



# High-performance electrochemical immunomagnetic assay for breast cancer analysis

Maria Freitas<sup>a</sup>, Henri P.A. Nouws<sup>a,\*</sup>, Elisa Keating<sup>b,c</sup>, Cristina Delerue-Matos<sup>a</sup>

<sup>a</sup> REQUIMTE/LAQV, Instituto Superior de Engenharia do Porto, Politécnico do Porto, Rua Dr. António Bernardino de Almeida 431, 4200-072 Porto, Portugal

<sup>b</sup> Department of Biomedicine – Unit of Biochemistry, Faculty of Medicine, University of Porto, Al. Prof. Hernâni Monteiro, 4200-319 Porto, Portugal

<sup>c</sup> CINTESIS – Center for Health Technology and Services Research, Rua Dr. Plácido da Costa, 4200-450 Porto, Portugal

## ARTICLE INFO

### Keywords:

Breast cancer  
HER2-ECD  
SK-BR-3  
Electrochemical immunoassay  
Magnetic beads  
Screen printed electrodes

## ABSTRACT

Despite the evolution of targeted therapies in oncology, some challenges such as screening and early diagnosis of cancer-related biomarkers still remain. The analysis of the Human Epidermal growth factor Receptor 2 (HER2) in biological fluids provides essential information for effective treatments. In this work we report the development of an electrochemical immunomagnetic bioassay for the analysis of the extracellular domain of HER2 (HER2-ECD) in human serum and cancer cells. Biomodified carboxylic acid functionalized magnetic beads (COOH-MBs) were used as the capture probe and an antibody labelled with alkaline phosphatase (AP) as the signalling probe. In the presence of HER2-ECD a sandwich complex was formed on the MBs, which were magnetically attracted to the surface of a screen-printed carbon electrode (SPCE). After the addition of 3-indoxyl phosphate and silver ions, used as the enzymatic substrate, the immunological interaction was detected by linear sweep voltammetry. Two linear concentration ranges were established: one between 5.0 and 50 ng/mL and another between 50 and 100 ng/mL. The developed assay provided a clinically useful detection limit (2.8 ng/mL) and has an adequate precision ( $V_{x0} < 5\%$ ). The assay provided accurate results and was selective towards the target biomarker. Additionally, CTCs were analysed in human serum and a detection limit of 3 cells/mL was achieved for the HER<sup>+</sup> breast cancer cell line SK-BR-3.

## 1. Introduction

Screening and early-stage diagnosis of oncological diseases and an adequate follow-up are critical for successful patient management. This promotes general public health and increases the survival rate [1,2]. The gold standard procedures established for screening and detection of breast cancer are based on imaging tools. However, these techniques have limitations such as the decrease of sensitivity when breast tissue density increases. In addition, invasive techniques (e.g. biopsies) are required to confirm the presence of the tumour [3,4]. Technological advances in this field include (bio)sensors/assays that provide rapid and accurate diagnosis and point-of-care detection possibilities by combining the selectivity of biomolecule interactions with the high sensitivity of modern analytical techniques for non-invasive analysis [5,6]. Therefore, electrochemical biosensors were already widely employed for tumour marker recognition and detection [7–9].

Human Epidermal growth factor Receptor 2 (HER2) is a specific cancer related biomarker used in clinical settings. Abnormal HER2 levels are particularly significant since its overexpression is related to

invasive and aggressive breast cancer-types [10]. HER2 is reported as a biomarker of interest in the development of non-invasive tests for diagnosis in serum samples by the European Group on Tumor Markers (EGTM) [11] and the Food and Drug Administration (FDA) [12]. Besides this, the analysis of circulating tumour cells has risen attention since biomarkers present on the cell surface can be detected through the analysis of their extracellular domains (ECDs) [13].

A wide diversity of electrochemical immunosensors for the analysis of HER2 in serum samples have been published [14–29]. Although simple strategies with non-modified transducers have been reported [14–18], versatile and innovative transducing platforms, modified and/or functionalized with nanomaterials [19–22], self-assembled monolayers [23,24], polymers [25] or sequential layer deposition [26–29] are also part of the described methodologies. However, this diversity leads to time-consuming sensor surface construction strategies. A variety of new smart materials, particularly nano- and micro materials, can vastly improve screening and diagnosis because they increase the assay's performance, including the reduction of the analysis time and the enhancement of the sensitivity and/or selectivity [30]. Therefore,

\* Corresponding author.

E-mail address: [han@isep.ipp.pt](mailto:han@isep.ipp.pt) (H.P.A. Nouws).

<https://doi.org/10.1016/j.snb.2020.127667>

Received 12 November 2019; Received in revised form 30 December 2019; Accepted 3 January 2020

Available online 07 January 2020

0925-4005/ © 2020 Elsevier B.V. All rights reserved.

magnetic nanoparticles (MNPs) and magnetic beads (MBs) have gained great interest as sensing platforms in electrochemical immunomagnetic assays for cancer diagnosis [1]. When using magnetic particles biomolecular interactions are improved, matrix effects are minimized through efficient washing steps [31] and preconcentration of the analyte is easily achieved. Thus, by immobilizing antibodies on their surfaces, the effective magnetic separation and the pre-concentration of an antigen from complex samples can be achieved. In the case of electrochemical magnetoassays an additional advantage is the fact that the assay is mostly performed away from the electrode (in microtubes), reducing possible electrode fouling. Only the detection strategy is carried out on the electrode surface using small magnets with the size of the working electrode for efficient magnetic attraction before the electrochemical measurement [32,33].

Because of the above-mentioned advantages, some electrochemical immunomagnetic assays have already been reported. These assays were based on the use of 'self-made' nanoparticles or commercially available MBs as sensing platforms, containing distinct functional groups or recognition elements on their surfaces [34–38]. In these works, modification of the magnetic particles' surfaces with the biorecognition element was achieved, either through covalent binding or affinity processes, in a short time (1 h) when compared to the reported immunosensors in which the immobilization procedure usually occurs overnight.

To avoid the misclassification of HER2-positive patients, the evaluation of circulating tumour cell overexpression is of utmost importance. Detection of HER2-positive cell-lines can greatly contribute to early status assessment and to monitor the patients' treatments. In this work we report the development of an electrochemical immunomagnetic assay for the detection of HER2-ECD in human serum using carboxylic acid functionalized magnetic beads (HOOC-MBs) and screen-printed carbon electrodes (SPCEs). The HOOC-MBs constituted a versatile tool for the construction of the magnetic immunosensing platform and stable immobilization of a large amount of antibodies was achieved through covalent binding. After this step, the sandwich assay consisted of the addition of HER2-ECD and biotinylated detection antibodies, which were then labelled with (streptavidin)-alkaline phosphatase (AP). This resulted in a bioconjugate that was attracted to the surface of the SPCE by placing a magnet ( $d = 4$  mm) below its WE. The combination of 3-indoxyl phosphate (the enzymatic substrate) and silver nitrate allowed the detection of the immunological interaction by linear sweep voltammetry (LSV) (Scheme 1). Additionally, the assay was also tested for the analysis of live breast cancer cells (HER2<sup>+</sup>: SK-BR-3; HER2<sup>-</sup>: MDA-MB-231). This is the first electrochemical immunomagnetic assay for HER2-ECD and cancer cell analysis using this detection strategy.

## 2. Materials and methods

### 2.1. Reagents and solutions

Albumin from bovine serum (BSA), 3-indoxyl phosphate (3-IP), ethanolamine (EA), streptavidin-alkaline phosphatase (S-AP) from *Streptomyces avidinii*, N-(3-dimethylaminopropyl)-n'-ethylcarbodiimide hydrochloride (EDC), N-Hydroxysuccinimide (NHS), MES monohydrate, Tween® 20, and tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma-Aldrich. Monoclonal capture and detection antibodies and recombinant HER2-ECD were purchased from Sino Biological Inc. Dynabeads™ MyOne™ Carboxylic Acid (MBs, 10 mg/mL) were acquired from Life Technologies.

The following solutions were used: 0.1 M MES pH 6 (buffer 1, B1), for MBs activation (200 mM EDC and 50 mM NHS) and to prepare the capture antibody (Ab-C) solution; PBS pH 8.3 (buffer 2, B2) to prepare the blocking solution (EA, 1 M); 0.1 M Tris-HNO<sub>3</sub> pH 7.4 (buffer 3, B3) to prepare working solutions of the detection (Ab-D) antibodies and the antigen (HER2-ECD); 0.1 M Tris-HNO<sub>3</sub> pH 7.4 with 1% BSA (m/V)

(buffer 4, B4) to prepare the S-AP solution; 0.1 M Tris-HNO<sub>3</sub> pH 9.8 containing Mg(NO<sub>3</sub>)<sub>2</sub> ( $2.0 \times 10^{-2}$  M) (buffer 5, B5) to prepare the solution containing 3-IP ( $1.0 \times 10^{-3}$  M) and silver nitrate ( $4.0 \times 10^{-4}$  M) (stored at 4 °C and protected from light). For the washing steps 0.01% of Tween 20 (T) was added to the distinct buffers. These buffers were used according to the specifications of the suppliers of the biomolecules and the MBs.

