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Nanostructured gold dsDNA sensor for early detection of breast cancer by beta protein 1 (BP₁)

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Abstract

Beta protein 1 (BP₁) is a homeobox protein expressed in 80% of breast cancer cells in either estrogen receptor (ER) positive or ER negative breast cancer. However, it is barely detectable in normal breast tissues. In this project we present an electrochemical DNA nanostructured gold biosensor for detection of BP₁. The gold sensor is first electrochemically nanostructured in 0.5 M sulfuric acid to reach superior conductivity, larger surface area, and higher stability.

Nanostructured gold surface was characterized by atomic force microscopy (AFM) and scanning electron microscopy (SEM). The nanostructured gold sensor is then modified with double-stranded (ds) DNA mapping the genomic sequence that contains the binding site for BP₁. A redox-active probe (methylene blue) was intercalated in dsDNA to monitor the binding event of BP₁. A linear correlation of the electrochemical response by concentration of BP₁ was obtained ($R^2 = 0.998$) with a limit of detection of 1.2 nM. This nanostructured gold dsDNA sensor is shown to be sensitive, selective, stable, and reusable allowing for its potential clinical use.

Graphical Abstract

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Keywords

Gold nanostructured sensor; beta protein 1; biosensor; DNA sensor

1. Introduction

Cancer is one of the major causing deaths in the United States. Statistically, one of four deaths in the United States is caused by cancer. The most common diagnosed cancer in woman is breast cancer. It is also the second leading cause of cancer death among women around the world [1]. During 2014, around 29% of the new cases of cancer in women were diagnosed to be breast cancer [2]. According to the American Cancer Society, over 231,840 new cases of invasive breast cancer are expected to be diagnosed in the United States in 2015. Additionally, 60,290 new cases of carcinoma *in situ* breast cancer, which is the earliest form of breast cancer and it is also non-invasive, will be diagnosed in 2015. Unfortunately, about 40,290 diagnosed women will die of this condition.

According to a statistical study, about 90% of the breast cancer patients diagnosed with carcinoma in stage 0 will survive, emphasizing the importance of early detection [3]. During the past decade researchers have driven their attention to search biomarkers for the early detection of breast cancer. In 2002, Sidney et al. reports a strong correlation between the expression of beta protein 1 (BP₁) and ER in breast cancer cells [4]. BP₁ is a member of the homeobox gene superfamily of transcription factors (TFs), which are expressed during embryonic development and are subsequently re-expressed by several different types of cancers [5]. This protein consists of a 60-amino acid helix-turn-helix structure with three alpha helices connected by short loop regions in which one of these interacts directly with DNA [6]. A remarkable feature of BP₁ is that it is expressed in 100% of ER– tumors and in 73% of ER+ tumors [4–7], with the former being more invasive and with a typical poor prognosis when compared with the latter. Meanwhile, lack of BP₁ expression was found in ER– normal breast cells and low-level expression in ER+ normal breast cells. In addition, frequent expression of BP₁ was also found in all tumor grades. All these properties make BP₁ an ideal biomarker for early detection of these types of breast cancer [4–7]. Recently researchers have driven their attention in the development of biosensors for breast cancer detection using different biomarkers [8–10]. One of the most promising biomarkers for breast cancer detection is the abovementioned BP₁ TF. The detection of breast cancer-

related TFs, such as BP₁, can be done using gel electrophoretic mobility shift assays [11] and DNase footprinting assays [12], which are based on migration retardation of protein-DNA complexes through gel electrophoresis. However, these methods require long and labor-intensive protocols and the use of an analytical laboratory with specialized personnel, making them neither feasible for routine biomarker determination nor applicable for point-of-care (POC) testing. Thus, the challenge on the detection of TFs is the development of sensitive and simple methods which allow quantification of them [13].

