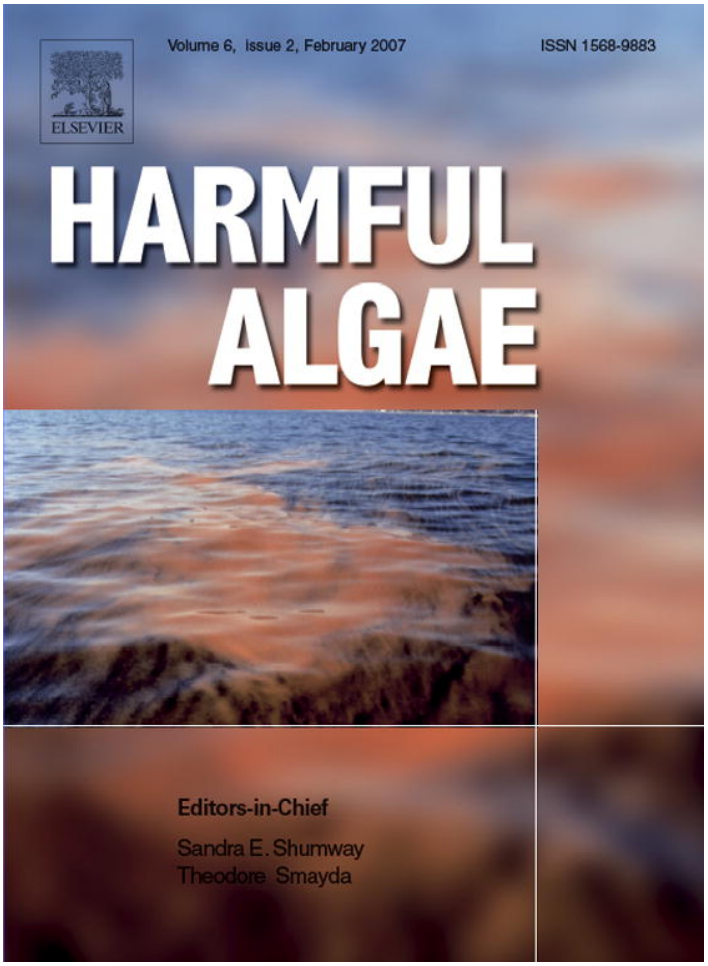

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Detection of the toxin domoic acid from clam extracts using a portable surface plasmon resonance biosensor

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Abstract

The amnesic shellfish poison domoic acid is produced by marine algae of the genus *Pseudo-nitzschia*. We have developed a portable surface plasmon resonance (SPR) biosensor system for the detection of domoic acid. Because of concerns with domoic acid contamination of shellfish, there is a need for rapid field quantification of toxin levels in both shellfish and seawater. Antibodies were raised against domoic acid and affinity purified. These antibodies were used to develop competition- and displacement-based assays using a portable six-channel SPR system developed in our laboratories. Standard curves for detection of domoic acid in phosphate buffered saline and in diluted clam extracts analyzed by the competition-based SPR assay demonstrated a limit of detection of 3 ppb (10 nM) and a quantifiable range from 4 to 60 ppb (13–200 nM). Comparison of analyses for domoic acid levels in Pacific razor clams, *Siliqua patula*, containing moderate to high levels of domoic acid by the standard HPLC analysis protocol and the SPR-based assay gave an excellent correlation. This same technology should also function for detection of domoic acid in concentrated algal extracts or high dissolved levels in seawater.

Keywords: Amnesic shellfish poisoning; Biosensor; Domoic acid; Surface plasmon resonance

1. Introduction

Domoic acid (DA) is a potent neurotoxin found in several species of the diatom genus *Pseudo-nitzschia* and is the causative agent for amnesic shellfish poisoning (ASP). The presence of domoic acid-producing algal blooms throughout the world has raised concerns with fisheries managers. ASP was first observed on Prince Edward Island, Canada, causing illness in 100 individuals and killing four elderly people (Perl et al., 1990). Symptoms of ASP include intestinal distress, facial grimaces, short-term memory loss, difficulty breathing and death (Ward et al., 1997). DA-induced lesions in the hippocampus have been observed in rats, humans as well as marine mammals (Debonnel et al., 1989; Silvagni et al., 2005). The main cause of toxicity results from the tight binding of DA to a subgroup of glutamate receptors,

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resulting in destruction of glutaminergic neurons particularly in the hippocampus (Todd, 1993; Hampson and Manalo, 1998). Toxic DA enters the food supply through shellfish species that consume contaminated algae, accumulate DA and are then harvested for human consumption. The currently accepted limit for DA is 20 ppm in shellfish tissue (Waldichuk, 1989).

