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Low fouling label-free DNA sensor based on polyethylene glycols decorated with gold nanoparticles for the detection of breast cancer biomarkers

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ABSTRACT

A label-free and low fouling biosensor based on functional polyethylene glycols selective for breast cancer susceptibility gene (BRCA1) is reported. Sensory interfaces were prepared through the modification of a glassy carbon electrode with highly cross-linked polyethylene glycol (PEG) film containing amine groups, followed by the self-assembly of gold nanoparticles and the immobilization of BRCA1 complementary single-strand 19-mer oligonucleotides. In the presence of a specific BRCA1 sequence capture and hybridization results in interfacial change sensitively monitored using electrochemical impedance spectroscopy. The combined utilization of a PEG polymer film and gold nanoparticle mixed interface enables very high levels of sensitivity and a highly effective assaying in patient samples. Assay linear range was from 50.0 fM to 1.0 nM, with a limit of detection of 1.72 fM. Furthermore, this label-free DNA sensor has been used for assaying BRCA1 in serum samples, showing its feasible potential for diagnostic applications in clinical analysis of breast cancer gene BRCA1. Foreseeable, this sensor made on this basis undoubtedly provide the most effective and sensitive detection for BRCA1.

1. Introduction

Recently, genome-wide association studies have led to the identification of numerous genes associated with an increased cancer risk (Li et al., 2012). Most human breast cancers are associated with specific mutations in the BRCAl anti-oncogene sequence on chromosome 17 at band 21 (Miki et al., 1994; Venkitaraman, 2004) responsible for the production of tumor suppressor proteins. As one of the three most common invasive cancer in females (Parkin et al., 2005), breast cancer mainly takes place in the inner lining of the milk ducts or lobules with different spread, aggressiveness and genetic makeup, and it is one of the leading causes of cancer mortality among women worldwide (Jemal et al., 2009). The value of identifying BRCA1 gene mutations in facilitating early diagnosis and intervention is well known (Sega and Low, 2008; Yucai et al., 2009) and the subject of intensive study and technological development (Balapanuru et al., 2010; Swierczewska et al., 2012).

The sequence-specific detection of nucleic acid targets is, of course, relevant to a multitude of research areas including diagnostics and forensics (Sassolas et al., 2008; Shuang et al., 2009). In the past few years, a number of methods have been specifically applied to the determination of BRCAl, including high performance liquid chromatography (Arnold et al., 1999), fluorescence conformation sensitive gel electrophoresis (Ganguly et al., 1998), single-strand conformation polymorphism assay (Rashid et al., 2006) and RNA/DNA sequencing (Jakubowska et al., 2001). However, there are certain limitations such as low sensitivity, high cost, time-consuming and complex procedures that prevent the broad practical application of these techniques.

Label-free DNA hybridization biosensors have received great attention in the past because of the simple fabrication procedure, rapid assay time and low cost. Typically, the fabrication of these involves the immobilization of a capturing single strand DNA (ssDNA) probe on a transducer surface (Liu et al., 2014; Lu et al., 2008), complementary sequence hybridization with the target probe, and an analysis based on fluorescence (Gerion et al., 2003; Taton et al., 2001), mass (quartz crystal microbalance) (Patolsky et al., 2001), local dielectric (surface plasmon resonance) (D'Agata et al., 2010; He et al., 2000) or steric bulk/electrostatics/conformational change (electrochemistry) (Ferapontova et al., 2010;

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Xia et al., 2010). The latter were initially explored by Millan and Mikkelsen (1993), and have subsequently attracted considerable attention by virtue of low associated assay cost and time and high levels of sensitivity (Kimmel et al., 2012; Tiwari and Gong, 2009). One key parameter in the efficacy of these approaches is the quantity of immobilized and available capture sequence (Du et al., 2009). Gold nanoparticles (AuNPs) have often been presented as accessible, chemically stable and effective entities in supporting high levels of biomolecular immobilization (Daniel and Astruc, 2004; Dreaden et al., 2012; Niu et al., 2011), including that associated with thiol-functionalized DNA in underpinning sequence-specific DNA assays (Ai et al., 2009; Jans and Huo, 2012; Pei et al., 2012). Analysis in real biological samples like serum, where plasma proteins and salts will typically initiate severe nonspecific adsorption and particle aggregation is, though, challenging (Henry et al., 2010; Kim et al., 2007; Zhang et al., 2009). PEG, a nontoxic and hydrophilic polymer (Ekblad et al., 2008; Goodwin et al., 2009), has been extensively applied in minimizing the nonspecific adsorption of proteins (Otsuka et al., 2003; Samantha et al., 2010; Zhang et al., 2009), within a range of biosensor configurations (Eck et al., 2008; Simpson et al., 2011).

