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Sensitive glyphosate electrochemiluminescence immunosensor based on electrografted carbon nanodots

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ABSTRACT

A novel electrochemiluminescence (ECL) immunosensor based on electrografted carbon nanodots (CND) is developed for the sensitive determination of glyphosate in soy milk and tea. Nitrogen rich CND were synthesized by microwave radiations using mild conditions and following the principles of green chemistry. L-arginine and 3,3'-diamino-N-methyldipropylamine were selected as precursors. CND were exhaustively characterized as well as the resulting nanostructured electrodes after CND electrografting. The high stability of CND nanostructured electrode together with the high electrical conductivity and the improvement of the electrochemiluminescent properties from the luminophore $[\text{Ru}(\text{bpy})_3]^{2+}$ makes it an excellent electrochemiluminescence detection platform for biosensing assays. The application to biosensors was assessed by combination with an immunoassay based on magnetic nanoparticles, in which anti-glyphosate-IgG coupled magnetic particles (MP-Ab) was used as recognition element of the analyte, glyphosate. The developed ECL immunosensor was successfully applied for the detection of glyphosate in a wide linear range from 28.9 to 200 pg/mL, a sensitivity of 3.38×10^{-3} mL/pg and a detection limit of 8.66 pg/mL. The immunosensor response is stable and reproducible and it has been applied to the determination of glyphosate in tea and soy milk, with results that agree with those provided by an ELISA kit involving the same immunoreagents.

KEYWORDS

Immunosensor; glyphosate; carbon nanodots; electrografting; electrochemiluminescence.

1. Introduction

Electrochemiluminescence (ECL) is a versatile analytical technique that converts electrical energy into radiative energy via the production of reactive intermediates from stable precursors on the surface of an electrode. Namely, after the application of a voltage the luminophore undergoes high-energy electron-transfer reactions to form electronically excited states that emit luminescent light when they return to the ground state [1, 2]. Due to its excellent advantages such as simple instrumentation, low cost, light source not required, fast and convenient measurement control, minimal electrode fouling, significant versatility and wide linear range, low background signal, high sensitivity, precision, reproducibility and stability, ECL analysis has attracted considerable attention and has become a powerful analytical tool in the design of biosensors for the detection of different analytes related to clinical diagnostics or environmental and food monitoring [2-7]. Nowadays, in order to improve the kinetics of electronic transfers and therefore the analytical performance of the developed devices, different kinds of nanomaterials are increasingly being incorporated in the ECL biosensor development. Moreover, the use of nanomaterials allows to improve the stability of the electrode surface as well as increasing the efficiency of ECL emission [8]. Among all different nanomaterials that have been employed, carbon nanomaterials and more specifically carbon nanodots (CND) have recently drawn the attention of researchers for the development of efficient ECL biosensors, not only for their photoluminescence but also for other excellent properties that they possess, such as low cytotoxicity, solubility in water, presence of different functional groups on their surface and good conductivity [9-12].

Among the aforementioned properties, one of the most interesting is the ease of modulating the functional groups that CND contain on their surface just by choosing the appropriate precursors. These functional groups (carboxyl, amine, hydroxyl, among others) can, on the one hand, facilitate the immobilization of biomolecules allowing a more efficient control of the biosensor development [13-15] and, on the other, enable the coupling of the nanomaterial to electrodes giving rise conductive nanostructured hybrid

