	STX 6.4, neo-STX <1	[106]
	mAb ic-SPR (GT-13a)	160 ppm	Shellfish	dcGTX2/3 111, C1/2 106, STX 95, dcSTX 79, GTX5 97, daSTX 49, dhSTX 75, dcSTX 79 ^A	[109]

	mAb MS-FC (GT-13a)	260 ppm	Shellfish	dc-GTX2/3 100 , C1/2 135 , GTX1/4 <1, STX 66, dcSTX 39, GTX5 78 ^A	[108]
	mAb ic- ELISA (GT-13a)	Not tested	Not tested	dc-GTX2/3 68, C1/2 54, GTX1/4 8, STX 6.9, neoSTX <1 ^A	[107]
DA					
20 ppm (mg/kg) [169]	scFv ELISA	156 ppb	Shellfish	Not reported	[101]
	scFv ELISA	0.3 ppb	Shellfish	Not reported	[100]
	mAb ELISA	0.15 ppb	Shellfish	Isodomoic acid -A 71, -B 1.3, -E < 1, -F 1.4, -G < 1, -H < 1, KA < 1, L-AA < 1, L-GA < 1	[170]
	pAb ELISA (AOAC official method)	0.3 ppb	Algae / shellfish / surface water	KA < 1	(Biosense Laboratories AS, Norway (ASP ELISA kit) [171])
	pAb ELISA	21-23 ppb	Shellfish	PSP toxins < 1, okadaic acid <1	Europroxima BV; The Netherlands (5191DOMO)

Abbreviations: microsphere-based flow cytometry (MS-FC), 3-acetyl-DON (3-Ac-DON), 3-acetyl-DON (15-Ac-DON), L-phenylalanine (L-Phe), zearalenol (ZEL), zearalanol (ZAL), ochratoxin (OTA), nodularin (Nod), decarbamoyl-GTX2/3 (dc-GTX2/3), N-sulfocarbamoyl-GTX2/3 (C1/2), saxitoxin (STX), dihydrochloride (STXdi-HCl), decarbamoyl saxitoxin (dcSTX), decarbamoyl neosaxitoxin (dcNEO), neosaxitoxin (neoSax), kainic acid (KA), L-aspartic acid (L-AA), L-glutamic acid (L-GA)

Table 4 Comparison of recombinant antibody performance to a selection of animal-derived conventional antibodies in environmental or food samples.

^A adapted by reviewer to represent relative cross reactivity to GTX2/3

^{N/A} Unknown if conventional antibody (cAb)-based assay is pAb or mAb as external publication not available

Future perspectives

To our knowledge every published rAb produced to hapten biotoxins have been generated using phage display technology despite the fact that the rAb with the highest known affinity to a hapten was produced using yeast display. A yeast-displayed scFv exhibiting subnanomolar affinity to the model hapten fluorescein was affinity matured *in vitro* resulting in creation of a “super antibody” with femtomolar (10^{-15} M) binding affinity similar to that of the streptavidin-biotin interaction [34, 172]. A significant limitation of phage display is the unpredictable expression of mammalian antibodies in bacterial expression systems commonly employed however eukaryotic systems such as yeast produce a higher ratio of functional antibody because they possess cellular mechanisms ensuring display of correctly folded rAb [173]. For this reason yeast display is considered a superior platform for surface displaying a fully representative functional rAb repertoire for affinity selection and post-production *in vitro* affinity maturation processes. Yeast display has produced hapten-specific rAb with nanomolar (thiazole orange) to picomolar (tacrolimus) affinity [174] and would be an ideal platform for generating high affinity binders to hapten biotoxins.

Due to the co-occurrence of multiple phycotoxins in shellfish or mycotoxin contaminations of plant materials the emphasis in biotoxin screening assays has moved towards simultaneous detection of several toxins in a single sample [156, 175, 176]. Antibody microarrays offer the prospect of rapid multiplex detection of analytes and require only a fraction of antibody reagent used in conventional immunoassay formats (pL droplet compared to μ l volumes for ELISA). The functional stability of conventional Abs in a microarray setting varies significantly but rAb have been found to constitute an excellent probe source for such applications. Some rAb-based phage libraries (e.g. n-CoDeR, Table 2) have been validated to provide probes displaying excellent on-chip performances all based on a single, fixed antibody scaffold which in some cases possess a shelf-life of over a year [177]. Other advantages are (1) rAb repertoires can be designed with affinity tags for immobilisation of antibodies to maximise antigen interactions, (2) their small size facilitates the production of high density arrays and (3) arrays based on a single, fixed scaffold means that all probes are homogenous (except for antigen-binding sites) which minimizes any probe-dependent assay variations.

In most cases reviewed herein success of affinity selection of hapten-specific rAbs was evaluated manually by ELISA which enables random screening of a relatively small proportion of hundreds of possible

valuable rAb clones. This “flashlight” approach opens the possibility of missing many highly valuable antibodies due to the workload required to screen every single clone. Automated robotic high-throughput screening [62] and next generation sequencing (NGS) of antibody genes have demonstrated the way forward in this regard. Deep sequencing analysis has been successfully carried out on peptide [178] and naïve scFv libraries [179] and can identify paratopes of potential hapten binders as well as help eliminate false-positive hits using only sequencing data. Replacement of conventional randomly picked scFv phage ELISA screening with in depth NGS analysis of CDR frequency from a scFv phage library following affinity selection retrieved the most potent target-specific scFv, some of which, were missed using classical *in vitro* screening [180]. With this in mind a low cost approach utilising open source software (AbMining ToolBox <http://sourceforge.net/projects/abmining/>) for H CDR3 deep sequencing outputs has recently been described and validated using a naïve and natural scFv repertoire [181]. Proteomics-based approaches marrying mass spectrometrical (LC-MS/MS) shotgun analysis of polyclonal serum antibodies (in particular HCDR3) produced following immunisation and V gene sequence database generated by NGS analysis of the same animals B cells has been used to rapidly identify heavily enriched populations of antigen-specific antibodies [182]. Such proteomics approaches avoid the often cumbersome technical and practical challenges involved in conventionally produced antigen-specific conventional and recombinant Abs and have resulted in the rapid production of recombinant mAbs with affinities surpassing that of the source pAb serum [183, 184].