Electrochemical assays based on DNA-mediated charge transport at microelectrodes offer an alternative approach which can be sensitive, specific, fast, simple, and low-cost, making them suitable for POC diagnostics and multiplexed platforms [14]. This type of approach has been useful in assays to detect mutations and lesions as well as for the detection of protein binding at self-assembled DNA monolayers. However, its use for the detection of protein binding utilizing double-stranded (ds) DNA monolayers has been scarce and can be divided into electrodes modified with a hairpin oligonucleotide with conjugated redox-active probes [15–21], or with dsDNA that bends upon protein binding [22–24]. For the first type, TF binding stabilizes an oligonucleotide conformation which brings the redox-active probe close to the electrode leading to a large increase in Faradaic current signal. The second type capitalizes on the fact that upon binding some TFs bend the immobilized dsDNA probe restricting the flow of electrons. Both alternatives function as a switch, in which electrons are either transported or not to the electrode.

Most nano-gold (NG) sensors modified with single-stranded DNA are designed to characterize the hybridization between the two complementary strands which allows electrochemical charge transport [14,20,24–27]. A series of conjugated charge donors or acceptors are employed as redox active probes interacting by π -stacked base pairs in dsDNA to monitor the electrochemical binding event [21,24,28–34]. In the case of TFs, the electrodes are modified with dsDNA containing the sequence which allows binding of a specific TF. Thus, this work proposes for the first time (1) the detection of a clinically-relevant transcription factor biomarker, (2) the electrochemical detection of a transcription factor using a double-stranded DNA probe on a nanostructured gold electrode, and (3) a limit of detection of 1.2 nM

2. Experimental section

2.1 Materials

Methylene Blue 82%, 6-mercapto-1-hexanol (MCH) 97%, and tris(hydroxymethyl)-aminomethane 99.9% were obtained from Sigma-Aldrich. The DNA sequences containing the BP₁ binding motif ATATATATG were as follows: Forward 5'-TTAAAAAGATATATATATATGTTTTCTAATGT-3', (modified with 5'end thiol C₆ linker) and reverse 5'-ACATTAGAAAAACATATATATATATCTTTT-TAA-3' [6,7]. The following sequences without the BP₁ binding sequence were used as the dsDNA negative control (Forward: 5'-TCTTAGAGGGAGGGCTGAGGGTTTGAAGTCCAA-CTCCTAAGCC-3'; Reverse: 5'-AGAATCTCCCTCCCGACTCCCAAACCTTCAGGTTG-AGGATTCGG-3') [6]. All DNA probes were obtained from Eurofins Genomics and the

BP₁ protein was obtained from Gen Way and used as received. Deionized water (18.2 MΩ) was used for all experiments.

2.2 Equipment

CP-II VEECO atomic force microscope (AFM) with scanning probe microscopy and lateral force microscopy and nanomanipulation capabilities with 100 × 100 lm scanning area capacity is used to characterize the surface morphology. Scanning electron microscope (SEM) is used to characterize the surface morphology of the nanostructured electrode using a Field Emission Scanning Electron Microscope Supra 35 (Zeiss). The equipment allows analysis of features in the nanometer range using low accelerating voltage (3kV). Electrochemical experiments were carried on an Epsilon Potentiostat/Galvanostat (Bioanalytical Systems, Inc.) using a specially designed three-electrode electrochemical cell having an analytical gold sensor as working electrode (0.02 cm²), a platinum wire as counter (auxiliary) and Ag/AgCl paste covered wire as reference electrode. SRS Quartz crystal microbalance (QCM) Ti/Au, 5MHz electrode used for surface characterization was purchased from Stanford Research Systems.