Many species of shellfish bio-accumulate domoic acid after consuming neurotoxin-containing phycoplankton or other contaminated organisms. Shellfish and planktivores known to have been contaminated with DA include cockles, crabs, mussels, clams and scallops (Wekell et al., 1994; Rhodes et al., 1998; Vale and Sampayo, 2001). The accumulation of DA varies greatly among shellfish species (Vale and Sampayo, 2001). Little is known about the process of elimination of the toxin in many shellfish, although it is known that many shellfish depurate the accumulated DA rapidly while others, particularly razor clams, may retain toxin for months (Wekell et al., 1994).

Detection of DA in shellfish has become an essential part of effective fisheries management. DA levels in shellfish may be determined by several analytical methods. The standard method used by regulatory agencies is high pressure liquid chromatography (HPLC) (Wekell et al., 1994). Other methods include rat bioassays (Beani et al., 2000), receptor binding assays (RBA) (Van Dolah et al., 1995), liquid chromatography combined with mass spectrometry (LCMS) (Furey et al., 2001), enzyme-linked immunosorbant assay (ELISA) (Branaa et al., 1999; Kawatsu et al., 1999) and two methods for DA analysis designed for field use, screen-printed electrode detection (Kreuzer et al., 2002) and a dipstick immunoassay available from Jellett Biotek Ltd. (Canada). Two reports describe the use of surface plasmon resonance (SPR) for detecting DA in buffer samples in the laboratory (Lotierzo et al., 2004; Yu et al., 2005), however, previous SPR detection instruments were not portable. Portability is important because researchers, governmental agencies as well as shellfish harvesters are especially interested in rapid methods for monitoring DA contamination in the field.

SPR technology is based on protocols that analyze changes in refractive index (RI) on thin gold surfaces in real time to detect intermolecular interactions (Fig. 1). Appropriate choice of binding partners allows for the development of methods for the detection of analytes that vary in size from small organics to whole microbes. For analysis of target molecules of sufficient size to cause a significant increase in RI on binding, receptors are covalently linked to the surface. Binding of analytes such as proteins, viruses, spores and microbes to specific antibodies immobilized on the sensor surface is readily followed in real time. No secondary label or amplification step is required for real-time detection. A general review of SPR applications can be found in a book edited by Davies (1996).

Small analytes cannot be easily detected using the direct approach since binding of small analytes to surface immobilized receptors results in very small changes in RI. Analysis of small molecules can be achieved by competition-based assays where analyte in solution prevents binding of antibodies to surface immobilized analyte. Displacement-based assays, where analyte present in the sample displaces antibodies bound to surface immobilized analyte, may also be used. As early as 1993, atrazine was detected using an SPR-based competition assay with an atrazine derivative bound to the gold film, and samples mixed with monoclonal atrazine antibodies. Atrazine in samples reduced binding of monoclonal antibodies to surface-immobilized atrazine (Minunni and Mascini, 1993). Similar assays for the analysis of fenitrothion, thyroxine and estradiol have been reported (Kaufman and Clower, 1995; Adamczyk et al., 1998; Miyashita

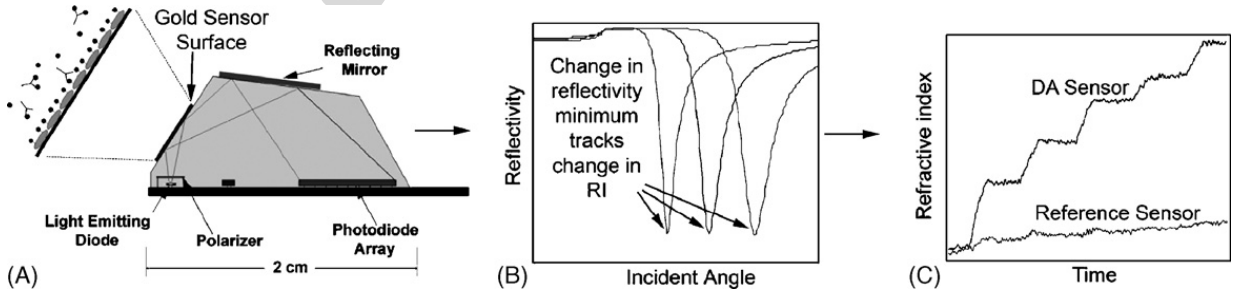

Fig. 1. The Texas Instruments Spreeta™ 2000 sensor chip uses a light emitting diode and polarizer to reflect light off an internal gold surface, a reflecting mirror and onto a photodiode array. The RI of the external gold surface determines the angle at which light that is not reflected but is instead converted to a surface plasmon wave (A). The intensity of light received at the photodiode array measures the angle of light of minimum reflection, the resonance angle of minimal reflected light intensity. As the RI of the external surface changes upon binding of biomolecules, the angle is shifted (B). The incident angle intensity minimum is converted to a RI and measured over time using the system software. A reference channel is used to compensate for bulk effects (C).

et al., 2005). Pharmaceutical drug interactions with proteins, lipids and DNA have also been analyzed using SPR (Liu et al., 2004; Baird et al., 2002; Carrasco et al., 2001). Detection of small molecules has also been accomplished using conformational changes that small molecules exert on proteins (Gestwicki et al., 2001).