Conclusion

The last two decades has seen the development of a number of rAb-based immunoassays for the rapid screening of hapten biotoxins in foods and environmental samples. The potential advantages of rAb over their conventional counterparts are as follows; (1) generating binders from naïve rAb repertoires circumvents the need for animal work in accordance with European legislation on animal protection [188], (2) the faster production pipelines (1-2 months from a naïve library) relative to conventional antibody production, (3) greater opportunities for improvement of rAb performance post-production by *in vitro* genetic manipulations, (4) haptens do not require the modifications (derivatives or conjugated to carrier molecules) necessary for animal immunisations in order to generate *in vitro* binders, (5) the antigen-driven inter-chain interaction of antibody V domains can be exploited in non-competitive immunoassays and, (6) rAb can be generated to extremely potent biotoxins *in vitro* that would not be possible *in vivo*. However in all the rAb-based assays reviewed herein there

is not a single example where a conventional Ab-based assay has not been published with equivalent or superior sensitivity or specificity (Tables 5 and 6). A note of caution is highlighted in the case of AFB1, the biotoxin to which most rAb have been produced to date, where of the thirteen AFB1-specific rAb-based immunoassays reported only two ELISAs [87, 120] and one SPR assay [90] were successfully validated using a relevant food matrix. This suggests that AFB1 was used more as a model ligand to validate the ability of a phage display library to generate hapten-specific rAb than to produce an immunoanalytical platform for AFB1 detection in food and feeds.

rAb possess several advantages over conventional Ab however most cases reviewed herein are produced from immunised animals thus necessitating animal work. The use of naïve natural and synthetic repertoires avoids animal use and can produce rAb in a shorter time period than the immune approach however affinities from these libraries are usually lower than those of immunised approach. It must also be noted that commercialised immunoassays employing rAbs isolated from commercially available naïve phage display libraries can be subject to licensing fees and some companies have restricted access to their libraries to protect intellectual property rights (Table 2). On the whole, rAb technology provides great potential in enhancing hapten biotoxin detection systems which to date has yet to be fully harnessed.

Hapten biotoxin	Conventional antibody	Recombinant antibody
Mycotoxins		
Aflatoxins	Validated commercial and “in house” pAb/mAb-based assays available capable of detecting sub-regulatory levels of -AFB1 -total aflatoxins (AFB1+B2+G1+G2) -AFM1/M2 (milk) In a wide range of foods, feeds & cereals	Two rAb-based ELISA and one SPR assays validated to detect AFB1 in cereals One scFv SPR validated to detect AFB2 in nuts No rAb –based assays commercially available
Tricothecenes (DON)	Validated commercial and “in house” assays available for DON testing in food, feed & drink	scFv ELISA capable of detecting sub-regulatory levels of DON in wheat- not commercially available
Fumonisin	Validated commercial and “in house” assays available immunoassays for -FB1 - total fumonisins (FB1,B2,B3) in a wide range of cereals	scFv ELISA capable of detecting sub-regulatory levels of FB1 in wheat- not commercially available
Ochratoxins	Validated commercially available and “in house” assays capable of detecting sub-regulatory levels of OTA in wide range of foods, beverages, cereals and feeds	VHH ELISA capable of detecting sub-regulatory levels of OTA in beverages- not commercially available
ZEN	Validated commercial available and “in house” assays capable of detecting sub-regulatory levels of OTA in wide range of cereals and feeds	scFv ELISA capable of detecting sub-regulatory levels of ZEN in maize- not commercially available

Aquatic & Marine Toxins		
ASP toxins (DA)	Validated commercial available and “in house” ELISAs capable of detecting sub-regulatory levels of DA in shellfish and water	Two scFv ELISAs capable of detecting sub-regulatory levels of DA in shellfish- not commercially available
Ciguatoxins (CTX)	mAb-based “in house” ELISAs reported No commercially antibody-based assays available	Fab-based assay possesses weak affinity for natural toxin
PSP toxins (GTX2/3)	Validated pAb/mAb-based assays capable of detecting greater number of PSP toxins at or below regulatory limit in shellfish	scFv OS-ELISA capable of detecting 4 PSP toxins at regulatory limit in shellfish- not commercially available
Microcystins	Validated commercial and “in house” assays available for detection of sub-regulatory levels of microcystins	Validated “in house” ELISAs- not commercially available Commercially available LFD available

Table 5. Summary of the progress of recombinant (rAb) and conventional Ab (pAb or mAb)-based immunoassays in detecting hapten biotoxins in food and environmental samples. Compiled from Table 5 and [77, 78, 110, 185–187]

Bibliography

1. Van Egmond HP (2004) Natural toxins: risks, regulations and the analytical situation in Europe. *Anal Bioanal Chem* 378:1152–60. doi: 10.1007/s00216-003-2373-4
2. Kantiani L, Llorca M, Sanchís J, et al. (2010) Emerging food contaminants: a review. *Anal Bioanal Chem* 398:2413–27. doi: 10.1007/s00216-010-3944-9
3. Hess P (2010) Requirements for screening and confirmatory methods for the detection and quantification of marine biotoxins in end-product and official control. *Anal Bioanal Chem* 397:1683–94. doi: 10.1007/s00216-009-3444-y
4. Di Stefano V, Avellone G, Bongiorno D, et al. (2012) Applications of liquid chromatography-mass spectrometry for food analysis. *J Chromatogr A* 1259:74–85. doi: 10.1016/j.chroma.2012.04.023
5. Rossini GP (2005) Functional assays in marine biotoxin detection. *Toxicology* 207:451–62. doi: 10.1016/j.tox.2004.10.012
6. Campbell K, Vilariño N, Botana LM, Elliott CT (2011) A European perspective on progress in moving away from the mouse bioassay for marine-toxin analysis. *TrAC Trends Anal Chem* 30:239–253. doi: 10.1016/j.trac.2010.10.010
7. Sheedy C, MacKenzie CR, Hall JC (2007) Isolation and affinity maturation of hapten-specific antibodies. *Biotechnol Adv* 25:333–52. doi: 10.1016/j.biotechadv.2007.02.003
8. Yau KYF, Lee H, Hall JC (2003) Emerging trends in the synthesis and improvement of hapten-specific recombinant antibodies. *Biotechnol Adv* 21:599–637.
9. Fodey T, Leonard P, O'Mahony J, et al. (2011) Developments in the production of biological and synthetic binders for immunoassay and sensor-based detection of small molecules. *TrAC Trends Anal Chem* 30:254–269. doi: 10.1016/j.trac.2010.10.011
10. Skerra A (2003) Imitating the humoral immune response. *Curr Opin Chem Biol* 7:683–93.
11. Nishimiya D (2014) Proteins improving recombinant antibody production in mammalian cells. *Appl Microbiol Biotechnol* 98:1031–42. doi: 10.1007/s00253-013-5427-3
12. Web of Science.
http://apps.webofknowledge.com/Search.do?product=UA&SID=N2JYPERxqToSQuxv2DD&search_mode=GeneralSearch&prID=f7835cc2-b4b4-45e0-882d-f9d16c41d8df.
13. Smith GP (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* 228:1315–7.
14. McCafferty J, Griffiths AD, Winter G, Chiswell DJ (1990) Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 348:552–4. doi: 10.1038/348552a0
15. Hoogenboom HR (2002) Overview of antibody phage-display technology and its applications. *Methods Mol Biol* 178:1–37.
16. Holliger P, Hudson PJ (2005) Engineered antibody fragments and the rise of single domains. *Nat Biotechnol* 23:1126–36. doi: 10.1038/nbt1142
17. Miersch S, Sidhu SS (2012) Synthetic antibodies: concepts, potential and practical considerations. *Methods* 57:486–98. doi: 10.1016/j.ymeth.2012.06.012

