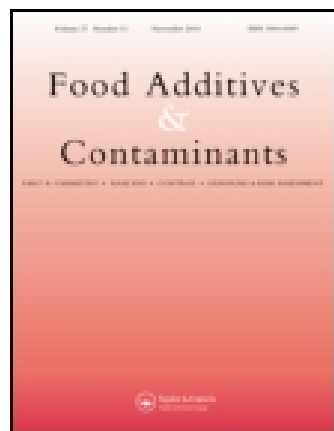




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Paralytic shellfish poisoning (PSP) toxin binders for optical biosensor technology: problems and possibilities for the future: a review

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Paralytic shellfish poisoning (PSP) toxin binders for optical biosensor technology: problems and possibilities for the future: a review

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This review examines the developments in optical biosensor technology, which uses the phenomenon of surface plasmon resonance, for the detection of paralytic shellfish poisoning (PSP) toxins. Optical biosensor technology measures the competitive biomolecular interaction of a specific biological recognition element or binder with a target toxin immobilised onto a sensor chip surface against toxin in a sample. Different binders such as receptors and antibodies previously employed in functional and immunological assays have been assessed. Highlighted are the difficulties in detecting this range of low molecular weight toxins, with analogues differing at four chemical substitution sites, using a single binder. The complications that arise with the toxicity factors of each toxin relative to the parent compound, saxitoxin, for the measurement of total toxicity relative to the mouse bioassay are also considered. For antibodies, the cross-reactivity profile does not always correlate to toxic potency, but rather to the toxin structure to which it was produced. Restrictions and availability of the toxins makes alternative chemical strategies for the synthesis of protein conjugate derivatives for antibody production a difficult task. However, when two antibodies with different cross-reactivity profiles are employed, with a toxin chip surface generic to both antibodies, it was demonstrated that the cross-reactivity profile of each could be combined into a single-assay format. Difficulties with receptors for optical biosensor analysis of low molecular weight compounds are discussed, as are the potential of alternative non-antibody-based binders for future assay development in this area.

Keywords: screening – biosensor; screening – immunoassays; toxicology; phycotoxins – PSP; seafood

Introduction

Paralytic shellfish poisoning (PSP) toxins are naturally occurring potent neurotoxins synthesised by microscopic dinoflagellates from the genii *Alexandrium*, *Gymnodinium* and *Pyrodinium* in marine and freshwater environments (Reis Costa et al. 2009). Increased temperatures, sunlight and nutrient-rich waters are considered to cause the rapid reproduction of dinoflagellate species and thereby lead to potential 'harmful algal blooms'. Climate change, increased ocean eutrophication and commercial shipping are believed to contribute to the increasing frequency and occurrence of these blooms worldwide (Botana et al. 2009). Filter-feeding organisms, such as molluscan shellfish, consuming dinoflagellates may thereby accumulate the PSP toxins and potentially transfer them through the trophic chain (Deeds et al. 2008). The toxins do not appear to harm shellfish directly, but are

potentially lethal to humans or other consumers such as marine mammals and birds (Huang et al. 1996).

No visible difference in appearance is observed between harmless and toxic shellfish. As the PSP toxins are heat stable they are not destroyed by cooking, but due to their solubility in water leaching into the cooking water occurs (Michalski 2007; Etheridge 2010), and canning procedures are reported to reduce toxin levels for this reason (Vieites et al. 1999). Following consumption the toxins bind with a high affinity to site 1 of the voltage-dependent sodium channel α -subunit of muscle and nerve cells, blocking the influx of sodium and preventing the generation and propagation of action potentials in these excitable cells (Cestele and Catterall 2000). Early symptoms following ingestion include tingling of the lips and tongue,

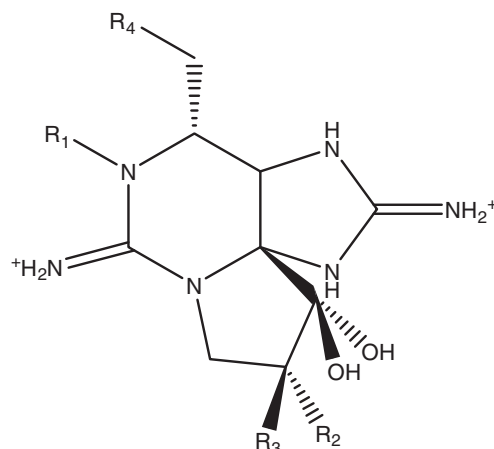
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which then progresses to the fingers and toes, followed by the loss of control of arms and legs. Difficulty in breathing ensues from paralysis of the muscles of the chest and abdomen, which results in death at high toxin doses. Fatal levels of less than 1 mg of the reference toxin saxitoxin (STX) have been reported (van Egmond et al. 2004). There is currently no antidote for intoxication with artificial respiration as the palliative care until the toxin is excreted from the body.

PSP has major implications to public health and thereby seriously threatening economic consequences in coastal communities and aquaculture industries worldwide. Therefore, globally shellfish destined for human consumption are routinely monitored for PSP toxins by regulatory bodies. The internationally accepted and European Union reference method of analysis for PSP toxins in shellfish is the Association of Analytical Communities (AOAC)-accredited biological method (AOAC 2005a) or more commonly referred to mouse bioassay. Using this method shellfish are deemed fit for consumption based on a regulatory limit, which in Europe is 800 µg of PSP toxins per kg of shellfish meat, a level which has been established for the prevention of acute poisoning (European Commission 2004). In some other countries this limit is lower (Campas et al. 2007). However, both ethical issues relating to the use of animals and technical concerns unfolding in relation

to interference and sensitivity of the assay, particularly if European Food Safety Authority (EFSA) recommendations for lowering the regulatory limit are to be considered, is advancing the search for alternative methods of analysis (EFSA 2009).

