# Evaluation of Surface Plasmon Resonance Biosensors for Detection of Tetrodotoxin in Food Matrices and Comparison to Analytical Methods

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Tetrodotoxin (TTX) is a low molecular weight neurotoxin found in a number of animal species, including pufferfish. One emerging method for TTX detection employs surface plasmon resonance (SPR) immunosensors. SPR, an optical technique that allows for label-free, real-time, multiplexed analysis, can have detection limits that rival many of the conventional transduction methods. Preliminary SPR approaches for TTX were successful, yet suffered from low throughput and used noncommercial instrumentation. To advance this method for broader use, the immunoassay was transferred to a commercial instrument and optimized for improved detection. This manuscript outlines the assay development and results for complex matrices relevant to seafood safety (pufferfish) and food adulteration (milk, apple juice). In addition, results are compared to those obtained using receptor binding assay, ELISA, HPLC-FD, and LC/MS/MS detection techniques. Results highlight the advantages of SPR assays, including rapid screening capability with low reagent consumption and low- to subppb detection limits.

KEYWORDS: Tetrodotoxin; surface plasmon resonance; immunoassay; food matrices

## INTRODUCTION

Tetrodotoxin (TTX) is a small molecular weight neurotoxin (319 Da) that has the ability to bind to site 1 of voltage-gated sodium channels (*1*). Upon binding, this toxin interrupts the passive influx of sodium ions and can result in numbness, tingling, respiratory paralysis, and even death of the affected individual. While most commonly associated with pufferfish (i.e., *fugu*), TTX is present in many other organisms including gobies, blue-ringed octopus, starfish, crabs, newts, frogs, some gastropods, and worms (*2*). Furthermore, TTX is listed as a U.S. Centers for Disease Control and Prevention (CDC) select agent (*3*), meaning that this toxin has been identified as one with the potential for intentional contamination of the food supply. TTX has an $LD_{50}$ in mammals of 2−10 $\mu$g/kg intravenously and 10−14 $\mu$g/kg subcutaneously (*4*). As such, this toxin poses a potential food safety and food defense risk.

Tetrodotoxin poisoning is most prevalent in Asian countries, but a limited number of events have also been reported in the United States. Multiple deaths occur annually due to TTX poisoning in Japan (*2*), where *fugu* is considered a delicacy. In the U.S., several deaths have been linked to the consumption of domestic pufferfish (*5*), where nontoxic, TTX containing, and saxitoxin (STX) containing species all occur (*6*). This highlights the need for rapid screening methods that can distinguish these two potent toxins. In the U.S. there has also been a limited number of incidents associated with the consumption of imported pufferfish, including the poisoning of three chefs who ate prepackaged *fugu* (*7*) and intoxication of five people in three states who consumed pufferfish that was imported illegally and mis-branded as monkfish (*8*). To address such species substitution concerns, there have been advances that allow for the rapid identification of fish species using methods such as DNA barcod-ing and real-time PCR (*9*, *10*). There remains a need for fast, high-throughput, sensitive screening techniques for the toxins these seafood products may contain.

The Japanese government has set regulatory limits for TTX in food (10 Mouse Units $g^{-1}$ (or 2 $\mu$g of TTX equivalents $g^{-1}$)) (*11*,*12*), whereas the United States does not have a stated action level due to the fact that no product sold legally in the U.S. is expected to contain this toxin. A single species of Japanese pufferfish (*Takifugu rubripes*) is allowed to be imported, under a restrictive set of conditions, but this product is processed in Japan and certified as safe prior to importation (*8*). For comparison, STX (*13*) is regulated by the U.S. at 80 $\mu$g of STX equivalents/100 g of tissue. While no AOAC approved methods exist for testing TTX in food samples, the mouse bioassay (MBA), as used in Japan, is considered the standard detection technique (*12*, *14*). In recent years, the MBA has come under scrutiny with respect to performance-related concerns (e.g., poor quantitation and low dynamic range, low sample throughput, and lack of specificity). Furthermore, with the move-ment for humane treatment of animals, alternative testing methods are being more seriously explored, developed, and implemented.

One alternative method, known as the receptor binding assay (RBA), uses sodium ion channels and radiolabeled toxin ($^3$H-STX or $^3$H-TTX) to determine a sample's toxicity in a competition based

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assay (*15*). Like the MBA, this assay cannot distinguish between TTX and STX (or its many congeners) and is further hindered by using expensive, scarce, and potentially deleterious radiolabeled materials. Additional techniques for detecting TTX have been developed, such as ELISA methods that use antibodies specific to TTX (*16*−*18*). These methods are toxin specific (TTX vs STX) and do not require the cumbersome safety approvals and regulations that are needed for the RBA, but they can suffer from long assay times, perishability of assay components, and excessive manual laboratory work. While there are a few ELISAs for the detection of TTX, none are commercially available that have been appropriately validated (e.g., AOAC, NSSP) for use in regulatory environments.

Chemical methods are becoming popular alternatives to immunological techniques, as high performance liquid chromatography with fluorescence detection (HPLC-FD) and liquid chromatography with mass spectrometric detection (LC/MS) can allow for simultaneous identification and quantitation of a toxin (*19*−*22*). There are drawbacks to using HPLC-FD and LC/MS, mainly in the need for instrumentation, trained personnel, and organic solvents. Furthermore, these methods can be laborious and are generally not applicable to high-throughput screening. Specifically, the current HPLC-FD method requires a complex post column oxidation step to create a fluorescent tetrodotoxin derivative (*21, 22*). An LC/MS/MS method for the detection of TTX and its analogues has been used successfully at FDA to detect TTX in pufferfish (*6, 8*), but this method can suffer from matrix suppression in certain complex matrices, particularly at low toxin concentrations when sample dilution is not possible. The method usually employed to account for these effects is the addition of stable isotope labeled standards, none of which are currently available for TTX, STX, or any of their analogues.