18. Xu JL, Davis MM (2000) Diversity in the CDR3 region of V(H) is sufficient for most antibody specificities. *Immunity* 13:37–45.
19. Wall JG, Plückthun A (1995) Effects of overexpressing folding modulators on the in vivo folding of heterologous proteins in *Escherichia coli*. *Curr Opin Biotechnol* 6:507–16.
20. Muyldermans S (2013) Nanobodies: natural single-domain antibodies. *Annu Rev Biochem* 82:775–97. doi: 10.1146/annurev-biochem-063011-092449
21. Wesolowski J, Alzogaray V, Reyelt J, et al. (2009) Single domain antibodies: promising experimental and therapeutic tools in infection and immunity. *Med Microbiol Immunol* 198:157–74. doi: 10.1007/s00430-009-0116-7
22. De Marco A (2011) Biotechnological applications of recombinant single-domain antibody fragments. *Microb Cell Fact* 10:44. doi: 10.1186/1475-2859-10-44
23. Vincke C, Muyldermans S (2012) Single Domain Antibodies. In: Saelens D, Muyldermans S (eds) *Humana Press*, Totowa, NJ, pp 15–26
24. Alvarez-Rueda N, Behar G, Ferré V, et al. (2007) Generation of llama single-domain antibodies against methotrexate, a prototypical hapten. *Mol Immunol* 44:1680–90. doi: 10.1016/j.molimm.2006.08.007
25. Dumoulin M, Conrath K, Van Meirhaeghe A, et al. (2002) Single-domain antibody fragments with high conformational stability. *Protein Sci* 11:500–15. doi: 10.1110/ps.34602
26. Harmsen MM, De Haard HJ (2007) Properties, production, and applications of camelid single-domain antibody fragments. *Appl Microbiol Biotechnol* 77:13–22. doi: 10.1007/s00253-007-1142-2
27. Muyldermans S, Cambillau C, Wyns L (2001) Recognition of antigens by single- domain antibody fragments : the superfluous luxury of paired domains. 26:230–235.
28. Spinelli S, Frenken LG, Hermans P, et al. (2000) Camelid heavy-chain variable domains provide efficient combining sites to haptens. *Biochemistry* 39:1217–22.
29. Spinelli S, Tegoni M, Frenken L, et al. (2001) Lateral recognition of a dye hapten by a llama VHH domain. *J Mol Biol* 311:123–9. doi: 10.1006/jmbi.2001.4856
30. Sheedy C, Yau KYF, Hiram T, et al. (2006) 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* 54:3668–78.
31. Kobayashi N, Oyama H (2011) Antibody engineering toward high-sensitivity high-throughput immunosensing of small molecules. *Analyst* 136:642–51. doi: 10.1039/c0an00603c
32. Chappey ON, Sandouk P, Scherrmann JM (1992) Monoclonal antibodies in hapten immunoassays. *Pharm Res* 9:1375–9.
33. Ponsel D, Neugebauer J, Ladetzki-Baehs K, Tissot K (2011) High affinity, developability and functional size: the holy grail of combinatorial antibody library generation. *Molecules* 16:3675–700. doi: 10.3390/molecules16053675
34. Boder ET, Midelfort KS, Wittrup KD (2000) Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity. *Proc Natl Acad Sci U S A* 97:10701–5. doi: 10.1073/pnas.170297297

35. Martin CD, Rojas G, Mitchell JN, et al. (2006) A simple vector system to improve performance and utilisation of recombinant antibodies. *BMC Biotechnol* 6:46. doi: 10.1186/1472-6750-6-46
36. Ueda H (2002) Open sandwich immunoassay: a novel immunoassay approach based on the interchain interaction of an antibody variable region. *J Biosci Bioeng* 94:614–9.
37. Oyama H, Tanaka E, Kawanaka T, et al. (2013) Anti-idiotypic scFv-enzyme fusion proteins: a clonable analyte-mimicking probe for standardized immunoassays targeting small biomarkers. *Anal Chem* 85:11553–9. doi: 10.1021/ac402868f
38. Abe R, Jeong H-J, Arakawa D, et al. (2014) Ultra Q-bodies: quench-based antibody probes that utilize dye-dye interactions with enhanced antigen-dependent fluorescence. *Sci Rep* 4:4640. doi: 10.1038/srep04640
39. Sherwood LJ, Hayhurst A (2012) Hapten mediated display and pairing of recombinant antibodies accelerates assay assembly for biothreat countermeasures. *Sci Rep* 2:807. doi: 10.1038/srep00807
40. Chen M, Wen K, Tao X, et al. (2014) Cloning, expression, purification and characterization of a bispecific single-chain diabody against fluoroquinolones and sulfonamides in *Escherichia coli*. *Protein Expr Purif* 100:19–25. doi: 10.1016/j.pep.2014.04.015
41. Zhang J, Tanha J, Hiramata T, et al. (2004) Pentamerization of single-domain antibodies from phage libraries: a novel strategy for the rapid generation of high-avidity antibody reagents. *J Mol Biol* 335:49–56.
42. Dimasi N, Gao C, Fleming R, et al. (2009) The design and characterization of oligospecific antibodies for simultaneous targeting of multiple disease mediators. *J Mol Biol* 393:672–92. doi: 10.1016/j.jmb.2009.08.032
43. Stone E, Hiramata T, Tanha J, et al. (2007) The assembly of single domain antibodies into bispecific decavalent molecules. *J Immunol Methods* 318:88–94. doi: 10.1016/j.jim.2006.10.006
44. Liu JL, Zabetakis D, Acevedo-Vélez G, et al. (2013) Comparison of an antibody and its recombinant derivative for the detection of the small molecule explosive 2,4,6-trinitrotoluene. *Anal Chim Acta* 759:100–4. doi: 10.1016/j.aca.2012.10.051
45. Zhou L-J, Zhen-Lin, Xu WH, Zhou, Li-Jun; Huang, Qi-Xin; Lei, Hong-Tao; Wang, Yu-Xiao; Wang, Qiang; Xu, Zhen-Lin; Wang, Hong; Sun Y-M (2012) Preparation of a Single-chain Variable Fragment for O,O-Diethyl Phosphorothioate Pesticides and Its Broad-specificity. *Chem J Chinese Univ* 33:2269–2274. doi: 10.7503/cjcu20120158
46. Park S, Lee D-H, Park J-G, et al. (2010) A sensitive enzyme immunoassay for measuring cotinine in passive smokers. *Clin Chim Acta* 411:1238–42. doi: 10.1016/j.cca.2010.04.027
47. Plana E, Moreno M-J, Montoya Á, Manclús JJ (2014) Development and application of recombinant antibody-based immunoassays to tetraconazole residue analysis in fruit juices. *Food Chem* 143:205–13. doi: 10.1016/j.foodchem.2013.07.121
48. Oyama H, Yamaguchi S, Nakata S, et al. (2013) “Breeding” diagnostic antibodies for higher assay performance: a 250-fold affinity-matured antibody mutant targeting a small biomarker. *Anal Chem* 85:4930–7. doi: 10.1021/ac3037602
49. Persson H, Wallmark H, Ljungars A, et al. (2008) In vitro evolution of an antibody fragment population to find high-affinity hapten binders. *Protein Eng Des Sel* 21:485–93. doi: 10.1093/protein/gzn024
50. Abe R, Ohashi H, Iijima I, et al. (2011) “Quenchbodies”: quench-based antibody probes that show antigen-dependent fluorescence. *J Am Chem Soc* 133:17386–94. doi: 10.1021/ja205925j

