Sensors & Actuators: B. Chemical 314 (2020) 128096

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

# Electrochemical immunoplatform to improve the reliability of breast cancer diagnosis through the simultaneous determination of RANKL and TNF in serum

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ARTICLE INFO

*Keywords:*
Dual MBs-based immunoplatform
RANKL
TNF
Amperometry
Serum
Breast cancer

ABSTRACT

This paper describes a dual immunosensor using neutravidin-functionalized magnetic microbeads (Neu-MBs) and dual screen-printed carbon electrodes (SPdCEs) for the simultaneous amperometric determination of two emerging biomarkers related to breast cancer (BC) and metastasis: Receptor Activator of Nuclear Factor-κB Ligand (RANKL) and Tumor Necrosis Factor alpha (TNF). In the implemented methodology, sandwich-type immunocomplexes, using biotinylated specific capture, detector antibodies and HRP-labeled secondary antibodies, are formed onto Neu-MBs. Electrochemical detection was performed by amperometry (−0.20 V *vs.* the Ag pseudo-reference) electrode using the $H_2O_2$/hydroquinone (HQ) system upon capturing the Neu-MBs modified with the sandwich immunocomplexes for each target biomarker on the corresponding working electrode (WE) of SPdCEs. The approach exhibits high sensitivity offering detection limits of 2.6 and 3.0 pg mL$^{-1}$ for RANKL and TNF, respectively, using simple protocols and taking 90 min as assay time. The usefulness of the dual immunoplatform was tested by determining RANKL and TNF levels in 5 μL of human serum from healthy controls and BC patients diagnosed with different HER2 subtypes. Results showed a higher expression of both biomarkers in BC patients (38 and 17 % higher for RANKL and TNF, respectively) and were in agreement to those obtained using the ELISA methodologies for each target biomarker involving the same immunoreagents. The obtained results show the potential of this immunoplatform to improve the reliability of BC diagnosis using fast and cost-effective procedures.

## 1. Introduction

Cancer is the second leading cause of death globally and their precise and early detection is key to controlling this disease and saving millions of lives each year [1]. In particular, breast cancer (BC) is the second most common malignancy and a leading cause of death in women population [2]. A primary luminal-like subtype breast tumor, characterized by estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) positive or negative statuses, is potentially curable. Several studies have shown that breast tumors with *HER2* gene amplification or overexpression are more aggressive [3]. Nevertheless, the therapy based on the incorporation of a monoclonal antibody (Trastuzumab) targeting the extracellular domain of the HER2 protein has demonstrated to be an efficient therapy for transforming outcomes of this particular BC subtype into one with a better prognosis [4]. In contrast, triple-negative (ER, PR and HER2, negative) BC is currently considered incurable whose median overall survival does not exceed 13 months [5]. These findings highlight the tremendous importance of reliable and preferably minimally invasive detection in early stages of the BC subtype for applying the most appropriate therapy.

Cytokines detection is important in diagnosis and prognosis of cancer and other diseases related to inflammation, immunological and atherosclerotic processes, since they play critical roles in repairing chemically-induced damaged tissue or controlling cell replication and apoptosis [6]. Consequently, the determination of circulating cytokines

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https://doi.org/10.1016/j.snb.2020.128096
Received 5 February 2020; Received in revised form 16 March 2020; Accepted 4 April 2020
Available online 13 April 2020

as tumor biomarkers is gaining more and more acceptance and relevance [7]. Within this context, circulating levels of receptor activator of nuclear factor-κB ligand (RANKL) and tumor necrosis factor (TNF) proteins in human serum are currently considered important prognosis biomarkers for different types of cancers [8,9].

RANKL, a homotrimeric type II membrane ligand protein of the TNF family, is the critical regulator of osteoclasts development and bone metabolism [10]. This ligand binds to its protein RANK on the surface of osteoclasts precursors and induces activation of osteoclasts and subsequent bone resorption [11]. Besides their essential function in bone, RANK and RANKL have been identified as the key factors for the formation of lactating mammary glands in pregnancy [12], to control the onset of hormone-induced BC through the expansion of mammary progenitor cells. The role of RANK/RANKL in BC is to confer resistance to irradiation-induced cell death in mammary epithelial cells, and to change cell adhesion and regulate self-renewal capacity of tumor stem cells, all of which might contribute to how RANKL/RANK drive mammary cancer development [13].

Regarding the multifunctional cytokine TNF, it is a 55 kDa biologically active molecule playing important roles in apoptosis and cell survival, and involved in various immune and inflammatory processes [14] for which it has become a good biomarker in relation to some pathologies [15]. Indeed, increased levels of TNF and other proinflammatory cytokines have been found for invasive breast carcinomas [16], and higher expression of intratumoral TNF is associated with lymph node metastasis and elevated tumor grade [17]. In this regard, it is worth mentioning that, among other types, human breast tumors have been shown to alter both the expression and function of TNF, providing soluble decoy receptors such as the RANKL-related osteoprotegerin (OPG) [18]. As it was recently demonstrated, OPG and RANKL are involved in the development of lactating breast glands and breast tumor growth and metastasis [12,19,20], whereas the RANKL/RANK system acts by providing to damaged mammary epithelium a growth and survival advantage, these being a prerequisite to the initiation of BC [21,22].