Thus, a screening method such as surface plasmon resonance (SPR) in tandem with confirmatory LC/MS could be an ideal approach to enhancing food safety/defense. Surface plasmon resonance occurs in thin conducting films (e.g., gold) at interfaces between media with different refractive indexes (e.g., sensor chip and sample solution) (*23*). In these optical sensors, SPR is observed as a drop in the intensity of reflected light at a specific wavelength, and the shift in this wavelength occurs upon interactions between an analyte from solution and a biomolecule immobilized on the sensor surface. SPR sensors allow for real-time and label-free analysis without the use of deleterious solvents, dangerous radiolabels, or animal systems. In addition to these advantages, SPR is also versatile and has been used for many analytes including bacteria, toxins, vitamins, drug residues, and allergens (*23*).

While an immunoassay for TTX in seafood matrices has been developed on a prototype instrument (*24*), it currently lacks the ability to be used in a broad range of food matrices that could be affected either by natural contamination or as an act of adulteration. Thus, this research focused on optimizing the immunoassay for common food matrices (pufferfish, milk, and apple juice) on a commercial platform. Pufferfish can be naturally contaminated with TTX, whereas milk and apple juice represent food commodities that could be purposefully contaminated with a select agent toxin such as TTX. These two beverages were specifically chosen, as they represent liquid matrices having high protein (milk) and high sugar/low pH (apple juice). In addition, SPR assay data were compared to those obtained with other analytical techniques both for naturally contaminated materials from a 2007 outbreak and for TTX-spiked milk solutions to understand how SPR can enhance current TTX detection capabilities.

## MATERIALS AND METHODS

**Materials.** Anti-TTX monoclonal antibody (1 mg) was obtained from Hawaii Biotechnology Group, Inc. (Aiea, HI), and TTX (10 mg) was acquired from Sankyo Co, Ltd. (Tokyo, Japan). TTX, obtained as a dry powder, was analytically weighed and dissolved in aqueous 1% acetic acid to a final stock solution of 1 mg/mL; LC/MS analysis was used to determine that the stock was >95% pure. 2,2′-(Ethylenedioxy)bis-(ethylamine), also known as Jeffamine, was purchased from Sigma-Aldrich (St. Louis, MO). HS-$(CH_2)_{11}$-$EG_4$-OH and HS-$(CH_2)_{11}$-$EG_6$-$NH_2$ hydrochloride were obtained from ProChimia Surfaces (Sopot, Poland). Standard laboratory reagents were acquired from Sigma-Aldrich, Pharmaco AAPER (Shelbyville, KY), and J.T. Baker (Phillipsburg, NJ). Millipore Milli-Q 18.2 MΩ·cm water was used for all solution preparations (Billerica, MA). Supplies for the commercial SPR instrument were obtained from GE Healthcare, Biacore (Piscataway, NJ). Gold substrates (1.5 nm Ti, 50 nm Au on Schott BK7 Glass) for the prototype instrument were provided by the University of Washington.

RBA $^3$H-STX was obtained from American Radiolabeled Chemicals (St. Louis, MO). Ultima Gold liquid scintillant was purchased from Perkin-Elmer (Gaithersburg, MD). Membrane preparations (*25*) were made with brains from male Holzman rats (Harlan Bioproducts, Indianapolis, IN). Standard laboratory reagents were obtained from Sigma-Aldrich. HPLC/MS solvents were obtained from commercial sources including J.T. Baker.

**Sample Preparation.** For complex matrix studies, skim milk and apple juice were purchased from commercial sources. Skim milk was used as received, whereas the apple juice samples required filtration with Amicon Ultra-4 Ultracel 3K centrifugal filters (unpublished data). The TTX standards were spiked into the milk matrix, as were four "unknown" (to the researchers performing detection) concentrations. For LC/MS/MS, an additional preparation step was required with 70% v/v acetonitrile in water added to precipitate the milk proteins followed by centrifugation (16000*g* for 30 s) and then analysis of the supernatant for the presence of TTX. Samples from a domestic, 2007 outbreak of pufferfish poisoning (*8*) for SPR analysis were run at no dilution, 1:10, 1:100, and 1:1000 (dilutions in running buffer), and the dilution within the linear range of the standard curve was then used for replicate measurements to determine the presence and concentration of TTX.

The procedure for extracting TTX from naturally contaminated samples was adapted from previous techniques and was reported to have 90% extraction efficiency (*19*). We previously obtained similar (86% extraction efficiency) results (*6*). First, 5 g of homogenized tissue was extracted twice with 10 mL of 1% acetic acid in methanol. This solution was then concentrated under vacuum to approximately 1 mL and redissolved in 5 mL of 1% acetic acid in HPLC grade water. After dissolution, the samples were defatted by adding 5 mL of chloroform and were separated by centrifugation. The aqueous layer was removed, and 5 mL of acidified water was added to the chloroform layer. Post centrifugation, the second aqueous layer aliquot was added to the first with the final extract equivalent to 0.5 g tissue/mL of extract. Pufferfish extracts (pufferfish matrix, PFM) for the generation of standard curves were also based on this procedure with the addition of filtration of the final extract (equivalent of 1 g tissue/mL matrix) via a Costar *μ*Star 0.22 *μ*m syringe tip filter (*26*).

