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Nanoimmunoassay onto a screen printed electrode for HER2 breast cancer biomarker determination

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ABSTRACT

A chip format sandwich-type immunoassay based on Nanobodies® (Nbs) with the Human Epidermal Growth Factor Receptor (HER2) extracellular domain as antigen model has been developed. The HER2 is considered as an important biomarker because its overexpression causes an aggressive type of breast cancer. Nbs are single domain antigen-binding fragments derived from camelid heavy-chain antibodies. The strategy of the presently developed sandwich immunoassay takes advantage of the small size of Nbs for the detection of the electroactive redox tracer onto the screen printed electrode (SPE). A capture anti HER2 Nb was covalently immobilized onto the SPE, and the detection Nb, raised against another epitope of HER2, was labeled with horseradish peroxidase (HRP). The biosensor signal corresponded to the electroreduction of para-quinone generated at the SPE by the HRP in the presence of hydroquinone and hydrogen peroxide. The best performing and optimized immunoassay conditions consisted of 2 and 20 min for the first and the second incubation times, respectively. The amperometric signal obtained was proportional to the logarithm of HER2 concentration between 1 and 200 μg/mL and the modified SPE storage stability lasted for at least three weeks. Determination of HER2 in human cells has been realized.

1. Introduction

Breast cancer is the most common cancer in women worldwide. The disease can be subdivided in four main classes, i.e. (i) hormone (estrogen or progesterone) receptor positive, (ii) HER2 positive, (iii) hormone receptor and HER2 positive and (iv) hormone receptor and HER2 negative breast cancers. The hormone receptor positive cancer responds to endocrine therapy and the prognosis is generally better than the other type of breast cancers [1]. Human epidermal growth factor receptors (HER/erbB) are involved in the normal growth and differentiation of cells. HER2 is the only receptor of this family which has no known ligand. The association of a specific ligand to HER1, HER3 or HER4 provokes a receptor dimerization, preferentially with HER2 as partner and induces cell signaling. A malignant growth occurs when HER2 is overexpressed giving rise to multiple HER2 heterodimers and to a stronger cell signaling, resulting in enhanced responsiveness to growth factors [2]. The HER2 overexpression is present in some cases of breast, ovarian, gastric, prostate and other cancers [3]. HER2 is a trans-membrane glycoprotein of 185 kDa with tyrosine kinase activity, encoded by a gene located on the long arm of chromosome 17 [4]. The amplification of this chromosome region leads to over-expression of the HER2 [5]. The HER2 is over-expressed in around 20–25% of invasive breast cancers and is associated with poor-prognosis as well as reduced survival [4]. The HER2 status of a breast cancer should be evaluated because therapeutic compounds that specifically target HER2 are available [5]. Trastuzumab (Herceptin®, Roche), a humanized monoclonal antibody against HER2 extracellular domain, is currently used but a significant proportion of patients with HER2 positive breast cancer display either primary or secondary resistance [6]. Trastuzumab combined with paclitaxel after a treatment with doxorubicin and cyclophosphamide reduce the rate of recurrence by half among women with operable HER2 positive breast cancer and the mortality was reduced by one third. The therapeutic results were similar for women with hormone-receptor-negative

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tumors and women with hormone-receptor-positive tumors [7]. Only patients with HER2 overexpression receive the anti-HER2 treatment because of the therapeutic cost and the side-effects. It is important to avoid false negatives so that only HER2 positive patients receive a treatment [8]. Immunohistochemistry is the primary technique applied to determine the HER2-status. The extracellular domain of HER2 (110 kDa) is released into the systemic circulation after HER2 cleavage by matrix metalloproteinase. Subsequently it can be determined in serum of patients. An enzyme linked immuno sorbent assay (ELISA) has been commercialized by Siemens, which has been approved by the FDA in 2000 [8]. The serum extracellular HER2 normal level limit is 15 ng/mL and it is a good indicator of the antitumor treatment efficiency. The extracellular domain level of HER2, however, is not always in correlation with the tumor response [9]. A high serum level of extracellular domain of HER2 could indicate resistance against trastuzumab [10].

Another HER2 immunoassay has been developed using two monoclonal antibodies directed against independent but adjacent target epitopes on the HER2 receptor [11]. One antibody is labeled with a fluorescent VeraTag™ and the other with a photosensitive molecule that releases free oxygen radicals upon photoactivation. The free oxygen radicals cleave the nearby VeraTag™ reporter which is subsequently collected and quantified using capillary electrophoresis [11]. Techniques such as real time PCR and microarray based sensors have been described to quantify the RNA level coding for HER2. Two techniques to measure DNA are approved by the FDA: a fluorescence in situ hybridization test and a chromogenic hybridization test [12].