As it is known, achieving an effective clinical diagnosis requires building reliable tools for the detection of biomarkers associated with malignant tumor growth in order to improve accuracy and minimize the false-positive rate [23]. Therefore, the determination of tumor biomarkers implied in tumor progression, aggressiveness and metastatic events is a primary task in the diagnosis of cancers as soon as possible in order to choose the most efficient therapy in the shortest time. However, given the poor specificity of biomarkers related to a type of cancer, the determination of a single tumor marker is facing great challenge [24]. Obviously, multianalyte approaches applied in this field for the purpose of determining multiple tumor biomarkers providing more information for clinical diagnostics by means of faster and low-cost assays are of great significance [25].

All these evidences highlight the interest in the development of novel, fast and accurate methodologies for the determination of RANKL and TNF simultaneously, whose monitoring shows some difficulties related to their low concentration ranges in biological samples, to improve the reliability in BC subtype diagnosis [6]. These target biomarkers are usually individually determined by ELISA tests (soluble fractions) [26] and immunohistochemistry [27]. Over these techniques, electrochemical immunosensors exhibit interesting advantages in terms of portability, miniaturization, on-site monitoring and multiplexing analysis [28]. Although the individual determination of TNF [29–32] and RANKL [33] have been reported, as far as we know, no bioelectroanalytical platform has been proposed for the determination of both cytokines simultaneously.

With the main purpose of contributing to the reliable, objective, quantitative and minimally invasive diagnosis of BC subtype and status within clinically actionable times, this work reports the preparation, characterization and application of a fast and user-friendly electrochemical immunoplatform for the accurate determination of serum circulating RANKL and TNF proteins recently related to this type of neoplasia. The proposed methodology involves the formation of sandwich immunocomplexes onto Neutravidin-functionalized magnetic beads (Neu-MBs) followed by the amperometric transduction at screen-printed dual carbon electrodes (SPdCEs). The immunoplatform was applied to the determination of both biomarkers in untreated human serum from BC patients.

## 2. Experimental

Used apparatus, electrodes, reagents and solutions are described in detail in the Supporting Information.

### 2.1. Experimental procedures

#### 2.1.1. Preparation of the dual immunoplatform

For the individual determination of each biomarker, a 3 μL-aliquot of Neu-MBs suspension was transferred into a 1.5 mL microcentrifuge tube and washed twice with 50 μL of 0.01 M phosphate buffer saline solution (PBS) pH 7.4. Then, after placed the microcentrifuge tubes in the magnetic separator, the MBs were concentrated during 3 min, and the supernatant was removed as well as after all incubation/washing steps. Bio-functionalization of MBs was carried out by incubating these particles with 25 μL of 5 μg $mL^{-1}$ RANKL or 1 μg $mL^{-1}$ TNF biotinylated capture antibodies ($bCAb_{RANKL}$ or $bCAb_{TNF}$) solutions prepared in 0.01 M PBS pH 7.4. The microcentrifuge tubes were placed in the incubator shaker during 45 min (25 °C, 950 rpm) to obtain, respectively, $bCAb_{RANKL}$-MBs and $bCAb_{TNF}$-MBs bioconjugates. After two washings with 50 μL of blocking buffer (BB) solution, the microcentrifuge tubes containing $bCAb_{RANKL}$-MBs were placed in the incubator shaker for 60 min (25 °C, 950 rpm) with 25 μL of a mixture solution containing RANKL standards (or the sample to be analyzed), 1 μg $mL^{-1}$ RANKL detector antibody ($DAb_{RANKL}$) and 0.5 μg $mL^{-1}$ HRP-anti-mouse IgG prepared in the BB solution. On the other hand, $bCAb_{TNF}$-MBs were incubated with 25 μL of TNF standards or the sample to be analyzed prepared in BB solution also containing 1 μg $mL^{-1}$ of TNF detector antibody ($DAb_{TNF}$) for 60 min (25 °C, 950 rpm). Next, the $DAb_{TNF}$-TNF-$bCAb_{TNF}$-MBs were washed twice with 50 μL of BB solution, and subsequently incubated for 30 min (25 °C, 950 rpm) with 25 μL of 0.5 μg $mL^{-1}$ HRP-anti-mouse IgG prepared in BB solution. Finally, the modified MBs were washed twice with BB solution and the amperometric measurements were performed after re-suspension in 5 μL of 0.05 M phosphate buffer solution (PB) pH 6.0.

#### 2.1.2. Amperometric measurements

The as-prepared 5 μL-aliquots of the modified MBs suspension were dropped onto the respective surface of the SPdCE working electrodes (HRP-anti-mouse IgG-$DAb_{RANKL}$-RANKL-$bCAb_{RANKL}$-MBs onto WE1 and HRP-anti-mouse IgG-$DAb_{TNF}$-TNF-$bCAb_{TNF}$-MBs onto WE2) previously placed in the poly(methyl methacrylate) (PMMA) casing with encapsulated neodymium magnets. Subsequently, the ensemble SPdCE/magnet holding block with the MBs immunoconjugates magnetically captured was immersed into 10 mL of 0.05 M PB pH 6.0 solution also containing 1 mM HQ (prepared just before the electrochemical measurement) in an electrochemical cell. Amperometric measurements were performed at room temperature in stirred solutions by applying −0.20 V (*vs.* Ag pseudo-reference electrode. After the background current was stabilized (~50 s), 50 μL of a recent 0.1 M $H_2O_2$ solution in 0.05 M PB pH 6.0 were added, and the current provided by the HRP reduction of $H_2O_2$ mediated by HQ, was recorded during ~100 s (time required for reaching the steady-state). All the amperometric data were calculated as the difference between both the steady-state and the background current, and the mean values of three replicates ($\alpha = 0.05$) were employed.

Each batch of modified MBs and SPdCEs was only used to perform a single measurement and they were discarded afterward.

