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Detecting DNA-DNA Hybridization at 3-mercaptopropionic acid Self- assembled on Tin-doped Indium Oxide Film with Electrochemical Measurement

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Abstract. Self-assembled monolayers (SAM) of 3-mercaptopropionic acid (MPA) were applied on tin-doped indium oxide (ITO) surfaces and used as a working electrode for sensing DNA hybridization. The concentration of probe single stranded DNA (ssDNA), complemented with target DNA, was optimized for the highest yield immobilization on MPA/ITO platform. The ssDNA/MPA/ITO was allowed to hybridize to target DNA prepared from PCR amplification that first tested by the synthesized complementary sequences. Both cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were employed for investigating probe ssDNA immobilization and target DNA hybridization. For fast and low concentration detecting purposes, methylene blue (MB) coupled with differential pulse voltammetry (DPV) was used for detecting the target DNA hybridization events.

1. Introduction

Electrochemical biosensors which rely on the conversion of the oxidation and/or reduction of electroactive species into a useful electrical signal are advancing rapidly, and recently are receiving greater attention as a valuable tool for medical diagnostic, especially microbial pathogen detection. Generally electrochemical measurement systems comprise three electrodes; reference, counter and working electrodes. Electrochemical reactions between recognition and target molecules take place on the working electrode which consequently transduces the concentration into an electrical signal for further analysis.

Tin-doped indium oxide (ITO) film has been used as the working electrode in many electrochemical biosensor systems for detecting coenzymes, antigens and deoxyribonucleic acid (DNA) due to its high electrical conductivity, wide electrochemical working window, excellent substrate adhesion, stable electrochemical and good optical properties [1]. ITO may be used for direct attachment sensing methods in which the probing molecule binds directly onto the ITO surface. This simplicity and high yield attachment has the drawback of easily releasing immobilized

molecules and difficulty in controlling their orientation. ITO may also be used for indirect attachment, where additional specific molecules or linker molecules are inserted between ITO surface and the probing molecule providing better adhesion through a covalent bonding.

Electrochemical signals in DNA sensing devices are usually obtained by measuring the current-voltage on a reversible redox couple such as ferricyanide, ruthenium complex and methylene blue (MB) which have a low redox potential [2]. The electrochemical signal is modulated by the DNA hybridization process leading to a sensitive method to quantify a specific target DNA. There have been previous attempts to use this approach. Indeed, Kelly and Barton [3] investigated the MB intercalation into double stranded DNA (dsDNA) to quantify hybridization and found an improvement of the cyclic voltammetry response of a DNA-immobilized electrode. The intercalating of multiple MB molecules per DNA copy helps improving the signal to noise ratio (S/N), and thus allows DNA detection sensitivities down to 10^{-10} mole using CV techniques [4] and 10^{-15} mole for DPV techniques [5].

In this work, thin ITO film was first modified with MPA. A ssDNA strand 23 oligonucleotides long was immobilized onto MPA/ITO electrodes to obtain a platform for detecting hybridization with the complementary DNA strand. The scanning electrochemical microscopy (SECM) was utilized for investigating the self assembled MPA on ITO. The MB redox couple was used to probe the presence of the extent and the characteristics of the DNA surface modification as well as to quantify the hybridization with the target DNA. Differential pulse voltammetry (DPV) was utilized to quantify and optimize probe ssDNA density for the highest hybridization yield. SECM was utilized for 3-MPA self-assembled on indium oxide doped tin (ITO) surface representation. The results showed that the self-assembled layer is an effective ssDNA immobilization platform. This detection platform was able to improve the detection time and increased the sensitivity for DNA-DNA hybridization by 2 orders of magnitude.