As a family group, both the chemical and biological properties of the PSP toxins categorise the task of developing an alternative method as extremely challenging from any analytical perspective. Chemically, there are more than 20 common PSP toxins (Figure 1) that vary in structure at four main sites (R1–R4) of the parent compound STX (Llewellyn 2006a). The toxins are classified into groups based on the chemical substitutions at R4 as carbamate, decarbamoyl, sulfocarbamoyl and deoxydecarbamoyl. The carbamoyl and decarbamoyl toxins are the most toxic, whereas the sulfocarbamoyl toxins have a much lower potency. In contrast to the leaching of PSP toxins from shellfish to water it is interesting to note, however, that in the processing of canned seafood contaminated with PSP toxins, the sulfocarbamoyl toxins may be hydrolysed to the more toxic groups, which will increase the overall PSP toxicity (Christian and Luckas 2008). In the last number of years due to advances in mass spectrometry new structural analogues are also being identified (Dell'Aversano et al. 2008). Biologically, each analogue displays a different



R ₁	R ₂	R ₃				N-Sulfocarbamoyl toxins			Decarbamoyl toxins			Deoxydecarbamoyl toxins		
			R ₄ : OCONH ₂	RMM	Toxicity Factor	R ₄ : OCONHSO ₃ ⁻	RMM	Toxicity Factor	R ₄ : OH	RMM	Toxicity Factor	R ₄ : H	RMM	Toxicity Factor
H	H	H	STX	299	1.00	B1 (GTX 5)	379.4	0.0632	dc-STX	256.3	0.7451	do-STX	240.3	ND
H	H	OSO ₃ ⁻	GTX 2	395.4	0.3382	C1	475.4	0.0047	dc-GTX 2	352.3	0.1625	do-GTX 2	336.3	ND
H	OSO ₃ ⁻	H	GTX 3	395.4	0.6005	C2	475.4	0.0754	dc-GTX 3	352.3	0.3978	do-GTX 3	336.3	ND
OH	H	H	NEO	315.3	1.0911	B2 (GTX 6)	395.4	0.0607	dc-NEO	272.3	0.7013			
OH	H	OSO ₃ ⁻	GTX 1	411.4	0.8993	C3	491.4	0.0101	dc-GTX 1	368.3	ND			
OH	OSO ₃ ⁻	H	GTX 4	411.4	0.6569	C4	491.4	0.0436	dc-GTX 4	368.3	ND			

Figure 1. Structure, relative molecular mass (RMM) and toxicity factor of paralytic shellfish poisoning (PSP) toxins.

binding affinity to the sodium channel receptors which results in different toxicities relative to saxitoxin (Genenah and Shimizu 1981). Therefore, the CONTAM Panel of EFSA recommended that the regulatory limit to be expressed as μg STX-equivalents per kg of shellfish meat (EFSA 2009), which was accepted by the European Community. This method of expression of total toxicity or total level of contamination is therefore a simple concept for the biological methods whereby toxin levels correlate to toxicity and therefore the regulatory limit directly. However, this causes severe complications for both biochemical and analytical methodologies whereby conversion factors are required especially when certain toxin standards are limited or unavailable for all detectable PSP toxins in a contaminated sample.

Toxicity-equivalence factors (TEFs) (Figure 1), originally determined by Oshima (1995) and provided as supplementary material for available toxin standards by the National Reference Centre in Canada (NRC), are used for the conversion of the individual toxin amounts to STXdiHCl equivalents. However, the EFSA working group on STX has recommended new TEFs that are due to be implemented later in 2010 in Europe with the major difference being that the TEF for dcSTX will double in value.

The use of TEFs has supported the development of analytical methods for PSP toxin analysis that can then be correlated to the mouse bioassay and the regulatory limits. Analytical methods such as high-performance liquid chromatography (HPLC) with fluorescence detection, using post-column (Oshima 1995; Asp et al. 2004) and pre-column oxidation methods (Lawrence and Niedzwiedek 2001; Lawrence et al. 2004, 2005), have been developed for the determination of PSP toxins. The method of Lawrence has now been AOAC accredited and accepted following rigorous validation studies by the European Union as a first action method for the toxins for which it is validated (AOAC 2005b). However, HPLC methods are slow, labour intensive and analytical standards are difficult to obtain. Oxidation of PSP toxins is required for HPLC with fluorescence detection because they lack a chromophore. For the AOAC HPLC method two oxidation steps (peroxide and periodate) are required resulting in two analytical runs. Lack of standard material, lengthy purification procedures and insufficient detectability has inhibited the progress in the development of other techniques such as mass spectrometry for routine monitoring. Though advances in mass spectrometry are being made through the use of a zwitterionic hydrophilic interaction chromatography column (Diener et al. 2007), nevertheless mass spectrometry methods are more suitable for confirmatory analysis rather than screening methods due

to their high expenditure and the requirement of dedicated skilled scientists for processing the data for quantitative analysis. Hence, alternative screening methods are still sought that are effective and suitable for routine on-site monitoring by unskilled personnel. Different functional assays, biochemical and biosensor-based technologies employing various binding molecules have shown some potential as alternative screening methods, but surface plasmon resonance (SPR) optical biosensor technology has shown considerable promise in this field in a relatively short period of time. This review describes SPR optical biosensor technology and the progress and problems to date in developing an SPR assay for PSP toxin detection for full acceptance as a regulatory tool. Highlighted is a feasibility study of how combining binders has the potential to overcome certain issues and discussed are the possibilities of new novel binders with this technology.

Surface plasmon resonance (SPR) optical biosensor technology

SPR is a powerful physical technique that measures changes in refractive index and, therefore, in the resonance angle by which polarised light is reflected from a surface which is in turn related to a change in mass on that surface (Subrahmanyam et al. 2002). It is a phenomenon used to measure biomolecular interactions in real time in a label-free environment. These biomolecular interactions can be used to identify the binding of two or more interactants to each other to determine the affinity of the interactions, to measure the actual association and dissociation rates of the interaction, and for the purpose of PSP toxin detection can be exploited to quantify the concentration of one of the interactants (Karlsson 2004).

Optical biosensors based on SPR technology are now an established tool in biomedical research for exploring molecular interactions but are increasingly being investigated as monitoring tools by academic and industrial scientists interested in food safety for the detection of additives and contaminants.