51. Hoogenboom HR, Griffiths AD, Johnson KS, et al. (1991) Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucleic Acids Res* 19:4133–7.
52. Perelson AS, Oster GF (1979) Theoretical studies of clonal selection: minimal antibody repertoire size and reliability of self-non-self discrimination. *J Theor Biol* 81:645–70.
53. Di Noia JM, Neuberger MS (2007) Molecular mechanisms of antibody somatic hypermutation. *Annu Rev Biochem* 76:1–22. doi: 10.1146/annurev.biochem.76.061705.090740
54. Mix E, Goertsches R, Zett UK (2006) Immunoglobulins--basic considerations. *J Neurol* 253 Suppl :V9–17. doi: 10.1007/s00415-006-5002-2
55. Griffiths a D, Williams SC, Hartley O, et al. (1994) Isolation of high affinity human antibodies directly from large synthetic repertoires. *EMBO J* 13:3245–60.
56. MacCallum RM, Martin a C, Thornton JM (1996) Antibody-antigen interactions: contact analysis and binding site topography. *J Mol Biol* 262:732–45. doi: 10.1006/jmbi.1996.0548
57. Persson H, Lantto J, Ohlin M (2006) A focused antibody library for improved hapten recognition. *J Mol Biol* 357:607–20. doi: 10.1016/j.jmb.2006.01.004
58. Braunagel M, Little M (1997) Construction of a semisynthetic antibody library using trinucleotide oligos. *Nucleic Acids Res* 25:4690–1.
59. Marks JD, Hoogenboom HR, Bonnert TP, et al. (1991) By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *J Mol Biol* 222:581–97.
60. Vaughan TJ, Williams AJ, Pritchard K, et al. (1996) Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nat Biotechnol* 14:309–14. doi: 10.1038/nbt0396-309
61. Pansri P, Jaruseranee N, Rangnoi K, et al. (2009) A compact phage display human scFv library for selection of antibodies to a wide variety of antigens. *BMC Biotechnol* 9:6. doi: 10.1186/1472-6750-9-6
62. De Wildt RM, Mundy CR, Gorick BD, Tomlinson IM (2000) Antibody arrays for high-throughput screening of antibody-antigen interactions. *Nat Biotechnol* 18:989–94. doi: 10.1038/79494
63. Lee CMY, Iorno N, Sierro F, Christ D (2007) Selection of human antibody fragments by phage display. *Nat Protoc* 2:3001–8. doi: 10.1038/nprot.2007.448
64. Strachan G, McElhiney J, Drever MR, et al. (2002) Rapid selection of anti-hapten antibodies isolated from synthetic and semi-synthetic antibody phage display libraries expressed in *Escherichia coli*. *FEMS Microbiol Lett* 210:257–61.
65. Silacci M, Brack S, Schirru G, et al. (2005) Design, construction, and characterization of a large synthetic human antibody phage display library. *Proteomics* 5:2340–50. doi: 10.1002/pmic.200401273
66. Pini a., Pini, A; Viti, F; Santucci, A; Carnemolla, B; Zardi, L; Neri PND (1998) Design and Use of a Phage Display Library. Human antibodies with subnanomolar affinity against a marker of angiogenesis eluted from a two-dimensional gel. *J Biol Chem* 273:21769–21776. doi: 10.1074/jbc.273.34.21769
67. Viti F, Nilsson F, Demartis S, Huber A ND, Viti F, Nilsson F, et al. (2000) Design and use of phage display libraries for the selection of antibodies and enzymes. *Methods Enzymol* 326:480–505.

