Voltammetric immunosensor for the simultaneous analysis of the breast cancer biomarkers CA 15-3 and HER2-ECD

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Abstract

Cancer Antigen 15-3 (CA 15-3) and the extracellular domain of the human epidermal growth factor receptor 2 (HER2-ECD) are independent breast cancer biomarkers. The combination of their profiles (presence and concentration) could provide an important contribution to diagnostics and patient follow-up. Therefore, a disposable electrochemical immunosensor for the simultaneous detection of CA 15-3 and HER2-ECD was developed in this work. The immunosensor was constructed on a customized dual screen-printed carbon electrode. The carbon working electrodes' surfaces were first modified with *in situ* electrodeposited gold nanoparticles and then individually coated with either a monoclonal anti-human CA 15-3 or a monoclonal anti-human HER2-ECD antibody. After incubation with the biomarkers and monoclonal biotin-labelled detection antibodies, the antigen-antibody interactions were detected by linear sweep voltammetric analysis of enzymatically (alkaline

phosphatase) generated metallic silver. The immunosensor's limits of detection for the selected biomarkers were 5.0 U mL^{-1} for CA 15-3 and 2.9 ng mL^{-1} for HER2-ECD. These values could allow the use of the sensor in the non-invasive control of these biomarkers in breast cancer patients.

Keywords: Multiplexed immunosensor, Electrochemical immunosensor, Breast cancer biomarker, CA 15-3, HER2-ECD

1. Introduction

Breast cancer is the top cancer in women both in the developed and the developing world [1]. Screening programs and the improvement in treatment and follow-up have been able to lower the mortality in the past few years [2]. However, breast cancer is still the most common cause of cancer-related mortality in women in less developed regions, and the second most common (after lung cancer) in developed regions [1].

Imagiology has been a helpful tool for screening in breast cancer, but it is very imprecise in the prediction of the biological behavior of the tumor. Therefore, important research has been conducted to identify suitable biomarkers, which has led to advances in its detection and treatment [3,4]. A cancer biomarker is a biological molecule found in tissues, cells or body fluids which presence and/or concentration level can be related to the presence of cancer [5,6]. Biomarker detection may be used for early diagnosis, staging of disease and monitoring of the response to treatment [3,7]. Breast cancer biomarkers include tissue markers such as estrogen and progesterone receptors and the human epidermal growth factor receptor 2 (HER2), and circulating markers such as carcinoembryonic antigen (CEA) and cancer antigen 15-3 (CA15-3) [7–9].

Although there are many newly suggested circulating breast cancer biomarkers, very few have been adopted into the clinic. So far, only the detection of CA 15-3 has been routinely adopted in practice.

CA 15-3 is the shed form of MUC-1, a protein whose normal function is cell protection and lubrication, but is typically seen to be overexpressed on breast tumor cells. CA 15-3 is therefore considered a circulating cancer biomarker that can be detected in serum [9,10]. Elevated levels of CA 15-3 are found in the majority of breast cancer patients with distant metastasis; about 60-75% of women with invasive breast cancer (metastasized cancer) present elevated levels of CA 15-3 [10,11]. High levels of CA 15-3 may also be found in patients that suffer several different types of advanced adenocarcinoma, such as ovarian, pancreatic, gastric or lung cancer [11]. Because CA 15-3 levels are only elevated in 10% of patients with stage 1 breast cancer, it has little value in early detection. However, CA 15-3 has been routinely adopted in practice to provide prognostic information, which is essential for optimum disease management, and also as a potential predictor of treatment [6,11]. A CA 15-3 concentration of 25 U mL^{-1} is considered a threshold value [7,10].

HER2 is a transmembrane protein of the epidermal growth factor receptors family that is overexpressed in approximately 15-30% of breast cancers [9,12]. HER2 overexpression has been related with accelerated growth, recurrence date, progressive metastatic disease and poor rate of disease-free survival [13,14]. This protein consists of three domains: an extracellular domain (ECD), a transmembrane domain and an intracellular tyrosine kinase domain [15]. The extracellular domain fragment of HER2 (HER2-ECD) is released from the surface of tumor cells into the blood stream. Thus, HER2-ECD levels are measurable in the serum fraction of blood and, therefore, HER2-ECD is a minimal-invasive (since it avoids HER2 status tests in tissues obtained by biopsy) and clinically relevant biomarker for breast cancer [12,13]. HER2 status characterization is now mandatory in breast cancer since, besides an important prognostic factor, it is also a target for specific immunotherapies [16]: for example, the use of Trastuzumab, which is a recombinant humanized monoclonal antibody against HER2-ECD, was approved by the Food and Drug Administration (FDA) in 1998 to treat metastatic breast cancer [13,15]. Therefore, the major advantage of HER2-ECD determination in

