DETECTION OF BIOLOGICAL AGENTS VIA A NOVEL BIOPOLYMERIC ASSEMBLY

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ABSTRACT

Nucleic acid-based biosensors can be very selective however it would be desirable to reduce the reagent load, remove unstable fluorescent labels and make the sensing element reusable. This may be achieved with electrochemical methods utilizing an electrode which is controlled by "molecular-scale gates" capable of being activated by a biological agent. In this project, chitosan covers the electrode and creates pathways for redox molecules to flow from the electrolyte to the electrode surface. Nucleic acid probes serve as switches, when unhybridized the pathways are open and will close upon the introduction of a complementary nucleic acid sequence from the analyte.

INTRODUCTION

There is a great need for sensitive sensors for genetic analysis to be used for varied purposes from monitoring gene expression in organisms to speciation of possible pathogens. An instrument capable of these tasks would be a great benefit for food and water safety, medical diagnostics and defense of military and civilian populations from biological threats. Traditional biosensors designed for this purpose are large, require significant volumes of liquid reagents and highly trained personnel. Typically, if any of these requirements are reduced then sensitivity and/or selectivity suffer greatly. Over the past several years researchers have been striving to improve this situation but many developments have been geared to large, array-based equipment to increase sensitivity or throughput in the laboratory setting.

There are many recognition elements, which have been utilized in biosensor design. Nucleic acid (e.g. DNA or RNA) hybridization assays have become popular techniques. Hybridization assays have been developed to interrogate samples for multiple analytes from a single sample.[1, 2] Nucleic acid based biosensors are very selective however have required multiple liquid reagents which must be stored in controlled environments and require fluorescent labels that can be unstable. It would be desirable to reduce the reagent load, remove the fluorescent labels and make the sensing element reusable. This may be achieved with constrained electrochemical methods[3], redox labels[4, 5] or even intrinsic redox character from guanine residues.[6, 7] A novel approach to this includes utilizing an electrode which is controlled by "molecular-scale gates" capable of being activated by a biological agent. In this project, chitosan (a primary amine containing biopolymer which is easily extracted from crab shells) will serve as a matrix to cover the electrode surface and create pathways for redox molecules to flow to the electrode surface. Nucleic acid probes will serve as switches, when unhybridized will leave these pathways open

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and will close upon the introduction of a complementary nucleic acid sequence from the biological analyte. The system will be interrogated by standard electrochemical techniques.

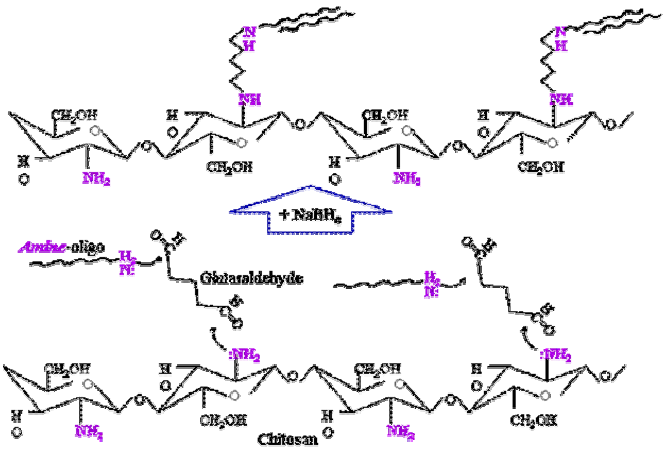

Figure 1. Diagram of chitosan, covalent coupling of modified ssDNA and hybridization.

