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Use of a novel micro-fluidic device to create arrays for multiplex analysis of large and small molecular weight compounds by surface plasmon resonance

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ABSTRACT

There is an increasing demand to develop biosensor monitoring devices capable of biomarker profiling for predicting animal adulteration and detecting multiple chemical contaminants or toxins in food produce. Surface plasmon resonance (SPR) biosensors are label free detection systems that monitor the binding of specific biomolecular recognition elements with binding partners. Essential to this technology are the production of biochips where a selected binding partner, antibody, biomarker protein or low molecular weight contaminant, is immobilised. A micro-fluidic immobilisation device allowing the covalent attachment of up to 16 binding partners in a linear array on a single surface has been developed for compatibility with a prototype multiplex SPR analyser.

The immobilisation unit and multiplex SPR analyser were respectively evaluated in their ability to be fit-for-purpose for binding partner attachment and detection of high and low molecular weight molecules. The multiplexing capability of the dual technology was assessed using phycotoxin concentration analysis as a model system. The parent compounds of four toxin groups were immobilised within a single chip format and calibration curves were achieved. The chip design and SPR technology allowed the compartmentalisation of the binding interactions for each toxin group offering the added benefit of being able to distinguish between toxin families and perform concentration analysis. This model is particularly contemporary with the current drive to replace biological methods for phycotoxin screening.

1. Introduction

The raised awareness to security and health concerns worldwide from reports of animal adulteration, chemical contamination and emerging toxic compounds has challenged regulatory authorities and scientific communities to improve monitoring regimes for assurance of food and water supplies. Many of the current monitoring procedures use either single analyte immunoassays, chromatography, mass spectrometry or biological methods which are either highly specific, time consuming, expensive and in the latter case unethical. Rapid and cost effective multiplexed detection technologies, capable of performing biomarker profiling or replacing contaminant or toxin sequential analysis with a single multi-assay format, are becoming a fundamental requirement in food safety and environmental analysis. Following the success of RNA chips for high throughput genome analysis, affinity protein micro-arrays for proteome and biomarker analysis (Sheridan, 2005; Wingren and Borrebaeck, 2006) and for toxin analysis have also been reported (Goldman et al., 2004; Ligler et al., 2003; Rowe-Taitt et al., 2000; Taitt et al., 2004). These array systems have an orderly arrangement of proteins, such as antibodies, that capture selected target proteins or toxins. In most cases, the capturing process is monitored through fluorescence detection which requires a time consuming direct or indirect protein labelling process. Consequently, there is a major interest in adopting label-free microarrays (Wingren and Borrebaeck, 2006) and a number of research groups have explored the use of surface plasmon resonance (SPR) based detection for biomarker concentration analysis (Kanda et al., 2004; Usui-Aoki et al., 2005; Yang et al., 2005; Lee et al., 2006) and multiplexing for antibiotic detection (Haasnoot, 2009).

SPR biosensor technology is a well-established label-free technique for the sensitive and real-time monitoring of molecular interactions with the binding measurement being responsive to a change in refractive index due to the change in mass on a sensor surface as result of the interaction. Detailed information on the principle behind SPR technology and its application of detecting both high and low molecular weight compounds of chemical and biological origin can be found in a recent review by Homola (2008). For high molecular weight compounds such as proteins either a direct or indirect inhibition assay is employed but for low molec-

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ular weight molecules such as toxins the preferred assay format is indirect inhibition. The key components to be considered for the design of a multi-analyte SPR biosensor assay are the biosensor chip surface, the microfluidic flow system and the SPR analyser. The binding partners immobilised on the sensor surface should be arranged with appropriate spatial separation for each binding assay. A microfluidic system, constructed to allow for compartmentalisation of the sensor surface, would allow sample flow over each immobilised partner and the interaction to take place. Additionally, this system would increase the capability of dissociating the multiple binding events and restoring the sensor surface for concentration analysis purposes. Each binding event must then be detectable and the responses distinguishable using the analyser and applied software.

Multiplexed immobilisation strategies are either based on spotting (Endo et al., 2006), advanced micro-fluidic devices that for example utilise hydrodynamic addressing (Säfsten et al., 2006), or different flow cell geometries for immobilisation and detection (Bravman et al., 2006). Of these strategies, spotting nanolitre size droplets through contact or micro-dispense printing offers the advantage that high density arrays can be produced. The drawback of spotting are the poor results with respect to the uniformity of the spotted surface (Deng and Zhu, 2006; Bouffartiques et al., 2007), stability of attachment, and relative low density of active ligands on the spotted chip (Chang-Yen et al., 2006). The design and generation of biosensor chips is considered a major challenge for microarray development but micro-fluidic devices can be used to transport reagents to the biosensor chip surface providing a controlled and uniform immobilisation reaction.

This present study describes the construction and performance characteristics of a low cost immobilisation device which was developed in combination with a prototype multiplex SPR analyser. The device was assessed in relation to its ability in immobilising ligands onto the sensor surface using a range of high (protein) and low (phycotoxins) molecular weight compounds as models.

