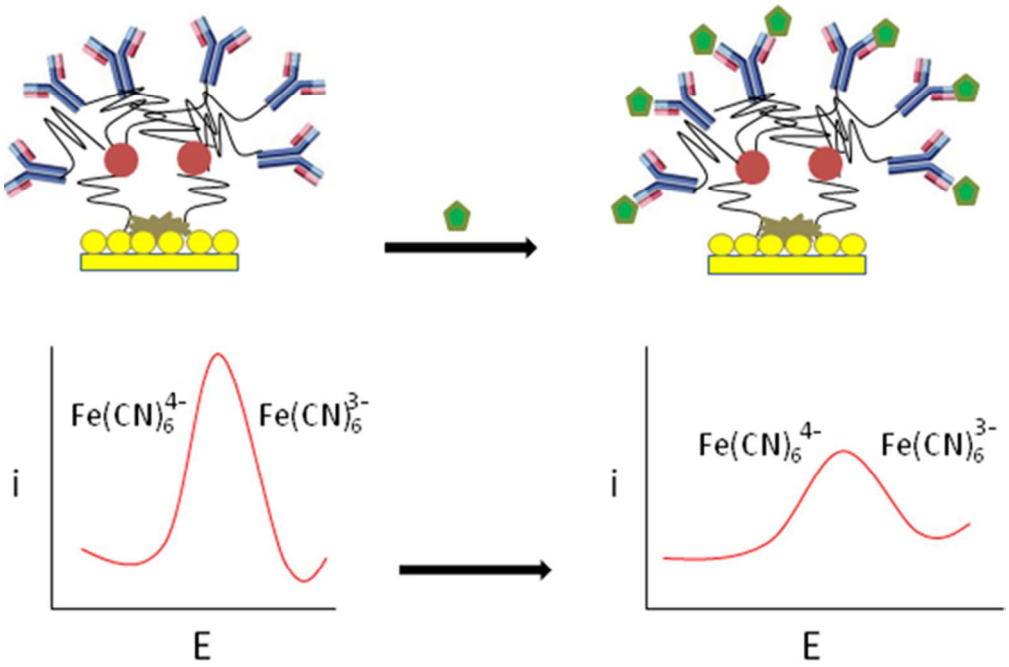
Analyst

Accepted Manuscript

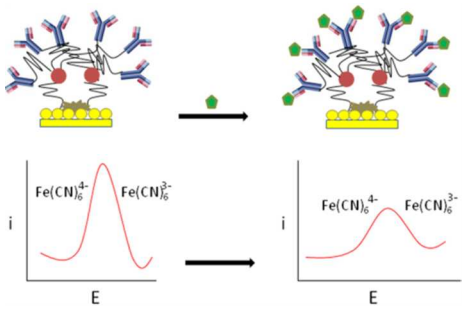
ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/analyst



Graphical Abstract
60x39mm (300 x 300 DPI)

This paper presents a facile and highly sensitive label free electrochemical immunosensor for breast cancer biomarker using antiHER2- Fe_3O_4 NP bioconjugate.



Electrochemical immunosensor for breast cancer biomarker based on antiHER2-Iron oxide Nanoparticle Bioconjugate

Mahdi Emami[a], Mojtaba Shamsipur[b], Reza Saber[c,d]*, Rasoul Irajirad[d]

[a]Schoolof chemistry, University college of science, University of Tehran, Tehran, Iran

[b]Department of chemistry, Razi university, Kermanshah, Iran

[c]Department of nanotechnology, School of Advanced Technologies in Medicine, Tehran university of medical science, Tehran, Iran

[d]Research center for science and technology in medicine, Imam Khomeini hospital, Tehran, Iran

*Corresponding Author. Tel. +98 21 66907525 fax: +98 21 66581533.

E-mail address: rsaber92@yahoo.com (R. Saber).

