Developing Gene-Based Remote Detection

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LONG-TERM GOALS

We seek to improve coastal water quality monitoring and ecological forecasting by incorporating the advantages of molecular biology into autonomous biosensors. Such “next generation” sensors will greatly enhance the power of ocean observing systems by relaying species-specific information in conjunction with more traditional environmental measurements. The biosensors will identify and quantify microbial species including harmful algae, fecal indicator bacteria, and human pathogens by electrochemical detection of nucleic acids (DNA or RNA). Autonomous, remote detection of biologics is needed to meet needs of natural resource management and the oceanographic and ecological sciences. This type of technology development also has relevance to medical and homeland security applications.

OBJECTIVES

The objectives of this Phase I study focus on three critical components required for gene-based remote biosensors: assessing whether electrochemical methods can be used to detect real nucleic acids (as

opposed to synthetic analogs) at relevant concentrations, determining the feasibility of using electrochemical methods for remote marine analyses, and developing remote nucleic acid extraction methods.

APPROACH AND WORK PLAN

Our approach is to use electrochemical methods and devices to meet the size, cost, power, and sensitivity demands of sensors that are to be deployed on buoys and autonomous vehicles. A major goal for the first year of the project was to evaluate and further develop electrochemical methods for detecting microbial nucleic acids, particularly methods that did not use PCR amplification. Tasks in the first year work plan included:

- Design nucleic acid probes for microbes that are important in coastal water quality monitoring, including sewage-indicating bacteria and harmful algae.
- Incorporate electrochemical techniques into the NOAA/University of Miami laboratories.
- Evaluate and select electrochemical instruments and sensors appropriate for remote use.
- Evaluate, select, and begin to optimize molecular biological methods coupled to electrochemical techniques to meet the demands of remote detection of marine microorganisms.
- Determine whether electrochemical techniques can achieve the needed sensitivity without using PCR amplification.

In the next year, we plan to further develop and optimize selected electrochemical assays, with focus on techniques that can be coupled to low cycle number PCR (see Results section); improve the sensitivity and quantification of microbes by electrochemical means; and optimize techniques using real-world environmental samples (seawater and beach sand). We will seek funding for Phase II of the project to allow automation of electrochemical detection assays. The long-term goal of Phase II is to build and ground-truth a prototype marine biosensor.

Our approach is to utilize an interdisciplinary team of academic, government, and industrial scientists to tackle the challenges of this project. The current NOPP partnership includes the NOAA Atlantic Oceanographic and Meteorological Laboratory (AOML), the University of Miami Rosenstiel School of Marine and Atmospheric Science (RSMAS), Arizona State University, Alderon Biosciences, and the Monterey Bay Aquarium Research Institute (MBARI). Drs. Kelly Goodwin (NOAA), Jack Fell (University of Miami), and Michael LaGier (University of Miami) coordinate the project, provide expertise in microbiology, molecular biology, and oceanography, and are responsible for carrying out the Phase I study objectives. Dr. Joseph Wang (Arizona State University) contributes electrochemical and biosensor expertise and is responsible for conducting basic research to develop more sensitive electrochemical methods. Dr. Peter Ortner (NOAA/AOML) provides guidance on buoy-deployed instrumentation. Dr. Chris Scholin (MBARI) provides guidance in the areas of remote nucleic acid isolation and detection techniques. Dr. Marek Wojciechowski and his team at Alderon Biosciences supply electrochemical instruments and sensors, contribute engineering knowledge in the area of remote, automated biosensing, and explore the commercialization of technology resulting from the project.