serum is the possibility of patient follow-up without the need of biopsies, as required by the established methods for the assessment of HER2 status (such as immunohistochemistry (IHC) and fluorescent *in situ* hybridization (FISH)) [8,12]. The normal serum HER2-ECD concentration is 15 ng mL^{-1} but a moderate increase (50 ng mL^{-1}) has also been described in the absence of cancer [12,13]. In the clinical field, determination of a single tumor marker often has limited diagnostic value because most biomarkers are not specific of a particular cancer. Moreover, most cancers have more than one associated biomarker. So, multi-biomarker analysis can be essential for the early diagnosis of disease onset [17,18]. As mentioned before, CA 15-3 and HER2-ECD are independent indicators for a worse disease free survival and the combination of both biomarkers is valuable for the identification of high-risk breast cancer patients [19]. Nevertheless, to the best of our knowledge, a sensor for the simultaneous determination of CA 15-3 and HER2-ECD has not been reported yet. Therefore, the purpose of the present work was to develop an electrochemical immunosensor for the simultaneous analysis of these biomarkers. Electrochemical sensors are excellent to be included into point-of-care devices because of their high selectivity and sensitivity, fast response, simplicity, low cost, easy miniaturization and integration into automatic systems [7,20,21]. For these reasons, several electrochemical immunosensors for CA 15-3 and HER2-ECD detection were previously developed. However, only a few of these are based on screen-printed electrodes [8,22–28], which are miniaturized transducers that allow the development of portable, simple and small-size sensors. Some electrochemical immunosensors are described in which CA 15-3 is analyzed together with other cancer biomarkers such as AFP, CEA, CA 125 or CA 19-9 [25–30]. However, and as mentioned before, an electrochemical sensor for the simultaneous detection of CA 15-3 and HER2-ECD has not been published.

In this work, the development of a multiplexed electrochemical immunosensor for the detection of CA 15-3 and HER2-ECD, based on dual screen-printed carbon electrodes (bi-SPCE) nanostructured

with *in situ* generated gold nanoparticles, is described. The applied immunosensing approach is based on a sandwich-type assay in which specific capture antibodies for these biomarkers are immobilized by adsorption onto each nanostructured working electrode. The antigen-antibody interaction is sensed using alkaline phosphatase as label on the detection antibodies and a mixture of 3-indoxyl phosphate with silver ions as substrate [31]. The signal is the peak current intensity of the enzymatically generated silver in a linear-sweep voltammogram. Since the signal results from the metallic silver deposited on the electrode surface at the site of the enzyme, cross-talk between electrodes is avoided, therefore it is possible to use the same label for the detection of both biomarkers [32,33]. Along the work, different immunoassay strategies and optimizations were carried out to obtain the highest and most reproducible analytical signal (i.e. the difference between the signal for the analytes and the background signal) for both biomarkers. The developed bi-immunosensor, besides detecting concentration ranges with clinical importance for both biomarkers, also shows important characteristics related to Green Analytical Chemistry [34] such as miniaturization, simplicity, use of low volume of reagents and sample, multi-analyte determination and possibility of *in situ* analysis.

2. Materials and methods

2.1. Equipment

The linear sweep voltammetric measurements and the *in situ* electrodeposition of gold nanoparticles were performed using a μStat 200 bipotentiostat from DropSens. The potentiostat was controlled by DropView (v2.0, DropSens) software, which was also used for data acquisition and treatment. Customized dual screen-printed carbon electrodes (bi-SPCE, DRP-X1110) and a connector (DRP-BICAST) to interface the bi-SPCE and the bipotentiostat were supplied by DropSens. The bi-SPCEs, printed on ceramic substrates (3.4×1.0×0.05 cm), incorporated two carbon working electrodes (elliptic-shaped, A = 6.3 mm^2), a silver pseudo-reference electrode and a carbon auxiliary electrode.

The electrochemical cell was delimited by an insulating layer printed around the electrodes. SEM images were obtained at the "Centro de Materiais da Universidade do Porto (CEMUP)" using FEI QUANTA 400 FEG / EDAX Pegasus X4M equipment.

2.2. Reagents

The immunoreagents for HER2-ECD analysis were purchased from Sino Biological: anti-human-HER2-ECD rabbit monoclonal antibody (anti-HER2; capture antibody for HER2-ECD), recombinant human HER2-ECD protein and anti-human-HER2-ECD mouse monoclonal biotinylated antibody (anti-HER2-bio; detection antibody for HER2-ECD). The antibodies for the CA 15-3 assay were purchased from Fujirebio Diagnostics: anti-human-CA15-3 mouse monoclonal antibody (anti-CA15-3; capture antibody for CA15-3) and anti-human-CA15-3 mouse monoclonal biotinylated antibody (anti-CA15-3-bio; detection antibody for CA 15-3). Human CA 15-3 protein was provided by MyBioSource.com.

The other reagents used throughout the work were: tetrachloroauric(III) acid ($H[AuCl_4]$) purchased from Merck; tris(hydroxymethyl)aminoethane (Tris), streptavidin from *Streptomyces avidinii* conjugated to alkaline phosphatase (S-AP), 3-indoxyl phosphate disodium salt (3-IP), β-casein from bovine milk, bovine serum albumin (BSA), magnesium nitrate hexahydrate, and nitric acid (≥ 65 %) were obtained from Sigma-Aldrich; and silver nitrate was supplied by Alfa Aesar.