Abstract

A label free immunosensor was designed for ultra-detection of human epidermal growth factor receptor 2 (HER2) in real samples using differential pulse voltammetry (DPV) method. In a separate process, anti-HER2 antibodies were attached to iron oxide nanoparticles (Fe_3O_4 NPs) to form stable bioconjugates which were later laid over the gold electrode surface. In this way, by the advantage of their long terminals, the bioconjugates provided the most possible space for the immune-reaction between biomolecules. Under optimal conditions, the immunosensor was responsive to HER2 concentrations over the ranges of 0.04 – 10 ng mL^{-1} and 10 – 100 ng mL^{-1} linearly and benefited from a satisfying detection limit as low as 0.995 pg mL^{-1} and a favorable sensitivity as sharp as 5.921 µA mL ng^{-1}. The reliability of the method in clinical analysis was proved by successful quantization of HER2 levels in serum samples obtained from patients. Furthermore, the precision and the stability of the method were evaluated and verified to be acceptable in immunoassay studies.

Keywords: Electrochemical immunosensor, HER2, bioconjugate, breast cancer, gold electrode

1. Introduction

To realize low-level of tumor biomarkers is vital for early awareness of cancers and commence the appropriate treatment processes. Human epidermal growth factor receptor 2 (HER2) as a key prognostic marker [1], is over-expressed in 15-25% of breast cancer [2] which is one of the most common malignant type of tumor in women [3]. To establish a fast technique sensitive to the low-levels of HER2 biomarker which results early diagnosis of the cancer is of great significance not only for increasing the survival rate, but also for saving cost and time in successful prognosis of disease.

For this purpose, several techniques [4-7] were developed focusing on detection of HER2 positive cells which are usually taken out in invasive methods like biopsy and are not available in human serum. In comparison to these techniques, electrochemical techniques by the use of bioconjugate modified electrodes are the most desired systems owing their excellent sensitivity, rapidity, low cost and easy

operation. Typically there are two kinds of electrochemical detection platforms for biomarker proteins. The first kind is labeled method known as sandwich type method in which an enzyme usually horse radish peroxidase, is attached to a secondary antibody (Ab). This labeled Ab remains tied to the biomarker attached to the primary Ab and usually catalyzes the reduction of hydrogen peroxidase to represent a measurable signal [8, 9]. However, other nanomaterials such as CdS and silver nanoparticles can also be attached to secondary antibodies and their stripping signals corresponding to the concentration of biomarkers be recorded subsequently [10, 11]. Although this strategy is assumed as a highly sensitive method, but problems such as sample pretreatment, separation and purification process of secondary Ab limit the approach. In the second kind, known as label free method, decrease in signal intensity of a redox probe is directly related to the concentration of biomarker, which is bound to a modified surface and hinder the electron transfer process [12, 13]. Eliminating time-consuming extra processes makes this method more simple, quick and desired.

Lately, functionalized nanopardicles specially functionalized iron oxide nanoparticles (Fe_3O_4 NPs) have attracted much attention in the fabrication of bio-sensing systems due to their unique properties such as biocompatibility, signal amplification and their ability to bind covalently to Abs via their functional groups [14, 15].

The extensive use of poly ethylene glycol (PEG), as a long compatible linker for nanoparticles, has been treated well [16-18]. The main advantage of using PEG is to provide enough space to bind more Abs to nanoparticles and allow them to stand aside and result more effective combination with the targets.

In this work, we attached different proportions of anti-HER2 Ab to the pegylated Fe_3O_4 NPs to form highly loaded bioconjugates. Designing a label free platform, the most appropriate bioconjugate was stabilized covalently on the surface of a gold electrode to assay ultra-low levels of HER2 antigen in serum samples. This highly sensitive and simple electrochemical analysis method holds great potential for detection of all other biomarkers in clinical diagnostics.

2. Experimental

2.1. Apparatus and conditions

CV and DPV measurements were conducted on a µAutolab Type III Potentiostat/Galvanostat. A three electrode cell system was used for the electrochemical experiments. A modified gold electrode was used as the working electrode. A platinum wire and Ag/AgCl (Saturated KCl) electrode were used as the counter electrode and the reference electrode, respectively. The EIS spectra were recorded with an Autolab Eco Chemie. B.V. Potentiostat/Galvanostat using the same electrode system.

The transmission electron microscopy (TEM) images were obtained from a TEM EM 208 Philips at an acceleration voltage of 100 kV. FT-IR spectra were recorded with a Bruker vertex 70v. The surface morphologies of GE and GNPs/GE were evaluated by field effect scanning electron microscopy (FESEM) at an accelerating voltage of 20 kV.

