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This is an **author produced version** of a paper published in:

Sensors and Actuators B: Chemical 343 (2021): 130096

DOI: <https://doi.org/10.1016/j.snb.2021.130096>

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**Bifunctional carbon nanodots for highly sensitive HER2 determination based on  
electrochemiluminescence**

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## **ABSTRACT**

Early detection of breast cancer increases the chances of achieving adequate and successful treatment as soon as possible. In this work, a promising disposable electrochemiluminescent immunosensor has been developed for simple, efficient detection of the HER2 protein, a breast cancer biomarker. Nitrogen-rich carbon nanodots were synthesized with two functions: to provide functional groups for covalent immobilization of HER2 antibodies and to act as co-reactants in the electrochemiluminescent process. The proposed immunosensor responded linearly to HER2 concentration over a wide range, showing a detection limit of  $20.4 \text{ pg mL}^{-1}$ . The reliability of this biosensor was confirmed by analyzing HER2 in the presence of another tumor biomarker (CEA), as well as various proteins and sugars. In addition, this proposed strategy presented good stability and applicability in the analysis of human serum samples, showing great potential for applications in the early diagnosis of breast cancer.

## **KEYWORDS**

Breast cancer, HER2, Immunosensor, Carbon nanodots, Electrochemiluminescence

## **1. Introduction**

Cancer is a major health problem today, with more than 100 types identified (National Cancer Institute, USA). While important scientific advances have recently allowed us to establish the mechanisms and causes that give rise to a great number of tumors, cancer is still one of the main causes of mortality in developed countries [1].

In the last few years, the number of breast cancer cases in the world has multiplied [2]. This disease almost exclusively affects women, although men can also suffer from it [3]. Therefore, breast cancer has become a serious health problem, mainly for women around the world. Recent studies have shown that early detection, prevention and treatment are effective for reducing patient mortality [4]. For this reason, the development of early detection systems is essential, and accurate prediction of the patient's chances of survival remains an important facet of the treatment of this disease.

In the early stages of the disease, cancer biomarkers usually have very low concentrations in biological fluids, making them difficult to detect. Therefore, very sensitive detection systems are required. Additional benefits of these systems are that they are also rapid, easy to handle and portable. Electrochemiluminescence (ECL) biosensing is a biomarker detection method that can combine all of the characteristics described above. In ECL, species generated at the electrode surface undergo electron transfer reactions, transforming them into excited states that generate light upon returning to ground state [5]. Luminophores such as ruthenium complexes or their derivatives are needed. The response of the sensing device is highly dependent on efficient transduction of the analyte recognition event. It has recently been reported that the performance of biosensors in general and ECL biosensors in particular is highly improved by the use of nanomaterials [6-9]. Specifically, novel

nanomaterials such as carbon nanodots (CNDs) have proven to be excellent co-reactants [10, 11] because of their high charge-transfer efficiency [12, 13].

Human epidermal growth factor receptor 2 (HER2) is a transmembrane enzyme and a common biomarker for breast cancer diagnosis [14]. It is overexpressed in approximately 25-30% of patients with this disease [15, 16]. This is associated with increased tumor activity, usually indicating an unfavorable course of disease. Patients with overexpression of HER2 generally have more aggressive forms of cancer, as well as increased resistance to conventional treatments [17], hence the great importance of early detection of this biomarker and the development of efficient diagnostic tests.

Although some tests for HER2 detection based on Surface Plasmon Resonance-based fiber optics [18] or on fluorescence [19] have been described, most reported HER2 biosensors are electrochemical. These include sensors based on molecularly imprinted polymers [20]; magnetic beads modified with an aptamer [21]; aptasensors combined with ferrocene-labeled DNA/Au nanospheres [22]; interdigitated gold electrodes [23]; electrochemical sandwich immunoassays based on CdSe/ZnS quantum dots [24, 25]; immunosensors based on a gold nanoparticle/multiwall carbon nanotube-ionic liquid electrode [26]; DNA-based immunosensors [27]; and classical immunosensors based on a sandwich enzyme [28], but ECL immunobiosensors have not previously been described. All these approaches are time-consuming and require laborious procedures to create the biosensor. In contrast, in this work we present an ECL immunosensor that can be produced in a few steps for the rapid determination of HER2, directly in human serum, with significant improvements in terms of sensitivity and speed compared to the sensors described above. This new device is based on nitrogen-rich CNDs that are synthesized with a dual purpose: acting as co-reactants in the ECL system and as support for the biorecognition layer (HER2

monoclonal antibody) through covalent bonds formed with their surface functional groups (see Scheme 1).

