Development of a direct detection method for Alexandrium spp. *Using surface plasmon resonance and peptide nucleic acid probes.*

Amber R. Bratcher*, Laurie B. Connell and Rosemary L. Smith
University of Maine
Orono, Maine, USA
*amber.bratcher@umit.maine.edu

***Abstract*—Marine dinoflagellates of the genus *Alexandrium* can produce dangerous amounts of paralytic shellfish toxins at such low cell densities that water discoloration often associated with harmful algal blooms is not always evident, making detection of blooms during early stages extremely important. Our research focuses on developing a new method of molecular detection for multiple species of *Alexandrium* using peptide nucleic acid (PNA) probes and surface plasmon resonance. Results thus far indicate that this method yields reliable detection of perfectly complimentary oligonucleotide sequences specific to *A. fundyense, A. tamarense, A. catenella,* and *A. ostenfeldii* with no detection of non-complimentary sequences, as well as very good discrimination between negative control PNA probes and *Alexandrium*-specific PNA probes. We have also been able to regenerate the probe layer on the sensor surface for use in multiple assays by removing the target DNA, making this method economical in the long run.**

I. Introduction

Paralytic shellfish poisoning (PSP) caused by consumption of shellfish that have fed on toxic algae is a major health issue worldwide [1]. Dinoflagellates of the genus *Alexandrium* can produce high amounts of paralytic shellfish toxins (PSTs) at such low densities that water discoloration often associated with Harmful Algal Blooms (HABs) is not always evident. Detection of blooms during early stages is extremely important for human health issues, effects on shellfish populations, bioaccumulation of toxin within the food web, and maintenance of fisheries [2, 3]. Species that produce PSTs are difficult to distinguish morphologically from non-PST producing species, and current identification methods are expensive, time-consuming, and require special training. Development of a rapid, low-cost and easy-to-use device to detect and monitor *Alexandrium* would be an important advancement as HABs vary interannually in location, intensity, and duration, making detection and prediction areas of necessary research [4].

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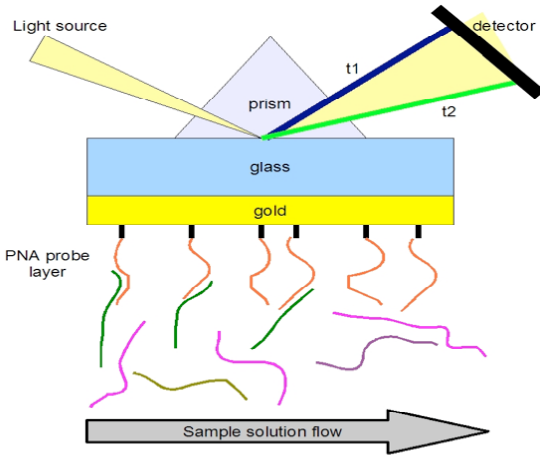

Fig. 1: SPR operation with PNA probe layer on gold surface. Binding of target molecules causes a change in refractive index.

Surface plasmon resonance (SPR) is a label-free, optical detection method that measures the change in refractive index after binding (hybridization) of target to probe on a surface [5] Fig. 1. As target molecules bind to a probe on a metal surface, the refractive index shifts, causing a change in the surface plasmon wave. SPR can monitor biological interactions in real-time, providing a distinct advantage over other types of detection.

Peptide nucleic acids (PNA) are short-sequenced DNA mimics where the negatively charged sugar-phosphate backbone is replaced by a neutral peptide chain. PNA probes have a high discrimination for mismatches, are resistant to protease and nuclease degradation, and will

Fig. 2: Custom-built SPR instrument developed at the University of Washington (SPIRIT, sold by Seattle Sensor Systems, Inc.). A=Buffer tube, B=Syringe in sample injection port, C=24-channel sensor bank, D=touch screen controls. Data outputs to directly to a PC software program that allows for real-time monitoring of the SPR response.

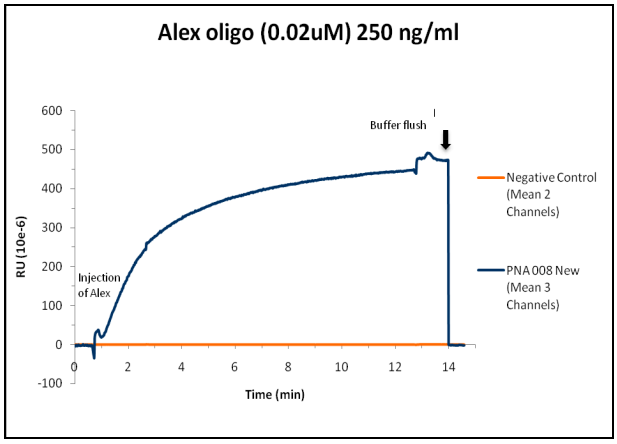

Fig. 3: SPR detection of *Alexandrium fundyense* oligonucleotide by PNA 008 probe (specific to this species). No detection of binding by negative control PNA probe.

hybridize in low salt concentrations, making them ideal for use in field settings [5, 6]. Thiolated PNA probes with spacer molecules inserted before the probe sequence hybridize readily to unmodified gold surfaces forming self-assembling monolayers (SAMs). SAMS of PNA bind to target sequences following Watson-Crick rules for base parings and with heightened specificity [7].