2. Experimental

2.1 Preparation of Patterned ITO-coated Glass

Glass substrates coated on one side with 140 nm thick tin-doped indium oxide (ITO) with resistivity of 10^{-4} Ω·cm were obtained from Luminescence Technology Corp. The ITO surfaces were cleaned in an ultrasonic bath, once with detergent, twice with de-ionized water, acetone and isopropanol, respectively. The cleaned surface was patterned by concentrated hydrochloric chemical etching to have a circular active area of 0.2 cm^2 . Before etching process, cleaned ITO was covered by negative photoresist film and then it was exposed UV-visible light through a mask. The exposed photoresist film was removed by sodium bicarbonate rinsed. The patterned ITO substrates were dried with a nitrogen stream and stored in a dry chamber for subsequent use.

2.2 Self Assembled of MPA on ITO

A self-assembled layer of MPA was deposited on the surface of the patterned area of the ITO-coated glass surface following previously published work [8]. Briefly, ITO was soaked in 5mM MPA diluted in analytical grade ethanol. The carboxylic end attaches to ITO via hydroxyl groups exposing the thiol on the other end which may be used for covalent bonding of thiolated probe ssDNA [9]. The MPA-coated ITO/glass or MPA/ITO glass can then be used for immobilizing probe ssDNA.

2.3 Immobilization of Probe ssDNA on MPA/ITO

The MPA/ITO was first reduced any possible disulfide bonds by 0.01 M $NaBH_4$ and rinsed copious amounts of water. Immediately after rinsing, the thiolated probe ssDNA was attached. A 50 µl drop of thiolated probe ssDNA at a concentration of 10^4 to 10^{10} copies/µl nM was then applied on the patterned area of the MPA/ITO glass and incubated 30 minutes at 37^{o}C in a humidity chamber. The unbound DNA was rinsed off by a mixture of 0.1% SDS and 2XSSC at 37^{o}C. In the remaining of this work, we refer to these patterned electrodes carrying immobilized ssDNA as ssDNA-MPA/ITO glass for short. To investigate the effect of ssDNA to MB, the negative control was prepared, 2XSSC on MPA/ITO, which incubated in the same environment of the ssDNA/MPA/ITOs.

2.4 Hybridization of Target DNA

The complementary target DNA was hybridized on active surface of ssDNA-MPA/ITO glass by incubating the modified ITO surfaces in 2XSSC at 42^{o}C for 30 min by using various synthesized target concentrations ranging from 10^6, 10^4, and 10^2 copies/μl and PCR product target ranging from 10^3, 10^2, and 10^1 copies/μl. The non-specific adsorption of target DNA was washed out by a sequence of three rinsing steps i) 2XSSC at 37^{o}C, ii)1XSSC and iii)0.5XSSC at 42^{o}C. These electrodes carrying target DNA hybridized on the patterned substrates are referred to by dsDNA/MPA/ITO glass in the remaining of this work.

2.5 Electrochemical Measurements

The scanning electrochemical microscopy experiments were carried out on a Sensolytic SECM with platinum tip diameter 25μm. Bulk electrochemical measurements were done with a PGSTAT12 galvanostat/potentiostat in a one compartment cell using a three electrode configuration. Both bare ITO as well as modified ITO glass plates were used as working electrodes while the reference and counter electrodes were Ag/AgCl and a platinum rod, respectively.

The supporting electrolyte for the two redox species, methylene blue (MB) and ferri/ferrocyanide, contained Tris-HCl:NaCl (10:1 v/v) pH 7.0. For all the electrochemical measurements with ferri/ferrocyanide, including the SECM experiments the ferri/ferrocyanide couple concentration was at 20 mM. The potential window for $[Fe(CN)_6]^{4-/3-}$ was -0.20 V to 0.65 V versus reference electrode and -0.40 V to 0.10 V for MB. Scan rates of a CV was varied from 20 to 100 mV/s. All CV measurements were repeated for 3 cycles. The SECM was operated at 0.00 V against pseudo Ag/AgCl reference electrode in 0.1 M KNO_3 supporting electrolyte.