Working solutions of casein, S-AP and the immunoreagents were prepared in a 0.1 M Tris-HNO_3 pH 7.2 buffer (buffer 1). A solution containing 1.0 mM 3-IP and 0.4 mM silver nitrate was prepared daily in a 0.1 M Tris-HNO_3 pH 9.8 buffer containing 20 mM $Mg(NO_3)_2$ (buffer 2), and stored in opaque tubes at 4ºC. Type I ultrapure water (resistivity = 18.2 MΩ.cm) was used throughout the work.

2.3. Electrodeposition of gold nanoparticles

It is well know that nanostructuration of SPCEs with AuNPs improves the biocompatibility of the transducer and facilitates electron transfer between the immobilized proteins and the electrode surface [35]. In this work gold nanoparticles were electrodeposited, at room temperature, on the surfaces of the carbon working electrodes of the bi-SPCEs (bi-SPCE-AuNP) following the method previously reported by Martínez-Paredes *et al*. [36], since the AuNPs thus generated have demonstrated a considerable improvement of the analytical signal for similar assays [37]. The nanostructuration procedure consisted of dropping 60 μL of a 0.1 mM $[AuCl_4]^-$ (in 0.1 M HCl) solution onto the bi-SPCE and applying a constant potential of -0.7 V during 240s. This was followed by the application of a constant potential of +0.1 V for 120s to desorb hydrogen formed during the previous step. After rinsing the bi-SPCE-AuNP (**Fig. S1**) with water they were ready to use.

2.4. Immunoassay

The following procedure, summarized in **Fig. 1**, describes an optimized assay. Each working electrode of the bi-SPCE-AuNP was coated with 4 μL of a capture antibody solution (50 μg mL^{-1} anti-HER2 or 100 μg mL^{-1} anti-CA15-3) and incubated overnight at 4 ºC. After the overnight incubation, the bi-SPCE-AuNP was washed using buffer 1. From this point on all the steps of the immunoassay were performed covering both working electrodes with the same solution. Free surface sites were blocked with a casein solution (60 μL, 2% w/v) during 30 min and afterwards the electrode was washed with buffer 1. Then the bi-immunosensor was incubated for 1 hour with 60 μL of a mixture (1 : 1 ratio) of a solution containing the antigens (HER2-ECD and CA 15-3) and a solution containing the anti-CA15-3-bio antibody (1 μg mL^{-1}) and BSA (1% w/v). After this step the sensor was washed with buffer 1 and a 60-μL aliquot of a solution containing the anti-HER2-bio antibody (0.5 μg mL^{-1}) and BSA (1% w/v) was placed on the sensor and left to react for 30 min. Finally, after

a washing step with buffer 1, 60 µL of a 0.5 nM S-AP solution containing 0.1 % BSA (w/v) was dropped on the sensor and left to react for 1 hour. Then, the electrode was washed with buffer 2 and the enzymatic reaction was performed by using a 60-µL aliquot of the 1.0 mM 3-IP/0.4 mM $AgNO_3$ solution. The enzymatic deposition of metallic silver catalyzed by alkaline phosphatase was already reported previously (**Fig. S2**) [31]. After 20 min a linear voltammogram between -0.02 and 0.4 V at 50 mV s^{-1} was recorded to obtain the electrochemical oxidation current of the enzymatically deposited silver. Since silver was reduced and deposited on the separate electrodes, no inter-electrode interferences were observed.

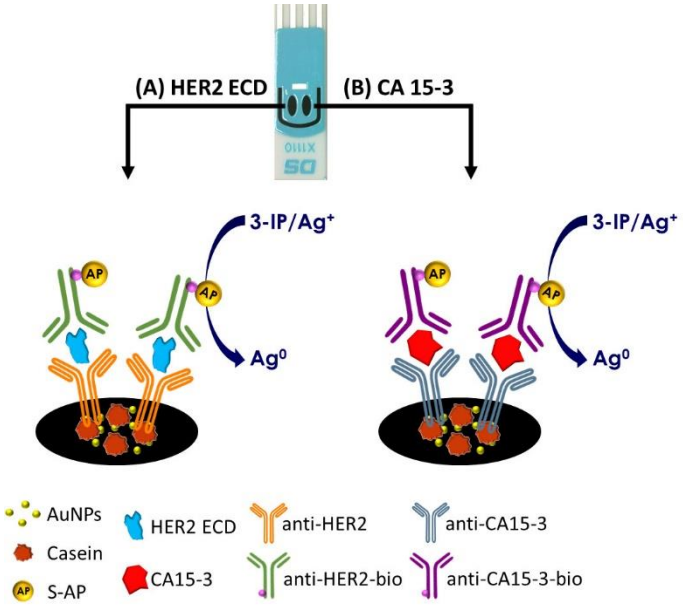

Fig.1. Schematic representation of the immunoassay for (A) HER2-ECD and (B) and CA 15-3 on a bi-SPCE-AuNP.

3. Results and discussion

3.1. Immunoassay optimization

The bi-immunosensor was developed based on previous studies of our group, using an SPCE with one working electrode, of individual electrochemical immunoassays for CA 15-3 (unpublished results) and HER2-ECD [8].