An electrochemical immunosensor has been developed by immobilizing trastuzumab on gold nanodisk electrodes. The interaction between HER2 and antibody is detected by suitable secondary antibodies labeled with HRP and methylene blue as the redox mediator. This immunosensor is used to determine HER2 in cell lysates and tumor lysates [13]. Another electrochemical immunoassay for HER2 detection is based on a sandwich format in which a primary monoclonal anti-HER2 antibody is coupled to protein A modified magnetic beads and the secondary antibody is labeled with biotin. After the two immunoreactions, alkaline phosphatase conjugated with streptavidin and 1-naphtyl-phosphate are used for the differential pulse voltammetry detection at screen printed electrodes [14]. An opto-fluidic ring resonator biosensor has also been developed to determine the HER2 extracellular domain in spiked serum [15]. A label free electrochemical immunosensor for HER2 based on antiHER2-iron oxide nanoparticle bioconjugates has recently been published. After the immunoreaction the nanoparticles are laid over the gold electrode surface for differential pulse voltammetry detection [16].

With respect to the development of a rapid immunoassay for HER 2 determination, it was of interest to exploit the advantages provided by Nanobodies® (Nbs). Also referred to as “VHH” because they consist of the variable part of heavy chain-only antibodies, they are the smallest known single entity with full antigen binding properties that are derived from functional antibodies [17]. Heavy chain-only antibodies were first discovered in serum of camel, but all species of the *Camelidae* contain these unique IgGs' in their serum[18]. Nbs have similar antigen binding properties compared to whole antibodies, but they offer multiple advantages over other antibody fragments e.g. they are readily produced by *E. coli* and are resistant to heat and reducing agents and are highly water soluble [17]. Their stability, size (ca 15 kDa) and antigen affinity and specificity make them highly attractive for developing novel analytical tools. In the biosensor field, the first reported application is a Nb-based chip for prostate-specific antigen immunoassay using a commercially available surface plasmon resonance (SPR) device [19]. An electrochemiluminescent immunosensor with Nbs immobilized onto a glassy carbon electrode was described for the highly sensitive assay of human procalcitonin in serum samples [20]. Subsequently we reported an amperometric immunoassay with nanobodies immobilized onto SPE for HER2 detection [21]. Two amperometric magnetoimmunosensors using nanobodies for fibrinogen detection in plasma [22] have also been described. The small size of the Nbs was instrumental for easy access to their target and for signal detection in close proximity of the transducer.

Here, we report for the first time the development of a sandwich type immunoassay (nanoimmunoassay) using Nbs linked to a carbon based screen printed electrode (SPE) for the HER2 extracellular domain determination. HER2 antigen was selected as a biomarker target model because of its availability together with a series of non-competitive anti HER2 Nbs [23]. The HER2 level was determined in human cells spiked with HER2 to mimic a cancerous lysate.

2. Material and methods

2.1. Reagents

N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-Hydroxysuccinimide (NHS), polysorbate 20 (tween 20), hydroquinone, Tris–Cl (Trizma® base) and Tergitol-type nonyl phenoxypolyethoxylethanol 40 (Nonidet P-40, NP40) were purchased from Sigma (Bornem, Belgium). EZ-link Plus Activated Peroxidase kit and BCA protein assay kit were from Pierce Biotechnology /Thermo scientific (Rockford, USA). Albumin from bovine serum (fraction V) (BSA) was from Fluka (Neu-Ulm, Germany). Recombinant Human ErbB2/Fc Chimera (HER2) was obtained from ImmunoSource (Schilde, Belgium). Hydrogen peroxide (30%) was from VWR International Inc (Leuven, Belgium). Citric acid monohydrate and sodium carbonate anhydrous were from Janssen Chimica (Beerse, Belgium). Sodium hydrogen carbonate was from Fisher Chemicals (Loughborough, United Kingdom). Potassium chloride and di-sodium hydrogen phosphate dihydrate were from Merck (Darmstadt, Germany). Potassium dihydrogen phosphate dihydrate was from Vel (Leuven, Belgium). Sodium chloride was from Carlo Erba (Rodano, Italy). The protease inhibitor cocktail tablets were from Roche (Mannheim).

Following buffers and solutions were prepared:

- Phosphate buffered saline pH 7.4 (PBS) containing 8 g/L of sodium chloride, 0.2 g/L of potassium chloride, 8 mM disodium hydrogen phosphate and 1.5 mM potassium dihydrogen phosphate for immunochemical reactions.
- MES buffer containing 10.6 g/L 2-(4-morpholino) ethanesulphonic acid adjusted to pH 5.5 for the formation of the peptide bond with EDC and NHS.
- PBS containing 0.5 g/L of polysorbate 20 as washing buffer.
- A 0.05 M citric acid solution was adjusted to pH 5.5 with a solution of sodium hydroxide for the electrochemical measurements.
- Lysis buffer containing 50 mM Tris–HCl pH 8.0, 0.4 M sodium chloride, 1% (v/v) NP40 and protease inhibitor cocktail (1 tablet for 10 mL) was used to lyse sample cells [13].