Here, we report the use of a portable SPR biosensor system to detect DA in standard buffer solutions as well as in clam extracts using competition and displacement-based assays.

2. Materials and methods

2.1. Preparation of conjugates and antibodies

DA-conjugated bovine serum albumin (BSA) was prepared using standard EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) coupling chemistry (Staros et al., 1986; Grabarek and Gergely, 1990). A solution of 200 μl containing 2.0 mg BSA was mixed with 1.0 ml conjugation buffer (0.1 M MES, 0.9 M NaCl, pH 4.7) containing 1 mg DA (Sigma, St. Louis, MO). Next, 10 mg EDC (Sigma) was added and the reaction was stirred for 2 h. To terminate the conjugation reaction and block unreacted groups, 100 μl of 1.0 M Tris, pH 8.5 was added. The DA-conjugated BSA was then dialyzed for 8 h against phosphate buffered saline, pH 7.4 (PBS).

Rabbit polyclonal anti-DA antibodies were prepared with ovalbumin conjugated to carboxyl groups of DA using the Imject Immunogen EDC Conjugation kit (Pierce, Rockford, IL). The resulting immunogen was injected subcutaneously into rabbits to elicit anti-DA antisera at R&R Research (Stanwood, WA). Initial injection was 0.5 mg/animal with incomplete Freund's adjuvant. Subsequent injections (0.25 mg without adjuvant) were administered at 3-week intervals. Bleeds were obtained 14 days after the third and each subsequent injection.

Affinity purification of anti-DA antibodies was performed using a column containing immobilized DA-conjugated BSA prepared with an AminoLink Immobilization Kit (Pierce). Rabbit serum (7 ml) was flowed over a 2 ml affinity column followed by washing with PBS. Bound antibodies were eluted with 10 mM glycine, pH 2.2 in 400 μl fractions and immediately mixed with 40 μl 10× PBS. ELISA analysis was used to analyze the column fractions.

Affinity purified rabbit anti-DA antibodies were first tested by examining the antibody binding to DA-conjugated BSA using a Vectastain ABC-AP ELISA kit following the procedures recommended by the manufacturer (Vector Laboratories, Burlingame, CA). Wells were coated with 10 μg/ml DA-conjugated BSA overnight and incubated with 4 ng/ml affinity purified rabbit anti-DA antibodies for the assays. Competition ELISAs were performed by pre-incubating dilutions of rabbit anti-DA antibodies with several dilutions of DA standards and comparing the binding with DA-free antibody solutions. The ability of DA to displace rabbit anti-DA antibodies from the DA-conjugated BSA wells was also examined by incubating ELISA wells coated with DA-conjugated BSA with affinity purified rabbit anti-DA antibodies followed by incubations with several dilutions of DA standards. The displacement ELISAs were performed to assess the potential of using these reagents in displacement assays in the future.

2.2. Dry storage reagent for SPR gold surfaces

Long-term storage of SPR sensors is a necessary component of accurate and reproducible detection. Dry storage of pre-functionalized SPR sensors with a general-purpose reagent was tested using 4 mm × 4 mm gold-coated slides. Slides were coated with gold binding protein (GBP) as described previously (Woodbury et al., 1998; Naimushin et al., 2002). Following activation of the GBP with a solution containing 20 mg/ml EDC and 5.5 mg/ml S-NHS (*N*-hydroxysulfosuccinimide) for 30 min (Staros et al., 1986), the slides were washed with dH_2O. For storage studies, rabbit anti-alkaline phosphatase antibodies were used instead of anti-DA antibodies to preserve costly DA reagents. Rabbit anti-alkaline phosphatase antibodies were covalently attached by incubating a 10 μg/ml solution of antibodies in 10 mM sodium acetate, pH 5.0, on the activated sensor surface for 10 min. Active esters were blocked using Tris-buffered saline before washing the slides with PBS. Slides were then coated with solutions containing 2.5% trehalose buffered with 10 mM Tris and either 2.5% dextran (average MW 500,000), 2.5% dextran (average MW 10,000) or no dextran and air dried to determine optimal conditions for stability. Slides were stored at room temperature for up to 11 months before analysis of antibody activity. After wetting with PBS, the slides were incubated with 0.1 mg/ml alkaline phosphatase for 30 min, washed four times with PBS and placed in a black 96-well plate for analysis of alkaline phosphatase activity on a Tropix TR717 Microplate Luminometer using Lumiphos Plus (Lumigen, Southfield, MI) reagent according to the manufacturer's instructions.