68. Lauer B, Ottleben I, Jacobsen H-J, Reinard T (2005) Production of a single-chain variable fragment antibody against fumonisin B1. *J Agric Food Chem* 53:899–904. doi: 10.1021/jf048651s
69. Prassler J, Thiel S, Pracht C, et al. (2011) HuCAL PLATINUM, a synthetic Fab library optimized for sequence diversity and superior performance in mammalian expression systems. *J Mol Biol* 413:261–78. doi: 10.1016/j.jmb.2011.08.012
70. Rothe C, Urlinger S, Löhning C, et al. (2008) The human combinatorial antibody library HuCAL GOLD combines diversification of all six CDRs according to the natural immune system with a novel display method for efficient selection of high-affinity antibodies. *J Mol Biol* 376:1182–200. doi: 10.1016/j.jmb.2007.12.018
71. Söderlind E, Strandberg L, Jirholt P, et al. (2000) Recombining germline-derived CDR sequences for creating diverse single-framework antibody libraries. *Nat Biotechnol* 18:852–6. doi: 10.1038/78458
72. Carlsson R, Söderlind E (2001) n-CoDeR concept: unique types of antibodies for diagnostic use and therapy. *Expert Rev Mol Diagn* 1:102–8. doi: 10.1586/14737159.1.1.102
73. Moghaddam A, Løbersli I, Gebhardt K, et al. (2001) Selection and characterisation of recombinant single-chain antibodies to the hapten Aflatoxin-B1 from naive recombinant antibody libraries. *J Immunol Methods* 254:169–81.
74. Van Wyngaardt W, Malatji T, Mashau C, et al. (2004) A large semi-synthetic single-chain Fv phage display library based on chicken immunoglobulin genes. *BMC Biotechnol* 4:6. doi: 10.1186/1472-6750-4-6
75. Tanha J, Xu P, Chen Z, et al. (2001) Optimal design features of camelized human single-domain antibody libraries. *J Biol Chem* 276:24774–80. doi: 10.1074/jbc.M100770200
76. Ohtani M, Hikima J, Jung TS, et al. (2013) Construction of an artificially randomized IgNAR phage display library: screening of variable regions that bind to hen egg white lysozyme. *Mar Biotechnol (NY)* 15:56–62. doi: 10.1007/s10126-012-9456-1
77. McElhiney J, Lawton LA (2005) Detection of the cyanobacterial hepatotoxins microcystins. *Toxicol Appl Pharmacol* 203:219–30. doi: 10.1016/j.taap.2004.06.002
78. Li P, Zhang Q, Zhang W (2009) Immunoassays for aflatoxins. *TrAC Trends Anal Chem* 28:1115–1126. doi: 10.1016/j.trac.2009.07.003
79. Maragos CM (2009) Recent advances in the development of novel materials for mycotoxin analysis. *Anal Bioanal Chem* 395:1205–13. doi: 10.1007/s00216-009-2728-6
80. Brichta J, Hnilova M, Viskovic T (2005) Generation of hapten-specific recombinant antibodies : antibody phage display technology : a review. 2005:231–252.
81. Dixon DE, Warner RL, Ram BP, et al. (1987) Hybridoma Cell Line Production of a Specific Monoclonal Antibody to the Mycotoxins Zearalenone and α -Zearalenol. 122–126.
82. Yuan Q, Clarke JR, Zhou HR, et al. (1997) Molecular cloning, expression, and characterization of a functional single-chain Fv antibody to the mycotoxin zearalenone. *Appl Environ Microbiol* 63:263–9.
83. Lee MG, Yuan QP, Hart LP, Pestka JJ (2001) Enzyme-linked immunosorbent assays of zearalenone using polyclonal, monoclonal and recombinant antibodies. *Methods Mol Biol* 157:159–70.
84. Wang S-H, Du X-Y, Lin L, et al. (2008) Zearalenone (ZEN) detection by a single chain fragment variable (scFv) antibody. *World J Microbiol Biotechnol* 24:1681–1685. doi: 10.1007/s11274-008-9657-y

85. European Commission (2007) Commission Regulation (EC) No 1126/2007 of 28 September 2007 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards Fusarium toxins in maize and maize products. Off J Eur Union L255:14–17.
86. Bennett GA, Nelsen TC, Miller BM (1994) Enzyme-linked immunosorbent assay for detection of zearalenone in corn, wheat, and pig feed: collaborative study. J AOAC Int 77:1500–9.
87. He T, Wang Y, Li P, et al. (2014) Nanobody-based enzyme immunoassay for aflatoxin in agro-products with high tolerance to cosolvent methanol. Anal Chem 86:8873–80. doi: 10.1021/ac502390c
88. Wang S-H, Du X-Y, Huang Y-M, et al. (2007) Detection of deoxynivalenol based on a single-chain fragment variable of the antideoxynivalenol antibody. FEMS Microbiol Lett 272:214–9. doi: 10.1111/j.1574-6968.2007.00765.x
89. Wang S, Zheng C, Liu Y, et al. (2008) Construction of multiform scFv antibodies using linker peptide. J Genet genomics 35:313–6. doi: 10.1016/S1673-8527(08)60045-4
90. Daly S, Dillon P, Manning B, et al. (2002) Production and Characterization of Murine Single Chain Fv Antibodies to Aflatoxin B 1 Derived From a Pre-immunized Antibody Phage Display Library System. Food Agric Immunol 14:255–274. doi: 10.1080/0954010021000096373
91. Trombley A, Fan T, LaBudde R (2011) Aflatoxin plate kit. Performance Tested Method 081003. J AOAC Int 94:1519–30.
92. Oguri H, Hiram M, Tsumuraya T, et al. (2003) Synthesis-based approach toward direct sandwich immunoassay for ciguatoxin CTX3C. J Am Chem Soc 125:7608–12. doi: 10.1021/ja034990a
93. Nagumo Y, Oguri H, Tsumoto K, et al. (2004) Phage-display selection of antibodies to the left end of CTX3C using synthetic fragments. J Immunol Methods 289:137–46. doi: 10.1016/j.jim.2004.04.003
94. Tsumoto K, Yokota A, Tanaka Y, et al. (2008) Critical contribution of aromatic rings to specific recognition of polyether rings. The case of ciguatoxin CTX3C-ABC and its specific antibody 1C49. J Biol Chem 283:12259–66. doi: 10.1074/jbc.M710553200
95. Lehane L, Lewis RJ (2000) Ciguatera: recent advances but the risk remains. Int J Food Microbiol 61:91–125.
96. Tsumuraya T, Takeuchi K, Yamashita S, et al. (2012) Development of a monoclonal antibody against the left wing of ciguatoxin CTX1B: thiol strategy and detection using a sandwich ELISA. Toxicon 60:348–57. doi: 10.1016/j.toxicon.2012.04.347
97. Tsumuraya T, Fujii I, Hiram M (2014) Preparation of anti-ciguatoxin monoclonal antibodies using synthetic haptens: sandwich ELISA detection of ciguatoxins. J AOAC Int 97:373–9.
98. Tsumuraya T, Fujii I, Hiram M (2010) Production of monoclonal antibodies for sandwich immunoassay detection of Pacific ciguatoxins. Toxicon 56:797–803. doi: 10.1016/j.toxicon.2009.06.003
99. Hu X, O'Dwyer R, Wall JG (2005) Cloning, expression and characterisation of a single-chain Fv antibody fragment against domoic acid in Escherichia coli. J Biotechnol 120:38–45. doi: 10.1016/j.jbiotec.2005.05.018
100. Shaw I, O'Reilly A, Charleton M, Kane M (2008) Development of a high-affinity anti-domoic acid sheep scFv and its use in detection of the toxin in shellfish. Anal Chem 80:3205–12. doi: 10.1021/ac7024199
101. Finlay WJJ, Shaw I, Reilly JP, Kane M (2006) Generation of high-affinity chicken single-chain Fv antibody fragments for measurement of the pseudonitzschia pungens toxin domoic acid. Appl Environ Microbiol 72:3343–3349. doi: 10.1128/AEM.72.5.3343