2. Materials and methods

2.1. Reagents

An amine coupling kit (containing 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS) and 1 M ethanolamine pH 8.5), 10 mM glycine–HCl pH 2.0, immobilisation buffer (10 mM sodium acetate buffer pH 5.5), 10 mM sodium acetate buffer pH 4.0 and HBS-EP+ buffer (pH 7.4, 0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate), Myoglobin and anti-myoglobin antibody were obtained from GE Healthcare. Saxitoxin dihydrochloride and neosaxitoxin were purchased from the National Research Council Canada (NRC), Halifax, Canada. Okadaic acid was obtained from LC Laboratories, USA. Domoic acid, formaldehyde (37%), 2,2-(ethylenedioxy)bis-(ethylamine) (Jeffamine), phosphate buffered saline (PBS), human serum albumin (HSA) and anti-HSA antibody were obtained from Sigma–Aldrich, UK. Recombinant bovine insulin-like growth factor-binding protein-2 (IGFBP-2) was obtained from Fusion Antibodies (Belfast, UK), and anti-IGFBP-2 rabbit polyclonal antibody (BC62) sourced from CER Groupe (Marloie, Belgium). Ultra pure water from a Milli-Q gradient set-up (Millipore) was used in all experiments.

2.2. Instrumentation

A prototype immobilisation unit and a prototype multiplex SPR analyser were developed within the BioCop project. Series S CM5 chips were obtained from GE Healthcare, Uppsala, Sweden. For reasons of comparison, other instruments capable of immobilising and detecting proteins on sensor surfaces were used. Biacore T100 (GE Healthcare, Uppsala, Sweden) was used to immobilise a single type of protein in a flow cell using a microfluidic system. Alternative multiplex immobilisation strategies were based on micro-dispense printing using a Nano-plotter from GeSiM,Germany and contact printing using an OmniGrid micro system (Genomics Solutions, Inc.) using a 255 μm ArrayIt brand stealth micro spotting pin from TeleChem International, Inc. Both printing technologies were used to immobilise proteins on gold affinity chips and immobilisation levels were measured using Flexchip from GE healthcare, Sweden.

2.2.1. Microfluidics for immobilisation unit

The integrated micro-fluidic system for the immobilisation unit contains 3 layers (Fig. 1). The top layer is an injection moulded part which contains 32 wells created from cutting a third of a 96-well microtitre plate (Fig. 1A). These wells have round bottoms to facilitate the usage of small volumes and removal of samples after usage and the well spacing and dimensions are the same as those of a 96-well microtitre plate. The bottom side is flat and the material thickness to the deepest point of the well is 0.1 mm. The flat bottom side was over moulded with a 0.2 mm thick polydimethylsiloxane (PDMS) layer. Connection between the wells and the flat bottom was produced by laser machining, thereby creating holes (diameter 0.3 mm) through the plastic and PDMS in the bottom of each well. Underneath the wells a two-layer microfluidic system was created from PDMS because of its favourable mechanical properties (Delamarche et al., 1997; Vickers et al., 2006; Yang et al., 2005). The first PDMS layer (Fig. 1B) contains 32 flow channels of equal length and the second layer contains 16 open immobilisation cells (Fig. 1C), each with a width of 100 μm and a depth of 90 μm. The in- and out-let of the flow cells are connected with the flow channels by laser ablating 200 μm holes.

2.2.2. Immobilisation unit

The integrated microfluidic system is inserted in the immobilisation unit and its position is controlled by two metal clips on the front side and one metal pin on the back side that can be retracted (Fig. 2B). A transparent PMMA lid is placed on the sample wells by lowering a metal arm which rests on a metal support on the front side of the immobilisation device and kept in place by a magnet (Fig. 2C). Within the lid, two rubber rings are inserted to create an air-tight sealing of the 16 wells on the left and right hand side, respectively. Pressurised air can be applied to the 16 wells on the left or the right hand side through two holes in the lid. A sensor chip docking station is positioned on rails and can be moved below the fluidic device. In the middle of the docking station, there is an open cylinder which, at the bottom, is connected to a tube that can deliver pressurized air. A metal ball with a diameter about 0.02 mm smaller than the cylinder is placed within (Fig. 2A). On top of the cylinder and ball, a plastic plate is positioned on which the sensor chip is placed (Fig. 2B). After inserting the sensor chip, the docking station is manually moved under the fluidic device and docking is performed by applying pressurized air (Fig. 2C). The ball will then move upwards, applying a force in the centre of the flow cell area. Resting on the spherical surface of the metal ball that can rotate frictionless, the plastic plate and sensor chip will align with the flow cell area. Immobilisation of proteins, washing and docking of the sensor chip is controlled by a unit that contains a programmable logic controller (AL2-24MR-D, Mitsubishi) which in turn controls the settings of an air pump (ASF-Thomas) and a few valves (ITV0010-34-Q, SMC). All are operated by a single 12 V power source. The status of the various functions of the immobilisation unit, i.e., sensor chip docking, washing and immobilisation, is visualised with a few LEDs. Addi-