2.3 Synthesis and characterization of gold nanoparticles

The gold electrode surface, before nanostructured, was polished prior to use with 0.5 μm alumina for 3 minutes and then cleaned in an ultrasonic bath with ethanol and deionized water for 5 minutes. The gold electrode is then electrochemically cleaned by cyclic voltammetry (CV) in 0.5 M sulfuric acid at 100 mVs⁻¹ scan rate, between -0.35 to 1.5 V until no changes in voltamograms. After electrochemical cleaning of the gold electrode, the thick hydrous gold oxide layer accumulated on the gold electrode is removed in 0.5 M sulfuric acid by using repetitive square-wave voltammetry (SWV), with a potential range between -0.8 V and 2.5 V (versus silver/silver chloride electrode) at 2,000 Hz for 5 minutes. In the following step, the potential is held at -0.8 V until complete electro-reduction of the hydrous gold oxide layer is accomplished. The NG surface was characterized by cyclic voltammetry in 0.5 H₂SO₄ between -0.35 to 1.5 V (versus silver/silver chloride electrode) at 100 mVs⁻¹. After synthesis of gold nanoparticles, the gold reduction peak current at 0.942 V increases considerably. SEM was used to characterize the surface morphology of the nanostructured electrode. In addition, atomic force microscopy (AFM) was used to measure the surface topography.

2.4 Immobilization of dsDNA onto gold nanostructured sensor

The BP₁ binding sequence oligonucleotides were resuspended in an annealing buffer consisting of 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA. Equimolar concentrations of the forward and reversed oligonucleotides were annealed at 94 °C for 4 minutes and then cooled at room temperature for 40 minutes. The resulting dsDNA was self-assembled into the NG surface for 18–24 h. After dsDNA addition to the NG surface, it was exposed to a solution of 1.0 mM mercaptohexanol (MCH) for 1 h (Scheme 1) to allow for surface passivation [35]. The co-adsorption of MCH onto DNA-modified gold surface removes the weakly, unspecifically adsorbed nucleic acids by forming a dense MCH sublayer. DNA sensor was then immersed in a solution on 20 μM MB for 20 minutes to intercalate it as redox-active probe [14,24]. Characterization of dsDNA and its binding to the NG surface

were performed using CV to monitor changes in MB redox cycle. The CV experiments were carried out between -0.450 to 0 V at 100 mVs^{-1} in 20 mM Tris, 100 mM NaCl, pH 7.5, at room temperature under an inert atmosphere. A three-compartment electrochemical cell is used with a Pt wire auxiliary electrode, silver/silver chloride electrode as reference, and a 0.02 cm^2 NG working electrode.

2.5 Electrochemical characterization of DNA-binding event of BP₁

The BP₁ binding event was characterized by CV between -0.450 to 0 V (vs. Ag/AgCl) in 20 mM Tris, 100 mM NaCl, $20\text{ }\mu\text{M}$ MB, pH 7.5 at 100 mVs^{-1} , using silver/silver chloride as reference, Pt wire as supporting electrode, NG/dsDNA/MCH as the working electrode and MB as redox-active probe. A stock solution of BP₁ was prepared dissolving 0.01 mg of the protein in buffer. For the binding event characterization, the redox cycle current of MB was monitored with different dilutions of the BP₁ stock solution (5.30 to 33.78 nM). The CVs were recorded after 2 minutes exposure time with the desired concentration. The NG/dsDNA/MCH was then incubated for two weeks and regeneration experiments were performed without loss of signal. Regeneration of the NG/dsDNA/MCH sensor was performed by a simple rinse in deionized water. Square wave voltammetry was also performed after regeneration to confirm the BP₁ binding event. This was carried out in 20 mM Tris, 100 mM NaCl, $20\text{ }\mu\text{M}$ MB, pH 7.5 from -0.6 to 0 V (vs. Ag/AgCl) at 600 Hz with an amplitude of 25 mV and a step size of 1 mV . It should be mentioned that both, CV and SWV can be used to monitor and quantify the BP₁ binding. The experiments were run in triplicates.

3. Results and discussion

3.1 Characterization of gold nanostructures

The gold electrode surface was nanostructured with a modified procedure [36] using SWV in 0.5 M H₂SO₄ at $2,000\text{ Hz}$ for five minutes followed by electro-reduction at a fixed potential of -800 V . Figure 1 shows the cyclic voltammograms in 0.5 M H₂SO₄ at 100 mVs^{-1} of the gold electrode before electroreduction (black) and after electroreduction (red). The cyclic voltammogram for the clean gold electrode shows two oxidation peaks related to the formation of gold oxides at 1.09 and 1.27 V and a reduction peak at 0.95 V [30]. At the end of the electroreduction process, the current of the reduction peak increases to $18\text{ }\mu\text{A}$ over the untreated gold electrode. The increase in reduction current of the gold reduction peak indicates the formation of gold nanostructures which increase both, the surface area and conductivity [30].