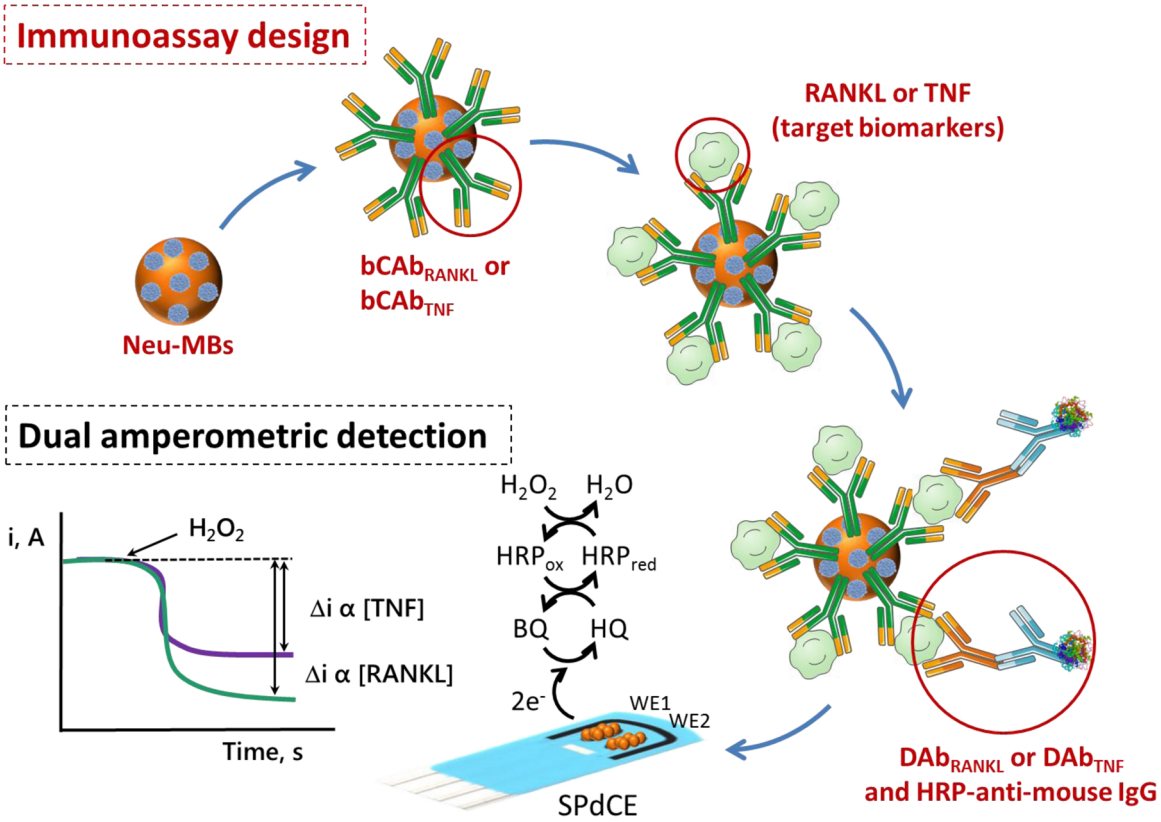


**Fig. 1.** Schematic display of the developed MBs-based immunoplatform for the dual amperometric determination of RANKL and TNF at SPdCEs.

*2.1.3. Analysis of sera*

Human serum samples from patients diagnosed with HER2-positive and HER2-negative BC and from healthy individuals (control group) were provided by Hospital Universitari de Sant Joan (Tarragona, Spain), and stored at −80 °C until use. In all cases, the ethical issues, relevant guidelines and regulations were accomplished for sample using and experiments performance. Furthermore, all individuals gave their written informed consent to participate in the study. The determination of RANKL and TNF was carried out in 5-times diluted serum samples in BB. Since no matrix effect was observed in the diluted serum samples, quantification of both biomarkers was performed by direct interpolation of the measured amperometric signals into the calibration plots constructed with RANKL and TNF standards. The results obtained with the developed dual immunoplatform were compared with those provided for the individual determination of each target analyte with the respective ELISA kit using the same immunoreagents and following the recommended protocols.

## 3. Results and discussion

Fig. 1 shows the steps involved in the preparation and functioning of the dual immunosensor, for the simultaneous amperometric detection of RANKL and TNF. Briefly, sandwich-type immunoassays were implemented onto the Neu-MBs by immobilizing the respective RANKL and TNF biotinylated capture antibodies (bCAbs). Neu-MBs were selected as solid support for the assays due to their advantages over streptavidin- or avidin-MBs in terms of higher binding affinity and lower non-specific adsorptions [34,35]. The target RANKL or TNF captured onto these immunocaptors were sandwiched with their corresponding detector antibodies (DAbs) enzymatically labeled with HRP-anti-mouse IgG. Thereafter, the MBs modified with the immunoconjugates were captured on the corresponding working electrode surface of the SPdCE (RANKL in WE1 and TNF in WE2). Amperometric measurements were carried out using the $H_2O_2$/HQ system and the reduction currents at −0.20 V (*vs.* Ag pseudo-reference electrode), were proportional to the concentration of RANKL and TNF.