Here, we report a label-free DNA hybridization biosensor for the detection of BRCA1 related sequence. In this biosensor system, highly cross-linked PEG films containing amine groups was used as the antifouling substrate, and AuNPs were then self-assembled onto the surface of the PEG film for the further immobilization of the DNA recognition probe. Faradaic electrochemical impedance spectroscopy (EIS), a non-destructive, label free and highly sensitive technique that has been used for the detection of a number of disease markers (Luo and Davis, 2013; Xu et al., 2013), was utilized herein as the target recruiting transducer. The marriage of hybrid PEGylated polymer–gold nanoparticle interface with EIS offers the biosensor several advantages such as facile fabrication, high sensitivity and selectivity, and acceptable reproducibility, and the biosensor is thus of great potential for future clinical application.

2. Experimental

2.1. Materials and apparatus

The 19-mer synthetic oligonucleotides related to BRCA1 breast cancer gene (Mohan et al., 2010) were supplied by Sangon Biotech (Shanghai, China) Co., Ltd., purified by high-performance liquid chromatography and used as received. And their sequences were as follows:

Immobilized probe (S_1): 5′ GAT TTT CTT CCT TTT GTT C 3′-SH
Target probe (Complementary) (S_2): 5′ GAA CAA AAG GAA GAA AAT C 3′
One-base mismatch (S_3): 5′ CAA CAA AAG GAA GAA AAT C 3′
Three-base mismatch (S_4): 5′ CAA CAA AAG CAA CAA AAT C 3′
Non-complementary (S_5): 5′ CCT TGT TGG ACT CCC TTC T 3′

Stock solutions of oligonucleotides were prepared with PBS (0.2 M, pH 7.4, we use NaCl in the buffer considering the ionic strength of the buffer) and stored in a freezer at 4 °C before use. 4-armed PEG-epoxide and 4-armed PEG-amine with molecular weights of 2000 g/mol were purchased from Creative PEGWorks USA, were separately dissolved in chloroform with concentrations 30 mg/mL. AuNPs (diameter of ~20 nm) and bovine serum albumin (BSA) were purchased from Aladdin Reagents (Shanghai, China). All other reagents were of analytical reagent grade and used directly without further purification. Millipore water from a Milli-Q water purifying system was used throughout. All experiments were performed at ambient room temperature.

Electrochemical experiments, which mainly including EIS, was performed with a CHI 760D electrochemical workstation (Shanghai Chenhua Instrument Co. Ltd., China). All experiments were carried out in a conventional three-electrode system consisting of a platinum wire counter electrode, a silver/silver chloride (Ag/AgCl, filled with 3.0 M KCl) reference electrode and a glassy carbon working electrode (GCE, diameter 3.0 mm). EIS measurements were recorded in 5.0 mM $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 M KCl with the direct current potential set at 0.25 V. The amplitude of the applied sine wave potential was 5 mV, the frequency range was from 0.01 Hz to 100 kHz. All experiments were conducted at ambient temperature. Field emission scanning electron microscope (SEM) was performed with a JEOL JSM-7500 F SEM instrument (Hitachi High-Technology Co., Ltd., Japan).