The NG surface topography was measured by AFM, and is shown in Figure 2. The gold surface topography before electroreduction (Figure 2A) shows a smooth surface topography with an average roughness of 2.089 nm while the NG electrode surface (Figure 2B) has a rough appearance with an average roughness of 7.202 nm . The increase in average roughness confirms the changes in surface area due to the formation of gold nanostructures. SEM of the clean gold electrode (Figure 2C) shows gold particles with a uniform size, while NG images (Figure 2D) shows small grains and nanoporous particles as reported in literature [30].

3.2 Immobilization of dsDNA onto gold nanostructured sensor

The complementary oligonucleotides with the BP₁ binding sequence were first annealed by mixing them in equimolar concentrations on a thermostatic bath at 95°C for 4 min and cooled to room temperature for 40 min. Subsequently, the annealed dsDNA was exposed to the NG electrode, forming a self-assembled monolayer through the interaction between the thiol linker molecule and the NG surface [24,28]. The presence of the dsDNA attached to the gold electrode was confirmed by CV of the redox-active probe intercalated into the dsDNA (Figure 3). Figure 3A shows the CV of MB using the NG/MCH electrode (blue, without dsDNA). The MB has an anodic peak in -0.188 V. When it is intercalated onto the dsDNA attached to the NG/dsDNA/MCH electrode, the anodic peak potential is shifted to -0.253 V (Figure 3A, pink). The anodic peak potential shift of the MB is due to its intercalation into dsDNA. An increase in anodic peak current was also observed because the DNA is facilitating the charge transport.

Hybridization of the complementary strands to form the dsDNA was verified to detect any mismatch in the double strand formation. The mismatch can be detected through reduction of MB in presence of potassium ferricyanide, K₃Fe(CN)₆. The current flows through the π -stacked base pairs in the dsDNA transferring 1 electron to the intercalated MB. The MB is then reduced to produce leucomethylene blue (LB) which reduces ferricyanide to reoxidate MB [37,38]. The electro-reduction cycle resulted in amplification of the MB signal (Figure 3B). Meanwhile, in the case that the dsDNA contains any mismatched base pairs, the MB reduction to LB does not occur resulting in an oxidation current decrease of the MB [14,25].

3.3 Characterization of the binding event of beta protein 1 (BP₁) to dsDNA

Characterization of the binding event of BP₁ to the dsDNA was done by CV and SWV monitoring of the MB redox-active probe [29,31,39,40]. The MB was intercalated onto a dsDNA base pairs and the TF BP₁ to the consensus binding sequence ATATATATG [6]. A negative control sequence, a dsDNA sequence without consensus motif, was also used to verify the selectivity. A schematic illustration of the binding event of BP₁ to the dsDNA is shown in Scheme 1. After nanostructuring the gold electrode, the dsDNA is linked to the NG surface and passivated with MCH to prevent direct interactions of the protein with the gold surface. The MB is then intercalated at the top of the dsDNA by incubation of the NG/dsDNA/MCH sensor. The MB intercalated NG/dsDNA/MCH sensor is then monitored by CV as a function of BP₁ concentration. When BP₁ binds the dsDNA, the charge transport through the DNA to the MB is interrupted attenuating the DNA-mediated reduction of the redox-active probe [24].

Figure 4 shows the cyclic voltammograms of MB intercalated on the dsDNA bound to the NG surface as a function of BP₁ concentration. The anodic peak current of MB decreases significantly after the addition of BP₁. The anodic peak current of MB continues to gradually decrease with BP₁ concentration. The percent response after the first addition of 5.30 nM BP₁ was 16% reduction in the anodic current of MB. Binding of the BP₁ TF to its target site, bends the DNA duplex and perturbs the base pair stack, attenuating the DNA-mediated reduction of the redox probe. The loss of signal becomes greater with concentration reaching a saturation point near 30 nM BP₁.