WORK COMPLETED

We have tested a variety of methods for potential compatibility with environmental sensor arrays that are presently deployed on ocean buoy platforms. Seven electrochemical approaches, two electrochemical instruments, and four electrode types were evaluated. Our efforts indicate that electrochemical assays can be developed for *in situ* deployment. We have demonstrated that target detection can be achieved by coupling molecular methods to electrochemical detection. Furthermore, the detection of nucleic acids directly from cell homogenates was demonstrated. We have shown the robustness of electrochemical techniques by targeting bacteria, dinoflagellates, and yeasts. In addition, we have detected nucleic acid signatures in environmental samples of seawater. Most importantly, we have zeroed in on two techniques that have the potential to meet the ultimate requirement of *in situ* biosensors -- quantitative detection. Throughout, we have focused on real world samples with an eye on developing methods that can be realistically integrated into ocean observing systems. Our work with the Cooperative Institute of Coastal and Estuarine Environmental Technology (CICEET) and the Alliance for Coastal Technology (ACT) has allowed us to maintain a dialog with stakeholders and end-users so that our developmental efforts will deliver useful products. Furthermore, we understand the challenges facing natural resource managers through our work on Oceans and Human Health (OHH) projects. We work jointly with scientists at the University of Miami OHH Center and Dr. Goodwin is a PI on a Hollings Marine Laboratory OHH project. Many of our efforts have been summarized in 6 publications.

RESULTS

Design of nucleic acid probes.

Nucleic acid probes were successfully designed for a variety of sewage indicating bacteria (*Escherichia coli, Enterococcus faecalis, Bacteroides distasonis, Bacteroides fragilis* group, the Enterobacteriaceae) and for toxic algae found in Florida red tide blooms (*Karenia brevis, Karenia mikimotoi*).

Evaluation of electrochemical instruments and sensors.

The incorporation of electrochemical techniques into autonomous sensing platforms requires instruments and sensors that are inexpensive, consume little power, and provide high quality data. We tested pyrolytic graphite electrodes and screen-printed electrodes custom made from the Wang laboratory and commercially available through Alderon Biosciences. Out of the sensors tested, we found the Alderon sensors to be the most reliable with regard to reproducibility and sensitivity. Our research indicates that adapting an electrochemical sensor/instrument package that is commercially available is the quickest route to meeting the challenges of oceanographic applications. The sensitivity, reproducibility, and quantitative characteristics of Alderon methods and instruments (Wojciechowski et al., 1999) are attractive for integration into remote sensing platforms. The Alderon instrument (http://www.alderonbiosciences.com) uses an ultra-sensitive and unique detection method (intermittent pulse amperometry) for DNA or RNA detection. It is compact, can be powered by a 9V battery, and reports data that does not require additional manipulations (**Figure 1**). The instruments also are compatible with 8 and 96-well sensor arrays, allowing for simultaneous detection of multiple organisms. The small size and low cost of the disposable carbon sensors are suited for platforms that will remain in an oceanic environment for extended periods of time (**Figure 2**). The use of disposable sensors avoids the chemicals and automation that would be needed to regenerate the electrodes.

Figure 1. Hand-held electrochemical instrument available through Alderon Biosciences.

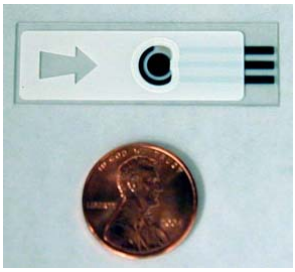

Figure 2. An Alderon miniaturized, disposable electrochemical sensor.

Evaluation of electrochemical techniques.

Detection of synthetic oligonucleotides. Work with synthetic DNA analogs is used to develop novel electrochemical methods to meet sensitivity and multiplexing needs. Four methods were used to achieve ultra-sensitive detection of synthetic DNA: 1) carbon-nanotubes loaded with CdS tags (Wang et al., 2003a), 2) carbon-nanotube-modified electrodes using an enzymatic amplified electrical signal (Wang et al., 2004a), 3) indium microrods (Wang et al., 2003b), and 4) nanocrystal-based bioelectronic coding (Wang et al., 2004b; Lui et al., 2005).