The first approach was as follows: after incubating the bi-SPCE-AuNP overnight at 4 ºC with the capture antibody solutions (4 μL each, 50 μg mL^{-1} anti-HER2 and 100 μg mL^{-1} anti-CA15-3), the electrodes were washed with buffer 1. The free surface sites were blocked with casein (60 μL, 2% w/v) during 30 min and the electrode was washed with buffer 1. Then the sensor was incubated for 1 hour with 60 μL of a mixture (1 : 1) of a solution containing the antigens and a solution containing the anti-CA15-3-bio antibody (2 μg mL^{-1}), the anti-HER2-bio antibody (1 μg mL^{-1}) and BSA (0.5% w/v). Another washing step with buffer 1 was performed and 60 μL of a 0.2 nM S-AP solution was placed on the sensor and left to react for 1 hour. Finally, the sensor was washed with buffer 2 and the enzymatic reaction was performed. The signal was recorded as indicated in **Section 2.4**. The results (**Fig. S3**) showed that the sensor could distinguish between different HER2-ECD concentrations but no variation was observed for different CA 15-3 concentrations.

Considering these results, it was suspected that non-specific binding of detection antibodies covered up the analytical signal for CA 15-3. Since only the analytical signal for HER2-ECD was observed, the results let to believe that the most important non-specific binding events were related to the anti-HER2-bio antibody. Therefore, an assay in which the concentration of this antibody was changed (from 0 to 2 μg mL^{-1}) was carried out (**Fig. 2A**). The results of this experiment showed that by decreasing (from 1.0 to 0 μg mL^{-1}) the anti-HER2-bio concentration the background signals also decreased. Furthermore, when no anti-HER2-bio was used the analytical signal for CA 15-3 was clearly observed, which did not occur when anti-HER2-bio was used.

In view of this, it was suspected that the anti-HER2-bio antibody interacted with the anti-CA15-3 antibody (capture antibody for CA 15-3). To verify this the same assay was performed but in this case the bi-SPCE-AuNPs were modified with only one of the capture antibodies, anti-HER2 or anti-CA15-3 (the unmodified working electrode was incubated overnight with 4 μL of buffer 1). Besides this, to evaluate the background signal tests without the capture antibodies were also carried out. The results

of this experiment, presented in **Fig. 2B**, showed that the peak current intensities are identical in the absence and presence of anti-CA15-3. So, it was concluded that anti-HER2-bio did not bind non-specifically to anti-CA15-3, but its presence did not allow to obtain an analytical signal for CA 15-3.

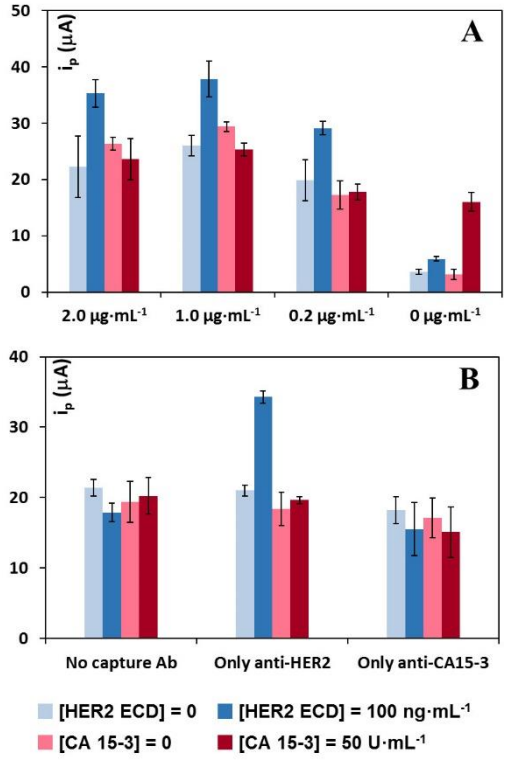

Fig. 2. (A) Effect of the concentration of anti-HER2-bio (using anti-CA15-3-bio 2 µg·mL^{-1}) in the absence and presence of the analytes ([HER2-ECD] = 100 ng mL^{-1} (blue bars) and [CA 15-3] = 50 U mL^{-1} (red bars)). (B) Peak current intensities in the absence of capture antibodies (no capture Ab) or in the presence of only one of them (anti-HER2 50 µg mL^{-1} or anti-CA15-3 100 µg mL^{-1}) using anti-CA15-3-bio 1 µg·ml^{-1} and anti-HER2-bio 1 µg·ml^{-1}. Analyte concentrations: [HER2-ECD] 0 and 100 ng mL^{-1} (blue bars) and [CA 15-3] 0 and 50 U mL^{-1} (red bars). Experimental conditions: anti-HER2 50 µg·mL^{-1}, anti-CA15-3 100 µg·mL^{-1}, casein 2% (w/v), BSA 1% (w/v) in solution of detection antibodies, S-AP 0.2 nM, 3-IP/$AgNO_3$ 1.0/0.4 mM. Average data ± *SD* are indicated (*n* = 3).