## Scheme 1.

## 2. Experimental

### 2.1 *Chemicals*

Tris(2,2-bipyridyl) dichlororuthenium(II) hexahydrate, urea, citric acid, potassium hexacyanoferrate(III), potassium hexacyanoferrate(II) trihydrate, acetic acid, 98% H<sub>2</sub>SO<sub>4</sub>, NaOH  $\geq$  98%, chitosan (chit), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC), N-hydroxysuccinimide (NHS), bovine serum albumin (BSA), 2-(N-morpholino)ethanesulfonic acid (MES), sodium phosphate dibasic  $\geq$  99%, sodium phosphate monobasic monohydrate  $\geq$  99%, potassium phosphate monobasic, potassium chloride, sodium chloride, Tween<sup>®</sup> 20, D-(+)-glucose, antigen p53, human carcinoembryonic antigen (CEA), IgG from human serum and human serum were obtained from Merck. The solutions were prepared using Milli-Q water.

Mouse anti-human HER2 monoclonal antibody (anti-HER2) and recombinant human HER2 standard were purchased as an ELISA kit (Human ErbB2/HER2 DuoSet ELISA DY1129B) from R&D Systems Europe, Ltd. Both were reconstituted with 0.01 M phosphate buffer saline solution (PBS), pH 7.4, containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub> and 1.5 mM KH<sub>2</sub>PO<sub>4</sub>. In the case of the antigen, the PBS contained 5% Tween<sup>®</sup> 20.

## 2.2 Instrumentation

Screen-printed carbon electrodes (SPCE) were supplied by DropSens-Metrohm. The counter electrode was carbon, with a silver pseudo-reference electrode.

The ECL experiments were performed in an ECL cell with an integrated Si photodiode and a potentiostat/galvanostat (potential range  $\pm 4$  V DC, maximum measurable current  $\pm 40$  mA) from DropSens-Metrohm. The volume of the ECL cell in which the electrodes were confined was 50  $\mu$ L. The ECL equipment was controlled by DropView 8400 software. The potentiostat was responsible for initiating the ECL reaction by applying pulses of voltage or current. The electrochemical and chemiluminescence responses were perfectly synchronized and displayed in real time. All ECL experiments were performed in 0.1 M phosphate buffer (PB), pH 8.0 and were carried out at room temperature in the absence of light.

Elemental analysis of the CNDs was performed using a PerkinElmer 2400 CHN elemental analyzer.

Transmission electron microscopy (TEM), Lacey carbon support film and copper grids (400 mesh, Electron Microscopy Sciences) were used.

X-ray powder diffraction spectra from a PANalytical X'pert PRO Theta/2Theta diffractometer were used.

Fourier transform infrared (FTIR) spectra were recorded from pressed KBr pellets of the solid material and precursors in the wavelength range 5000–500  $\text{cm}^{-1}$  using a Bruker IFS60v spectrometer.

UV-visible absorption spectra and fluorescence spectra were recorded in aqueous solutions using a quartz cell with a 1.0-cm optical path using a double beam PharmaSpec UV-1700 series spectrometer (Shimadzu) and a Varian Cary Eclipse spectrofluorophotometer, respectively.

Electrochemical Impedance Spectroscopy (EIS) measurements were recorded in the frequency range of  $10^5$  to  $1 \times 10^{-2}$  Hz, with a sinusoidal potential modulation of  $\pm 5$  mV in amplitude superimposed onto the formal potential of the redox probe. EIS experiments were carried out in 0.1 M PB, pH 7.0 in the presence of 0.1 M KCl containing  $1.0 \times 10^{-2}$  M  $\text{K}_3\text{Fe}(\text{CN})_6:\text{K}_4\text{Fe}(\text{CN})_6$  (1:1).