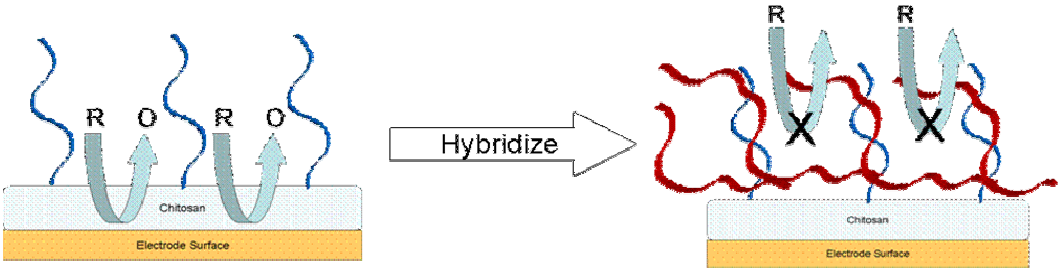

Figure 2. Diagram of the operation of the chitosan based "molecular-scale gates". Before hybridization electrochemical signal is generated. After hybridization transport of the redox species and thus the signal generation is blocked at a given electrode potential.

Electrochemical deposition of chitosan was performed on gold electrodes as recently described.[8, 9] The ability for the chitosan layer to block the oxidation of a redox molecule (potassium ferricyanide) was characterized. The blocking ability will also be affected by the cross-linking of the chitosan through glutaraldehyde treatment. This step needs to be understood as the immobilization of the nucleic acid capture strands to the chitosan also requires a linker like glutaraldehyde.

Once the blocking ability of the chitosan layer is understood it is necessary to control the flow of redox species to the electrode surface through nucleic acid hybridization. This requires a relatively short (20-30 DNA bases long) recognition sequence to be bound to the chitosan. Immobilization and hybridization has recently been demonstrated in separate experiments for fluorescently based systems[10] but needs to be verified that the nucleic acid sequences are viable and hybridization causes efficient blocking.

After hybridization the signal must change dramatically. In the first generation of the device this will be accomplished by using a sequence of complementary, synthetic oligonucleotide. In actual samples encountered following a cell rupture technique the genetic sequence would be quite long (on the order of thousands of DNA base pairs). Therefore when hybridization with the complementary sequence immobilized on the chitosan occurs the large analyte will block a large percentage of the pathways leading to the electrode surface. This will affect the ability for charged species from the solution to reach

the electrode and dramatically change the impedance (increase) and the voltammetric signal (decrease the peak height and shift the redox potential positive). This should be a linear effect depending on the quantity of oligonucleotide captured at the chitosan interface.

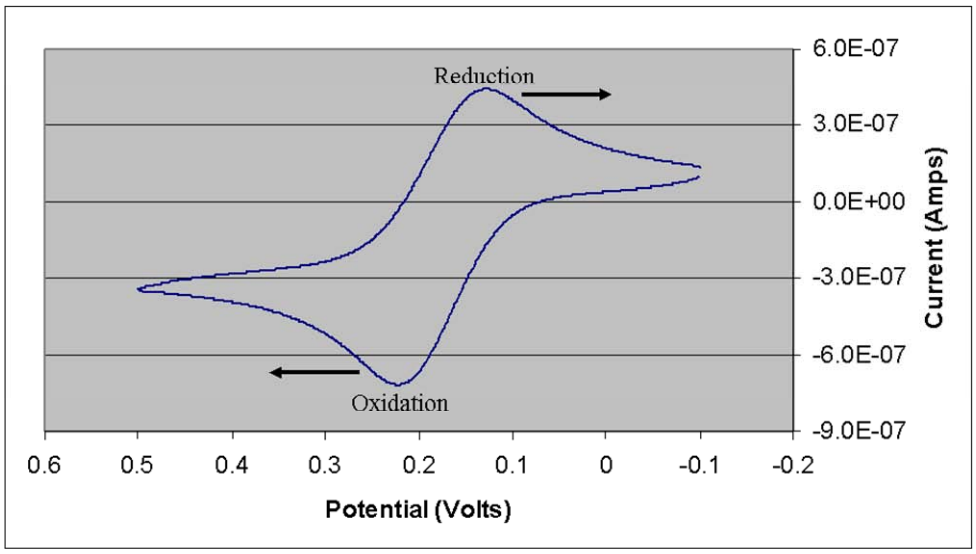

Figure 3. Schematic of the effect of closing the "molecular-scale gates". Oxidation peak shifts more positive, reduction peak shifts more negative. It takes more energy to move redox molecule to the surface of the electrode.