2.2. Instrumentation

The electrochemical measurements were performed using an LC Epsilon computer-controlled potentiostat with Epsilon EC software. The screen printed electrodes (SPE) and the adapted connector were purchased from DropSens (Oviedo, Spain). SPE had the following dimensions: $3.4 \times 1.0 \times 0.05$ cm^3 (length × width × height). Working (4 mm diameter) and counter electrodes were made of carbon, pseudoreference electrode and electric contacts were made of silver.

The LC-QTOF analyzes were performed with a QTOF 6520 (Agilent technologies, Palo Alto, CA, USA).

2.3. Anti-HER2 Nb production

The anti-HER2 Nbs 2Rs15d, 1R143c and 1R59b were produced and purified as described previously [23]. In short, their DNA fragments were re-cloned in vector pHEN6 and expressed in *E. coli* WK6 after isopropyl-β-D-thiogalactoside (IPTG) induction to obtain His_6-tagged Nbs in the periplasm. The Nbs were extracted from the periplasm by osmotic shock and purified by immobilized metal affinity chromatography (IMAC) on Ni-NTA resin (Sigma-Aldrich, St Louis, MO, USA), eluting with 0.5 M imidazole in PBS pH 7.5 and containing 80 g/L NaCl, and further purified by molecular sieving on Superdex 75 h 16/60 (Pharmacia, Gaithersburg, USA) in PBS. Molecular weights, the number of lysines and the extinction coefficients at 280 nm were calculated from their amino acid sequence analysis, and binding properties were determined by SPR (Table 1).

2.4. Surface plasmon resonance (SPR)

To confirm the mutual non-competitiveness of the utilized anti-HER2 Nbs, single solutions and paired mixtures were subjected to SPR performed on a Biacore T100 instrument (GE Healthcare, Little Chalfont, UK), with the recombinant human ErbB2/Fc fusion protein immobilized on a CM-5 gold layer chip. In practice, the first 600 s binding phase occurred with a single Nb, and was followed by a second 600 s binding phase with a mixture of the Nb and one other HER2 Nb. Control curves were measured with the single Nb in both phases and analyzed using Biacore's evaluation software (not shown).

2.5. Peroxidase labeled anti-HER2 Nbs

The procedure recommended by the kit supplier was followed except that glycine was used instead of ethanolamine (see reason below) to neutralize the excess of EZ-Link™ Plus activated peroxidase, and was optimized in terms of pH and incubation time. The sugars of native HRP were oxidized by periodate to generate aldehyde groups, and the oxidized HRP was then crosslinked to the anti-HER2 Nbs by forming Shiff-bases between the aldehydes on HRP and the primary amines present on the Nb. Sodium cyanoborohydride was added to reduce the link and form a stable HRP-Nb conjugate. (i) A volume of 100 μl of oxidized HRP (1 mg/100 μL) and 10 μL of sodium cyanoborohydride (5.0 M) were added to 500 μL PBS containing 0.3 mg of Nb; (ii) this solution was incubated overnight under stirring at room temperature; (iii) a 20 μL volume of glycine 3 M was added and reacted during 15 min at room temperature. At the end, the HRP labeled Nb (Nb-HRP) was purified as described above for free Nbs by IMAC (to remove free HRP) and size exclusion chromatography (to remove free Nb). The efficiency of the labeling was tested by SDS-PAGE. The SDS-PAGE experiments were carried out on precast gels (NuPAGE Novex 4–12% Bis–tris with MES SDS running buffer) using the X-cell SureLock Mini-cell from Invitrogen. The running conditions were 200 V, 50 mA at constant temperature 25 °C during 45 min. The Novex Sharp Protein standard from Invitrogen consisted of a mixture of 12 pre-stained standards (3.5, 10, 15, 20, 30, 40, 50, 60, 80, 110, 160, 260 kDa) for protein molecular weight comparison. Protein detection was performed using Coomassie Blue staining (ethanol 40% (v/v), acetic acid 10% (v/v) and Coomassie Brillant R250 0.2% (w/v)).

Table 1
Properties of selected Nbs.

Nanobody	SPR			MW	Lys #	ε ($M^{-1}cm^{-1}$)
	Ka ($M^{-1}s^{-1}$)	kd (s^{-1})	K_D (nM)			
2Rs15d[a]	2.14×10^5	5.71×10^{-4}	2.70	13453	6	25690
1R59b[a]	5.01×10^5	2.43×10^{-3}	4.90	13361	3	11710
1R143c[a]	6.27×10^5	4.23×10^{-3}	6.80	14540	6	20650

[a] Non-competitiveness between these Nbs was demonstrated with SPR.