**Detection Methods.** *Prototype SPR System.* This instrument was designed by Homola and co-workers at the Institute of Photonics and Electronics (IPE, Prague, Czech Republic) for FDA evaluation. The novel, custom-built instrument has been described previously for both the four-channel used herein and the eight-channel configuration (*27*−*29*). This SPR sensor is based on the Kretschmann geometry of attenuated total reflection and uses wavelength interrogation for signal transduction. Data acquisition was performed by the corresponding SPRSpectral program (version 2.03.1).

The methods for preparing the immunoassay surface for the prototype SPR system have been detailed in previous publications (*24, 30*). Briefly, after ozone cleaning the gold substrates (20 min, NovaScan PSD-UV, Ames, IA), the slides were immersed for 18 h in a mixture of 4.65 mL of ethanol, 150 *μ*L of triethylamine, 5 *μ*L of 5 mM HS-$(CH_2)_{11}$-$EG_6$-$NH_2$ in ethanol, and 195 *μ*L of 5 mM HS-$(CH_2)_{11}$-$EG_4$-OH in ethanol. After formation of the mixed thiolate monolayer, the slides were rinsed with a series of ethanol and ethanol/acetic acid solutions and finally dried with $N_{2(g)}$. TTX was covalently linked to the surface by immersing the slides in a mixture of 284 *μ*L of 100 mM pH 7.0 phosphate buffer, 6 *μ*L of 10 mg/mL TTX dissolved in 0.1 N acetic acid, and 10 *μ*L of formaldehyde. After 72 h

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at 37 °C the slides were rinsed with DI water, dried with $N_{2(g)}$, and stored at 4 °C until use. For use, a slide was then coupled to the SPR prism with Cargille Type A Immersion Oil ($n_D$ 1.5150, Cedar Grove, NJ). Finally the 4-channel, 50 $\mu$m Mylar gasketed flow cell was mated to the slide, and the fluidic lines were purged with the running buffer.

Inhibition immunoassays on the prototype instrument were performed to detect TTX with modifications made to the previously reported procedure (*24*). After stabilizing the system to a temperature of 22.5 °C and conditioning the chip for 15 min with 50 mM NaOH, a baseline was established by flowing buffer over the sensor surface. Prior to introduction of samples, 1 $\mu$g/mL anti-TTX was flowed over all channels to account for differences in surface conjugation and sensor response. For all experiments, there were four channels in the SPR instrument, one of which was always reserved as an antibody reference to allow for normalization of the data. Solutions were mixed for 11 min prior to introduction into the biosensor with a ratio of 1 part TTX sample to 9 parts 1.1 $\mu$g/mL anti-TTX in PBS for PBS, milk, and apple juice standards or 2.2 $\mu$g/mL in PBS for pufferfish samples. Standard curves were performed with TTX concentrations between 0 and 100,000 ng/mL in the appropriate matrix. The anti-TTX/TTX mixture was flowed over the SPR biosensor surface for 15 min at a flow rate of 30 $\mu$L/min. At that time, any unbound antibody in the mixture was available to bind to the sensor surface, which would yield a shift in the SPR wavelength. Following sample introduction, buffer was flowed over the surface for 7 min and then 50 mM NaOH was introduced for 15 min to regenerate the sensor for further use. The running buffer for each matrix was as follows: 10 mM PBS, 10% skim milk in PBS, or 10% pufferfish muscle extract in PBS.

For the prototype instrument, raw data sensorgrams (wavelength shift vs time) were processed by first normalizing the channels to account for sensitivity differences (*24*, *26*). Next, the individual runs were normalized to the antibody reference in each run. The response for each sample was then determined by subtracting the baseline prior to injection of the anti-TTX/TTX solution from the stability point obtained four minutes after switching back to buffer.

*Biacore T100*. The Biacore T100 (GE Healthcare) is a commercial instrument designed for concentration analysis and kinetic characterization. The instrument has four flow cells, an in-line buffer degasser, sample compartment and analysis temperature control, and automated run capability. In this instrument, the SPR signal is a measure of the angle of minimum reflected intensity and is defined as 1 Response Unit (RU) corresponding to a refractive index change of $10^{-6}$ (i.e., $10^{-4}$ degrees). This instrument is controlled using the Biacore T100 Controller Software v. 2.0.

The Biacore chips for the direct transfer study (Bia IPE, Bia standing for Biacore and IPE for Institute of Photonics and Electronics chip design) were prepared by following the same standard protocol described above for the prototype instrument. After thiol conjugation, the gold chip was adhered to the Series S support following the SIA kit instructions. The TTX was conjugated by reacting the substrate with 47.3 $\mu$L of phosphate buffer, 1 $\mu$L of 10 mg/mL TTX, and 1.7 $\mu$L of formaldehyde. Upon removal the chip was rinsed, dried, inserted in the sensor chip holder, and placed in the instrument.

The direct transfer of the immunoassay to the Biacore system (Bia IPE) has slight modifications due to the instrument design differences. The flow cell and sample compartments were set at 23 and 10 °C, respectively. All flow rates were 30 $\mu$L/min with 10 mM PBS as the running buffer. The TTX sample and antibody solutions were mixed for 11 min prior to injection, run with a contact time of 11 min, and dissociated for 5 min. Finally, the chip was regenerated for 10 min with 50 mM NaOH.