This technique, if successful, will lay the ground work to build a low cost, small size and low power biosensor platform. The reagents are very inexpensive and stable. The electronics to operate a sensor of this nature could be very low power and cost and it has the possibility of being comparable to fluorescent techniques. This paper will discuss chitosan coating conditions, cross-linking with glutaraldehyde, immobilization of the ssDNA, and hybridization on chitosan-coated electrodes.

EXPERIMENTAL METHODS

Chitosan-coated electrodes were prepared by dissolving appropriate quantities of chitosan flakes in dilute HCl. Films were electrodeposited by placing a 2 V bias between a gold working electrode and a platinum counter electrode while immersed in 0.25% w/v chitosan solution at pH 2.5. Deposition times were varied from 1 minute to 30 minutes. The films were then neutralized by soaking in 1M NaOH. The excess base and salt formed were then removed by soaking in deionized water. Coated electrodes were stored in deionized water.

The primary amine groups on the chitosan film were then modified using glutaraldehyde. The chitosan-coated electrodes were exposed to concentrations of 0.05% - 4.0% v/v glutaraldehyde aqueous solutions. The electrodes were then rinsed in deionized water to remove unreacted glutaraldehyde. Concentrations of 4.0% were used to demonstrate the closing of the pores in the chitosan lattice as an irreversible process. It was found that concentrations of 0.05% were adequate for the immobilization of amine-terminated oligonucleotides for hybridization experiments while having no significant effect on the electrochemistry of the chitosan coated electrodes.

DNA oligonucleotide sequence of the *E. coli* gene *dnaK* (5'-NH2-CTT TCG CGT TGT TTG CAG AA) with its complementary target sequence (5'-TTC TGC AAA CAA CGC GAA AG) was utilized. This 20-mer target sequence is located near the 3' end of the *dnaK* gene and was selected because the specific region had little homology to other *E. coli* genomic sequences.

In order to immobilize oligonucleotides the chitosan-coated and 0.05% glutaraldehyde-treated electrodes were soaked in 2 μg/mL of amine terminated oligonucleotide. This solution was prepared in 6x saturated sodium citrate (6xSSC) buffer and allowed to react overnight at 4 C.

Hybridizations were preformed using 10 nanomolar complement in 6xSSC buffer. The reaction was allowed to take place for 2 hours at 4 C but could be much more rapid at room or elevated temperatures. The electrodes were extensively washed with buffer at room temperature to remove any unbound oligonucleotides.

All electrochemistry was performed on a CH Instruments 660a electrochemical workstation. The electrodes were 2.0 mm gold disk working, platinum wire counter and Ag/AgCl reference. Cyclic voltammograms (CVs) at a sweep rate of 10 mV/sec were taken at every step of electrode modification as well as before and after hybridization. The electrolyte was 6xSSC and 0.1 mM potassium ferricyanide as the redox molecule.

RESULTS AND DISSCUSSION

To demonstrate the concept of the "molecular-scale gates" and its utility as a biosensor requires four main tasks: 1) Electrochemically deposit chitosan films on electrode surfaces, 2) Chemically "closing the gates", 3) Immobilize a capture strand of oligonucleotide to the surface of the chitosan, and 4) Hybridize a target sequence to the capture strand of oligonucleotide. In this section, these tasks and representative data will be discussed.

Chitosan must first be deposited on the electrode surface. This was achieved by using electrochemical deposition. Two electrodes were immersed in a solution (0.5-0.25%) of chitosan at pH 2.5. A bias of 2 V was applied across the electrodes and held for various amounts of time. Figure 4 shows the CVs of gold electrodes before, after 10 minutes and after 20 minutes of deposition. As the potential is swept positive the current starts to flow as the potassium ferricyanide is oxidized. This happens very easily (low potential) at the bare electrode and becomes increasingly more difficult (peak shifts positive) as more chitosan is deposited onto the surface. This is a clear demonstration that the flow of redox molecules to the electrode surface is not stopped, but is dramatically hindered by the chitosan lattice.