All solutions were prepared in Type I purified water (resistivity = 18.2 MΩ cm).

### 2.2. Modification of the HOOC-MBs and immunoassay

All the steps of the immunomagnetic assay were performed at room temperature, under continuous vortex stirring (950 rpm) and protected from light. The washing steps consisted of the addition of 100 μL of the adequate buffer containing Tween-20 and subsequent continuous stirring for 2 min. After each washing step, the MBs were attracted using a magnetic rack and the supernatant was discarded after 1 min. A 6-μL aliquot of the HOOC-MBs suspension (10 mg/mL) was placed in a 1.5-mL tube and the MBs were washed once with B1 (100 μL, 5 min) before proceeding.

The main steps of the assay are represented in Scheme 1.

The MBs' biomodification consisted of the following steps: (i) activation of the MBs by adding EDC/NHS (100 μL, 15 min), followed by a single washing step with B1-T; (ii) addition of 100 μL of a Ab-C solution (25 μg/mL, 60 min) (this leads to an average amount of 0.047 μg antibody per MB, which is in accordance with the amount recommended by the MBs supplier) followed by washing with B1-T and B2-T; (iii) incubation of the MBs with EA (100 μL, 10 min) after washing steps with B2-T and B3-T. The MBs were resuspended in B3-T and stored at 4 °C until use.

For the optimized immunoassay (iv) HER2-ECD or cancer cells (variable concentration) and Ab-D (2 μg/μL) were previously mixed and a 100-μL aliquot of this mixture was added for 60 min. Then, the MBs were washed with B3-T. Afterwards, the MBs were incubated with (v) a S-AP solution ( $5.0 \times 10^{-10}$  M in B4, 100 μL, 30 min), and finally washed twice with B5-T and resuspended in 50 μL of B5.

### 2.3. Electrochemical measurements

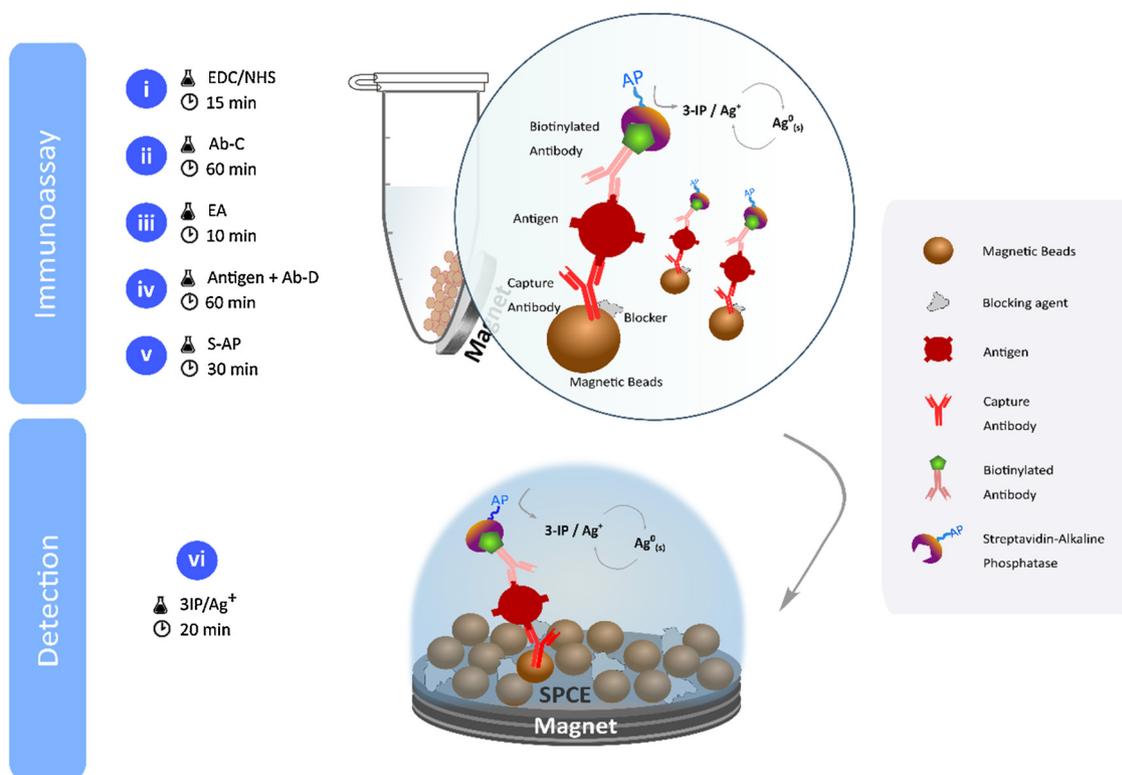
The MBs were attracted to the WE of the SPCE (DRP-110, DropSens) using a 4-mm magnet. A 25-μL aliquot of the re-suspended MBs (corresponding to 30 μg of MBs) was placed on the SPCE and the MBs were magnetically attracted for 2 min. The solution was then removed and (vi) 40 μL of the enzymatic substrate solution (3-IP,  $1.0 \times 10^{-3}$  M) containing silver nitrate ( $4.0 \times 10^{-4}$  M) was added for 20 min. The detection was performed by linear sweep voltammetry (LSV) through the analysis of the deposited silver (potential range:  $-0.03$  V to  $+0.4$  V, scan rate: 50 mV/s). A potentiostat/galvanostat (PGSTAT101, Metrohm Autolab) and the NOVA software package (v.1.9; Metrohm Autolab) were used to record the voltammograms.

### 2.4. Analyses of human serum samples

To evaluate the applicability of the developed assay, Human serum (from male AB clotted whole blood, Sigma-Aldrich) was spiked with HER2-ECD (1 μL of a HER2-ECD standard solution was added to 49 μL of serum) and analysed without any pre-treatment or further dilution. The results were compared with the ones obtained with a commercial Human ErbB2 (HER2) ELISA kit (Thermo Scientific, Invitrogen).

### 2.5. Cell culture and detection

The breast cancer cell line MDA-MB-231 (HER2-negative cancer cells) was obtained from ATCC® and SK-BR-3 cells (HER2-positive cancer cells) were provided by the Department of Biomedicine — Unit



**Scheme 1.** Representation of the immunoassay.

of Biochemistry of the Faculty of Medicine of the University of Porto. The cells were cultured in RPMI medium. For detection experiments, cells were seeded on 21 cm<sup>2</sup> plastic cell culture dishes (TPP®). On the day of the experiment, the cells were harvested with Trypsin-EDTA 0.25% and counted using an automated cell counter (Countess™, Thermo Scientific, Invitrogen). A trypan-blue exclusion assay was performed using automatic cell counting to confirm cell viability, which was between 91% and 96%. The distinct cells concentrations ( $1 \times 10^2$ – $1 \times 10^5$  cells/mL) were prepared in human serum and analysed using the optimized immunoassay (Sections 2.2 and 2.3).

## 2.6. Magnetic bead and cell analysis

FEI Quanta 400FEG ESEM/EDAX Genesis X4M equipment was used to obtain the SEM images. ImageJ open source software was used to determine the particles' average size and histograms were obtained with SPSS (v.20.0; SPSS Inc.). The cell lines used in this study were imaged by a Nikon TMS microscope.

## 3. Results and discussion

### 3.1. Electrode surface characterization

The MBs were magnetically attracted to the SPCE and the electrode surface was characterized by SEM (Fig. 1). In the obtained images no agglomeration of the magnetic particles was observed, and they were perfectly distributed on the electrode surface. The average size was  $1094 \pm 32.5$  nm which is in agreement with the size indicated by the supplier. The organization and linear distribution of the MBs on the electrode's surface are excellent features for the detection of the biomarker.

### 3.2. Optimization of the experimental parameters of the immunoassay

The effect of the amount of MBs used in the assay was evaluated

between 7.5 and 90 µg using the following conditions: Ab-C: 25 µg/mL; EA: 1 M; HER2-ECD: 0 (blank) and 50 ng/mL; Ab-D: 2 µg/mL; S-AP:  $5.0 \times 10^{-10}$  M with BSA 1% (m/V). The obtained results are presented in Fig. 2A. With the increase of the amount of MBs, from 7.5 to 45 µg, an increase of the analytical signal (peak current intensity ( $i_p$ )) was observed, after which it slightly decreased. The lowest amount of MBs (7.5 µg) led to the lowest  $i_p$  due to the reduced amount of capture antibodies. However, for the highest amount of MBs (90 µg) a slight decrease of the peak current intensity was observed, which can be due to a higher electron transfer resistance and thus lower current intensity. The best signal-to-blank ratio (S/B) was verified for 30 µg of MBs. This amount was used for the optimization of the other experimental parameters.