The redesigned Biacore chip (Bia Ref, Bia standing for Biacore and Ref for the use of a reference flow cell) was functionalized using a dual approach of online linker modification followed by on-bench conjugation with TTX. A CM5 chip was placed into the Biacore instrument, and Jeffamine was immobilized following the standard amine chip conjugation (e.g., flow cell one was a reference cell, and flow cells two through four were active channels). For the active channels, the CM5 carboxylmethyl dextran surface was reacted with 0.4 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and 0.1 M *N*-hydroxysuccinimide (NHS) to activate succinimide ester groups. These groups then reacted with primary amines of Jeffamine to form amide bonds (0.1 M in borate buffer, 420 s, 10 $\mu$L/min). Finally deactivation of unreacted succinimidyl esters was achieved with 1 M ethanolamine-HCl, pH 8.5. The chip was then removed from the instrument,

and 1 $\mu$L of TTX (10 mg/mL), 1.7 $\mu$L of formaldehyde, and 47.3 $\mu$L of phosphate buffer were added to the chip surface for 15 h at 37 °C. The chip was then rinsed with water, dried with $N_{2(g)}$, and inserted back into the instrument for the immunoassays.

For the redesigned Biacore assay (Bia Ref), the running buffer was changed to HBS-EP+ (a buffer optimized for the Biacore T100 instrument fluidics and similar in pH to the PBS used previously), and the assay times were decreased. The temperatures were 25 °C for the flow cells and 10 °C for the sample compartment. Using all four channels, three conditioning cycles (30 s 50 mM NaOH pulses) were performed to remove any unbound TTX. To perform the immunoassay, antibody at 1.1 $\mu$g/mL was mixed (90% fraction) with the TTX samples for 4 min. The flow rate was 20 $\mu$L/min with a contact time of 70 s, a 30 s dissociation, and a 120 s regeneration.

Data processing on the Biacore instrument was performed with the Biacore T100 Evaluation Software v. 2.0. The instrument and sensor chip were normalized prior to sample evaluation following standard protocol. The shift for each sample was obtained by subtracting the baseline window 10 s prior to injection from the stability window 15 s after the sample injection was complete; both windows were set to 5 s. These data were normalized to the trial run with only antibody, and the normalized signals were plotted versus the original solution concentration.

*ELISA*. The solid phase competitive inhibition enzyme immunoassay for TTX was developed specifically for FDA by Hawaii Biotech, Inc. under contract and employs the same antibody used in the SPR immunoassay. The assay is similar to the one described in Raybould et al. (*17*) and is not available commercially. The plates were prepared by coating each well with TTX-protein coating antigen in PBS for 1 h, washing the plates with 10 mM Tris buffered saline (0.05% Tween-20, 0.02% sodium azide), and blocking with 1% w/v bovine serum albumin in PBS for 1 h. Following washing with the Tris buffer, the TTX samples/standards and anti-TTX, alkaline phosphatase conjugate were added to the wells and incubated for 1 h. After washing with Tris buffer, enzyme substrate solution (*p*-nitrophenylphosphate) was allowed to react for 1 h at room temperature. The final color was read with an ELISA plate reader with the reference wavelength at 650 nm and the sample wavelength at 405 nm.

*RBA*. The procedure for the receptor binding assay for paralytic shellfish poisoning toxicity has been described in detail elsewhere (*15*, *25*, *31*). To apply this assay to TTX, standard solutions of TTX were substituted in the protocol where STX was called for. Briefly, 35 $\mu$L of TTX standard/sample, 35 $\mu$L of $^3$H-STX solution, and 140 $\mu$L of synaptosome preparation (*25*) were placed into each well of a 96-well microtiter filtration plate; each standard/sample was run in triplicate. The milk samples were incubated for 1 h at 4 °C, and pufferfish samples were incubated for 45 min at room temperature, and then each well was rinsed with 200 $\mu$L of HEPES buffer using a vacuum manifold. Finally, 25 $\mu$L of scintillant were added to each well and incubated for 30 min at room temperature. All measurements were taken on a 1450 Microbeta scintillation counter (Perkin-Elmer, Wallac Inc., Gaithersburg, MD). GraphPad Prism (v. 5.0, La Jolla, CA) was used to fit the standard curve.

