Effectiveness of hairpin probe in increasing the limit of detection for gold nanowire based-biosensor

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Received 15 October 2014
Accepted for publication 27 October 2014
Published 27 November 2014

Abstract
Electrochemical DNA (E-DNA) sensors, which are rapid, reagentless and readily integrated into microelectronics and microfluidics, appear a promising alternative to optical methods for the detection of specific nucleic acid sequences. In keeping with this, a large number of distinct E-DNA architectures have been reported to date. Most, however, suffer from one or more drawbacks, including low signal gain, signal-off behavior or instability. To remedy these problems, we report here the development of a signal-on E-DNA architecture that achieves both high signal gain and good stability. This new sensor employs a commercially synthesized, asymmetric hairpin DNA as its recognition and signaling probe, the shorter arm of which is labeled with a redox reporting methylene blue at its free end. Unlike all prior E-DNA architectures, in which the recognition probe is attached via a terminal functional group to its underlying electrode, the probe employed here is affixed using a thiol group located internally, in the turn region of the hairpin. Hybridization of a target DNA to the longer arm of the hairpin displaces the shorter arm, allowing the reporter to approach the electrode surface and transfer electrons. The observed signal gain is sufficient to achieve a demonstrated detection limit of 25 pM.

Keywords: E-DNA sensor, hairpin probe, biosensor, interleukin-8 mRNA, oral cancer

Mathematics Subject Classification: 6.09

1. Introduction

Early diagnosis of cancer is crucial for the successful treatment of the disease. Highly sensitive methods are urgently needed for measuring cancer diagnosis markers present at ultra-low levels during early stages of the disease. Such methods should facilitate early detection and an adequate selection of the treatment of diseases and should lead to increased patient survival rates. Existing diagnostic tests (e.g., ELISA) are not sensitive enough and detect proteins at levels corresponding to advanced stages of the disease. Smaller, faster, and cheaper (one-step) devices are highly desired for replacing time-consuming laboratory analyses. Making analytical results available at the patient’s bedside within a few minutes will greatly improve the monitoring of cancer progress and patient therapy.

Advances in molecular biology have led to greater understanding of potential biomarkers that can be used for cancer diagnosis. The realization of point-of-care cancer diagnostics thus requires proper attention to the major challenge of multi-target detection. Arrays of biosensors, detecting protein signature patterns or multiple DNA mutations, can be used to help screening and guide treatment. Innovative biosensor strategies would allow cancer testing to be performed more rapidly, inexpensively, and reliably in a decentralized setting.

Over the past three decades we have witnessed a tremendous amount of activity in the area of biosensors.
Biosensors are small devices employing biochemical molecular recognition properties as the basis for a selective analysis. The major processes involved in any biosensor system are analyte recognition, signal transduction, and readout. Due to their specificity, speed, portability, and low cost, biosensors offer exciting opportunities for numerous decentralized clinical applications, ranging from ‘alternative-site’ testing (e.g., physician’s office), emergency-room screening, bedside monitoring, or home self-testing.

Electrochemical devices have traditionally received the major share of attention in biosensor development. Such devices produce a simple, inexpensive and yet accurate and sensitive platform for patient diagnosis. The name electrochemical biosensor is applied to a molecular sensing device which intimately couples a biological recognition element to an electrode transducer. The purpose of the transducer is to convert the biological recognition event into a useful electrical signal. Electrochemical DNA (E-DNA) biosensors operate by applying a constant potential and monitoring the current associated with the reduction or oxidation of an electroactive species involved in the recognition process. An E-DNA may be more attractive because of its high sensitivity and wide linear range. Elegant research on new sensing concepts, coupled with numerous technological innovations, has opened the door to widespread clinical applications. The high sensitivity, specificity, simplicity, and inherent miniaturization of modern electrical bioassays permit them to rival the most advanced optical protocols. Such miniaturization allows packing of numerous microscopic electrode transducers onto a small footprint of a biochip device, and hence the design of high-density arrays.