As Biacore[®] biosensors had been proven to be a successful platform for detecting low-level chemical contaminants and toxins in food products, the Biacore Q platform was selected for assay development for the monitoring of PSP toxins (Campbell et al. 2007; Fonfria et al. 2007). This biosensor-based assay measured the real-time interaction of a specific biological recognition element or binder with a target toxin immobilised onto the sensor chip surface. A typical sensorgram, a measure of the binding response against time, is illustrated in Figure 2 (Abery 2001). The running buffer flowing over the

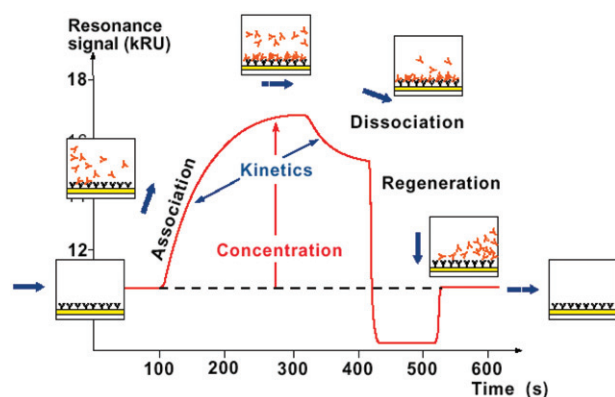


Figure 2. SPR optical biosensor sensorgram of the molecular interaction between the binder and immobilised surface. Source: Abery (2001). Courtesy: Biacore International AB, GE Healthcare.

surface established the baseline value and as the sample solution passed over the sensor surface, the profile of the molecular interaction appeared. During injection the observed binding rate was the difference between the molecules binding to the surface and molecules leaving the surface. Consequently, binding appears fastest at the beginning of the injection as the binder in the sample readily interacts with the high concentration of available toxin-binding partners on the sensor surface. As the injection continues, the observed rate of binding decreased as fewer toxin molecules are available on the surface. After the injection was complete, the binder dissociated and was washed away by the continuous flow of buffer. The sensor surface was regenerated back to the baseline value for the analysis of the next sample (Campbell et al. 2007).

The SPR optical biosensor method for PSP toxins was designed as an inhibition assay. A set amount of PSP binder was mixed with the sample and injected over the surface of a sensor chip to which toxin was immobilised. When no PSP toxins were present in the sample, the binder was then free to bind to the immobilised toxin producing a response (Figure 3a) (Campbell et al. 2007). When PSP toxins were present in the sample, these then bound to the binder, which inhibited the binder from binding to the immobilised toxin on the chip surface and a lower response was produced (Figure 3b) (Campbell et al. 2007). For most biomolecules the change in response is proportional to the mass of the material that has bound to the surface. Therefore, for binders to elicit a change in response from the baseline, they must be greater than 1000 daltons and hence this is why the low molecular weight toxins are immobilised to the surface. Using this assay format, the level of binding to the surface was inversely proportional to the concentration of PSP toxins present in the sample. Figure 4 shows a

typical response against a concentration calibration curve.

Further developments in optical SPR biosensor technology, particularly the progression of multichannel and multiplex instruments such as the Biorad Proteon XPR36 (<http://www.bio-rad.com>) or Biacore prototype developed as part of the European Union 6th Framework Project BioCop (<http://www.biocop.org>), provide additional options for dealing with toxicity factors of toxins and cross-reactivity issues of binders. Within the European Union 7th Framework programme the project CONFIDENCE is evaluating the BioCop multiplex optical SPR for developing a single assay for multiple toxin groups legislated by the European Union and emerging toxins to European Union waters (<http://www.confidence.eu>). This multiplex prototype device is a four-channel instrument with four detection spots within each channel allowing for the simultaneous analysis of up to 16 different analytes in a sample. This increases the scope of binder interactions that could be employed to detect the different PSP analogues. An ideal scenario would be to have a highly specific binder for each of the main PSP toxin groups. Each binder assay for each group could be isolated in separate channels for simultaneous detection in a sample, and then not only would the analyst be able to determine if PSP toxin was present, but also to which group it belonged and a correlation made of the toxic potency level for that sample.

Binders for SPR analysis of PSP toxins

A number of different types of binder have been exploited in the development of various methodologies for the detection of PSP toxins. These include receptors such as the sodium channel receptor (SCR) and saxiphilin receptor, polyclonal and monoclonal antibodies raised against various toxin analogues and chemical binders (Llewellyn and Doyle 2001; Usleber et al. 2001; Gawley et al. 2007; van Dolah et al. 2009). The general advantages and disadvantages of binders that have been previously investigated in the development of assays for SPR analysis for PSP toxins are highlighted in Table 1.

Receptors

Receptors can be either cell membrane bound or cytoplasmic proteins that may be exploited for their ligand-binding capabilities for screening methodologies based on their functional response. SCRs from crude rat brain membrane preparations have been used in radioligand binding assays (Wiegele and Barchi 1978) and in recent years they have been configured into a microtitre plate format (Powell and Doucette 1999;

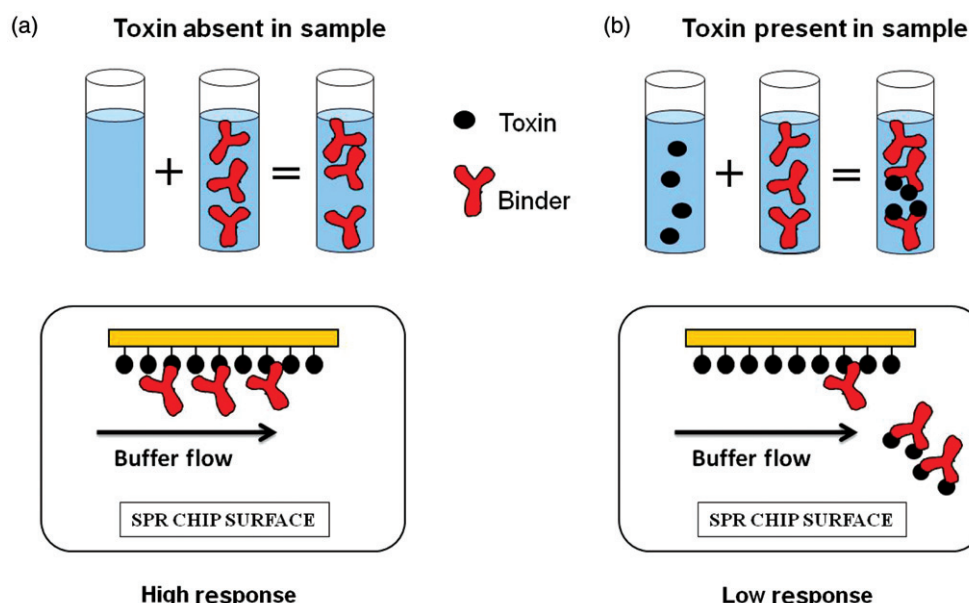


Figure 3. SPR optical biosensor inhibition assay format.