2.2. Materials and reagents

Anti-HER2 Ab (Herceptin, 150 mg) was purchased from F. Hoffman-La Roche Ltd (Switzerland). Active HER2, 5 µg, was obtained from Biovision Inc. (USA). Poly (ethylene glycol) - maleimide *ƒ* -NHS ester (Mal- PEG-NHS, MW „ 2000) was purchased from NANOCS (USA). Bovine serum albumin (BSA), N-hydroxy succinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), cysteamine (Cys), 2-iminithiolane (Traut…s reagent), gold (III) chloride hydrate, sodium phosphate dibasic, potassium phosphate monobasic and 3-mercaptopropionic acid (MPA) were purchased from Sigma-Aldrich ltd (USA). Iron (III) chloride hexahydrate, iron (II) chloride tetra hydrate, hydrochloric acid (37%), methanol, toluene, 3-aminopropyltrimethoxysilane (APTMS) and ammonium hydroxide (32%) were purchased from Merck (Germany). Phosphate buffered solutions (PBS) were prepared using 0.1M Na_2HPO_4 and 0.1M KH_2PO_4. All other chemicals and reagents were of analytical grade and were prepared using redistilled water.

2.3. Production of functionalized Fe_3O_4 NPs

2.3.1. Synthesis of bare Fe_3O_4 NPs

Fe_3O_4 NPs were synthesized based on the most common method [19]. Briefly, $FeCl_2.4H_2O$ (0.397 g in 1 mL of 2 M hydrochloric acid) was added to $FeCl_3.6H_2O$ (1.08 g in 4 mL of 2 M hydrochloric acid) under strong stirring over a magnetic stirrer. Then, 50 mL of 0.7 M ammonium hydroxide was added to the mixture drop wisely. At the end of the reaction, the black suspension of iron oxide was separated by a permanent magnet and then redispersed in 10 mL of methanol.

2.3.2. Synthesis of the amine-Fe_3O_4 NPs (APTMS-coated Fe_3O_4 NPs)

Bare Fe_3O_4 NPs tend to aggregate and therefore it is better to start the modification process of the surface immediately. 35 mL of toluene and 25 µL of APTMS were added to 0.1 g of bare Fe_3O_4 NPs. The mixture was sonicated in a bath sonicator for 30 min. Afterwards, the mixture was heated in an oven (60 °C) for 7 h. Finally, the obtained APTMS-coated Fe_3O_4 NPs was separated by a permanent magnet and redispersed in 50 mL of methanol.

2.3.3. Synthesis of PEG-maleimide-coated Fe_3O_4 NPs

The resulted nanoparticles were introduced with NHS-PEG2000-Mal to obtain sulfhydryl-reactive pegylated nanoparticles through amidization of the surface amine groups. For this purpose, 31 mg of NHS-PEG2000-Mal was added to 10 mL of redistilled water containing 10 mg of nanoparticles. After sonication for 30 min, the mixture was stirred vigorously for 12 h. Finally, pegylated nanoparticles were separated by magnet and redispersed in 5 mL of redistilled water.

2.4. Preparation of the bioconjugates

The bioconjugates were prepared based on a reported method [20] with some modifications. Scheme 1 shows the bioconjugate preparation procedure.

Scheme 1 Preparation of the bioconjugate

2.4.1. Thiolation of anti-HER2 Abs

A solution of anti-HER2 Ab (1 mg mL^{-1}) in 0.1 M PBS pH 8 was prepared first. For thiolation of Abs, it was followed by adding 100-fold molar excess of 2-iminithiolane to the prepared solution. To protect the thiol groups from oxidation, 5 mM EDTA was also added to the mixture. The mixture was left for 1 h under constant stirring at room temperature. Afterwards, thiolated Abs were purified by dialysis against 20 mL of PBS pH 8, 5 times each for 1 h.

2.4.2. Attachment of anti-HER2 Abs to the NPs

The Abs-labeled nanoparticle conjugate was prepared through the following approach: 1 mL of activated nanoparticles (5 mg mL^{-1}) was incubated with different aliquots (50, 100, 200 and 300 µL) of thiolated Abs (1 mg mL^{-1}) over night at room temperature under constant shaking. The thiol groups of Abs were covalently attached to the unsaturated bond of maleimide linked to the nanoparticle to form the bioconjugate. The bioconjugates, then, were separated by magnet and redispersed in 1 mL of PBS pH 7.2.