The Ab-C and Ab-D concentrations were subsequently optimized using the following conditions: HOOC-MBs: 30 µg; Ab-C: 10 and 25 µg/mL; HER2: 0 (blank) and 50 ng/mL; Ab-D: 1, 2 and 4 µg/mL; S-AP:  $5.0 \times 10^{-10}$  M with BSA 1% (m/V). As can be seen in Fig. 2B, the highest  $i_p$  was obtained for Ab-C 25 µg/mL and Ab-D 4 µg/mL, however with a lower precision when compared to the other tested combinations. Although the combination of Ab-C 25 µg/mL and Ab-D 2 µg/mL provided a slightly lower sensitivity, a better precision of the results was achieved. Therefore, this combination was used in the subsequent optimizations.

To improve the total assay time, the incubation times of the several reagents were tested using the 'step-by-step' approach (SI Fig. S1): (1) HER2-ECD 30 min, Ab-D 30 min, S-AP 30 min; (2) HER2-ECD 30 min, Ab-D 60 min, S-AP 60 min; (3) HER2-ECD 60 min, Ab-D 60 min, S-AP 60 min; (4) HER2-ECD 30 min, Ab-D 60 min, S-AP 30 min. The alternatives 3 and 4 led to the highest sensitivity, however, in approach 4 the shorter incubation time of the enzyme led to a lower blank signal. To further reduce the blank signal, and using alternative 4, 0.5% (m/V) of BSA was added to the following solutions: (i) antigen (HER2-ECD), (ii) Ab-D and (iii) both the antigen and the Ab-D, with the purpose of blocking nonspecific adsorption. The addition of BSA 0.5% (m/V) to the Ab-D solution (alternative (ii)) clearly reduced the blank signal while

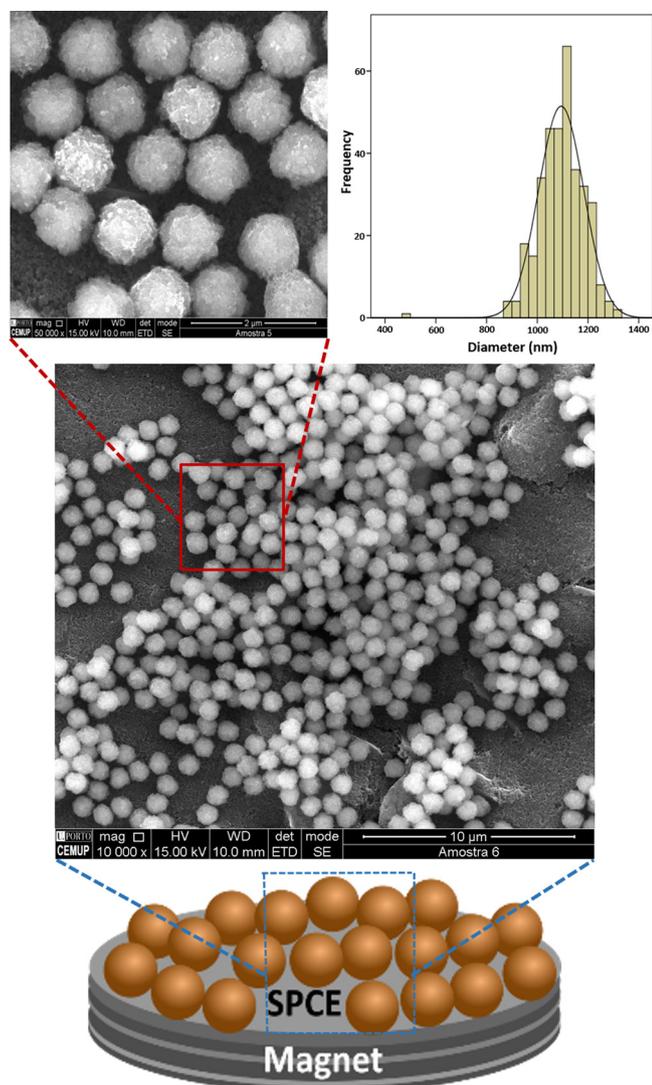


Fig. 1. SEM image of the SPCE covered with HOOC-MBs and the respective size distribution.

maintaining the signal for the analyte (50 ng/mL), leading to the best S/B ratio (SI Fig. S2).

Furthermore, in immunoassay protocols where enzyme conjugates are used, the addition of a blocking agent is usually advantageous to improve the S/B ratio. For this purpose, DEA 0.1 M and BSA 1% (m/V) were tested, according to previously published works [19,37]. As can be seen in SI Fig. S3A, although the use of both blockers resulted in excellent blank values, the highest analytical signal was obtained when BSA 1% (m/V) was used. Subsequently, four different S-AP solutions (with BSA 1% (m/V)) were tested:  $2.0 \times 10^{-10}$ ;  $5.0 \times 10^{-10}$ ;  $1.0 \times 10^{-9}$  and  $5.0 \times 10^{-9}$  M (SI Fig. S3B). As can be observed, higher S-AP concentrations led to higher  $i_p$  values. The  $5.0 \times 10^{-10}$  M concentration was chosen because it provided the best S/B ratio.

In order to reduce the analysis time of the immunoassay even further, a combination of several steps, by pre-incubation of the reagents, was studied. The tested alternatives were: (A) step-by-step assay, (B) pre-incubation of HER2-ECD + Ab-D during 60 min and (C) 120 min; (D) pre-incubation of Ab-D + S-AP (60 min); and (E) pre-incubation of HER2-ECD + Ab-D + S-AP (60 min). The obtained  $i_p$  values are presented in Fig. 3. Alternatives B and C led to the highest analytical signals, with alternative B providing a better precision among these two alternatives. On the other hand, the results obtained for alternatives D and E revealed a low signal and are not suitable for appropriate HER2-

ECD detection. Thus, the pre-incubation of HER2-ECD and Ab-D (containing BSA 0.5% (m/V)) for 60 min was used for the analysis of HER2-ECD in Human serum. To complete the optimization of the experimental parameters, the assay was tested at different temperatures (20, 25 and 30 °C). As can be seen in SI Fig. S4A the optimum temperature was 30 °C. The affinity of the MBs to the antigen, i.e. without the use of capture antibody, was also tested and no significant interaction was observed (SI Fig. S4B). Table S1 presents the main experimental variables, the tested range and the selected values.

### 3.3. Analytical characteristics of the assay

The analytical performance of the assay was evaluated under the optimized conditions. After verification of the suitability of the magnetic immunoassay to detect HER2-ECD in buffer solutions (0.1 M Tris-HNO<sub>3</sub> pH 7.4 (buffer 3); linear range 7.5–75 ng/mL (n = 5),  $i_p = 1.05 \pm 0.08$  [HER2-ECD] +  $55.2 \pm 3.5$ ,  $r = 0.993$  (SI Fig. S5)), experiments using Human serum as the matrix were carried out. The precision of the assay was evaluated for the analysis of 50 ng/mL HER2-ECD using three different SPCEs, on the same day and different days. Relative standard deviations (RSDs) of 4.0% and 3.4% were obtained, indicating that the developed assay provided precise results.

For calibration purposes, the electrochemical signal for different HER2-ECD concentrations (5–100 ng/mL) were studied (Fig. 4). The peak current intensity increased proportionally to the HER2-ECD concentration in two distinct ranges: between 5.0 and 50 ng/mL (1) and between 50 and 100 ng/mL. At the lower HER2-ECD concentrations the sensitivity was higher, which implies that the developed method can detect small variations in the cancer biomarker concentrations at the early stage of the disease. For the higher concentration range a different analytical behaviour was observed which can be explained by the near saturation of the antibody binding sites. The figures of merit for both ranges are indicated in Table 1. The linear relationship between  $i_p$  and [HER2-ECD] were: (1)  $i_p = 0.81 \pm 0.03$  [HER2-ECD] +  $2.46 \pm 0.94$  ( $r = 0.997$ ) and (2)  $i_p = 0.19 \pm 0.01$  [HER2-ECD] +  $32.6 \pm 1.1$  ( $r = 0.997$ ). The limits of detection (LOD) and quantification (LOQ) were calculated using the respective calibration data, using the equations:  $LOD = 3 s_b/m$  and  $LOQ = 10 s_b/m$ , where  $s_b$  is the standard deviation of the blank and  $m$  is the slope of the calibration plot. The obtained LODs were 2.8 ng/mL and 11.8 ng/mL and the LOQs were 9.3 ng/mL and 39.2 ng/mL, for concentration ranges 1 and 2, respectively. For both ranges the LODs were clearly lower than the established cut-off value (15 ng/mL). The analytical characteristics are better for range 1, however, the usefulness for both ranges are evident since they allow primary diagnosis (screening and/or early detection) and the evolution of the patient's treatment (follow-up). The coefficient of variation of the method ( $V_{x0}$ ) was 1.20% and 0.29% for the ranges 1 and 2 respectively, demonstrating adequate precision of the method ( $V_{x0} < 5\%$ ).