Our research combines the use of SPR and PNA probes to develop direct detection sensors not currently feasible for field use or not sensitive enough with presently available molecular probes. This technology will fill a current gap in the ability to easily monitor potential HABs on-site with little sample processing.

II. MATERIALS AND METHODS

A. *Probe and Target Development*

Multiple 16-mer PNA probes were developed by optimizing previously designed DNA probe sequences ATNA1 for the *Alexandrium tamarense/fundyense/catenella* species complex [7] and AOST01 for *A. ostenfeldii* [8], as well as a universal negative control probe sequence that does not match any sequence found in GenBank. Probe sequences were derived from large-subunit ribosomal RNA sequences for these species and are specific to target ribosomal RNA [7, 8]. Each probe was modified on the 3' terminus with a cysteine and spacer groups of 1.3 nm long ethylene glycol linkers were inserted before the probe sequence. The cysteine provides a thiol group that allows for absorption directly onto the gold sensor surface. A 15-mer target oligonucleotide sequence was designed as an exact DNA compliment to the probe PNA 008 and a 43-mer target oligonucleotide sequence was designed with a 15-mer sequence at the end as an exact compliment to the probe PNA 013. PNA probes were obtained from Panagene (Daejeon, Korea), and DNA oligonucleotide sequences were obtained from IDT Technologies (Coralville, IA, USA). PNA probes were prepared as stock solutions of 250 µM in milli-Q H_2O and stored at -20 ^oC until use. DNA target oligonucleotide sequences were also prepared as stock solutions of 250 µM in milli-Q H_2O and stored at -20 ^oC until use.

B. *SPR Instrument*

We are using a portable SPR instrument, SPIRIT, (Fig. 2) developed with collaborators at the University of Washington in Seattle, Washington, that employs a miniature sensor module with a modifiable gold-coated surface (Spreeta, by Icx Nomadics) to which the probes are covalently bound. The Spreeta SPR chips contain a light source and array detector integrated with the gold

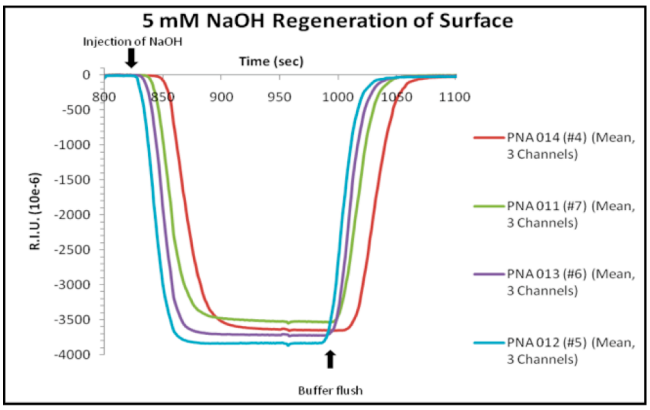

Fig. 4: Regeneration of probe layer with 5 mM NaOH. Return of RIU to pre-experimental baseline level indicates successful removal of bound material.

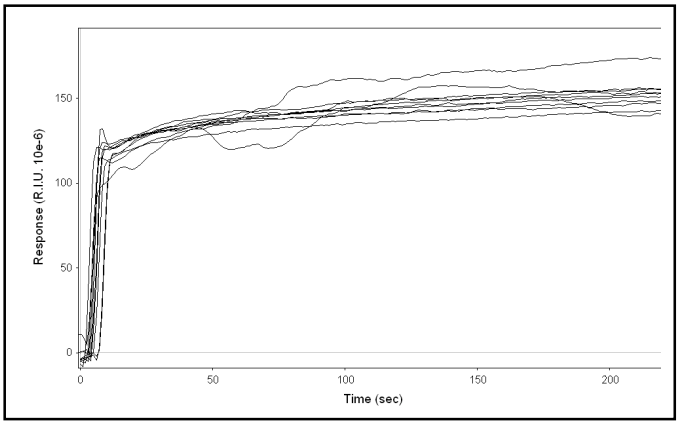

Fig. 5: SPR detection of *Alexandrium fundyense* oligonucleotide by PNA probe 008, with the reference channel subtracted. A total of 40 experimental cycles of introducing oligonucleotide complimentary to the PNA probe and then regenerating the surface by removal of bound material with 5 mM NaOH were performed before seeing a 10% loss of R.I.U.