2.5. Fabrication of the immunosensor and electrochemical procedure

The fabrication process of the immunosensor is represented in scheme 2. Firstly, the gold electrode (GE) surface (r = 3 mm) was polished with 0.1 µm alumina slurry on a polishing cloth for 1 min and was then ultrasonically cleaned with ethanol and redistilled water each for 3 min respectively. Afterwards, the electrode was immersed in piranha solution (H_2SO_4 : H_2O_2, 3:1) for 5 min and then was washed with pure water several times. The gold nanoparticles (GNPs) was electrodeposited on the cleaned electrode surface from solution containing 0.06 mM $HAuCl_4$ and 0.1 M KCl by cycling potential between −0.5 and 0.5 V for 20 times with the scan rate of 0.2 mV s^{-1}. The gold modified electrode was immerged in 0.1 M MPA for 12 h at room temperature to form MPA/GNPs/GE. The resulted electrode was rinsed with redistilled water to remove physically adsorbed materials. Thereafter, 30 µL of solution containing 0.1 M EDC + 0.1 M NHS was dropped and maintained onto the surface 2 h at room temperature to activate the carboxyl groups. After being washed with pure water, the electrode was immersed into 0.1 M Cys Solution for 8 h at room temperature to form Cys/MPA/GNPs/GE via amide formation. Subsequently, after being rinsed with redistilled water, to prepare Bioconjugate/Cys/MPA/GNPs/GE, the obtained electrode was introduced to 30 µL of bioconjugate and left for 8 h in 4 ºC. The double bond in free maleimides of bioconjugate readily reacts with thiol groups from Cys to form a stable carbon-sulfur bond [21]. Excessive and physically adsorbed bioconjugates were washed away with PBS (0.1, pH 7.2) and redistilled water respectively. The process was followed by casting 20 µL of BSA (1 mg mL^{-1}) over the electrode and keeping for 45 min at 37 ºC to block any possible nonspecific binding sites. Finally

BSA/Bioconjugate/Cys/MPA/GNPs/GE was rinsed with PBS (0.1 M, pH 7.2) and redistilled water respectively. The resulting electrode was then incubated with 20 µL of different concentrations of HER2 antigen for 30 min at 37 ºC and was washed again with PBS (0.1 M, pH 7.2) and redistilled water respectively. Finally, DVPs of the redox probe solution (PBS 0.05 M, pH 7.2 containing 5 mM of $Fe(CN)_6^{3-/4-}$) were recorded from -0.2 to 0.5 V using HER2/BSA/Bioconjugate/Cys/MPA/GNPs/GE.

Scheme 2. Graphical illustration of the construction procedure of the immunosensor and HER2 detection.

2.6. Patient serum analysis

Fresh serum samples, collected from patients in different stages of the cancer, were obtained kindly from the central clinical laboratory of Imam Reza hospital, Kermanshah, Iran. Serum samples were diluted with PBS (0.1M, pH 7.2) for 20 times and then, 20 µL of the samples was dropped onto the prepared

electrode and kept for 30 min at 37 ºC. Finally, DPVs of the probe solution were recorded after rinsing the electrode with PBS and redistilled water respectively. HER2 levels for three replicates were calculated using calibration regression equations.

2.7. Recovery test

Fresh serum samples of two healthy females were obtained from Imam Khomeini hospital. After 20 times dilution with PBS (0.1M, pH 7.2), serum samples were spiked with two different concentrations of HER2. The spiked concentrations were assayed using standard addition method.

2.8. Electrochemical measurements

Cyclic voltammograms (CVs) were recorded between -0.2 and 0.5 V with the scan rate of 20 $mV s^{-1}$ in 0.1 M PBS pH 7.2 containing 10 mM $Fe(CN)_6^{3-/4-}$. The parameters for DPVs, taken from the same probe solution, were: pulse width of 0.06 s, pulse increment of 5 mV, pulse period of 0.1 s, pulse amplitude of 55 mV and scan rate of 50 $mV s^{-1}$.

EIS measurements were conducted for 0.1 M PBS pH 7.2 containing 2 mM $Fe(CN)_6^{3-/4-}$ in a frequency range from 0.1 to 100 kHz. The amplitude of the applied sine wave was 10 mV with the direct current potential set at 0.2 V.