*HPLC*. High performance liquid chromatography (HPLC) with post-column oxidation and fluorescence detection (FD) was performed similarly to Sohji et al. (*21*). TTX from naturally contaminated samples, described previously (*8*), were analyzed using an Agilent 1200 series HPLC system equipped with a model G1321A fluorescence detector (Wilmington, DE). Toxins were separated on a Develosil 5 $\mu$m C30-UG 100A column (Phenomenex, Torrance, CA) eluted isocratically using an aqueous solution containing 1% (v/v) acetonitrile, 20 mM ammonium heptafluorobutyrate, and 10 mM ammonium formate (pH adjusted to 5.0 with 5% ammonium hydroxide) at 30 °C at a flow rate of 0.4 mL/min. The eluted compounds were mixed postcolumn with 4 N sodium hydroxide using an Alltech model 301 pump with an Alltech online degasser (Grace, Deerfield, IL) at a flow rate of 0.7 mL/min. The combined eluate was heated to 100 °C in a postcolumn reaction system prior to flowing into the FD system. The postcolumn reaction system consisted of a 10 m long, 0.2 mm i.d. × 1/16 in. o.d. Teflon tube (Upchurch Scientific, Oak Harbor, WA) housed in a 1 L trinecked round glass flask containing mineral oil. The oil was heated to 100 °C using a 520 W Glas-Col heating mantle thermostated with a Glas-Col Mantle Minder II (Terra Haute, IN). To maintain temperature stability after initial heating, the heating mantle wattage was reduced to 30% of maximum using a Powerstat (Type 116B,
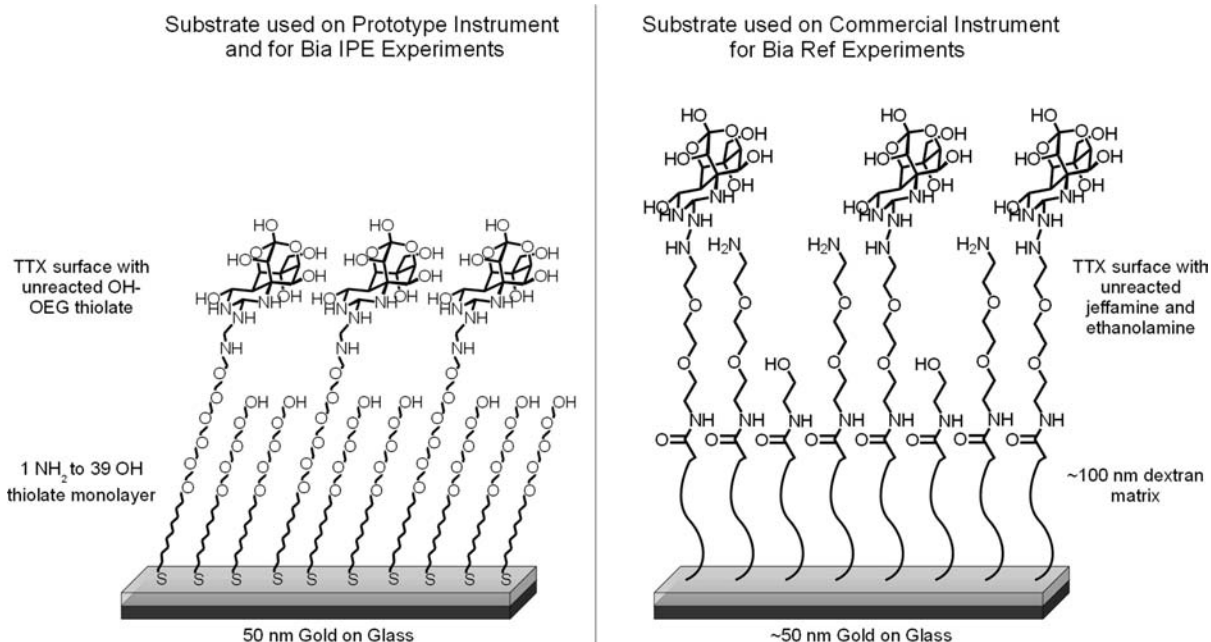
**Figure 1.** Sensor surface designs for the (**a**, left) prototype instrument and (**b**, right) commercial (Biacore) instrument.

Superior Electric Co., Bristol, CT). Fluorescent products of TTX and its congeners were monitored at an excitation wavelength of 365 nm and an emission wavelength of 510 nm. TTX was quantified from peak areas matching the retention time of standard TTX based on a linear regression of the following standards: 10, 1, and 0.1 μg/mL TTX in 1% aqueous acetic acid. Regression analysis was performed using GraphPad Prism (v.4.03) software.

*LC/MS/MS.* Methods for determining TTX using LC/MS/MS have been previously established (*8*). In the present study, electrospray MS/MS was performed on an ABSciex API5000 (Foster City, CA) triple quadrupole mass spectrometer with a turbospray ion source. An Agilent 1100 series HPLC provided the chromatographic separation using a TSK-GEL amide-80 HILIC column (2 × 250 mm × 5 μm) from Tosoh Bioscience (Montgomeryville, PA) which was heated to 35 °C and an isocratic mobile phase of 2 mM ammonium formate and 3.6 mM formic acid in 65:35 acetonitrile/water pumped at 300 μL/min. The mass spectrometer was operated in the positive ion multiple-reaction monitoring (MRM) mode with MRM conditions optimized to produce the maximum intensity or signal for the ions monitored (data not shown). Three fragment ions were chosen to be monitored ($m/z$ transitions: 320 → 302, 320 → 256, 320 → 162), which provided sufficient evidence to prove and quantify TTX if present in the test portion. Standards were run in duplicate, and the unknown values were compared to the linear curves based on peak value, and the concentrations were determined.

## RESULTS AND DISCUSSION

**Immunoassay Substrates.** The sensor surfaces for the TTX immunoassay followed two different designs: one optimized for the prototype instrument and the second for the commercial Biacore instrument. As shown in **Figure 1**, these surfaces share many similarities. First, each chip is based on a glass slide coated with a thin layer of gold that allows for generation of the SPR signal. Next, the thiolate monolayer allows for attachment of the ligand molecule, TTX in this case, to the gold surface. For the prototype instrument, a mixed monolayer of thiols is used with the OH terminated thiol decreasing nonspecific interactions (*32*, *33*), the NH₂ thiol providing a covalent linking site for the TTX via formaldehyde coupling chemistry (*17*, *34*), and the triethylamine additive improving the smoothness of the monolayer (*35*) (**Figure 1a**). Recent studies using external reflection infrared spectroscopy have shown that this surface formation

(1) does indeed occur and that the monolayer does conjugate TTX, (2) can specifically bind anti-TTX, and (3) has little nonspecific binding on a non-TTX conjugated monolayer (data not shown).