2.3. Surface plasmon resonance surface preparation

Texas Instruments Spreeta™ 2000 sensors contain a gold surface slide attached to a detection chip (Fig. 1A). The gold surfaces were functionalized using DA-conjugated BSA. After washing the gold surface sequentially with 10% nitric acid for 10 min, 95% ethanol for 15 s, then distilled water for 15 s, 100 μg/ml DA-conjugated BSA was incubated on the gold surface for 1 h. The sensor gold surface was then washed with PBS. The sensor gold surface was incubated with 0.1% casein in PBS for 30 min to block non-specific binding during experiments, followed by neat fetal bovine serum (Sigma) for 30 min. Following a final wash with PBS, the sensor gold surfaces were covered with drying reagent and dried to generate a carbohydrate glass. Reference sensors were prepared as above using cortisol-conjugated BSA (Fitzgerald, Concord, ME).

2.4. Preparation of clam samples

Extracts from Pacific razor clams, *Siliqua patula*, contaminated with varying levels of domoic acid were prepared using standard methods (Hatfield et al., 1994). Briefly, two grams of clam tissue were homogenized in 50% MeOH and centrifuged. The supernatant was filtered through a 0.45 μm HA Millex filter (Millipore, Billerica, MA), flowed through a Strata SAX column (Phenomenex, Torrance, CA) and DA was eluted with 0.5 M NaCl in 10% acetonitrile.

HPLC was carried out using the method of Quilliam et al. (1991). Conditions were isocratic at 40 °C with a flow rate of 0.3 ml/min using a Waters 600 HPLC (Waters, Milford, MA). Samples were analyzed on a 2.1 mm × 25 mm reverse phase C18 column (Vydac, Hesperia, CA) with a mobile phase of H_2O/MeOH/TFA (90/10/0.1). The domoic acid was monitored at 242 nm and had a retention time between 6 and 9 min. DA levels were determined by comparing peak areas of the razor clam samples with the peak areas of standards (Wekell et al., 1994).

2.5. SPR biosensor

The six-channel SPR biosensor used in these experiments was designed and built by our research group and has been described previously (Furlong et al., 2005) (Fig. 2). The SPR module contains two three-channel Texas Instruments' Spreeta™ 2000 sensor chips (Chinowsky et al., 2003; Melendez et al., 1996). Integrated fluidics allow for sample introduction by injection through a chromatography-like port into a 1 ml in-line sample loop. Unless otherwise stated, the temperature of the module was maintained at 23 ± 0.01 °C and samples were introduced at a flow rate of 25 μl/min. Data were transmitted to a laptop computer and analyzed using Texas Instruments Multispr software version 10.82.

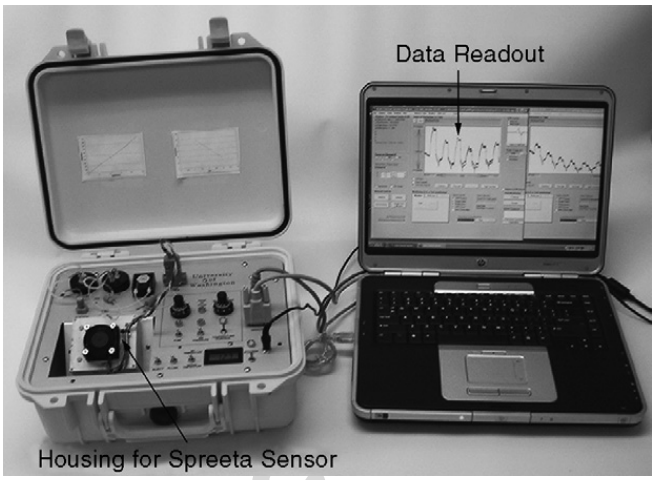

Fig. 2. The six-channel SPR biosensor. With an injection port, integrated fluidics, temperature control and a nine-pin ports for interfacing with a laptop computer, this prototype biosensor is capable of near-real time detection of domoic acid using Spreeta™ 2000 Texas Instruments sensor chips with the protocols described here.