3. Results and discussion

3.1. Characterization of Fe_3O_4 NPs

The morphology and size distribution of Fe_3O_4 NPs was characterized by TEM. As can be observed in Fig.1A, spherical nanoparticles with the average size of about 20 nm were distributed uniformly. SEM was also used to confirm the electrodeposition process. Figs.1B and C exhibit the surface of the electrode before and after electrodeposition of the nanoparticles, respectively. A rough and stony surface is obtained in consequence of gold electrodeposition.

Fig. 1. (A) TEM image of Fe_3O_4 NPs, (B and C) FESEM images of gold electrode surface before and after GNPs electrodeposition, respectively (D) IR spectra of APTMS coated Fe_3O_4 NPs, (E) CVs of 0.1 M PBS pH 7.2 containing 10 mM $Fe(CN)_6^{3-/4-}$ at a: MPA/GNPs/GE and b: Fe3O4 NPs/ MPA/GNPs/GE

FT-IR spectroscopy was operated to validate the presence of APTMS at the surface of APTMS-coated Fe_3O_4 NPs. The spectrum in Fig. 1D shows a sharp band around 530 cm^{-1} for iron oxide NPs and three moderate bands at 1100, 1620 and 2930 cm^{-1} that can be assigned to vibration of C-Si, N-H and stretching of C-H, respectively.

To characterize the APTMS-coated Fe_3O_4 NPs electrochemically, MPA/GNPs/GE was modified with the nanoparticles through combining carboxyl and amine groups, and its performance was observed before and after modification practice using CV technique. As can be seen in Fig. 1E, the presence of the NPs intensifies the redox signal distinctly. As an explanation for this event, the intrinsic properties of nano-sized iron oxide particles decorated with functional groups facilitate the electron transfer (ET) process between the probe and the electrode. This desirable quality of the functionalized Fe_3O_4 NPs makes them proper candidates for Abs to be loaded over and to form bioconjugates for using in electrochemical systems.

3.2. Characterization of the immunosensor

The most common technique CV was initially used to monitor each step of modification of the gold electrode surface (Fig. 2A, B). As can be seen, a pair of redox peaks is observed for 10 mM of $Fe(CN)_6^{3-/4-}$ in PBS (0.1 M, pH 7.2) at bare gold electrode (a), (E_p = 94 mV). Sharper redox peaks with less difference in peak potential (ΔE_p = 76 mV) were noticed for the electrode after electrodeposition of gold nanoparticles (b). Subsequently, at the end of immersing of GNPs/GE in MPA solution to obtain MPA/GNPs/GE through formation of Au-S bonds, redox peaks increased again clearly (c). In here, dangling carboxyl groups at the surface may facilitate the electron transfer (ET) process between the probe and the electrode. Further modification of the electrode with Cys reduced the redox peaks (d). It is probably due to the ET blockage in consequence of increasing the length of carbon chain as well as

turning carboxyl groups to terminal sulfhydryl groups. Afterward, although Fe_3O_4 NPs, because of their intrinsic properties, intensify the redox signal which has been investigated in section 3.1, but placing of the bioconjugates at the surface reduced the redox peaks since more interruption in ET process happens by the presence of the huge biomolecules (Abs) on the nanoparticles (e). Thereafter, in order to block the possible nonspecific bonding sites at Bioconjugate/Cys/MPA/GNPs/GE, BSA was applied to the surface of the electrode and thus an obvious decrease in redox peaks occurred in (f). Finally, introducing of HER2 (10 ng mL^{-1}) to the resulted electrode made more decrease in redox peaks (g).

Furthermore, electrochemical impedance spectra (EIS, Nyquist plots) were also conducted to monitor the performance of the immunosensor through construction (Fig. 2C). Semicircle part of the Nyquist plots at higher frequencies is related to the ET limited process, so it is possible to investigate the surface change at each step of the modification process by measuring the semicircle diameter which equals the ET resistance (R_{et}). Spectrum (a) shows a tiny semicircle for bare gold electrode. Modification of the electrode with GNPs and MPA gave smaller and quite smaller values of faradic impedance for GNPs/GE and MPA/GNPs/GE, respectively (b, c) indicating that these modifications increase the ET process of the system. After modification of the resulted electrode with Cys, semicircle diameter was extended and thus an initiated impedance was observed in (d). Predictably, after loading of the surface with bioconjugates, R_{et} increased sharply because of the main hindrance against ET process caused by spacious Abs (e). Comparing with other works [8, 13], in this step, less increase in R_{et} was observed that is probably due to the advantageous use of iron oxide NPs with their positive effect on ET process. Blocking some parts of the surface, further modification of the electrode by BSA resulted an additional increase in R_{et} (f). Finally, specific coupling of HER2 with anti-HER2 Ab at the surface of the immunosensor made more interference in the ET process and thus increased R_{et} in consequence (g).