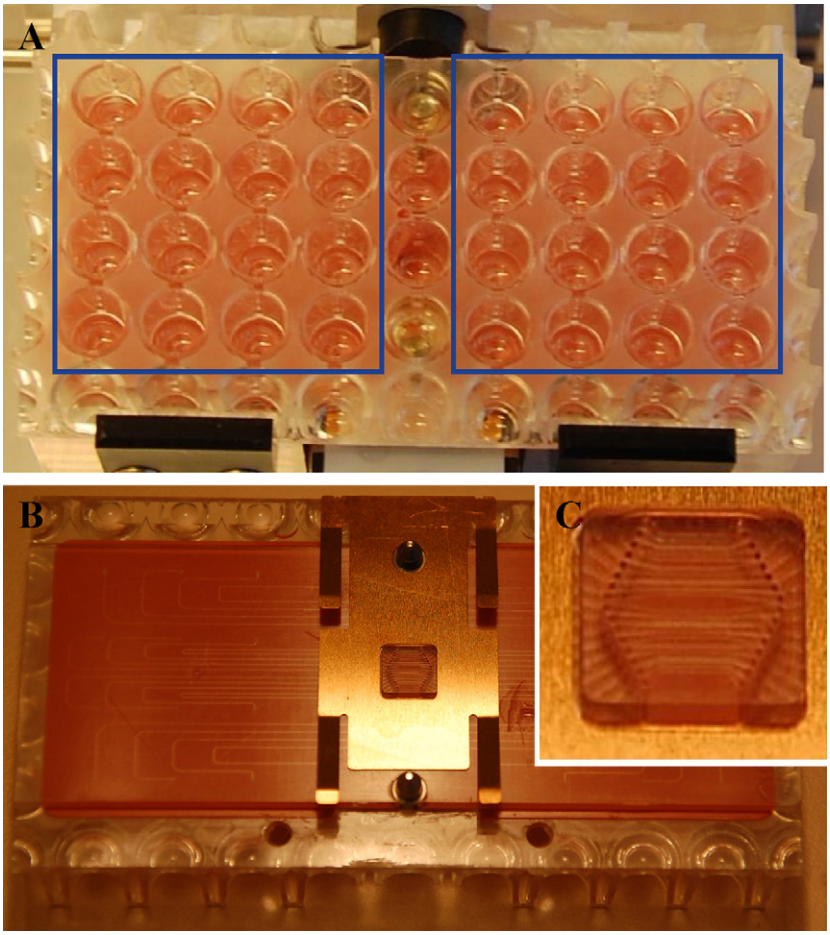


Fig. 1. (A) Top view of microfluidic immobilisation system showing sample wells created by cutting a 96-well microtiter plate (9 cm × 5 cm). (B) Bottom view of microfluidic immobilisation system showing 16 fluidic channels of equal length on the left hand side that connect sample wells (A) and flow cells that are located in the centre. The right hand side contains another 16 channels, symmetric to the 16 channels on the left hand side. The metallic cover and pins are used to guide and align docking of a sensor surface. (C) Close up of second microfluidic layer that contains 16 open flow cells (6.4 mm × 6.4 mm × 1.3 mm). Upon docking a sensor chip, flow channels are closed. Pressurized air is used to push samples that were placed in the 16 wells inside the square on the left hand side (A) through the flow channels (B) and flow cells (C) towards 16 wells on the right hand side (A) to create immobilised patterns as shown in Fig. 3.

tionally, a pressure meter (Mano 2000, Keller) is connected to the tubing that applies pressure on the sample wells.

2.2.3. *Multiplex SPR analyser*

The BioCop SPR analyser, compatibly designed to the immobilisation unit, is a molecular interaction array system that can monitor up to 16 interactions simultaneously. The instrument consists of a processing unit with liquid and sample handling and an SPR detection system. The detection system is based on the Kretschmann configuration (Kretschmann, 1971) as used in all current commercially available Biacore™ instruments. The SPR signal is proportional to the change in refractive index close to the sensor surface and is expressed in resonance units (RU). For proteins, one RU corresponds approximately to 1 pg of material bound per square millimetre of surface area (Stenberg et al., 1991).

The system is designed for rapid screening of multiple ligands (up to 16) simultaneously employing a 4 × 4 format with four 0.6 mm^2 flow cell areas each containing four SPR sensing spots. This multichannel format enables assays with either multi-ligand or multi-sample focus. When a Series S CM5 chip with 16 immobilised ligands is docked, four independent parallel flow cells are formed by the sensor chip pressing against moulded channels on the integrated microfluidic cartridge (IFC). The response is measured from four detection spots in each flow cell. The liquid handling system comprises of three peristaltic pumps, the IFC, the needle block with four injection needles and the solvent block. During an experiment, the sample pump aspirates either running buffer (up to 4, one per flow cell) from the solvent block, samples and reagents from the rack tray or air, with a constant flow rate through the flow cells simultaneously.

Samples are aspirated with four needles that address four adjacent wells of a 96-well microtitre plate and are guided to the four flow cells where the multiple antibody-ligand interactions are monitored. The needles are fixed and the sample rack moves into position as assigned. The instrument is designed to accommodate microtitre and reagent plates. A rack tray holds a 96-well microtitre plate (standard or deep-well) and a proprietary large volume (4 mL) reagent plate (8 × 3 format). The injection of samples from the 96 (8 × 12) well plate is designed that samples lifted from columns A and E are injected over flow cell 1, columns B and F over flow cell 2, columns C and G over flow cell 3 and columns D and H over flow cell 4. Therefore, each flow cell has dedicated sample wells on the microtitre plate. After each interaction measurement, the sensor surface is regenerated and prepared for the next measurement. In a single cycle there is the potential to analyse one sample on sixteen ligands, two samples on eight ligands, or four samples on four ligands, respectively. Assay conditions such as sample, dilution rate, buffers and regeneration solutions can be optimized for each of the four flow cells. All data presented in this paper are obtained under optimised assay conditions.