2.2. Sensor surface modification

The preparation process is schematically summarized in Fig. S1. GCE was polished, cleaned and electrochemically pretreated in phosphate buffered saline (PBS) according to a previous report (Luo et al., 2007). The pre-treated GCEs were then drop-coated with 2.0 μL chloroform solution containing 1.0 mg/mL 4-armed PEG-amine and 0.6 mg/mL 4-armed PEG-epoxide by pipette prior to gentle heating in an oven at 80 °C for ~3 h under nitrogen atmosphere. A highly non-fouling cross linked hydrated polymeric network is formed across the electrode by this (catalyst and by-product-free) process (Meyerbröker et al., 2013). After washing and soaking in PBS (0.2 M, pH 7.4) for 12 h to remove non-cross-linked material and equilibrate the PEG film. PEG/AuNPs composite films were prepared by immersion of the PEG films into a freshly prepared (stored at 4 °C) solution of citrate-stabilized AuNPs for 6 h, followed by extensive rinsing with water and drying in a nitrogen stream. The obtained electrodes were denoted as PEG/AuNPs/GCEs. The PEG/AuNPs/GCEs were then incubated in 1.0 μM thiol-functionalized oligonucleotides solution (0.2 M PBS, pH 7.4) for 12 h to allow the covalent attachment of oligonucleotides to the surface of the AuNPs via the formation of Au–S bond. The obtained electrodes were denoted as S_1/PEG/AuNPs/GCEs.

2.3. Hybridization studies

The hybridization experiments were carried out by immersing the S_1/PEG/AuNPs/GCEs in PBS (0.2 M, pH 7.4) containing different concentrations of target analyte (S_2, S_3, S_4, S_5, BSA) for 2 h at ambient room temperature. And then, the hybridized electrode was next rinsed with PBS (0.2 M, pH 7.4) for several times to remove the non-specifically bound target analyte (S_2, S_3, S_4, S_5, BSA). The electrodes thus obtained were denoted as S_1–S_2 (S_3, S_4, S_5, BSA)/PEG/AuNPs/GCEs.

3. Results and discussion

3.1. SEM characterization of the modified electrodes

The morphology and microstructures of the prepared films (PEG and PEG/AuNPs) were characterized by SEM. As shown in Fig. 1, a layer of PEG was formed on the electrode, and the surface of the PEG film (Fig. 1a and b) was relatively uniform and smooth. After the self-assembly of AuNPs, a layer of nanoparticles with a diameter of approximately 20 nm were homogeneously distributed among the PEG film (Fig. 1c and d), indicating successful attachment of AuNPs onto the PEG film. Due to the rich terminal amine groups of the PEG film formed on the electrode, AuNPs can easily attach to the electrode surface through their interaction with the amine groups, providing a considerably large surface area