Detection using real cells and environmental samples. The great majority of previous efforts have used electrochemical techniques to detect artificial nucleic acids. To date, only a handful of scientists worldwide have reported electrochemical detection of nucleic acids isolated from cells (http://www.alderonbiosciences.com; Barken et al., 2004; Gabig-Ciminska et al., 2004a, 2004b; Metfies et al., 2005). An important step in this project was to test whether electrochemical methods could detect nucleic acids isolated from actual cells relevant to the needs of our potential end-users (e.g., fecal contaminants and harmful algae). We demonstrated that electrochemical methods can be used to specifically detect nucleic acids isolated from actual targets of interest. In fact, the methods were robust in that detection of both RNA and DNA was successfully achieved from a variety of prokaryotic and eukaryotic organisms. We detected RNA (LaGier et al., 2005a) and DNA of the fecal bacteria *Escherichia coli* and *Enterococcus faecalis.* In addition, a group specific probe was designed to detect total coliforms, and RNA from the Enterobacteriaceae group was detected (Goodwin et al., 2004). Detection of the toxic dinoflagellate, *Karenia brevis* (LaGier et al., 2004c; LaGier et al., 2005a) and the yeast, *Cryptococcus neoformans* (LaGier et al., 2004b) was also achieved. Furthermore, targets were detected from environmental seawater samples. For example, sensitive electrochemical

detection of *K. brevis* from water samples was achieved using the Alderon Rapid PCR method (**Figure 3**).

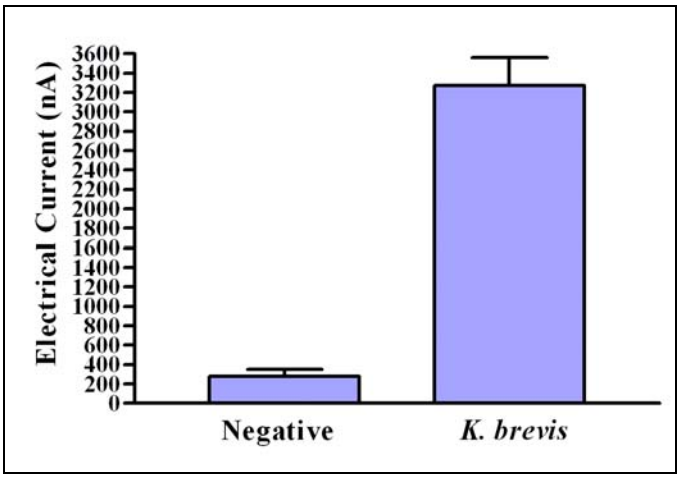

Figure 3. Successful detection of the toxic dinoflagellate, K. brevis, from a seawater sample containing less than 1000 K. brevis cells per liter.

Detection without nucleic acid clean-up. In contrast to mainstream thinking, we (LaGier et al., 2005a,b; http://www.alderonbiosciences.com) and one other group (Gabig-Ciminska et al., 2004b) have demonstrated that electrochemistry can be used to detect nucleic acids directly from cell homogenates. We detected *E. coli* from crude lysate – no purification of the nucleic acid was necessary. This was accomplished using two separate methods, both guanine oxidation (LaGier et al., 2005a) and the Alderon Direct Detect assay (**Figure 4**). These findings are encouraging from the perspective of being able to simplify automation. The potential impact is reduced engineering costs for integrating biosensors into marine monitoring platforms.

Detection without PCR amplification. We used several electrochemical techniques to detect both DNA and RNA without using PCR amplification. All of the methods used nucleic acid hybridization to achieve specific detection of the target.

Detection of isolated *E. coli* DNA was demonstrated using microspheres impregnated with an electroactive marker. The results were quite encouraging – detection of DNA isolated from 100 *E. coli* cells was achieved without using PCR amplification (LaGier et al., 2004a). However, quantification was inconsistent due to instability of the electroactive beads. This method holds promise if bead manufacture can be improved.

Detection of RNA without PCR amplification was achieved using guanine oxidation. This method tracks the intrinsic electrical properties of RNA by oxidizing the guanine in the nucleic acid. The resulting electrical signal was proportional to the amount of guanine, and thus to the number of target cells. Specific, quantitative detection of *E. coli* was achieved from crude lysate. Although this technique demonstrated important concepts for the project, sensitivity was lacking (10^7 *E. coli* cells per reaction).