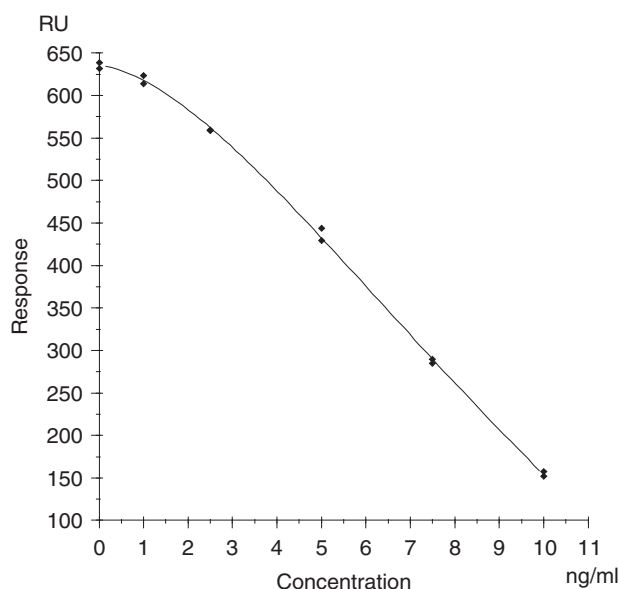


Figure 4. Typical response versus PSP toxin concentration calibration curve using a polyclonal antibody binder.

Ruberu et al. 2003). This microtitre plate format has recently undergone successful single-laboratory validation (van Dolah et al. 2009). The fact that all STX analogues bind to SCRs with affinities that vary according to their toxic potency (Doucette et al. 1997) indicates that receptor-based competitive binding assays provide a measure of a sample's toxicity, irrespective of which PSP toxin(s) is present. The saxiphilin receptor, occurring naturally in various amphibians and terrestrial invertebrates, has also been integrated into a competitive binding assay using a microtitre plate format (Llewellyn and Doyle

2001). Llewellyn (2006b) studied the behaviour of mixtures of PSP toxins in these two unrelated competitive binding assays and found that the most potent toxins affect the toxicity of the mixture to the greatest extent with the less active toxins present required to be several orders of magnitude greater for the mixture to reflect their potency.

Receptor ligand binding assays using SPR technology had previously been investigated (Gestwicki et al. 2001; De Jong et al. 2005). As receptor binders for PSP toxins reflect toxicity in a similar manner to the mouse bioassay, it was envisaged that they could be an ideal candidate for the development of an SPR assay to replace the mouse bioassay. Campbell et al. (2007) examined crude extracts of SCR from rat brains using SPR analysis, but technical difficulties were encountered with the SCR in that this preparation appeared to be stable for only a short time at 4°C, which in effect was detrimental to reliability and reproducibility. Another issue with developing SPR assays for small molecular weight toxins is that when the toxin is immobilised onto the surface, the toxin may then be orientated in such a manner that it no longer locks into the binding site of the receptor pocket, making the toxin unrecognisable with minimal to no binding occurring. As SPR is dependent on changes in mass, for the receptor to be immobilised on the toxin surface the toxin binding in isolation is not of a large enough mass to achieve a change in response and the signal would need to be amplified. Amplification could be performed in a manner similar to a sandwich assay by using a secondary antibody. For small molecular weight compounds this is difficult to achieve due to a

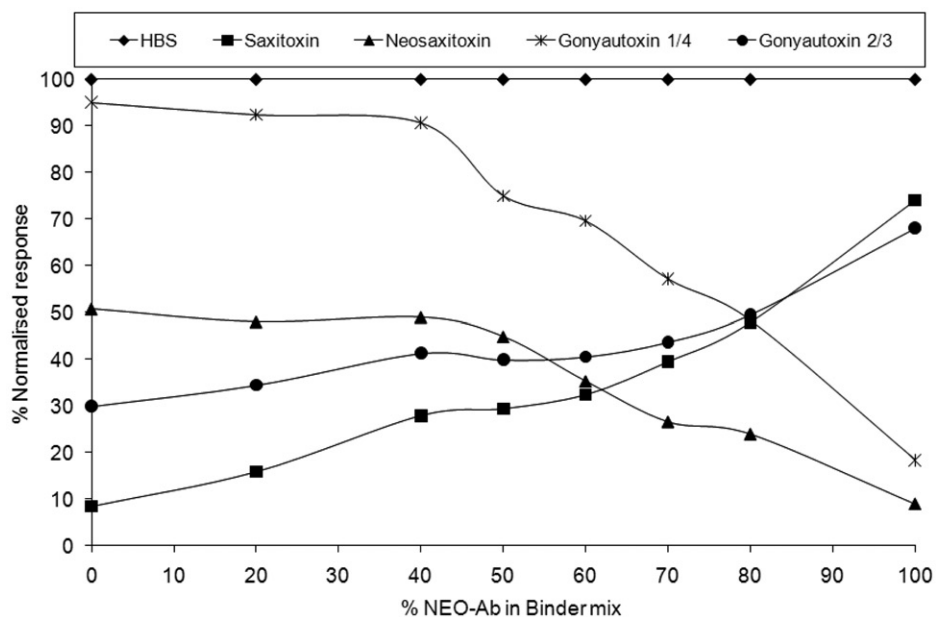


Figure 5. Trends in carbamate toxin response at a fixed concentration of 10 ng ml^{-1} with increasing percentage of NEO antibody in STX/NEO antibody binder mix. Source: Campbell et al., unpublished data.

limited numbers of available epitopes that are not already encapsulated in the receptor pocket. A different approach that could be investigated is using a ligand of a high molecular weight that would compete and dissociate appropriately with the toxin for the receptor site when the receptor is immobilised on the surface (Karlsson 1994). This ligand would provide the molecular mass necessary for the SPR response, but sourcing a suitable competitor to PSP toxins may be difficult. The limited availability of the saxiphillin receptor to only a few research groups has inhibited the characterisation of this binder in the role of PSP toxin detection by SPR. Research is on-going into the synthesis of cloned receptors that would prove beneficial in both functional assays and as more stable alternative binders to antibodies for sensor technologies.

Antibodies

Antibodies unlike receptors are biochemical binders and bind selectively to chemical moieties on the structure of the toxin to which they are produced. These binders do not show a similar correlation between the toxicity of a toxin and binding affinity in the same manner as receptors for PSP toxins. The difficulty in trying to detect such small molecular weight compounds using antibodies is that there are limited functional groups to conjugate a protein for antibody production based on site specificity and, therefore, few chemical reactions can be utilised for immunogen synthesis. Restricted and limited

availability of toxins makes the synthesis of alternative derivatives impractical unless sufficient yield can be achieved for immunogen production. The formation of the toxin protein conjugate must also reduce the toxicity of the toxin or when immunised the animal may be affected with PSP symptoms. The first antibodies produced against PSP toxins were reported by Johnston et al. (1964). For most PSP toxin antibodies produced since then either a modification of the Mannich reaction or reductive alkylation chemistry (Bürk et al. 1995) has been employed for the synthesis of the protein conjugate. As highlighted by Usleber et al. (2001) during these reactions there are three potential reactive sites for the carbamate toxins. These include the amino, guanidine and imine groups, but it is difficult to characterise which is the predominant orientation of the toxin to the protein conjugate or the ratio of protein conjugation to all three sites.

