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A label-free electrochemical affisensor for cancer marker detection: The case of HER2

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ABSTRACT

In this paper, we report the development of a sensitive label-free impedimetric biosensor based on the use of affibody as bioreceptor and gold nanostructured screen-printed graphite as a sensor platform for the detection of human epidermal growth factor receptor 2 (HER2). The affisensor is realized by immobilizing a terminal cysteine-modified affibody on gold nanoparticles. The sensor was characterized by electrochemical techniques and scanning electron microscopy (SEM). Furthermore, surface plasmon resonance (SPR) technology was also applied to explore the potential of affibodies as small-molecule discriminating tools. Using optimized experimental conditions, a single-use affisensor showed a good analytical performance for HER2 detection from 0 to 40 μg/L. The estimated limit of detection was 6.0 μg/L. Finally, the realized affisensor was applied to human serum samples.

1. Introduction

Breast cancer is one of the most frequently diagnosed diseases among women in the world (http://www.who.int/cancer/en/). Detecting cancer at an early stage is one of the largest factors associated with successful treatment outcome [1]. Confirmation of malignancy is performed by invasive techniques based on taking a biopsy and then by tissue examination, using cell fixation and morphology approaches to identify and detect cancer cells. These tests, however, are not individually conclusive as tissue removal can miss cancer cells at the earlier onset of the disease [2]. The use of genomic assays to create molecular signatures of the disease has been employed recently. Knowing whether certain genes are present or absent, overly active or not active enough, can help doctors in making treatment decisions, such as whether or not chemotherapy should be part of the treatment plan. Three genomic assays are currently in use for breast cancer: Oncotype DX, Mammostrat and MammaPrint. Particularly, MammaPrint test (Agendia, The Netherlands) has been approved in 2007 by the Food Drug Administration for clinical use [3]. It is based on the analysis 70 gene-related breast cancer using DNA microarray on frozen fresh sample obtained from patient biopsy. The resulting 70 gene-expression profile allows the classification of breast cancer patients into two groups: one called "good profile" with low possibility to develop metastases; the other "poor profile" with high risk of developing distant metastases. These techniques are producing data, which are useful in understanding the disease, but are complex and require data management as well as linking to patient information. Rapid diagnosis by evaluation of cancer biomarkers in body fluids remains a complementary and necessary analysis for diagnostic and theranostic purposes. Recently, advancements in molecular biology have led to the discovery of novel biomarkers that can show the stage of the disease [4,5]. One of the most used biomarker is human epidermal growth factor receptor 2 (HER2) that can be measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or of pharmacologic responses to therapeutic intervention. Specifically, the HER2 proto-oncogene is amplified and/or over-expressed in approximately 30% of invasive breast cancers [6]. Normal individuals have a HER2 concentration in the range between 2 and 15 μg/L in the blood. The screening of HER2 on tumor biopsies is often used to evaluate whether a patient may successfully respond to therapy with trastuzumab (Herceptin), a monoclonal anti-HER2 antibody [7,8].

Electrochemical biosensors could be adequate alternatives as their high selectivity and sensitivity allow early detection of many diseases. The instrumentation needed to operate and analyze the results obtained by these biosensors has been reduced to pocket-size dimensions, which makes them ideal for incorporating in point-of-care devices. For this reason, the development of electrochemical affinity biosensors for the detection and monitoring of cancer biomarkers is a major area of recent researches [9–17]. Various strategies have been developed for

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improving the analytical performance of the biosensor such as the use of screen-printed electrodes [18,19] and nanostructures [20–24]. In addition, it is extremely important that the ligand is specific and stable for protein expression analysis in complex matrices such as blood, other body fluids, or tissue. Therefore, the improvement of existing ligands and the identification of new ligand scaffolds are of great importance in increasing biosensor sensitivity [25–28].

Several electrochemical immunosensors for human epidermal growth factor receptor detection have been published. Al-Khafaji et al. developed a magnetic-bead based electrochemical biosensor [9]. The immunoassay was based on a sandwich format in which a primary anti-HER2 monoclonal antibody was coupled to protein A-modified magnetic beads. The modified beads were then used to capture the protein from the sample solution followed by the addition of a secondary anti-HER2 monoclonal antibody labeled with biotin to complete the sandwich assay. The enzyme alkaline phosphatase conjugated with streptavidin and its substrate were then used for the electrochemical detection by differential pulse voltammetry. The immunoassay was able to detect HER2 with a detection limit of 6 μg/L and a linear range of 0–35 μg/L. Emami et al. [29] proposed an immunosensor based on iron oxide nanoparticles. HER2 cancer biomarker was determined by DPV measurement in two linear ranges between 0.01 and 10 ng/mL and between 10 and 100 ng/mL. A sandwich based-immunoassay was proposed by Marques et al. [30]; the immunosensor was assembled on the surface on AuNP modified graphite screen-printed electrodes, showing a linear range between 15 and 100 μg/L with a detection limit of 4.4 μg/L. An electrochemical aptasensor was described by Chun et al. [31]. The DNA aptamer was immobilized on the nanostructured gold electrode surface; HER2 biomarker in buffered solutions was then determined by EIS measurements in a linear range between 10^{-5} and 100 ng/mL. Patris et al. [32] reported the use of a new class of bioreceptors called Nanobodies® (Nbs). The proposed immunoassay was based on the immobilization of a primary anti-HER2 nanobody on the surface of graphite screen-printed electrode followed by the reaction with the antigen and with a horseradish peroxidase (HRP)-labeled secondary anti-HER2 nanobody. The detection of HER2 cancer biomarker was achieved by differential pulse voltammetry (DPV) measurements in a linear range from 1 to 200 mg/L with a detection limit of 1 mg/L.

