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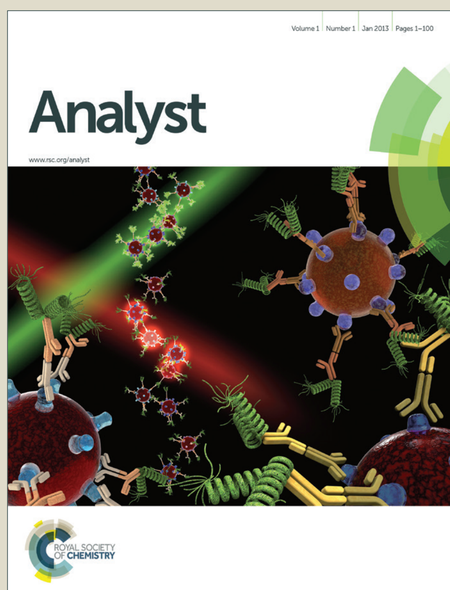

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Electrochemical immunosensor for sensitive determination of the anorexigen peptide YY at grafted reduced graphene oxide electrode platforms

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Abstract

The first electrochemical immunosensor for the determination of peptide YY is reported in this paper. A novel electrochemical platform prepared by electrochemical grafting of diazonium salt of 4-aminobenzoic acid onto a reduced graphene oxide-modified glassy carbon electrode is used, where covalent immobilization of the specific anti-PYY antibody is accomplished. The HOOC-Phe-rGO/GCEs were characterized by cyclic voltammetry and electrochemical impedance spectroscopy. The different variables affecting the preparation of the modified electrodes and the performance of the immunosensor were optimized. Under the optimized conditions, a calibration plot for PYY showing a linear range extending between 10^{-4} and 10^{2} ng/mL was found. This range is adequate for the determination of the protein in real samples, since the expected concentrations in human serum are around 100 pg/mL. The limit of detection was 0.01 pg/mL PYY. The immunosensor exhibited a good reproducibility for the PYY measurements, excellent storage stability and selectivity as well as a shorter assay time than that of ELISA kits. The usefulness of the immunosensor for the analysis of real samples was demonstrated by analyzing human serum samples spiked with PYY at three concentration levels.

Keywords: Peptide YY, graphene, electrochemical immunosensor, food intake, polypeptide hormone

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Introduction

Peptide YY is a potent anorexigen belonging to the pancreatic polypeptide family. It is produced in the gut by the L cells of the terminal ileum and colon and is secreted to the circulation in response to food[1,2]. There are two endogenous forms of the hormone: $PYY_{1\text{-}36}$ and $PYY_{3\text{-}36}$, the released $PYY_{1–36}$ being rapidly metabolized by dipeptidyl peptidase-IV to active $PYY_{3–36}$ by removal of two *N* terminal amino acids from the fulllength form[3,4]. Although both forms are biologically active, $PYY_{3–36}$ (hereinafter PYY) is the main storage and circulating form and is thought to more actively control food intake[5]. PYY stimulates gastrointestinal absorption of fluids and electrolytes, reduces gastric and pancreatic secretions, and delays emptying[2]. The effects of PYY on satiety, food intake and body weight have been investigated[6,7]. Although to date, contradictory results have been published concerning relationship between PYY and body weight[5], it is well known that PYY reduces food intake acting on the arcuate nucleus in the hypothalamus, possibly by inhibiting neuropeptide Y neurons and stimulating POMC expressing neurons via Y2 receptors[8]. This behavior has led PYY to become a therapeutic target to reduce hunger and caloric intake[1].

In spite of its importance, methods for determining PYY are restricted to RIA or ELISA immunoassays. A variety of ELISA commercial kits are available. These are mainly based on competitive schemes involving specific PYY antibodies or biotinylated PYY binding, as well as HRP-labeled avidin or streptavidin conjugates and colorimetric detection after hydrogen peroxide and TMB addition. These assays allow the determination of PYY at concentration ranges between 0.1 - 1 pg/mL to 100 - 1000 pg/mL, with minimum detectable concentration of 0.5 pg/mL to approximately 3 pg/mL. Times required for these assays take around 2.5 - 3.5 h.