### *2.3 Procedures*

#### *2.3.1 Carbon nanodot synthesis*

The CNDs were synthesized by a previously-described method [29]. Citric acid (3 g) and urea (3 g) were weighed and dissolved in 10 mL of distilled water to form a colorless solution. The solution was heated in a domestic microwave at 750 W for 4 min, resulting in the solution changing color to dark brown and finally in the formation of a dark solid, indicating the formation of the CNDs. The solid was dissolved in 20 mL of distilled water and centrifuged (10000 rpm, 10 min) to remove agglomerations and large particles. The precipitate was removed, and the supernatant was purified for 1.5 h with a dialysis membrane. The final concentration of CNDs was  $1.78 \pm 0.04$  mg/mL and they were stored at 4°C.

#### *2.3.2 Preparing the immunosensor*

Firstly, as shown in Scheme 1, the surface of a SPCE was modified by drop casting with 10  $\mu\text{L}$  of CNDs. When the CNDs had dried (CNDs/SPCE), 5  $\mu\text{L}$  of chitosan ( $1.0 \text{ mg mL}^{-1}$  in 3% acetic acid) were dropped onto the surface and left overnight at 4°C. On the next day, the chit/CNDs/SPCE was washed with plenty of Milli-Q water, then the carboxyl groups of the CNDs were activated by adding 10  $\mu\text{L}$  of EDC/NHS solution ( $50 \text{ mg mL}^{-1}/50 \text{ mg mL}^{-1}$  in 0.025 M MES buffer, pH 5.0) and incubated for 30 min in a wet chamber. After that, the chit/CNDs/SPCE was washed with 0.025 M



MES buffer, pH 5.0, and the anti-HER2 was covalently bound to the activated carboxyl groups by dropping 5  $\mu\text{L}$  of antibody solution ( $5.0 \mu\text{g mL}^{-1}$  in 0.025 M MES buffer, pH 5.0) and incubating for 60 min in a wet chamber. After 60 min, the anti-HER2/chit/CNDs/SPCE was washed with 0.025 M MES buffer, pH 5.0. Finally, to prevent nonspecific binding, 10  $\mu\text{L}$  of BSA solution (1% in 0.1 M PB, pH 7.4) were added and kept for 30 min in a wet chamber. After washing with 0.1 M PB, pH 7.4, the BSA/anti-HER2/chit/CNDs/SPCE was incubated for 30 min with 5  $\mu\text{L}$  of different concentrations of recombinant human HER2 in 0.01 M PBS, pH 7.4, and 5% Tween<sup>®</sup> 20. Subsequently, the HER2/BSA/anti-HER2/chit/CNDs/SPCE was washed with 0.01 M PBS, pH 7.4, and 5% Tween<sup>®</sup> 20 and covered with 5  $\mu\text{L}$  of 0.1 M PB, pH 8.0, until the immunosensor response was measured.

#### *2.3.3 Detection of HER2*

The HER2/BSA/anti-HER2/chit/CNDs/SPCE was placed in the ECL cell and the measurement was performed by adding 50  $\mu\text{L}$  of 7.0 mM  $[\text{Ru}(\text{bpy})_3]^{2+}$  in 0.1 M PB, pH 8.0, and applying voltametric cycles between +0.40 V and +1.25 V at 0.010  $\text{Vs}^{-1}$ .

#### *2.3.4 Detection of HER2 in human serum*

The immunosensor produced was used to determine HER2 protein in human serum samples. To verify the results, they were compared with those obtained using a commercial colorimetric ELISA kit. The human serum samples were diluted 100-fold before use and subsequently spiked with a known concentration of HER2 protein.