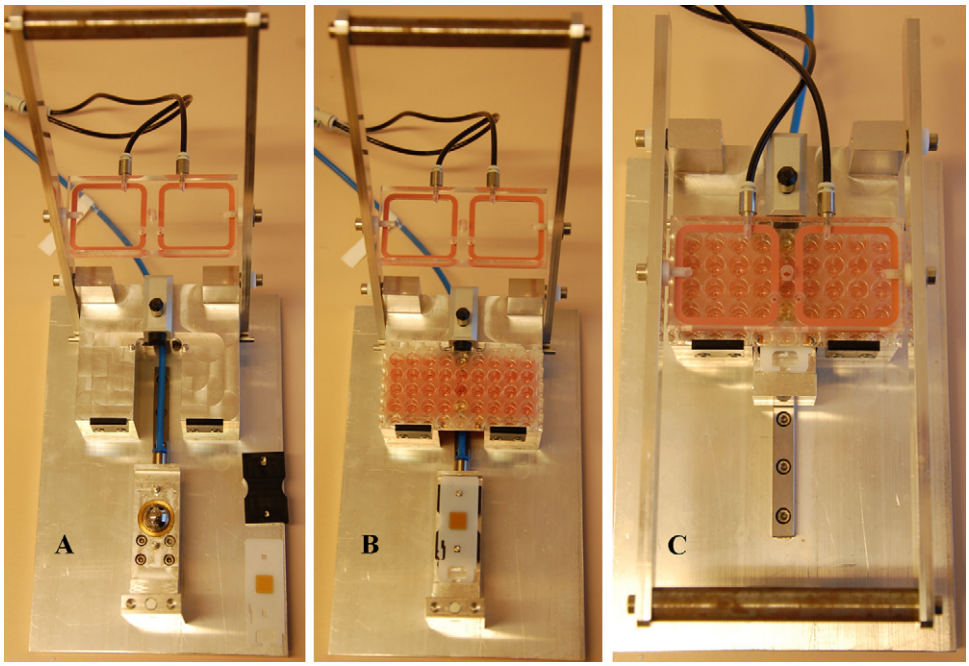


Fig. 2. (A) Immobilisation unit for the immobilisation of 16 ligands on SPR sensor chips. (B) A plastic plate and sensor chip are positioned on the docking station (bottom) and the microfluidic system is placed in its holder. (C) The docking station (on rails) is moved below the microfluidic system. A transparent lid is placed on the sample wells by lowering the metal arm that rests on a metal support. Within the lid, two rubber rings (red coloured) are inserted to create an air-tight sealing of the 16 wells on the left and right hand side, respectively. Pressurised air can be applied to the 16 wells on the left or the right hand side through two holes in the lid and are connected to the black tubes. Docking of the sensor chip is performed by applying pressurized air (blue tube) under the metal ball of the docking station. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2.3. Performance characterization of sensor chip immobilisation

For characterizing the performance of the immobilisation unit three interaction systems were used: human serum albumin (HSA) and anti-HSA antibody, myoglobin and anti-myoglobin antibody and IGFBP-2 and anti-IGFBP-2 antibody. For immobilisation of Anti-HSA and anti-myoglobin antibodies, carboxymethylated sensor (CM5) surfaces were activated by applying 50 μL of a freshly mixed activation solution (EDC/NHS (1:1, v/v)) over the whole sensor chip surface area for 10–15 min. During activation the sensor chip was placed in a petri dish together with water saturated tissue to limit evaporation of the EDC/NHS mixture. After activation, the surface was rinsed with water, dried under a stream of nitrogen and docked in the immobilisation unit. 16 antibody containing ligand solutions (typically 20–50 μL of a 0.5–10 μg/mL protein solution in immobilisation buffer) were pipetted into the sample wells on the left hand side of the immobilisation unit. Upon pressing the immobilisation button (IMM) on the control unit, ligand solutions are pressed into the fluidic channels by applying 0.1 bar to the ligand containing wells for a few seconds. The flow of ligand solution is then continued for 8 min at 0.02 bar. After every 2 min the flow direction was reversed. As a final step in the immobilisation procedure, the sample is pushed back into the sample wells and removed using a 16-tip pipette connected to a vacuum source. The flow system is washed using water to remove non-bound ligands from the flow cell areas. The flow profile of this washing step (1× WASH button) is essentially identical to that of the immobilisation step except for reducing the 0.02 bar flow duration to 1 min. After washing, the sensor chip is undocked, rinsed with water, dried and the surface is deactivated by placing a 50 μL drop of deactivation solutions (1 M ethanolamine) on the sensor surface area for 10–15 min. Again the chip is rinsed with water and dried with a stream of nitrogen. A similar procedure was performed for immobilisation of IGFBP-2, except that surface activation and deactivation was also performed using the immobilisation unit by applying 50 μL of the activation and deactivation solution to the corresponding well of the microfluidic immobilisation system, respectively. Both activation and deactivation lasted 8 min (1× of IMM button) while immobilisation of IGFBP-2 lasted 24 min (3× of IMM button). In between protein immobilisation and deactivating the spots were washed with 50 μL of water for 1 min (1× WASH button). The performance of the immobilisation unit was characterized with a number of investigations:

(a) To evaluate the precision of immobilising specific regions of the sensor chip with various ligands HSA antibodies were immobilised on odd numbered spots and myoglobin antibodies were immobilised on even numbered spots. Immobilisation areas were inspected visually and the potential risk of cross contamination of immobilised antibodies was evaluated by measuring binding responses of HSA and myoglobin to all spots.
(b) To determine the reproducibility of immobilisations, 4 μg/mL anti-myoglobin antibody was immobilised in all flow cells on seven different sensor surfaces and the variation in immobilisation levels was evaluated.
(c) The effect of ligand concentrations on immobilization levels and relation between immobilisation levels and analyte binding responses was established by immobilising with 0.5, 2, 5 and 10 μg/mL anti-myoglobin antibody solutions and measuring anti-myoglobin immobilisation and myoglobin binding levels. Myoglobin binding levels were obtained by injecting 0.5 μg/mL Myoglobin in HBS-EP+ buffer at a flow rate of 40 μL/min for four minutes over the immobilised spots. The average binding response of 6 repetitive myoglobin injections was calculated. In between each myoglobin injection, the surface was regenerated using 20 μL glycine–HCl pH 2.0.
(d) To investigate long-term performance of immobilised sensor chips to measure analyte concentrations, IGFBP-2 was immobilised on 3 spots within one flow cell and the response of a positive control sample was followed during a 30 day period.