Fig. 2. (A and B) CVs of 0.1 M PBS pH 7.2 containing 10 mM $Fe(CN)_6^{3-/4-}$ at a: Bare GE, b: GNPs/GE, c: MPA/GNPs/GE, d: Cys/MPA/GNPs/GE, e: Bioconjugate/Cys/MPA/GNPs/GE, f: BSA/Bioconjugate/Cys/MPA/GNPs/GE and f: HER2/BSA/Bioconjugate/Cys/MPA/GNPs/GE. (C) Nyquist plots for 0.05 M PBS pH 7.2 containing 2 mM $Fe(CN)_6^{3-/4-}$ obtained at different electrodes (as A and B)

3.3. Optimization of analytical variables

3.3.1. The quantity of the stabilized Abs on the NPs

The effect of the amount of the Abs loaded on Fe_3O_4 NPs was investigated over the response of the immunosensor. For this purpose, various loaded bioconjugates, prepared by different quantities of thiolated anti-HER2 (50, 100, 200 and 300 µg), were used to construct the immunosensor. As can be seen in Fig. 3A, the current change is intensified by increasing the anti-HER2 Abs amount up to 200 µg. Using more amount of Abs probably results in saturation of the nanoparticle surface and interferes the effective binding of the bioconjugate to the surface of the electrode, so the optimum amount of the anti-HER2 was selected to be 200 µg.

3.3.2. Incubation time

Since the formation of the covalent bonds in the modification steps is slow, the incubation time in each relevant step plays an important role and needs to be optimized. Fig. 3B shows the effect of incubation times of three modifier components on the immunosensor performance. The optimum incubation time for MPA, Cys and anti-HER2 Ab were obtained to be 12, 8 and 8 h respectively. More incubation times had no positive effect on the signal most likely due to the saturation of the electrode surface.

Fig. 3. Optimization of analytical variables. (A) Current change vs. Ab amount (n = 3), (B) Effect of incubation time over the response of the immunosensor (n = 3).

3.4. Analytical performance of the immunosensor

Under the most advantageous constructing conditions, the response of the immunosensor towards different concentrations of HER2 was studied by recording DVPs of PBS (pH 7.2) containing 5 mM $Fe(CN)_6^{3-/4-}$ with the result shown in Fig. 4A. The resulting calibration curve is linear over two concentration ranges from 0.01 to 10 ng mL^{-1} and 10 to 100 ng mL^{-1} (Fig. 4B). The corresponding calibration regression equations for lower and higher concentration ranges are: $I\ (\mu A) = (5.921 \pm 0.091)$ [HER2] (ng mL^{-1}) + (11.507 ± 0.511), $R^2 = 0.9981$ and $I\ (\mu A) = (0.300 \pm 0.008)$ [HER2] (ng mL^{-1}) + (69.357 ± 0.412), $R^2 = 0.9913$ respectively.

Fig. 4. (A) DPVs of the probe solution taken at the immunosensor after incubation with a: 0, b: 0.01, c: 0.4, d: 1, e: 2, f: 4, g: 8, h: 10, i: 25, j: 40 and k: 100 ng mL^{-1} of HER2. (B) Calibration graph (current change vs HER2 concentrations), (n = 4)

The detection limit (DL) was evaluated as $3s/m$ and determined to be (0.995 ± 0.022) pg mL^{-1} where s is the standard deviation of the peak current (n = 5) and m is the slope of the calibration curve for lower

concentration range. The sensitivity of this method (5.21 ± 0.091 A mL ng^{-1}) is more than five times that of the most recent method for quantification of HER2 biomarker [10]. Beside this advantage, the more convenient fabrication process and simplicity of the method would make it comparable with other methods which were reported sensitive to HER2. To corroborate the superiority of this method, a comparative study was done with the results shown in Table 1.