To solve this problem, a new procedure was tested in which the anti-HER2-bio antibody was added independently, i.e. after the incubation with the analytes and anti-CA15-3-bio. Furthermore, the incubation time of this antibody was only 30 min in order to minimize non-specific binding. Like this, the assay procedure was as follows: after blocking the surface with casein, the immunosensor

was incubated for 1 hour with a 60-µL aliquot of a mixture (1 : 1) of a solution containing CA 15-3 and HER2-ECD and a solution containing the anti-CA15-3-bio antibody and BSA (1 % w/v). Then, after a washing step, a 60-µL aliquot of an anti-HER2-bio solution containing BSA (0.5 % w/v) was added and left for react for 30 min. In this procedure different concentrations of anti-CA15-3-bio and anti-HER2-bio were tested (**Fig. 3**).

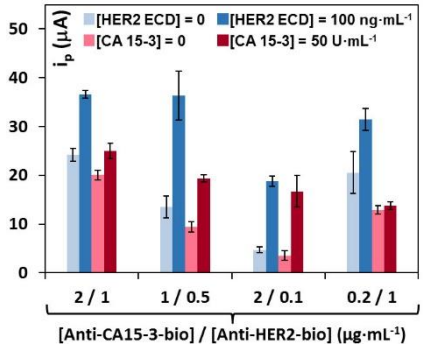

Fig. 3. Effect of anti-CA15-3-bio and anti-HER2-bio concentrations when anti-HER2-bio was added in an independent step. Analyte concentrations: [HER2-ECD] 0 and 100 ng mL^{-1} (blue bars) and [CA 15-3] 0 and 50 U mL^{-1} (red bars). Experimental conditions: anti-HER2 50 µg mL^{-1}, anti-CA15-3 100 µg mL^{-1}, casein 2% (w/v), BSA 1% (w/v) in anti-CA15-3-bio solution, BSA 0.5% (w/v) in anti-HER2-bio solution, S-AP 0.2 nM, 3-IP/$AgNO_3$ 1.0/0.4 mM. Average data ± *SD* are indicated (*n* = 3)

By comparing **Fig. 3** with **Fig. 2A** it could be concluded that the addition of the anti-HER2-bio antibody in an independent step resulted in a signal for CA15-3 that is clearly different from the background signal. For example, for anti-CA15-3-bio and anti-HER2-bio concentrations of 2 and 1 µg·mL^{-1}, respectively, in the case of **Fig. 3**, a slight analytical signal for CA15-3 was observed while in **Fig. 2A** no signal for this analyte was obtained. Comparing the anti-CA15-3-bio and anti-HER2-bio concentrations of 2 and 0.2 µg mL^{-1}, respectively, in **Fig. 2A**, with the anti-CA15-3-bio and anti-HER2-bio concentrations of 2 and 0.1 µg mL^{-1}, respectively, in **Fig. 3**, the background signal decreased enormously and an important analytical signal was obtained for CA15-3. Thus, it was concluded that this assay procedure could allow the simultaneous detection of both biomarkers.

Fig. 3 was also useful to study the effect of the detection-antibody concentrations on the analytical signal. As can be seen, the best detection-antibody concentrations are either 1 and 0.5 $\mu g \cdot mL^{-1}$ or 2 and 0.1 $\mu g \cdot mL^{-1}$ (anti-CA15-3-bio and anti-HER2-bio, respectively). Using these concentrations, an analytical signal for CA15-3 was observed and the background signals were lower than with the other concentrations tested. The 1 and 0.5 $\mu g\ mL^{-1}$ antibody concentrations were chosen for further studies because they provided the highest analytical signals, despite the higher background signals. Therefore, the concentration of BSA in the anti-HER2-bio solution was increased, the S-AP concentration was increased and BSA was added to the S-AP solution. The results of these experiments are summarized in **Fig. 4A**. This figure shows that increasing the BSA concentration in the anti-HER2-bio solution (see **Fig. 3**, anti-CA15-3-bio and anti-HER2-bio concentrations 1 and 0.5 $\mu g \cdot mL^{-1}$, respectively) decreased the background signals for both biomarkers. Moreover, increasing the concentration of S-AP solution to 0.5 nM and adding BSA (0.1% w/v), the background signals for both antigens decreased even more. So, finally, the chosen concentrations of both detection antibodies and S-AP were: anti-CA15-3-bio 1 $\mu g \cdot mL^{-1}$ with BSA 1 % (w/v), anti-HER2-bio solution 0.5 $\mu g \cdot mL^{-1}$ with BSA 1 % (w/v) and S-AP 0.5 nM with BSA 0.1 % (w/v).

Once all these conditions were optimized, the effect of the capture antibody concentration was evaluated. **Fig. 4B** summarizes the results using different concentrations of capture antibodies. For the sake of economy and sensitivity, the concentrations chosen as optimal for anti-CA15-3 and anti-HER2 were 100 and 50 $\mu g\ mL^{-1}$, respectively.