3.4 Analytical performance of the biosensor

The analytical performance of the NG/dsDNA/MCH sensor was evaluated using three independent measurements using the same electrode. The NG/dsDNA/MCH sensor was incubated for 2 minutes on the desired concentration before the CV was recorded. A significant attenuation of the MB current response was observed while the BP₁ concentration increases. The percent of current attenuation follows a good linear relation with BP₁ concentrations (Figure 5), with a linear range from 5.3 to 23.6 nM with a correlation coefficient of 0.998 and a limit of detection of 1.2 nM (based on a linear regression). There is reproducibility higher than 90% on independent measurements. The NG/dsDNA/MCH biosensor also demonstrates to be stable and reusable with reproducible results. Comparing the NG/dsDNA/MCH with other biosensors (Table 1), our biosensor based on protein interaction is specific for breast cancer cells, identifying the BP₁ protein, does not require pre-conditioning, can take instant measurements and is reusable.

3.5 Regeneration of the fabricated DNA sensor

The NG/dsDNA/MCH is a simple and sensitive biosensor for the early detection of breast cancer through the recognition of the BP₁ TF. This TF biosensor is not only simple to use but it can be easily regenerated through a 30 s rinse with deionized water, removing all the protein bound to the dsDNA, allowing the reconstitution of the MB signal [21,24]. Additional measurements of BP₁ binding were realized by SWV to prove the stability of the TF biosensor. Selection of the square wave frequency was performed to determine the MB redox signal at different frequencies after regeneration. The frequency which demonstrated the strongest MB signal was 600 Hz (Figure 6). Different SWV experiments were carried out as a function of BP₁ concentration. A rapid response for the lowest concentration of BP₁ was observed with a signal of about 16% less than the MB initial signal. Subsequent attenuation response with BP₁ concentration is shown in Figure 7.

3.6 Selectivity of NG/dsDNA/MCH biosensor

SWV was also used to assess the selectivity of the NG/dsDNA/MCH biosensor. A sequence of dsDNA without the BP₁ binding motif was used as a negative control [6]. Figure 8 shows the SWV of MB in the TF biosensor modified using the negative control sequence. The MB intercalated onto the negative control sequence (black) shows a higher current reduction than the MB intercalated into the dsDNA BP₁ binding motif (blue). However, upon addition of BP₁ only a 3% of signal attenuation was observed with the negative control sequence (red) compared to a 16% with the genomic sequence containing the BP₁ binding motif at the same TF concentration (cyan). No significant changes in attenuation response were observed at higher BP₁ concentrations. These results suggest that the NG/dsDNA/MCH biosensor has a good selectivity due to the specific sequence for the BP₁ binding site. In addition, the NG/dsDNA/MCH biosensor remained stable after two weeks of its preparation.

4. Concluding remarks

A prototype NG/dsDNA/MCH biosensor using MB as redox-active probe was fabricated in a simple manner affording a sensitive BP₁ biosensor. The NG/dsDNA/MCH sensor demonstrates to have rapid and sensitive response range from 5.30 to 33 nM of BP₁ and a

limit of detection of 1.2 nM. Women with breast cancer have BP₁ levels higher than 39 ng/mL [45]. Thus, our biosensor has the potential to be a tool for breast cancer detection. Also, it has been demonstrated that NG/dsDNA/MCH biosensor is stable and can be regenerated. Regeneration of the sensor provides the advantage of a reusable sensor. Through the selective detection of BP₁ at low concentrations, the NG/dsDNA/MCH biosensor is suitable for early detection of breast cancer. Although it has not been tested on real blood samples (complex matrix), the nanostructured gold dsDNA sensor presented here has shown to be sensitive, selective, stable, and reusable allowing its potential use for POS testing. This biosensor is a *proof-of-concept* that NG/dsDNA/MCH biosensor with the correct oligonucleotide sequence to recognize BP₁ can be fabricated and has the potential to become a simple tool for breast cancer detection.