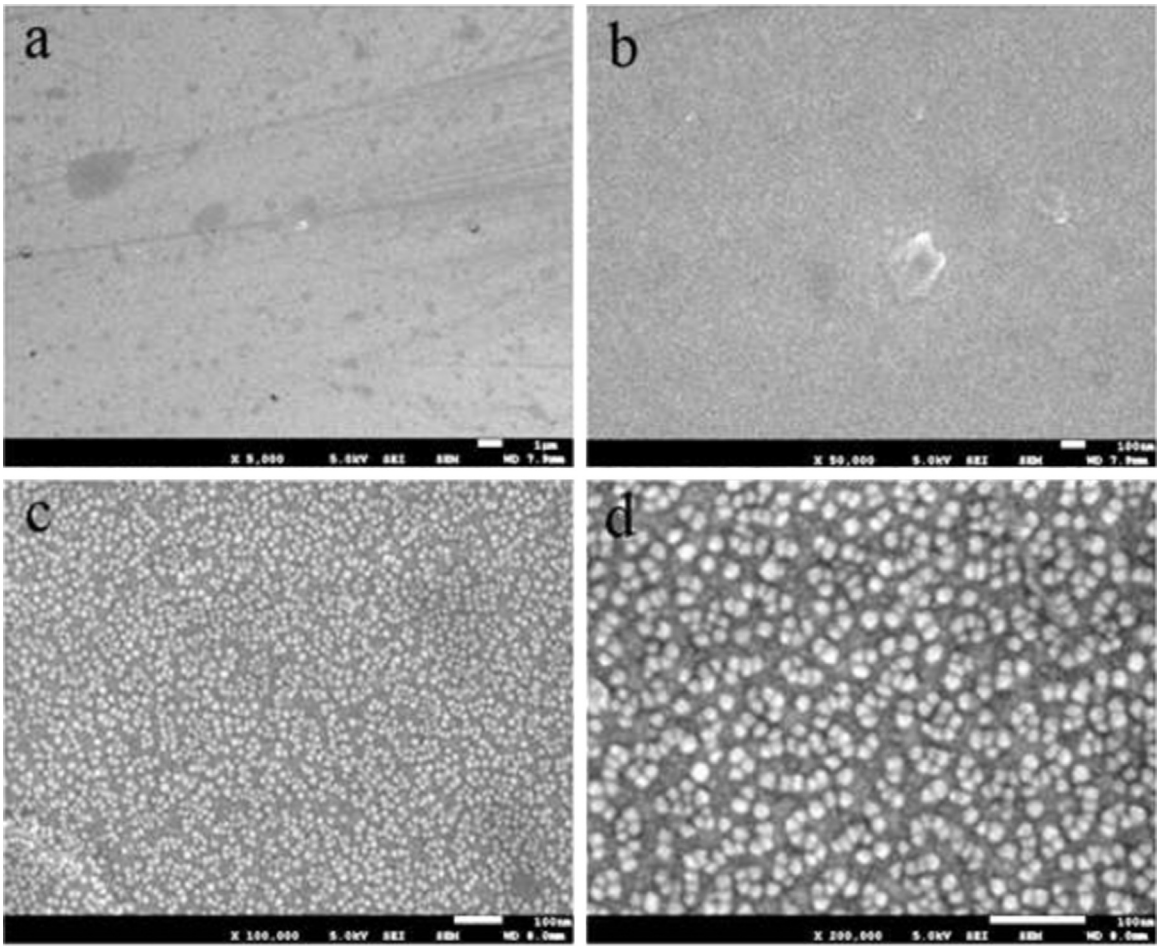

Fig. 1. SEM images of the PEG films (a, b) and the PEG/AuNPs composite films (c, d). Panels (a, c) are of lower magnification and (b, d) are of higher magnification.

for the immobilization of the DNA recognition probe (to the AuNPs surface).

3.2. EIS characterization of the biosensor fabrication process

EIS is a useful technique for characterizing the stepwise fabrication of a modified electrode (Bryan et al., 2013; Ciani et al., 2012; Elshafey et al., 2015; Elshafey et al., 2013). Nyquist impedance plots, including a semicircle portion at higher frequencies and a linear portion at lower frequencies, represent fingerprints of charge transfer and diffusion limited processes respectively (Oliveira et al., 2011; Xu et al., 2013). The former can be quantified through the semicircle diameter as the charge-transfer resistance (R_{ct}) of the modified electrode, when fitted using a standard Randles equivalent circuit (Bonanni and del Valle, 2010; Guo et al., 2012; Wu et al., 2011). Predictably, there are sharp increases in R_{ct} as the receptor layer is fabricated on the initially pristine gold surface. The R_{ct} significantly increases after the formation of the PEG film (~8000 Ω, curves b, Fig. 2), and decreases (because of the conductive path provided) when AuNPs were added to the film (curves c, Fig. 2) (Arotiba et al., 2008; Fayazfar et al., 2014; Wang et al., 2014). Subsequent capture probe immobilization will increase the R_{ct} (Dinçkaya et al., 2011; Fayazfar et al., 2014; Nascimento et al., 2011; Wang et al., 2014; Yan et al., 2013) (curve d, Fig. 2) because its negatively charged phosphate skeletons characteristically repels the negatively charged $[Fe(CN)_6]^{3-/4-}$ probe from approaching the electrode surface (Zhang and Jiang, 2012). Interestingly, a further significant increase in R_{ct} (curve e, Fig. 2) could be observed when the modified interface interact