Differential pulse voltammetry (DPV) technique was used to follow the concentration of MB on surface of various working electrodes; 3-mercaptopropionic acid self assembled on ITO (MPA/ITO), single stranded DNA immobilized on MPA/ITO (ssDNA-MPA/ITO) and hybridized DNA with probe ssDNA on MPA/ITO (dsDNA-MPA/ITO). The 100 μM MB was diluted in supporting electrolyte. All electrodes were immersed in the MB solution then measured reduction current of MB via DPV every minute for ten times. The potential window was -0.40V to 0.00V versus Ag/AgCl reference electrode.

The single stranded DNA probe and its complementary target DNA, a 23-base long oligonucleotide were sequences corresponding to the WSSV virus of the peneaid shrimp, and were produced at the Center of Excellence for Shrimp Molecular Biology and Biotechnology (CENTEX), Mahidol University. The probe had the sequence: 5'- ATG AGA ATG AAC TCC AAC TTT AA -3' and it was modified at the 5'-end with a thiol group via a C6 linker. The complementary target had the sequence: 5'- TTA AAG TTG GAG TTC ATT CTC AT -3'. The disulfide of thiol modifier was reduced by 1,4-Dithio-DL-theritol (DTT) and evaluated its concentration via absorbance at 260 nm before immobilization. The synthesized complementary target DNA was used the ssDNA-MPA/ITO probe modified electrodes to test their response at various probe surface concentrations. Then optimized ssDNA surface was selected and used to detect PCR amplified target from a virus infected shrimp sample. Both probe and target DNAs were suspended in a buffer solution of 0.3M sodium chloride, 0.015M sodium citrate tribasic (2XSSC). Sodium Dodecyl Sulfate (SDS) was purchased from BIOBASIC INC, 1,4-Dithio-DL-threitol and 3-mercaptopropionic acid were purchased from Fluka. Methylene blue, potassium ferricyanide and sodium citrate tribasic were obtained from Sigma-Aldrich, sodium chloride was purchased from Lab Scan and sodium borohydride was purchased from Laboratory Chemistry Inc.

(a) (b)

Figure 1 Experiment apparatus for Cyclic and Differential Pulse Voltammetry (a) and Scanning Electrochemical Microscopy (b) in one compartment.

3. Results and Discussion

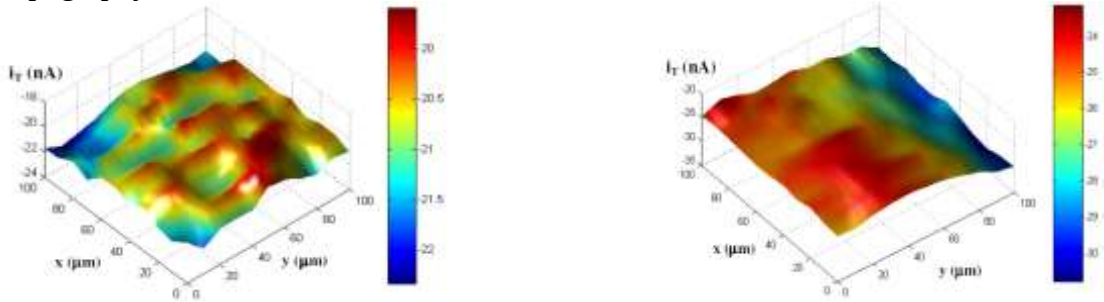
3.1. Characterization of the MPA/ITO Platform

3.1.1 Cyclic Voltammetry

The MPA layer deposited on the ITO surface is less than 1.0 nm in thickness [10]. Therefore the cyclic voltammetry for the $[Fe(CN)_6]^{4-/3-}$ redox couple looks very similar for bare ITO and for MPA coated electrode. Measurements of the capacitive currents yielded capacitance of 2.86 μF/cm^2 for bare ITO and 1.71 μF/cm^2 for MPA modified ITO electrodes. Thus, assuming that the relative dielectric constant for the MPA monolayer is 2.7 we estimate that the average MPA thickness is 0.54 nm. This is mean that the MPA surface coverage is 60% of the ITO electrode area. Coverage of nearly 80% are common for self assembled monolayers of thiols on gold electrodes.