Immunochemical methods are frequently used for qualitative, semi-quantitative and quantitative measurements of many chemical substances with high biological impact. These methods are well established in clinical chemistry and endocrinology and have become in recent years more sophisticated in their use for food and environmental analysis and toxicology screening due to their ability to function effectively in complex biological matrices without major purification. From the production of the first antibodies for PSP toxins, it was decades later when immunoassays were actually utilised to monitor PSP toxins in shellfish. Immunoassay methods for PSP toxins have been reviewed in the past (Usleber et al. 2001) and several antibody-based approaches for detecting these

Table 1. General advantages and disadvantages of binders previously assessed for SPR analysis of PSP toxins.

Binder	Advantages	Disadvantages
Receptors	Have a binding affinity that generally correlates with the biological response or toxicity of the toxins Can be isolated rapidly compared with antibody production Good sensitivity	Wider scope of binding to all compounds that act on the same receptor Have to be isolated from biological tissues and in some cases the species of origin is difficult to obtain May display poor stability May be temperature sensitive for analytical purposes
Monoclonal antibodies	Constant renewable source of binder once produced with minimal batch to batch variation During production less immunizing agent is required compared with polyclonal production and it is possible to select for specific epitope specificities and generate antibodies against a wider range of antigens React with a single epitope on an antigen with high specificity Binding affinity for the immunizing antigen is generally better than a receptor Less background signal than polyclonal antibodies	For production, time, effort and commitment is high and use animals On average the binding affinity of a monoclonal antibody is lower than a polyclonal antibody As monoclonal antibodies are highly specific to a single epitope they may lose affinity to other antigens within a group with minor molecular modifications The binding affinity of an antibody does not correlate with biological response for a toxin group Antibodies sometimes display unexpected cross-reactivity with unrelated antigens in biological matrices
Polyclonal antibodies	For production relatively quick and inexpensive to produce compared with monoclonal antibodies Polyclonals will recognize multiple epitopes on any one antigen and are therefore more tolerant of minor changes in the antigen than monoclonal antibodies providing better cross-reactivity to structural antigens within a group The recognition of multiple epitopes generally provides more robust detection Binding affinity for the immunizing antigen is generally better than a monoclonal antibody or receptor	Animals are required and are prone to batch to batch variability They produce large amounts of non-specific antibodies which can sometimes give background signal in some applications Multiple epitopes make it important to check for any cross-reactivity in biological matrices The binding affinity of an antibody does not correlate with biological response for a toxin group

toxins have been described since that time. These include radioimmunoassay (Carlson et al. 1984), immunoaffinity columns (Dietrich et al. 1996), lateral flow devices (Jellett et al. 1998; Laycock et al. 2010) and numerous competitive enzyme immunoassays (EIAs). Indirect EIAs have been developed for STX (Chu and Fan 1985; Cembella et al. 1990; Micheli et al. 2002), neosaxitoxin (NEO) (Chu et al. 1992; Bürk et al. 1995) and gonyautoxin (GTX) 2/3 (Kawatsu et al. 2002) and direct EIAs for STX and NEO (Usleber et al. 1991; Schneider et al. 1995; Chu et al. 1996; Huang et al. 1996; Dubois et al. 2010) and GTX 2/3 (Kawatsu et al. 2002). These assays all utilise antibodies produced using the previously mentioned protein conjugation chemistries with the primary modification being the PSP toxin to which the protein is conjugated. Although several EIAs and lateral flow test kits are commercially available (Laycock et al. 2010; <http://www.abraxiskits.com>; <http://www.biosense.com>; <http://www.r-biopharm.com>), one shortcoming is that they are highly specific to

certain toxins. This specificity, corresponding mainly to chemical structure but not proportionately to toxicity, limits an accurate measure of the total toxicity in a sample as required by regulatory authorities. For such diverse potency within this relatively large toxin group, whose small molecular weight structures are highly similar, it is extremely difficult to match the cross-reactivity profile of an antibody to the potency of each toxin for similar comparability as a receptor assay to the mouse bioassay. Usleber et al. (2001) state that from an immunological point of view the PSP toxin family must be subdivided into STX (non-hydroxylated) and NEO (hydroxylated) groups. Previous ELISA studies have demonstrated that when the antibodies are specific to either group they are less specific to the other. Therefore, to incorporate the full spectra of PSP toxins to an equivalent extent, antibodies produced to both the non-hydroxylated (STX) or hydroxylated toxins (NEO) will have to be utilised. Other drawbacks to these commercial kits are the high costs, primarily due to the availability and

quantity of toxin required for producing either coating antigen or enzyme labels, and the potentially subjective interpretation of results.

Current progress of SPR analysis of PSP toxins using antibody binders

To date although antibodies may have specificity issues relating to the analysis of such a large toxin group they are the most successful binder in terms of sensitivity for the detection of PSP toxins. For this reason antibodies were chosen as the model binder to demonstrate the feasibility of SPR optical biosensor technology for PSP toxin analysis. The applicability of SPR analysis to this task was demonstrated using both a monoclonal antibody (GT-13A) raised to gonyautoxin 2/3 and a polyclonal antibody (R895) raised to STX (Campbell et al. 2007). When an STX surface was employed, which can be regenerated over 1000 times for reuse conserving toxin supplies and reducing cost, these two antibodies displayed vast differences in their cross-reactivity profiles. The GT-13A binder showed a high degree of segregation between non-hydroxylated and hydroxylated toxins indicating that when samples are analysed and compared with the mouse bioassay using this binder the toxin content of a sample would be underestimated if the toxin composition is predominately the hydroxylated toxins, NEO or GTX1/4 (Campbell et al. 2007). However, PSP toxins such as C1/C2 and GTX5 displayed high specificity, although their toxic potency is relatively low to STX. Therefore, samples containing levels of these toxins will overestimate compared with the mouse bioassay. For regulatory purposes, underestimation could have severe health implications to the consumer whereby contaminated material is declared safe for consumption, whereas overestimation could cause detrimental economic losses to the industry through the unnecessary closure of harvesting beds. This GT-13A binder was further assessed by SPR to determine its tolerance capabilities to various shellfish matrices and PSP toxin extraction solvents (Fonfria et al. 2007). Fonfria et al. applied different extraction procedures, previously employed for PSP toxin analysis, to different shellfish species and they highlighted that by either using an ethanolic extraction procedure reported by Garthwaite et al. (2001) or the AOAC HPLC acetic acid extraction (AOAC 2005b), the determination of PSP toxins in shellfish could be performed by SPR. The Garthwaite method was recommended for further evaluation based on analysis time, but only 50% recovery of STX was achieved using this extraction procedure. However, samples containing C1/C2 toxins overestimated compared with HPLC and mouse bioassay results due to the low toxic potency but high specificity of this toxin. As a proof of concept this monoclonal antibody was