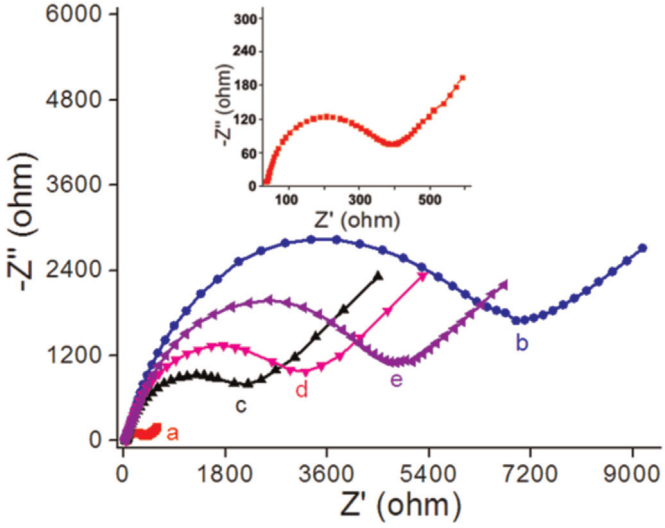

Fig. 2. Nyquist plots of the EIS recorded in PBS (0.2 M, pH 7.4) solution containing 5.0 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl: (a) bare GCE; (b) PEG interfaces; (c) PEG/AuNPs/GCE and subsequently prepared capture probe modified interfaces. Inset: the magnified portion of nyquist plots for the bare GCE.

with its complementary target DNA, which might be ascribed to more negative charges on the surface and larger interfacial steric bulk after hybridization (Bonanni and del Valle, 2010; Kowalczyk et al., 2011; Nascimento et al., 2011; Wang et al., 2014; Zhang et al., 2011). Therefore, a selective and sensitive DNA biosensor can be

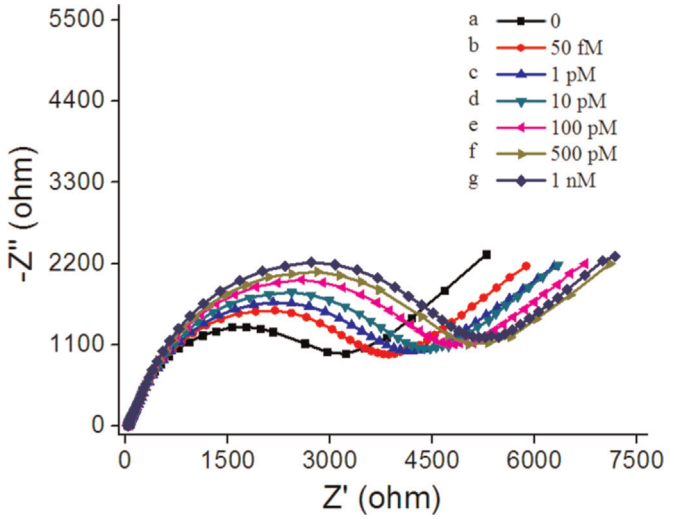

Fig. 3. Typical Faradaic impedance spectra corresponding to the biosensor after incubation in PBS (0.2 M, pH 7.4) of different target probe concentration: curves from inner to outer represent 0 fM, 50 fM, 1 pM, 10 pM, 100 pM, 500 pM and 1 nM target probe, respectively.

developed based on the sensitive impedance change associated with the specific DNA hybridization.

3.3. Sensing performance

The sensing performance of the biosensor for the label free determination of BRCA1 related sequence was initially investigated in phosphate buffered saline (PBS, 0.2 M, pH 7.4) using EIS. As shown in Fig. 3, along with the increase of the target concentration, the R_{ct} changes accordingly, and the binding efficacy and interfacial sensitivity resolved herein is sufficient for the PEG based surface to be detectably responsive to BRCA1 at low femtomolar range levels. It is likely that this sensitivity is, at least partially, aided by the strong negative charge of the DNA target and the resulting electrostatic repulsion of the redox probe. More detailed analyses (Fig. 4) reveal that R_{ct} has a good linear correlation (R^2=0.995) with the logarithmic value of the complementary oligonucleotides concentration, a linear range from