For concentration analysis of IGFBP-2, an indirect inhibition assay in HBS-EP+ buffer was constructed. This assay included a negative control (1:1 ratio of specific binding partner to buffer) and a positive control (1:1 ratio of specific binding partner to fixed protein concentration). The flow rate was set at 40 μL/min with a sample injection time of 180 s. Following the injection of the negative control the chip surface was regenerated to allow the injection of the positive control. These injections were repeated 16 times during a one month period. During this time the prepared biosensor chip was routinely undocked, washed and re-docked and the instrument had undergone routine maintenance procedures. Additionally, the concentration of IGFBP-2 in at least 150 bovine plasma samples have been analysed with the same sensor chip during this period.

2.4. Multiplexed phycotoxin analysis

The performance of the immobilisation unit in combination with the SPR analyser was assessed for phycotoxin concentration analysis. The parent toxins from the different classifications of shellfish toxin were evaluated. Domoic acid was assessed for amnesic shellfish poisoning (ASP) toxins and okadaic acid for diarrheic shellfish poisoning (DSP) toxins. Immunologically, the paralytic shellfish poisoning (PSP) toxins may be subdivided into the R1-non-hydroxylated and hydroxylated toxins due to the cross-reactivity profiles of antibodies raised to either saxitoxin and neosaxitoxin, respectively (Usleber et al., 2001). Therefore, both of these PSP toxins were assessed. These parent toxins from the three phycotoxin groups are all relatively low molecular weight compounds (<1000 Da) showing considerable variation in structure between groups.

2.4.1. Antibody production

The preparation of the immunogens and the production of the domoic acid antibody (DA-Ab) (Traynor et al., 2006); okadaic acid antibody (OA-Ab) (Llamas et al., 2007); neosaxitoxin antibody (NEO-Ab) (Bürk et al., 1995) and saxitoxin antibody (STX-Ab) (Campbell et al., 2007) have been described in detail in the corresponding publications.

2.4.2. Phycotoxin chip surface production

Using the prototype immobilisation unit the four target toxins were immobilised onto one spot of each flow cell of a Series S CM5 chip. The first spot of each flow cell was treated similarly with the only difference for each being the toxin immobilisation step.

Four spots on the chip surface were washed three times with 50 μL of deionised water and then activated using 50 μL of EDC/NHS (1:1, v/v) for 16 min. An amine linker was attached to each activated spot by reacting 50 μL of jeffamine (20%) in borate buffer pH 8.5 for 16 min. The immobilisation step for each toxin was as follows:

(a) Flow cell 1: The carboxylic acid groups of domoic acid (50 μg) were activated using 20 μL of EDC/NHS (1:1, v/v) in 0.01 M PBS pH 7.2 (20 μL).
(b) Flow cell 2: The carboxylic acid group of okadaic acid (50 μg) was activated using 10 μL of EDC/NHS (1:1, v/v) in 0.01 M PBS pH 7.2 (30 μL).
(c) Flow cell 3: For neosaxitoxin the spot was washed four times with 50 μL of deionised water to prevent cross-linking of the jeffamine in the immobilisation unit. Neosaxitoxin (20 μg) in deionised water was activated using 37% formaldehyde (12.5 μL).
(d) Flow cell 4: Saxitoxin was immobilised in the same manner as neosaxitoxin.

A total volume of 50 μL of each activated toxin was applied to the corresponding spot over a 4–5 h period. Each spot was then washed three times with 50 μL of deionised water and deactivated by exposure to 50 μL of 1 M ethanolamine for 16 min followed by washing five times with 50 μL of deionised water. The chip was removed from the immobilisation unit, washed and dried using a stream of nitrogen gas, and stored desiccated at 4–8 °C when not in use.

2.4.3. Evaluation of toxin chip and production of calibration curves using SPR analyser

Serial dilutions of each antibody, to each of the toxins on the chip surface, were assessed to determine a suitable antibody titre (dilution) for further assay development. Different regeneration solutions were investigated for the displacement of the antibodies from the surface to re-establish the baseline response.

In order to plot calibration curves, the sensitivity of each antibody relative to the corresponding toxin, over the concentration range of 0–1000 ng/mL, was determined using toxin standards prepared in HBS-EP+ buffer. Each toxin standard (40 μL) was mixed manually in the appropriate wells of the microtitre plate with an equal volume of antibody (40 μL) previously diluted in HBS-EP+ buffer and then the plate containing 80 μL per well was loaded onto the machine. The solutions were then automatically injected over all the corresponding flow cells of the sensor chip surface at a flow rate of 20 μL/min with a contact time of 180 s. Report points were recorded 10 s before and 30 s after each injection and the relative response units determined for each spot on the chip. Each flow cell was regenerated with the regeneration solution most appropriate for each antibody at a flow rate of 20 μL/min for 90 s. A typical analytical cycle for all four flow cells was completed in approximately 8 min. Each standard was analysed in triplicate and calibration curves were plotted based on antibody binding response versus toxin concentration. The response units relative to the toxin concentration curves were evaluated using a 4-parameter fit function using BIAevalution software (version 4.1) which was also used to determine the IC_{20}, IC_{50} and IC_{80} values.