2.6. Immobilizing capture anti-HER2 on the SPE (Fig. 1.1)

The SPE surface was modified according to the following procedure: (i) the SPE was activated in the presence of 50 μL of 2 M H_2SO_4 dropped onto the SPE and by applying a voltage of +1.60 V for 150 s, then the SPE was washed with distillated water, (ii) 10 μL of 40 mM EDC and 40 mM NHS solution in MES buffer was spiked onto the pretreated SPE surface during 1 h, (iii) the SPE was again thoroughly washed with distilled water and dried to remove excess reagent and 10 μL of 10 μg/mL (6.6×10^{-7} M) anti-HER2 Nb were spiked onto the electrode surface, (iv) after 1 h the non-linked Nbs were eliminated by gently washing the SPE with distillated water. Any remaining surface free sites were blocked by 10 μL of BSA solution in PBS (1% m/v) for 30 min. Finally the SPE was washed with water. All the incubations and reactions occurred at room temperature and in a humid atmosphere.

2.7. Nanoimmunoassay (Fig. 1.2 and 1.3)

The SPE surface modified with anti-HER2 Nb was spiked with 10 μL of HER2 solution in PBS for 2 min at room temperature (Fig. 1.2a). After this incubation period, the SPE was rinsed with water and 10 μL Nb-HRP solution was added and left reacting for 20 min (Fig. 1.2b). The SPE was washed twice with 1 mL of washing buffer then, 40 μL of citrate buffer containing 2.5 mM H_2O_2 was added onto the SPE for the amperometric detection. A potential of −280 mV was applied and 10 μL HQ (0.1 M) was spiked onto the electrode and the reduction current was monitored (Fig. 1.3). All the electrochemical parameters were optimized in a previous study [24].

2.8. Sample assays

HER2 negative (MCF7) cells were cultured in a T80 flask in 15 mL of the following medium: RPMi, 10% FBS heat inactivated, 2% Pen-strep (10,000 Um/L), 2% L-glutamine (200 mM) and 0.2% Gentamicine (50 mg/mL). The cells were collected when the confluence reached 85–90%. The cell lysates were prepared using 500 μL of lysis buffer. After an incubation time of 30 min at 4 °C, the lysates were collected, sonicated, and centrifuged. A volume of 70 μL of lysate was spiked with 30 μL HER2 and 10 μL of this sample served for the HER2 nanoimmunoassay ($n=3$). The total protein concentration was estimated by a Bradford assay before adding HER2. The HER2 concentration in the samples was determined by referring to a calibration curve realized daily in PBS.

3. Results and discussion

3.1. Anti-HER2 Nb selection

Three different Her2-specific Nbs were selected based on their known binding rate constants and mutual non-competitiveness (Table 1). For stability reasons, the Nb 2Rs15d having the lowest k_d, would be the first choice as capture Nb (immobilized onto the SPE), to be combined with Nb 1R59b having the second best kd and K_D as detecting Nb (HRP conjugated) in a in HER2 nanoimmunoassay. The 1R59b Nb however contains only three lysines

