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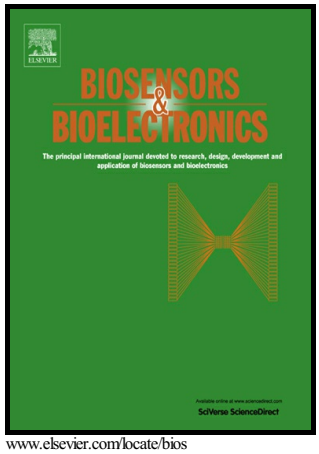

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A highly sensitive and reliable detection of CA15-3 in patient plasma with electrochemical biosensor labeled with magnetic beads

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Abstract

An early on-time detection of breast cancer can effectively affect the outcome of the treatment. Here, we developed an ultrasensitive, simple and reliable immunosensor to detect the lowest alteration of CA 15-3, the standard biomarker of breast cancer patients. The proposed immunosensor was achieved by modification of gold electrode by streptavidin to immobilize the biotinylated anti-CA 15-3 monoclonal antibody (mAb). Bovine serum albumin was used to prevent nonspecific binding. To improve the sensitivity of modified immunosensor, the sandwich signal enhancer consisting of streptavidin-coated magnetic beads conjugated with biotinylated horseradish peroxidase (HRP) and anti-CA 15-3 biotinylated mAb was applied. The electrochemical measurements were obtained in the presence of hydroquinone as a redox agent and H_2O_2 as the activating agent of HRP. Under optimized condition and using square wave voltammetry, the lower limit of quantification was obtained as 15×10^{-6} U/mL and the linear CA 15-3 concentration range was 50 to 15×10^{-6} U/mL. While showing significant stability, the immunosensor displayed an excellent sensitivity and specificity for the detection of CA 15-3 even in the human serum as compared to the enzyme-linked immunosorbent assay (ELISA) as a gold standard method. Based on our findings, the engineered immunosensor is proposed as a robust diagnostic tool for the clinical determination of CA 15-3 and other cancer biomarkers.

Keywords: CA15-3; Electrochemical biosensor; Immunosensor; Magnetic bead; Breast cancer; Serum biomarker.

1. Introduction

To date, cancer has been considered as one of the main health burden worldwide with a high rate of mortality. Based on the latest cancer statistics, the current growth rate of cancer cases has been

predicted to grow significantly within the next few years, resulting in a substantially high rate of mortality and morbidity in the United States (Siegel et al. 2015, 2018). Of various types of malignancies, breast cancer is the most prevalent tumor among the female population. The occurrence rate was estimated ~25- 30%, while its mortality rate was reported to be around 14.7% (Siegel et al. 2015, 2018). Based on the recent cancer statistics, the new cases of breast cancer in the US are estimated about 266 thousand individuals with about 41 thousand death cases (Siegel et al. 2018), while the International Agency for Research on Cancer (IARC) reported 500 thousand death cases worldwide in 2012 (Ferlay et al. 2015). An early on-time diagnosis of the breast cancer can lead to a marked decrease in the aforementioned statistics.

The precise detection of the relevant biomarkers provides key information regarding the presence or severity of disease conditions and leads to the development of new approaches or platforms for the early diagnosis of the breast cancer (Strimbu and Tavel 2010). To date, different biomarkers have been introduced for the detection or prediction of the breast cancer, including cancer antigen (CA15-3, CA 27-29), carcinoembryonic antigen (CEA), human epidermal growth factor receptor 2 (HER2), cytokeratins and mammaglobin (Dorvillius et al. 2002; Liu et al. 2018; Nicolini et al. 2015; Perrier et al. 2018; Rack et al. 2010; Tang et al. 2016; Wang et al. 2014a; Zehentner et al. 2004; Zhu et al. 2009). Among them, the CA 15-3 is a product of mucin-1 gene family, which has extensively been studied for the breast cancer. The mucin-1 is a glycoprotein consisting of three domains, including extracellular, membrane-spanning and cytoplasmic domains (Gendler and Spicer 1995). An association between the functional expression of the CA 15-3 and the prognosis of the breast cancer has been reported, in which the higher the level of the biomarker is, the worse the outcome of cancer will be (Duffy et al. 2004). The CA 15-3 has routinely been used in the clinic as the biomarker for the prediction of the disease outcome and

monitoring of chemotherapy treatment modalities or even the relapse of the disease. In another word, the CA 15-3 is considered as the standard practical serum biomarker of the breast cancer (Busetto et al. 1995; Duffy 1999).

To meet the growing demands for the detection of CA15-3, a number of techniques have been developed and applied for the quantitative determination of this cancer marker, including immunohistochemistry (IHC) (Sandri et al. 2012), enzyme-linked immunosorbent assay (ELISA) (Soheila Nasizadeh 2005), and enzyme immunoassay (EIA) methods (Liu et al. 2008). These techniques appear to be complex, laborious, time-consuming and fail to provide a real-time detection, as a result, demands for a simple cost-effective and highly sensitive approach for the evaluation of diseases biomarkers have continuously been increased. Among various methods used for the detection of biomarkers including CA 15-3, the electrochemical techniques have gained the attention of a large number of researchers worldwide, in large part due to their advantages together with high sensitivity and low limit of detection (LOD) for the detection of various biomarkers (Kavosi et al. 2015; Zhang et al. 2016).