2.6. Analysis of DA by competition assay

SPR competition assays are similar to competitive enzyme linked immunosorbant assays (cELISA), however, the results are generated in near-real time. After placing the DA-conjugate coated Spreeta™ 2000 sensor and reference sensor into the biosensor module, the sensors were calibrated by exposing the sensor surfaces to aqueous solutions of known RI as described previously (Naimushin et al., 2002). PBS was then flowed over the sensor surfaces to generate a baseline. Next, PBS containing 1:3000 diluted affinity purified rabbit anti-DA antibodies (33 ng/ml final concentration) was flowed over the sensor surfaces for 5 min followed by PBS. The sensor surfaces were then regenerated by flowing 100 mM glycine, pH 2.2 over the sensors for 5 min which released bound antibodies. The antibody binding procedure was then repeated using 1:3000 diluted affinity purified rabbit anti-DA antibodies spiked with DA samples. DA solutions of known concentration were used to generate a standard curve. DA standard solutions were added to clam extracts from Pacific razor clams known to have undetectable quantities of DA (analyzed by HPLC) to generate a standard curve for DA in clam extracts. Extracts from

six clams known to be contaminated with DA were then analyzed using the same procedure.

2.7. Data analysis

Standard curves and DA competition values were generated after raw data were collected using a Multispr program provided by Texas Instruments. RI values for reference channels were averaged and subtracted from the DA channel values to compensate for changes in RI due to any differences in bulk solution RI and nonspecific binding to the gold sensor surfaces. Next, the initial slope of RI change versus time was calculated for each addition of antibody without DA or antibody with DA in samples. The initial slopes for each sample were converted to a percentage of the DA-free samples by comparing slopes of samples containing DA to the average slopes of the DA-free samples detected immediately preceding and following injection of a specific sample. All DA samples were analyzed in triplicate and standard deviations were calculated.

3. Results

3.1. Optimization of slide storage conditions

Optimal conditions for storing antibody-derivatized sensor elements were established by coating gold slides with GBP-linked rabbit anti-alkaline phosphatase antibody and drying the slides after coating with trehalose-dextran solutions. The slides stored under carbohydrate glasses were tested for stability following varying periods of storage on the bench top at temperatures that fluctuated from 18 to 32 °C. The drying reagent containing 2.5% trehalose, 2.5% dextran (average MW 500,000) and 10 mM Tris, pH 8.0 showed no loss in antibody activity in luminometry assays after storage for as long as 11 months (Fig. 3). Spreeta™ sensor chips with the gold surfaces functionalized for DA detection were stored for over a month without loss of activity using the optimized drying reagent (data not shown).

3.2. Characterization of anti-DA antibodies

Rabbit anti-DA antibodies were affinity purified and tested for potential effectiveness in SPR experiments by first examining the antibody binding to DA-conjugated BSA using ELISAs (Fig. 4). Half-maximum competition of antibody binding was observed at a concentration of approximately 400 ng/ml DA. Half-maximum displacement of antibodies from the well surface was seen at a concentration of approximately 100 ng/ml DA (Fig. 4).

3.3. Detection of DA by SPR competition assay

The competition detection assay relies on measuring the reduction of antibody binding to the gold sensor surface as a result of DA interfering with antibody interaction with the immobilized DA-conjugated BSA

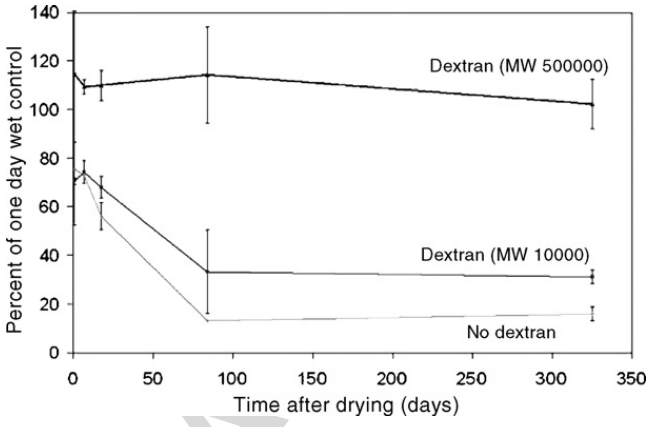

Fig. 3. Determination of optimal storage conditions for surface-functionalized gold slides. Slides with surface-immobilized anti-alkaline phosphatase antibody were coated with Tris-buffered solutions (pH 8.0) containing trehalose and either dextran (average MW 500,000), dextran (average MW 10,000) or no dextran. After storing for up to 11 months, slides were wetted and analyzed for alkaline phosphatase binding activity as described in the materials and methods. Error bars represent standard deviations for experiments run in triplicate.