platforms that present better yields, greater surface area, decreased work potential and higher electron transfer and diffusion coefficients [16, 17]. However, in spite of their interesting properties, their potential application as surface modifiers in the field of ECL biosensors is currently limited [18, 19]. Specifically, previous works reported on direct adsorption of CND on glassy carbon electrodes for the development of Alpha protein and Human IgG immunosensors. Nevertheless, the development of new materials and procedures that allow electrode surface modification by a simple and stable way is a research area of great interest. In this sense, our group has recently reported on a rapid and facile strategy to covalently immobilize diazotized nitrogen-doped CND onto carbon electrodes based on an electrografting methodology, and the resulting platforms were applied to the development of a taurine ECL sensor [20]. The electrografting of diazonium salts has proven to be a smart and efficient way of introducing different functional groups in order to decorate a variety of electrode surfaces, obtaining an organic layer covalently attached to the electrode surface in a controlled, reproducible, uniform and stable way. In this regard, electrodes modified with aryl diazonium salts have been used for the immobilization of different biomolecules for the development of biosensors [21-23] and, more specifically, of immunosensors [24-26]. However, this methodology has been scarcely employed to decorate electrode surfaces with nanomaterials.

Glyphosate [N-(phosphonomethyl)glycine] is a non-selective herbicide with a wide range of application that has become the most widely used worldwide. Its use is allowed in agricultural, urban and domestic activities. However, its massive and uncontrolled application in agriculture has raised environmental and health concerns and has caused water, soil and food samples contamination [27, 28]. In fact, the European Commission (EC), the Food and Drug Administration (FDA) and the U.S Environmental Protection Agency (EPA), have established different maximum residue limits (MRLs) for different matrixes [28, 29]. Therefore, it is of great interest to develop methods of analysis that require a simple and inexpensive instrumentation, provide high sensitivity, rapid response and can be directly applied to environmental and

food samples. Biosensors can meet these expectations and several authors have previously reported on optical or amperometric biosensors for the determination of glyphosate [30-34]. ECL biosensors by taking the advantage of the selectivity of the biological recognition elements and the high sensitivity of ECL technique are emerging as a powerful analytical device for ultrasensitive detection of wide range of biomolecules and metals ions [3]. However, few works can be found in the literature concerning the application of these devices to glyphosate determination [35, 36]. In fact, to the best of our knowledge, the development of ECL immunosensors for glyphosate determination has not yet been described.

In this work, we have developed a fast, selective, sensitive and label-free ECL immunosensor for glyphosate determination. It combines the selectivity of the anti-glyphosate-antibody-immobilized on magnetic particles and the sensitivity of ECL technique, enhanced by the presence of Nitrogen rich CND covalently bonded to screen-printed carbon electrodes by an electrografting process. The applicability of the device has been demonstrated by the determination of the glyphosate content in different food samples.

2. Experimental

2.1. Chemicals

L-arginine, 3,3'-diamino-N-methyldipropylamine, $[\text{Ru}(\text{bpy})_3]^{2+}$, sodium phosphate dibasic $\geq 99\%$, sodium phosphate monobasic monohydrate $\geq 99\%$, carbendazim, acetochlor, phosmet, glufosinate, N-(phosphonomethyl)iminodiacetic acid hydrate, (aminomethyl)phosphonic acid, D-(+)-glucose, calcium chloride, sodium nitrate and potassium sulfate were purchased from Merck (Darmstadt, Germany). 37% (w/w) hydrochloric acid was obtained from Scharlau (Barcelona, Spain). Sodium nitrite was purchased from Riedel-de-Haën (Seelze, Germany). Anti-glyphosate-IgG coupled magnetic particles (MP-Ab), glyphosate standards, diluent solution, washing solution, buffer assay and the derivatization reagent diluent, which is used to modify glyphosate in the standard solutions as well as in the spiked samples

immediately before use, were purchased with the Glyphosate HS Kit from Eurofins Abraxis (Northampton (PA), USA). Purified water in a Millipore Milli-Q system was used in all experiments.

2.2. Instrumentation

Carbon nanodots (CND) were synthesized using a CEM Discover microwave system (Matthews (NC), USA). A dialysis membrane tubing cutoff in the range of 0.1-0.5 kDa was provided by Spectrum Laboratories (Piraeus, Greece). CND were freeze-dried using Labconco FreeZone freeze dry system (-50°C, 0.03 mbar pressure).