The aim of this study is the development of a new, rapid, single-use and label free biosensor for HER2 determination in human serum samples using electrochemical impedance spectroscopy and an engineered binding protein called an affibody as the bioreceptor. We report, for the first time, an electrochemical affisensor with high affinity for HER2 for applications in early diagnosis and therapy monitoring of breast cancer.

Affibodies have received particular attention recently and found application in several studies especially for in vivo diagnostic imaging [33,34] and targeted cancer therapy applications [35]. Affibody molecules are an engineered version (Z domain) of one of the five stable three-a-helix bundle domains from the immunoglobulin Fc-binding region of staphylococcal protein A. They are constituted by only 58 amino acids without disulfide bonds and can therefore be produced in a simpler organism such a prokaryote, rather than the animal system required in antibody synthesis. Moreover, due to their small size they can also be chemically synthesized using solid phase peptide synthesis (SPPS), which means that specific site modification can be easily performed. In fact, these biomolecules can include specific labels, such as fluorophores, radioactive labels and other moieties, such as biotin or cysteine groups, which can be used to couple the affibody to other molecules, or for the immobilization on different sensor surfaces [36].

In this work, the terminal cysteine-modified affibody molecules were easily immobilized on gold nanostructured screen-printed sensors. The experimental conditions for constructing the affisensor were optimized by means of electrochemical impedance spectroscopy (EIS); preliminary experiments in human serum sample spiked with HER2 protein were also performed. Surface plasmon resonance (SPR) technology has also been applied to explore the potential of affibodies as small-molecule discriminating tools.

2. Materials and methods

2.1. Chemicals

Sodium chloride, tetrachloroauric acid ($HAuCl_4$), 6-mercapto-1-hexanol (MCH), dithiothreitol (DTT), tris(hydroxymethyl)aminomethane hydrochloride (TRIS), polyethylene glycol sorbitan monolaurate (Tween® 20), Bovine Serum Albumin (BSA), IgG from rabbit serum (rIgG), human vascular endothelial growth factor (VEGF), non-fat dried bovine milk and human serum were obtained from Sigma-Aldrich (Milan, Italy).

Sulfuric acid, hydrochloric acid, potassium chloride, potassium ferrocyanide ($K_4[Fe(CN)_6]$), potassium ferricyanide ($K_3[Fe(CN)_6]$), disodium hydrogen phosphate (Na_2HPO_4), and sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$) were purchased from Merck (Milan, Italy).

Human Epidermal growth factor Receptor 2 (HER2) protein (R&D Systems) was obtained from Space S.r.l. (Milan, Italy). Anti-HER2 affibody was purchased from Affibody AB, Sweden. Illustra™ NAP-5 column was obtained from GE Healthcare Italy (Milan, Italy).

The following buffers were used in the experiments:

- 0.1 M phosphate buffered saline (PBS), 0.1 M NaCl, pH 7.4,
- 0.1 M phosphate buffered saline (PBS) 0.1 M NaCl, pH 7.4 added with 0.005% w/w Tween
- 0.01 M TRIS buffer pH 8.5.

All the solutions were prepared using water from Milli-Q Water Purification System (Millipore, UK).

2.2. Apparatus

Electrochemical experiments were performed in a digital potentiostat/galvanostat AUTOLAB PGSTAT 30(2)/FRA2 controlled with the General Purpose Electrochemical System (GPES) and Frequency Response Analyzer (FRA2) 4.9 software (Eco Chemie, Utrecht, The Netherlands). The affisensor was assembled using screen-printed cells, comprising a graphite working electrode (2.5 mm in diameter), counter graphite electrode and a silver/silver chloride (Ag/AgCl) pseudo-reference electrode [9]. Screen-printed cells were produced in house on a DEK 248 screen-printing machine (DEK, Weymouth, UK). The printing was performed on a polyester film (Autostat CT5) from Autotype (Milan, Italy) using polymeric inks (Electrodag PF-410 (silver)) and (Electrodag 423 SS (graphite)), which were purchased from Acheson (Milan, Italy). Vinylfast 36–100 was used as the insulting ink and was obtained from Argon (Lodi, Italy).

SPR measurements were all carried out on Biacore X™ using bare gold sensor chip (General Electric Healthcare Bio-Sciences AB; Uppsala, Sweden).

A Zeiss Merlin Scanning Electron Microscope (SEM) (Zeiss, Oberkochen, Germany) equipped with an Energy Dispersive X-ray Spectroscopy (EDS) detector (LINCA X-Max, Oxford, UK), was used for electrode surface characterization. SEM images were acquired using the following parameters: acceleration voltage 20 kV, resolution 0.8 nm at 15 kV, magnification 5 kX.

