MINIATURIZED DNA BIOSENSOR SYSTEM FOR DETECTING *CRYPTOSPORIDIUM* IN WATER SAMPLES

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ABSTRACT

This project led to the development of novel biosensing protocols for detecting *Cryptosporidium* in water samples. The effort focused on new miniaturized biosensors, that convert the DNA recognition (hybridization) process into electrical or frequency signals. Special attention has been given to the introduction of new nucleic-acid recognition elements that enhance the specificity and sensitivity of the assay and to new strategies for transducing the hybridization event into useful analytical signals. Current efforts in this laboratory, aimed at integrating flow-through hybridization biosensors with on-chip sample handling and amplification, should facilitate the on-site testing of *Cryptosporidium* (and other waterborne pathogens) in water samples.

Keywords: biosensors, *Cryptosporidium,* environmental monitoring

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JUSTIFICATION FOR THE WORK

This project addresses the urgent needs for improved analytical methods for detecting *Cryptosporidium* in water samples. The microbial pathogen *Cryptosporidium* has been recognized as a serious public health threat. Infections with this protozoan can lead to acute gastroenteritis and diarrhea that can become life threatening in individuals with weakened immune systems. *Cryptsporidium* can spread in humans through direct contact, or through waterborne outbreaks. Due to this major health threat, there are growing demands for a routine test that reliably and rapidly detects the presence of *Cryptsporidium* in water supplies. On-site analysis, in a field setting, would be preferred as it provides the option of rapid warning. Yet, despite these major concerns and increasing efforts by the U.S. Environmental Protection Agency (EPA), no analytical method satisfactorily detects the *Cryptsporidium* in water samples.

The primary goal of this project is to develop and test a cost-effective, user-friendly, portable analytical system, based on new DNA biosensor technology, for rapid on-site detection of *Cryptsporidium* in water samples. The resulting easy-to-use hand-held PCR/sensor system will address various deficiencies of current tests for *Cryptosporidium*, and should accelerate the realization of wide-scale screening for this deadly pathogen in water samples.

METHODS USED

Advanced biosensor technology has been used to achieve this project's goal. Biosensors, which intimately couple specific biological recognition elements with physical transducers, show great promise for numerous on-site environmental monitoring applications. The new DNA hybridization sensing technology relies on the conversion of specific DNA base-pair recognition events into useful electrical signals. Oligonucleotide probes, specific to *Cryptosporidium*, have been immobilized onto various transducers (electrodes, piezocrystals). Their interfacial hybridization reaction with the complementary *Cryptosporidium* target sequence has been monitored via a proper electrochemical or frequency signal (with or without an indicator). Hence, the sensing protocol consists of the probe immobilization, the hybridization event, and detection of the duplex formation. Because of the miniaturized character and low-power requirements of modern biosensors, such devices hold great promise for the proposed detection of waterborne pathogens in a field setting.

RESULTS AND THEIR SIGNIFICANCE

Our work has focused on the development of new strategies for the biosensing of DNA sequences specific to *Cryptosporidium parvum* based *on* converting the hybridization event into electrical or frequency signals. As desired for a field-deployable miniaturized biosensor system, the new protocols greatly simplify the detection of *Cryptosporidium* DNA sequences while enhancing the sensor sensitivity and specificity. Attention has thus been given to both DNA recognition and signal transduction processes. This brief WRRI-sponsored effort has already resulted in seven papers in major international journals (see References section).

Particularly attractive for the proposed miniaturized biosensor analyzer is our new indicator-free hybridization biosensor. Such novel protocol eliminates the common use of external redox indicators, and the additional indicator association step. The new indicator-free operation relies on the intrinsic DNA signal, associated with the electroactivity of the guanine bases. It involves the immobilization of a guanine-free (inosine substituted) probe onto the carbon surface, and direct electrochemical detection of the duplex formation via the appearance of the guanine peak of the *Cryptosporidium* target. The new indicator-free approach not only eliminates the need for external indicators, but offers a more reliable detection of the duplex formation, as it relies on the appearance of a new (guanine) peak – without a background signal – rather than on an increased indicator response.