Sensors capable of detecting specific DNA and RNA sequences have attracted significant research interest because of their importance in basic research in genetic diseases, medical diagnosis and treatment [1]. Among the DNA sensing platforms currently available, a specific class of DNA sensors, namely the electrochemical DNA (E-DNA) sensor, has gained substantial popularity in recent years [2].

Although E-DNA sensors have a wide variety of positive attributes, many of the diverse E-DNA probe architectures described to date operate in a signal-off fashion, meaning that the measured current decreases as the concentration of analyte DNA increases. For example, first generation E-DNA sensors employ a stem-loop architecture [3] such that, when a complementary target oligonucleotide is introduced, hybridization causes the stem to open. This moves the redox reporter further from the electrode surface, reducing electron transfer. This signal-off mechanism significantly limits the gain of the sensor: the maximum possible signal change (the change in current between a blank measurement and saturating target concentrations) can never exceed 100% (i.e., the current cannot decrease below zero). A second limitation of signal-off sensors is that probe degradation can be misinterpreted as an authentic response to target. Signal-on sensors, in contrast, can achieve much improved signaling; as the background current observed in the absence of target is reduced, the gain of such a sensor, at least in theory, increases without limit [4].

Thus motivated, we and others have explored signal-on E-DNA architectures, an inverted stem-loop.

2. Experimental

2.1. Material

A signaling strand (15 base) is modified at its 5’-end with a methylene blue redox reporter (figure 1) and capture strand (34 base), probe DNA were synthesized by Biosearch Technologies (Novato, CA), where they were purified by HPLC and used as-received. The probe molecules employed in our sensors were stored dry at −20 °C until the day of their use. The sequences of these oligomers used are as follows: 5′- MBTTTITGAAGACCCCGA-(CH2)6-S-S-(CH2)6-AGCCCTCTT-CAAAAAATTTTCTCCCAACCCCTCTGC-3′.

Target is produced by RT-PCR (with the primers pairs IF: CCAAGGAAAACTGGGTTGCA and IR: CTTGGA-TACCACAGAGAATGAATTTTT) [5] from mRNA of Interleukin-8 without further purification process (provided by Center for Molecular Biomedicine, The University of Medicine and Pharmacy at Ho Chi Minh City).

Target DNA sequences are: 5′-CCAGGAAACTGGGTGCAAGAGGTTGGAGAA- GAAGTTTTTGAAGGAGGCTGTAGAGATTTTCA- TAAAAATTTTATCTGTGATCCCAG-3′.

The 6-mercaptopentanol (MCH), tris (2-carboxyethyl) phosphine hydrochloride (TCEP), potassium ferricyanide, phosphate buffer saline (PBS) (pH 7.4) solutions were used as-received (Sigma Aldrich, St. Louis, MO). All other reagents were of analytical grade and used without further purification. Deionized and sterilized water (resistance 18 MΩ cm) was used throughout the experiments. The E-DNA sensor was fabricated by using an array of gold (Au) nanowires electrodes (length: 1000 μm, width: 20 μm, height: 60 nm) (figure 2) [6]. Binding curves were obtained by interrogating with square wave voltammetry (SWV) each...
sensor in buffer solution (background signal) and at different target concentrations until signal suppression was stable.

2.2. Sensor preparation

A stock solution of the probe (100 μm) was first reduced in 10 mM TCEP, this solution was left at 4°C for 1 h to reduce the disulfide bonds of the probe DNA (TCEP selectively and completely reduces even the most stable water-soluble alkyl disulfides over a wide pH range). The solution was diluted with a 10 mM PBS (pH 7.4) (as was used in all experiments unless otherwise noted). The freshly cleaned electrodes were immersed into the appropriate concentration of hairpin probe for 30 h for probe immobilization. Following incubation and self-assembled monolayer (SAM) formation, remove any excess probe DNA physically adsorbed on electrode surface via a room temperature-deionized water rinse (30 s). The stream of deionized water should flow over the modified electrode. At this point, the probe DNA will attach to the gold electrode surface via the formation of a thiol-on-gold SAM. The probe DNA concentrations applied in this step range from 100 nM to 1 μM, with 500 nM being a typical value. The modified electrodes were dried using nitrogen and immediately transfer to 2 mM MCH solution in water for 30 min (1 μm). Gently wick off any excess water from the edge of the electrode using a laboratory wipe (no nitrogen needed). Transfer the electrode directly to PBS for storage.