2.3. Electrochemical measurements

Faradic impedance measurements were carried out in the presence of 0.01 M $[Fe(CN)_6]^{3-/4-}$ redox probe (equimolecular mixture prepared in 0.1 M KCl or in 0.1 M PBS buffer pH 7.4). A voltage of 10 mV in amplitude (peak-to-peak), within the frequency range of 100 kHz–10 mHz, was superimposed on the applied bias potential. The DC

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potential was set to +0.13 V (vs. Ag/AgCl pseudo-reference electrode), the formal potential of $[Fe(CN)_6]^{3-/4-}$ redox probe. Experimental spectra, presented in the form of complex plane diagrams (i.e. Nyquist plot), were fitted with proper equivalent circuits using the facilities of FRA2 4.9.004 (EcoChemie) software. Both charge transfer resistance (R_{ct}) and charge transfer resistance difference (ΔR_{ct}) values were taken as analytical signals. The quality of the fit to the used electrical equivalent circuit was determined using the chi-square value and the error distribution vs. frequency dependence comparing the experimental with simulated data. The standard deviation of chi-square value (χ^2) was of the order of 10^{-3} and the relative error was less than 4% for all the tested electrodes.

Electrodeposition of gold nanoparticles onto graphite screen-printed electrodes was obtained by cyclic voltammetry in the range of −0.2–+1.6 V (vs. Ag/AgCl pseudo-reference electrode) at 0.1 V/s scan rate using 0.6 mM $HAuCl_4$ solution prepared in 0.5 M H_2SO_4.

Cyclic voltammetry measurements for surface characterization were carried out in a range of potentials between −0.8 and +0.8 V (vs. Ag/AgCl pseudo-reference electrode) in the presence of 0.01 M $[Fe(CN)_6]^{3-/4-}$ (equimolecular mixture in KCl 0.1 M) at various scan rates. The experiments were carried out at room temperature (25 °C).

The entire procedure was repeated at least 10 times using different screen-printed cell and biochemical (proteins and bioreceptors) batches. Relative Standard Deviation % (%RSD) was calculated as a measure of inter-assay reproducibility.

2.4. Surface plasmon resonance (SPR) measurements

Firstly, the gold sensor chip was cleaned with a solution consisting of H_2O_2 (30%), NH_3 (30%) and Milli-Q water in a 1:1:5 ratio for 10 min and then thoroughly washed with Milli-Q water. After the cleaning step, a monolayer of affibody molecules was prepared by their direct immobilization onto the bare gold sensor chip. In particular, 1 mg/L of C-terminal cysteine affibody solution was added on the surface of the gold sensor chip and incubated overnight in a wet chamber. The sensor chip was then washed with Milli-Q water and treated with 1 mM (1 mL) blocking thiol solution (MCH) at room temperature for a further hour in the dark. The free binding-sites of the sensor were blocked by incubation in non-fat milk powder (1% w/v in 0.1 M PBS buffer, pH 7.4) for 30 min. After the washing step, the biosensor was inserted into the instrument.

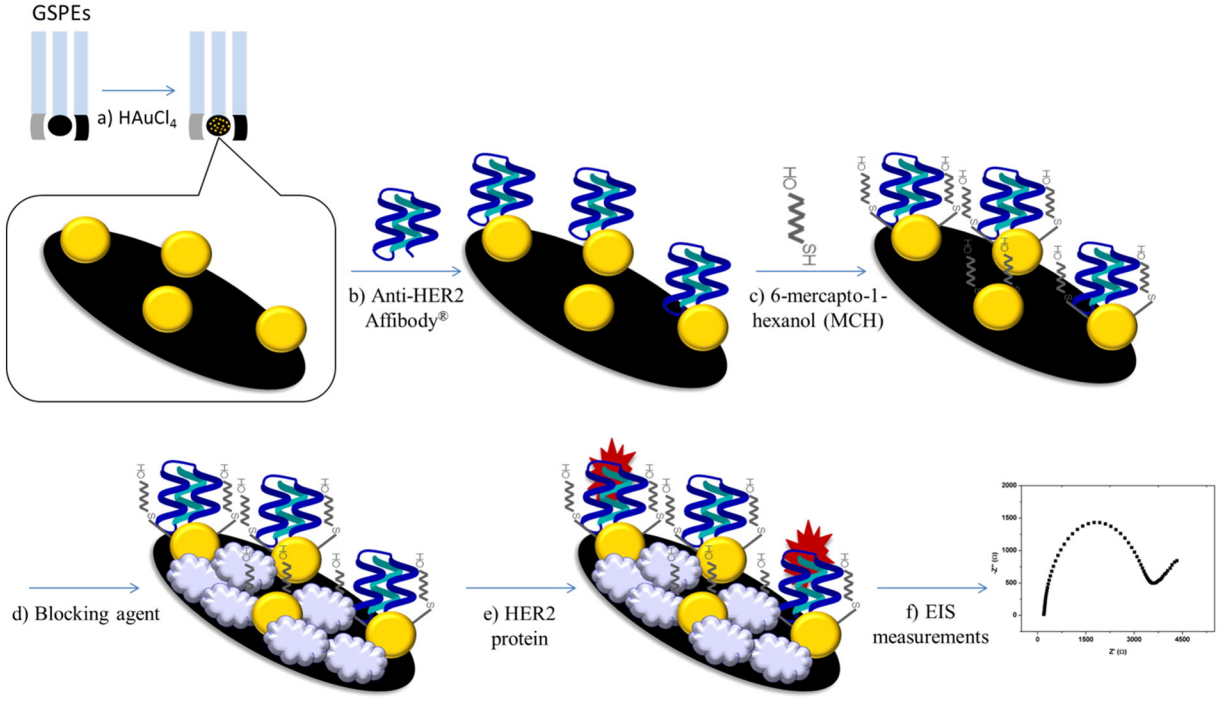
The affibody-HER2 affinity reaction was evaluated by injecting HER2 buffered solutions (prepared in 0.1 M PBS, pH 7.4) at different concentrations (20, 30, 40 mg/L) and using 0.1 M PBS buffer pH 7.4 (added with 0.005% w/w Tween) as running buffer. All the measurements were performed at 25 °C with the following experimental parameters: association phase 180 s, dissociation phase 180 s and flow 5 μL/min. After each measurement, in order to remove any remaining analyte from the sensor surface, a regeneration step was introduced using 2 mM HCl solution. The results were elaborated by BIAevalutation 3.1 software (sensorgrams not shown).