The scientific and technological importance acquired by graphene in recent years is enormous. Owing to its remarkable physical properties, this material has largely proven to be extremely suitable and versatile for electroanalytical applications[9]. The use of graphene in the preparation of electrochemical biosensors commonly requires modifying the electrode surface using colloidal suspensions of this material. Graphene suspensions are prepared from graphene oxide (GO) by chemical methods which typically involve reduction of dispersed single-layer GO sheets to form stable rGO

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3 suspensions. In this paper we have introduced a green alternative for rGO preparation
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5 by using the natural antioxidant ascorbic acid as reducing agent which demonstrated
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7 good efficiency for such purpose[10]. Glassy carbon electrodes were then modified with
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9 the as prepared rGO and the resulting rGO/GCEs were used as platforms for the
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11 development of the first electrochemical immunosensor for PYY. The protocol for the
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13 immobilization of immunoreagents on the rGO/GCEs involved grafting of free radicals
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15 on the electrode surface[11,12]. Specifically, the diazonium salt of 4-amino benzoic acid
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17 (4-ABA) was electrochemically reduced at the electrode surface, this resulting in
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19 covalent attachment of 4-carboxy phenyl to rGO/GCE[13]. Then, anti-PYY antibodies
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21 were covalently immobilized onto the modified electrode, and a competitive
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23 immunoassay involving PYY and biotinylated PPY (Biotin-PYY) was performed. The
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25 determination of PYY was carried out by differential pulse voltammetry using alkaline
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27 phosphatase-labeled streptavidin (AP-Strept) and 1-naphthyl phosphate (1-NPP) as the
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29 enzyme substrate. AP catalyzes hydrolysis of 1-NPP to 1-naphtol and the
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31 electrochemical oxidation of this compound on the electrode surface is measured by
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33 DPP.
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37 ## Experimental
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39 ### Reagents and solutions
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41 Peptide YY (PYY) (3-36) (human) purified IgG antibody (antiPYY), peptide YY (PYY) (3-
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43 36) (human) (PYY), and biotinylated-PYY (3-36) (human) (Biotin-PYY) were purchased
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45 to Phoenix Pharmaceuticals, Inc. Graphene oxide (NIT.GO.M.140.10) was from
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47 Nanoinnova Technologies. Alkaline phosphatase labelled-streptavidin (AP-Strept), 1-
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49 naphthyl phosphate (1-NPP), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydro-
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51 chloride (EDC), and N-hydroxysulfo-succinimide (NHSS) were from Sigma. 4-
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53 aminobenzoic acid (ABA) was from Across. Ethanolamine (ETA, Aldrich), bovine serum
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55 albumin (BSA) from Gerbu, and casein from Thermo Scientific were used as blocking
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57 agents. A 0.1 M phosphate buffer solution of pH 7.4 (PBS) was prepared from sodium
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59 di-hydrogen phosphate and di-sodium hydrogen phosphate (Scharlau). A 50 mM Tris
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(tris(hydroxymethyl)amino-methane, Sigma) buffer solution containing 10 mM $MgCl_2$

(Panreac) of pH 9.6 (Trizma) was also used. All reagents solutions were prepared in 0.1

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M PBS except AP-Strept and 0.05 M 1-NPP solutions which were prepared in Trizma buffer. Insulin, human growth hormone (hGH) and follicle stimulating hormone (FSH), all from Sigma-Aldrich, adiponectin (APN, Abnova), ghrelin (GHRL) and des-acyl-ghrelin (da-GHRL, Anaspec) were tested as potentially interfering compounds. De-ionized water was obtained from a Millipore Milli-Q purification system (18.2 MΩ cm).

Apparatus

Voltammetric measurements were carried out using a BAS (Bioanalytical System) 100 B potentiostat provided with BAS 100/W software for electrochemical analysis. A three electrodes (BAS VC-210-mL) glass electrochemical cell was used. Modified 3-mm diameter CHI 104 glassy carbon electrodes from CH Instruments were used as working electrodes. The reference electrode was an Ag/AgCl/KCl 3M BAS MF 2063 and the auxiliary electrode was a BAS MW 1032 Pt wire. A P-Selecta ultrasonic bath, a Vortex (Heidolph) stirrer and a precision Metrohm Herisau E-510 pH-meter were also used.

Procedures

Preparation of rGO

2 mL from a 1 mg/mL GO aqueous dispersion were sonicated for 120 min and then centrifuged at 10,000 g for 10 min. The precipitate was discarded and the supernatant treated with a 25 % NH_3 solution up to pH 9-10. Further, the reduction of GO was performed by adding solid ascorbic acid up to a 2 mM final concentration and letting to react at 100 ^{o}C for 15 min. The resulting rGO dispersion was left in the dark at room temperature. The product was replenished every week although is was stable for at least two weeks.