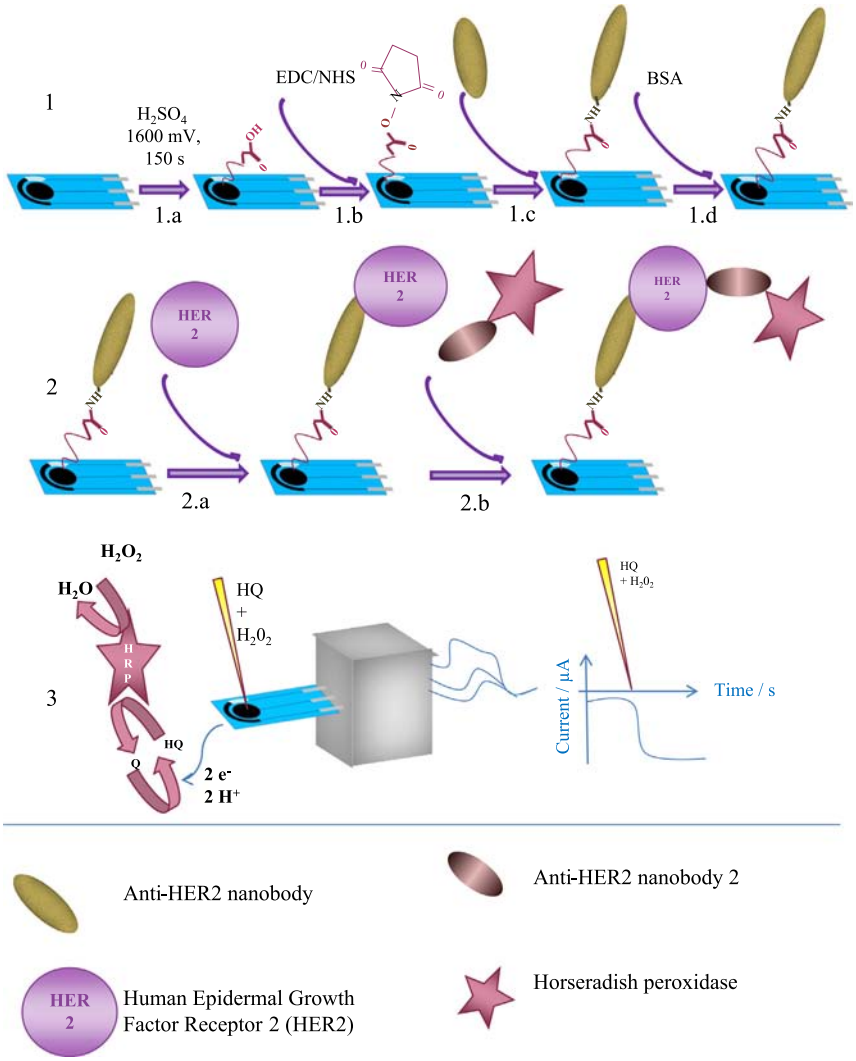

Fig. 1. Schematic drawing of SPE modification. (1) and nanoimmunoassay (2 and 3). 1. SPE oxidation to generate carboxylic groups (1a), activation of the carboxylic groups with EDC and NHS (1b), immobilization of the anti-HER2 Nb (1c), blocking of the free sites by BSA (1d); 2. Sandwich nanoimmunoassay: HER2 sample incubation (2a), Nb-HRP incubation (2b); 3. Amperometric detection of p-quinone (Q) generated by HRP in the presence of HQ and H_2O_2.

compared to 6 for the other two Nbs, and thus has less free amine groups available for conjugation with HRP.

Nbs 2Rs15d and 1R59b were tested as capturing Nb (immobilized onto the SPE) and Nb 2Rs15d and Nb143c were labeled with HRP. The pair 1R59b as capturing Nb and 2Rs15d-HRP gave the highest amperometric signal during the nanoimmunoassay, and was selected for further application of nanoimmunoassay. The SPR results (Table 1) suggest that 2Rs15d is the best capturing Nb because of its low dissociation rate, but the HRP labeling of 1R59b, containing only three lysines, was unsuccessful. Nb143c-HRP as detecting Nb probably suffered from its less favorable binding properties.

3.2. Peroxidase labeled anti-HER2 Nb

Anti-HER2 Nb 2Rs15d and Nb 1R143c were crosslinked with activated HRP. The reaction products were followed by SDS-PAGE. Fig. 2 shows the results for Nb 2Rs15d. The 40 kDa band of HRP was observed as well as two additional bands (Fig. 2: Nb-HRP 5 and 6). These two bands corresponded to the mass of HRP plus one (Fig. 2: Nb-HRP 5) or two (Fig. 2: Nb-HRP 6) times the mass of Nb 2Rs15d. The bands were identified by LC-QTOF [25] by excising gel pieces subsequently submitted to in gel trypsin digestion [26]. The LC-QTOF results confirmed the presence of HRP and 2Rs15d in the two additional bands. The reaction product was further purified in two steps to isolate the HRP-2Rs15d conjugates by IMAC and size exclusion chromatography, where both HRP-Nb (considered as major component) and HRP-Nb_2 eluted in the same peak. This mixture was further used for nanoimmunoassay.

3.3. Anti-HER2 Nb immobilization

The optimal oxidation conditions for surface activation were evaluated by voltammetry. A progressive increase in the pseudocapacitive current (i.e. higher amount of charged functionalities) occurred till 150 s and then stabilized (figure not shown), and the SPE surface was therefore oxidized at 1.6 V during 150 s in 2 M H_2SO_4.The classical immobilization procedure with EDC and NHS was used to link, via a peptide bond, the carboxyl groups generated at the SPE surface with an amine function of the nanobody. The activated groups, however, were not blocked by ethanolamine since some remaining ethanolamine was found to react with the HRP generated quinone in the amperometric assay. During the detection step, hydroquinone was oxidized to p-quinone by HRP and the p-quinone was electrochemically reduced. Ethanolamine interfered in this process due to its reaction with p-quinone. The reaction of a quinone with amines was described in the literature [27]. This interaction was verified by cyclic voltammetry (CV) of HQ and of

HQ in the presence of ethanolamine; it involves the formation of a compound which cannot be reduced at the electrode. The CV of HQ showed a quasi-reversible behavior (Fig. 3 curve A). The addition of ethanolamine regenerated a HQ form giving rise to a catalytic signal with peak increase and shift in potential (Fig. 3 curve B) while the Q reduction peak had disappeared. Because of its interference, the use of ethanolamine was avoided in the immunosensor preparation. No such interference was observed with BSA or glycine. The quenching solution containing ethanolamine provided with EZ-link Plus Activated Peroxidase kit was replaced by a solution of glycine or BSA.