In the recent decades, electrochemical approaches have paved the way towards detection of various substances and molecules with extremely low detection limits in industry and clinic (Aliakbarinodehi et al. 2018; Khalilzadeh et al. 2016; Saberian-Borujeni et al. 2014; Yamada et al. 2017). Of the electrochemical sensors, immunosensors, which are performed based on the reaction of antibodies and antigens, have played important role in the detection of different biomarkers (Afsharan et al. 2016b; Johari-Ahar et al. 2015). An electrochemical immunosensor can reach to optimum selectivity if the sandwich approach is applied in its development. In another word, the use of a secondary antibody engineered with signal enhancers can lead to a proper selectivity with good signal amplification. Among different particles used in the sandwich

assay in immunosensors, magnetic nanoparticles (MNPs) or beads (MBs) are used to enhance the detection signal, in large part due to their large surface to volume ratio. Besides, they could be coated with biomolecules such as streptavidin to improve the conjugation of antibodies (Lacharme et al. 2009; Rida and Gijs 2004).

Given the fact that the early and on-time quantification of CA 15-3 in breast cancer patients can lead to a beneficial cancer therapy, in the present study, we have developed a simple, reliable and ultrasensitive electrochemical immunosensor for the detection of CA 15-3. Streptavidin was used to immobilize the CA 15-3 specific monoclonal antibody (mAb) on the surface of the gold electrode. Applying streptavidin-biotin strategy improves the specificity and effectiveness of interactions between the antigen and the biotinylated mAb. Besides, streptavidin can significantly affect the antibody immobilization (Zhu et al. 2015). To increase the selectivity, the biotinylated mAb conjugated with the streptavidin-magnetic beads and biotinylated horseradish peroxidase (HRP) were used to amplify the electrochemical signals. Having considered the previously reported CA 15-3 immunosensors, to the best of our knowledge, this current immunosensor provides a reliable and ultrasensitive detection of the CA 15-3 in the human serum.

2. Experimental

Materials and reagents, apparatus and procedures have been described in the supplementary materials.

2.1. Preparation of signaling label

Signaling label for the utilization in the sandwich approach immunosensor was prepared as described in the following context. First, 7.5 µL of biotinylated HRP was mixed with 5 µL streptavidin-coated magnetic (beads Strp-MB) and incubated at 37 °C for 2 hours. Then, the obtained mixture was washed to remove the unconjugated HRP and beads according to the manufacturer of the magnetic beads. Afterward, 1 µL of biotinylated anti-CA 15-3 mAb was added to the mixture and incubated at 37 °C for another 2 hours. It should be noted that, during both incubation periods, the mixture was gently mixed four times to provide the maximum reaction possibility for the reactants. The obtained signaling label was prepared prior to use each time.