Preparation of the immunosensors

Figure 1 shows the steps involved in the modification of the electrodes and preparation of immunosensors. Firstly, diazonium salt was prepared by adding dropwise 2 mM $NaNO_2$ aqueous solution to a 1 mg/mL ABA solution prepared in 1 M HCl and cooled with ice (38 mL $NaNO_2$ for each 200 mL ABA). The reaction was allowed to proceed for 10 min under stirring. Separately, glassy carbon electrodes were

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3 polished with 0.3 µM alumina slurries for 1 min, sonicated for 30 s in water and dried in air. Then, 10 µL of 0.5 mg/mL rGO suspension were deposited onto the electrode surface and, after drying at room temperature, rGO/GCEs were immersed into 450 µL of the diazonium salt. Ten successive voltammetric cycles from 0 to -1.0 V vs. Ag/AgCl (ν = 200 mV/s) were carried out to allow electrochemical grafting. The resulting HOOC-Phe-rGO/GCE modified electrodes were washed thoroughly with water and ethanol and dried at room temperature.

Activation of carboxylic groups was performed by dropping 10 µL of an EDC/NHSS (0.1 M each) aqueous solution onto HOOC-Phe-rGO/GCEs and left to react for 1 h in the dark. After rinsing with water and methanol and allowing drying, 10 µL of a 20 µg/mL anti-PYY solution were casted onto the electrode and left to stand for 1 h at 37 ^{o}C. Then, 20 µL of a 0.2 % casein blocking solution were deposited onto the electrode and incubation was allowed for 1 h at 37 ^{o}C. Competitive immunoassay was performed by spotting 10 µL of a mixture solution of PYY (or the sample) and 100 ng/mL Biotin-PYY on the anti-PYY-Phe-rGO/GCE, allowing incubation for 30 min at 37 ^{o}C. Thereafter, 10 µL of a 5 µg/mL AP-Strept were added to the Biotin-PYY-anti-PYY-Phe-rGO/GCE allowing incubation for 30 min at 37 ^{o}C. Finally, the immunosensor was immersed in 450 µL of 50 mM Trizma buffer solution, and 50 µL of 0.05 M 1-NPP solution were added. After a delay time of 5 min for the enzyme reaction to take place, differential pulse voltammograms were recorded over the -0.15 to + 0.70 V range to obtain the electroanalytical signals, using ΔE = 50 mV and ν = 20 mV/s.

Determination of PYY in spiked serum samples

Lyophilized human serum S-7394 from Sigma containing no PPY was reconstituted in 1 mL of 0.1 M PBS solution of pH 7.4 by mixing up to total dissolution and subsequently spiked with the target analyte at 0.35, 3.5 and 35 pg/mL concentration levels. PYY determination was performed by applying the procedure described above, and the peak current values measured by DPV were interpolated into the linear portion of the calibration plot constructed with PYY standard solutions.

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Results and discussion

In order to develop the first electrochemical immunosensor for PYY, we have designed an approach whose rationale is based on the immobilization of specific capture antibodies, using a grafting strategy via the diazonium salt of 4-amino benzoic acid, on a tailor-made electrode surface modified with rGO to take advantage of the well known properties of this nanomaterial (high conductivity, large specific surface area) to enhance the performance of electrochemical biosensors. Moreover, the electrode modification involves a green route using ascorbic acid as reducing agent.

Preparation of rGO

The procedure described in the Experimental section was followed for the preparation of rGO from graphene oxide. In this procedure, ascorbic acid was used as the reducing agent due to the high efficiency demonstrated in the reduction of GO[10] together with the advantage of substituting other commonly used toxic reagents such as hydrazine, hydroxylamine or sodium borohydride for this natural and inexpensive antioxidant. Furthermore, it is well known that the electronic properties of rGO can be tuned on the basis of the extent of the reduction process[14]. In this sense, the use of a mild reducing agent such as ascorbic acid allows an ease optimization of the experimental conditions for the reduction reaction to get optimal electrocatalytic properties[15,16].