102. Wang T, Ding H, Yang L, et al. (2009) Screening and identification of a single chain antibody fragment. *Acta Microbiol Sin* 49:135–40.
103. Yang L, Ding H, Gu Z, et al. (2009) Selection of single chain fragment variables with direct coating of aflatoxin B1 to enzyme-linked immunosorbent assay plates. *J Agric Food Chem* 57:8927–32. doi: 10.1021/jf9019536
104. Yang L, Zhang Y, Ding H, et al. (2009) Expression and optimization of anti-AFB1 scFv in *Escherichia coli*. *Acta Microbiol Sin* 49:880–8.
105. Rangnoi K, Jaruseranee N, O’Kennedy R, et al. (2011) One-step detection of aflatoxin-B(1) using scFv-alkaline phosphatase-fusion selected from human phage display antibody library. *Mol Biotechnol* 49:240–9. doi: 10.1007/s12033-011-9398-2
106. Hara Y, Dong J, Ueda H (2013) Open-sandwich immunoassay for sensitive and broad-range detection of a shellfish toxin gonyautoxin. *Anal Chim Acta* 793:107–13. doi: 10.1016/j.aca.2013.07.024
107. Kawatsu K, Hamano Y, Sugiyama A, et al. (2002) Development and application of an enzyme immunoassay based on a monoclonal antibody against gonyautoxin components of paralytic shellfish poisoning toxins. *J Food Prot* 65:1304–8.
108. Fraga M, Vilariño N, Louzao MCC, et al. (2012) Detection of paralytic shellfish toxins by a solid-phase inhibition immunoassay using a microsphere-flow cytometry system. *Anal Chem* 84:4350–6. doi: 10.1021/ac203449f
109. Fonfría ES, Vilariño N, Campbell K, et al. (2007) Paralytic shellfish poisoning detection by surface plasmon resonance-based biosensors in shellfish matrixes. *Anal Chem* 79:6303–11. doi: 10.1021/ac070362q
110. Campbell K, Rawn DFK, Niedzwiadek B, Elliott CT (2011) Paralytic shellfish poisoning (PSP) toxin binders for optical biosensor technology: problems and possibilities for the future: a review. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* 28:711–25. doi: 10.1080/19440049.2010.531198
111. European Commission (2004) Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for on the hygiene of foodstuffs. *Off J Eur Union L139*:55–206.
112. European Food Safety Authority (EFSA) (2009) Scientific opinion of the panel on contaminants in the food chain on a request from the European Commission on marine biotoxins in shellfish – saxitoxin group. *EFSA J* 1019:1–3. doi: 10.2903/j.efsa.2010.1677. Available
113. Suzuki T, Munakata Y, Morita K, et al. (2007) Sensitive Detection of Estrogenic Mycotoxin Zearalenone by Open Sandwich Immunoassay. *Anal Sci* 23:65–70. doi: 10.2116/analsci.23.65
114. Kobayashi N, Iwakami K, Kotoshiba S, et al. (2006) Immunoenzymometric assay for a small molecule, 11-deoxycortisol, with attomole-range sensitivity employing an scFv-enzyme fusion protein and anti-idiotypic antibodies. *Anal Chem* 78:2244–53. doi: 10.1021/ac051858f
115. Ihara M, Suzuki T, Kobayashi N, et al. (2009) Open-sandwich enzyme immunoassay for one-step noncompetitive detection of corticosteroid 11-deoxycortisol. *Anal Chem* 81:8298–304. doi: 10.1021/ac900700a
116. Dong J, Ihara M, Ueda H (2009) Antibody Fab display system that can perform open-sandwich ELISA. *Anal Biochem* 386:36–44. doi: 10.1016/j.ab.2008.11.045
117. Liu X, Eichenberger M, Fujioka Y, et al. (2012) Improved detection sensitivity and selectivity attained by open-sandwich selection of an anti-estradiol antibody. *Anal Sci* 28:861–7.

118. Huovinen T, Syrjänpää M, Sanmark H, et al. (2013) Two ScFv antibody libraries derived from identical VL-VH framework with different binding site designs display distinct binding profiles. *Protein Eng Des Sel* 26:683–93. doi: 10.1093/protein/gzt037
119. Perkin Elmer (2010) Unmatched Sensitivity Time after Time. DELFIA TRF Technol 1–12.
120. Edupuganti SR, Edupuganti OP, Hearty S, O’Kennedy R (2013) A highly stable, sensitive, regenerable and rapid immunoassay for detecting aflatoxin B1 in corn incorporating covalent AFB1 immobilization and a recombinant Fab antibody. *Talanta* 115:329–35. doi: 10.1016/j.talanta.2013.05.012
121. Dunne L, Daly S, Baxter A, et al. (2005) Surface Plasmon Resonance-Based Immunoassay for the Detection of Aflatoxin B 1 Using Single-Chain Antibody Fragments. *Spectrosc Lett* 38:229–245. doi: 10.1081/SL-200058689
122. European Commission (2010) Commission Regulation (EU) No 165/2010 of 26 February 2010 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins. *Off J Eur Union* L50:8–12.
123. Alexander J, Benford D, Cockburn A, et al. (2009) Effects on public health of an increase of the levels for aflatoxin total from 4 µg / kg to 10 µg / kg for tree nuts other than almonds , hazelnuts and pistachios. *EFSA J* 1–11.
124. Edupuganti SR, Edupuganti OP, O’Kennedy R (2013) Biological and synthetic binders for immunoassay and sensor-based detection: generation and characterisation of an anti-AFB2 single-chain variable fragment (scFv). *World Mycotoxin J* 6:273–280. doi: 10.3920/WMJ2012.1523
125. Zhang D, Li P, Zhang Q, et al. (2009) Production of ultrasensitive generic monoclonal antibodies against major aflatoxins using a modified two-step screening procedure. *Anal Chim Acta* 636:63–9. doi: 10.1016/j.aca.2009.01.010
126. Li X, Li P, Lei J, et al. (2014) A simple strategy to obtain ultra-sensitive single-chain fragment variable antibodies for aflatoxin detection. *RSC Adv* 3:22367. doi: 10.1039/c3ra42706d
127. Fischer WJ, Garthwaite I, Miles CO, et al. (2001) Congener-independent immunoassay for microcystins and nodularins. *Environ Sci Technol* 35:4849–56.
128. Zeck A, Weller MG, Bursill D, Niessner R (2001) Generic microcystin immunoassay based on monoclonal antibodies against Adda. *Analyst* 126:2002–7.
129. McElhiney J, Drever M, Lawton LA, Porter AJ (2002) Rapid Isolation of a Single-Chain Antibody against the Cyanobacterial Toxin Microcystin-LR by Phage Display and Its Use in the Immunoaffinity Concentration of Microcystins from Water. *Appl Environ Microbiol* 68:5288–5295. doi: 10.1128/AEM.68.11.5288
130. Alvarenga LM, Muzard J, Ledreux A, et al. (2014) Colorimetric engineered immunoprobe for the detection and quantification of microcystins. *J Immunol Methods* 406:124–30. doi: 10.1016/j.jim.2014.02.014
131. Zou L, Xu Y, Li Y, et al. (2014) Development of a single-chain variable fragment antibody-based enzyme-linked immunosorbent assay for determination of fumonisin B1 in corn samples. *J Sci Food Agric* 94:1865–71. doi: 10.1002/jsfa.6505
132. European Commission (2002) Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off J Eur Communities* L221:8–36.