### **3. Results and discussion**

#### *3.1 Synthesis and characterization of carbon nanodots*

CNDs with high nitrogen content were synthesized in a microwave oven, using citric acid and urea as precursors. The idea was to obtain CNDs with amino groups on their surfaces, since these play an important role in ECL systems by acting as co-reactants [5]. Figure 1A shows a TEM image of the prepared nanomaterial. It can be seen that most of the purified CNDs are practically spherical, with a diameter of approximately 5 nm. The presence of nitrogen groups on the surface of each nanoparticle could improve their stability and prevent aggregates. The inset of Figure 1A shows an individual nanoparticle observed by TEM. It does not have a defined structure, which suggests that the purified nanoparticles have a high level of amorphousness in their structure. The X-ray diffraction (XRD) pattern of the CNDs included a broad band at  $26.2^\circ$ , indicating and confirming the presence of a carbon nucleus in the amorphous phase (Figure 1B).

#### **Figure 1.**

Elemental analysis of the synthesized CNDs revealed the following composition: 36.03% carbon, 5.33% hydrogen, 19.69% nitrogen and 28.95% oxygen (calculated by difference). To determine and assign the functional groups present in the CNDs, the FTIR spectra of the CNDs and their precursors were obtained (Figure 1C). The CND spectrum is clearly different from the spectra of their precursors. It has the characteristic OH and NH<sub>2</sub> stretching bands as a broad band centered at  $3433\text{ cm}^{-1}$ ; the bands observed around  $2942\text{ cm}^{-1}$  derive from the C-H bond stretching vibration. C=O stretching vibrations appear at  $1715\text{ cm}^{-1}$ . At  $1450\text{ cm}^{-1}$  and  $1377\text{ cm}^{-1}$ , a band related to C-N bonds is visible. These results confirm the high nitrogen content in the CNDs' structure.

The CNDs' optical properties were also explored. The UV-visible spectrum (Figure 1D) shows an absorption band at 270 nm, attributed to the  $\pi\rightarrow\pi^*$  transition of the conjugated C=C units and the  $n\rightarrow\pi^*$  transition of C=O from the carbon core present in the functional groups [30]. There is a

broad absorption band at 339 nm, attributed to the different functional groups present on the CNDs' surfaces. Specifically, amino groups produce an absorption band at 409 nm due to  $n \rightarrow \pi$  transitions [10]. The fluorescence spectrum of the CNDs shows maximum emission at 527 nm when the solution is excited at 420 nm. The emission intensity gradually decreases as the excitation wavelength increases from 420 to 500 nm. This behavior, characteristic of CNDs, is mainly due to the presence of different functional groups within the synthesized CNDs themselves [31]. As can be seen in the inset of Figure 1D, the CND solution shows blue fluorescence when irradiated with a UV lamp, while the solution of a mixture of the precursors does not.

### 3.2 Electrochemiluminescent behavior of carbon nanodots

As a preliminary step, we studied the electrochemical behavior of the synthesized CNDs in 0.1 M PB, pH 8.0 and in 0.1 M  $\text{H}_2\text{SO}_4$  (see the inset of Figure 2A, curves a and b, respectively). An oxidation process can be observed, centered at 0.86 V, which we attribute to the oxidation of amine groups that are present on the CNDs' surfaces. The anodic peak potential shifts toward more positive values (curve b) on decreasing the pH, as one would expect from the voltametric behavior of amines after protonation [32]. In the presence of 7.0 mM  $[\text{Ru}(\text{bpy})_3]^{2+}$  (Figure 2A, curve c), there is a considerable increase in the anodic current concomitant with a decrease in the cathodic current, in contrast to the cyclic voltametric response of the  $[\text{Ru}(\text{bpy})_3]^{2+}/[\text{Ru}(\text{bpy})_3]^{3+}$  system observed in the absence of CNDs (curve d). This effect indicates an electrocatalytic process in which the oxidized form of Ru is reduced by the CNDs and re-oxidized on the electrode's surface, starting a new cycle, which gives rise to the catalytic current observed. The background current of the electrode in 0.1 M PB, pH 8.0, before CND immobilization is shown as curve e of Figure 2A. The ECL *versus* potential profiles, which were performed simultaneously, perfectly synchronized

with the electrochemical response and displayed in real time, are shown in Figure 2B. The high ECL response in the presence of CNDs (see curve c of Figure 2B) can be attributed to their high electrical conductivity and excellent optical properties. These preliminary results suggest that the CNDs can act as co-reactants and amplification agents, allowing considerable enhancement of the ECL response of  $[\text{Ru}(\text{bpy})_3]^{2+}$ .