3.1.2 Scanning Electrochemical Microscopy

SECM was used to investigate the formation of the self-assembled monolayer of MPA on the ITO electrode. Both ITO and MPA/ITO electrodes showed positive feedback and the same manner in electrochemical reactivity. With this behavior, the reactivity of bare and modified ITO was not taken into account therefore the images come from their topographies. The Pt micro-tip was controlled to move 10 μm for each approaching step. The x-y plane area was 100 μm×100 μm. The topographies were performed in Fig. 2 (a) and (b), ITO show crystallized structure, and MPA totally changed topography of ITO.



(a) (b)

Figure 2 Topography imagig via SECM of bare ITO (a) and MPA self-assembled on ITO (b); the micro-tip diameter is 25 μm operated in 0.1 M KNO_3 using 20 mM of $[Fe(CN)_6]^{3-/4-}$ as redox specie.

3.1.2 Differential Pulse Voltammetry

Methylene blue (MB) is an organic dye that binds DNA oligonucleotides with great affinity. In this work MB was utilized with two purposes; first, the specific binding with ssDNA and, second, its sensitive electrochemical detection with low reduction potential. In a series of experiments, MB 100 μM was diluted in supporting electrolyte (20 mM Tris-HCl:NaCl (10:1 v/v) pH 7.0) and various modified ITO electrodes were immersed and collected data every one minute. Observing data from voltammograms (data was not shown), MPA cannot block all area of bare ITO.

The ratio of bare and blocked area is 0.38 that differ from barrier factor which obtained from CV. This agrees with our observations of electron transfer via tunneling in SECM experiments. Hence remaining signal of MB on MPA/ITO for further modified surfaces, ssDNA, had to subtract this background and compared the DPV signal to the same ITO substrate.

3.2 Negative and Positive Controls

A series of negative controls for the constructed sensor were carried out in the absence of immobilized ssDNA, replacing it with a solution of sodium citrate (2XSSC), which is used as background of the measurement for comparing electrochemical signal with positive control, where all the components are present (ssDNA-MPA/ITO).

3.2.1Differential Pulse Voltammetry

Strong and specific interactions between MB and ssDNA increase the effective MB surface concentration. In a sequence of experiments with ITO, and MPA/ITO electrodes, the differential current for MB first decreased. Then reduced MB increased again after ssDNA was immobilized on the MPA/ITO. The higher cathodic current in the voltammograms confirmed that a larger amount of MB occurred on the ssDNA modified electrodes relative to the negative control. Besides the higher reduced MB on ssDNA/MPA/ITO via DPV, the adsorption isotherm of adsorbed MB on ssDNA-MPA/ITO surface as shown in Fig. 3 gave maximum adsorbed MB equal to 1.49×10^{14} molecules/cm^2 with Langmuir equilibrium constant (K) was 1.79×10^{-6} cm^2/µl then they would give a ratio 2.67 of MB per one oligonucleotide strand that close to guanine number in 447F1 sequence. This result also confirmed MB-guanine binding of ssDNA in high ionic strength (sodium chloride 20 mM) [11, 12, 13].

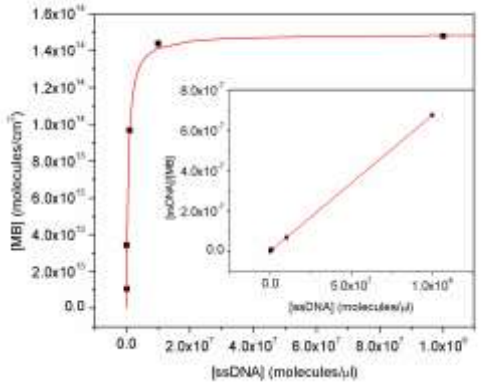


Figure 3 Adsorbed concentration of MB on ssDNA-MPA/ITO surfaces as changed ssDNA concentration in solution.