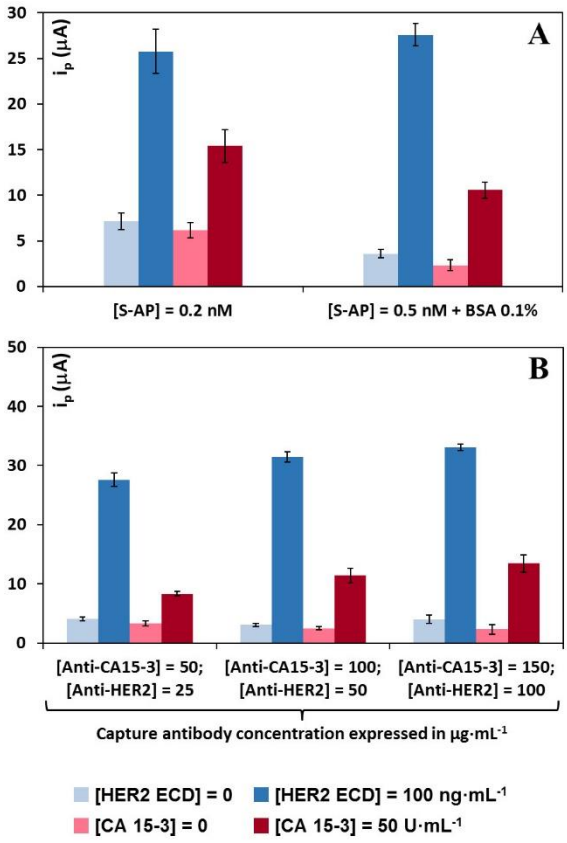

Fig. 4. (A) Effect of the concentration of S-AP and the use of BSA in the S-AP solution (using anti-HER2 50 µg mL^{-1} and anti-CA15-3 100 µg mL^{-1}). (B) Effect of the concentration of the capture antibodies. Analyte concentrations tested: [HER2 ECD] 0 and 100 ng·mL^{-1} (blue bars) and [CA 15-3] 0 and 50 U·mL^{-1} (red bars). Experimental conditions: casein 2% (w/v), anti-CA15-3-bio 1 µg·mL^{-1} with BSA 1%, anti-HER2-bio solution 0.5 µg·mL^{-1} with BSA 1% (w/v) (independent step), S-AP 0.2 nM with BSA 0.1% (w/v), 3-IP/$AgNO_3$ 1.0/0.4 mM. Average data ± *SD* are indicated (*n* = 3).

3.2. Analytical characteristics of the immunosensor

Under the optimized conditions (indicated in **Section 2.3.2.**), the response of the dual immunosensor to different concentrations of HER2-ECD and CA 15-3 was evaluated (concentrations between 0 and 100 ng mL^{-1} for HER2-ECD and between 0 and 100 U mL^{-1} for CA 15-3). The figures of merit of the method are indicated in Table S1. A linear relationship between peak current intensity and antigen concentration was found between 10 and 50 ng·mL^{-1} for HER2-ECD and between 20 and 70 U·mL^{-1} for CA 15-3 according to the following equations (**Fig. 5**): *i* (µA) = 0.366 [HER2-ECD] (ng mL^{-1}) + 4.00; *R* = 0.998, *n* = 5; *i* (µA) = 0.157 [CA 15-3] (U mL^{-1}) + 1.57; *R* = 0.998, *n* = 5. The limits of

detection (LOD) and limits of quantification (LOQ) were calculated from the calibration plot using the equations LOD = 3 s_b/m and LOQ = 10 s_b/m, where s_b is the standard deviation of the intercept and m is the slope of the calibration plot. In the case of HER2-ECD, the LOD was found to be 2.9 ng mL^{-1} while for CA 15-3, the LOD was 5.0 U mL^{-1}. The obtained LOQs were 9.8 ng·mL^{-1} and 16.6 U·mL^{-1} for HER2-ECD and CA 15-3, respectively. Considering the cut-off values for HER2-ECD 15 ng mL^{-1} and for CA 15-3 25 U mL^{-1}, the LOQs are clearly lower than the cut-off values, so the developed bi-immunosensor could be useful in the diagnostic, treatment and follow-up of breast cancer patients. The precision of the method for the analysis of both biomarkers is also adequate because $V_{x0} \approx 5\%$. Although electrochemical immunosensors were previously published for the individual or simultaneous (together with other cancer biomarkers, but not including the CA15-3 & HER2-ECD pair) detection of CA 15-3 and HER2-ECD, in several cases achieving lower LODs than in the present work (**Table 1**), the simplicity of the proposed bi-immunosensor makes it attractive, even more since the concentrations it covers is in the clinical useful range. Furthermore, the immobilization of the capture Ab by simply adsorption obviates the need of use cross-linkers, polymers or self-assembly monolayers, reducing the operation complexity and assay cost.