When the calibration plot was constructed in serum a clear matrix effect was observed. The slopes of the calibration plots in serum were 1.3 (range 1) and 5.6 (range 2) times lower than the slope obtained with the measurements in buffer. This could be due to globulins, especially immunoglobulins G (IgG) present in the serum [39,40].

To evaluate the accuracy of the developed immunoassay, spiked sera with 15 and 50 ng/mL HER2-ECD were tested and recoveries were found to be 98.7% and 95.3%, respectively. The recovery obtained with the ELISA kit was 111.3% for 15 ng/mL (Table 2) (note: the sample containing 50 ng/mL could not be analysed using the ELISA kit because the measured absorbance was outside the calibration range). When comparing the results of both assays a relative deviation of  $-11.4\%$  was obtained, which confirmed that the developed assay provides accurate results.

The storage stability of the MBs, modified with the capture antibody and blocked with ethanolamine, was also tested. The anti-HER2-ECD-MBs were stored at 4 °C in 100  $\mu$ L of PBS-T. The immunomagnetic assay

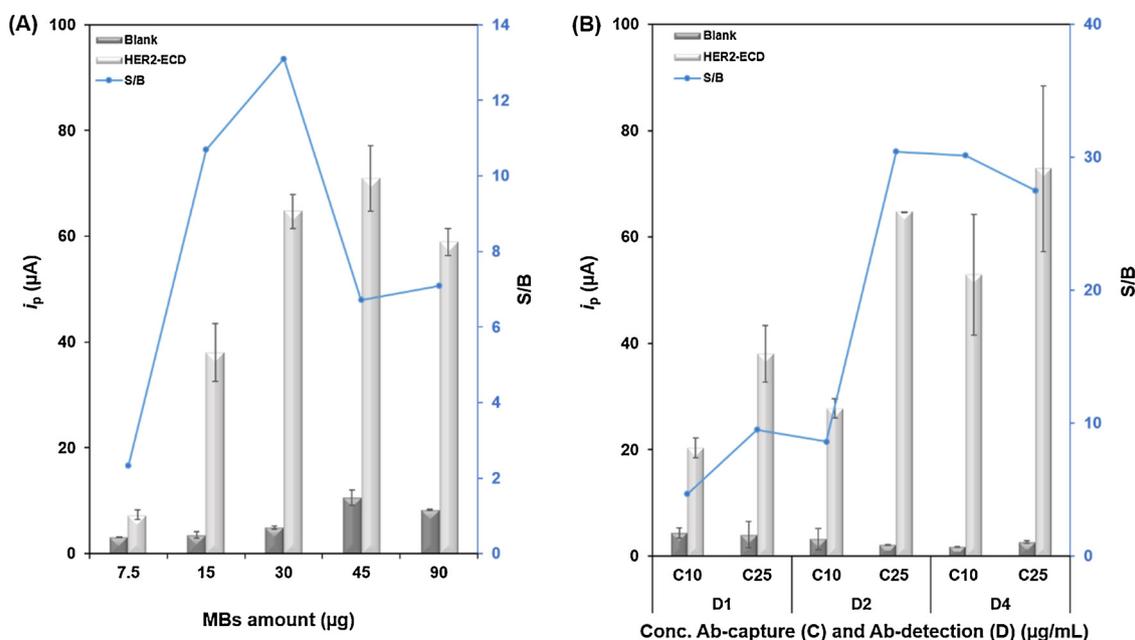


Fig. 2. Optimization of (A) the amount of magnetic beads and (B) the Ab-C and Ab-D concentrations (HER2-ECD: 0 and 50 ng/mL).

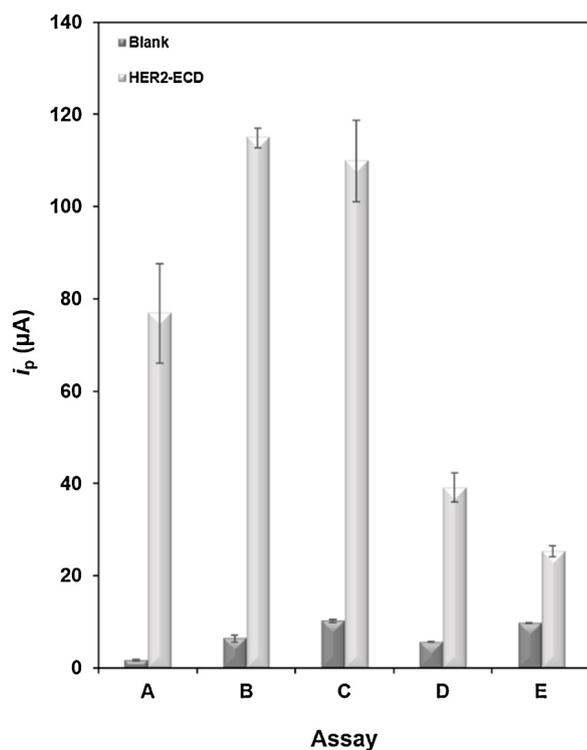


Fig. 3. Results of different assay strategies. (A) step-by-step assay, (B) pre-incubation of HER2 + Ab-D (60 min) and (C) pre-incubation of HER2 + Ab-D (120 min); (D) pre-incubation of Ab-D + S-AP (60 min); and (E) pre-incubation of HER2 + Ab-D + S-AP (60 min).

(in buffer solution) was performed on the same day and after 1, 7, 15, 21, 30 and 60 days using 0 and 50 ng/mL HER2-ECD. No significant difference in the measured blank and analytical signals was apparent over 60 days, obtaining 99.7% of the initial signal (SI Fig. S6), which indicates the stability of the MBs during this period. Furthermore, the use of these previously prepared MBs reduces the time required for the biomarker detection to 110 min.

The selectivity of the assay towards HER2-ECD was tested with

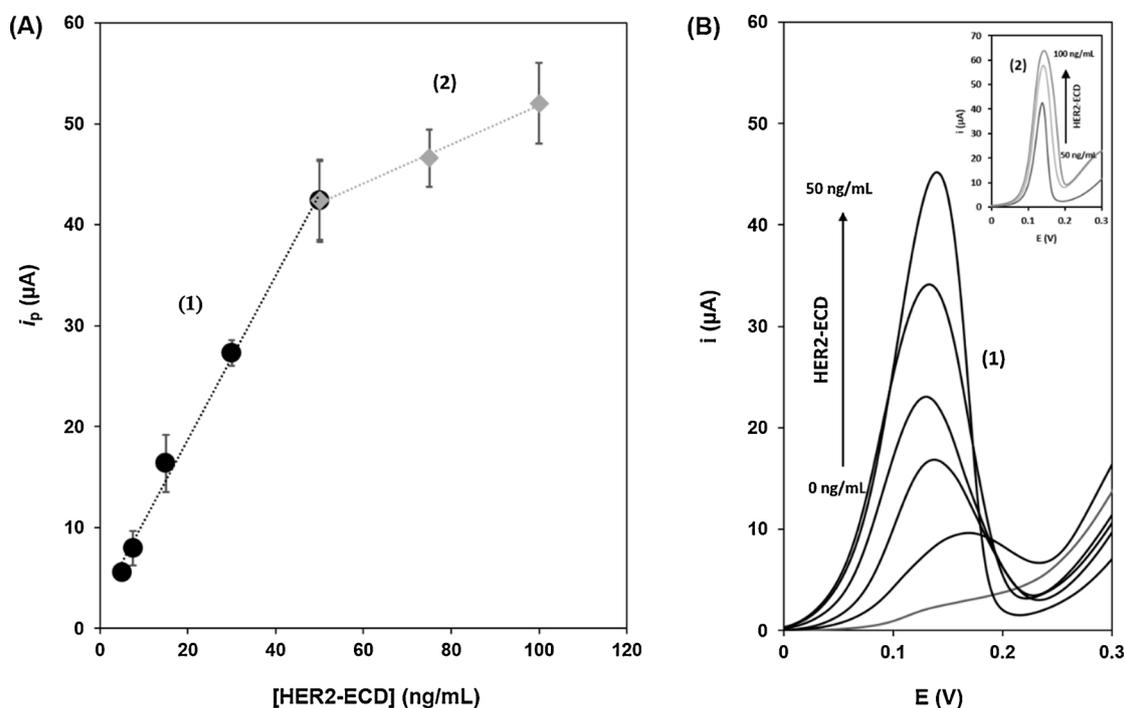
distinct human proteins (analysed in human serum): an analogous breast cancer biomarker (CA 15-3, 30 U/mL), a kidney function biomarker (cystatin C, 565 ng/mL) and Human serum albumin (HSA, 35 mg/mL). As can be seen in Fig. 5, the tested proteins showed an extremely low electrochemical signal, even at higher concentrations, confirming the selectivity of the assay towards HER2-ECD. In addition, this study allowed to confirm the specificity of the monoclonal antibodies.