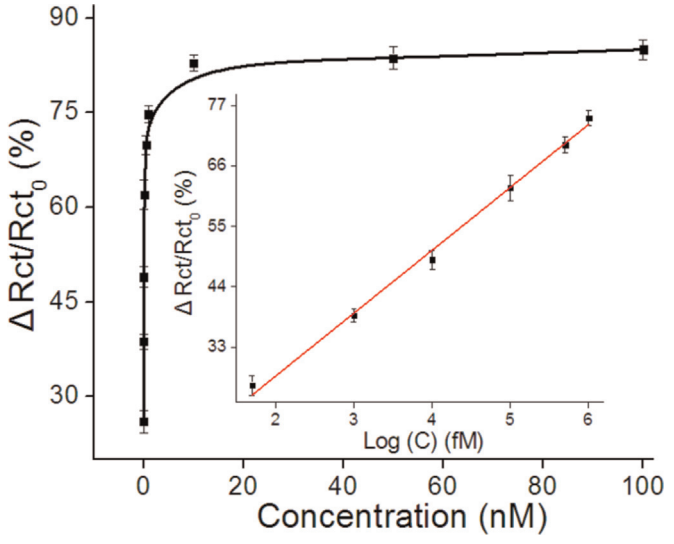

Fig. 4. Normalized charge-transfer resistance (R_{ct}) changes of the biosensor as a function of analyte concentration. The inset shows the corresponding calibration curve for the BRCA biosensors. Error bars represent the standard deviations across five repeats.

50.0 fM to 1.0 nM. The regression equation was $\Delta R_{ct}/R_{ct0}(\%)=11.45 \log C\ (pM)+4.84$, and the detection limit was calculated to be 1.72 fM (based on a signal-to-noise ratio of 3, with noise was determined as the standard deviation of 5 EIS measurements of the blank buffer solution) (as shown in Fig. 4). Notably, this lies much lower than that of previous assays for BRCA1 related sequence (Table 1), and can comfortably meet the requirements of clinically relevant levels of BRCA1 in biological samples.

3.4. Selectivity of the gene sensor

In the presence of a large excess of nonspecific protein and DNA sequences, the direct and sensitive detection of disease biomarkers is extremely demanding, particularly for low molecular weight targets present at sub-picomolar levels with an unamplified label free approach. In order to test the interfacial selectivity, the prepared gene sensor responses were assessed in solutions containing bovine serum albumin (BSA), single-base mismatched DNA sequences, three-base mismatched DNA sequences or non-complementary DNA sequences either separately or combined, respectively. As shown in Fig. 5, the result revealed that an excellent selectivity toward target probe S_2 was achieved. By contrast, immobilized probe (S_1), one-base mismatch (S_3), three-base mismatch (S_4) and non-complementary (S_5) DNA sequences, and bovine serum albumin (BSA) show negligible response at artificially high concentrations (For S_1, 100,000 times higher than target probe S_2). In addition, the influence of all the 5 compounds in solutions could also be neglected. The assayed EIS signal differed by only 1.18% from that associated with a pure S_2 solution at equivalent concentration. Hence, the as-prepared gene sensor possesses an excellent selectivity toward target probe S_2, which may be ascribed to the good anti-fouling ability of the PEG film that effectively prevents the nonspecific adsorption onto the sensing interface.

3.5. Reproducibility of the biosensor

Sensory systems are of practical application only if their performance is reproducible. To evaluate the reproducibility of the BRCA1 biosensor, five interfaces were fabricated independently and analyzed against 5.0 pM S_2. The ΔR_{ct} values ($\Delta R_{ct}=R_{ct}-R_{ct0}$) obtained were 2180.74 Ω, 2242.17 Ω, 2293.42 Ω, 2262.86 Ω and 2287.17 Ω, respectively. The average value is 2253.27 Ω, and the relative standard deviation (RSD) obtained was 2.01%, suggesting a satisfactory reproducibility.

3.6. Clinical application

The practical application of the proposed DNA hybridization sensor was evaluated by assaying BRCA1 related sequence within human serum samples. Samples were, specifically, prepared by doping specific concentrations of BRCA1 into human serum samples (obtained from The Eighth People's Hospital of Qingdao, China). Human serum samples were prepared by centrifugation of fresh blood for 5 min at 10,000 rpm, the supernatants were collected without dilution afterwards. The analytical results are shown in Table S1, where it is evident that quantification is robustly retained (assay results being 92.55–100.57% of doped levels), with the relative standard deviation (RSD) ranging from 1.77% to 7.74%. We believe these observations, which compose the most effective electroanalytical detection of BRCA1 in any spiked serum sample, are the result of excellent DNA specificity in terms of recruitment and perfect antifouling property of the PEG/AuNPs nanocomposite.