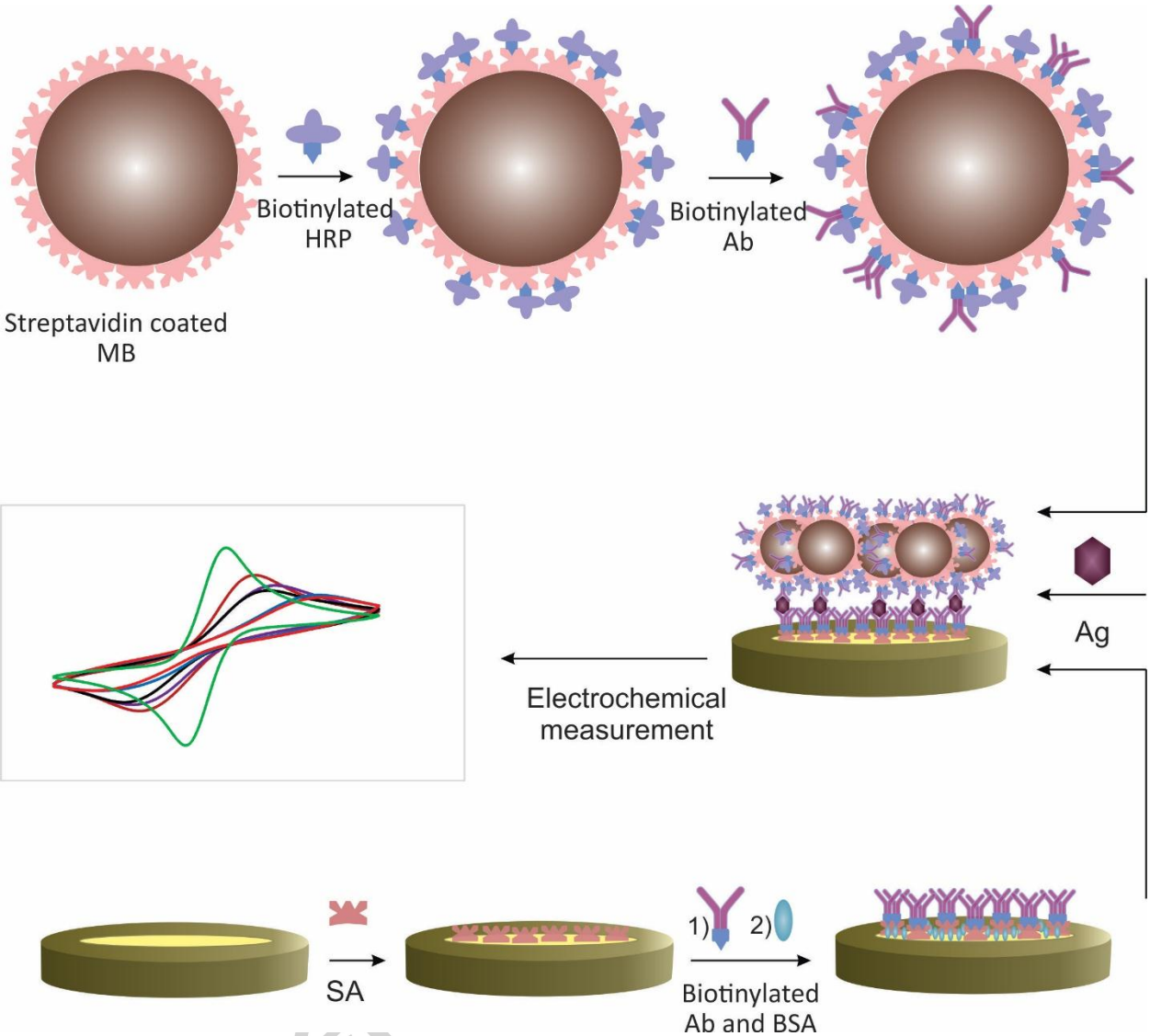
2.2. CA 15-3 immunosensor fabrication

The details of the preparation step of the immunosensor are illustrated in **Fig. 1.** Briefly**,** the bare Au electrode (GE), 2 mm in diameter, was firstly rinsed in double distilled deionized water followed by a polishing process using a polishing pad and alumina powder (0.03 mm and 0.5 mm). The electrode was then washed twice with 2% V/V nitric acid and double distilled water. Afterward, to remove particles from the surface of the electrode, the electrode was sunk in the double distilled water for 10 min and pure ethanol for 10 min – both under the sonication. Finally, the surface of the electrode was gently dried by N_2 stream. Subsequently, 10 µL of streptavidin (Strp) (0.1 mg/mL) was cast on the electrode and incubated at 4 °C for 12 hours. Then, 10 µL of biotinylated mAb of CA 15-3 was cast on the electrode and kept for another 12 hours at 4 °C. To prevent the unspecific binding, 10 µL of BSA 5% + 0.5 % tween 20 were dropped on the modified electrode and kept at room temperature for 1 hour, and followed by washing of the electrode with washing buffer (PBS pH 7 + 0.05% tween 20). Then, 10 µL of CA

15-3 antigen was dropped onto the electrode and incubated at 37 °C for 2 hours. To complete the sandwich assay in the preparation of CA15-3 immunosensor, 5μL of freshly prepared signaling label was dropped on the modified electrode and incubated at 37 °C for 2 hours. It should be mentioned that among each step of the preparation of immunosensor and prior to the next step of the experiments, the modified electrode was washed with PBS (0.1M, pH 7) for 10 min.

2.3. Detection procedures of CA 15-3

The CA 15-3 was detected using the GE/ Strp/ biotinylated mAb / CA15-3/ biotinylated mAb/ Strp-MB/ biotinylated HRP immunosensor as a working electrode in the presence of various concentration of CA 15-3. The electrochemical experiments were performed in the electrochemical cell containing PBS (0.1 M, pH 7) with hydroquinone and H_2O_2. The potential was swiped between -0.6 and 0.2 V at the scan rate of 0.125 V/S.