3. Results and discussion

3.1. Performance characterization of sensor chip immobilisation

Fig. 3 shows the immobilisation pattern obtained after immobilising with 10 μg/mL HSA (odd spot numbers) and myoglobin (even spot numbers) antibodies on the sensor chip surface when viewed using a contrast microscope. The 16 linear array pattern produced by the immobilisation unit on the chip surface is clearly evident and within the flow cell areas the width of each immobilised area is 100 μm. This indicates that immobilised areas on the sensor chip match the flow cell areas of the immobilisation device and that no spreading of ligands outside the immobilisation flow cell areas occurs. It is only when the chip is docked onto the IFC of the SPR analyser that four independent flow cells are formed that cover 4 immobilised spots each as depicted in Fig. 3. Spots 1–4 are located in flow cell 1, 5–8 are in flow cell 2, 9–12 are in flow cell 3 and 13–16 are in flow cell 4.

When HSA or myoglobin was injected in the four flow cells, only binding was observed to spots that contain anti-HSA or myoglobin antibodies. This demonstrates that no contamination of ligands to adjacent spots occurs during immobilisation. When a mixture of HSA and myoglobin was injected, binding levels were similar to those obtained when the individual proteins were injected indicating that the presence of other proteins do not interfere with the specific antibody–antigen binding.

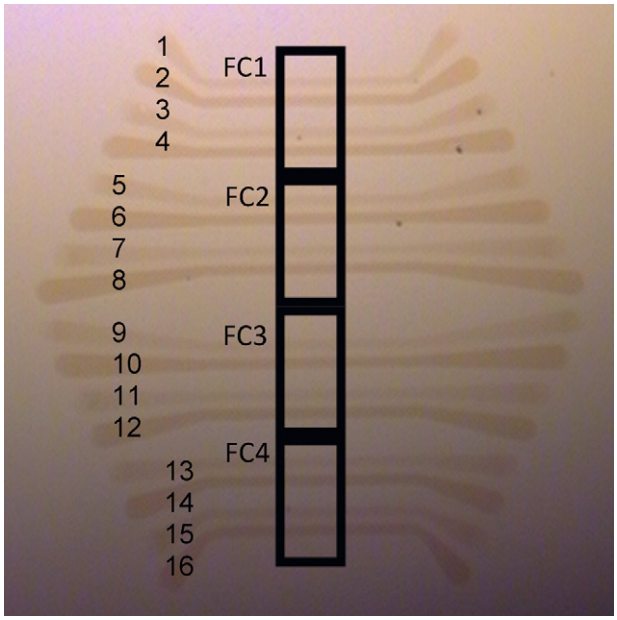


Fig. 3. Contrast microscope image of a sensor chip on which 16 lanes with immobilised antibodies were created using the immobilisation unit. The immobilised areas are darker than the unmodified sensor chip surface. Upon docking the sensor chip in the SPR analyser, four flow cells (denoted FC1, FC2, FC3, and FC4) are created that cover 4 immobilised spots each and these flow cell areas are drawn in the figure. The inner dimensions of these flow cells are 0.5 mm × 1.1 mm and within these flow cells the width of the immobilised area of each spot is 100 μm.

To characterize the reproducibility of immobilisations, 4 μg/mL anti-myoglobin antibody was immobilised on all spots of seven sensor surfaces. The average immobilisation level of these 112 spots was 6404 RU and the coefficient of variation (CV) was 17%. The average spot-to-spot variation in immobilisation levels within one chip yielded the same CV of 17%. When calculating the variation in immobilisation levels between the seven sensor surfaces but for the same spot number an average CV of 11% was obtained. This implies that the immobilisation device has a small variation in immobilisation efficiency between the 16 spots. To compare these variations in immobilisation levels with other techniques, anti-HSA antibodies were immobilised on CM 5 sensor chips (7 min EDC/NHS activation followed by 7 min immobilisation of 60 μg/mL anti-HSA in 10 mM acetate buffer pH5.5) using Biacore T100 and on Flexchip gold affinity chips from 200 μg/mL anti-HSA in 10 mM PBS pH 7.4 using contact and micro-dispense printing. Resulting CV values were 3% (n = 7), 14% (n = 54) and 10% (n = 16). This indicates that the variation of immobilisation levels is more in line with printing technologies than with microfluidic immobilisation where injection of solutions is controlled by syringe pump rather than by pressurized air. When the anti-myoglobin concentration was varied between 0.5 and 10 μg/mL during immobilisation, the immobilisation levels varied accordingly as shown in Fig. 4A. Immobilisation levels varied from 1400 to 14,000 RU which corresponds to 1.4–14 ng anti-myoglobin per mm^2 (Stenberg et al., 1991). Thus by varying the ligand concentration, a broad immobilisation density range can be obtained. The highest immobilisation level to the dextran covered sensor surface is more than twice the amount that can be accommodated in a densely packed monolayer on flat surfaces (Buijs et al., 1995).