#### 3.4. Analysis of live breast cancer cells

Monitoring HER2-overexpressed cancer cells is actually a huge challenge from the clinical point of view. To test the performance of our immunoassay, two distinct breast cancer cell-lines were used: the HER2<sup>+</sup> SK-BR-3 cell line and the HER2<sup>-</sup> MDA-MB-231 cell line. As can be observed in Fig. 6, HER2<sup>+</sup> cancer cells provided a concentration-dependent signal which was 5x higher than the signal obtained with HER2<sup>-</sup> cells, confirming the high selectivity of the optimized assay for the detection of HER2 biomarkers. The calibration curve ( $i_p$  vs. log [cells]) for SK-BR-3 cells was established in the linear range between  $1 \times 10^2$ – $1 \times 10^5$  cells/mL ( $i_p = 3.38 \pm 0.15 \log[\text{SK-BR-3}] - 2.81 \pm 0.55$ ),  $r = 0.996$ ). The coefficient of variation of the method ( $V_{x0}$ ) was found to be 2.4% and a limit of detection of 3 cells/mL was achieved.

#### 3.5. Comparison with other electrochemical immunomagnetic assays

The developed immunomagnetic assay was compared with other electrochemical methodologies for cancer biomarker analysis (HER2, ErbB2, EGFR, CA 15-3, exosomes and  $\alpha$ -LA) using a wide variety of functionalized MBs (e.g. protein A functionalized MBs (ProtA-MBs), streptavidin functionalized MBs (Strep-MBs), carboxylic acid functionalized MBs (HOOC-MBs)) or 'self-made' iron nanoparticles ( $\text{Fe}_3\text{O}_4$  NPs) [34–38,41–45] (Table 3). The 'self-made' magnetic particles required a time-consuming procedure and expensive characterization techniques leading to lengthy and costly preparation of the material. This is a key factor in favouring the use of commercially available MBs, enabling not only a faster but also a cheaper and more sustainable sensor preparation. Moreover, compared with the construction of immunosensors, magnetic assays can improve the antibody orientation on



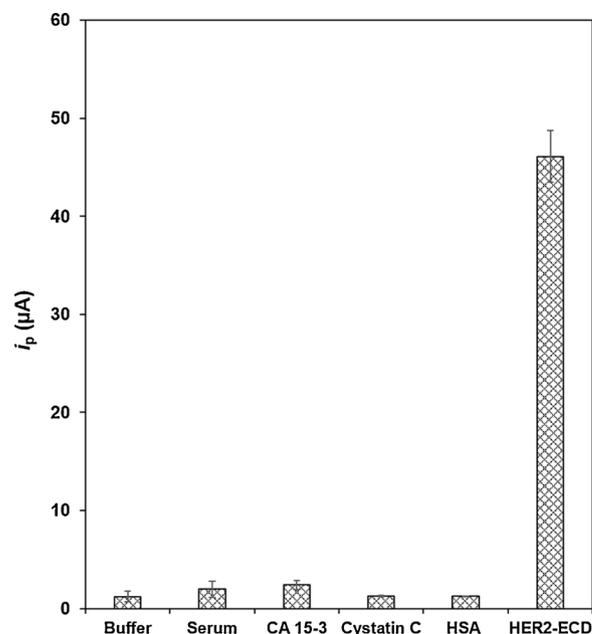
**Fig. 4.** Analysis of HER2-ECD in undiluted Human serum (A) Calibration plots and (B) Representative linear sweep voltammograms ([HER2-ECD] (ng/mL): (1) 0, 5, 7.5, 15, 30 and 50; (2) 50, 75 and 100).

**Table 1**

Figures of merit of the developed magnetic immunoassay for the analysis of HER2-ECD in Human serum.

Figure of merit	1	2
Concentration interval (ng/mL)	5.0–50	50–100
Correlation coefficient (r)	0.997	0.997
Slope (m) ( $\mu\text{A} / (\text{ng/mL})$ )	0.81	0.19
Standard deviation of the slope ( $S_m$ ) ( $\mu\text{A} / (\text{ng/mL})$ )	0.03	0.01
Intercept (b) ( $\mu\text{A}$ )	2.46	32.6
Standard deviation of the intercept ( $S_b$ ) ( $\mu\text{A}$ )	0.94	1.09
Standard deviation of the linear regression ( $S_{y/x}$ )	1.29	0.49
Standard deviation of the method ( $S_{x0}$ )	0.26	0.16
Coefficient of variation of the method ( $V_{x0}$ ) (%)	1.20	0.29
Limit of detection (LOD) (ng/mL)	2.8	11.8
Limit of quantification (LOQ) (ng/mL)	9.3	39.2

the particles' surfaces in a rapid manner (1 h), allowing appropriate antigen binding. In addition, the described assays allow studies to be carried out using (extremely) low volumes and concentrations, and, due to the efficient washing steps and analyte pre-concentration, reliable analysis in serum samples can be achieved with excellent limits of detection. Although only a few studies concerning cell analysis were reported [38,44], CTC analyses for the evaluation of HER2-positive patients are of utmost importance to help the decisions of clinical teams for effective personalized therapy. The immunomagnetic assay developed in this work can effectively contribute to distinguish positive and negative HER2 cell-types with a competitive assay time that is



**Fig. 5.** Selectivity studies using non-target proteins (the signal for HER2-ECD is included for comparison).

**Table 2**

Recovery values, relative standard deviations (RSD) and relative deviation obtained in the analysis of HER2-ECD using the developed immunoassay and a commercial ELISA kit.

Technique	Added (ng/mL)	Found (ng/mL)	Recovery (%)	RSD (%)	Relative deviation (%)	Sample dilution
Elisa Kit	15	16.7	111.3	0.033	-11.4	4-fold
Electrochemical immunoassay	50	47.6	95.3	3.7	*	Undiluted

\* The absorbance value for 50 ng/mL obtained using the ELISA kit was outside of the calibration range.

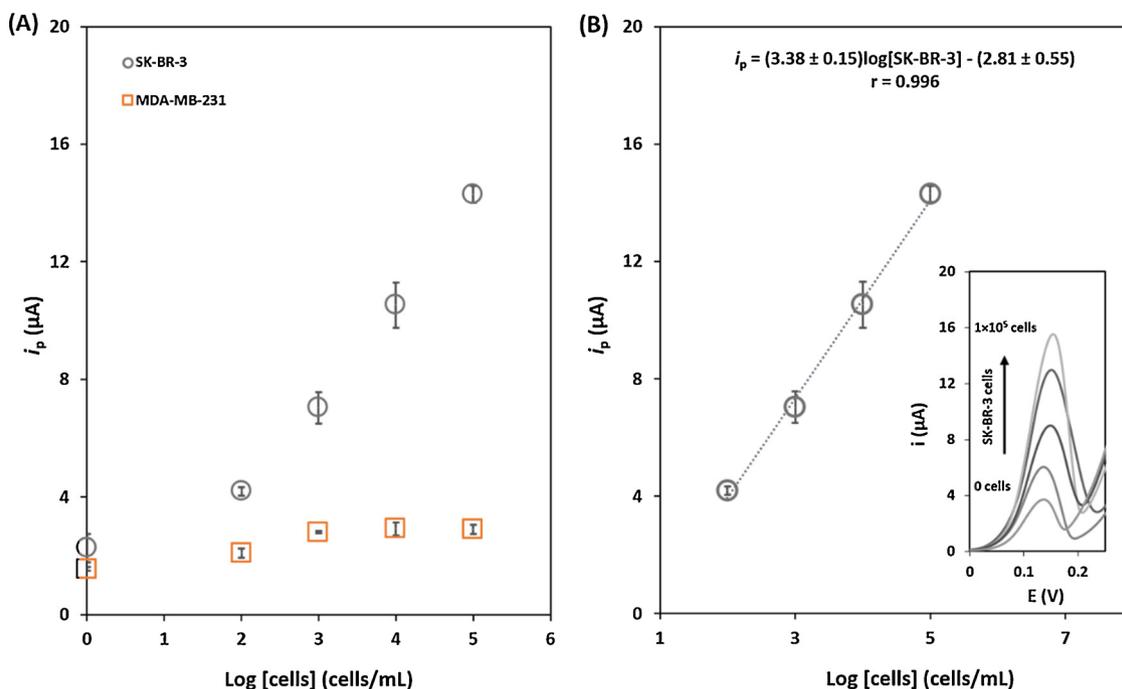


Fig. 6. (A) Calibration plots for the analysis of SK-BR-3 and MDA-MB-231 breast cancer cell lines in human serum; (B) Calibration straight for the analysis of SK-BR-3 (HER2<sup>+</sup> cancer cells):  $1 \times 10^2$ ,  $1 \times 10^3$ ,  $1 \times 10^4$  and  $1 \times 10^5$  cells/mL, Inset: Examples of linear sweep voltammograms.

surpassed only by two works [38,41]. However, one of these works [38] didn't include a stability study and the other showed a much shorter stability [41].