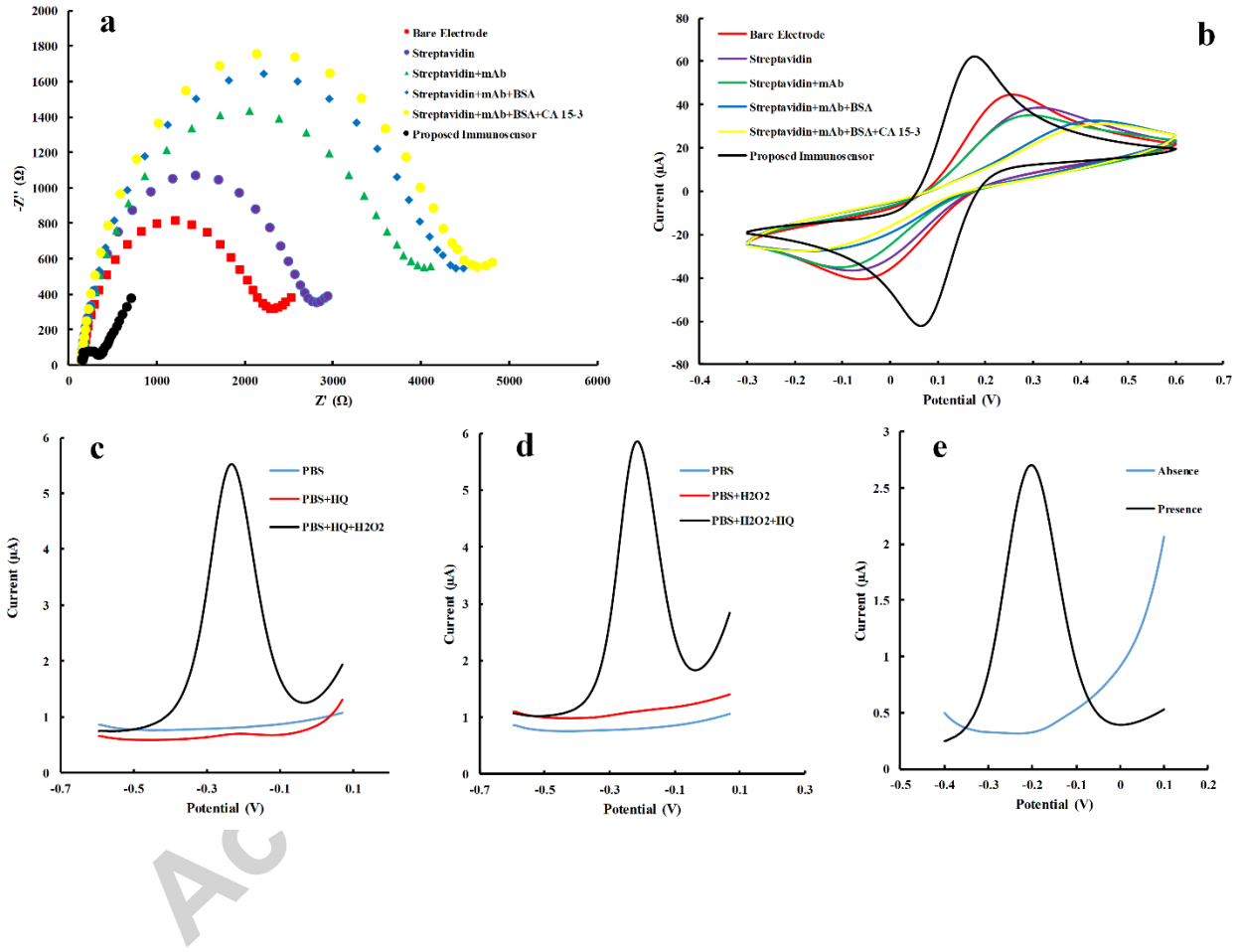
3. Results and discussion

3.1. Electrochemical characterization of immunosensor preparation steps

The preparation steps of the modified electrode were evaluated by Cyclic voltammetry (CV) and Electrochemical impedance spectroscopy (EIS) techniques using the electrochemical standard solution, i.e., 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) and KCl 0.1 M.

As a well-established method, the self-assembly of materials could be detected by EIS via changes in the conductivity. At the higher frequencies, the semicircle segment of the Nyquist plot demonstrated a growth in the diameter which is reversely related with the electron transfer. On the other words, the electron transfer was reduced as a result of electrode modification and indeed, the size of semicircles of Nyquist plots increased. As shown in **Fig. 2a**, the bare GE displayed the minimum electron-transfer resistance (R_{et}). By successfully self-assembling of streptavidin on the surface of the GE, the R_{et} was increased while after casting mAb, the amount of resistance elevated much more. This might be explained by the self-assembly of mAb with streptavidin which might lead to an electron transfer prevention. This trend continued when BSA has used to prevent non-specific binding. The R_{et} reached its maximum value while the antigen (CA15-3) was cast on the electrode, in another word, the GE/ Strep/ mAb/ BSA/ CA15-3 modified electrode showed the highest resistance among others. This latter phenomenon could be described by the blockade of the electron transfer, which consequently confirmed the presence of self-assembled biomolecules on the electrode. Finally, after the addition of the signaling label, due to the paramagnetic effect of MBs, the conductivity of the engineered immunosensor was increased. The decrease in the R_{et} amount was completely confirming that the signaling label was attached to the electrode surface and the electrode preparation steps were accomplished successfully and our findings are totally in agreement the results of others (Liu et al. 2017a; Wang et al. 2014b). Further, the CV was performed to investigate the electrode preparations steps. The CVs were obtained using 5 mM K3[$Fe(CN)_6$]/K4 [$Fe(CN)_6$] (1:1) and KCl 0.1 M solution at the potential range of -0.6 V to 0.2 V and the scan rate of 0.1 V/s. As described in **Fig. 2b**, the CV of the bare GE displayed the maximum growth. Casting various biomolecules have caused a progressively decreasing at oxidation and reduction peaks of CVs. The GE/ Strp/

mAb/ BSA/ CA15-3 showed the lowest peak height as compared to other preparation steps of the electrode. This might be interpreted due to the presence of multiple biomolecules on the electrode surface. Furthermore, the preparation steps of the engineered electrode were also evaluated in the electrochemical cell containing PBS, HQ, and H_2O_2. Interestingly, the obtained data confirmed the results obtained from the electrochemical standard solution **(Fig. S1).**



3.2. Principals of proposed immunosensor

Among various enzymes used to enhance the sensitivity of electrochemical biosensors due to their catalytic activity, the HRP is one of the most commonly recruited enzymes for the signal amplification purposes. A number of researchers utilized the HRP conjugated nanoparticles in the presence of different electron mediators such as hydroquinone (HQ), methylene blue and

thionine to improve the sensitivity of biosensors and meet very low LODs (Ge et al. 2012; Liu et al. 2017b; Pakchin et al. 2017; Zhao et al. 2011). Considering the fact that hydrogen peroxide can accelerate the electrochemical reduction of hydroquinone via electro-catalytic effect, the proposed immunosensor shows an excellent electrocatalytic effect in the presence of HQ and H_2O_2. Reaction 1 displays the electrochemical mechanisms of HQ reduction in the presence of the modified electrode with HRP and H_2O_2 (Afsharan et al. 2016a; Khalilzadeh et al. 2015; Yao et al. 2005).

Reaction 1:

(1-1) HRP (Fe^{III}) + H_2O_2 /HRP (Fe^{IV}) + H_2O

(1-2) HRP (Fe^{IV}) + HQ (R) /HRP (Fe^{III}) + HQ (O)

(1-3) HQ (O) + $2H^+$ + $2e^-$ /HQ (R)

R=reduce, O=Oxidize

3.3. Electrochemical characterization process

Three approaches were used to investigate the electrochemical response of the modified immunosensor. In the first approach **(Fig. 2c)**, the engineered immunosensor was tested using (**I)** PBS (pH 7, 0.1M), (**II)** PBS containing 2.5mM HQ and (**III)** PBS containing 2.5 mM HQ and 2.5mM H_2O_2. For the PBS alone, the obtained peak currents did not exhibit any specific peaks. Although a small peak was expected to appear after the addition of HQ in association with hydroquinone oxidation. By adding 2.5 mM H_2O_2 to the electrochemical cell containing PBS and HQ, a significant increase in the peak current was observed. This increase in the peak current height might correspond to the reduction of HQ in the presence of H_2O_2. This indicates that the electron transfers between HRP and the electrode surface could be accelerated when H_2O_2