2.5. HER2 affisensor protocol

In this work, a label-free impedimetric affisensor for HER2 cancer biomarker determination was developed as illustrated in Scheme 1. The protocol involves the following steps: a) electrodeposition of gold nanoparticles (AuNPs) on graphite screen-printed electrodes (GSPEs); b) functionalization with anti-HER2 affibody molecules; c) mixed SAM formation with MCH; d) blocking step with non-fat milk; e) affinity reaction with HER2 protein and f) HER2 determination in buffered solutions and human serum samples by electrochemical impedance spectroscopy (EIS) measurements.

2.5.1. Electrodeposition of gold-nanoparticles onto graphite screen-printed electrodes

Electrodeposition of gold nanoparticles (AuNPs) on the surface of graphite screen-printed electrodes (GSPEs) was realized in accordance with the optimized procedure reported in our previous work [37].



Scheme 1. Scheme of affisensor for HER2 detection: a) electrodeposition of gold nanoparticles (AuNPs) on graphite screen-printed electrodes (GSPEs); b) immobilization of anti-HER2 affibody molecules); c) mixed SAM formation with MCH; d) blocking step with non-fat milk; e) affinity reaction with HER2 protein; f) electrochemical impedance spectroscopy (EIS) measurements.

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Briefly, 50 μL of 0.6 mM of $HAuCl_4$ solution, prepared in 0.5 M H_2SO_4, was placed onto GSPE surface. Subsequently, the potential was cycled from −0.2 to +1.6 V (vs. Ag/AgCl pseudo-reference electrode) for 25 times using a scan-rate of 0.1 V/s. Successively, AuNP-modified GSPEs were washed 3 times with Milli-Q water. Finally, electrochemical measurements (performed by cyclic voltammetry and impedance spectroscopy) and SEM/EDS analysis were carried out for surface characterization.

Various GSPEs were modified with gold nanoparticles on the same day and stored under dry conditions at 4 °C. The stability of different AuNPs/GSPEs was evaluated by means of EIS measurements under the conditions described in Section 2.3. The average reproducibility of the nanostructured sensors remains below 6% RSD for at least two weeks.

2.5.2. Affibody immobilization

Anti-HER2 affibody molecule contains a C-terminal cysteine ideal for direct chemical modifications. However, tail-to-tail dimers are spontaneously generated via a disulfide bridge between the C-terminal cysteine. Prior to use, as suggested by the supplier, the affibody molecules were treated with dithiothreitol (DTT) in order to cleave the –S–S– bonds eventually present due to the oxidative coupling of two molecules. In particular, the lyophilized affibody molecules were dissolved in 0.02 M DTT solution (prepared in 0.01 M TRIS buffer, pH 8.0) and left to incubate for 120 min at room temperature. The solution was then purified by elution through a NAP-5 column with 0.1 M PBS buffer pH 7.4. The affibody stock solution (100 mg/L) was then divided in aliquots and stored frozen (−20 °C) for further experiments.

The affibody immobilization on the AuNP-modified electrode surface was performed in a single step by chemisorption, permitting also the correct orientation of the bioreceptor for the recognition of the target protein. 10 μL of 1 mg/L of anti-HER2 affibody solution was placed on the surface of the nanostructured electrodes and incubated overnight in a home-made wet chamber at 25 °C. The affibody-modified sensors were then rinsed three times with 0.1 M PBS pH 7.4 buffer in order to remove the unbound molecules.

Mixed SAM formation was then carried out incubating the sensor surface with 10 μL of 1 mM MCH solution (prepared in a mixture of 4:1 in volume of ethanol and water) for 30 min at 25 °C. Subsequently, the sensors were washed three times with 0.1 M PBS pH 7.4 buffer.

After the mixed SAM formation, in order to limit the non-specific interactions on the free sites eventually present onto the graphite sensor surface, a blocking step was introduced. For this purpose, the affisensors were incubated with non-fat milk solution (1% w/v in 0.1 M PBS buffer, pH 7.4) for 30 min. Then, the sensors were rinsed in 0.1 M PBS buffer pH 7.4. All steps of electrode surface modification were characterized by EIS measurements.

2.5.3. HER2 detection

To perform the calibration curve, the proposed affisensors were incubated with different concentrations of HER2 protein in the concentration range of 0–40 μg/L (prepared in 0.1 M PBS buffer, pH 7.4) for 60 min at room temperature. The affisensors were then rinsed with 0.1 M PBS buffer pH 7.4 solution. Finally, EIS spectra were recorded under the conditions described in Section 2.3.