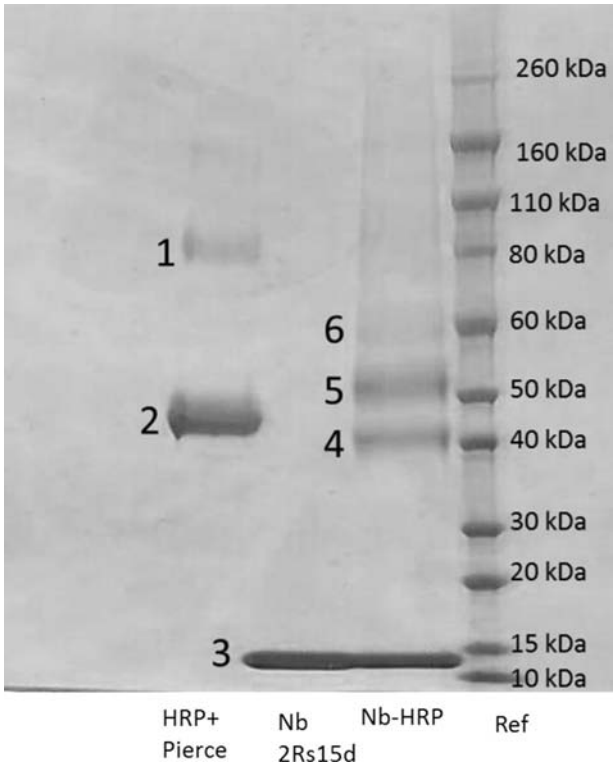

Fig. 2. SDS-page electrophoresis of oxidized HRP, Nb and the mix of oxidized HRP and Nb. Peptide sequences of peroxidase C1A were identified in bands 1, 2 and 4. Band 2 and 4 corresponded to HRP and band 1 corresponded to HRP-dimer. Peptide sequences of Nb 2Rs15d were identified in band 3 and peptide sequences of peroxidase C1A and Nb 2Rs15d were identified in bands 5 (HRP-Nb) and minor band 6 (HRP-Nb2).

To control the efficiency of Nb immobilization onto the SPE, the response of the modified electrode was compared to the response obtained with a SPE modified only with BSA. The signal obtained in presence of capturing Nb was 4 times higher than in its absence.

To follow the SPE surface modifications and to evaluate any diffusional restriction, cyclic voltammetric experiments of HQ (0.1 mM) were realized after each modification step. As shown in Fig. 4, after the surface of the electrode has been oxidized, the pseudocapacitive current increased substantially and the voltamperogram became quasi- reversible but the anodic peak current and the cathodic peak current decreased (Fig. 4B) ($\Delta Ep = 300$ mV became $\Delta Ep = 100$ mV). After each modification step a slight signal decrease was observed, while the addition of Nb-HRP caused a potential shift and an increase of the capacity current.

The same experiment was carried out with trastuzumab, a "classical" antibody against HER2, as capturing element. The electrode was modified with the same amount of antibody as for the nanobody in terms of recognition element i.e. two recognition sites by antibody and one by nanobody. After the immobilization of trastuzumab onto the SPE, a 27% decrease of the anodic peak was observed while was only 15% in the case of the modification by the nanobodies. This permitted to infer a less important surface access restriction of the HQ mediator when using Nbs comparing to the "classical" antibody.

3.4. Blocking step

To minimize unspecific interaction (interaction of Nb-HRP without HER2 link) i.e. different blocking agents were considered such as BSA and no-fat milk. BSA with an incubation time of 30 min was selected and two concentrations (1 and 3% m/v) were tested. BSA 1% (m/v) gave lowest unspecific and highest specific responses and was therefore employed in further experiments.

3.5. Nanoimmunoassay incubation time

The incubation time between the antigen and the modified SPE was varied between 0 and 60 min (Fig. 5A). The magnitude of the signal resulted from the extent of interaction between HER2 and anti-HER2 Nb immobilized on the SPE. The response was very quickly at its maximum. An incubation time of 2 min was selected. The incubation time of the Nb-HRP was varied between 10 and 40 min (Fig. 5B). The non-specific interaction between the HRP conjugate and the SPE increased with time (blank) while the