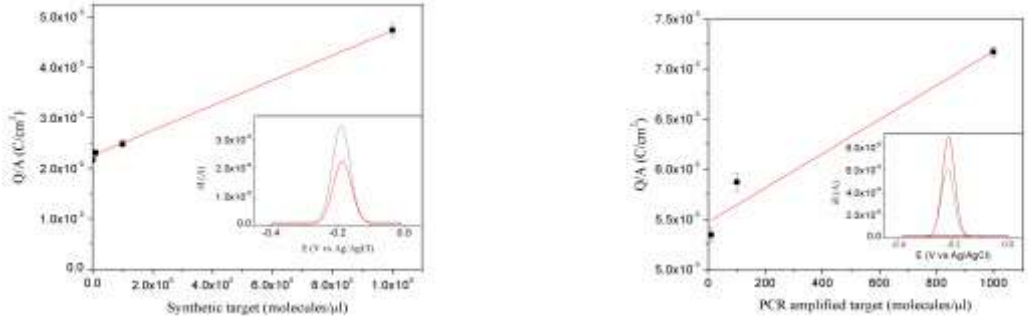
3.3 Optimization of Probe Single Stranded DNA Immobilization

The thin DNA blocking layer could be evidenced via cyclic voltammetry. However, differential pulse voltammetry could distinguish between the negative control (without probe ssDNA) and positive control (with probe ssDNA). When the dye methylene blue (MB) was included in the system, the cathodic current for the redox species can be associated with the ssDNA probe concentration. The optimum concentration for binding of the single stranded DNA probe onto the MPA/ITO electrode was established by quantifying the charge in the cathodic peak for MB of via DPV. However MB has a much stronger interaction with the guanine in ssDNA that it does in dsDNA where the guanine binding sites are hybridized to cytosine. Because the ssDNA probe contains 3 guanines, we expect to find about 3 MB molecules per ssDNA probe. Fig. 4 shows the results of DPV experiments with methylene blue on MPA/ITO electrodes that have been exposed to various concentrations of ssDNA probe molecules. We find the optimum ssDNA concentration when the plot reaches the saturation plateau. The insert in Fig. 3 shows a Langmuir plot from which we obtain the maximum MB number of MB molecules bound to the ssDNA on the electrode. Using the highest concentration in our experiments where the DPV charge nears saturation, we estimate that there are about 2.67 molecules of methylene blue per ssDNA. This number is in reasonable agreement with our expectation based on the guanine content of the ssDNA probes.

3.4 Electrode Response to Synthetic and PCR Amplified Target DNA Hybridization

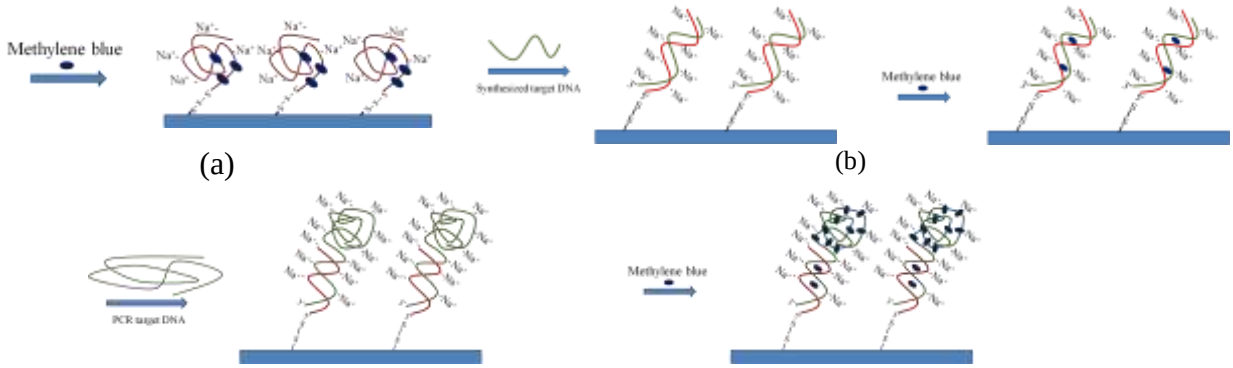
In this work, the hybridization interaction between probe ssDNA and complementary target DNA was investigated through CV and DPV. The utilized targets were synthetic ssDNA and PCR amplified DNA. The synthetic target DNA contained 23 oligonucleotides while PCR amplified contained 1447 dsDNA and mixed with the other dsDNAs. The comparison between negative and positive control was taken both CV and DPV (dtat were not shown). Slightly differences in redox currents, the voltammograms of ssDNA (both CV and DPV), before and after hybridizing step, were similar, and the redox potentials were reliable at the same position due to electrochemical mechanism occur on the same ITO substrate.