Control experiments were performed using human vascular endothelial growth factor (VEGF) protein to investigate the non-specific signal of the affisensor. VEGF is used as a biomarker for diagnosis and prognosis with a clinical cut-off of 1 μg/L in the blood. Its level increases in patients with breast cancer with HER2 levels [38].

2.5.4. Serum sample analysis

Preliminary experiments for the determination of HER2 protein in human serum samples were also performed. The affisensors were tested in 1/200 diluted (in 0.1 M PBS buffer, pH 7.4) and filtered (0.45 μm) serum samples spiked with standard addition of HER2 protein (10, 20, 30 μg/L). Affisensor response was then determined by EIS measurements, under the same conditions used for HER2 calibration curve.

3. Results and discussion

3.1. Electrochemical characterization of AuNP-modified graphite screen-printed electrodes

Graphite screen-printed electrodes (GSPEs) were modified by electrodeposition of gold nanoparticles (AuNPs) using cyclic voltammetry in accordance with the optimized procedure reported previously [37]. The graphite screen-printed electrodes and gold nanostructured screen-printed electrodes were analyzed by cyclic voltammetry using 0.01 M $[Fe(CN)_6]^{3-/4-}$ as redox probe at different scan-rates (ranging from 0.025 to 0.4 V/s). The results obtained are shown in Fig. 1A and B. The cathodic and anodic current intensity peaks of AuNPs/GSPE are higher than GSPE as well as the reversibility. This demonstrated the formation of AuNPs on the graphite sensor surface.

The electrochemical properties of the sensor were also characterized using the EIS technique. EIS provides detailed information on electrode surface changes during the modification processes. Therefore, the impedance spectra of GSPEs and AuNPs/GSPEs in 0.01 M $[Fe(CN)_6]^{3-/4-}$ equimolecular mixture in 0.1 M KCl were determined. R_{ct} values of gold-nanostructured GSPEs were about 30 times lower with respect to bare GSPEs (respectively 650 ± 50 Ω and 20,000 ± 250 Ω; EIS spectra not shown). This demonstrates that the electrode surface has been successfully modified with AuNPs and provides the necessary conduction pathways, besides

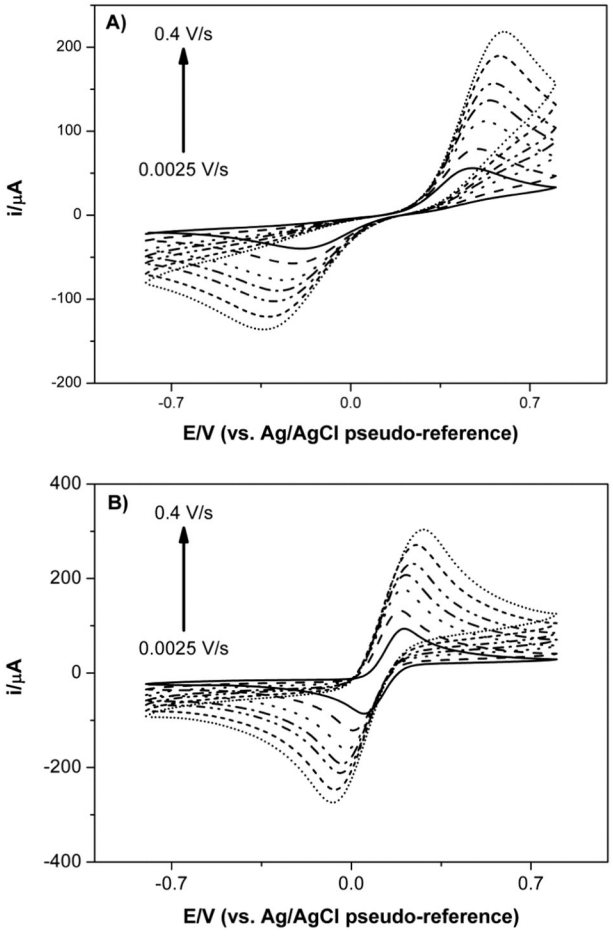

Fig. 1. Cyclic voltammetry scans of GSPEs (A) and AuNPs/GSPEs (B) performed using 0.01 M $[Fe(CN)_6]^{3-/4-}$ in 0.1 M KCl as redox probe at different scan-rates.

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acting like a nanoscale electrode in promoting electron transfer between the analyte and the electrode surface.

3.2. Scanning electron microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDS) surface characterization

SEM analysis of gold nanoparticle-modified graphite screen-printed electrodes (AuNP/GSPEs) was performed. Fig. 2A shows SEM image of AuNP-modified GSPEs. Clearly visible is the presence of randomly distributed AuNPs on the entire surface of the graphite screen-printed electrode with dimensions about 100 nm, as reported in our previous work [37]. Moreover, Energy Dispersive X-ray Spectroscopy (EDS) analysis of AuNPs/GSPEs (Fig. 2B) was carried out. Three characteristic gold bands centered at 2.2, 9.7 and 11.5 keV were found when the AuNP modified screen-printed electrode was investigated, confirming the presence of gold nanostructures on the sensor surface.

3.3. Optimization of the affisensor development

The HER2 affisensor was optimized with respect to several experimental conditions such as affibody concentration, the type of blocking agent, its incubation time and HER2 protein incubation time by changing one parameter at a time. For this purpose, EIS measurements were performed using 0.01 M $[Fe(CN)_6]^{3-/4-}$ as redox probe prepared in PBS buffer (0.1 M, pH 7.4).