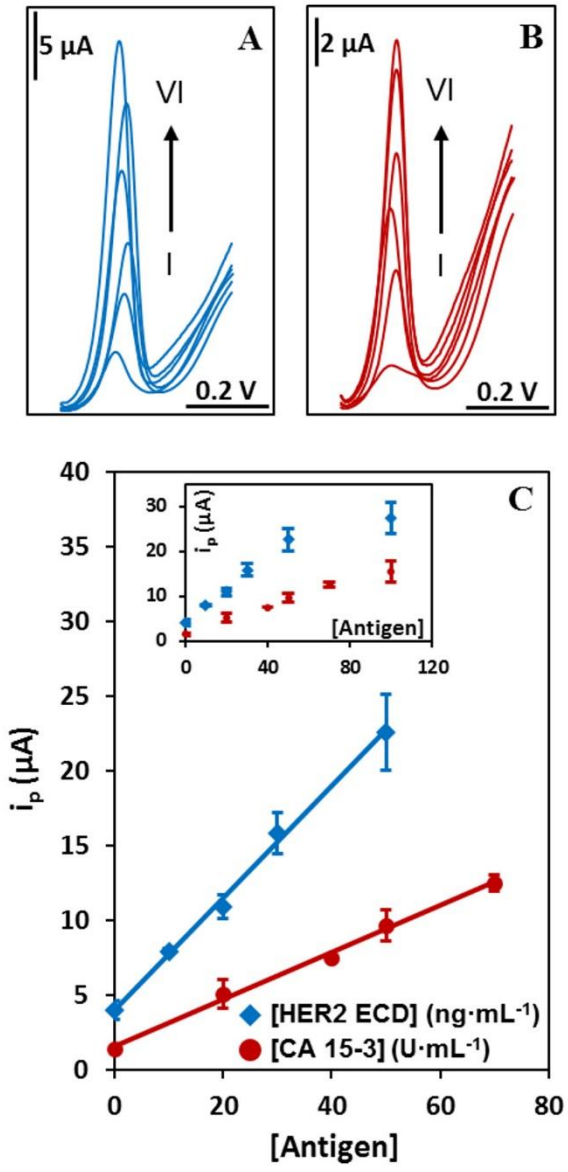

Fig. 5. Simultaneous determination of HER2-ECD and CA 15-3 using the developed immunosensor. Linear sweep voltammograms (-0.02 to +0.4 V) for the analysis of (A) HER2-ECD and (B) CA 15-3; [HER2-ECD] (I to VI): 0, 10, 20, 30, 50, 100 ng mL^{-1}; [CA 15-3] (I to VI): 0, 20, 40, 50, 70, 100 U mL^{-1}. (C) Calibration curves obtained for HER2-ECD (from 0 to 50 ng mL^{-1}) and CA 15-3 (from 0 to 70 U mL^{-1}); inset: the peak current intensity values obtained for HER-ECD (from 0 to 100 ng mL^{-1}) and CA 15-3 (from 0 to 100 U mL^{-1}). Error bars correspond to the standard deviation of 3 measurements. Experimental conditions: anti-HER2 50 µg·mL^{-1}, anti-CA15-3 100 µg·mL^{-1}, casein 2% (w/v), anti-CA15-3-bio 1 µg·mL^{-1} with BSA 1% (w/v), anti-HER2-bio solution 0.5 µg·mL^{-1} with BSA 1% (w/v), S-AP 0.5 nM with BSA 0.1% (w/v), 3-IP/$AgNO_3$ 1.0/0.4 mM.

Table 1. Comparison of analytical properties of recent electrochemical immunosensors for CA15-3 and HER2-ECD detection.

Biomarker	Construction	Detection	Concentration range	LOD	Ref.
CA 15-3, HER2-ECD	Sandwich-type assay for simultaneous determination immobilizing capture Abs on bi-SPCE- AuNPs. Sandwich-type assay. AP as label.	LSV	CA 15-3: 0 - 70 $U \cdot mL^{-1}$; HER2-ECD: 0 - 50 $ng \cdot mL^{-1}$	CA 15-3: 5.0 $U \cdot mL^{-1}$; HER2-ECD: 2.9 $ng \cdot mL^{-1}$	This work
HER2-ECD	Label-free using affibody immobilized on AuNPs/GSPE as bioreceptor.	EIS	0 - 40 $ng \cdot mL^{-1}$	6.0 $ng \cdot mL^{-1}$	[38]
HER2-ECD	Sandwich-type assay on SPCE-AuNPs immobilizing capture Ab by adsorption. AP as label.	LSV	15 - 100 $ng \cdot mL^{-1}$	4.4 $ng \cdot mL^{-1}$	[8]
HER2-ECD	Sandwich-type assay coupling capture Ab to protein A modified magnetic beads. SPCE as transducer and AP as label.	DPV	0 - 15 $ng \cdot mL^{-1}$	6 $ng \cdot mL^{-1}$	[23]
HER2-ECD	Sandwich-type assay using Nanobodies. SPCE as transducer and HRP as label.	Amperometry	1 - 200 $\mu g \cdot mL^{-1}$	1 $\mu g \cdot mL^{-1}$	[22]
HER2-ECD	Label free. Capture Ab attached to iron oxide nanoparticles which are laid over a gold electrode.	DPV	0.01 - 10 $ng \cdot mL^{-1}$; 10 - 100 $ng \cdot mL^{-1}$	0.995 $pg \cdot mL^{-1}$	[39]
HER2-ECD	Sandwich-type assay. Capture Ab immobilized on AuNPs-modified glassy carbon electrode. For detection a conjugate of hydrazine-AuNP-aptamer is used.	SWSV	0.1 $pg \cdot mL^{-1}$ – 10 $ng \cdot mL^{-1}$	0.037 $pg \cdot mL^{-1}$	[40]
HER2-ECD	Label free. Capture Ab immobilized on AuNPs/HDT/AuNPs@MWCNT-CIL electrode.	EIS	10 – 110 $ng \cdot mL^{-1}$	7.4 $ng \cdot mL^{-1}$	[41]
CA 15-3	Nanoporous gold/graphene modified glassy carbon electrode. Sandwich type assay using HRP-encapsulated liposomes as labels.	DPV	$2 \cdot 10^{-5}$ – 40 $U \cdot mL^{-1}$	$5 \cdot 10^{-6}$ $U \cdot mL^{-1}$	[42]
CA 15-3	Label free. Capture Ab attached onto *N*-doped graphene sheets and then immobilizing on a glassy carbon.	DPV	0.1 – 20 $U \cdot mL^{-1}$	0.012 $U \cdot mL^{-1}$	[43]
CA 15-3	Sandwich-type assay. Capture Ab immobilized on graphene-oxide-modified gold electrode. For detection detection Ab/MWCNT/ferritin bioconjugated is used.	DPV	0.05 – 100 $U \cdot mL^{-1}$	0.009 $U \cdot mL^{-1}$	[44]
CA 15-3	Label free. Capture Ab immobilized on a glassy carbon electrode modified with ferrocene/electrochemically reduced graphene oxide/electrochemically generated AuNPs composited used as immobilizin platform and signal probe.	DPV	0.05 – 2.0 $U \cdot mL^{-1}$; 2.0 – 25.0 $U \cdot mL^{-1}$	0.015 $U \cdot mL^{-1}$	[45]
CA 15-3	Sandwich-type assay immobilizing capture Ab on glassy carbon electrode modified with AuNPs functionalized graphene. Detection Ab adsorbed on grafting polystyrene-based polymer chains with pendant Ru(II) luminophore on the surface of palladium nanocages is used for detection.	ECL	0.01 – 120 $U \cdot mL^{-1}$	0.003 $U \cdot mL^{-1}$	[46]