133. McElhiney J, Lawton LA, Porter AJR (2000) Detection and quantification of microcystins (cyanobacterial hepatotoxins) with recombinant antibody fragments isolated from a naive human phage display library. *FEMS Microbiol Lett* 193:83–88. doi: 10.1111/j.1574-6968.2000.tb09406.x
134. Choi G-H, Lee D-H, Min W-K, et al. (2004) Cloning, expression, and characterization of single-chain variable fragment antibody against mycotoxin deoxynivalenol in recombinant *Escherichia coli*. *Protein Expr Purif* 35:84–92. doi: 10.1016/j.pep.2003.12.008
135. Doyle PJ, Arbabi-Ghahroudi M, Gaudette N, et al. (2008) Cloning, expression, and characterization of a single-domain antibody fragment with affinity for 15-acetyl-deoxynivalenol. *Mol Immunol* 45:3703–13. doi: 10.1016/j.molimm.2008.06.005
136. Van Houwelingen A, De Saeger S, Rusanova T, et al. (2008) Generation of recombinant alpaca VHH antibody fragments for the detection of the mycotoxin ochratoxin A. *World Mycotoxin J* 1:407–417. doi: 10.3920/WMJ2008.1070
137. Lawton L a, Chambers H, Edwards C, et al. (2010) Rapid detection of microcystins in cells and water. *Toxicon* 55:973–8. doi: 10.1016/j.toxicon.2009.05.030
138. Min W-K, Cho Y-J, Park J-B, et al. (2010) Production and characterization of monoclonal antibody and its recombinant single chain variable fragment specific for a food-born mycotoxin, fumonisin B1. *Bioprocess Biosyst Eng* 33:109–15. doi: 10.1007/s00449-009-0350-9
139. Min W-K, Kweon D-H, Park K, et al. (2011) Characterisation of monoclonal antibody against aflatoxin B1 produced in hybridoma 2C12 and its single-chain variable fragment expressed in recombinant *Escherichia coli*. *Food Chem* 126:1316–1323. doi: 10.1016/j.foodchem.2010.11.088
140. Tuomas H, Yuan LIU, Xian-jin LIU, et al. (2012) Screening and Identification of Single-Chain Antibodies Against Microcystin-LR by Magnetic Beads and Time Resolved Fluorescence Immunoassay. 45:330–337. doi: 10.3864/j.issn.0578-1752.2012.02.015
141. McGrath TF, Elliott CT, Fodey TL (2012) Biosensors for the analysis of microbiological and chemical contaminants in food. *Anal Bioanal Chem* 403:75–92. doi: 10.1007/s00216-011-5685-9
142. Lacy A, Dunne L, Fitzpatrick B, et al. (2006) Rapid analysis of coumarins using surface plasmon resonance. *J AOAC Int* 89:884–92.
143. Little M, Welschhof M, Braunagel M, et al. (1999) Generation of a large complex antibody library from multiple donors. *J Immunol Methods* 231:3–9.
144. Daly SJ, Keating GJ, Dillon PP, et al. (2000) Development of surface plasmon resonance-based immunoassay for aflatoxin B(1). *J Agric Food Chem* 48:5097–104.
145. Hu X, Spada S, White S, et al. (2006) Adsorption and activity of a domoic acid binding antibody fragment on mesoporous silicates. *J Phys Chem B* 110:18703–9. doi: 10.1021/jp062423e
146. Hu X, O'Hara L, White S, et al. (2007) Optimisation of production of a domoic acid-binding scFv antibody fragment in *Escherichia coli* using molecular chaperones and functional immobilisation on a mesoporous silicate support. *Protein Expr Purif* 52:194–201. doi: 10.1016/j.pep.2006.08.009
147. Hortigüela MJ, Wall JG (2013) Improved detection of domoic acid using covalently immobilised antibody fragments. *Mar Drugs* 11:881–95. doi: 10.3390/md11030881
148. Li P, Zhang Z, Zhang Q, et al. (2012) Current development of microfluidic immunosensing approaches for mycotoxin detection via capillary electromigration and lateral flow technology. *Electrophoresis* 33:2253–65. doi: 10.1002/elps.201200050

149. WHO (2008) WHO guidelines for drinking-water quality. In: WHO Geneva (ed) WHO Chron., 3rd Editio. WHO Geneva, Geneva, p 195
150. WHO (1999) Guidelines for safe recreational water environments Volume 1 : Coastal and fresh waters. Geneva, pp 136–158
151. Kim YM, Oh SW, Jeong SY, et al. (2003) Development of an ultrarapid one-step fluorescence immunochromatographic assay system for the quantification of microcystins. *Environ Sci Technol* 37:1899–904.
152. Lattanzio VMT, Nivarlet N, Lippolis V, et al. (2012) Multiplex dipstick immunoassay for semi-quantitative determination of *Fusarium* mycotoxins in cereals. *Anal Chim Acta* 718:99–108. doi: 10.1016/j.aca.2011.12.060
153. Food and Drug Administration (FDA) (2000) Guidance for Industry : Action Levels for Poisonous or Deleterious Substances in Human Food and Animal Feed. In: Food Guid. Doc. <http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/ChemicalContaminantsMetalsNaturalToxinsPesticides/ucm077969.htm>.
154. FAO (2004) 3.4.1 Worldwide limits for aflatoxins. *Worldw. Regul. mycotoxins food Feed 2003*. Food and Agriculture Organisation of the United Nations, pp 17–22
155. Yu F-Y, Gribas A V, Vdovenko MM, Sakharov IY (2013) Development of ultrasensitive direct chemiluminescent enzyme immunoassay for determination of aflatoxin B₁ in food products. *Talanta* 107:25–9. doi: 10.1016/j.talanta.2012.12.047
156. Van der Gaag B, Spath S, Dietrich H, et al. (2003) Biosensors and multiple mycotoxin analysis. *Food Control* 14:251–254. doi: 10.1016/S0956-7135(03)00008-2
157. European Commission (2006) Commission regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Off J Eur Union L70*:12–34.
158. FAO (2004) 3.4.2 Worldwide limits for other mycotoxins. *Worldw. Regul. mycotoxins food Feed 2003*. Food and Agriculture Organisation of the United Nations, pp 23–26
159. Food and Drug Administration (FDA) (2008) Chapter 7. Molecular biology and natural toxins. *FDA Compliance Progr. Guid. Man. Progr. 7307.001*. pp 1–3
160. Sheng Y, Jiang W, De Saeger S, et al. (2012) Development of a sensitive enzyme-linked immunosorbent assay for the detection of fumonisin B₁ in maize. *Toxicon* 60:1245–50. doi: 10.1016/j.toxicon.2012.08.011
161. Shiu C-M, Wang J-J, Yu F-Y (2010) Sensitive enzyme-linked immunosorbent assay and rapid one-step immunochromatographic strip for fumonisin B₁ in grain-based food and feed samples. *J Sci Food Agric* 90:1020–6. doi: 10.1002/jsfa.3911
162. European Commission (2010) Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Off J Eur Union L364*:5–24.
163. Zhang A, Ma Y, Feng L, et al. (2011) Development of a sensitive competitive indirect ELISA method for determination of ochratoxin A levels in cereals originating from Nanjing, China. *Food Control* 22:1723–1728. doi: 10.1016/j.foodcont.2011.04.004
164. Yu F-Y, Chi T-F, Liu B-H, Su C-C (2005) Development of a sensitive enzyme-linked immunosorbent assay for the determination of ochratoxin A. *J Agric Food Chem* 53:6947–53. doi: 10.1021/jf0513922