As far as we know, to date no electrochemical immunomagnetic assays were reported for the detection of HER2-ECD using screen-printed carbon electrodes, MBs-COOH, 3-indoxyl phosphate (3-IP) and silver ions ( $\text{Ag}^+$ ). Only the magnetic bioassay for the assessment of ErbB2 status directly in intact breast cancer cells employed SPCE and HOOC-MBs, achieving an excellent limit of detection [38]. The assay construction and the simplicity of this method can be compared with the present work and allows the use of a small-size (portable) equipment. This allows in situ analysis, requiring reduced reagent/sample volumes. Like this, the assay developed in this work could allow non-invasive screening and follow-up, according to the requirements of the clinical teams, and facilitates the monitoring of patients with reduced mobility or difficult access locations.

The FDA-approved HER2 diagnostic tests and the recently updated guidelines from the European Group on Tumor Markers (EGTM) reported that the main tests available to measure HER2 gene amplification/protein overexpression are immunohistochemistry (IHC), in situ hybridisation (ISH) and ELISA [11,12]. These tests are mostly performed by clinical professionals from oncological centres or specialized clinics, which makes their accessibility limited to the availability of specific technicians and / or equipment. Despite the evolution in this area and the significant investments and continuous development, it is still verified that the available tests have high costs for the national healthcare systems. Furthermore, only three biomarkers are mandatory to be analysed for all patients with breast cancer (oestrogen receptor (ER) and progesterone receptor (PR) for endocrine therapy and HER2 for the anti-HER2 therapy). Recommendations for further research not only involve the identification of additional markers but also analytical techniques capable of detecting the disease. Therefore, the constant development of electrochemical biosensors/assays could contribute to this field of research.

#### 4. Conclusions

An electrochemical magnetic immunoassay, using HOOC-MBs and a

disposable SPCE as transducer, for the detection of HER2-ECD, a breast cancer biomarker, was developed. Sensitive and precise detection of the biomarker and an LOD well below the cut-off value was achieved in a total assay time assay of 110 min (actual hands-on-time: 20 min). The applicability and selectivity of the bioassay was demonstrated through the analysis of spiked human serum samples and distinct non-target proteins and possible serum interferents: Cancer Antigen 15-3 (CA 15-3), Cystatin C and Human Serum Albumin (HSA). The storage stability of the MBs was at least 60 days, which is much better than previously reported. The immunomagnetic assay exhibited an excellent analytical performance and was successfully applied to spiked serum samples and may be applicable in the clinical practice. Additionally, the assay was also tested for the analysis of live breast cancer cells (HER2<sup>+</sup>: SK-BR-3; HER2<sup>-</sup>: MDA-MB-231) and it was possible to distinguish the different HER2 expression levels, showing high selectivity for HER2-positive cells.

#### Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Acknowledgements

The authors are grateful for the financial support from the Fundação para a Ciência e a Tecnologia (FCT)/the Ministério da Ciência, Tecnologia e Ensino Superior (MCTES) through national funds (Portugal) (LAQV - UID/QUI/50006/2019 and CINTESIS - UID/IC/4255/2019). Maria Freitas is grateful to FCT for her PhD grant (SFRH/BD/111942/2015), financed by POPH-QREN-Tipologia 4.1-Formação Avançada, subsidized by Fundo Social Europeu and the MCTES. The authors are also thankful to Rui Rocha and CEMUP "Centro de Materiais da Universidade do Porto" for the SEM work.

#### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the

**Table 3**  
Electrochemical immunoassays for breast cancer detection using magnetic beads (MBs).

Sensing surface construction		Preparation time*	Stability	Analyte	Assay strategy	Assay time	LOD	Cell analysis	Ref
Transducer	Detection scheme								
SPCE/ HOOC-MBs	LSV performed after addition of 3-IP/Ag <sup>+</sup> . AP used as label	1 h 15 min	60 days	HER2-ECD		1 h 50 min	2.8 ng/mL	SK-BR-3; MDA-MB-231	<b>This work</b>
GrE/ Nitrocellulose- Strep-MBs	CC recorded before and after the nitrocellulose-modified electrode exposure to cellulase	2 h	n.d.	HER2		3 h	1 fM	–	[34]
GCE/ Fe <sub>3</sub> O <sub>4</sub> -APTMS	Hyd-AuNPs-Fe <sub>3</sub> O <sub>4</sub> NPs used as label. Silver detection by DPV	> 5 h	10 days	HER2		2 h	2.0 × 10 <sup>-5</sup> ng/mL	–	[35]
8 × SPE/ Strep-MBs or ProtA-MBs	Hydrolysis of 1-NP catalysed by AP and detected by DPV	12 h 15 min 1 h	1 week	HER2		2 h 51 min 1 h 51 min	1.8; 2.6; 3.4 ng/mL	–	[36]
SPCE/ ProtA-MBs	Detection of 1-NP performed by DPV. AP used as label.	1 h	10 days	HER2		2 h 05 min	6.0 ng/mL	–	[37]
SPCE/ HOOC-MBs	HRP used as label. HQ allow to obtain the amperometric response	1 h 40 min	n.d.	ErbB2		1 h	26 pg/mL	SK-BR-3; MCF-7; MDA-MB-436	[38]
GCE/ Strep-MBs	AuNPs used as signalling probe. Detection accomplished by DPV	1 h	7 days	EGFR		1 h	50 pg/mL	–	[41]
Au microelectrodes/ Strep	Strep-MBs conjugated with biot-HRP. HQ measured by SWV	> 24 h	15 days	CA 15-3		5 h	15 × 10 <sup>-6</sup> U/mL	–	[42]
SPCE/ P(1.5DAN)-PPy NWS	Strep-MBs used as carriers and HRP as label. DPV performed to measure HQ	> 14 h	n.d.	CA 15-3		1 h 30 min	0.02 U/mL	–	[43]
GCE/ Strep-MBs	Cd <sup>2+</sup> detection carried out by SWASV. CdSe QDs used as label	30 min	n.d.	Exosomes		1 h 50 min	100 exosomes/ $\mu$ L	BT474; SW-48	[44]
SPCE/ Lys-Fe <sub>3</sub> O <sub>4</sub> NPs	Ferrocene-modified AuNPs used to obtain the amperometric response	13 h	1 month	$\alpha$ -LA		4 h	0.07 ng/mL	–	[45]

$\alpha$ -LA –  $\alpha$ -lactalbumin; 1-NP – 1-naphthol; 3-IP – 3-indoxyl phosphate; Ag<sup>+</sup> – silver ions; AP – alkaline phosphatase; APTMS – 3-aminopropyltrimethoxysilane; AuNPs – gold nanoparticles; CA 15-3 – cancer antigen 15-3; CC – chronocoulometry; CdSe QDs – Cadmium selenide quantum dots; Cd<sup>2+</sup> – cadmium ions; DPV – differential pulse voltammetry; ECD – extracellular domain; EGFR – epidermal growth factor receptor; Fe<sub>3</sub>O<sub>4</sub> – magnetite nanoparticles; GCE – glassy carbon electrode; GrE – graphene electrode; HER2, ErbB2 – human epidermal growth factor receptor 2; Hyd – hydrazine; HOOC-MBs – carboxylic acid functionalized magnetic beads; HQ – hydroquinone; HRP – horseradish peroxidase; Lys – lysozyme; LSV – linear sweep voltammetry; n.a. – not applicable; P(1.5DAN) – poly(1,5diaminonaphthalene); PPy – NWS polypyrrole nanowires; ProtA-MBs – Protein A-modified magnetic beads; SPE – screen-printed electrode; Strep-MBs – streptavidin-modified magnetic beads; SWASV – square wave voltammetry; SWV – square wave anodic stripping voltammetry.