The commercial, CM5 Biacore chip is coated with a carboxymethylated dextran matrix which allows for electrostatic concentration and immobilization of ligands while also creating freedom of movement for the attached biomolecules (*36*). For these commercial chips, unreacted amine groups after ligand conjugation are blocked with ethanolamine, thus providing a surface resistant to nonspecific adsorption. Initial experiments with the commercial chip showed that directly coupling TTX to the activated surface yielded a less stable surface that was prone to degradation upon regeneration. To overcome this, a Jeffamine linker was used during the instrument immobilization step, the remaining surface sites were blocked with ethanolamine, and then the TTX was coupled via formaldehyde outside the instrument thus yielding the surface depicted in **Figure 1b**. The improvements in the Bia Ref chip conjugation method over the Prototype/Bia IPE substrate include enhanced stability of the chip surface, better immunoassay performance (as detailed below), and reduced time (one day from four days) to create the assay platform.

**Transfer of Immunoassay to a Commercial Platform.** Initial assay design for PBS and pufferfish matrices has been described previously (*24*, *26*). For this assay, direct detection of TTX via SPR is not possible due to the small size of this molecule being at or below the general limit of detection. Furthermore, traditional sandwich immunoassay is not a good format, as the small TTX cannot easily bind two antibodies. Thus, an inhibition immunoassay format is used for this research. The antibody concentration for these assays was chosen based on achieving a high enough SPR shift to allow for detection, yet not high enough for surface saturation. Such a moderate antibody concentration allows for sufficient reaction with the substrate, while also allowing for complete antibody inhibition when high TTX concentrations are present in solution.

While a viable instrument for food-borne pathogen and toxin detection, the prototype instrument, previously used for the initial TTX assay design, is not available to the general public at this time. As such, it is necessary to transfer the established protocol to
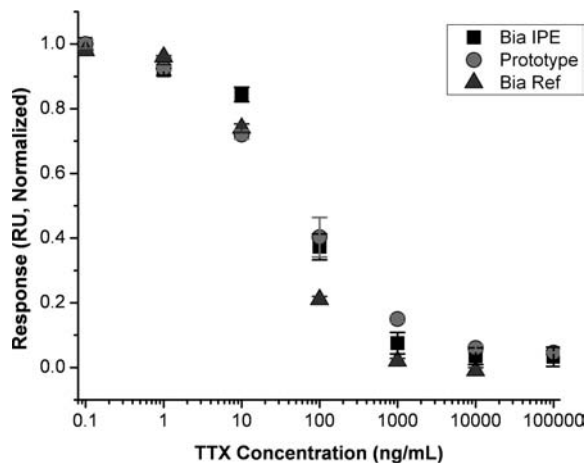
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**Figure 2.** Transfer of TTX immunoassay to a commercial platform (Biacore T100). Comparison of prototype instrument (circles) assay, mixed monolayer substrate and immunoassay on Biacore T100 (squares, Bia IPE), and a redesigned assay taking advantage of the commercial CM5 substrates and improved reaction conditions (triangles, Bia Ref). All curves show approximately the same trend with the redesigned Biacore assay having improved standard deviations ($N = 3$).

a commercially available instrument, such as the Biacore T100. With this in mind, a direct transfer experiment was performed. By using the prototype surface design and a similar protocol, an immunoassay was performed with TTX spiked PBS. In addition, the chip surface and assay procedure were redesigned to take advantage of the commercial instrument capabilities (as described in the Materials and Methods and in **Figure 1**).

Upon plotting the results for these assays, it is seen in **Figure 2** that the direct assay transfer to the commercial instrument went smoothly. These curves follow the expected sigmoidal, inhibition immunoassay curve shape. The highest signal occurs when there is no TTX in solution, and the signal decreases as the amount of tetrodotoxin in solution increases, thus consuming the antibody in solution and decreasing the amount available to bind to the substrate until complete inhibition (0.05 response at 100,000 ng/mL) is seen. All the curves demonstrate similar trends with potential differences in curve shape most likely due to the differences in surface design (TTX coverage and orientation), buffer conditions (PBS for the prototype protocol and HBS-EP+ for the redesigned assay), reaction times, and flow rates for the individual immunoassays.

**Complex Matrix Immunoassays.** To expand the assay system to food matrices, the optimized assay for PBS and pufferfish muscle were initially studied. Furthermore, analysis of milk and apple juice was performed to determine the assay applicability to potential food adulteration matrices. Many recent studies have investigated using SPR to detect materials in milk, and a few examples include staphylococcal enterotoxin B (*37*), *Salmonella* (*38*), beta-casein (*39*), whey proteins (*40*), and antibiotic residues (*41*). In these and other previous studies using milk matrices and SPR, milk has shown little interference with well-designed assay substrates and low limits of detection with few or no sample preparation steps. With this in mind, we investigated TTX spiked into milk to determine if sample preparation would be needed for small molecule toxins.