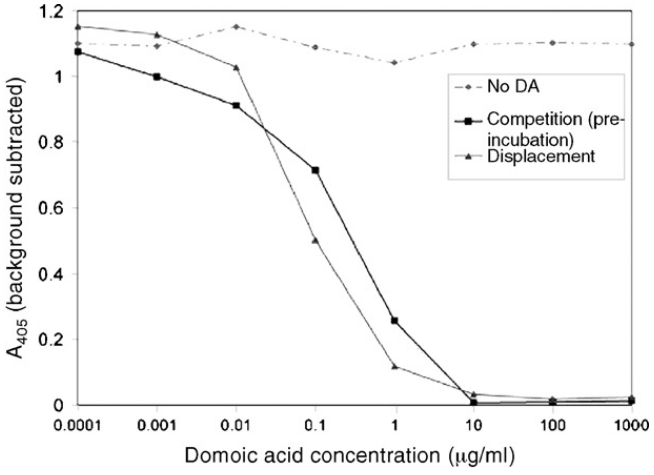

Fig. 4. ELISA testing of affinity-purified rabbit anti-DA antibodies. Wells of a 96-well plate were coated with DA-conjugated BSA. For the competition assay, antibodies were pre-incubated with dilutions of DA and then placed in wells for analysis. For the displacement assay, wells were incubated with antibodies, washed and then incubated with dilutions of DA for analysis. After alkaline phosphatase-conjugated secondary antibodies were added, wells were washed and exposed to 4-nitrophenyl phosphate for color development.

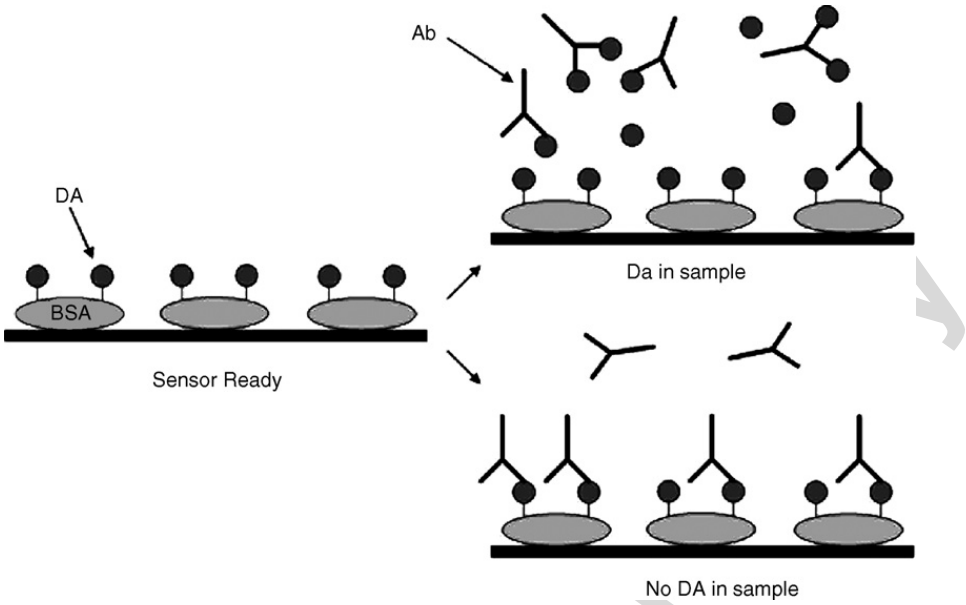

Fig. 5. Competition detection of a small molecule using SPR. A schematic of the sensor surface shows the DA-conjugated BSA on the surface of a sensor in the 'sensor ready' state. The quantity of antibodies that binds to the surface during exposure to sample is inversely correlated to the amount of DA in the sample.

(Fig. 5). The rates of binding of antibodies to surface immobilized DA in the absence and presence of DA were measured. Standard curves were generated by analyzing rates of binding in the absence and presence of DA-containing samples. The average of the reference channel RIs was subtracted from the RI of DA sensing channels to compensate for bulk RI changes, temperature fluctuations and non-specific binding (Fig. 6A). Temperature was maintained at 23 ± 0.01 °C.

A standard curve for DA detection was generated by analyzing triplicate channels exposed to 10 DA concentrations ranging from 2.9 to 605 ng/ml DA. A log–log plot of the rate data revealed a linear range of detection between 4 and 60 ng/ml DA in PBS (Fig. 6B).

3.4. Analysis of DA in clam extracts

Clam extracts were generated as described above. The extraction protocol resulted in a dilution of the DA by 25-fold from the initial clam tissue. Extracts were diluted an additional 100-fold into a solution of 33 ng/ml affinity purified rabbit anti-DA antibodies for analysis. The standard curve for DA concentrations in clam was generated by analysis of clam extracts with no detectable DA spiked with 1.5–145 ng/ml final concentration of DA. Alternating cycles of DA-free antibodies and antibodies with known levels of DA were carried out with a low pH regeneration step between each detection (Fig. 7A). Assays were run in triplicate. Rates of RI change versus time were used to generate a standard curve (Fig. 7B).