The as obtained rGO was characterized by UV-vis spectrophotometry. Figure S1 in the Supplementary material shows two peaks at 230 and 300 nm corresponding to $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions, respectively, in the unreduced GO spectrum[17]. Upon reduction of GO with ascorbic acid, the $n \rightarrow \pi^*$ peak partially disappeared and a red-shift of the $\pi \rightarrow \pi^*$ peak to 241 nm takes place. This peak position has been used as a convenient probe of the reduction degree achieved with different reducting reagents[10]. Furthermore, aqueous ascorbic-reduced GO suspensions are clearly different than those of unreduced GO (inset in Figure S1), and exhibited long-term stability without observing precipitation for several weeks. These results demonstrated the efficiency of the method used for the reduction of GO.

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Preparation of the modified electrodes

The preparation of the modified electrodes implied optimization of: a) loading of rGO on the GCE surface, b) ABA concentration and c) number of cycles and potential scan rate used in the electrochemical grafting of rGO/GCE. Detailed information on the optimization of these steps can be found in Supplementary material.

Once prepared, rGO/GCEs were characterized by cyclic voltammetry and electrochemical impedance spectroscopy (EIS) using 5 mM $Fe(CN)_6^{3-/4-}$ as redox probe in 0.1 M KCl. Figure 2a displays typical cyclic voltammograms recorded at (1) bare GCE, (2) rGO/GCE, (3) HOOC-Phe-rGO/GCE, and (4) HOOC-Phe-rGO/GCE activated with EDC/NHSS. As it can be seen, slightly larger voltammetric peaks and smaller electron transfer resistance (Figure 2b) were observed at rGO/GCE with respect the unmodified GCE which could be attributed to both the larger conductivity and/or the porous diffusion effects of the functionalized graphene electrodes[18]. Conversely, the electrochemical grafting resulted in a poorer voltammetric behavior and in a large increase in the electron transfer resistance as a consequence of the electrostatic repulsion between the redox probe and the negatively charged carboxylate groups. However, when carboxyl moieties were activated using EDC/NHSS reagents, a semicircle with a shorter diameter appeared as a consequence of the neutralization of negative charge on carboxylate groups (curve 4). An equivalent behavior can also be observed by cyclic voltammetry. These results confirmed the successful modification of the electrode surface by electrochemical grafting of diazonium salt.

Immunosensor preparation

All the experimental variables involved in the immunosensor construction and therefore affecting its analytical performance were investigated. These variables were: a) loading of anti-PYY and incubation time; b) the blocking step; c) loading of biotin-PYY and incubation time; d) loading of AP-Strept and incubation time, and e) concentration of 1-NPP and time for the enzyme reaction to proceed. Detailed information on the results obtained in these studies can be found in Supplementary material and are summarized in Table S1.

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Analytical characteristics of the immunosensor for PYY

Once all the working conditions were optimized, a calibration plot for PYY with the AP-Strept-Biotin-PYY-anti-PYY-Phe-rGO/GCE immunosensor was constructed showing the expected inverse peak current vs. log PYY concentration relationship for competitive immunoassay (Figure 3a). The i_p vs. PYY concentration curve was fitted by non-linear regression using the Sigma Plot data analysis software. Error bars displayed were calculated from measurements carried out with three different immunosensors and the concentrations tested ranged between 10^{-7} and 10^{4} ng/mL PYY. The corresponding equation was:

$$y = \frac{i_{\max} - i_{\min}}{1 + (EC_{50} / x)^{h}} + i_{\min}$$

The maximal and minimal current values were: i_{max} = 2.40 ± 0.09 µA and i_{min} = 0.57 ± 0.08 µA. The EC_{50} value, corresponding to PYY concentration for a fifty per cent competition, was 0.08 ng/mL, and the Hill slope was h = -0.24 ± 0.04. A linear range (r=0.995) between 10^{-4} and 10^{2} ng/mL PYY was observed. This range is adequate for the clinical determination of PYY taking into account the expected concentrations in human serum (around 100 pg/mL)[3,19]. The limit of detection was calculated to be 0.01 pg/mL PYY according to the equation:

$$LOD = EC_{50}\left(\frac{i_{\max} - i_{\min}}{i_{\max} - i_{\min} - 3s} - 1\right)^{-1/h}$$

where s is the standard deviation, ±0.06 µA, for i_p values measured for solutions (n = 10) containing no PYY (zero value).

Some of the recorded DP voltammetric traces are displayed in Figure 3b. The reproducibility of the peak current measurements for solutions containing no PYY and containing 1.0 ng/mL PYY, was tested using different immunosensors on the same day and on different days. A new anti-PYY-Phe-rGO/GCE conjugate was assembled for each measurement. Relative standard deviation (RSD) values of 4.0 and 5.0 % (n = 5) were found, respectively, for the assays performed on the same day, whereas the RSD values were 5.5 % in the absence of PYY and 3.2 % for 1.0 ng/mL PYY when the

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measurements were carried out on different days. These results revealed the good reproducibility achieved in the fabrication and functioning of the proposed immunosensing platform.