*3.1. Evaluation of the experimental variables*

The working protocol for the individual amperometric determination of RANKL and TNF at SPCEs was optimized using the larger ratio between the amperometric responses obtained in the presence of 100 pg mL$^{-1}$ RANKL or TNF standard solutions (signal, S) and in their absence (blank, B) (S/B ratio), as the selection criteria. Table S1 summarizes the tested variables, and the results obtained are displayed in Figs. S1 and S2 (in the Supporting Information), respectively. Other used experimental variables such as the detection potential (−0.20 V *vs.* the Ag pseudo-reference electrode), or the concentrations of $H_2O_2$ and HQ, and the pH value and composition of the solution where the amperometric measurements were made, were those optimized in previous works [36–38].

The number of incubation steps (30 and 60 min each for RANKL and TNF, respectively) required for modification of the bCAb-MBs upon target addition was optimized for both immunoassays. Figs. S1c and S2c show the responses obtained with the prepared immunosensors by implementing the following protocols: (I) One single incubation step performed in a mixture solution which contained the target biomarker (RANKL or TNF), the DAb and HRP-anti-mouse IgG; (II) protocols involving two successive incubation steps, a first step in a mixture solution of the target biomarker and the detector antibody (DAb), followed by incubation in the HRP-anti-mouse IgG solution (IIA); a first incubation step in the biomarker solution and a subsequent incubation performed in a mixture solution containing DAb and HRP-anti-mouse IgG (IIB); (III) three successive incubation steps in RANKL or TNF standard, DAb and HRP-anti-mouse IgG solutions, respectively. With the aim of developing simpler and shorter methods without compromising sensitivity, protocols involving 1 incubation step for RANKL and 2 incubation steps (first incubation with biomarker and DAb, and then

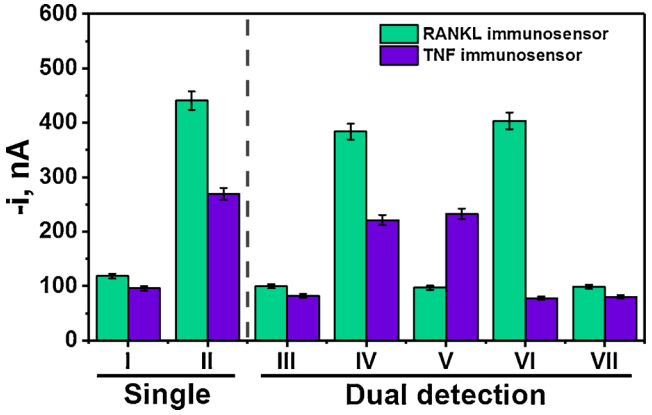


**Fig. 2.** Comparison of the amperometric currents measured with single and dual immunosensors for mixture solutions containing: 0 pg mL$^{-1}$ of both RANKL and TNF (I and III); 100 pg mL$^{-1}$ RANKL and TNF (II and IV); 0 pg mL$^{-1}$ RANKL and 100 pg mL$^{-1}$ TNF (V); 100 pg mL$^{-1}$ and 0 pg mL$^{-1}$ TNF (VI); and 100 pg mL$^{-1}$ RANKL (with $bCAb_{TNF}$-MBs and $DAb_{TNF}$) and 100 pg mL$^{-1}$ TNF (with $bCAb_{RANKL}$-MBs and $DAb_{RANKL}$) (VII). Error bars were estimated from three replicates as three times the calculated standard deviation.

with HRP-anti-mouse IgG, protocol IIA) for TNF were selected to develop the immunosensors.

### 3.2. Simultaneous determination of RANKL and TNF with the dual immunoplatform

The optimized individual methodologies for RANKL and TNF were integrated at the dual electrochemical SPdCEs platform. The possible loss of sensitivity compared with the individual determinations and the eventual cross-talking that could occur between the neighboring electrodes were evaluated by evaluating the amperometric responses measured for RANKL and TNF standards prepared at different concentrations. Fig. 2 shows a decrease of about 12 and 17 % in the amperometric responses recorded at the SPdCEs for 100 pg mL$^{-1}$ RANKL and TNF standards, respectively, attributed to the higher surface area of the working electrodes in the single SPCEs (12.6 mm$^2$) compared to those of SPdCEs (6.3 mm$^2$ each). In addition, no significant cross-talking was apparent between the two adjacent electrode surfaces due to the presence of the non-target biomarker. As Fig. 2 shows, the currents measured when a 100 pg mL$^{-1}$ RANKL standard was incubated with $bCAb_{TNF}$-MBs and $DAb_{TNF}$ (V), or when a 100 pg mL$^{-1}$ TNF standard was incubated with $bCAb_{RANKL}$-MBs and $DAb_{RANKL}$ (VII) were not significantly different from those measured in the absence of each target biomarker incubated with its corresponding immunoreagents (IV and VI). These results demonstrated the usefulness of the developed strategy for the sensitive and selective simultaneous determination of RANKL and TNF.

Under the optimized working conditions, the calibration plots for RANKL and TNF obtained at the SPdCEs are displayed in Fig. 3. Linear regressions adjusted to the equations: -i, nA = (1.9 ± 0.1) nA mL$^{-1}$ pg [RANKL] + (117 ± 9) nA ($R^2$ = 0.992) and -i, nA = (1.10 ± 0.06) nA mL$^{-1}$ pg [TNF] + (91 ± 5) nA ($R^2$ = 0.991) were obtained. The LOD (2.6 pg mL$^{-1}$ for RANKL and 3.0 pg mL$^{-1}$ for TNF) and LQ (8.6 pg mL$^{-1}$ and 9.9 pg mL$^{-1}$ for RANKL and TNF, respectively) values were estimated according to the $3 \times s_b/m$ and $10 \times s_b/m$ criteria, being $s_b$ the standard deviation obtained from 10 amperometric measurements carried out in the absence of each biomarker (B signals) and $m$ the slope of the respective standard calibration plot.