also employed to demonstrate that SPR biosensor-based biochemical analysis could be combined with state-of-the-art mass spectrometry chemical analysis for joint screening and confirmatory determination of PSP toxins (Marchesini et al. 2009).

The R895 binder showed a much more diverse but narrower pattern in cross-reactivity profile with improved sensitivities to all toxins compared with GT-13A, but the hydroxylated toxins dcNEO and GTX1/4 will require further improvement. Rawn et al. (2009) performed an evaluation of both binders by SPR using the Garthwaite extraction procedure for analysing 88 natural samples and compared the results with the AOAC HPLC method (AOAC 2005b). This study demonstrated that when using this extraction procedure, different shellfish matrix effects were considerable compared with a buffer curve, and if a buffer curve were used for analysis, this would cause an overestimation in results of 50%. Therefore, natural samples were evaluated using an extracted mussel calibration curve for better correlation in recovery and matrix effects. In general, the polyclonal antibody correlated better with HPLC than the monoclonal antibody due to the differences in cross-reactivity profiles. However, it was recommended that improved antibody response to the hydroxylated toxins would be necessary if SPR were to be a replacement for the mouse bioassay for regulatory testing. As a screening tool, it was suggested that this SPR method using either antibody could eliminate greater than 80% of samples from the mouse bioassay at the current regulatory limit. In relation to the three Rs of replace, reduce and refine at this stage, the SPR assay may not be a replacement tool but a reduction tool for the number of animal assays performed for regulatory purposes.

Further work was then performed on the extraction procedure in order to calibrate the SPR assay using a buffer curve to eliminate the requirement of sourcing uncontaminated material and using relatively excessive quantities of toxin in the preparation of a calibration curve (Campbell et al. 2009). This was achieved in a comparative study using a second polyclonal binder raised to STX (BC67) for SPR analysis compared with EIA, HPLC and mouse bioassay (Campbell et al. 2009). This study demonstrated that the key for immunological assay development was the binder and highlighted that the advantages of using SPR over all the other methods were simplicity, ease of use and speed of analysis. This new extraction procedure using pH 5 sodium acetate buffer followed by centrifugation followed by dilution and analysis was then applied to the STX antibody and a single laboratory validation of this SPR method performed highly satisfactorily (Campbell et al. 2010). An inter-laboratory study between seven international laboratories is currently underway. Threshold values for the assay have been

Table 2. Sensitivity and specificity data for both STX and NEO antibody on a NEO chip surface (Campbell et al., unpublished data).

Chip surface	Neosaxitoxin chip surface					
Antibody	STX			NEO		
Antibody dilution in HBS-EP	1/250			1/50		
Antibody ratio to standard	1:1			1:1		
Flow rate ($\mu\text{l min}^{-1}$)	25			25		
Contact time (min)	2			2		
PSP toxin analogue	IC ₅₀ (ng ml ⁻¹)	Percentage cross-reactivity	Dynamic range, IC ₂₀ to IC ₈₀ (ng ml ⁻¹)	IC ₅₀ (ng ml ⁻¹)	Percentage cross-reactivity	Dynamic range, IC ₂₀ to IC ₈₀ (ng ml ⁻¹)
Saxitoxin dihydrochloride	4.8	100	2.7–8.1	41.8	100	6.6–203
Neosaxitoxin	13.4	35.8	3.7–84.8	2.4	1742	1.1–5.5
Gonyautoxin 1/4	474 ^b	1.0 ^b	77–2780 ^b	4.1	1032	1.6–14.9
Gonyautoxin 2/3	7.0	68.7	2.1–22.0	19.6	213	4.1–91.6
Decarbamoyl saxitoxin	2.9	165.1	1.3–6.3	>1000	<4.2	n.d. ^a
Decarbamoyl neosaxitoxin	100	4.8	14.4–200	192.1	21.8	41.9–512
Decarbamoyl gonyautoxin 2/3	18.7	25.8	4.4–62.3	>1000	<4.2	n.d.
C1/C2	14.8	32.6	3.3–53.6	>1000	<4.2	n.d.
Gonyautoxin 5	4.1	117	2.7–7.7	>1000	<4.2	n.d.
C3/C4	206 ^b	2.3 ^b	27–590 ^b	100	41.3	n.d.

Notes: ^an.d., Not determined due to lack of the standard.

^bValues calculated based on extrapolation of the four-parameter fit applied to the standard curve due to availability of the standard.

discussed for regulatory purposes, but this level could vary depending on geographical location and the risk-management strategies that have to be considered whether by industrial or by regulatory monitoring laboratories. To date there has been no reports of PSP toxins occurring in isolation, but if GTX1/4 were to do so, this method could then become invalid even as a screening tool due to the lack of sensitivity of this binder to this toxin. Therefore, alternative binders are still being sought with improved sensitivity towards the hydroxylated toxins and different heterologous assay formats are being investigated that could improve the overall toxicity determination of PSP toxins in a sample.