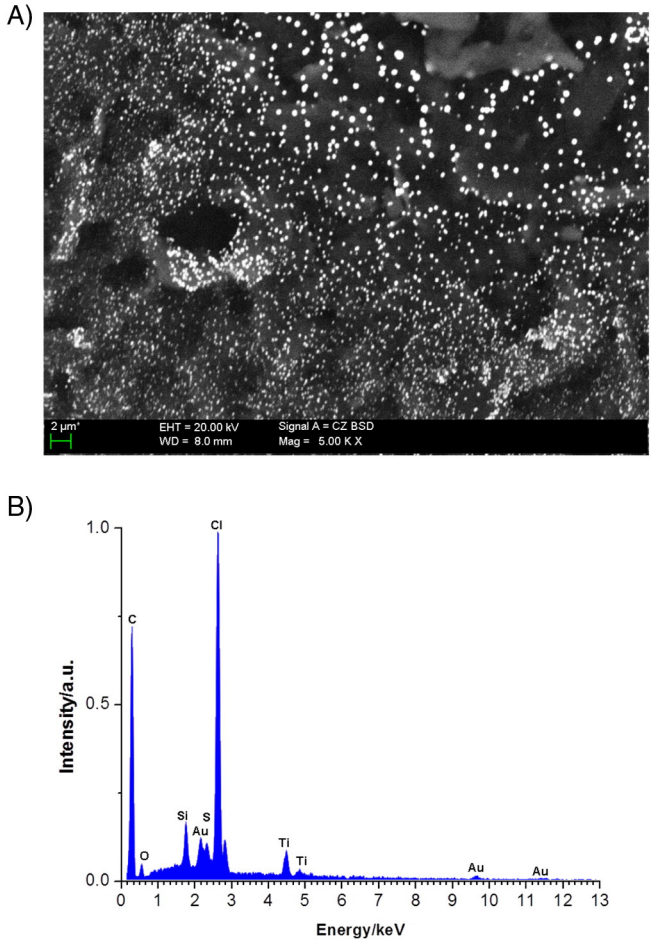

Fig. 2. SEM/EDS measurements of screen-printed electrodes performed in accordance with Section 2.2. (A) SEM image of gold nanoparticle-modified graphite screen-printed electrode; (B) EDS spectrum of gold nanoparticle-modified graphite screen-printed electrode.

Table 1
Experimental parameter optimization. The R_{ct} ratio represents the $R_{ct,\ HER2}/R_{ct,\ blank}$ ratio between the resistance charge transfer values obtained using respectively 30 µg/L and 0 µg/L HER2 buffered solutions. The letters of assay step column are in accordance to Scheme 1. Average %RSD values were calculated as reported in Section 2.3.

Assay step	Parameter optimization		R_{ct} ratio	Average %RSD
b	Affibody concentration (incubation time: overnight)	0.5 mg/L	1.1	8
		1.0 mg/L	1.5	9
		2.0 mg/L	1.4	9
d	Blocking agent (incubation time: 30 min)	1% w/v non-fat milk	1.6	10
		1% w/v BSA	1.2	15
		10 mg/L rIgG	1.1	18
	1% w/v non-fat milk incubation time	30 min	1.6	13
		60 min	1.4	16
		30 min	1.2	9
e	HER2 incubation time	60 min	1.5	7
		120 min	1.6	10

The optimized parameters were chosen taking into consideration the charge transfer resistant ratio between 30 µg/L HER2 ($R_{ct,\ HER2}$) and the blank ($R_{ct,\ blank}$) and the best reproducibility in terms of % Relative Standard Deviations (%RSD) (n = 10). The results are displayed in Table 1.

The immobilization of the affibody on the gold nanostructured screen-printed electrode surface, with an appropriate orientation and flexibility (for binding the target protein), is of paramount importance for the performances of the sensor. In order to optimize the affibody immobilization, the AuNP-modified electrodes were incubated overnight with affibody solution at different concentrations (0.5, 1.0 and 2.0 mg/L) followed by MCH treatment, blocking step and affinity reaction with the antigen. The best R_{ct} ratio and reproducibility were obtained using an affibody concentration of 1.0 mg/L, which was then selected for further experiments (Table 1, assay step b).

To minimize non-specific adsorption on the free sites of the sensor surface, the effect of different blocking agents used after the modification of the sensor with affibody was investigated. Blocking agents such as BSA (1% w/v in PBS buffer), non-fat milk (1% w/v in PBS buffer) and 10 mg/L rIgG solution were studied in accordance with other immunosensors reported in the literature [10]. The best result was obtained when non-fat milk was used. The different incubation times of the blocking agent were also studied. The results showed that 1% non-fat milk solution used with incubation time of 30 min ensured an optimum surface blocking (Table 1, assay step d).

The best conditions for binding HER2 to the immobilized affibody were then investigated. The affibody-modified sensors were incubated with the protein target for 30, 60 and 120 min. The incubation time of 30 min was not sufficient to bind the whole amount of the protein, whereas a similar higher signal to blank ratio is being obtained after 60 and 120 min. Moreover, a better reproducibility was obtained using an incubation time of 60 min, which was then selected for further experiments (Table 1, assay step e).