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

For transmission electron microscopy (TEM), Lacey carbon support film copper grids (400 mesh, Electron Microscopy Sciences) were used. Images were recorded with a JEOL JEM 2100 electron microscope.

Powder X-ray diffraction spectra were obtained from freeze-dried CND powder using an X-pert PRO Theta/2Theta diffractometer from Panalytical.

Elemental analysis of CND was performed using a Perkin-Elmer 2400 CHN elemental analyzer.

Zeta potential measurements were carried out with Zetasizer Nano ZS instrument (Malvern Instrument Ltd.).

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

Electrochemical measurements were performed with a Metrohm-Autolab potentiostat PGSTAT 302N, using integrated screen-printed carbon electrodes (SPCE, DRP-110, Metrohm-DropSens) that include a carbon ink counter electrode and a silver pseudoreference electrode.

EIS experiments were performed in 0.1 M phosphate buffer (PB) (pH 7.0) in presence of 0.1 M KCl containing an equimolar mixture of 1.0×10^{-2} M $\text{K}_3\text{Fe}(\text{CN})_6$ / 1.0×10^{-2} M $\text{K}_4\text{Fe}(\text{CN})_6$. Impedance measurements were recorded in the range of frequency between 10^5 and 1×10^{-2} Hz, with a sinusoidal potential modulation of ± 5 mV in amplitude superimposed onto the formal potential of the redox probe.

Atomic Force Microscopy (AFM) images were taken on HOPG electrodes with an Agilent 5500 microscope and Olympus cantilevers (RC800PSA, 200_20 mm) operating in tapping mode in air.

Scanning electron microscopy (SEM) images were registered using a Hitachi S-3000N microscope with EDX analyzer Model XFlash 6I30, from Bruker. A Philips XL30 S-FEG microscope was used when better resolution was needed.

ECL experiments were performed using a bipotentiostat/galvanostat (± 4 V DC potential range, ± 40 mA maximum measurable current) combined with a Si-Photodiode integrated in an electrochemiluminescence cell (50.0 μL) from Metrohm-DropSens.

2.3. Procedures

2.3.1. Carbon nanodots (CND) synthesis.

CND were synthesized following a similar procedure to that previously described by F. Arcudi et al. [37] with variations. In a typical synthesis, 87 μg L-arginine and 86 μL 3,3'-diamino-N-methyldipropylamine and 100 μL Milli-Q water were irradiated in a microwave system at a constant temperature of 235 $^\circ\text{C}$ and a maximum pressure of 20 bar during 180 s. Then, the brown solid obtained was dissolved in 10 mL of Milli-Q water and filtered using 0.1 μm porous filter. Finally, the solution was dialyzed in a 0.1-0.5 kDa dialysis membrane for 7 days. The final concentration of as prepared CND was 1.52 mg/mL. The resulting solution was stored at 4 $^\circ\text{C}$. A fraction of the CND suspension was freeze-dried in order to carry out XRD and FT-IR.

2.3.2. CND diazonium salt electrografting.

The CND were diazotized by mixing 0.76 mg/mL CND in 0.5 M HCl and 1.5×10^{-2} M NaNO₂ for 60 min. in an ice bath. The electrografting process of the in situ generated CND diazonium salt was performed by adding the reaction mixture on carbon electrodes (SPCE and HOPG) and cycling the potential between 0.0 V and -0.8 V at 0.10 V/s. Finally, the resulting CND modified electrode (CND₆₀/SPCE or CND₆₀/HOPG) was washed with purified water.