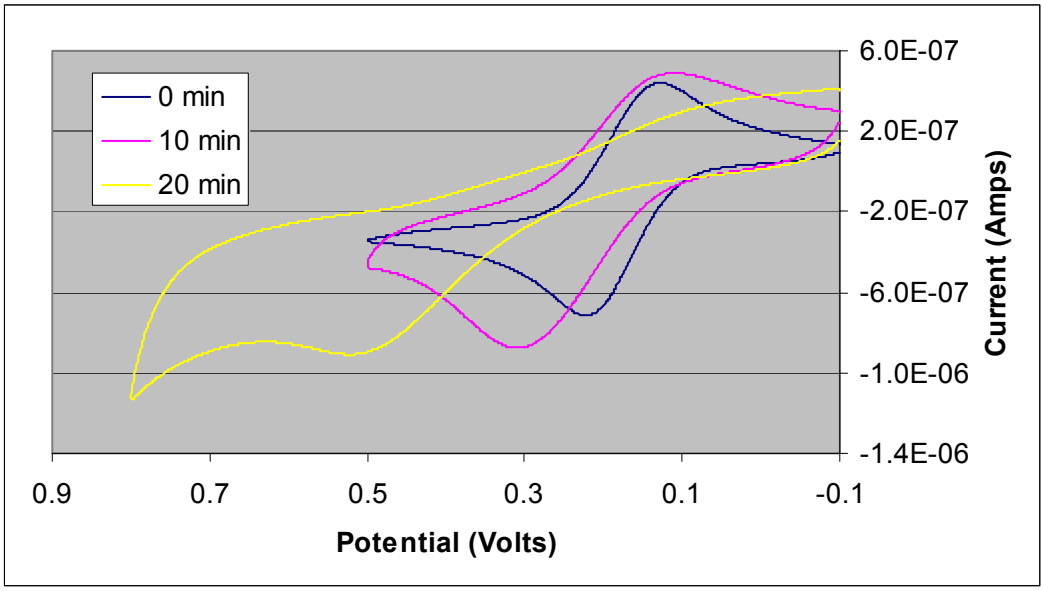

Figure 4. Cyclic voltammograms of potassium ferricyanide (0.1mM) on chitosan (0.5%) coated (2 V) electrodes. (Peak Potentials: 0 Min = 223 mV; 10 Min = 309 mV; 20 Min = 522 mV)

The next step is to determine if it is possible to further influence the transport of the redox molecules in solution by means other than thicker chitosan depositions. Figure 5 shows this is possible by chemically constricting the pores in the chitosan lattice. Glutaraldehyde, a dialdehyde, was selected to react with the primary amines in chitosan to form covalent, imine bonds. When added in high concentrations (single percents by volume) the glutaraldehyde can react at two different primary amines on the chitosan surface. This further restricts redox molecule flow to the electrode surface confirmed by hundreds of millivolt shifts in the oxidation peak. This demonstrates that it is possible to chemically influence the size of the pathways in the chitosan and therefore the electrochemistry meaning a sensor should be possible.