Extracts from six razor clams contaminated with DA were analyzed using both HPLC and SPR assays. Extracts of clams harvested from coastal areas of the Washington State containing measurable DA were assayed by both HPLC and SPR analysis. Comparison of the two analytical methods showed an excellent correlation ($r^2 = 0.99$) (Fig. 8).

4. Discussion

The ability to detect DA in the field or in the laboratory in near-real time has a number of important applications. Shellfish harvesters and health departments can benefit from the development of instrumentation and protocols for rapid analysis of DA in shellfish and the marine environment. The six-channel SPR biosensor uses robust Spreeta™ 2000 Texas Instruments sensor chips enclosed in a portable, rugged biosensor system. The competition assays are inexpensive and generate multiple replicates. Unlike ELISA assays, the sensor chips may be regenerated and used many times. The presence of six channels also allows for detection of multiple targets simultaneously depending on the pattern of antibodies or receptors immobilized on the sensor surfaces and the conformation of the fluidics. Earlier versions of this system have been used for detection of other small molecules, proteins, microbes and viruses (Naimushin et al., 2002; Furlong et al., 2005). SPR has proven robust for detection in the field and has been used for detection of aerosols from a rear-prop airplane during flight

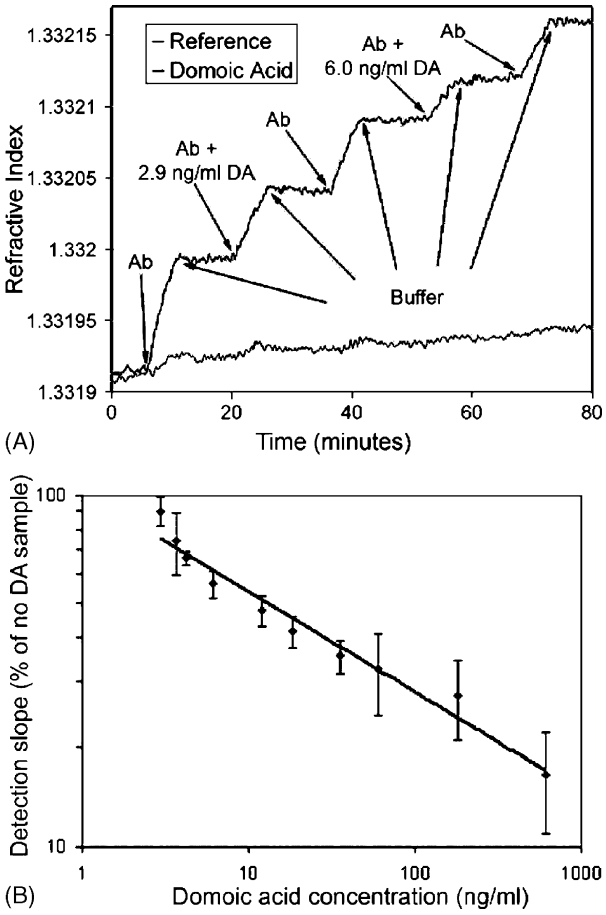

Fig. 6. SPR competition assay for domoic acid. Affinity purified rabbit anti-DA antibodies were introduced to the sensors, alternating with buffer alone. At 20 and 55 min antibodies were introduced that were first pre-incubated with DA. Reductions in slopes during antibody binding to the DA-conjugated BSA surface were used to determine DA concentration. The data from three sensor channels were averaged for both the DA and reference (A). The log–log plot shows competition data for detection of DA in PBS. Error bars represent standard deviations for experiments run in triplicate (B).