In Fig. 4B, the myoglobin binding levels are plotted as function of anti-myoglobin immobilisation levels. This resulted in a clear linear relationship indicating that the activity of anti-myoglobin is independent of the immobilisation levels studied. In another study a similar linear relationship between antibody immobilisation levels and antigen binding levels has been reported when a different micro-fluidic device was used (Natarajan et al., 2008). In the same study, it was shown that immobilisation with a pin printer on the same SPR sensing surface resulted in no significant antigen binding when antibodies were immobilised with a concentration below 10 μg/mL and a severe spreading of the antibody over the surface for concentrations above 10 μg/mL.

By performing concentration analysis measurements over a one month period, both the long term stability of sensor chips and its usage for determining analyte concentrations over a longer time period could be evaluated. IGFBP-2 was immobilised on 3 of the 4 spots in one flow cell. Immobilisation levels were: Spot9 = 650 RU, Spot10 = 550 RU, Spot11 = 500 RU, Spot12 = 180 RU. Negative controls containing only anti-IGFBP-2 antibody were used to normalise binding responses for all concentration measurements. This is typically performed to account for signal variation caused by reagent variation, non-specific binding, and chip surface degradation. The average normalised response of 16 positive control samples and their CVs were Spot9 = 575 RU (2.6%CV), Spot10 = 542 RU (2.9%CV), Spot11 = 498 RU (2.5%CV), Spot12 = 184 RU (6.4%CV).

The reproducibility of the signal response, over the month, for immobilised spots (9–11) in a flow cell was very satisfactory. The most noteworthy of which are length of the experiment, the number of runs and the total number of cycles of unrelated samples (n > 150) that have also been injected over the flow cell. Spot 12 has no immobilised protein hence spot 12 shows lower response values. Ideally spot 12 would have a value of zero (no binding)

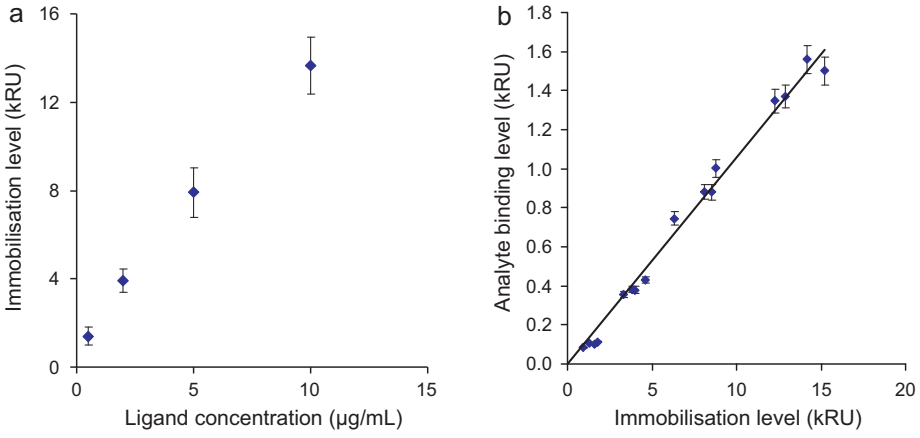
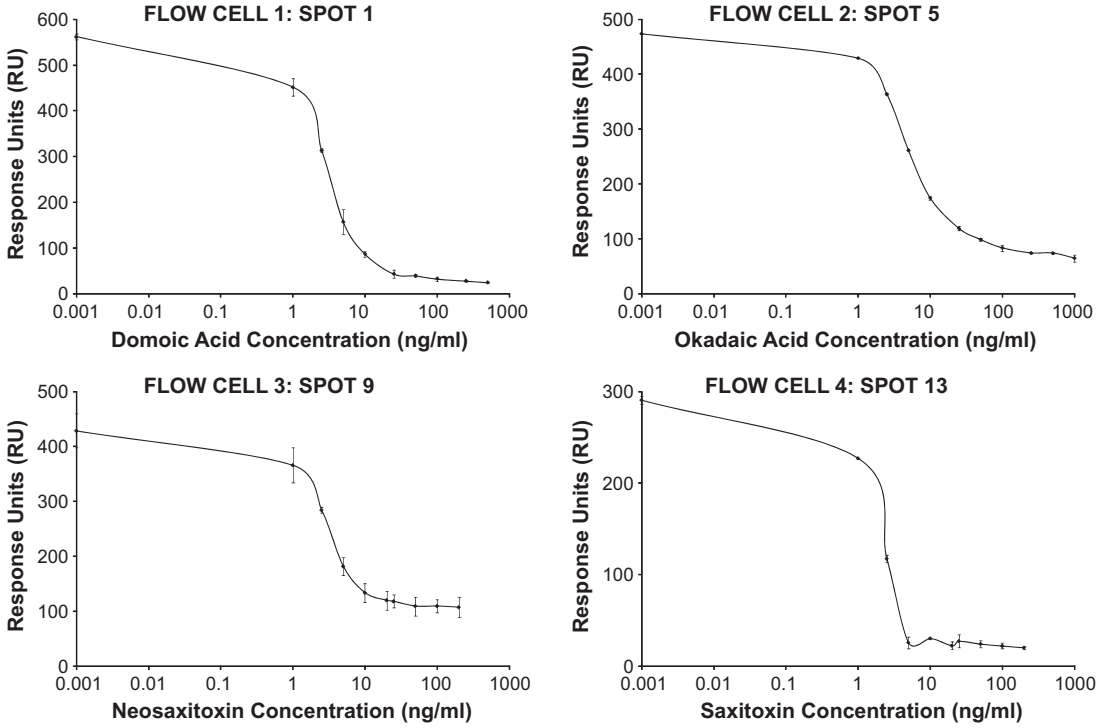


Fig. 4. (A) Immobilisation levels (n = 4) as function of the ligand anti-myoglobin concentration during immobilisation and (B) binding levels (n = 6) of the analyte myoglobin as a function of anti-myoglobin immobilisation levels. Myoglobin binding levels increase linearly with anti-myoglobin immobilisations levels and the results of a linear fit (x = 0.106y, R^2 = 0.99) is displayed in the figure.