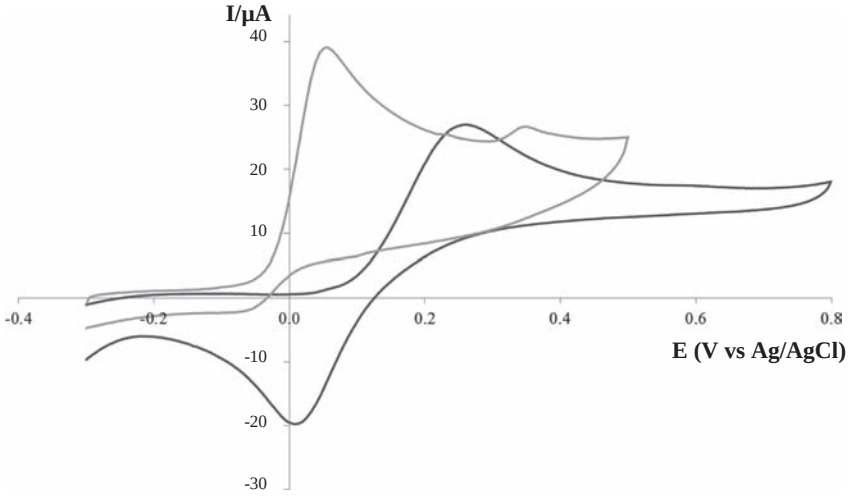

Fig. 3. Cyclic voltammetry of hydroquinone (2 mM) (dark gray, curve A) and hydroquinone (2 mM) with ethanolamine (200 mM) (light gray, curve B) carbon SPE, scan rate: 10 mV/s, citrate buffer pH 5.5 (-300 mV$\rightarrow+500$ mV$\rightarrow-300$ mV).

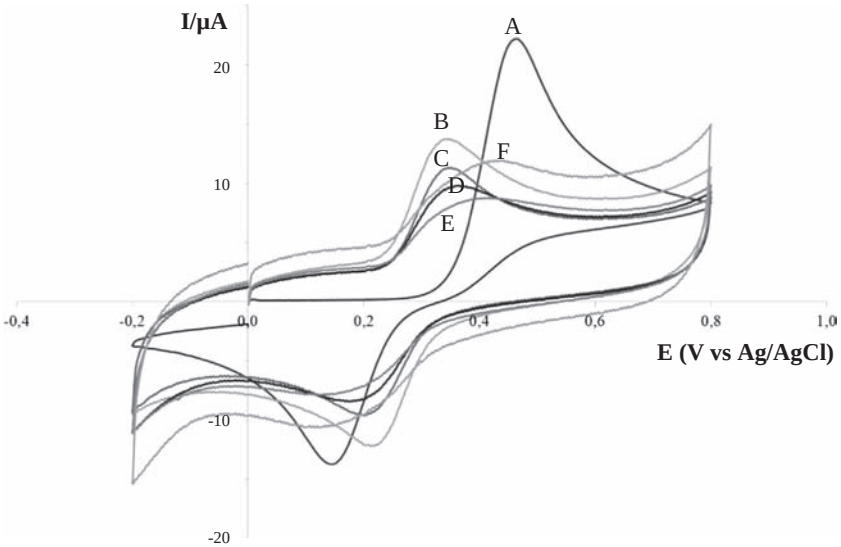

Fig. 4. Evaluation of SPE surface diffusional restriction. Cyclic voltammetry of HQ (0.1 mM) in citrate buffer (0.00 to 0.80, 0.80 to −0.20 and −0.20 to 0.00 V, scan rate: 100 mV/s). Unmodified SPE (A), oxidized SPE (B), after EDC-NHS step (C), after capturing Nb (10 µg/mL) immobilization (D), after blocking step (BSA 1%, 30 min) (E) and after HER2 and Nb-HRP incubation (F).

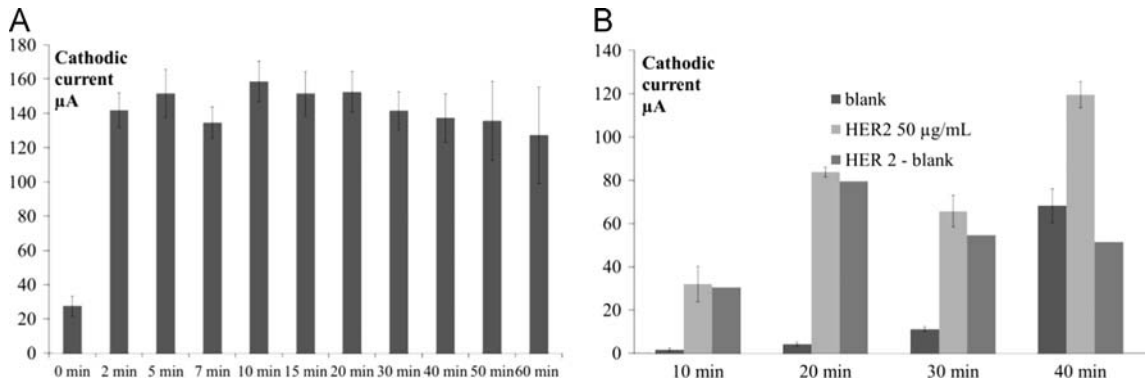

Fig. 5. Influence of incubation time (in PBS) on the EC response. $E_{app} = -280$ mV, HQ 20 mM, H2O2 25 mM. (A) Comparison between different HER2 incubation times. HER2 50 µg/mL, incubation volume 10 µL, HRP labeled anti-HER2 Nb incubation volume 10 µL; the first incubation time was varied between 0 and 60 min and the second incubation time was 20 min. (B) Comparison between different HRP labeled Nb incubation times. HER2 50 µg/mL, incubation volume 10 µL, HRP labeled anti HER2 Nb incubation volume 10 µL; the first incubation time was 20 min and the second incubation time was varied. The assay blank was realized in the absence of HER2 ($n=3$).