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Research Highlights

Fabrication of re-usable nanostructured gold dsDNA sensor for early detection of breast cancer

Nanostructured gold dsDNA biosensor for breast cancer beta protein 1 (BP₁)

NG-dsDNA sensor has a linear electrochemical response with a minimum detection of 5.30 nM

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Figure 1. Cyclic voltammograms of gold clean (black) and gold nanostructured sensor (red) performed in 0.5 M H_2SO_4 at a scan rate of 100 mVs^{-1} . Methylene blue (MB) is the probe.

Figure 2.

A) AFM image of clean gold electrode, B) AFM image of gold nanostructured electrode, C) SEM image of clean gold electrode, and D) SEM image of NG electrode.

Figure 3.

Cyclic voltammograms of; A) NG/MCH sensor in 20 mM Tris, 100 mM NaCl, 20 μM MB (blue) and NG/dsDNA/MCH with MB intercalated into dsDNA (pink). B) CV of NG/dsDNA/MCH in 20 mM Tris, 100 mM NaCl, 20 μM MB (pink) and with 0.1 M K₃Fe(CN)₆. MCH = 6-mercapto-1-hexanol.

Figure 4.

Cyclic voltammograms of 20 μM MB, in 20 mM tris, 100 mM NaCl at 100mVs⁻¹ in presence of different concentrations of BP₁ using the NG/dsDNA/MCH as working electrode and MB as redox-active probe.

Figure 5.
Calibration curve illustrating the percent of current change vs BP₁ concentration (nM).

Figure 6. Square wave voltammetry of MB in NG/dsDNA/MCH sensor after regeneration at different frequencies. SWV was carried out in 20 mM tris, 100 mM NaCl from 60 Hz to 600 Hz with amplitude of 25 mV and a step size of 1 mV.

Figure 7. Square wave voltammetry of MB in NG/dsDNA/MCH sensor after regeneration at different concentrations of BP₁. SWV was carried out in 20 mM tris, 100 mM NaCl at 600 Hz with amplitude of 25 mV and a step size of 1 mV.

Figure 8.

Square wave voltammetry of MB in NG/dsDNA/MCH sensor modified with the negative control DNA (black and red) and NG/dsDNA/MCH with the genomic sequence (blue and cyan). SWV was carried out in 20 mM tris, 100 mM NaCl at 600 Hz with amplitude of 25 mV and a step size of 1 mV.

Table 1

Comparison of analyte detection, linear range, advantages or disadvantages of electrochemical biosensors for breast cancer.

Modified electrodes	Analyte	Linear range	Advantages/disadvantages	References
anti-HER-3	HER-3	0.2 to 1.4 pg/mL	HER-3 is present in a wide range to tumors cell lines Incubation of 1 h with HER-3 sample Single use electrode	[41]
SAM-assisted SiNW	Era	1 pM to 1 fM	Era is present in breast cancer cell lines and non-tumorigenic breast cells Incubation of 1 h with cells sample Single use electrode	[42]
Avastin-MGO-Au	VEGF	31.25 – 2000 pg/mL	VEGF is present in many human cancers Incubation of 30 min with sample Reusable	[43]
VEGF-R1		10 – 70 pg/mL	VEGF is secreted by tumor cells Incubation of 1 h with VEGF sample Single use electrode	[44]
NG/dsDNA/MCH	BP ₁	5.3 – 23.6 nM	BP ₁ is expressed in Era ⁺ and Era [−] breast cancer cells Instant sample measurements Reusable	This work

HEGF-3 = Human epidermal growth factor-3, SAM = self-assembled monolayer, SiNW = silicon nanowire, ERE = estrogen receptor element, Era = Estrogen receptor alpha MGO = magnetic graphene oxide, VEGF-R1 = vascular endothelial growth factor receptor 1