A good repeatability between the amperometric responses was obtained for both biomarkers using ten different dual immunosensors prepared at the same day. The RSD values (3.8 % and 4.1 % for RANKL and TNF, respectively) confirmed the high reproducibility of the working experimental procedure, which includes the preparation of the sandwich immunocomplexes on the MBs followed by their magnetic capture on the SPdCEs working surfaces and the dual amperometric measurements.

The developed dual immunoplatform (the first one described until now for the simultaneous determination of RANKL and TNF) provides quite similar LOD values (indicated in parenthesis) for both biomarkers than those claimed for previously reported single immunosensors such as an integrated immunoplatform for RANKL involving AuNPs/MWCNTs hybrid nanocomposites (3.1 pg mL$^{-1}$) [32], a MBs-based immunosensor for TNF (2.0 pg mL$^{-1}$) [29], a label-free design involving Ag@Pt core–shell nanoparticles supported on MWCNTs for TNF (1.6 pg mL$^{-1}$) [30], or a dual nanostructured immunoplatform for the simultaneous determination of TNF and IL-1β (0.83 pg mL$^{-1}$ for TNF) [31]. Importantly, the methodology reported in this paper requires the shortest assay time (2 h 15 min) for the determination of both target biomarkers simultaneously, in just 90 min counting since the preparation of the bCAb-MBs.

The analytical characteristics of the dual immunoplatform were also compared with those claimed for the commercial ELISA kits used for the individual determination of each biomarker which involve the same immunoreagents employed in this work. The ELISA kits required a much longer assay time with an “overnight” step for immobilization of the capture antibody, 1 h for blocking and nearly 5 h to complete the immunoassay after target addition. In addition, ELISA kits demand 100 µL of biological sample to each well, and provide logarithmic calibration plots (non-linear) with dynamic ranges from 78 to 5000 pg mL$^{-1}$ for RANKL and 16 – 1,000 pg mL$^{-1}$ for TNF, as well as minimum detectable concentrations of 20 pg mL$^{-1}$ and 5 pg mL$^{-1}$, respectively. Conversely, as stated above, the dual bioplatform is not restricted to the determination of a single biomarker like the ELISA kits, allowing the simultaneous determination of both biomolecules in the above cited total assay time of 90 min counting upon target addition. Furthermore, the immunosensor demands only 25 µL of solution containing the biomarker for the suitable determination of each compound, and provides non-logarithmic linear ranges between 8.6 and 1000 pg mL$^{-1}$ for RANKL (LOD of 2.6 pg mL$^{-1}$) and 9.9 – 1,000 pg mL$^{-1}$ for TNF (LOD of 3.0 pg mL$^{-1}$). In addition, a better precision, with RSD values around 4 %, was achieved for the amperometric measurements carried out with the dual immunoplatform than that reported for individual ELISA kits (RSD = 10 %). All these advantages together with the inherent characteristics of portability and cost-effectiveness of the electrochemical instrumentation required make the developed dual immunoplatform an ideal device to perform routine determinations of RANKL and TNF biomarkers at the point of attention.

#### 3.2.1. Storage stability of the bCAb-MBs bioconjugates

In order to evaluate the storage stability of the $bCAb_{RANKL}$-MBs and $bCAb_{TNF}$-MBs bioconjugates (*i.e.* before the addition of the biomarker/sample), different batches of bCAb-MBs were prepared in the same day and stored at 4 °C in 1.5 mL microcentrifuge tubes resuspended in 50 µL of sterilized 0.01 M PBS pH 7.4. Then, the amperometric responses were measured with the immunosensors prepared using the stored bCAb-MBs, in the absence and in the presence of both biomarker standards (100 pg mL$^{-1}$ RANKL and TNF), with the results plotted in Fig. S3 (Supporting Information). As it can be seen, a good storage stability of bCAb-MBs bioconjugates was apparent, allowing the accurate determination of RANKL and TNF without significant differences in sensitivity during approximately 20 days after the preparation of MBs bioconjugates.

#### 3.2.2. Dual immunosensor selectivity

The selectivity of the dual immunoplatform was tested by comparison of the amperometric responses for 0 and 100 pg mL$^{-1}$ RANKL and TNF standard solutions, prepared in the absence and in the presence of

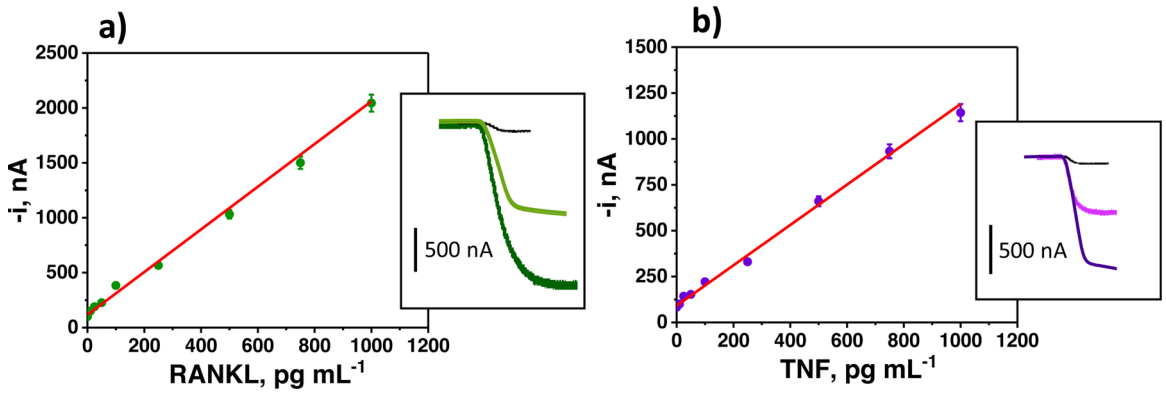


**Fig. 3.** Calibration plots and real amperometric responses (0, 500 and 1,000 pg mL$^{-1}$) obtained with the dual amperometric immunoplatform for RANKL (a) and TNF (b) standard solutions. Error bars were estimated from three replicates as three times the calculated standard deviation.