A combination of two binders, one raised to STX (Campbell et al. 2007), the other to NEO (Bürk et al. 1995), were assessed on a NEO chip surface to determine the feasibility of combining the cross-reactivity profiles of each in order to be able to detect the full range of PSP toxins in isolation at the regulatory level (Campbell et al., unpublished data). The sensitivity and specificity of each binder relative to STX and its analogues were assessed. The cross-reactivity profile of the STX and NEO antibodies on the NEO surface was favoured to the non-hydroxylated and hydroxylated toxins at the R1 position respectively (Table 2). The percentage cross-reactivity of STX antibody in relation to STXdiHCl was variable in relation to toxin structure with the NEO surface. Toxins with modifications in the R4 position (Figure 1) displayed the highest percentage cross-reactivity followed by those

with modifications to the R2 and R3 positions and then those toxins hydroxylated in the R1 position. Combinations of modifications showed an additive decrease in percentage cross-reactivity with the outcome for GTX1/4, which is modified at the R1, R2 and R3 positions, displaying the lowest percentage cross-reactivity at $\leq 1.0\%$. For the NEO antibody binder the chemical reaction used to prepare the chip surfaces was different from the reaction used to prepare the toxin protein conjugate used as the immunogen. A change in orientation of the toxin conjugated to the surface could explain the relatively low antibody titres required to produce a response on this NEO chip surface in relation to the titre of this antibody reported in the ELISA study (Bürk et al. 1995). This antibody was significantly more sensitive to the R1 hydroxylated toxins compared with the corresponding non-hydroxylated toxins as in agreement with the ELISA study.

Initially for the carbamate toxins different ratios of STX antibody to NEO antibody titres were assessed with the most suitable for the mix study being 1/500 and 1/50, respectively. Different percentage ratios of STX to NEO antibody at these dilutions were assessed using the neosaxitoxin chip surface and each of the carbamate toxins at a concentration of 10 ng ml^{-1} (Figure 5). The full cross-reactivity profile was determined using an optimum 20% STX and 80% NEO antibody (Table 3) as this ratio showed the lowest differential in cross-reactivity for the carbamate toxins. On comparison with the cross-reactivity profile of STX and NEO antibody on their own with this surface,

Table 3. Sensitivity and specificity of the STX/NEO antibody mix for each PSP toxin (Campbell et al., unpublished data).

	STX			1/500		
Antibody dilution in HBS-EP	NEO			1/50		
Antibody mix	20% STX/80% NEO					
Antibody ratio to standard	1:1					
Flow rate (μl min ⁻¹)	12					
Injection volume (μl)	60					
Contact time (min)	5					
	Toxin concentration (ng ml ⁻¹)			Toxin concentration as STXdiHCl equivalents (ng ml ⁻¹)		
	IC ₅₀ (ng ml ⁻¹)	Percentage cross-reactivity	Dynamic range, IC ₂₀ to IC ₈₀ (ng ml ⁻¹)	IC ₅₀ (ng ml ⁻¹)	Percentage cross-reactivity	Dynamic range, IC ₂₀ to IC ₈₀ (ng ml ⁻¹)
PSP toxin analogue						
Saxitoxin dihydrochloride	2.8	100	0.2–68.8	2.8	100	0.20–68.8
Neosaxitoxin	2.8	100	0.8–9.9	3.1	90.3	0.87–10.8
Gonyautoxin 1/4	4.4	63.6	0.8–25.3	4.0	70	0.72–22.8
Gonyautoxin 2/3	4.9	57.1	0.9–28.0	2.9	96.6	0.54–16.8
Decarbamoyl saxitoxin	0.4	700	0.1–1.2	0.3	933	0.07–0.9
Decarbamoyl neosaxitoxin	9.1	30.7	1.7–48.7	6.4	43.8	1.19–34.2
Decarbamoyl gonyautoxin 2/3	17.1	16.4	3.1–94.7	6.8	41.1	1.23–37.7
C1/C2	14.5	19.3	2.0–102.7	1.1	255	0.15–7.7
Gonyautoxin 5	0.4	700	0.1–1.5	0.03	9333	0.01–0.1
C3/C4	91.8	3.0	24.0–198	4.0	70	1.03–8.5

the cross-reactivity profile has been amalgamated to produce a potential single-assay format for all PSP toxins. With the exceptions of GTX5 and dcSTX, which will overestimate based on this cross-reactivity profile, this antibody mix correlates much better than either binder individually with the mouse bioassay toxicity factors (Figure 1) for each of the toxins. This is illustrated in Table 3 as the cross-reactivity profile in STXdiHCl equivalents.

Consequently, by using optical SPR biosensor analysis, there is the potential to resolve cross-reactivity issues that arise using a single binder. Combining the two polyclonal antibodies STX and NEO with a common denominator of the NEO surface enabled the design of a single-assay format for both hydroxylated and non-hydroxylated toxins. In addition, as the cross-reactivity is more in line with the toxicity profile for the mouse bioassay, this is an advantage in having one screening test for all PSP toxins compared with two ELISA tests. This approach of mixing antibodies raised in the same species could be used for other families of chemical contaminants or toxins where there is significant variability in the cross-reactivity profile as long as there is a common denominator in the family for chip surface immobilisation.

Potential binders for future optical SPR analysis of PSP toxins

A wide range of non-antibody-based binders have been reviewed previously as alternative molecules for the

detection of pathogens (Ngundi et al. 2006). The most promising non-antibody binders worth investigating in their role for PSP toxin analysis by SPR are chemosensors (Bell and Hext 2004), molecular imprinted polymers (MIPs) (Hall et al. 2005) and aptamers (Tombelli et al. 2005). The advantages and disadvantages of these particular binders are highlighted in Table 4.

Chemosensors

Chemists have used a variety of approaches to design artificial receptors or chemosensors capable of selectively binding analytes. Chemosensors are molecules specifically designed for the qualitative and quantitative monitoring of analytes and are being researched in the areas of biological and analytical chemistry, medicine and environmental sciences (Bell and Hext 2004; Minkin et al. 2008). These molecules bind to the target analyte through non-covalent interactions and produce a change in light absorption or fluorescence. Molecular structures that have displayed some potential as chemosensors are polyalcohols, crown ethers, calixarenes, helicenes, sterically geared tripods, metal complexes, pinwheels, porphyrins and fused ring heterocycles. Most chemosensors described do not display sufficient sensitivity or selectivity to their targets for practical applications, and they usually require synthetic modification for immobilisation onto a sensor surface. However, chemosensors such as crown ether sensors like calix[4]arene have been investigated as alternative binders for STX detection

Table 4. Advantages and disadvantages of potential binders for SPR analysis of PSP toxins.