Detection without PCR amplification was also tested using the Alderon Direct Detect method. In this technique, hybridized RNA is detected by tracking the electrical current produced when horseradish peroxidase reduces an electroactive substrate. The technique is specific because the enzyme can only act upon the substrate in the presence of the target nucleic acid. In the absence of a PCR step and without optimizing the reaction conditions, we found that 10^6 *E. coli* cells per reaction could be detected with low levels of background noise (**Figure 4**). This level of sensitivity was achieved from whole cells and without thorough optimization of the reaction conditions. We are currently modifying this technique to improve sensitivity and adapting it to detect *K. brevis* and *E. faecalis*.

Out of the techniques tested that did not use a PCR amplification step, the Alderon method showed the most promise because it offered the least engineering challenges with regard to automation and simultaneous detection of multiple species. It also offered substantially improved reproducibility compared to electroactive microspheres. Based on our findings, we anticipate that this method requires a 100-1000 fold increase in sensitivity to be useful in oceanic applications. We have discussed several ideas with Alderon that might be used to increase sensitivity.

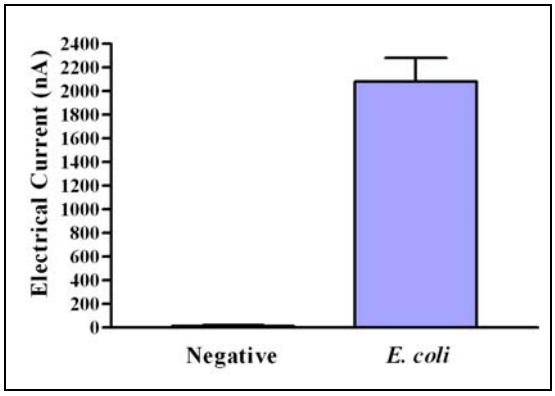

Figure 4. Electrochemical detection of a fecal bacterium (E. coli).

Detection with PCR amplification. We evaluated two electrochemical methods that utilize PCR amplification -- the Alderon Rapid and Hybrid-PCR techniques. Both techniques are sensitive, quantitative, specific, and flexible. For example, we were able to detect 10 *K. brevis* cells per reaction by using a PCR amplification step prior to electrochemical detection (**Figure 5**). As shown in **Figure 5**, the assay has the potential to be at least semi-quantitative since the electrochemical signal is directly related to the concentration of cells amplified by PCR. We also observed that sensitive detection of targets can be achieved from environmental samples. For example, electrochemical detection (Alderon Rapid PCR) of *K. brevis* from a representative water sample is demonstrated in **Figure 3**. A potential limitation of assays using PCR is they are not quantitative due to saturation of the amplification reaction. Our preliminary results indicate that we may address this issue by limiting the PCR cycle number to include only the exponential phase of amplification. This approach is a key component in the work plan of the coming year. Our preliminary efforts have already achieved the discrimination of cell concentrations differing by an order of magnitude or less.

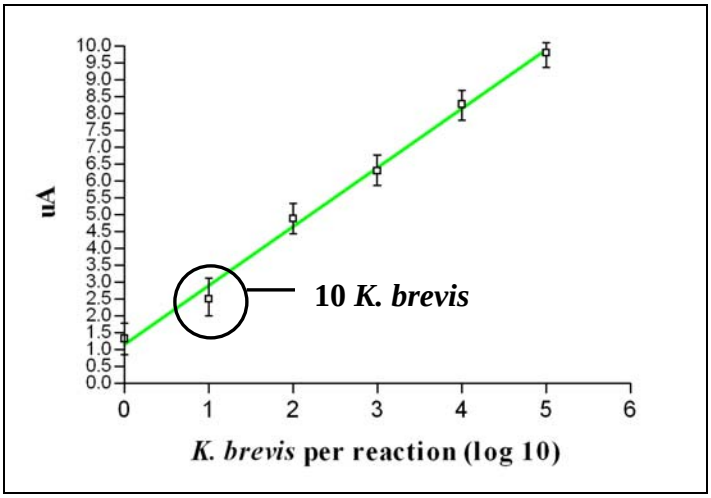

Figure 5. Quantitative, electrochemical detection of a harmful alga (K. brevis).