## Figure 2.

The CND-modified electrodes have high electrical conductivity, as confirmed by EIS. As shown in the Nyquist plots (Figure S1), after modifying the SPCE with CNDs, the charge-transfer resistance decreased from 550  $\Omega$  (a) to 350  $\Omega$  (b). Therefore, based on both the best conductivity and the highest relative active surface area, as well as the improved ECL performance, CNDs/SPCEs should be a suitable electrochemical platform for the development of immunosensors.

### 3.3 Electrochemiluminescence-based HER2 immunosensor

Synthesized CNDs, besides acting as co-reactants, have functional groups such as carboxylate that enable the covalent immobilization of biomolecules. Hence, CND-modified electrodes (CNDs/SPCEs) were used to develop a HER2 immunosensor. As shown in Scheme 1, the CNDs/SPCE is used as a platform for the covalent immobilization of anti-HER2, using EDC and NHS in MES buffer, pH 5.0, to activate the carboxylate groups found on the CNDs' surfaces and form amide bonds with the anti-HER2's amino groups. MES buffer was the optimal choice, as the activation buffer must not contain any primary amine or carboxyl groups that could compete with

the activation reaction or reduce the reactivity of the EDC. By following this stepwise strategy, oriented and stable anti-HER2 immobilization is achieved (see Scheme 1).

The successive steps followed to produce the immunosensor were characterized. IR measurements were carried out to confirm the formation of amide bonds between the anti-HER2 amino groups and the carboxylate groups present on the CNDs' surfaces. Figure 3 shows the IR spectra of the CNDs, anti-HER2 and anti-HER2-modified CNDs. The latter spectrum is clearly different from the other two. It has characteristic amide bands at  $1680\text{ cm}^{-1}$  corresponding to C=O and at  $1640\text{ cm}^{-1}$  associated with the N-H bending of amines [33], confirming the binding of CNDs to anti-HER2.

### Figure 3.

Immunosensor response was monitored by ECL in the presence of the luminophore  $[\text{Ru}(\text{bpy})_3]^{2+}$ . Hence, detection of the immunological recognition event is based on the change in the ECL signal produced by the luminophore  $[\text{Ru}(\text{bpy})_3]^{2+}$ . Figure 4 shows the ECL response obtained at each step in the process of preparing the immunosensor (Figure S2 shows the corresponding ECL *versus* potential profiles). The bare SPCE exhibits a small baseline signal (a), and no significant increase is observed after the addition of chitosan (b), while ECL emission increases when the electrode is modified with CNDs alone (c). This result agrees well with the description given above, since the CNDs act as amplifying agents for the ECL signal. However, in the presence of both CNDs and chitosan, the ECL response decreases (d), despite the presence of primary amines that could improve ECL emission. This can be due to chitosan impairs electron transfer. The addition of BSA to prevent nonspecific interactions has a slight effect on ECL response, but the covalent immobilization of anti-HER2 gives rise to a dramatic signal increase (f), due to the amino and alcohol groups of the protein that behave as co-reactants [5, 6]. HER2 capture (g) causes a

significant decrease in ECL signal, due to major steric hindrance of the ECL probe  $[\text{Ru}(\text{bpy})_3]^{2+}$ , and enables the detection of HER2 [34, 35].

As a control, the platform was also prepared without the addition of the antibody (h). In this case, the ECL response after incubation with HER2 was similar to the background level (e), which confirms that the immunosensor response is due to HER2 recognition.

#### **Figure 4.**

Extensive optimization was carried out, including several parameters that play key roles in the performance of the final immunosensing device (see Figure S3). This consisted of evaluating the quantity of CNDs, the concentrations of chitosan and anti-HER2, and the incubation times of anti-HER2 and the HER2 protein. From the study, we can conclude that the best response is obtained with 10  $\mu\text{L}$  CNDs, 1.0  $\text{mg mL}^{-1}$  chitosan and 5  $\mu\text{g mL}^{-1}$  anti-HER2, with incubation times of 60 min for the antibody and 30 min for the HER2 protein.