After hybridized with synthetic target ssDNA, the salt ion in buffer solution will neutralize negative backbone of dsDNA that cause MB intercalated into duplex as described in Fig. 5 (b). With DPV result, the ssDNA and dsDNA on MPA/ITO surface could be discriminated. Methylene blue intercalation gave lower cathodic current that was shown insert in Fig. 4. Although the voltammograms are clearly separation among those ssDNA/MPA/ITO and dsDNA/MPA/ITO surfaces, the probe ssDNA on MPA/ITO platform give quite high limit of detection for synthetic target that is equal to 1.02×10^5 molecules/μl (Fig. 4 (a), S/N = 3). Whereas the limit of detection for PCR amplified target is equal to 475 molecules/μl (Fig. 4 (b), S/N = 3). The working principle of PCR amplified target was also shown in Fig. 5 (c) which described MB binding guanine at the left end. This kind of binding will promote a cathodic of MB again after hybridization (insert in Fig. 5 (b)). Because of more complicated than synthetic probe ssDNA, PCR amplified target fail to make a Langmuir plot.



(a) (b)

Figure 4 Charge density on dsDNA/MPA/ITO as various concentration of synthetic target and inserted **v**oltammograms of probe ssDNA before (black line) and after hybridized with (a) synthetic target DNA (red line) and (b) with PCR amplified target DNA in Tris-HCl:NaCl (10:1) pH 7.0 by using 100 μM MB as redox specie.



(a) (b)

(c)

Figure 5 Working principle diagram of electrochemical DNA sensor on MPA/ITO platform; (a) specific binding of MB to guanine base of probe ssDNA, (b) intercalating of MB into duplex structure of dsDNA and (c) MB specific binding to guanine of left end free and intercalating in the duplex.

4. Conclusions

This work have investigated electrochemical responses of MPA-attached ITO thin film in electrochemical measurement; cyclic voltammetry (CV), scanning electrochemical microscopy (SECM) and differential pulse voltammetry (DPV) for estimating surface coverage and verifying attachment purposes. Then subsequent ssDNA was immobilized on such electrode and was optimized its density on modified electrode. Its complementary stranded DNA was applied to hybridize on this recognized electrode and study their hybridization interactions via completely hybridized and left unhybridized end such PCR amplified of target DNA. MPA sharply reduces an amount of MB on the surface compared to the bare ITO surface. The immobilization of ssDNA and its following interaction to form dsDNA become different MB concentration. Differential pulse voltammetry measurements also show clear separation before and after probe ssDNA immobilization and also before and after complementary target DNA hybridization. The results confirmed that MB interacts with ssDNA via guanine binding while with dsDNA via intercalating. The optimized probe ssDNA on MPA/ITO was successfully applied to sense both synthetic and PCR amplified target DNA at the limit of detection of synthetic target is equal to 0.169 pM and of PCR amplified target is equal to 0.789 fM.

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