In summary, our Phase I studies indicate:

- Electrochemical methods have been and continue to be developed with the ability to detect synthetic DNA at ultra-sensitive levels.
- Electrochemistry is a plausible and promising approach for detecting and quantifying nucleic acids from actual cells in real environmental samples.
- Electrochemical detection of biologics does not require the isolation of nucleic acids, and thus automation should be able to avoid nucleic acid purification steps.
- Of the electrochemical methods tested, the instruments, reagents and assays developed by Alderon Biosciences showed the most promise for meeting the demands of *in-situ* biosensors.
- If it is desired to avoid upstream PCR amplification, additional time needs to be invested in research to improve the sensitivity of electrochemical methods.
- Sensitive detection of targets can be achieved from environmental samples using electrochemical detection of PCR products. The electrochemical quantification of PCR products may be improved by using a low cycle number PCR approach. This approach may be the fastest route to achieve project goals. In particular, this method offers the robustness and flexibility of design needed to detect actual pathogens, freeing detection schemes from the limitations associated with indicator species.

IMPACT AND APPLICATIONS

National Security

In situ, autonomous detection of biologics can be applied to drinking water, freshwater, and coastal environments. This technology could be used to provide early warning of harmful or deadly pathogens introduced into these systems.

Economic Development

The developed biosensors and associated assays will have applications in diverse markets including marine biology, clinical microbiology, environmental microbiology, homeland security, and food safety. The implementation of remote biosensors in coastal waters will result in economic benefits by

providing local governments and the seafood industry the real-time data they need to make rationale decisions about the management of coastal and aquaculture resources.

Quality of Life

Coastal areas in the U.S. comprise less than one-fifth of the contiguous United States land area but account for over one-half of the nation's population and housing supply; therefore, coastal zone water quality is critical to quality of life. In addition to direct effects on human health, quality of life is lessened by the nuisances associated with deteriorating water quality. *In situ* biosensors can provide early detection of harmful algae and sewage contamination so that human health can be protected. In addition, *in situ* sensors identify biologics in conjunction with measurements of physical variables and this can lead to a better understanding of the processes that impact aquatic ecosystems, including the initiation of harmful algal blooms and the spread of microbial pollutants. An increased understanding of aquatic ecosystems will contribute to improved ecological forecasting and resource management strategies. The rapid identification of human pathogens and harmful algae by autonomous biosensors can provide public health benefits by reducing the incidence of diseases derived from consumption of contaminated seafood or from swimming in polluted waters.

Science Education and Communication

Biosensor concepts, designs, and data will be available on the Internet for use in educational activities. This will be achieved by leveraging resources through the PIs involvement in Ocean and Human Health Centers. The concept of automated, robotic devices as vehicles of scientific discovery, e.g. the Mars Rovers (http://marsrovers.jpl.nasa.gov/home), captures the imagination. The autonomous biosensor and its associated data will be a unique and intriguing way to engage students in biotechnology, oceanography, and microbiology, and molecular biology.

TRANSITIONS

Economic Development

Progress made during the first year of the project has had a positive impact on the biotechnology industry by the development of novel applications for instrument platforms commercialized by Alderon Biosciences (http://www.alderonbiosciences.com). A commercial spin-off of the remote biosensor research will be the creation of portable instruments and assays for on-site detection of aquatic microorganisms.

RELATED PROJECTS

We have demonstrated previous success using the principles of molecular biology and biotechnology to develop novel methods for the improved detection of harmful algae (Goodwin et al., 2005) and fecal pollution indicator bacteria (http://www.ciceet.unh.edu/bulletins/fell.html) from marine sources. The knowledge and materials gained from these related projects allowed for rapid progress to be made on the NOPP remote detection project. In addition, this project shares field efforts, DNA, and molecular biological information (e.g., success and failures of probes and PCR primers) with several projects at the University of Miami's NIEH/NSF Center for Oceans and Human Health (http://www.rsmas.miami.edu/groups/ohh/) and with a project at the NOAA Center for Oceans and Human Health at the Hollings Marine Laboratory (http://www.nccos.noaa.gov/about/hml.html; http://www.nccos.noaa.gov/news/jan05.html).

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