CA 15-3	Sandwich-type assay. Capture Ab labeled graphene oxide immobilized on SPCE. Peroxidase-like magnetic silica nanoparticles/graphene oxide/detection Ab composites are used for detection.	DPV	10^{-3} - 200 $U \cdot mL^{-1}$	$2.8 \cdot 10^{-4}$ $U \cdot mL^{-1}$	[24]
CA 15-3, CA 125, CEA	Sandwich-type assay for simultaneous determination immobilizing capture Ab on graphene modified SPCE with three WEs. Mesoporous platinum NPs as label.	DPV	CA 15-3: 0.008 – 24 $U \cdot mL^{-1}$	CA 15-3: 0.001 $U \cdot mL^{-1}$	[25]
CA 15-3, CA 125, CEA	Sandwich-type assay for simultaneous determination immobilizing capture Ab on a AuNPs/graphene modified SPCE with three WEs. A conjugate AP/detection Ab/Au clusters/graphene is used for detection.	LSV	CA 15-3: $5 \cdot 10^{-3}$ - 50 $U \cdot mL^{-1}$	CA 15-3: $1.5 \cdot 10^{-3}$ $U \cdot mL^{-1}$	[26]
CA 15-3, CA 125, CA 19-9	Sandwich-type assay for simultaneous determination immobilizing capture Ab on magnetic beads and using PAMAM dendrimer-metal sulfide QD as distinguishable label. An *in situ* formed mercury drop electrode on a glassy carbon as WE.	SWSV	CA 15-3: 0.01 - 50 $U \cdot mL^{-1}$	CA 15-3: 0.005 $U \cdot mL^{-1}$	[29]

WE: working electrode; LSV: linear sweep voltammetry; DPV: differential pulse voltammetry; SWSV: square wave stripping voltammetry; EIS: electrochemical impedance spectroscopy; ECL: electrochemiluminescence; GSPE: graphite screen-printed electrode; HDT: 1,6-hexanedithiol; MWCNT: multiwalled carbon nanotube; CIL: carbon ionic liquid; AP: alkaline phosphatase; QD: quantum dots; CEA: carcinoembryonic antigen; NP: nanoparticles.

4. Conclusions

The current trends in analytical chemistry are focused on developing simple and *in situ* analysis devices. Moreover, it is still an important challenge to be able to simultaneously determine several analytes with the same device. Therefore, in this work the first multiplexed electrochemical immunosensor for the simultaneous detection of two breast cancer biomarkers (CA 15-3 and HER2-ECD) was developed. Several aspects of the immunoassay were studied in order to achieve an immunosensor able to detect both markers in the range of concentration with clinical importance. An immunosensor with limits of detection of 2.9 ng mL^{-1} for HER2-ECD and 5.0 U mL^{-1} for CA 15-3 was achieved. Considering the cut-off values, 15 ng mL^{-1} and 25 U mL^{-1} for HER2-ECD and CA 15-3 respectively, the usefulness that this immunosensor could have in clinical analysis is clear. The possibility to simultaneously analyze two biomarkers increases the sensor's utility. Moreover, this

sensor can be included in a portable and ready-to-use device due to the use of SPE as transducer. Thus, this sensor could be a value tool for a rapid and non-invasive screening breast cancer test and control of these biomarkers in breast cancer patients under treatment.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/

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