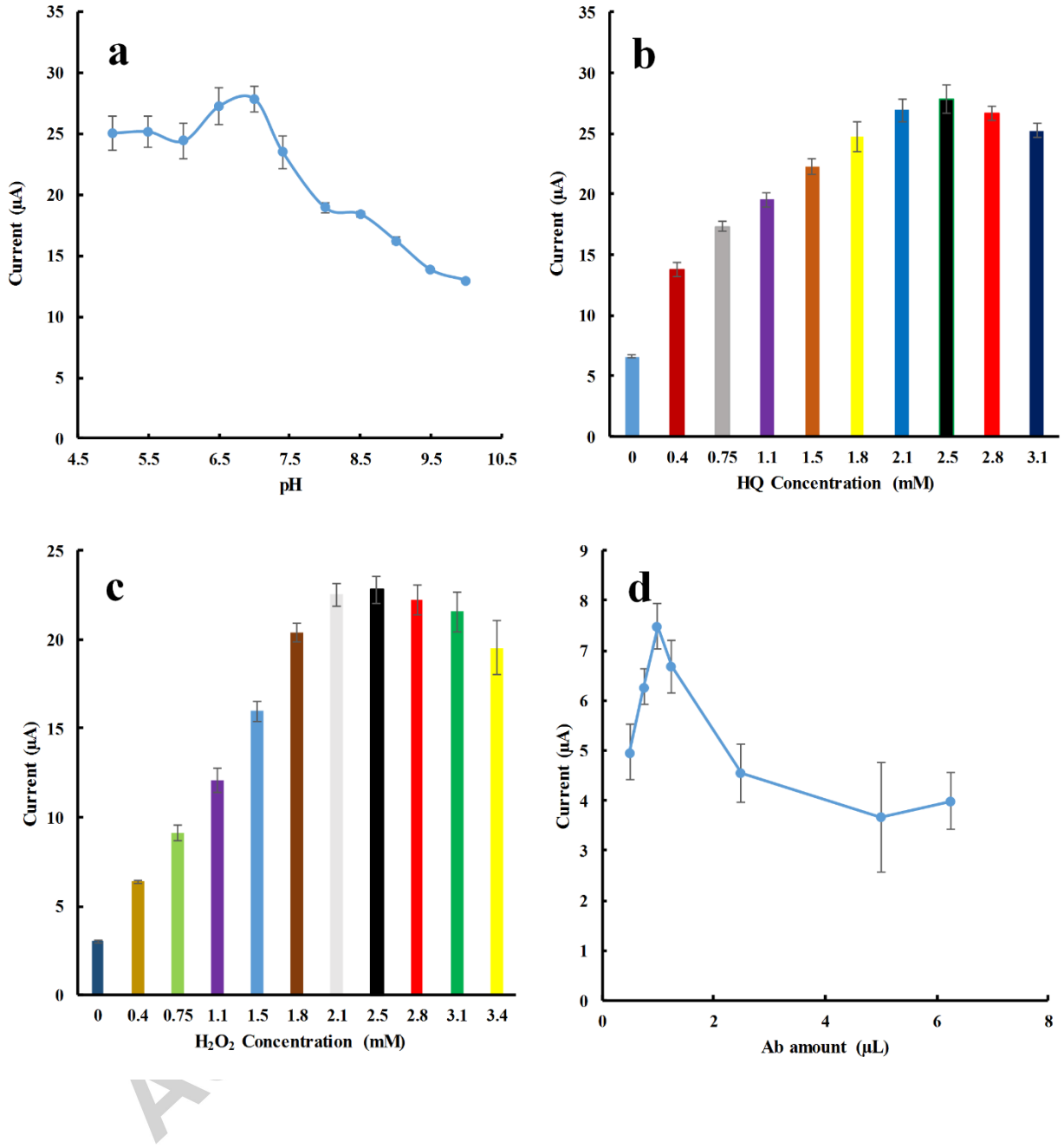
injected into the cell. In the second method, the modified electrode was tested using (**I)** PBS, (**II)** PBS + H_2O_2, and (**III)** PBS + H_2O_2 + HQ. As illustrated in **Fig. 2d**, the peak current of PBS showed no specific redox signals, while after adding H_2O_2, no specific peak appeared. By adding HQ to the aforementioned cell media, the peak current was significantly increased. Having taken all these results into consideration, it can be concluded that the electron transfers between HRP and electrode surface increases in the presence of H_2O_2 and HQ.

In the third approach, the electrode has electrochemically evaluated in the electrochemical cell containing PBS (0.1M, pH 7) plus 2.5 mM HQ and 2.5 mM H_2O_2 in the absence of signaling label. The results revealed no catalytic peak for the use of H_2O_2. Following the addition of the label to complete the sandwich assay and after incubation for 2h, the procedure was repeated under the same condition. The results presented a meaningful peak current corresponding to the presence of HRP in the signaling label. **Fig. 2e** illustrates the electrochemical response of modified immunosensor.

3.4. Optimization of CA 15-3 immunosensor experimental conditions

To get the best performance of the CA 15-3 immunosensor, different factors, which might influence the sensitivity of biosensor, were optimized.