By following the standard assay protocol, TTX was spiked into milk and run on the prototype instrument. As shown in **Figure 3a**, there was little difference between PBS, PFM, and milk matrices. This confirms that there are no interferences in the 10% skim milk solutions and that no special sample preparation is needed for this
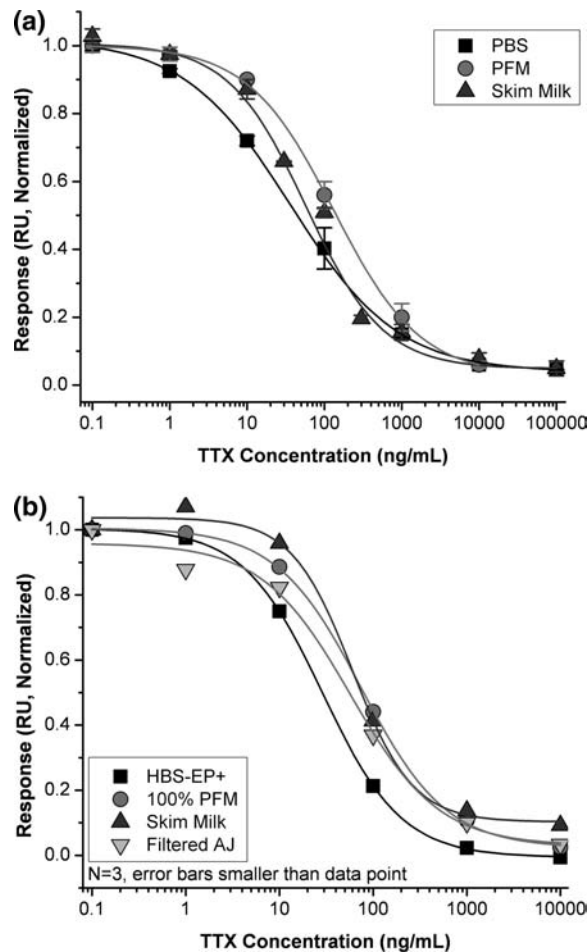


**Figure 3.** (**a**) Standard curves from the prototype instrument for PBS, pufferfish matrix (PFM), and skim milk matrices. $N = 3$ for each data point. (**b**) Complex matrix assays using the redesigned assay and Biacore T100 system (Bia Ref and AJ = apple juice).

small molecule toxin. The differences in curve shape are attributed to the varied pH of these solutions affecting analyte/antibody binding kinetics, and these curves exemplify the need for matrix matching. Research is currently underway to better understand such pH effects.

In addition, the redesigned, optimized Biacore assay was performed with the two complex matrices previously studied (PFM and skim milk) as well as extended to filtered apple juice (AJ), and the resultant calibration curves are shown in **Figure 3b**. Apple juice was chosen as an additional matrix to study due to the limited number of SPR assays using this matrix, and the few studies that have been performed have focused on large analytes (i.e., bacteria) (*42*) and the need to adjust solution pH (*43*). Optimization studies using apple juice proved that this matrix, unlike milk and PFM, required filtration to decrease nonspecific adsorption and chip degradation most likely due to background carbohydrates and sugars (data not shown).

The most noteworthy difference between the prototype and commercial platform is the decreased standard deviation for the complex matrix assays run on the Biacore instrument. This decrease in error leads to improved limits of detection (LODs) for the optimized Biacore assay (Bia Ref) by a factor of 1.1 to 1.9 compared to the prototype system and a factor of 9.0 over the Bia IPE configuration (**Table 1**). The dynamic range, however, is slightly larger for the prototype instrument.

While the prototype and Bia IPE configurations performed well compared to the Bia Ref assay, there are other advantages to

**Table 1.** Comparison of TTX Assay Performance in Different Matrices and on Different Instruments[a]

| instrument | matrix | LOD (ng/mL) | dynamic range (ng/mL) | $IC_{50}$ (ng/mL) |
|---|---|---|---|---|
| Prototype | PBS | 0.49 | 5.0−495.2 | 44.5 |
| | PFM | 6.13 | 25.1−881.5 | 142.1 |
| | milk | 5.02 | 13.0−391.9 | 65.5 |
| Bia IPE | PBS | 3.43 | 13.7−250.3 | 60.4 |
| Bia Ref | HBS-EP+ | 0.38 | 7.8−107.2 | 28.9 |
| | PFM | 3.25 | 22.4−392.2 | 91.5 |
| | milk | 4.51 | 28.8−335.2 | 82.6 |
| | apple juice | 0.08 | 5.5−398.2 | 46.9 |

[a] Limit of detection (LOD) is determined by taking the normalized response (1.0) minus three times standard deviation of the lowest concentration (0.1 ng/mL, 3 replicates). Dynamic range is the linear range between $IC_{20}$ (0.8 signal) and $IC_{80}$ (0.2 signal), while the $IC_{50}$ indicates the response at 50% inhibition.

**Table 2.** Comparison of Detection Methods for Analyzing TTX-Spiked Milk[a]

| prepared (ng/mL) | LC/MS/MS (ng/mL) | RBA (ng/mL) | SPR (ng/mL) | av (ng/mL) |
|---|---|---|---|---|
| 80.0 | 66.8 | 89.3 | 155.9 | 104.0 ± 46.3 |
| 8.0 | 7.1 | 12.4 | 9.0 | 9.5 ± 2.7 |
| 0.80 | ND | 1.10 | 0.79 | 0.95 ± 0.22 |

[a] SPR was performed on the Prototype instrument. Milk was spiked at 80, 8, and 0.8 ng/mL and analyzed with SPR, RBA, and LC/MS/MS. ND indicates that TTX was not detected.

using the optimized Biacore assay. First and foremost is the savings in time and reagents achieved by using an automated sample platform and decreased mixing, contact, and regeneration times. The total time saved in the immunoassay is 40 min (48 min for prototype assay versus 8 min for optimized Biacore assay). Furthermore, a 2 min regeneration, decreased from 15 min, was sufficient for complete regeneration and allowed for more immunoassay cycles (200+) due to the reduced regeneration time. In addition, the optimized assay utilized a sensor chip that contains a reference flow cell that allows for monitoring of chip degradation, potential nonspecific binding, and instrument characteristics. These give the user further confidence in the data obtained and increase the ability to reuse the chip fully, but within acceptable degradation limits (e.g., less than 20% change in RU for antibody standards and equivalent normalized calibration curves). Further studies focusing on implementing this optimized immunoassay on less expensive SPR instrumentation and validating the technique for potential use in analytical laboratories and industrial settings are currently in progress.