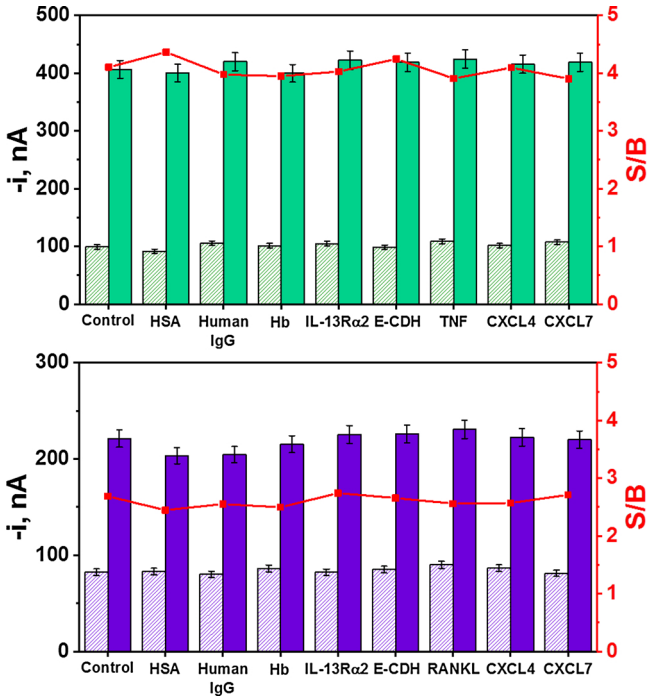


**Fig. 4.** Amperometric measurements obtained with the dual immunoplatform for 0 (stripped bar, B) and 100 pg mL$^{-1}$ RANKL (green) or TNF (purple) (solid bar, S) prepared in the absence (Control) and in the presence of 50 mg mL$^{-1}$ HSA; 1 mg mL$^{-1}$ Human IgG; 5 mg mL$^{-1}$ Hemoglobin (Hb); 50 ng mL$^{-1}$ IL-13Rα2; 10 ng mL$^{-1}$ E-CDH; 100 pg mL$^{-1}$ RANKL (purple) or TNF (green); 500 ng mL$^{-1}$ CXCL4 and 500 ng mL$^{-1}$ CXCL7. Red circles indicate the respective S/B ratio and error bars are estimated as three times the standard deviation value of three replicates (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

various non-target cancer biomarkers as well as other proteins coexisting in serum samples. Fig. 4 shows as no significant interference was found for the determination of RANKL and TNF coexisting with other serum proteins (assayed at the expected concentration levels for healthy patients), which evidenced the high specificity of the bCAbs and DAbs employed and the excellent selectivity of the dual immunoplatform.

The dual immunoplatform was applied to the quantification of RANKL and TNF standards prepared in lyophilized human serum. Fig. 5 compares the immunosensor responses for 0, 500 and 1000 pg mL$^{-1}$ RANKL or TNF standards prepared in buffered solutions or in undiluted and (2 or 5-times) diluted serum (in BB solution). As it can be seen, no significant differences could be observed for the amperometric responses obtained in buffered solutions and in 5-times diluted serum samples. Therefore, at such dilution level, matrix effects were no significant, which allows the determination of RANKL and TNF in 5-times diluted serum samples just by simple interpolation of the amperometric signals into the respective calibration plot constructed with the standards of both biomarkers.

### 3.3. Applicability to the analysis of RANKL and TNF in human serum from BC patients

The applicability of the dual immunoplatform to determine RANKL and TNF levels in human serum from healthy individuals (control group) and BC patients diagnosed with different HER2 subtypes was evaluated. It is important to note that since serum samples were 5-times diluted and an incubation volume of 25 µL was used (see detailed protocol in sections 2.1.1 and 2.1.3) just 5 µL of clinical sample are required for each target biomarker determination. Moreover, the possibility to perform the determination by simple interpolation of the measured amperometric responses into the calibration plots prepared with RANKL and TNF standard solutions greatly simplifies the protocols and reduces the assay times. Data obtained for the quantification of both biomarkers in the different human serum samples analyzed are displayed in Fig. 6. As expected, a higher expression of RANKL (38 % higher) and TNF (17 % higher) was found in BC patients compared with control group. Despite the small cohort of patients analyzed, the results obtained were in agreement with the reported oncogenic role of both biomarkers and their key actuation in the BC progress HER2 subtype identification.

Table 1 compares the concentrations of RANKL and TNF found in the samples of human serum for three replicates using the dual immunosensor with those provided by the individual commercial ELISA kits involving the same immunoreagents. As it is shown in Fig S4, an excellent correlation between both methods occurred.