Electrochemical impedance spectroscopy was used to characterize the layer-by-layer formation of the affisensor. The Nyquist plots after each assembly step, under the optimized conditions, are shown in Fig. 3. For analyzing EIS data, they were fitted with the equivalent circuit model reported in Fig. 3 inset.

After the modification of AuNPs/GSPEs with anti-HER2 affibodies, an increase of R_{ct} values respectively from 650 ± 50 Ω to 1300 ± 80 Ω is observed. Results are consistent with the presence on the AuNP/GSPE surface of a layer of protein that reduces (because of steric hindrance and electrostatic repulsion) the electron transfer from the redox probe solution to the electrode surface. The gold nanostructured affisensors were then incubated with 10 µL of 1 mM MCH solution for 30 min (in order to obtain a mixed-SAM formation) and with non-fat milk solution (1% w/v for 30 min). An increase of R_{ct} values was observed (R_{ct} = 1600 ± 70 Ω) which confirms the realization of the functionalization step.

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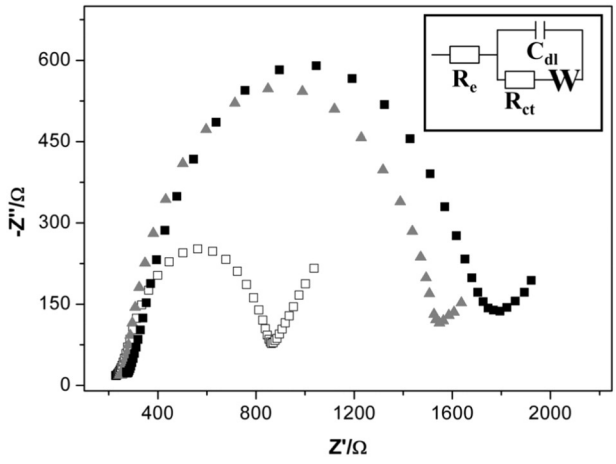

Fig. 3. Nyquist plots after each assembly step under the optimized conditions obtained in 0.01 M $[Fe(CN)_6]^{3-/4-}$ equimolecular mixture in 0.1 M PBS, pH 7.4: (□) gold nanoparticle-modified graphite screen-printed electrodes (AuNPs/GSPEs); (▲) affibody modified sensors (Af/AuNPs/GSPEs); (■) mixed SAM formation with 6-mercapto-1-hexanol (MCH/Af/AuNPs/GSPEs). Inset: Randles equivalent circuit used to fit EIS measurements (R_e: electrolyte resistance, C_{dl}: double layer capacitance, R_{ct}: charge transfer resistance, W: Warburg impedance). The measurements were repeated at least 10 times using different affisensors as reported in Section 2.3.

3.4. Label-free detection of HER2 antigen

The affisensor analytical response was evaluated by means of electrochemical impedance spectroscopy (performed in 0.01 M $[Fe(CN)_6]^{3-/4-}$ 0.1 M PBS buffer solution pH 7.4) analyzing the changes in R_{ct} values obtained after the affinity reaction with HER2 buffered solution; a proportional increase of R_{ct} values with respect to antigen concentration was observed. This can be explained by the formation of a kinetic barrier for the electron transfer due to the increase of HER2 molecules bound to the immobilized affibodies (Fig. 4A).

The calibration curve for the detection of HER2 biomarker under optimized conditions was reported in Fig. 4B (black squares, ■). A linear relationship ($y = 20x$, $R^2 = 0.98$) between the ΔR_{ct} (calculated as $\Delta R_{ct,HER2} - \Delta R_{ct,0}$) and concentration of HER2 was obtained in the range of 0 and 40 μg/L with a limit of detection of 6.0 μg/L (calculated as $LOD = 3S_{blank} / Slope$).

In order to evaluate the selectivity of the immunoassay, the effect of VEGF protein as non-specific antigen was studied. As can be observed in Fig. 4B (white circles, ○), very similar R_{ct} values compared to the blank solution were achieved. This result confirms that the observed relative change in impedance originated from specific affibody/HER2 interactions, showing appropriate affisensor selectivity for HER2 detection. The reproducibility of the affisensor was analyzed by the relative standard deviation (%RSD) using10 different electrodes for each concentration of antigen. The %RSD for 20 μg/L was 10%. Thus, the proposed sensor presents a good reproducibility, providing a convenient platform for HER2 analysis.

Additionally, the non-specific adsorption of HER2 cancer biomarker on the nanostructured sensor surface was evaluated. In particular, after the electrodeposition of AuNPs, the sensors were modified with MCH and non-fat milk solution, as reported in the Materials and methods section. The sensors were then incubated with HER2 solution (20 μg/L) or with 0.1 M PBS buffer (as control experiments).

No difference in R_{ct} values between the sensors (in the presence of HER2 protein) and the blank was observed, proving proper blocking of the nanostructured electrode surface (data not shown).

3.5. Surface plasmon resonance (SPR) experiments

In order to verify and characterize the affinity binding between theLabel-free affisensor was applied to HER2 detection in human serum samplesbioreceptor (anti-HER2 affibody) and the antigen (HER2 cancer biomarker) SPR measurements were performed using Biacore X™ instrumentation under the conditions described in Section 2.4.