Binder	Advantages	Disadvantages
Chemosensors	Non-animal-based binders Can be incorporated into self-assembly monolayers for sensing on a surface	Lower binding affinity than antibodies No correlation between the binding intensity and biological response or toxicity
Molecular imprinted polymers (MIPS)	Non-animal-based binder Target analyte defines its own recognition site Stable at extreme temperature and pH Show specificity in natural systems Adaptable and flexible in their use Low cost and ease of manufacture May be used in non-aqueous media or aggressive environments	May be sensitive to small alterations in target analyte structure and properties Show non-specific binding Diversity of binding sites Template bleeding requires suitable template analogue for imprinting and affects quantitative applications Generally lower binding affinities than antibodies
Aptamers	Nucleic acid aptamers are non-animal-based binders produced by chemical synthesis resulting in limited batch to batch variation Selectively bind to low molecular weight compounds Binding affinities of aptamers are in the μM to nM range and kinetic parameters can be altered on demand Toxins that do not illicit a good immune response can be used to generate high-affinity aptamers Aptamers are stable for long-term storage and can be transported at ambient temperature Selection conditions can be manipulated to obtain aptamers with properties desirable for <i>in vitro</i> assays, e.g. non-physiological buffers, solvents, etc. Reporter molecules can be attached to aptamers at precise locations so as not to affect binding	Aptamers are costly to generate and long aptamer sequences are difficult to achieve DNA aptamers have a smaller range of three-dimensional structures obtainable compared with RNA aptamers but they can bind their target to the entire sequence DNA and RNA aptamers are susceptible to enzymatic degradation by nucleases, thus requiring highly pure environments. This can be overcome Peptide aptamers are constrained to the scaffold protein so they are less flexible, which may affect their effectiveness. Only the variable range is used for binding Peptide aptamers require biological systems for selection purposes

(Chen et al. 2007). These binders are showing considerable promise in detecting STX using fluorescence, but as yet the sensitivity is five orders of magnitude lower than that achievable using the mouse bioassay (Gawley et al. 2002, 2007). For naturally contaminated shellfish samples there was no correlation between the intensity of absorption and toxicity of the sample. However, SPR spectroscopic detection using these binders increased the sensitivity up to 1000-fold (Chen et al. 2007). Shellfish matrices have not as yet been evaluated.

Molecular imprinted polymers (MIPs)

MIPs have received controversial exposure over the past 10 years from researchers and industries in their potential role in the environmental and food analysis

of chemical contaminants and toxins (Lotierzo et al. 2004; Henry et al. 2005; Baggiani et al. 2008). They are artificially created ligand binding sites that can selectively recognise a target molecule. The synthesis involves the polymerisation of functional and cross-linking monomers around the target of interest or a structural mimic template through non-covalent interactions or reversible covalent bonds or both with the functional groups of the template. These three-dimensional polymer-based systems can be optimised to yield recognition sites highly specific and selective to the target of interest in the same manner as either a polyclonal or a monoclonal antibody. However, the mechanism of interaction or recognition of a particular analyte to the polymer is still not completely comprehended. Unlike antibodies, these are fairly rigid macroscopic structures are insoluble in any solvent. MIPs therefore are more commonly marketed and used for

selectively separating molecules for clean-up and pre-concentration of analytes from complex samples, but they have also been demonstrated in detection assays (Ramstrom et al. 2001; Chianella et al. 2003). The applicability of MIPs as a surface template in the SPR analysis of domoic acid has also been demonstrated (Lotierzo et al. 2004). Mahony et al. (2005) stated that combinatorial chemistry and computational molecular modelling are the most promising concepts for the development of MIPs for enhanced recognition properties. Using this technology with advances in polymeric materials, in the future there is the potential to develop and design binders with the highest selectivity to the PSP toxin family as a whole. However, the elimination of problems associated with template leaching for quantitative analysis would have to occur.

Aptamers

Aptamers, first described by Tuerk and Gold (1990), are creating a new wave of enthusiasm as alternative binders to antibodies due to progress in aptamer screening methods (Wang and Jia 2009) and their potential in the field of aptasensors (Wilner and Zayats 2007). Aptamers can be divided into those created from polymers of nucleic acids (RNA and DNA) or amino acids (peptides). Nucleic acid aptamers are small synthetic oligonucleotides of up to 100 base pairs that can bind specifically to a variety of targets, such as whole cells, proteins, drugs, toxins and low molecular weight compounds, with affinities in the nanomolar and sub-nanomolar ranges. They can bind with a high affinity and specificity to a target molecule through complementary shape interactions as a result of their three-dimensional shape. Nucleic acid aptamers are produced by an *in vitro* process called systematic evolution of ligands by exponential enrichment (SELEX) (Stoltenburg et al. 2007). These aptamers can provide high molecular discrimination in being able to distinguish differences in a methyl group between two compounds (Jenison et al. 1994). Aptamers offer several distinct advantages compared with antibodies or receptors. They are isolated *in vitro*, and the targets are of a broad spectrum. Following aptamer selection, DNA aptamers with a long shelf life can be prepared with high batch consistency and low cost by automated DNA synthesis, and RNA aptamers can be prepared by simple *in vitro* transcription. Aptamers are also simple to modify and introduce various reactive groups, affinity tags or reporting moieties essential for biosensor applications. Nucleic acid aptamers have a limited number of structures compared with peptide aptamers, but offer better structural stability. Protein scaffolds are an adaptation of peptide aptamers that may overcome stability issues. Peptide aptamers still require biological systems for

selection purposes. Aptamers have been demonstrated in therapeutic applications, in the field of separation chemistry, in environmental and food analysis of chemical contaminants and toxins, and in a number of aptasensor applications (Tombelli et al. 2007; Wilner and Zayats 2007; Stead et al. 2010). These binders are now developing rapidly and may be the one binder group that in future replaces antibodies for diagnostic and detection applications. To date, they offer the best opportunity of finding a single binder for the detection of the whole PSP toxin family or individual binders for each of the varying toxic groups within this family.

Conclusion

Optical SPR biosensor analysis for PSP toxin detection has been demonstrated over the past five years to be a highly effective rapid screening method that has the potential to reduce the vast number of mouse bioassays performed worldwide. This review has highlighted the key problems associated with antibody cross-reactivity in over- and underestimating total toxicity and how, by using a dual antibody binder system with a generic surface, there is the potential to adjust the cross-reactivity profile to help overcome this problem. Alternative non-antibody-based binders, however, may offer a complete non-animal-based methodology for the detection of PSP toxins by SPR. In addition, advances in SPR technology, with the development of multiplexing multichannel instruments, could help resolve some of the difficulties in binder specificity not relating to toxicity. Multiplex multichannel SPR biosensors with highly designed binders such as nucleic acid aptamers (Mok and Li 2008) may be the way forward not only for monitoring phycotoxins, but also for a wide variety of food additives and contaminants.

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