The calculated RANKL and TNF expression level in healthy individuals are in accordance with reported values [32,33,39–44]. As commented above, higher concentrations of both biomarkers were found in BC patients compared with the control group. Importantly, while similar TNF concentrations were found for HER2-positive and negative BC patients (48 *vs.* 40 pg mL$^{-1}$), a significantly higher RANKL concentration was apparent for HER2-positive BC group compared to the HER2-negative BC patients (550 *vs.* 339 pg mL$^{-1}$). These results demonstrate the independent prognostic factor value of both cancer biomarkers, where high concentration levels of TNF are related with larger stages of BC and lymph node metastasis [43] and overexpression of RANKL in serum, closely related with the BC subtype (HER2 positive), is indicative of cancer progression and aggressiveness and a higher probability of the metastatic process to bone [45].

Regarding TNF expression in BC patients (Fig. 6b), circulating biomarker levels found in serum were threefold higher for BC than for the

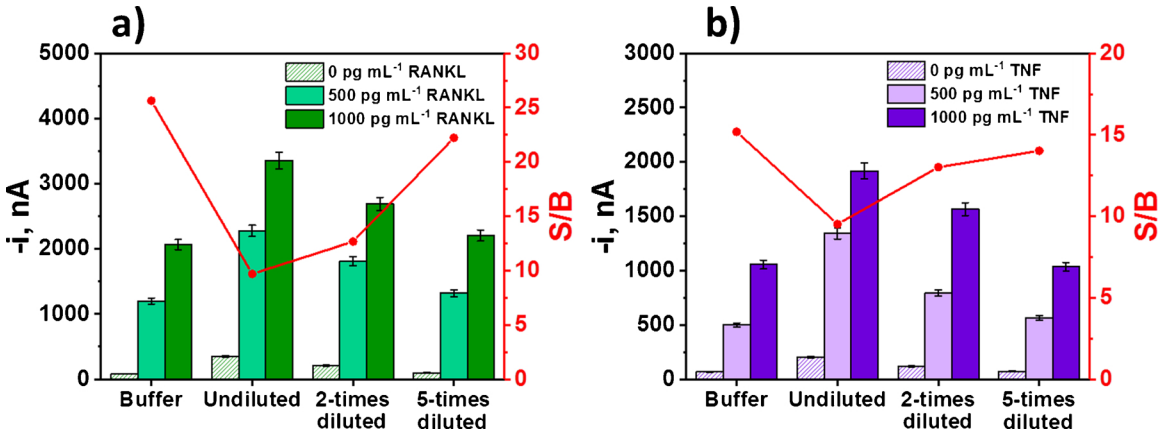


**Fig. 5.** Amperometric measurements provided with the dual immunoplatform for standard solutions of RANKL (a) and TNF (b) prepared in buffer solution and in human serum samples (undiluted and diluted as indicated). Error bars are estimated as three times the standard deviation of three replicates. S/B ratios are estimated as the ratios of the amperometric signals obtained in the absence and in the presence of 1,000 pg mL$^{-1}$ of the target biomarker.

control group. These results agree with those found in the literature for TNF expression in BC [43]. However, data obtained indicated that the TNF levels in serum between HER2 positive and HER2 negative BC patients were not significantly different. Therefore, it can be concluded that TNF levels in BC are associated with tumor aggressiveness but not with HER2 expression [43]. Conversely, significant differences were found for RANKL expression in BC patients with different HER2 expression (Fig. 6a), with mean values of 339 and 550 pg mL$^{-1}$ for HER2 negative and HER2 positive BC patients, respectively. This suggests that HER2 expression in BC may promote the production of RANKL, as increased levels of RANKL in the bone microenvironment can be directly regulated by HER2 overexpression [45]. In BC patients, HER2 oncogene amplification (HER2 positive) is strongly associated with enhanced disease aggressiveness and a poor prognosis due to that breast carcinoma frequently metastasizes to bone [46]. Moreover, the increased circulating levels of RANKL found in BC patients compared with healthy individuals, coupled with the high levels obtained for the aggressiveness cancer biomarker TNF, indicate advanced stages of BC that may disseminate to bone [21,46].

Despite the higher overexpression of RANKL and TNF in serum from patients diagnosed with BC should be exhaustively evaluated with more samples from different patient cohorts, the results obtained with the developed dual immunoplatform make RANKL and TNF attractive circulating biomarkers for the reliable detection of BC and HER2 subtype.

## 4. Conclusions

In this work, the first electrochemical immunoplatform for the simultaneous determination of RANKL and TNF, two relevant biomarkers related with BC and HER2 subtype, is reported. The developed methodology relies on the preparation of sandwich type configurations for the target biomarkers between the capture antibodies immobilized onto MBs-based immunoplatforms and detector antibodies labeled with HRP-conjugated secondary antibodies. The developed immunosensor is sensitive (LODs of 2.6 pg mL$^{-1}$ and 3.0 pg mL$^{-1}$ for RANKL and TNF, respectively) and selective, allowing the simultaneous and accurate determination of the biomarkers in 5-times diluted serum samples. Importantly, the determinations can be completed in about 90 min (starting from the prepared bCAb-MBs) using simple protocols and small sample amounts (5 µL of human serum per target biomarker determination). The developed methodology is competitive with respect to the commercial ELISA kits for single determinations, in terms of assay time, precision, portability and cost for the determination of the endogenous content of the target proteins in serum. Furthermore, the developed methodology using the MBs-based electrochemical design may be easily adapted to the determination of other proteins, and to fabricate an electrochemical array useful for the determination of multiple circulating biomarkers in a single test. Among their advantages, this novel device involving affordable and portable