2.3.3. ECL immunosensor (MP-Ab/CND₆₀/SPCE)

10 μ L of glyphosate standard or the beverage sample were pipetted into an Eppendorf tube. Then, 40 μ L of assay buffer and 4 μ L of the derivatization reagent diluent were added. After 10 min., when the derivatization reaction was finished, 100 μ L of MP-Ab were added and the mixture was incubated for 30 min. at room temperature. After 30 min., each Eppendorf tube was placed into a magnetic separator DynaMag™-2 (Thermo-Fisher Scientific) for 2 min., the solution was removed and 200 μ L of washing solution were added. This procedure was repeated twice. Finally, MP-Ab with captured glyphosate were resuspended in 20 μ L of 0.1 M PB (pH 8.0) and the solution was transferred onto CND₆₀/SPCE working electrode (MP-Ab/CND₆₀/SPCE) using a magnet. The quantification of glyphosate was achieved by recording the changes in ECL signal in the presence of 2.0×10^{-3} M [Ru(bpy)₃]²⁺ (see Scheme 1).

2.3.4. Analysis of food samples

Glyphosate was determined in food samples using the developed ECL immunosensor and the results were compared with those obtained using a commercial ELISA kit. Soy milk and tea were purchased in a local store and were analyzed without any pre-treatment other than dilution in diluent solution. Samples were spiked by adding suitable amounts of the analyte.

3. Results and discussion

3.1. Synthesis and Characterization of CND

Fluorescent Nitrogen rich CND have been synthesized in a microwave reactor, using L-arginine and 3,3'-diamino-N-methyldipropylamine as precursors as described in detail in the Experimental Section.

Synthesized CND have been exhaustively characterized. The TEM image (Fig. 1A) shows CND as quasi-spherical nanoparticles. Their size distribution obtained by measuring the average size of around 100 CND (see histogram at Fig. S1A) indicates a size of 2.1 nm ranging from 1 to 3 nm in diameter. No aggregates were observed. Powder X-ray diffractogram of CND (Fig. 1B) shows a broad band at 18.9° degree, consistent with the amorphous morphology of the nanomaterial. Regarding the composition of CND, elemental analysis shows %C: 50.29, %H: 9.04, %N: 21.93, %O: 18.74 (calculated). As can be observed, the as prepared CND have an important percentage of nitrogen. To determine the functional groups, present in CND, the FTIR spectra of CND and their precursors were carried out (Fig. 1C). CND present a spectrum clearly different compared with the spectra of their precursors, pointing out the synthesis of a new compound. It exhibits the characteristic stretching band of OH and NH₂ as a broad band centered at 3436 cm⁻¹, the bands observed around 2930 cm⁻¹ derive from the C-H bond stretching vibration. C=O stretching vibrations appear at 1644 cm⁻¹. At 1562 cm⁻¹ appears a strong band ascribed to the C=N stretching, while the bands at 1457 cm⁻¹ and 1384 cm⁻¹ are related with C-N bonds. The 1071 cm⁻¹ bands correspond to the C-O stretching vibrations. Hence, we infer the presence of mainly hydroxyl and amine groups besides carbonyl and imine groups. In addition, the zeta potential value measured at different pH (Fig. S1B) suggests the presence of negative charges at the CND surface, most likely in the form of carboxylate groups.

The UV/Vis spectrum (Fig. 1D) shows an absorption band at 288 nm, ascribed to the π - π^* transition of the conjugated C=C units and the n - π^* transition of the C=O from the carbon core [38]. A broad emission peak at 383 nm is observed when excited at 320 nm [39].

FIGURE 1

3.2. Electrografting of CND onto carbon electrodes

The CND, among other nanomaterials, have previously used as co-reactants in the presence of $[\text{Ru}(\text{bpy})_3]^{2+}$ [40]. In addition, we recently demonstrated that CND with an abundance of aromatic primary amines on their surfaces has a good solubility in aqueous solution and can be electrografted on carbon electrodes. The resulting nanostructured electrochemical platforms amplify the ECL signal of the emitter $[\text{Ru}(\text{bpy})_3]^{2+}$ [20]. Inspired by this, we have synthesized aromatic primary amines rich CND that will directly be electrografted onto the electrode surface.