2.3. Target preparation and hybridization

Target solution was diluted to concentrations corresponding to 1 pM, 10 pM, 25 pM, 50 pM, 100 pM, 150 pM, 200 pM, 250 pM and 300 pM in 10 mM PBS pH 7.4. The target was in the form of double-strand DNA, so we proceed to denature the DNA by raising the temperature to 95°C for 5 min to double-strand DNA of IL-8 completely denatured into single strands of DNA, then quickly cooled it down to 0°C in ice. Then, electrodes were incubated in 200 μl of the target DNA sample for 2 h at 2°C before target detection measurements were performed.

2.4. Electrochemical measurements

The reported electrochemical experiments were conducted using SWV and electrochemical impedance spectroscopy on an AUTOLAB PGSTAT12/30/302 (Netherlands), and all experiments were carried out with a conventional three-electrode system in a 15 ml electrochemical cell. Unless otherwise stated, SWV measurements were obtained in the range of -0.2 V to 0.6 V at 60 Hz with an amplitude of 25 mV, and a step width of 4 mV. All of the experiments were conducted with a platinum wire or platinum disk counter electrode and an Ag/AgCl (saturated with 3 M NaCl) electrode served as the reference electrode in 10 mM PBS, pH 7.4, containing 5 mM potassium ferricyanide [K₃Fe(CN)₆] at room temperature. Signal gain was computed by the relative change in SWV peak currents with respect to background current (SWV peak current in the absence of target). In addition to long-term stability, sensor robustness to multiple testing cycles were assessed by subjecting the sensors to a repeated cycle of testing in buffer (no target), testing in saturating target, and regeneration with a 30 s deionized water rinse [8, 9].

The electrochemical impedance spectroscopy (EIS) measurements were performed at a fixed potential of 0.2 V versus Ag/AgCl (the reduction potential of ferricyanide ions) and in the frequency range of 0.1 Hz to 100 kHz. The impedance spectra were recorded in the form of Nyquist plots and they were fitted to an equivalent circuit, shown in figure 3, based on the Randles and Ershler model [10–12].

In the presence of a redox probe, the model circuit consists of the resistance of the electrolyte solution (RS), the Warburg resistance (ZW) resulting from the diffusion of the redox probe, the charge-transfer resistance (RCT) of the electrode/electrolyte interface on the surface of the working electrode, and a non-ideal capacitance represented by the constant phase element (CPE) due to the non-homogeneity of the interface on the working electrode surface [12–14]. CPE and RCT represent parameters that are dependent on the solution–electrode interface, thus the nature of the film covering the gold surface. For non-homogeneous films and rough surfaces the impedance data do not fit the theoretical behaviour predicted by the equivalent circuit made of ideally behaving components [11, 15]. For such cases, the capacitance can be replaced by CPE [12–14] and any changes in
surface coverage are determined by observing changes in the RCT values [12, 16–20]. When molecules with hydrophobic moieties adhere to the gold surface, they form an insulation layer which perturbs the interfacial electron transfer between the electrode and the electroactive species in solution, thereby increasing the charge-transfer resistance. The same effect is observed when charged molecules, such as DNA oligonucleotides, are deposited on the electrode surface. This is attributed to the electrostatic repulsion between the negatively charged DNA layer and the ferricyanide ion, $\text{Fe(CN)}_6^{3-}$.

3. Results and discussion

The signal-on E-DNA sensor is based on a target-induced strand-displacement mechanism (figure 1). The sensor is composed of two parts. The first part is a single-stranded ‘capture probe’ covalently attached by a 5’ thiol to a gold electrode using standard self-assembled monolayer chemistry [21].

