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

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### **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  $\leq 1000$  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].

### **Naive 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 naive (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  $\mu$ 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 ( $8.6 \times 10^{-8}$  M  $K_D$ ) but considerably weaker affinity to the free ABCD fragment ( $2.4 \times 10^{-5}$  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  $1.3 \times 10^{-5}$  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 IC<sub>10</sub> & IC<sub>80</sub> values) with an LOD of 0.5 ng/ml (IC<sub>20</sub> 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 AFB<sub>1</sub>-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 AFB<sub>1</sub> 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 AFB<sub>1</sub> in buffer described to that date (Table 4) and exhibited some cross reactivity to other aflatoxins (AFG<sub>1</sub> (42%) > AFB<sub>2</sub> (12%) > AFG<sub>2</sub> (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-E<sub>2</sub>) 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 IC<sub>50</sub>) 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 IC<sub>50</sub> 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 IC<sub>50</sub>) or AFM1 alone (clone 4F3; 0.27 ng/ml IC<sub>50</sub>) [125]. The same group subsequently constructed a phage scFv library composed of the VH and VL of almost 20 hybridomas secreting mAbs (including clones 1C11 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 IC<sub>50</sub> in buffer) and was highly specific to AFB1. The second clone (1A7) also exhibited excellent affinity to AFB1 (0.02 ng/ml IC<sub>50</sub> 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 (( $\text{IC}_{50} = 2.97 \text{ }\mu\text{M}$  & LOD ( $\text{IC}_{10}$ ) of  $0.25 \text{ }\mu\text{M}$  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 ( $2.1\text{-}76.45 \text{ }\mu\text{g/L}$ ) was marginally higher than that of the parent 1D11 ( $1.67\text{-}61.74 \text{ }\mu\text{g/L}$ ). 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  $6.30 \text{ }\mu\text{g kg}^{-1}$  due to the crude nature of the matrix and LOD ( $8.32 \text{ }\mu\text{g/kg}$ ) were calculated from the same corn samples spiked with known concentrations of FB1 with intra- ( $9.72\text{-}10.26 \text{ \% CV}$ ) and inter-assay ( $11.28\text{-}14.03 \text{ \% CV}$ ). 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 ( $\text{CC}\beta$ ) ( $15 \text{ }\mu\text{g/kg}$ ) must be less than or equal to the regulatory limit set which worldwide ranges from  $1000\text{-}4000 \text{ }\mu\text{g/kg}$  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 (IC<sub>50</sub>) to the ic-ELISA mentioned previously but didn't publish the value however the K<sub>D</sub> for soluble AFB1 in buffer was determined using the Langmuir 1:1 model to be  $1.2 \times 10^{-12}$  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 IC<sub>50</sub>) than in an almond matrix (11 ng/ml IC<sub>50</sub>) 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<sup>-1</sup> 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<sup>-1</sup> [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 desalination 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 x 10 <sup>-6</sup> 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 x 10 <sup>-9</sup> M (K <sub>D</sub> )	[73]	Only semi-synthetic library generated high affinity scFv
2002	MC LR	Human semi-synthetic (Griffin.1)	scFv (3A8)	ELISA	4.5 x10 <sup>-9</sup> M (IC50)	[129]	Incorporated into immunoaffinity column for concentration of MC LR from water
	MC LR	Human semi-synthetic (Griffin.1)	scFv	ELISA	13 x10 <sup>-9</sup> 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	$3.7 \times 10^{-6} \text{M}$ ( $K_D$ )	[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 ( $10^{-5} \text{M}$ ) 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	$4.08 \times 10^{-7} \text{M}$ ( $K_D$ )	[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	$9.6 \times 10^{-10} \text{M}$ ( $K_{\text{aff}}$ )	[89]	Bispecific scFv retained affinity of monomeric scFv
	15-ac-DON	Immunised (Llama)	VHH (NAT-267 monomer or pentamer)	FPIA	0.5 $\mu\text{M}$ (monomer) 1.24 $\mu\text{M}$ (dimer) (IC50)	[135]	Monomer & pentamer affinity comparable to parent pAb (1.42 $\mu\text{M}$ )
	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) $1.2 \times 10^{-12} \text{M}$ ( $K_{\text{D}}$ )	[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	$1.16 \times 10^{-7}$ M ( $K_D$ )	[139]	Parent mAb 17-fold better affinity ( $6.95 \times 10^{-9}$ M) than 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
	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)
2013	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 $\mu$ M (IC50)	[130]	Moderate 3A8 affinity due to heterologous scFv population
	FB1	Immunised (Hybridoma)	scFv	ELISA	12.67 $\mu$ g/L (IC50)	[131]	Similar affinity (10.16 $\mu$ 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
<b>AFB1</b>					
<b>12-0.1 ppb (µg/kg)</b> [122]  <b>300-20 ppb</b> [153]  <b>200-1 ppb</b> [154]	Fab ic-ELISA	12 ppb (35 ppb IC50)	grain	<b>AFB2 2, AFG1 18, AFG2 1</b>	[120]
	VHH ic-ELISA (clone Nb26)	3 ppb IC50	Peanut, rice, corn, feedstuff	<b>AFB2 1, AFG2 2, AFM1 11</b>	[87]
	pAb c-ELISA	8 ppt	Rice, beans	Not reported	[155]
	cAb-ELISA <sup>N/A</sup>	20 ppb	Corn, wheat, peanut butter & feeds	Total Aflatoxins ( <b>AFB1+B2+G1+G2</b> ) but % cross reactivity not reported	[126][102]Neo gen Corp., USA (Agri-Screen for aflatoxin)
	cAb-ELISA <sup>N/A</sup>	1.75 ppb	Cereals & feed	Total Aflatoxins <b>AFB1 100, AFB2 48, AFG1 75, AFG2 18</b>	Biopharm AG, Germany (RIDASCREE N® Aflatoxin Total)
	pAb c-ELISA	25 ppt 2.5 ppb	cereals & nuts animal feed	<b>AFB2 20, AFG1 17, AFG2 4</b>	Europroxima BV; The Netherlands (5121AFB)
	scFv ic-SPR (clone C4)	0.75 ppb	grain	<b>AFB2 53, AFG1 76, AFG2 68, AFM1 50, AFM2 23, AFB2a 18, AFG2a 22</b>	[90]
	mAb ic-SPR	0.2 ppb	grain	Not reported	[156]
	pAb Ic-SPR	3 ppb	No	<b>AFB2 68, AFG1 66, AFG2 50, AFM1 35, AFM2 2, AFB2a 33, AFG2a 16</b>	[144]
<b>AFB2</b>	scFv SPR (E9)	0.9 ppb	Almonds	<b>AFB1 0, AFG1 0, AFG2 0, AFM1 0</b>	[124]

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

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

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

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

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

<sup>A</sup> adapted by reviewer to represent relative cross reactivity to GTX2/3

<sup>N/A</sup> 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  $\mu$ 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 naive 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
<b>Mycotoxins</b>		
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” 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

<b>Aquatic &amp; Marine Toxins</b>		
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]



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