Another novel biosensing strategy developed during the project is the use of new probes based on dendritic nucleic acids. DNA dendrimers are highly branched structures, possessing multiple single-stranded arms, capable of hybridizing with a complementary nucleic acid sequence. We collaborated with Polyprobe Inc. and designed 4-layer dendrimer structures, possessing about 30 single-stranded arms, specific to *Cryptosporidium*. The immobilization of these tree-like polyprobe structures onto the transducer surface greatly increases the hybridization capacity, and thus led to substantially higher sensitivity and extended linear range of the resulting *Cryptosporidium* biosensor. Such attention to the hybridization capacity and to the assay sensitivity is crucial for monitoring *Cryptosporidium* in water

samples. The resulting devices still lack the sensitivity needed for routine *Cryptosporidium* testing.

The reusability of DNA hybridization biosensors requires 'removal' of the bound *Cryptosporidium* target in connection with time-consuming and partially effective thermal and chemical renewal steps. Our recent effort introduced a simpler and faster regeneration procedure in connection with a mechanical polishing of the probe-modified composite electrode. For this purpose, the *Cryptosporidium* probe was uniformly dispersed within the interior of a carbon composite matrix that served as a 'reservoir' for the hybridization activity. The new mechanical polishing scheme rapidly erased memory effects and allowed numerous hybridization/ measurement cycles.

We also coupled the remarkable specificity of the DNA mimic peptide nucleic acid (PNA) probes with the high sensitivity of mass transducers. The thiol-derivatized probe was immobilized by self assembly onto the gold surface. The new hybridization biosensor offered unusual distinction between closely related oligomers and was not affected by a large excess of a single-base mismatch sequence. It also eliminates non-specific adsorption effects through the judicious use a densely packed PNA layer and of hydrophilic ethylene glycol linker.

We also demonstrated the utility of conducting polymer modified electrodes for sensitive, fast, low-potential, universal amperometric detection of nucleic acids. The unique response of these polypyrrole-coated electrodes is attributed to the adsorption/desorption of the nucleic acid during the passage of the sample plug over the modified flow detector. The new scheme offers great promise for detection following microscale separations and in connection to on-chip PCR amplification. Such coupling and miniaturization currently is being examined in this laboratory in connection to new integrated capillary-electrophoresis/ electrochemical-detector chips.

PRINCIPAL FINDINGS

The research described in this report resulted in several protocols for the electrochemical biosensing of DNA hybridization. Special attention has been given to the introduction of new nucleic-acid recognition elements (dendrimers and PNA) that enhance the specificity and sensitivity of the assay, and to new label-free strategies for transducing the hybridization event into useful analytical signals. These new electrochemical routes offer similar analytical performance characteristics (sensitivity, specificity and reproducibility), and can be adapted for on-site testing of *Cryptosporidium* in water samples. Such suitability is attributed to the inherent miniaturization and low-power requirements of electrochemical devices. The realization of routine on-site environmental applications would require further attention to various challenges, particularly matrix effects and the assay sensitivity. Lower detection limits and/or proper amplification would thus be required to meet the requirements of direct detection of *Cryptosporidium* in water samples. Current efforts in this laboratory are aimed at integrating flow-through electrochemical hybridization biosensors with on-chip sample collection, preparation/handling and amplification (using a 'Lab-on-a-Chip' format). Advanced micromachining technologies are being used for providing the microfluidic network essential for such integration as well as for microfabricating the PCR microchambers. By performing all the steps of the biological assay on a single chip platform, we expect significant advantages in terms of cost, speed, and simplicity.

SUMMARY

DNA biosensors offer many great possibilities for environmental monitoring and control. The new protocols developed in this project greatly enhance the power of DNA hybridization biosensors for *Cryptosporidium*, as they result in greatly enhanced sensitivity and specificity, while simplifying their operation. Such developments would greatly facilitate the field testing and screening of *Cryptosporidium* (and other waterborne pathogens) in water samples. Yet, prior to routine environmental applications of these devices, it would be necessary to complete their integration with the on-chip sample-handling platform that would address the remaining challenges of matrix effects and insufficient sensitivity.

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