As a measure of the surface coverage of this capture probe, the second sensor component is a ‘signaling probe’ with a methylene blue (MB)-redox moiety covalently attached to its 5’ terminus. In the absence of target, the two double-stranded regions formed between the capture and signaling probes sequester the MB from the electrode surface, producing a relatively small MB redox current. When the sensor is challenged with a 34-base complementary target, the observed MB redox current increases significantly. The proposed mechanism underlying this signaling is as follows. Target hybridization displaces the 10 hybridized bases at the 5’ terminus of the signaling probe. This displacement liberates the MB-modified end of the signaling probe, generating a flexible, single-stranded end that allows the MB to collide with the electrode surface and transfer electrons (figure 1, right).

Figure 4(A) shows the electrochemical impedance spectra of gold slides recorded in the form of Nyquist plots, prior to surface modification (Φ bare gold), after surface modification with the mixed monolayer of 500 nM thiolated probe (□) and 2 mM 6-mercaptop-1-hexanol (◊), and following hybridization with 1 nM full match target oligonucleotide (●). Impedance measurements were performed in 5 mM $\text{K}_3\text{Fe(CN)}_6$ and 100 mM KCl solution prepared in 10 mM phosphate buffer, pH 7.4. The spectra were obtained in the frequency range from 100 kHz to 0.1 Hz at a fixed dc potential of 0.2 V. Inset: impedance spectrum of the bare gold slide at a smaller scale. (B) Square wave voltammetry of (a) bare gold, (b) surface incubated in 500 nM thiolated probe oligonucleotide, (c) surface incubated in 2 mM 6-mercaptop-1-hexanol and (d) surface incubated in 1 nM full match target oligonucleotide (hybridized).
advantages of reagentless, reusable operation without compromising sensitivity or selectivity.

The E-DNA sensor also offers significant advantages over optically detected molecular beacons. For example, whereas the most highly optimized optical approaches can detect a femtomolar target in the laboratory, the picomolar sensitivity of the E-DNA sensor is comparable to or significantly better than the fluorescence-based techniques used in the ‘real world’ (i.e., with lower-power light sources and off-the-shelf detectors [25, 26]. Moreover, the sensitivity of the E-DNA sensor exceeds that of typical, charge-coupled device-based fluorescent detectors by at least an order of magnitude [25]. The E-DNA sensor also vastly surpasses solid-state optical molecular beacons, for which an ~1 nM detection limit is reported [27]. However, we note that the current E-DNA sensor, like all other state-of-the-art electrochemical or optical DNA sensors, does not meet the high-end requirements of many real-world gene-targeting applications, whereas picomolar sensitivity is orders of magnitude more sensitive than necessary for the detection of amplified targets (and offers the very real possibility of replacing cumbersome gel- or optical-based detection schemes in this role), picomolar sensitivity is not sufficient for the unamplified detection of most pathogens. By employing electrochemical amplification methods, such as coupling with electrocatalysis [28] or extreme catalysis [23], it may be possible to increase the E-DNA signal sufficiently to enable direct pathogen detection.

Given the extremely low limits of detection that are necessary to identify pathogens in biological samples, these sensors will invariably be used in combination with an amplification technique such as loop-mediated isothermal amplification (LAMP) or polymerase chain reaction (PCR). Doing so will be straightforward, since these sensors function well when employed directly in PCR mix. This is a significant advantage over optical DNA detection systems, such as microarrays, which require lengthy incubation and washing steps following the PCR reaction. Furthermore, the cost of amplification systems can be quite low [29, 30], as is the cost of E-DNA supporting electronics [31], suggesting that such a ‘marriage’ could provide an inexpensive and robust system for pathogen detection.

4. Conclusion

By attaching probes with hairpin structure onto gold nanowires array chip, we have reached the limit of detection at 25 pM. This can be considered as a demonstrated detection performance for a sensor signal-on mechanism. We developed an effective method for electrochemical detection of mRNA using hairpin probe with high sensitivity. These results also indicate that the E-DNA sensor could provide fast and effective measurements for directly detecting salivary mRNA biomarker.

Acknowledgments

This work was supported by Laboratory for Nanotechnology, Vietnam National University in Ho Chi Minh City and Center for Molecular Biomedicine, The University of Medicine and Pharmacy in Ho Chi Minh City.

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