Toxin	Regulatory Limit (μg/ml)	Antibody	Titre	IC_{50} (ng/ml)	$IC_{20} - IC_{80}$ (ng/ml)	Regeneration Solution
Domoic Acid	20000	DA-Ab	1/200	2.6	1.0 – 6.4	75mM sodium hydroxide
Okadaic Acid	160	OA-Ab	1/4000	4.9	1.7 -14.4	180mM sodium hydroxide with 15% acetonitrile
Neosaxitoxin	800 STXeqs	NEO-Ab	1/25	2.6	1.1 – 6.0	100mM Hydrochloric acid
Saxitoxin	800	STX-Ab	1/1000	1.9	1.0 – 3.7	50mM Hydrochloric acid

Fig. 5. Calibration curves of antibody binding response against toxin concentration for each phycotoxin. For each analysis $n = 3$.

but in this case there is a response of ~180 RU consistently on the different days. This binding activity on the blank spot is most likely caused by non-specific binding of the antibody to the dextran layer.

3.2. Multiplexed phycotoxin analysis

Domoic acid, okadaic acid, neosaxitoxin and saxitoxin were immobilised onto the first number spot within flow cells 1–4, respectively. In the case of the formaldehyde reaction for the PSP toxins a longer reaction time is generally required to achieve optimum immobilisation. As the molecular weight of the toxins is relatively small the resultant change in mass on the chip surface was negligible with minimal resultant change in baseline response in comparison to when the proteins were immobilised.

The sensitivity and specificity of each antibody to the corresponding toxin on each flow cell were assessed and optimised. The antibody titre was determined from the antibody dilution in HBS-EP+ which provided a response of greater than 280 response units over the 180 s contact time. The regeneration solution for each of the antibody toxin interactions was optimised to maintain the baseline of the flow cells on the chip. At the optimised antibody titres, calibration curves illustrating the antibody binding response against toxin concentration for each toxin, are illustrated in Fig. 5. In addition, the mid-point (IC_{50}) and the dynamic range (IC_{20}–IC_{80}) (Usleber et al., 2001; Taylor et al., 2008) of each of the calibration curves are provided. For each of the toxins analysed an IC_{50} of less than 5 ng/mL was achievable. Based on the action limits currently set for these toxin groups, as outlined in Fig. 5, this level of sensitivity would mean that there is considerable scope for sample preparation and dilution of matrix or solvent effects. The theoretical lower limits of detection, based on the IC_{20} values, achieved were between 1.0 and 1.7 ng/mL for each of the toxins.

This initial study shows the feasibility for the technology transfer of three existing Biacore Q SPR methods previously developed for phycotoxin analysis onto a multiplex SPR format. The ability to perform such multiplex analysis on a single chip surface has the advantage of decreased assay time compared to the sequential analysis of each toxin. The current four cell flow system allows for the multiplexing of the four assays whilst isolating each assay into compartments. This separation avoids any interference between binding partners in a flow cell and allows for the optimization of

the regeneration solution for each toxin and antibody combination. This compartmentalization of samples onto the different flow cells would also have the potential for allowing the end-user to identify which toxin was present in a sample. With the development of a single sample preparation method for the toxins shellfish monitoring regimes could be combined into a single screening assay.

4. Conclusion

The purpose of this study was three fold: to demonstrate the applicability of a novel immobilisation unit for immobilising up to 16 ligands on a chip surface using both high and low molecular weight compounds; to assess the ability of the prototype SPR analyser to determine and differentiate binding events occurring at each of the 16 spots; and to demonstrate the technology's ability for the simultaneous concentration analysis of four phycotoxins.

The micro-fluidic immobilisation unit performed effectively in immobilising both high and low molecular weight compounds. This system allowed multiple chemical steps to be performed on the immobilisation of the low molecular weight toxins. The study of the immobilised proteins showed that different proteins could be immobilised on specific regions of the sensor area and that cross-contamination between spots was negligible. Immobilisation levels could be steered by varying the ligand concentration during the immobilisation process and a good correlation between ligand immobilisation levels and analyte binding capacities was obtained. Immobilised chips could be used for concentration analysis over a one month period with excellent reproducibility in analyte responses.

The system was evaluated with regards its ability to incorporate ASP, DSP and PSP toxin analysis into a single assay format. The sensitivity and repeatability of the assay shows that this technology has the potential to be fit for purpose for the multiplexing of all legislative phycotoxins onto a single chip format whilst still being able to distinguish between toxin families. The levels of detection for each toxin are comparable with the previously developed single channel SPR assays (Traynor et al., 2006; Llamas et al., 2007; Campbell et al., 2007). The multi-channel setup of the analyser has advantages over single channel multi-arrays in that different sample preparations can be injected over each channel therefore removing one of the biggest multi-analyte limitations in food analysis.

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