Table 1

Comparison of HER2 results for the recent proposed methods.

method	Linear Range (ng mL^{-1})	Detection limit (pg mL^{-1})	reference
PEMS[1]	0.05 – 2	25.3	22
OFRR[2]	13 – 100	13000	23
ST-ECIS[3]	6 – 30	6000	24
LF-ECIS[4]	0.01 – 10 10 – 100	0.995	This work

[1] Piezoelectric microcantilever sensor

[2] opto-fluidic ring resonator

[3] Sandwich type electrochemical immunosensor

[4] Label free electrochemical immunosensor

3.5. Real sample analysis

The proposed method was applied to quantify HER2 concentrations in serum samples collected from several patients. Results obtained by this method were compared with those obtained by the expensive reference method, enzyme linked immunosorbent assay (ELIZA) method, (Table 2). From the good agreement between two methods, it can be deduced that this method can be assumed as a replacing applicable method for quantization of the biomarker.

Table 2

HER2 levels in patient serum samples obtained by the proposed and ELIZA method

Serum sample no.	Proposed method (ng mL^{-1}), (n =3)	ELIZA method (ng mL^{-1})	Relative error (%)
1	13 ± 0.5	12.7 ± 0.3	2.36
2	26 ± 0.9	24.9 ± 0.1	4.41
4	24.5 ± 0.6	23.5 ± 0.3	4.25
5	61 ± 0.8	62.1 ± 0.4	-1.77
6	84.8 ± 0.9	86.1 ± 0.3	-1.51

3.6. The precision and stability study

To evaluate the precision of the method, serum samples were spiked with HER2 and the recoveries were extracted using standard addition method. The percentage recoveries for replicates of added 2 and 20 ng mL^{-1} HER2 were assayed to be (101 ± 0.9) % and (101.8 ± 1.2) % respectively. Satisfactory recoveries verified the good precision of the method.

The stability of the method was also tested by recording the results of the immunosensor which has been already kept in 0.1 M PBS pH 7 for three weeks at 4 ºC. A (9 ± 1) % decrease in primary results indicated that the method benefits from acceptable stability (n =3).

4. Conclusion

A facile and novel electrochemical immunosensor for detection of breast cancer biomarker was constructed based on anti-HER2-Fe_3O_4 NP bioconjugate. The use of bioconjugate not only facilitates the electron transfer in redox probe due to the good nature of the nanoparticles, but also increases the sensitivity of the method by loading as much Abs on the board and keeping them far enough from the electrode surface to interact with biomarkers more efficiently. The method has low detection limit with the excellent sensitivity and seems to be unique and comparable with other methods responsive to HER2

and could be assumed as a promising replacing technique for cancer recognition. Fe_3O_4 NPs can be anchored with various Abs, so this immunoassay method holds great potential for detection of all other biomarkers in clinical diagnostics.

References

[1] M.A. Owens, B.C. Horten, M.M. Da Silva, HER2 amplification ratios by fluorescence in situ hybridization and correlation with immunohistochemistry in a cohort of 6556 breast cancer tissues, Clin. Breast Cancer 5 (2004) 63–69.

[2] D.J. Slamon, G.M. Clark, S.G. Wong, W.J. Levin, A. Ullrich, W.L. McGuire, Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene, Science, 235 (1987) 177–182.

[3] P. Boyle, B. Levin, World Cancer Report, International Agency for Research on Cancer, 2008.

[4] W. Lu, S.R. Arumugam, D. Senapati, A.K. Singh, T. Arbneshi, S.A. Khan, H. Yu, P.C. Ray, Multifunctional oval-shaped gold-nanoparticle-based selective detection of breast cancer cells using simple colorimetric and highly sensitive two-photon scattering assay, ACS Nano 4 (2010) 1739–1749.

[5] K. Li, R. Zhan, S.S. Feng, B. Liu, Conjugated polymer loaded nanospheres with surface functionalization for simultaneous discrimination of different live cancer cells under single wavelength excitation, Anal. Chem. 83 (2011) 2125–2132.

[6] M.C. Tsai, T.L. Tsai, D.B. Shieh, H.T. Chiu, C.Y. Lee, Detecting HER2 on cancer cells by TiO_2 spheres Mie scattering, Anal.Chem. 81 (2009) 7590–7596.

[7] J. Gao, K. Chen, Z. Miao, G. Ren, X. Chen, S.S. Gambhir, Z. Cheng, Affibody based nanoprobes for HER2-expressing cell and tumor imaging, Biomaterials 32 (2011) 2214–2148.