A key aspect in the in situ generation of the diazonium salt is the diazotization reaction time. To optimize this parameter in the case of CND, times of 40, 50 and 60 min were assayed. After the diazotization reaction, the diazonium salt of CND was electrografted on the carbon electrode by applying 10 potential cycles between 0.0 V and -0.8 V. The first cyclic voltammogram for the electrografting process, following the different diazotization reaction times is shown in Fig. 2A. In all cases, a reduction process appears that increased in current as the diazotization reaction time increases. This process is attributed to the reduction of aryl diazonium groups, giving highly reactive nucleophilic aryl groups that are rapidly electrografted on the electrode surface. In successive potential cycles, a gradual reduction of the cathodic current is observed. This behavior is characteristic of the electrografting processes [41].

Control experiments were carried out under the same conditions but using solutions of non-diazotized CND. In this case, no reduction process is observed (data not shown). These results confirm that the synthesized CND contain aromatic amines on their surface that allow, after diazotization, their immobilization by electrografting, leading to the formation of a covalent bond.

To confirm the electrografting of CND, electrodes were subjected to AFM. Fig. 2B (1 and 2) shows topographic AFM images of a HOPG surface after CND electrografting, when the reaction time to form

the CND diazonium salt was 60 min. (CND₆₀/HOPG). It can be observed that the surface is decorated with a film composed of irregular globular features randomly distributed along the surface. The topographic profile (Fig. 2B (2, inset)) shows particles of ~ 60 nm in diameter. This value is approximately 25 times larger compared to the diameter determined by TEM. This is probably due to the aggregates formed by spontaneous reaction of the diazonium groups with other CND during the diazotization process [42, 43], giving rise to larger CND. In fact, when the reaction time to form the CND diazonium salt is reduced to 40 min., the AFM images (Fig. S2) after electrografting (CND₄₀/HOPG) show particles of ~ 40 nm in diameter. The amount of electrografted CND is also less. Compared to the image of Fig. 2B, a much less dense surface is observed.

FIGURE 2

3.3. Amplified ECL by CND

Fig. 2C shows the ECL response of luminophore [Ru(bpy)₃]²⁺ at SPCE modified with electrografted CND. As can be seen, compared to that observed at bare SPCE, CND behaves as an ECL signal amplifier. In addition, the ECL signal increases on increasing the reaction time to form the CND diazonium salt, which in turn results in a dense layer of electrografted CND, as AFM images demonstrated. Based on this results, 60 min. (CND₆₀/SPCE) was chosen as optimal CND diazotization reaction time.

The storage stability of the proposed ECL system was evaluated by measuring the ECL emission with [Ru(bpy)₃]²⁺ for 10 days. After this period, it lost only 3% of its initial response.

3.4. Fabrication of an ECL immunosensor

The CND modified electrodes were employed as ECL platform to fabricate an immunosensor for selective glyphosate determination (see Scheme 1). Commercial magnetic particles with glyphosate-IgG specific antibodies (MP-Ab) were just added to the CND modified electrode surface in a drop-assay type and

retained over the electrode surface using a magnet (MP-Ab/CND₆₀/SPCE). After glyphosate addition, the immune-recognition event resulted in a decrease of the ECL signal measured at 620 nm, due to the greater steric hindrance suffered by the [Ru(bpy)₃]²⁺ probe to reach the electrode surface.

SCHEME 1

The commercial anti-glyphosate-IgG coupled magnetic particles (MP-Ab) and the resulting immunosensor platform, after their immobilization on the CND modified electrode (MP-Ab/CND₆₀/SPCE), were morphologically characterized by SEM (Fig. 3A). In order to check the nanometric size of the magnetic particles, higher resolution SEM images were also obtained (Fig. S3E). As can be observed, the almost spherical ferrite particles have an average size of 50 nm with a homogeneous dispersion. The images show also a uniform coverage, with some areas resulted in agglomerates of magnetic particles. As it is shown in the inset of Fig. 3A, EDX-map analysis confirms the presence of Fe atoms (in blue color). The rest of elements present as C and O are also evident in the EDX-map (Fig. S3A-D). These results confirm the presence of ferrite and therefore the presence of antibodies, since EDX does not allow to determine the presence of nitrogen related to the antibody composition.