\* Overnight incubations were considered as a 12 h period for comparison purposes.

online version, at doi:<https://doi.org/10.1016/j.snb.2020.127667>.

## References

- [1] M. Freitas, H.P.A. Nouws, C. Delerue-Matos, Electrochemical biosensing in cancer diagnostics and follow-up, *Electroanalysis* 30 (2018) 1576–1595, <https://doi.org/10.1002/elan.201800193>.
- [2] F. Puglisi, C. Fontanella, G. Numico, V. Sini, L. Evangelista, F. Monetti, S. Gori, L. Del Mastro, Follow-up of patients with early breast cancer: is it time to rewrite the story? *Crit. Rev. Oncol. Hematol.* 91 (2014) 130–141, <https://doi.org/10.1016/j.critrevonc.2014.03.001>.
- [3] A. Ravelli, J.M. Reuben, F. Lanza, S. Anfossi, M.R. Cappelletti, L. Zanotti, A. Gobbi, C. Senti, P. Brambilla, M. Milani, D. Spada, P. Pedrazzoli, M. Martino, A. Bottini, D. Generali, Breast cancer circulating biomarkers: advantages, drawbacks, and new insights, *Tumor Biol.* 36 (2015) 6653–6665, <https://doi.org/10.1007/s13277-015-3944-7>.
- [4] S. Mittal, H. Kaur, N. Gautam, A.K. Mantha, Biosensors for breast cancer diagnosis: a review of bioreceptors, biotransducers and signal amplification strategies, *Biosens. Bioelectron.* 88 (2017) 217–231, <https://doi.org/10.1016/j.bios.2016.08.028>.
- [5] S. Campuzano, M. Pedrero, J.M. Pingarrón, Non-invasive breast cancer diagnosis through electrochemical biosensing at different molecular levels, *Sensors (Switzerland)* 17 (2017) 1993, <https://doi.org/10.3390/s17091993>.
- [6] M. Labib, E.H. Sargent, S.O. Kelley, Electrochemical methods for the analysis of clinically relevant biomolecules, *Chem. Rev.* 116 (2016) 9001–9090, <https://doi.org/10.1021/acs.chemrev.6b00220>.
- [7] A.J. Wang, X.Y. Zhu, Y. Chen, X. Luo, Y. Xue, J.J. Feng, Ultrasensitive label-free electrochemical immunoassay of carbohydrate antigen 15-3 using dendritic Au@Pt nanocrystals/ferrocene-grafted-chitosan for efficient signal amplification, *Sens. Actuators B Chem.* 292 (2019) 164–170, <https://doi.org/10.1016/j.snb.2019.04.128>.
- [8] R. Wang, J.J. Feng, Y. Xue, L. Wu, A.J. Wang, A label-free electrochemical immunosensor based on AgPt nanorings supported on reduced graphene oxide for ultrasensitive analysis of tumor marker, *Sens. Actuators B Chem.* 254 (2018) 1174–1181, <https://doi.org/10.1016/j.snb.2017.08.009>.
- [9] Y. Chen, P.X. Yuan, A.J. Wang, X. Luo, Y. Xue, L. Zhang, J.J. Feng, A novel electrochemical immunosensor for highly sensitive detection of prostate-specific antigen using 3D open-structured PtCu nanoframes for signal amplification, *Biosens. Bioelectron.* 126 (2019) 187–192, <https://doi.org/10.1016/j.bios.2018.10.057>.
- [10] A.C. Wolff, M. Elizabeth Hale Hammond, K.H. Allison, B.E. Harvey, P.B. Mangu, J.M.S. Bartlett, M. Bilous, I.O. Ellis, P. Fitzgibbons, W. Hanna, R.B. Jenkins, M.F. Press, P.A. Spears, G.H. Vance, G. Viale, L.M. McShane, M. Dowsett, Human epidermal growth factor receptor 2 testing in breast cancer: American society of clinical oncology/college of American pathologists clinical practice guideline focused update, *J. Clin. Oncol.* 36 (2018) 2105–2122, <https://doi.org/10.1200/JCO.2018.77.8738>.
- [11] M.J. Duffy, N. Harbeck, M. Nap, R. Molina, A. Nicolini, E. Senkus, F. Cardoso, Clinical use of biomarkers in breast cancer: updated guidelines from the European Group on Tumor Markers (EGTM), *Eur. J. Cancer* 75 (2017) 284–298, <https://doi.org/10.1016/j.ejca.2017.01.017>.
- [12] A.K. Füzéry, J. Levin, M.M. Chan, D.W. Chan, Translation of proteomic biomarkers into FDA approved cancer diagnostics: issues and challenges, *Clin. Proteomics* 10 (2013) 13, <https://doi.org/10.1186/1559-0275-10-13>.
- [13] M. Hasanzadeh, N. Shadjou, M. de la Guardia, Early stage screening of breast cancer using electrochemical biomarker detection, *Trends Anal. Chem.* 91 (2017) 67–76, <https://doi.org/10.1016/j.trac.2017.04.006>.
- [14] M. Freitas, M.M.P.S. Neves, H.P.A. Nouws, C. Delerue-Matos, Quantum dots as nanolabels for breast cancer biomarker HER2-ECD analysis in human serum, *Talanta* 208 (2020) 120430, <https://doi.org/10.1016/j.talanta.2019.120430>.
- [15] Z.M.A.N.H. Lah, S.A.A. Ahmad, M.S. Zaini, M.A. Kamarudin, An electrochemical sandwich immunosensor for the detection of HER2 using antibody-conjugated PbS quantum dot as a label, *J. Pharm. Biomed. Anal.* 174 (2019) 608–617, <https://doi.org/10.1016/j.jpba.2019.06.024>.
- [16] S.D. Tallapragada, K. Layek, R. Mukherjee, K.K. Mistry, M. Ghosh, Development of screen-printed electrode based immunosensor for the detection of HER2 antigen in human serum samples, *Bioelectrochemistry* 118 (2017) 25–30, <https://doi.org/10.1016/j.bioelechem.2017.06.009>.
- [17] S. Patris, P. De Pauw, M. Vandepuy, J. Huet, P. Van Antwerpen, S. Muyldermans, J.M. Kauffmann, Nanoimmunoassay onto a screen printed electrode for HER2 breast cancer biomarker determination, *Talanta* 130 (2014) 164–170, <https://doi.org/10.1016/j.talanta.2014.06.069>.
- [18] S.P. Mucelli, M. Zamuner, M. Tormen, G. Stanta, P. Ugo, Nanoelectrode ensembles as recognition platform for electrochemical immunosensors, *Biosens. Bioelectron.* 23 (2008) 1900–1903, <https://doi.org/10.1016/j.bios.2008.02.027>.
- [19] M. Freitas, H.P.A. Nouws, C. Delerue-Matos, Electrochemical sensing platforms for HER2-ECD breast cancer biomarker detection, *Electroanalysis* 31 (2019) 121–128, <https://doi.org/10.1002/elan.201800537>.
- [20] E. Arkan, R. Saber, Z. Karimi, M. Shamsipur, A novel antibody-antigen based impedimetric immunosensor for low level detection of HER2 in serum samples of breast cancer patients via modification of a gold nanoparticles decorated multiwall carbon nanotube-ionic liquid electrode, *Anal. Chim. Acta* 874 (2015) 66–74, <https://doi.org/10.1016/j.aca.2015.03.022>.
- [21] A. Ravalli, C.G. da Rocha, H. Yamanaka, G. Marrazza, A label-free electrochemical affisensor for cancer marker detection: the case of HER2, *Bioelectrochemistry* 106 (2015) 268–275, <https://doi.org/10.1016/j.bioelechem.2015.07.010>.
- [22] R.C.B. Marques, S. Viswanathan, H.P.A. Nouws, C. Delerue-Matos, M.B. González-García, Electrochemical immunosensor for the analysis of the breast cancer biomarker HER2 ECD, *Talanta* 129 (2014) 594–599, <https://doi.org/10.1016/j.talanta.2014.06.035>.
- [23] M. Jarczewska, A. Trojan, M. Gałala, E. Malinowska, Studies on the affinity-based biosensors for electrochemical detection of HER2 cancer biomarker, *Electroanalysis* 31 (2019) 1125–1134, <https://doi.org/10.1002/elan.201900041>.
- [24] S. Carvajal, S.N. Fera, A.L. Jones, T.A. Baldo, I.M. Mosa, J.F. Rusling, C.E. Krause, Disposable inkjet-printed electrochemical platform for detection of clinically relevant HER-2 breast cancer biomarker, *Biosens. Bioelectron.* 104 (2018) 158–162, <https://doi.org/10.1016/j.bios.2018.01.003>.
- [25] E. Chocholova, T. Bertok, L. Lorencova, A. Holazova, P. Farkas, A. Vikartovska, V. Bella, D. Velicova, P. Kasak, A.A. Eckstein, J. Mosnacek, D. Hasko, J. Tkac, Advanced antifouling zwitterionic layer based impedimetric HER2 biosensing in human serum: glycoprofiling as a novel approach for breast cancer diagnostics, *Sens. Actuators B Chem.* 272 (2018) 626–633, <https://doi.org/10.1016/j.snb.2018.07.029>.
- [26] S. Augustine, P. Kumar, B.D. Malhotra, Amine-Functionalized MoO<sub>3</sub>@RGO Nanohybrid-based biosensor for breast cancer detection, *ACS Appl. Bio Mater.* 2 (2019) 5366–5378, <https://doi.org/10.1021/acsbm.9b00659>.
- [27] S. Sharma, J. Zapatero-Rodríguez, R. Saxena, R. O’Kennedy, S. Srivastava, Ultrasensitive direct impedimetric immunosensor for detection of serum HER2, *Biosens. Bioelectron.* 106 (2018) 78–85, <https://doi.org/10.1016/j.bios.2018.01.056>.
- [28] M. Emami, M. Shamsipur, R. Saber, R. Irajirad, An electrochemical immunosensor for detection of a breast cancer biomarker based on antiHER2–iron oxide nanoparticle bioconjugates, *Analyst* 139 (2014) 2858–2866, <https://doi.org/10.1039/c4an00183d>.
- [29] Y. Zhu, P. Chandra, Y.B. Shim, Ultrasensitive and selective electrochemical diagnosis of breast cancer based on a hydrazine-Au nanoparticle-aptamer bioconjugate, *Anal. Chem.* 85 (2013) 1058–1064, <https://doi.org/10.1021/ac302923k>.
- [30] P. Yáñez-Sedeño, S. Campuzano, J.M. Pingarrón, Magnetic particles coupled to disposable screen printed transducers for electrochemical biosensing, *Sensors (Switzerland)* 16 (2016) 1585, <https://doi.org/10.3390/s16101585>.
- [31] E. Paleček, M. Fojta, Magnetic beads as versatile tools for electrochemical DNA and protein biosensing, *Talanta* 74 (2007) 276–290, <https://doi.org/10.1016/j.talanta.2007.08.020>.
- [32] V. Mani, B.V. Chikkaveeraiha, J.F. Rusling, Magnetic particles in ultrasensitive biomarker protein measurements for cancer detection and monitoring, *Expert Opin. Med. Diagn.* 5 (2011) 381–391, <https://doi.org/10.1517/17530059.2011.607161>.
- [33] F. Ricci, G. Adornetto, G. Palleschi, A review of experimental aspects of electrochemical immunosensors, *Electrochim. Acta* 84 (2012) 74–83, <https://doi.org/10.1016/j.electacta.2012.06.033>.
- [34] K. Malecka, D. Pankratov, E.E. Ferapontova, Femtomolar electroanalysis of a breast cancer biomarker HER-2/neu protein in human serum by the cellulase-linked sandwich assay on magnetic beads, *Anal. Chim. Acta* 1077 (2019) 140–149, <https://doi.org/10.1016/j.aca.2019.05.052>.
- [35] M. Shamsipur, M. Emami, L. Farzin, R. Saber, A sandwich-type electrochemical immunosensor based on in situ silver deposition for determination of serum level of HER2 in breast cancer patients, *Biosens. Bioelectron.* 103 (2018) 54–61, <https://doi.org/10.1016/j.bios.2017.12.022>.
- [36] H. Ilkhani, A. Ravalli, G. Marrazza, Design of an affibody-based recognition strategy for human epidermal growth factor receptor 2 (HER2) detection by electrochemical biosensors, *Chemosensors* 4 (2016) 23, <https://doi.org/10.3390/chemosensors4040023>.
- [37] 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) 735–742, <https://doi.org/10.1002/elan.201100501>.
- [38] U. Elctxigerra, J. Martinez-Perdiguero, S. Merino, R. Barderas, R.M. Torrente-Rodríguez, R. Villalonga, J.M. Pingarrón, S. Campuzano, Amperometric magnetosensitized immunosensor for ErbB2 breast cancer biomarker determination in human serum, cell lysates and intact breast cancer cells, *Biosens. Bioelectron.* 70 (2015) 34–41, <https://doi.org/10.1016/j.bios.2015.03.017>.
- [39] S.E.F. Melanson, M.J. Tanasijevic, P. Jarolim, Cardiac troponin assays: a view from the clinical chemistry laboratory, *Circulation* 116 (2007) e501–e504, <https://doi.org/10.1161/CIRCULATIONAHA.107.722975>.
- [40] J.T. Busher, Serum albumin and globulin, in: H.K. Walker, W.D. Hall, J.W. Hurst (Eds.), *Clinical Methods: The History, Physical, and Laboratory Examinations*, third ed., Butterworths, Boston (USA), 1990 (Chapter 101), <http://www.ncbi.nlm.nih.gov/pubmed/21250048>.
- [41] H. Ilkhani, M. Sarparast, A. Noori, S.Z. Bathaie, M.F. Mousavi, Electrochemical aptamer/antibody based sandwich immunosensor for the detection of EGFR, a cancer biomarker, using gold nanoparticles as a signaling probe, *Biosens. Bioelectron.* 74 (2015) 491–497, <https://doi.org/10.1016/j.bios.2015.06.063>.
- [42] S. Akbari Nakhjavani, B. Khalilzadeh, P. Samadi Pakchin, R. Saber, M.H. Gahremani, Y. Omid, A highly sensitive and reliable detection of CA15-3 in patient plasma with electrochemical biosensor labeled with magnetic beads, *Biosens. Bioelectron.* 122 (2018) 8–15, <https://doi.org/10.1016/j.bios.2018.08.047>.
- [43] V.A. Nguyen, H.L. Nguyen, D.T. Nguyen, Q.P. Do, L.D. Tran, Electrosynthesized poly(1,5-diaminonaphthalene)/polypyrrole nanowires bilayer as an immunosensor platform for breast cancer biomarker CA 15-3, *Curr. Appl. Phys.* 17 (2017) 1422–1429, <https://doi.org/10.1016/j.cap.2017.08.002>.
- [44] K. Boriachek, M.N. Islam, V. Gopalan, A.K. Lam, N.T. Nguyen, M.J.A. Shiddiky, Quantum dot-based sensitive detection of disease specific exosome in serum, *Analyst* 142 (2017) 2211–2219, <https://doi.org/10.1039/c7an00672a>.