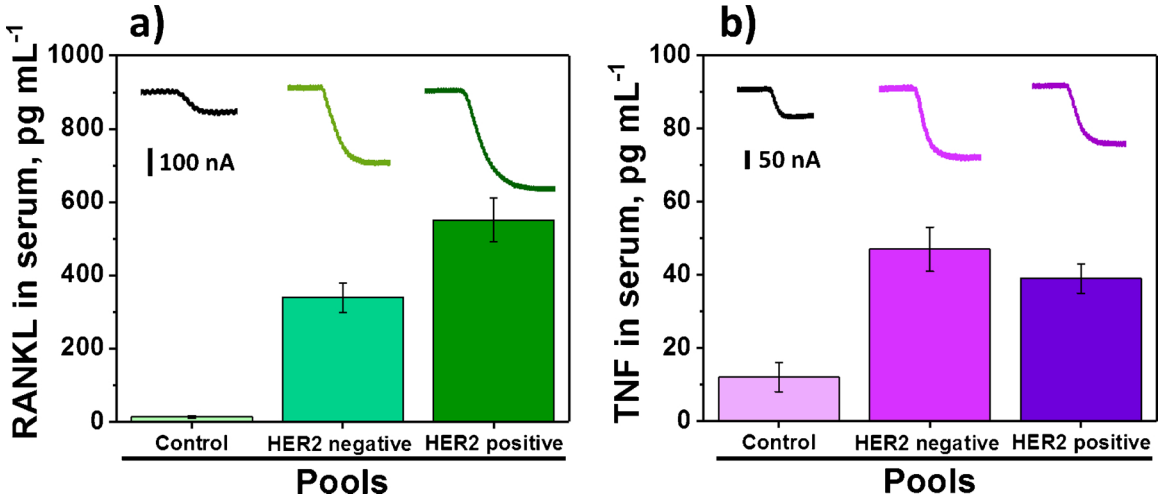


**Fig. 6.** RANKL (a) and TNF (b) levels in serum samples grouped into pools of healthy individuals (control) and BC patients regarding HER2 status measured with the developed dual immunosensor. Representative real amperometric traces are shown above the bars for each clinical group. Error bars are estimated as three times the standard deviation value of three replicates.

**Table 1**
Determination of RANKL and TNF in human serum samples from healthy individuals and patients diagnosed with opposite BC HER2 subtypes using the dual immunoplatform and the individual ELISA kits.

| Group | Patient | RANKL, pg $mL^{-1}$ | | TNF, pg $mL^{-1}$ | |
|---|---|---|---|---|---|
| | | Immunoplatform | ELISA | Immunoplatform | ELISA |
| Healthy individuals | 1 | 12 ± 4 | 12 ± 10 | 14 ± 5 | 15 ± 9 |
| | 2 | 14 ± 5 | 14 ± 12 | 11 ± 4 | 15 ± 11 |
| HER2 | 3 | 377 ± 20 | 323 ± 59 | 44 ± 9 | 50 ± 10 |
| negative (BC) | 4 | 302 ± 23 | 262 ± 51 | 49 ± 8 | 47 ± 8 |
| | 5 | 338 ± 24 | 334 ± 40 | 48 ± 9 | 45 ± 12 |
| HER2 | 6 | 508 ± 23 | 474 ± 50 | 40 ± 8 | 41 ± 11 |
| positive | 7 | 616 ± 20 | 578 ± 54 | 37 ± 5 | 40 ± 9 |
| (BC) | 8 | 528 ± 19 | 517 ± 51 | 38 ± 9 | 44 ± 10 |

Mean value ± ts/√n (n = 3; α = 0.05).

instrumentation, allows to perform the much-in-demand in-situ determinations of these emerging relevant biomarkers. It is worth to mention that the simultaneous determination of RANKL and TNF biomarkers in a single experiment is expected to be decisive in improving the reliability in BC detection and HER2 subtype classification.

## 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.

## CRediT authorship contribution statement

**Alejandro Valverde:** Investigation, Data curation, Writing - original draft. **Verónica Serafín:** Investigation, Data curation, Writing - original draft. **Jesús Garoz:** Investigation, Data curation. **Ana Montero-Calle:** Investigation, Resources. **Araceli González-Cortés:** Supervision, Data curation, Writing - original draft, Writing - review & editing, Funding acquisition. **Meritxell Arenas:** Resources. **Jordi Camps:** Resources. **Rodrigo Barderas:** Supervision, Resources, Writing - review & editing, Writing - original draft, Funding acquisition. **Paloma Yáñez-Sedeño:** Conceptualization, Supervision, Writing - review & editing, Writing - original draft, Funding acquisition. **Susana Campuzano:** Conceptualization, Supervision, Writing - review & editing, Writing - original draft, Funding acquisition. **José M. Pingarrón:** Writing - review & editing, Writing - original draft, Funding acquisition.

## Acknowledgements

The financial support of CTQ2015-64402-C2-1-R (Spanish Ministerio de Economía y Competitividad), RTI2018-096135-B-I00 (Spanish Ministerio de Ciencia, Innovación y Universidades) Research Projects, PI17CIII/00045 Grant from the AES-ISCIII Program and the TRANSNANOAVANSENS-CM Program from the Comunidad de Madrid (Grant S2018/NMT-4349) are gratefully acknowledged. A.V. was supported by contract of the S2018/NMT-4349 Project. J.G. acknowledges the financial support from MINECO through the “Juan de la Cierva” program. A.M-C. is supported by a FPU predoctoral contract supported by the Spanish Ministerio de Educación, Cultura y Deporte.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.snb.2020.128096.

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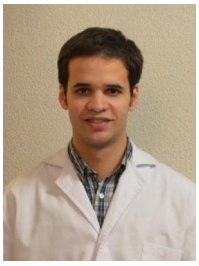

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