HER2–anti HER2 interaction tended to decrease (HER2-blank). An incubation time of 20 min was selected. This time was a good compromise for a high signal related with the antigen-Nb interaction and a small blank signal.

3.6. Nanoimmunoassay performances

Calibration curves were realized with the modified SPE, the first incubation time being 2 min and the second 20 min. The Nb-HRP was used at 30 µg/mL. The signal was proportional to the logarithm of HER2 concentration between 1 and 200 µg/mL with a limit of detection (LOD) of 1 µg/mL and a limit of quantification (LOQ) of 4.4 µg/mL. The LOD and the LOQ were calculated from the signal given by the blank. The latter was the signal obtained without HER2. The means and the standard deviation were calculated from 3 assays. The equations applied to determine the LOD and the LOQ were the following:

$$LOD = m_{bl} + 3SD_{bl}$$

$$LOQ = m_{bl} + 10SD_{bl}$$

with m_{bl}: mean of 3 blank assays and SD_{bl}: standard deviation of 3 blank assays. The LOD value was adapted to the studied calibration curve.

At higher concentrations, a saturation of the antibody link sites was inferred since the signal no longer increased. At lower concentration, no signal was measured (Fig. 6).

3.7. Stability

The storage stability of the modified SPE under dry conditions at 4 °C was evaluated for a period of 3 weeks. Various SPEs were prepared on the same day as reported in the experimental section, stored and used in different days. The nanoimmunoassay response remained within the limits (set at 3 times the standard deviation of the measurements ($n=3$) realized the first day) during 3 weeks and more. The non-specific response of the Nb-HRP, evaluated by the blank, also remained constant during this period.

3.8. Application to HER2 spiked cells

The total protein concentration in the studied samples was measured by Bradford technique at 3 mg/mL. Three cell samples spiked with increasing amounts of HER2 were analyzed with the

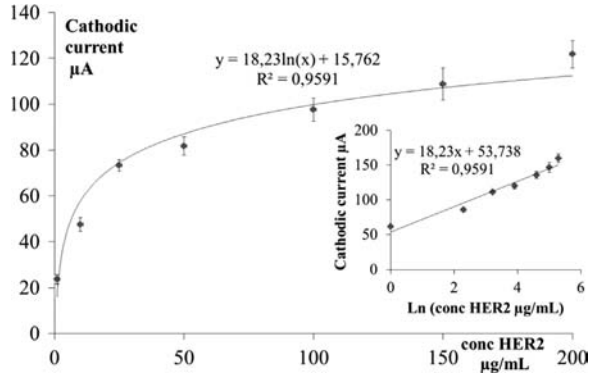

Fig. 6. Calibration curve. Representative results obtained with SPE modified by EDC/NHS, anti-HER2 (1R59b) Nb and BSA 1%, incubation HER2 2 min; Nb-HRP (2Rs15d) incubation 20 min. Blank signal was subtracted.

Table 2
Recoveries, biases, RSDs for HER2 determination in cell lysates using the developed HER2 nanoimmunosensor.

HER2 spiked (μg/mL)	HER2 found (μg/mL)	Recovery (%)	Bias (%)	RSD (%)
25	22	88	−12	8
50	41	82	−18	6
75	66	88	−12	14

nanoimmunoassay ($n=3$ for each sample). A blank sample containing no HER2 was also tested. The signal obtained with this sample was similar to the signal obtained with the PBS buffer. This indicated an absence of interference in our nanoimmunoassay and reflects the specificity of the test. The results obtained with the spiked samples are shown in Table 2. An acceptable negative bias was found for all samples, possibly due to the high protein concentration in tested cell lysates.

4. Conclusion

This work reported the successful SPE modification by Nb. It permitted a new type of immunoassay called nanoimmunoassay onto the SPE. A high stability of Nb was inferred since the storage stability of the SPE was higher than 3 weeks. Very short incubation times were sufficient to obtain a satisfactory response. The nanoimmunoassay allowed determining HER2 spiked in cell lysates. Future work, however, is needed in order to increase the sensitivity of the nanoimmunoassay for serum sample applications at physiological relevant values and for cell lysates assays after eventual dilution.

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