- [45] A. Mars, S. Ben jaafar, A.B.A. El Gaid, N. Raouafi, Electrochemical immunoassay for lactalbumin based on the use of ferrocene-modified gold nanoparticles and lysozyme-modified magnetic beads, *Microchim. Acta* 185 (2018) 449, <https://doi.org/10.1007/s00604-018-2986-0>.

**Maria Freitas** obtained her degree in Biochemistry in 2010 from the University of Trás-os-Montes and Alto Douro (Portugal) and her MSc degree in Quality Control in 2012 from the Faculty of Pharmacy, University of Porto (Portugal). Currently she is a Ph.D. student in the doctoral program on Sustainable Chemistry at REQUIMTE and her research interests include the development of electrochemical methodologies (immunosensors and MIPs) for the analysis of cancer biomarkers, and synthesis of nanomaterials, mainly magnetic core/shell nanoparticles.

**Henri P.A. Nouws** obtained his PhD in Chemistry in 2007 (Faculty of Sciences, University of Porto, Portugal). Currently he is an auxiliary professor at the Chemical Engineering Department of the School of Engineering, Polytechnic Institute of Porto (ISEP-IPP, Portugal). He is also an integrated research member of REQUIMTE and his

present research interests include the development of analytical (electrochemical) methodologies for pharmaceutical, clinical, and food analysis.

**Elisa Keating** obtained her PhD from the Faculty of Medicine, University of Porto (Portugal). Currently she is an Assistant Professor of the Department of Biomedicine and a member of the Pedagogical Council of the Faculty of Medicine of the University of Porto. She is the principal investigator of the ProNutri Group at CINTESIS, where she focuses on nutrition throughout life and metabolic programming.

**Cristina Delerue-Matos** obtained her PhD in Chemical-Physics, specialty in electrochemistry, in 1990. At the moment she is principal coordinator professor at the Chemical Engineering Department of the School of Engineering, Polytechnic Institute of Porto (ISEP-IPP, Portugal) and also coordinates the REQUIMTE/ISEP research group ([www.graq.issep.ipp.pt](http://www.graq.issep.ipp.pt)). Her research interests include the development of analytical methodologies for environmental, food, pharmaceutical, biochemical and industrial control. She is co-author of more than 250 publications in scientific journals.