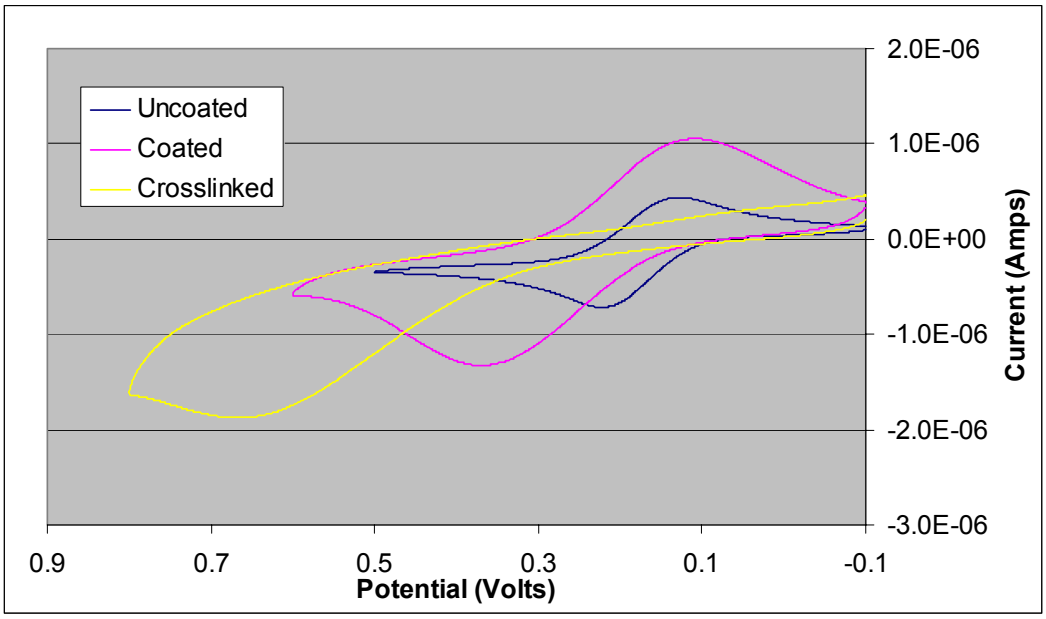

Figure 5. Cyclic voltammograms of potassium ferricyanide (0.1mM) and effect of 4% glutaraldehyde. (Peak Potentials: Uncoated = 223 mV; Coated = 366 mV; Cross-linked = 667 mV)

In order to make this a biosensor, a recongnition element, in this case a capture strand of oligonucleotide, needs to be placed on the chitosan surface without hindering the electrochemistry to the extent further change would not be apparent after hybridization. This was accomplished using the glutaraldehyde just as before however in much lower concentrations. Figure 6 shows CVs of potassium ferricyanide before and after functionalization of the chitosan with 0.1% glutaraldehyde. Note that the oxidation peak actually shifted negative after the reaction with the chitosan. This indicates that the use of low concentrations of chitosan should not have a deleterious effect on the electrochemistry and it was determined that 0.05% glutaraldehyde is sufficient for oligonucleotide immobilization.

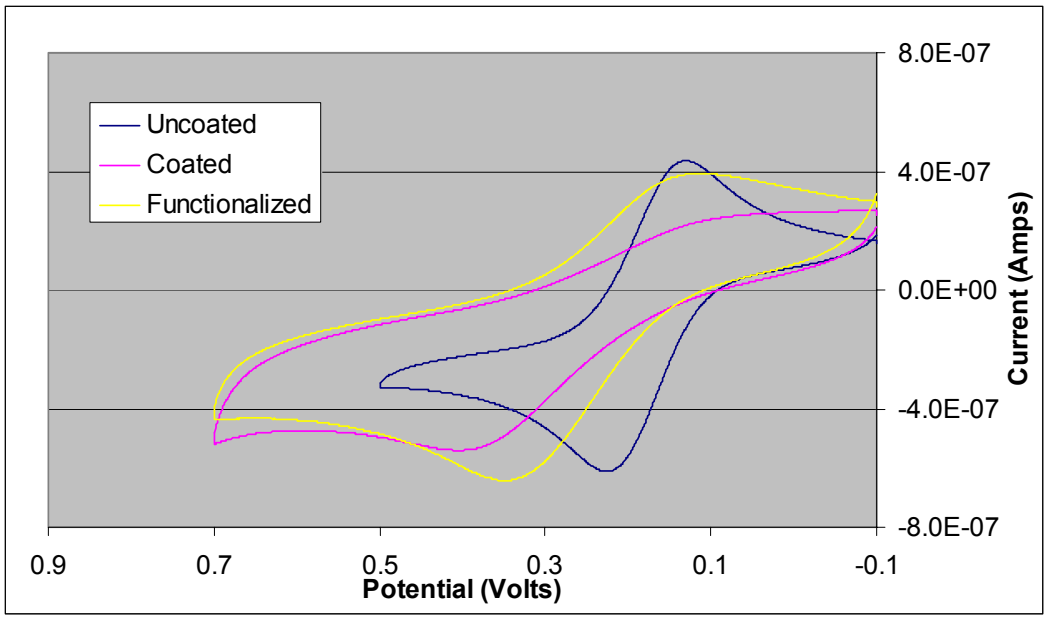

Figure 6. Cyclic voltammograms of potassium ferricyanide (0.1mM) and effect of 0.1% glutaraldehyde. (Peak Potentials: Uncoated = 224 mV; Coated = 402 mV; Functionalized = 352 mV)