FIGURE 3

Electrochemical impedance spectroscopy (EIS) analysis has been carried out to study the interfacial properties of the developed surfaces. Fig. 3B shows the Nyquist diagrams obtained at SPCE, CND₆₀/SPCE and MP-Ab/CND₆₀/SPCE. Impedance analysis have been performed using [Fe(CN)₆]^{3-/4-} in 0.1 M PB (pH 7.0) containing 0.1 M KCl, as redox probe. After CND were electrografted on the SPCE, the charge

transfer resistance (R_{CT}) decreased considerably from 260 Ω to 10 Ω , which suggests that CND₆₀/SPCE is much more conductive than bare SPCE. This result agrees well with that obtained by us in a previous work [20], confirming that coating the electrode surface with CND makes it more conductive.

It is also observed in Fig. 3B that after modifying the CND₆₀/SPCE with MP-Ab, there is no significant increase in R_{CT} , the value is practically the same, approximately 15 Ω . This is due to the MP-Ab deposited on the electrode surface are made of ferrite, a highly conductive material.

Results presented above confirm that we have prepared an immunosensing electrochemical platform with enhanced ECL properties. We then proceeded to apply it to the development of an ECL-based immunosensor for glyphosate determination. Experimental variables involved in the immunosensor development were optimized considering the highest ratio between the ECL response of as prepared immunosensor in the absence (S_0) or in the presence (S) of 75 pg/mL of glyphosate. The optimization study involved evaluation of the pH solution, $[Ru(bpy)_3]^{2+}$ concentration, potential scan rate and the volume of MP-Ab used. Data are shown in Fig. 4. The highest ECL response was obtained using 0.1 M PB (pH 8.0), 2.0×10^{-3} M $[Ru(bpy)_3]^{2+}$, a scan rate of 0.03 V/s and 100 μ L of MP-Ab. These optimized conditions will be used to develop the immunosensor.

FIGURE 4

3.5. Analytical performance of the ECL immunosensor

The analytical parameters of the glyphosate immunosensor were evaluated. Fig. 5A shows the immunosensor response from ECL measurements vs. glyphosate concentration in 0.1 M PB (pH 8.0) under the optimal experimental conditions. It can be observed that ECL intensity decreases on increasing the glyphosate concentration as it is observed for other ECL immunoassays [11]. The reason is the great steric hindrance suffered by the ECL probe ($[Ru(bpy)_3]^{2+}$) to reach the electrode surface when increasing

amounts of glyphosate are linked with the capture antibody. The calibration curve is displayed in the inset of Fig. 5A. A linear dependence between the normalized ECL signal vs. the glyphosate standard concentration up to 200 pg/mL ($r^2=0.996$), which fitted to the equation: ECL, a.u. $=-(3.38\pm0.08)\times10^{-3}$ [glyphosate], pg/mL + (1.006 ± 0.009) was found. Data presented are the average value of three determinations. Detection and quantification limits were determined from the standard deviation of the background signal (S_b) and the slope of the calibration curve (m), following the $3 S_b/m$ and $10 S_b/m$ criteria, respectively. Values of 8.66 and 28.9 pg/mL, for detection and quantification limit were found. Considering the established value of 0.1 $\mu\text{g/L}$ pesticide in food by the EU [29], the developed immunosensor seems to be compatible with practical applications. Compared with other previously published glyphosate biosensors (Table 1), the proposed immunosensor presents one of the lowest detection limit and high sensitivity, attributed to the high electron transfer capability, increased surface area and good biocompatibility of the nanomaterial used, CND. In addition, the biosensor has a rapid response, being it less than one minute.

Table 1. Analytical parameters of other biosensors for glyphosate detection.