Table 1
Comparison of sensing performances for the detection of BRCA1.

Materials used	Detection method	Linear range	LOD	Reference
Biochip	Fluorescence	0.2–5 μM	70 nM	Culha et al. (2004)
NGS-HPCS[a]	CV	0.001–35 ng/mL	0.33 pg/mL	Ren et al. (2014)
PNA[b] electrode	enzyme-catalyzed signal amplification	1 nM–100 μM	1 nM	Won et al. (2008)
PVP-GS/TH[c] electrode	Amperometric	0.01–15 ng/mL	4.86 pg/mL	Cai et al. (2011)
Gold electrode	EIS	0.1–10 nM	0.05 nM	Xu et al. (2012)
MCN–TB[d]/GCE	SWV	0.01–15 ng/mL	3.97 pg/mL	Fan et al. (2013)
cDNA/CHIT-*co*-PANI/ITO	CV	0.05–25 fM	0.05 fM	Tiwari and Gong (2009)
Hemin/G-Quadruplx DNAzyme nanowires	Enzyme-free amplified	0.1 pM–1 μM	0.1 pM	Shimron et al. (2012)
PEG/AuNPs	EIS	50.0 fM–1.0 nM	1.72 fM	This study

[a] N-doped grapheme- hydroxypropyl chitosan.
[b] Peptide nucleic acid.
[c] Polyvinylpyrrolidone-graphene sheets /thionine.
[d] Mesoporous carbon nanospheres-toluidine blue.

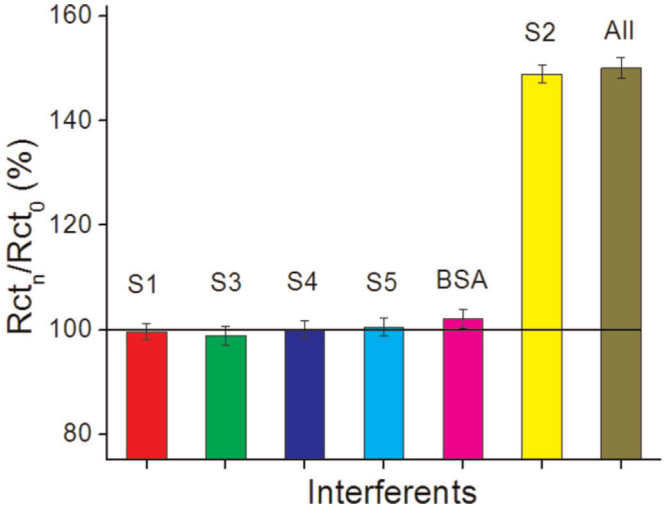

Fig. 5. Responses of the BRCA1 biosensor to 1.0 M S_1, 0.1 μM S_3, 0.1 μM S_4, 0.1 μM S_5, 0.1 μM BSA, 10.0 pM S_2 and a mixture of all the above mentioned substances.

4. Conclusion

A novel label-free and low fouling electrochemical DNA hybridization biosensor with ultrahigh sensitivity and selectivity has been developed based on a BRCA1 related 19-mer oligonucleotide sequence. The readily prepared interfaces are based on the immobilization of DNA capture probe onto an underlying AuNPs, which was adsorbed onto the surface of a PEG film with excellent anti-fouling property. The impedance based assay of BRCA1 related sequence was realized with a linear range from 50 fM to 1.0 nM with a detection limit of 1.72 fM. The sensor was efficient enough to detect DNA mismatch, and it was rapid, simple and free from the complex chemistry of redox indicator and markers for DNA hybridization detection. Notably this ability is retained in serum samples and supports a quantification that is accurate across a broad clinically relevant concentration range. Moreover, this biosensor has good specificity, low detection limit, good biocompatibility and facile preparation process. It is foreseeable that low fouling biosensor made on this basis will undoubtedly provide greater convenience for the detection of human disease markers in the future.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.04.018.

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