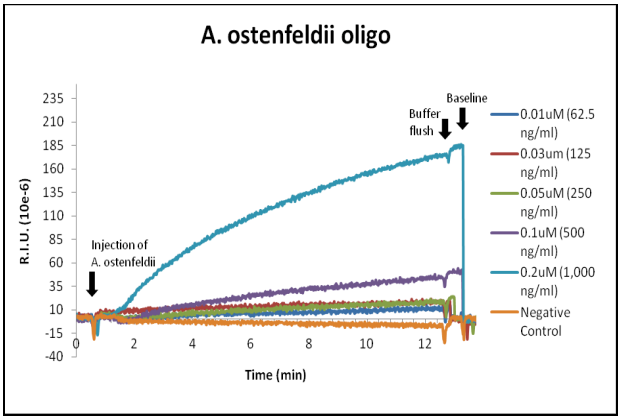

Fig. 6: SPR detection of *Alexandrium ostenfeldii* oligonucleotide by PNA 013 at multiple concentrations. This method can still detect quantities of this oligonucleotide down to concentrations of 0.01uM, though quantities below 0.05uM where undistinguishable from lower concentrations. No detection of binding with negative control PNA probe.

sensor surface [9-11]. Samples are flowed over the sensor surface and subsequent binding of target to probe is visible on a connected laptop within seconds. Details of this SPR instrument have been previously described by Soelberg and coworkers [12].

C. Immobilization of Probes on Sensor Surfaces

Spreeta sensors were initially cleaned by gently drawing a lens paper wetted with 50% ethanol across the surface. For immobilization of PNA probes on the sensor surfaces, working stocks of probes were diluted to concentrations of 250 ng/mL in milli-Q H_2O. A 30µl droplet of probe solution was spread across the gold surface layer of the sensor and allowed to incubate outside of the SPR instrument in a humid chamber at 25°C for 3.5 hours, after which the surface was rinsed vigorously with dH_2O before inserting sensors into the SPR instrument.

D. Experimental Procedure

All experiments were carried out in PBS-Tween (0.1%) running buffer. For the first set of experiments a 1.5 mL oligonucleotide sample was flowed across the sensor surface at a rate of 57 uL/min for a total of 15 minutes. After 15 minutes running buffer was flushed through the sample loop followed by a 2 minute regeneration step, consisting of 1.5 mL of 5mM NaOH being flowed over the sensor surface at 57 uL/min to remove any bound target oligonucleotides while leaving the PNA probe layer intact.

III. RESULTS AND DISCUSSION

Results indicate that this method provides reliable detection of synthetic oligonucleotides. Fig. 3 shows the response of a sensor coated with the PNA probe specific for *A. fundyense/tamarense/catenella* (PNA 008) to a 43-mer oligonucleotide sequence designed with the first 15 bases to be exactly complimentary to this probe. This probe can detect the target oligonucleotide sequence with no binding to a sensor coated with a universal negative control probe. After detection the target can be removed with 5mM NaOH, restoring the probe layer to pre-experimental conditions (Fig. 4), regenerating the sensor surface for instant reuse. A second, bench-top, SPR instrument (SenisQ, by Icx Nomadics, Oklahoma City, OK, USA) was used in parallel. It was found that a total of 40 experimental cycles (consisting of first binding a target to the probe surface and then regenerating the surface by removing any bound target) could be run on a sensor before a 10% loss of intensity occurred (Fig. 5).

Further, we can detect *A. ostenfeldii* oligonucleotide in buffer solution down to concentrations of 0.01µM (Fig. 6) without secondary amplification. Though concentrations of oligonucleotide less than 0.05µM show similar responses, they are well above the background signal shown by a sensor coated with the negative control probe. The longer length of the oligonucleotide specific to PNA 008 yields an increased shift in refractive index in comparison to the shorter length of the oligonucleotide specific to PNA 013, as was expected. The ability to detect such minute quantities is extremely important, as harmful blooms often are not high in cell number and small concentrations of RNA would thus be extracted from a water sample. Based on predicted RNA levels [14] this would yield detection levels as low as 200 cells without any secondary amplification or concentration, though this number would also be dependent on the volume of seawater processed for a sample.

Future steps for this research include investigating the effect of secondary amplification for increasing the sensitivity of this method of detection. Harmful blooms of *Alexandrium* can contain as few as 200 cells per liter, and because of the advantages of detecting blooms before they produce dangerous amounts of toxin it is important to have a highly sensitive assay. Also, a method of rapid RNA extraction for *Alexandrium* is currently being investigated. Preliminary experiments have confirmed the ability to detect extracted RNA from cultures using this method without purification or amplification steps.

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