Detection technique	Surface chemistry	Assay type	Detection limit (pg/mL)	Ref.
White light reflectance spectroscopy	Chip SiO ₂ /Si functionalized with glyphosate-protein conjugate	Competitive immunoassay	10	[30]
Fluorescence	Anti-glyphosate Ab immobilized on Si substrate	Competitive immunoassay	45	[34]
Amperometry	Carbon nano-onion/tyrosinase conjugate on chitosan matrix on SPE	Enzyme inhibition	1086	[32]
Chronoamperometry	HRP-conjugate glyphosate-TMB/H ₂ O ₂ on SPCE	Competitive immunoassay	5	[33]
Electrochemiluminescence	HRP-assisted in situ generation of ZnS quantum dots on GCE	Enzyme inhibition	17	[35]
Electrochemiluminescence	Lu-Au-Lcys-Cu(II)/AChE-ChOx/rGO-Au/GCE	Direct measurement	83.5	[36]
Electrochemiluminescence	MP-Ab/CND ₆₀ /SPCE	Direct immunoassay	8.66	This work

FIGURE 5

The reproducibility of the ECL glyphosate immunosensor was evaluated from the response of five electrodes, constructed as described above, to 75.0 pg/mL glyphosate. The RSD calculated was 2.6%. This result shows that the proposed ECL immunosensor has good reproducibility.

One of the key aspects to consider for any analytical application of immunosensors is the study of the effect of potential interfering compounds that may be present in the samples. For this reason, the selectivity of the proposed ECL immunosensor was tested against various potentially interfering compounds, such as (aminomethyl)phosphonic acid (AMPA), phosmet, glufosinate, carbendazim, acetochlor, N-(phosphonomethyl)iminodiacetic acid hydrate (PMIDA), D-(+)-glucose, calcium chloride, potassium sulfate and sodium nitrate. The immunosensor response to 75.0 pg/mL glyphosate was obtained (under the optimized experimental conditions) in the absence and presence of each interfering compound at the concentration of 75.0 pg/mL. As can be seen in Fig. 5B, no significant changes in ECL intensity in the presence of potential interfering compounds are observed. Results indicate that the developed ECL immunosensor can be used for the highly selective determination of glyphosate.

In addition, the stability of the immunosensor was tested. The ECL response was measured under 10 consecutive cyclic potential scans from +0.40 to +1.25 V in 0.1 M PB (pH 8.0) containing 2.0×10^{-3} M $[\text{Ru}(\text{bpy})_3]^{2+}$ and 75.0 pg/mL glyphosate. After 10 cycles, the immunosensor kept 96% of its initial response (Fig. S4).

3.6. Determination of glyphosate in food samples

The developed ECL immunosensor was applied to the direct determination of glyphosate in two different food samples: tea and soy milk. The only pretreatment of samples was just 10-fold dilution in the diluent solution. The ECL response obtained directly without normalizing is presented in Fig. S5. Table 2 summarizes the amount and the recovery of glyphosate obtained from triplicate analysis. As can be seen,

recoveries from 106% to 109% were obtained. To validate the method, results were compared to those obtained by the commercial colorimetric ELISA kit (see Table 2). The average value of glyphosate concentration agrees well with that obtained by the kit, demonstrating that the developed magnetic immunoplatfrom can be used for the determination of glyphosate in food samples. Furthermore, the proposed immunosensor fabrication strategy can also be applied to develop other ECL immunosensors for the detection of trace-level of different analytes of interest.

Table 2. Determination of glyphosate in different samples with the magnetic ECL immunosensor and with a commercial ELISA kit (n=3).