[8] X. Zhua, J. Yangb, M. Liua, Y. Wua, Z. Shena, G. Li, Sensitive detection of human cancer cells based on aptamer–cell–aptamer sandwich architecture, Anal. Chim. Acta 764 (2013) 53–59

[9] L. Wang, X. Jia, Y. Zhou, Q. Xie, S. Yao, Sandwich-type amperometric immunosensor for human immunoglobulin G using antibody adsorbed Au/SiO_2 nanoparticles, Microchim. Acta 168 (2010) 245–251.

[10] Y. Zhu, P. Chandra, Y. Shim, Ultrasensitive and selective electrochemical diagnosis of breast cancer based on a hydrazine–Au nanoparticle–aptamer bioconjugate, Anal. Chem. 85 (2013) 1058–1064.

[11] J. Wang, Electrochemical biosensors: Towards point-of-care cancer diagnostics, Biosens. Bioelectron. 21 (2006) 1887–1892.

[12] K.J. Huang, J. Li, Y.Y. Wu, Y.M. Liu, Amperometric immunobiosensor for α-fetoprotein using Au nanoparticles/chitosan/TiO_2-graphene composite based platform, Bioelectrochem. 90, (2013) 16–23.

[13] Sh. Eissa, L. L'Hocine, M. Siaj, M. Zourob, A graphene-based label-free voltammetric immunosensor for sensitive detection of the egg allergen ovalbumin, Analyst 138 (2013) 4378–4384.

[14] C. Liu, Q. Jia, C. Yang, R. Qiao, L. Jing, L. Wang, C. Xu, M. Gao, Lateral flow immunochromatographic assay for sensitive pesticide detection by using Fe3O4 nanoparticle aggregates as color reagents, Anal. Chem. 83 (2011) 6676–6684.

[15] Y. Zhuo, P.X. Yuan, R. Yuan a, Ya.Q. Chai, C.L. Hong, Bienzyme functionalized-layer-by-layer composite magnetic nanoparticles for electrochemical immunosensors, Biomaterials 30 (2009) 2284–2290.

[16] A.S. Karakoti, S. Das, S. Thevuthasan, S. Seal, PEGylated inorganic nanoparticles, Angewandte Chemie 50 (2011) 1980–1994.

[17] H. Otsuka, Y. Nagasaki, K. Kataoka, PEGylated nanoparticles for biological and pharmaceutical applications, Adv. Drug Deliv. Rev. 64 (2012) 246–255.

[18] J. Suh, K.L Choy, S.K. Lai, J.S. Suk, B.C. Tang, S. Prabhu, J. Hanes, PEGylation of nanoparticles improves their cytoplasmic transport, Int. J. Nanomedicine 2 (2007) 735–741.

[19] I. Martinez-Mera, M.E. Espinosa, R. Perez-Hernandez, J. Arenas-Alatorre, Synthesis of magnetite (Fe_3O_4) nanoparticles without surfactants at room temperature, J. Mater. Lett. 61 (2007) 4447–4451.

[20] C.M. Niemeyer, Bioconjugation protocols. New Jersey: Humana; 2004.

[21] C.W. Scales, A.J. Convertine, C.L. McCormick, Fluorescent labeling of RAFT-generated poly(N-isopropylacrylamide) via a facile Maleimide–Thiol coupling reaction, Biomacromolecules 7 (2006) 1389–1392.

[22] L. Loo, J.A. Capobianco, W. Wu, X. Gao, W.Y. Shih, W.H Shih, K. Pourrezaei, M.K. Robinson, G.P. Adams, Highly sensitive detection of HER2 extracellular domain (ECD) in the serum of breast cancer patients by piezoelectric microcantilevers (PEMS), Anal. Chem. 83 (2011) 3392–3397.

[23] J.T. Gohringa, P.S. Daleb, X. Fan, Detection of HER2 breast cancer biomarker using the thiol-ene clickchemistry functionalized fluidic optical ring resonator biosensor, Sens. Actuat. B 146 (2010) 226–230

[24] Q.A.M. Al-Khafaji, M. Harris, S. Tombelli, S. Laschi, A.P.F. Turner, M. Mascini, G. Marrazza, An Electrochemical Immunoassay for HER2 Detection, Electroanalysis 24 (2012) 737–745