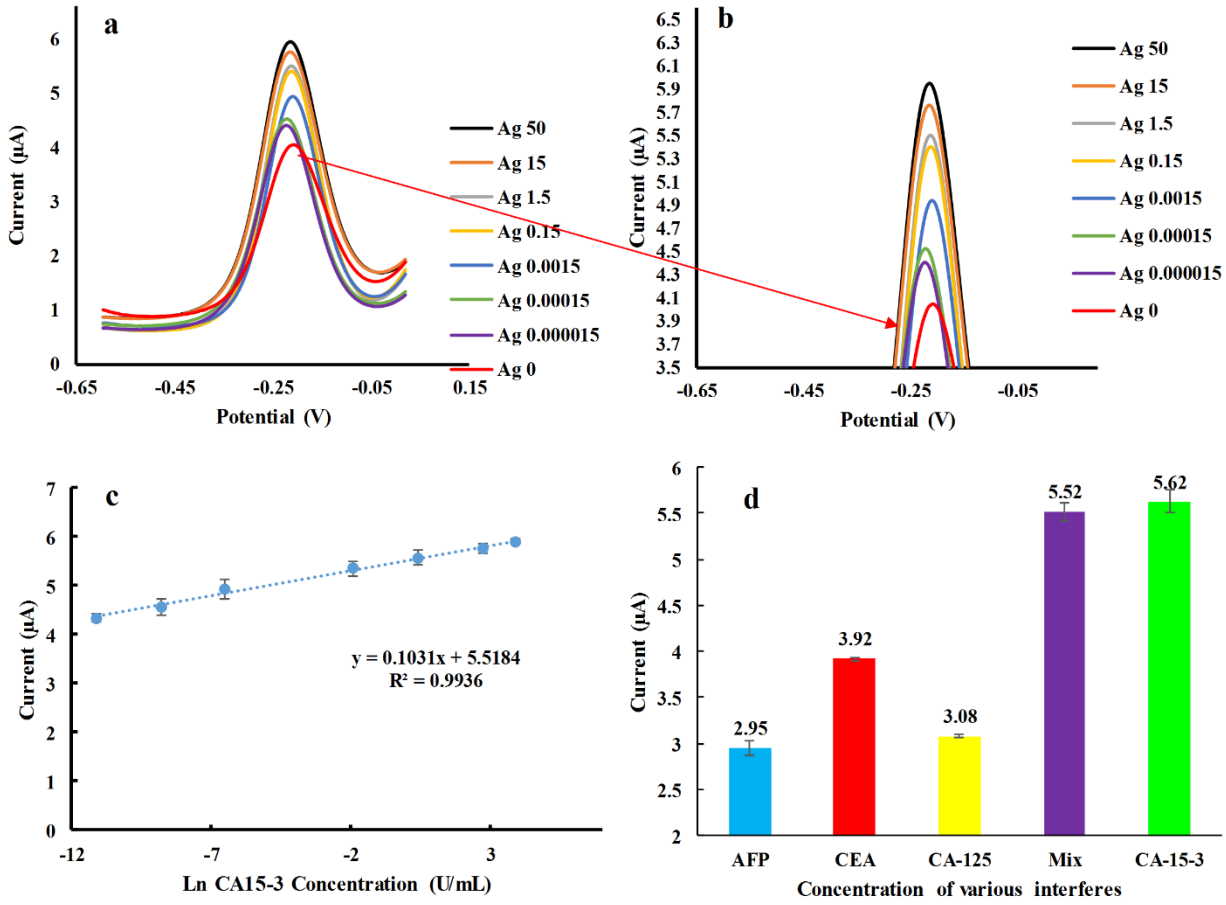
Optimization studies of pH, HQ concentration, H_2O_2 concentration and optimization of the ratio of mAb to HRP conjugated magnetic beads were described in details in the supplementary information. The results of optimization studies are shown in **Fig 3**.



3.5. Analytical performance of engineered immunosensor for quantification of CA 15-3

The electrochemical performance of the proposed modified electrode was investigated using the SWV technique under the aforementioned optimized conditions in the presence of various concentrations of CA 15-3. **Fig. 4a and b** present the SWV voltammograms of the modified

immunosensor for different concentrations of CA 15-3 from 0 to 50 U/mL. It is noteworthy that the detection range of the proposed immunosensor outstrips the previously reported studies (Table 1). The obtained current peaks demonstrated that increasing the CA 15-3 concentration led to an increase in the electrochemical response of immunosensor. In other words, under the optimized circumstances, there was a relationship between the concentration of CA 15-3 and the current peak. The linear relation of CA 15-3 immunosensor response could be described by the equation of I (μA) = 0.1031 [Ln (CA 15-3 concentration)] (U/mL) + 5.5184 with a coefficient correlation of 0.9936 **(Fig. 4c)**. LOQ is the lowest amount of analyte that can reliably practically be detected while LOD is the lowest calculated concentration of the analyte (Armbruster and Pry 2008). The lower limit of quantification (LLOQ) of the proposed immunosensor was practically found to be about 15×10^{-6} U/mL. This LLOQ could outstrip the other CA 15-3 detecting methods, including the commercial methods in addition to previously reported immunosensors (**Table 1**). The proper sensitivity together with the broad linear range of the engineered CA 15-3 immunosensor could be explained considering its signaling label and streptavidin modification of the electrode surface. Basically, possessing four binding capacities, streptavidin can interact with four biotinylated mAbs. Thus, the concentration of capture antibody can reach a dense-loading level. Having capitalized on streptavidin-coated magnetic beads as the signaling label, a significant elevation was seen in both the electrochemical conductivity and the loading capacity(Chikkaveeraiah et al. 2012). Under the normal conditions, only one HRP happen to interact with a secondary mAb, as shown in the routine ELISA as well as other immunosensors. Besides, a further signal amplification of the immunosensor might occur, in large part due to the existence of both HQ and H_2O_2 in the analyzing cell together with the existence of HRP on the signal label.