#### ***3.4 Analytical performance of the HER2 immunosensor***

The analytical parameters of the proposed immunosensing method were studied under the experimental conditions previously selected as optimal. Figure 5A shows the immunosensor's response to increasing amounts of HER2 protein. It can be observed that the immunosensor's response decreases as the concentration of HER2 protein increases. Figure 5B shows that the observed decrease is linear ( $r^2=0.993$ ) up to a HER2 protein concentration of 900  $\text{pg mL}^{-1}$ , with a slope value of  $-1.12 \pm 0.03 \times 10^{-3}$  (ECL, a.u./[HER2],  $\text{pg mL}^{-1}$ ) and an intercept value of  $1.10 \pm 0.02$  (ECL, a.u.). The limits of detection (LOD) and quantification (LOQ) were estimated using the

3  $S_b/m$  and 10  $S_b/m$  criteria, respectively, where  $m$  is the slope of the calibration curve and  $S_b$  is the normalized standard deviation of the reference signal (the BSA/anti-HER2/chit/CNDs/SPCE signal), which is 0.007. Values of 20.4 and 67.9  $\text{pg mL}^{-1}$  were calculated for the LOD and LOQ, respectively.

## Figure 5.

Another important analytical parameter, reproducibility, was estimated by quantifying 400  $\text{pg mL}^{-1}$  HER2 protein with five different devices prepared in the same manner. The calculated relative standard deviation (RSD) was 5.5%.

The stability of the HER2 immunosensor was evaluated by applying ten consecutive potential scan cycles from +0.40 to +1.25 V in 0.1 M PB, pH 8.0, containing 7.0 mM  $[\text{Ru}(\text{bpy})_3]^{2+}$  and 400  $\text{pg mL}^{-1}$  HER2 protein. The recorded signal of the HER2 immunosensor kept about 95% of the initial value (Figure 5C). The storage stability of the BSA/anti-HER2/chit/CNDs/SPCE was also evaluated. The immunosensor was stored at 4°C for 47 days and kept 92% of the initial ECL response (Figure S4).

In addition, the selectivity of the proposed HER2 immunosensor was tested against several potential interfering compounds that may be present in serum samples. The concentration of HER2 tested was 400  $\text{pg mL}^{-1}$ , in the presence or absence of 50  $\text{mg mL}^{-1}$  BSA, 50  $\text{mg mL}^{-1}$  glucose, 1  $\text{ng mL}^{-1}$  p53, 100  $\text{ng mL}^{-1}$  CEA and 10  $\mu\text{g mL}^{-1}$  IgG. As can be seen in Figure 5D, the study confirmed the high selectivity of the method.

The proposed ECL-based HER2 immunosensor was compared with other, previously-described HER2 immunosensors based on detection techniques other than ECL, because no other ECL-based immunosensors have been developed for HER2 to date (Table S1). From the comparison, we can

confirm that our sensor has one of the lowest LODs, which is probably due to its high electron transfer capability, its increased surface area and the good biocompatibility of the CNDs.

### 3.5 *Determination of HER2 in serum samples*

We evaluated the real usefulness of the immunosensor by using it to directly determine HER2 protein in human serum. Samples were diluted 100-fold in 0.01 M PBS, pH 7.4, before use. As shown in Table 1, the results obtained by the immunosensor were compared with those obtained by a commercial colorimetric ELISA kit to validate the method. The recovery obtained by three different immunosensors in the three serum samples assayed agreed closely with that obtained by the ELISA kit, which shows that this immunosensor can be used for the quantitative direct determination of HER2 protein in human serum samples.

**Table 1.** Determination of HER2 in human serum samples with the ECL immunosensor and with the commercial ELISA kit (n=6).