**Detection Method Comparison for TTX-Spiked Milk and Pufferfish Outbreak Samples.** To determine the applicability of this assay for food samples, two analyses were performed and compared to conventional detection techniques. The first study focused on determining the amount of TTX spiked into milk. **Table 2** shows the results for the analysis of these samples by SPR, RBA and LC/MS/MS. The values determined by each technique compare well and are near the expected value for most samples, indicating that there were minimal matrix effects in milk for the techniques and that SPR could be a viable alternative for analysis of complex samples. An advantage of SPR over LC/MS/MS in this case is the lack of sample preparation for analysis. Furthermore, while SPR has a LOD above 0.8 ng/mL in milk matrices (5.02 ng/mL), the value for this sample (0.79 ng/mL) matches well with the expected level, indicating that detection was possible below the statistically determined LOD. We attribute this to the larger standard deviations associated with the milk assay that make the LOD a factor of 10 higher than for an assay in the "clean" PBS matrix. This capability could prove valuable for

**Table 3.** Comparison of Methods Used for Detecting TTX (All Values Reported in $\mu$g/100 g) in the Naturally Contaminated and 2007 Outbreak Samples[a]

| sample | LC/MS/MS (8) | ELISA | HPLC | RBA | SPR | av |
|---|---|---|---|---|---|---|
| fish flesh 1 | 374 | 438 | 373 | 456 | 377 | 404 ± 40 |
| fish flesh 2 | 655 | 721 | 655 | 590 | 815 | 687 ± 85 |
| soup broth | 361 | 402 | 366 | 304 | 193 | 325 ± 82 |
| puffer 1 | 10 | 4 | NA | 18 | 7 | 10 ± 6 |
| puffer 2 | 214 | 268 | NA | 352 | 247 | 270 ± 59 |
| puffer 3 | 876 | 1412 | NA | 1050 | 709 | 1012 ± 301 |
| puffer 4 | 961 | 1248 | NA | 1324 | 1074 | 1152 ± 165 |
| puffer 5 | 10 | 2 | NA | 16 | 7 | 9 ± 6 |
| monkfish 1 | 0 | 0 | NA | ND | ND | |
| monkfish 2 | 0 | 0 | NA | ND | ND | |

[a] SPR was performed on the Prototype instrument. ND indicates tetrodotoxin was not detected or was below the detection limit for the method, while NA indicates the samples were not analyzed with this method.

potential food adulteration, in which any toxin present in a commodity like milk would indicate an act of purposeful contamination. Finally, the RBA suffers from cross reactivity of the sodium channels to other small molecule toxins, such as saxitoxin and related congeners. This SPR biosensor does not suffer from cross reactivity, as the antibody is specific to TTX over common seafood toxins (e.g., STX, okadaic acid, domoic acid; data not shown) and thus could be a more specific screening method for samples.

To further evaluate the SPR method, naturally contaminated pufferfish as well as samples from a 2007 outbreak were analyzed for the presence of TTX. In these assays, monkfish was used as a control, as this fish is not known to harbor TTX. SPR measurements were performed with sample dilutions of no dilution, 1:10, 1:100, and 1:000 in order to ensure that one set of measurements was within the dynamic range of the assay. The normalized SPR response indicated the presence of TTX in each of the outbreak and pufferfish samples, as the signals showed inhibition to antibody binding (response below 0.95) while the monkfish controls did not indicate tetrodotoxin presence. After calculating the concentration for each sample in units of $\mu$g toxin per 100 g of tissue and comparing the results to the results from other methods commonly used for TTX detection, the SPR results agreed well with the other detection methods (**Table 3**), further indicating that SPR is a viable alternative for TTX detection in food samples.

As all techniques show comparable performance for detecting TTX in complex matrices, the method of choice will depend mainly on the user and facility needs with manual labor, operation simplicity, sample preparation, throughput, cost, and sensitivity the figures of merit evaluated. With respect to these criteria, the SPR biosensor offers a rapid (triplicate measurement of a sample in less than eight minutes) analysis of samples with little need for sample cleanup and detection limits in the low- to sub-ppb range. As such, the TTX SPR immunoassay would be useful for screening samples. Specifically, SPR biosensors have (1) instrumentation that can be relatively inexpensive and possibly portable, (2) low reagent consumption, (3) a flow system that allows for continuous monitoring, (4) analysis that is not affected by fluorescence quenchers or naturally fluorescent compounds, (5) use of nonradioactive, nonorganic solvents, (6) potential multiplexing capability, and (7) antibodies that may have less cross reactivity than other bioreagents (e.g., sodium channel receptors). Work is underway to investigate the transferability of this SPR assay to other commercial instrumentation as well as to conduct studies that further evaluate the interlaboratory reproducibility of this newly redesigned method.

Article

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