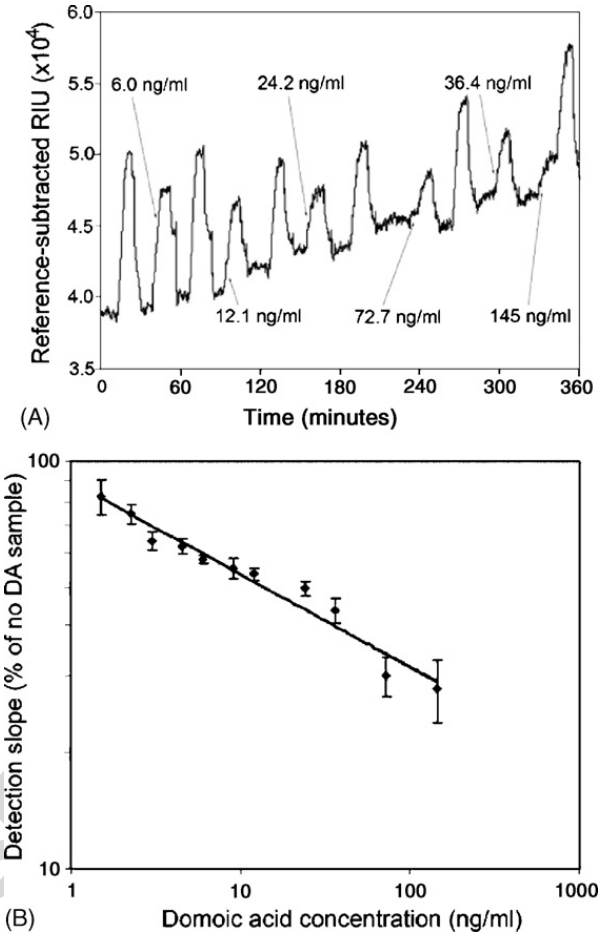

Fig. 7. SPR competition assays for detection of DA in spiked clam extracts. A DA-negative clam extract (by HPLC analysis) was spiked with DA standard and used to create a standard curve for DA detection. Affinity purified rabbit anti-DA antibodies were introduced to the sensors in 1:100 diluted clam extract, alternating with buffer alone. DA-spiked samples were included in alternating antibody samples. Sensors were regenerated between each clam extract assay. The data from three sensor channels were averaged and subtracted from the averaged reference channels (A). A standard curve log–log plot showing rates of antibody binding in SPR competition analyses in diluted clam extracts with differing levels of DA was generated (B). Error bars represent standard deviations for experiments run in triplicate.

(Naimushin et al., 2005). Our next generation system will be a 24-channel unit capable of either multi-analyte analysis or processing of numerous samples in parallel.

The current method for field detection of DA uses methanol extractions of pureed clams that are analyzed using a dipstick competition immunoassay to yield a yes/no result with 1 to 2 μg/ml sensitivity (Jellett Biotek Ltd., Canada). Modifying the field extraction protocols for DA detection in shellfish tissues by adding an in-line anion exchange purification will make SPR detection of DA possible in the field with quantifiable results. We envision that the SPR biosensor system will also be used in a modified format for shipboard, mooring buoy, dock and beach detection of DA.

A major advantage of SPR technology is the stability of the sensor during continuous use. The percent reduction in slope for detection of DA remained consistent from one DA-conjugated BSA sensor chip to another (data not shown). Also, the sensor surfaces were highly robust to regeneration using 100 mM glycine, pH 2.2 to release bound antibody and ready the sensor for additional analyses. Several sensors were used almost daily alternating from room temperature with exposure to assay reagents to nightly storage at 4 °C with little loss in the initial slope of antibody binding or in the sensitivity of DA detection after more than 50 regenerations (data not shown). An additional advantage of the competition assay is that multiple detections can be carried out with very low concentrations of antibody before the surface needs to be regenerated.

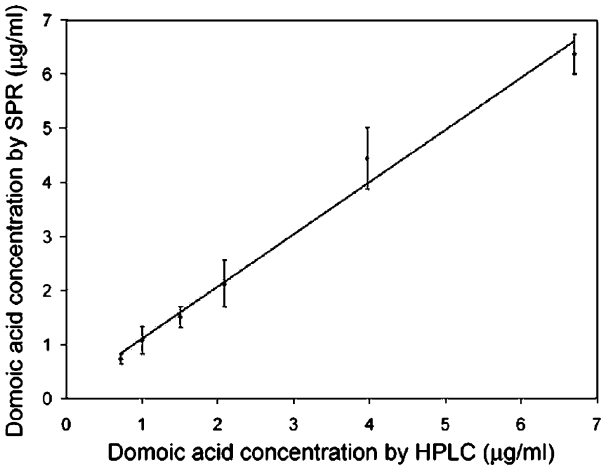

Fig. 8. Comparison of HPLC and SPR analyses using clam extracts. The HPLC analytical protocol and the SPR-based assay protocol were used to measure DA from clam extracts contaminated with varying levels of DA.

The extraction protocol used for the production of clam extract combined with dilution of the extract before introduction to the SPR biosensor resulted in a 2500-fold dilution of the DA in samples when compared to the original clam tissue. Despite this dilution, DA in clams at levels below the regulatory limit of 20 μg/ml DA was easily detected with the competition assay. With refinement of the extraction protocols and generation of higher affinity monoclonal antibodies, detection of much lower levels of DA should be assayable with the SPR system and protocols described here. Also, detection of low levels of dissolved DA in seawater using automated biosensors onboard ships and mooring buoys should be possible after a concentration step or with the use of higher affinity antibodies. While *Pseudo-nitzschia* blooms have often resulted in levels of 1–2 ppb dissolved DA in seawater (V. Trainer, pers. commun.), concentrations below the current detection limit of the SPR, detection of dissolved DA at levels up to ∼100 nmol/l (Wells et al., 2005) in some of the highest concentration blooms may soon be possible.

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