Sample	Added (pg/mL)	Immunosensor		ELISA kit	
		Found (pg/mL)	Recovery (%)	Found (pg/mL)	Recovery (%)
Serum 1	400	$(4.1 \pm 0.3) \times 10^2$	102	$(4.1 \pm 0.1) \times 10^2$	101
Serum 2	400	$(3.9 \pm 0.1) \times 10^2$	97	$(3.9 \pm 0.3) \times 10^2$	98
Serum 3	400	$(4.2 \pm 0.3) \times 10^2$	105	$(4.3 \pm 0.2) \times 10^2$	107

## Conclusions

In this work, a CND-based nanostructured electrochemical platform was used as the basis for the development of a selective ECL-based HER2 immunosensor. For this purpose, nitrogen-rich CNDs with two functions, as support for the covalent immobilization of HER2 antibodies and as co-reactants in the ECL process, were synthesized. The disposable HER2 immunosensor showed



broad linear response with a LOD of 20.4 pg mL<sup>-1</sup> and high selectivity, reproducibility and stability. Finally, the applicability of this immunosensor was demonstrated by using it in human serum samples for the direct determination of HER2, and the results were validated by an ELISA kit.

### **Acknowledgments**

This work has been supported by the Spanish Ministry of Science, Innovation and Universities (CTQ2017-84309-C2-1-R; RED2018-102412-T) and by the Autonomous Community of Madrid (Talent Attraction Project 2017-T1/BIO-5435 and the P2018/NMT4349 TRANSNANOAVANSENS Program).

## Figure captions

**Scheme 1.** Preparation of an immunosensor (HER2/BSA/anti-HER2/chit/CNDs/SPCE) for HER2 detection.

**Figure 1.** (A) TEM image of CNDs. The inset of Figure 1A shows the amplification of a single CND. B) XRD pattern of powdered CNDs. (C) FTIR spectra of CNDs (c) and the precursors citric acid (a) and urea (b). (D) UV-visible absorption spectrum (a) and emission spectra (successive measurements at different excitation wavelengths in a range from 420 to 500 nm) of CNDs ( $17.8 \mu\text{g mL}^{-1}$ ) in aqueous solution. The inset shows photographs of the precursors (left) and the CND solution (right) under UV light at 365 nm.

**Figure 2.** (A) Cyclic voltammograms of a SPCE in the presence of  $0.89 \text{ mg mL}^{-1}$  CNDs in 0.1 M PB, pH 8.0 (curve a); in 0.1 M  $\text{H}_2\text{SO}_4$  (curve b); in a mixture of CNDs and  $7.0 \text{ mM } [\text{Ru}(\text{bpy})_3]^{2+}$  in 0.1 M PB, pH 8.0 (curve c) and in  $7.0 \text{ mM } [\text{Ru}(\text{bpy})_3]^{2+}$  (curve d). Background current of the bare electrode in 0.1 M PB, pH 8.0 (curve e). (B) ECL *versus* potential profiles of the same solutions as in Figure 2A. Scan rate:  $v = 0.010 \text{ V s}^{-1}$ .

**Figure 3.** FTIR spectra of anti-HER2 (a), CNDs (b) and anti-HER2 modified CNDs (c).

**Figure 4.** ECL responses of a bare SPCE (a), chit/SPCE (b), CNDs/SPCE (c), chit/CNDs/SPCE (d), BSA/chit/CNDs/SPCE (e), BSA/anti-HER2/chit/CNDs/SPCE (f), HER2/BSA/anti-HER2/chit/CNDs/SPCE (g) and HER2/BSA/chit/CNDs/SPCE without antibody (h) in 0.1 M PB, pH 8.0, in the presence of  $7.0 \text{ mM } [\text{Ru}(\text{bpy})_3]^{2+}$ . Number of replicate measurements,  $n=3$ .

**Figure 5.** (A) ECL immunosensor responses to increasing HER2 protein concentrations; (B) calibration curve obtained from three consecutive measurements; (C) ECL immunosensor response to  $400 \text{ pg mL}^{-1}$  HER2 protein measured under continuous cycles; (D) immunosensor response to  $400 \text{ pg mL}^{-1}$  HER2 protein measured in the presence of different potential interfering compounds.

The measurements were performed in 0.1 M PB, pH 8.0, in the presence of 7.0 mM  $[\text{Ru}(\text{bpy})_3]^{2+}$ .

Scan rate:  $v = 0.010 \text{ V s}^{-1}$ . Number of replicate measurements,  $n=3$ .

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