3.6. Selectivity and interfere study of modified immunosensor

The specificity of immunosensor is considered one of the most important factors that could affect its performance. To demonstrate the selectivity of the immunosensor, its performance was investigated in the presence of 5 ng/mL of α-fetoprotein (AFT), CEA and 10 U/mL of CA 125 and CA 15-3 individually and mixed approach. The results revealed that the engineered immunosensor presented substantially high selectivity towards CA 15-3 (**Fig. 4d**). Further, the presence of other proteins could not markedly influence the specificity of the designed

immunosensor and the impacts of these interfering proteins on the detection CA 15-3 were found to be insignificant.

Table 1. Different studies designed biosensors for detection of CA15-3

Electrode	Detecting method	LOD/ LLOQ	Linear range	Ref
GE/DpAu/Ab/CA 15-3/S-Ab/Co/Nf/TiO_2	DPV	0.3 U/mL	1-100 U/mL	(Wang et al. 2013)
GCE/NGS/Ab/BSA/CA-15-3	DPV	0.012 U/mL	0.1-20 U/mL	(Pressly et al. 2013)
GSPE/CuS-RGO nanocomposite	DPV	0.3 U/mL	1.0-150 U/mL	(Amani et al. 2017)
Au/ZnO Thin fim	SPR	0.025 U/mL	1-40 U/mL	(Chang et al. 2010)
GCE/PEI-CNTs/AuNPs/Ab1/BSA/CA15-3/Ab2/PtNPs/Ru-L-lys/Mn-ZnONRs	ECL	0.014 U/mL	0.05 -120 U/mL	(Zhang et al. 2015)
GCE/GS-Ab1/BSA/CA15-3/Ab-2-f-TiO2-Cd^{2+}	CV, SWV	0.008 U/mL	0.02-60 U/mL	(Zhao et al. 2014)
GCE/OrgSi@Cs-CNT/Pt NCs/GOD/Pt NCs/Ab/GOD/CA 15-3	CV	0.04 U/mL	0.1- 160 U/mL	(Chang et al. 2010)
GCE/DpAu/Ab1/BSA/CA15-3/ZNs-PAMAM-luminul-Ab2	ECL	0.033 U/mL	0.1-120 U/mL	(Jiang et al. 2015)
GCE/$Ru(bpy)_3^{2+}$-Ag@SBA-15/Ab/chit-IL/BSA/CA15-3	ECL	0.004 U/mL	0.01-100 U/mL	(Li et al. 2013)
GCE/GNs?NPG/Thi/CA 15-3/Lip/HRP	DPV	5×10^{-6} U/mL	40-2×10^{-5} U/mL	(Ge et al. 2012)
Current work	SWV	15×10^{-6} U/mL	50-15×10^{-6} U/mL	

LOD: Limit of detection, LLOQ: Lower limit of quantification, GE: Gold electrode, DpAu: Deposition of Au, Ab: Antibody, S-Ab: Secondary antibody, Co: Cobalt, Nf: nafion, TiO2: Titanium oxide, DPV: Differential pulse voltammetry, GCE: Glassy Carbone electrode, NGS: N-doped graphene sheets, BSA: Bovine serum albumin, GSPE: Graphite screen printed electrode, CuS: Copper Sulfide, RGO: Reduced graphene oxide, PEI: Poly ethyleneimine, CNT: Carbon nanotube, Pt: Platinum, Ru: Ruthenium, Mn: Manganese, ZnONRs: ZnO nanorads, GS: Graphene sheet, f-TiO2: functionalized TiO2 , Cd: Cadmium, NP: Nonprecious, ECL: Electrochemiluminescence, SWV: Square wave voltammetry, CV: Cyclic voltammetry, OrgSi@Cs: Organosilica chitosan, NCs: Nanocluster, GOD: glucose oxidase, ZNs: ZnO nanorads, PAMAM: Polyamidoamine, Ag: silver, SBA: Santa Barbara Amorphous-15, Chit: Chitosan, IL: Ionic liquid, GNs: graphene nanosheets, NPG: nanoporous gold, Thi: Thionine, Lip: liposome.

3.7. Reproducibility, repeatability, and stability of immunosensor

Reproducibility of the proposed immunosensor was studied with different approaches. The detection precision of the CA 15-3 immunosensor was investigated via intra-assay and inter-assay methods. The intra-assay approach was evaluated by preparing the CA 15-3 immunosensor at the concentration of 15 U/mL two times (n=4). The results indicated similar responses while the coefficient of variation (CV) calculated to be as low as 2.25%. The precision of the immunosensor for inter-assay was calculated by preparing the CA 15-3 immunosensor at the concentration of 1.5 U/mL with three individually electrodes (n=4). The results showed similar electrochemical responses with an acceptable coefficient of variation (3.16%). The obtained results demonstrated an acceptable precision and reproducibility for the engineered CA 15-3 immunosensor. Taking the previously reported studies into consideration, the obtained results are comparable to with their findings (2.9, 2.51 and 3.8%) (Amani et al. 2017; Babamiri et al. 2018; Khoshroo et al. 2018). The repeatability of immunosensor was investigated by repeating the measurement of fabricated CA 15-3 immunosensor at the concentration of 15 U/mL for 30 times at the same condition. The results indicated similar electrochemical responses while the coefficient of variation was calculated to be 2.44%. The stability of engineered immunosensor was investigated by its storage in PBS (pH 7; 4 °C) for 15 days. The results revealed that during this period of storage, the immunosensor did not show noteworthy changes in its primary responses. Further, the CA 15-3 immunosensor showed 99% and 96% of its initial response respectively on days 11 and 15 of the storage period, indicating excellent stability (**Fig. S3**). In comparison with previously published studies showing with the stability of 10 (94% of initial response) to 30 (90% of initial response) days (Ao et al. 2014; Zhao et al. 2014), the proposed immunosensor in this study displayed an acceptable stability.