Sample	Added (pg/mL)	Found (pg/mL)	Recovery (%)	Found (pg/mL)	Recovery (%)
		(ECL Magnetic Immunosensor)		(ELISA kit)	
Tea	100	109±5	109	110±5	110
Soy milk	100	106±3	106	108±4	108

4. Conclusions

In this study, a nanostructured electrochemical platform, based on electrografted CND, with extraordinary ECL properties was developed. Nitrogen rich CND, with a high content of aromatic amines, have been synthesized with the aim of being covalently attached to carbon electrodes by electrografting. The resulting CND nanostructured electrochemical platform amplifies the $[\text{Ru}(\text{bpy})_3]^{2+}$ ECL signal besides to show a high stability, good conductivity and electronic transfer. Hence, combined with magnetic nanoparticles modified with anti-glyphosate antibodies, it has been applied to the development of a disposable ECL-based immunosensor for glyphosate determination. The immunosensor showed a wide linear response with a detection limit of 8.66 pg/mL and a RSD of 2.6%. Finally, it was applied to the direct determination of glyphosate in tea and soy milk.

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Figure captions

Scheme 1. Scheme of the magnetic immunosensor for glyphosate detection.

Fig. 1. TEM micrograph (A), powder X-ray diffractogram of CND (B), FTIR spectra of CND (blue) and the precursors L-arginine (black), and 3,3'-diamino-N-methyldipropylamine (red), UV-Vis absorption spectrum (blue) and emission spectrum (red) using 320 nm as excitation wavelength of CND (15.2 $\mu\text{g/mL}$) in aqueous solution (D). Inset of Fig. 1D shows photographs of CND solution (left) and CND solution under UV light at 365 nm (right).

Fig. 2. (A) CV of the electrografting of the CND diazonium salt (0.76 mg/mL) prepared as indicated in Experimental Section by reacting with 1.5×10^{-2} M NaNO_2 in 0.5 M HCl at different reaction times: 40 (a, red line), 50 (b, green line) and 60 (c, blue line) min. at SPCE. The first CV of the 10 cycles performed by each electrografted process has been represented. Scan rate: 0.01 V/s. (B) AFM topographic image (1 and 2) and profile (inset) of $\text{CND}_{60}/\text{HOPG}$ from image 2. (C) ECL response at bare SPCE, $\text{CND}_{40}/\text{SPCE}$, $\text{CND}_{50}/\text{SPCE}$ and $\text{CND}_{60}/\text{SPCE}$ in 0.1 M PB (pH 8.0) in the presence of 7.0×10^{-3} M $[\text{Ru}(\text{bpy})_3]^{2+}$.

Fig. 3. (A) SEM images of $\text{MP-Ab}/\text{CND}_{60}/\text{SPCE}$. Inset: EDX-map analysis of Fe. (B) Nyquist diagrams obtained in 0.1 M PB (pH 7.0) (with 0.1 M KCl) in presence of 1.0×10^{-2} M $\text{K}_3\text{Fe}(\text{CN})_6$ / 1.0×10^{-2} M $\text{K}_4\text{Fe}(\text{CN})_6$ for SPCE (blue), $\text{CND}_{60}/\text{SPCE}$ (red) and $\text{MP-Ab}/\text{CND}_{60}/\text{SPCE}$ (black).

Fig. 4. Optimization of different experimental variables: (A) pH, (B) $[\text{Ru}(\text{bpy})_3]^{2+}$ concentration, (C) potential scan rate and (D) volume of MP-Ab. ECL responses (left) measured in the absence (light grey bars) or in the presence (dark grey bars) of 75 $\mu\text{g/mL}$ glyphosate, and the resulting S_0/S ratio (right).

Fig. 5. (A) ECL immunosensor responses (obtained from 3 consecutive measurements) to increasing glyphosate concentrations in 0.1 M PB (pH 8.0) with 2.0×10^{-3} M $[\text{Ru}(\text{bpy})_3]^{2+}$. Scan rate 0.03 V/s. Inset: Calibration curve. (B) Immunosensor responses to 75.0 $\mu\text{g/mL}$ glyphosate in the absence or in the presence of different potential interfering compounds at the concentration of 75.0 $\mu\text{g/mL}$ in 0.1 M PB (pH 8.0), containing 2.0×10^{-3} M $[\text{Ru}(\text{bpy})_3]^{2+}$.