165. Cha S-H, Kim S-H, Bischoff K, et al. (2012) Production of a highly group-specific monoclonal antibody against zearalenone and its application in an enzyme-linked immunosorbent assay. *J Vet Sci* 13:119–25.
166. Metcalf J., Bell S., Codd G. (2000) Production of novel polyclonal antibodies against the cyanobacterial toxin microcystin-LR and their application for the detection and quantification of microcystins and nodularin. *Water Res* 34:2761–2769. doi: 10.1016/S0043-1354(99)00429-7
167. Rapala J, Erkomaa K, Kukkonen J, et al. (2002) Detection of microcystins with protein phosphatase inhibition assay , high-performance liquid chromatography – UV detection and enzyme-linked immunosorbent assay Comparison of methods. *Anal Chim Acta* 466:213–231.
168. Sheng J-W, He M, Shi H-C (2007) A highly specific immunoassay for microcystin-LR detection based on a monoclonal antibody. *Anal Chim Acta* 603:111–8. doi: 10.1016/j.aca.2007.09.029
169. European Commission (2002) Commission Decision establishing special health checks for the harvesting and processing of certain bivalve molluscs with a level of amnesic shellfish poison (ASP) exceeding the limit laid down by Council Directive 91/492/EEC. *Off J Eur Comm* L75:65–66.
170. Kawatsu K, Hamano Y, Noguchi T (1999) Production and characterization of a monoclonal antibody against domoic acid and its application to enzyme immunoassay. *Toxicon* 37:1579–89.
171. Garthwaite I, Ross KM, Miles CO, et al. (1998) Polyclonal antibodies to domoic acid , and their use in immunoassays for domoic acid in Sea Water and Shellfish. *Nat Toxins* 6:93–104.
172. Midelfort KS, Hernandez HH, Lippow SM, et al. (2004) Substantial energetic improvement with minimal structural perturbation in a high affinity mutant antibody. *J Mol Biol* 343:685–701. doi: 10.1016/j.jmb.2004.08.019
173. Pepper LR, Cho YK, Boder ET, Shusta E V (2008) A decade of yeast surface display technology: where are we now? *Comb Chem High Throughput Screen* 11:127–34.
174. Boder ET, Raeeszadeh-Sarmazdeh M, Price JV (2012) Engineering antibodies by yeast display. *Arch Biochem Biophys* 526:99–106. doi: 10.1016/j.abb.2012.03.009
175. Capriotti AL, Caruso G, Cavaliere C, et al. (2012) Multiclass mycotoxin analysis in food, environmental and biological matrices with chromatography/mass spectrometry. *Mass Spectrom Rev* 31:466–503. doi: 10.1002/mas.20351
176. McNamee SE, Elliott CT, Delahaut P, Campbell K (2012) Multiplex biotoxin surface plasmon resonance method for marine biotoxins in algal and seawater samples. *Environ Sci Pollut Res Int*. doi: 10.1007/s11356-012-1329-7
177. Borrebaeck CAK, Wingren C (2011) Recombinant antibodies for the generation of antibody arrays. *Methods Mol Biol* 785:247–62. doi: 10.1007/978-1-61779-286-1_17
178. 't Hoen PAC, Jirka SMG, Ten Broeke BR, et al. (2012) Phage display screening without repetitious selection rounds. *Anal Biochem* 421:622–31. doi: 10.1016/j.ab.2011.11.005
179. Glanville J, Zhai W, Berka J, et al. (2009) Precise determination of the diversity of a combinatorial antibody library gives insight into the human immunoglobulin repertoire. *Proc Natl Acad Sci U S A* 106:20216–21. doi: 10.1073/pnas.0909775106
180. Ravn U, Gueneau F, Baerlocher L, et al. (2010) By-passing in vitro screening--next generation sequencing technologies applied to antibody display and in silico candidate selection. *Nucleic Acids Res* 38:e193. doi: 10.1093/nar/gkq789

181. D'Angelo S, Glanville J, Ferrara F, et al. (2014) The antibody mining toolbox: An open source tool for the rapid analysis of antibody repertoires. *MAbs* 6:160–72. doi: 10.4161/mabs.27105
182. Heffner KM, Hizal DB, Kumar A, et al. (2014) Exploiting the proteomics revolution in biotechnology: from disease and antibody targets to optimizing bioprocess development. *Curr Opin Biotechnol* 30:80–86. doi: 10.1016/j.copbio.2014.06.006
183. Cheung WC, Beausoleil S a, Zhang X, et al. (2012) A proteomics approach for the identification and cloning of monoclonal antibodies from serum. *Nat Biotechnol* 30:447–52. doi: 10.1038/nbt.2167
184. Wine Y, Boutz DR, Lavinder JJ, et al. (2013) Molecular deconvolution of the monoclonal antibodies that comprise the polyclonal serum response. *Proc Natl Acad Sci U S A* 110:2993–8. doi: 10.1073/pnas.1213737110
185. Meneely JP, Ricci F, van Egmond HP, Elliott CT (2011) Current methods of analysis for the determination of trichothecene mycotoxins in food. *TrAC Trends Anal Chem* 30:192–203. doi: 10.1016/j.trac.2010.06.012
186. Weller M (2013) Immunoassays and Biosensors for the Detection of Cyanobacterial Toxins in Water. *Sensors* 13:15085–15112. doi: 10.3390/s131115085
187. Meulenber EP (2012) Immunochemical methods for ochratoxin A detection: a review. *Toxins (Basel)* 4:244–66. doi: 10.3390/toxins4040244
188. European Commission (2010) Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. *Off J Eur Union* L276:33–79.