3.8. Real Sample Analysis of CA 15-3 in patient's serum

The applicability of the engineered CA 15-3 immunosensor for the detection of CA 15-3 in real samples was also investigated. To meet this goal, four serum samples of patients were used to evaluate the accuracy and potential of the engineered CA 15-3 immunosensor in the clinical practice. The results were compared with the ELISA as the gold-standard method of detection (**Table 2**). The immunosensor showed excellent detection potential with improved LOD towards detecting CA 15-3 in the patient's serum samples as compared to the ELISA results. Based on these findings, the proposed immunosensor could be potentially used in the clinic to evaluate the amount of CA 15-3 in patients with breast cancer.

Table 2. Detection of CA 15-3 with proposed immunosensor and ELISA in real samples

Sample	**Proposed immunosensor (U/mL)***	**ELISA (U/mL)**
S1	34.28±0.07	34
S2	17.74±0.36	18.8
S3	25.9±0.12	26.6
S4	20.23±0.18	20.5

*number of measurement (n=3).

Conclusion

Here, we reported a reliable and ultrasensitive sandwich-type electrochemical immunosensor for the detection of CA 15-3, a breast cancer biomarker, in patients with the breast cancer. To engineer the immunosensor, the least surface modification of the electrode was applied, while magnetic bead-HRP linked anti-CA 15-3 mAb was used as the signaling label. Streptavidin facilitated the assembly of the biotinylated anti-CA 15-3 mAb on the surface of the gold

electrode while BSA prevented from non-specific binding of other interferers. The electrochemical signals were improved by utilizing HRP-linked MBs with anti-CA 15-3 mAb (i.e., biotinylated HRP/ streptavidin coated magnetic beads/ biotinylated mAb). The magnetic beads caused the maximum loading capacity of anti-CA 15-3 mAb linked with HRP, and consequently, the interaction between CA 15-3 and anti-CA15-3 mAb reached its highest level. The engineered immunosensor presented remarkable analytical detection efficiency for the CA 15-3 with excellent sensitivity and specificity, even in the presence of other proteins' interference. As the final assessment, the potential application of the immunosensor in terms of the detection of the CA 15-3 in the human serum was examined with results showing excellent detection potential as compared to the results of ELISA as the gold standard method. Taken all into the considerations, this proposed immunosensor may be counted as a simple, ultrasensitive, and specific method for the detection of CA 15-3 in the clinical samples of the breast cancer patients and used for the early diagnosis of the disease.

Ethical issues

This study was ethically approved (IR.TUMS.REC.1394.1273) by Tehran University of Medical Sciences. All human samples were collected with patients' consent by signed forms.

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Conflict of interests

The authors have no conflict of interest to declare relevant to this article.

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Fig. 1. The developing steps of immunosensor. Streptavidin was immobilized onto the gold electrode, followed by introducing (1) CA15-3 monoclonal antibody and (2) BSA for blocking non-specific binding, then CA 15-3 as target analyte was put on the electrode. Streptavidin-coated magnetic beads were conjugated with biotinylated HRP and CA15-3 mAb, and then, cast on the electrode. Finally, the electrode was measured electrochemically in PBS containing optimized amounts of HQ and H_2O_2. SA: Streptavidin, Ag: CA 15-3, Ab: Anti body, BSA: Bovine serum albumin, MB: Magnetic beads, HRP: Horse radish peroxidase.

Fig. 2. Preparation steps of immunosensor in impedance standard solution and electrochemical evaluation of the immunosensor. (a) Impedance curves of preparation steps. (b) CVs of preparation steps of immunosensors. (c) The first approach in PBS with HQ followed by adding H_2O_2. (d) The second approach in PBS with H_2O_2 followed by adding HQ. (e) The third approach; modified electrode in the absence and presence of the signaling label in detecting solution, PBS with HQ and H_2O_2.

Fig. 3. Optimization of proposed CA15-3 immunosensor. (a) pH optimization in a constant amount of HQ and H_2O_2. (b) HQ concentration in optimized pH. (c) H_2O_2 concentration in an optimized pH and HQ. (d) The selected amount of CA 15-3 mAb (2.5 µg/mL) based on the optimized ratio of magnetic beads, HRP, and pH, HQ and H_2O_2.

Fig. 4. The square wave voltammetry (SWV) analysis and interfaces study. (a) SWV of the engineered immunosensor after incubation with 50, 15, 1.5, 0.15, 0.0015, 0.00015, 0.000015 and

0 U/mL of CA 15-3 in PBS (pH 7, 1mM) containing 2.5mM HQ and 2.5mM H_2O_2. (b) A magnified view of the voltammograms. (c) The plot of current responses of immunosensor vs. Ln CA 15-3 concentration. (d) Interface analysis of proposed immunosensor with different interferes, including AFP, CEA, CA 125 and CA 15-3 and their mixture.

Highlights:

- An ultrasensitive and reliable immunosensor was developed for the detection of CA 15-3.
- An enzymatic sandwich approach was applied to enhance the signals of the biosensor.
- A significant lower limit of quantification was achieved.
- An excellent performance of proposed immunosensor was observed compared to ELISA as the reference method.