The last step was to actually perform oligonucletide-oligonucleotide hybridization on the modified chitosan surface. A target strand which was a matched-length, perfect complement to the capture strand was reacted in the refrigerator for two hours to complete the hybridization. The results of two CVs are shown in Figure 7. The hybridization did not give a dramatic shift on either the oxidation or reduction peak but the shift was reproducible in triplicate. It is probably best described by peak splitting or the difference in voltage between the location of the maximum oxidation and reduction currents.

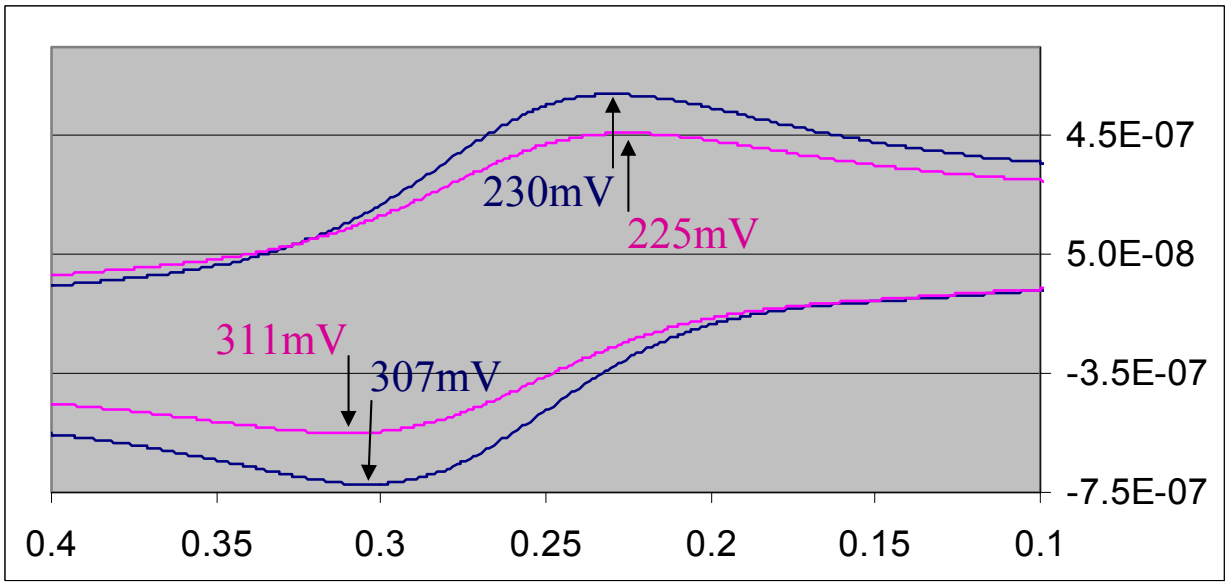

Figure 7. Magnification of cyclic voltammograms of potassium ferricyanide (0.1mM) and effect of hybridization (Blue = Unhybridized, Pink = Hybridized). Note the 9mV increase in peak splitting.

The difference in peak splitting was consistently 9 mV which is not extremely large but is easily measurable on the electrochemical workstation used. If a larger oligonucletide, genomic DNA or fragments were used as the target this splitting should become much more dramatic.

CONCLUSIONS

In conclusion, this work is preliminary but shows promise in the development of low cost, low power biosensors. These results show slight potential shifts but in a real world application will actually show more dramatic signal changes. Electrochemistry holds many advantages over optical techniques including no optical elements to align, ability to operate in turbid media such as blood or waste water, as well as capitalize on the vast electronics processing industry for electrode arrays and control electronics. This is not a polished technique as of yet, but could be used to develop sensors which produce a signal upon recognition and label-free designs through minor modifications.

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