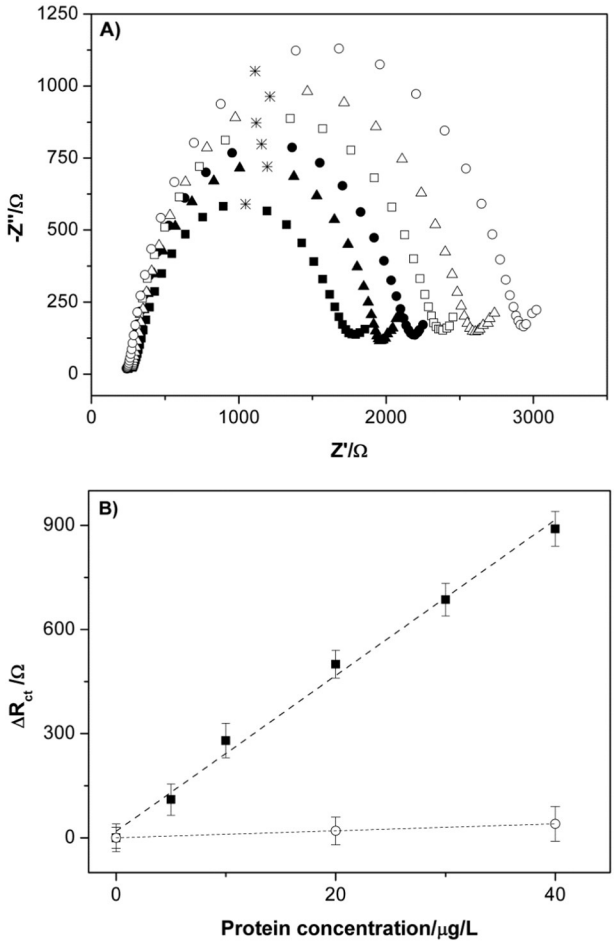

Fig. 4. (A): Nyquist plots of affibody-HER2 affinity reaction obtained in 0.01 M $[Fe(CN)_6]^{3-/4-}$ equimoleculear mixture in 0.1 M PBS, pH 7.4; HER2 concentrations: (■) 0 μg/L, (▲) 5 μg/L, (●) 10 μg/L, (□) 20 μg/L, (△) 30 μg/L, (○) 40 μg/L. Highlighted dot (∗) corresponds to impedance response obtained applying a frequency of 115 Hz. (B) Calibration curves obtained under optimized conditions for HER2 analysis (■); aspecific interactions with VEGF protein (○). The measurements were repeated at least 10 times using different affisensors. Further details are available in the Materials and methods section.

The kinetic rate constant (k_a and k_d) as well as the equilibrium dissociation constant K_D were estimated by the fitting analysis of titration curves using 1:1 Langmuir interaction model. Results confirm the fast "on rate" ($K_a = 3.6 \cdot 10^5$ 1/M s) properties and their moderate "off rate" ($2.4 \cdot 10^{-3}$ 1/s) of these affibody molecules (Table 2) [37].

3.6. Serum sample analysis

Once the suitability of the affisensor to detect HER2 in standard solutions was verified, experiments on spiked commercial serum samples were carried out. HER2 was added to commercially available serum after filtration and dilution 1/200 in 0.1 M PBS buffer solution pH 7.4. The serum sample signal increases with respect to the blank solution

Table 2
Kinetic and thermodynamic parameters for anti-HER2 affibody–HER2 affinity reaction.

k_a (10^5 1/M s)	k_d (10^{-3} 1/s)	K_D (nM)	χ^2
3.6	2.4	8.4	0.351

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Table 3
Recoveries, biases, %RSDs for HER2 determination in HER2 protein spiked serum samples. Each concentration was repeated at least 5 times using different affisensors.

HER2 spiked (μg/L)	HER2 found (μg/L)	Recovery (%)	Bias (%)	%RSD
10	9.5	95	−5	8
20	22	110	+10	9
30	33	110	+10	10

in accordance with the HER2 concentration in the analyzed serum sample. The same behavior was observed on increasing the concentration of HER2 in spiked serum samples, indicating that it is possible to establish a direct correspondence. When control experiments were carried out with VEGF protein (40 μg/L), no increase of R_{ct} signal was observed with respect to serum sample signal (data not shown), proving the high selectivity of the developed affisensor for the HER2 antigen.

The HER2 concentration in the serum samples was determined by referring to a calibration curve obtained daily in PBS. The responses obtained with these samples were similar to the signals obtained in phosphate buffer, indicating an absence of matrix interference in HER2 detection and reflecting the specificity of the developed affisensor. Furthermore, as reported by Orlova et al. [39] no interactions between affibody and IgG molecules were demonstrated avoiding a further blocking step when using serum samples.

The results obtained with the spiked samples are shown in Table 3. An acceptable bias ranging −5% to 10% was found for spiked serum samples confirming the possibility to use the affisensor in clinical samples.

4. Conclusions

A label-free single-use impedimetric biosensor based on the easy immobilization of anti-HER2 affibody bioreceptor on gold nanostructured graphite screen-printed electrode for the detection of HER2 cancer biomarker was developed. A good linear relationship between the electron transfer resistance difference and HER2 concentration in the range of 0–40 μg/L was found.

In comparison with the conventional HER2 detection methods typically based on antibodies, the impedimetric affisensor showed a fast, sensitive and specific detection of HER2 in real samples with a very low detection limit (6.0 μg/L) yielding promising results for the use of newly-engineered proteins in biosensor technology for clinical applications.

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