The comparison of the analytical performance of the AP-Strept-Biotin-PYY-anti-PYY-Phe-rGO/GCE immunosensor versus that reported for commercial ELISA kits allow us to point out the following advantages. Firstly, the assay time is considerably shorter. In fact, once the antibody was immobilized, measurements can be made in two hours in contrast with the colorimetric kits which take longer than three hours. Examples are the Enzyme Immunoassay Kit from RayBio® or Abnova KA1686 for human, mouse or rat PYY that require 3h 45 min. Furthermore, the accessible dynamic range with these kits ranges from 1 to 1000 pg/mL This is a much narrower range than that achieved with the developed immunosensor. Moreover, the minimum detectable concentration achievable with such commercial kits, 2.84 pg/mL, is more than three hundred times larger than the detection limit achieved with the immunosensor, and the precision of these kits, with CV<10% (intra-assay) or CV<15% (inter-assay), is worse than that obtained in this work. Another noticeable advantage is the reusability of the electrochemical platforms employed for the construction of the immunosensor. Regeneration of the GCE surface could be accomplished simply by polishing for one minute with 3-micron alumina and rinsing for 30 s each with water and methanol under ultrasonic stirring followed by drying under IR radiation.

The storage stability of the anti-PYY-Phe-rGO/GCE conjugate was also investigated. In order to do that, different bioelectrodes were prepared on the same day and stored in a refrigerator at 4°C. Each bioelectrode was used to construct the corresponding immunosensor and to measure the voltammetric response for 5 mM 1-NPP in the absence of PYY. The results obtained (not shown) revealed a high stability for this configuration, since the measured peak currents remained within the control limits set at ±3 x standard deviation of the responses obtained on the first day of the study for at least 12 days (no longer period of storage time was tested).

Selectivity of the immunosensor

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Various proteins: adiponectin (APN), ghrelin (GHRL), desoctanoyl ghrelin (do-GHRL), insulin (INS) human growth hormone (hGH), and follicle stimulating hormone (FSH) were tested as potential interfering substances for the determination of PYY using the developed immunosensor. Selectivity evaluation was accomplished by comparing the immunosensor responses for 0 µg/mL PYY with those measured in the presence of each tested compound at a concentration of 1 µg/mL. Figure 4 clearly shows that no significant differences in the measured response were apparent in any case thus demonstrating the high selectivity of the proposed configuration for PYY determination.

Determination of PYY in spiked serum samples

The applicability of the immunosensor for the determination of PYY in human serum was demonstrated by analyzing a commercial human serum sample containing no PYY spiked with the hormone at three different concentration levels: 0.35, 3.5 and 35 pg/mL. As it was described in the Experimental section, the samples were analyzed without any treatment except dilutions. In order to evaluate potential matrix effect, a calibration plot for PYY in serum was constructed by appropriate dilution. Figure S8 shows a comparison between the calibration plots from standard PYY solutions and from the diluted samples. The slope value calculated for the linear portion of this calibration was 0.19 ± 0.01 µA per decade of concentration. A statistical comparison using the Student t-test with the slope value of the linear range corresponding to the calibration graph prepared with PYY standards, 0.198 ± 0.002 µA per decade of concentration, showed that t_{exp}, 0.853, was lower than the tabulated value 3.355, for n=8, at a 0.05 significance level, indicating that no significant differences existed between both slope values. Accordingly, significant matrix effect could be discarded and the determination of PYY in human serum could be carried out simply by interpolation of the amperometric measurements for the samples into the calibration plot constructed with standards. Table 1 summarizes the results obtained. As it can be observed, satisfactory recoveries were obtained for four replicates and for all the tested concentration levels, with recoveries ranging between 99 ± 3 and 102 ± 3 %.

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Conclusions

The first electrochemical immunosensor for the determination of the anorexigen PYY is described in this work. The immunosensor design involved: a) (c) the use of these modified electrodes as scaffolds to covalently immobilize the capture antibodies; (d) competitive immunoassay involving PYY and biotinylated PPY. The immunosensor exhibited an excellent analytical performance with a broad calibration linear range between 10^{-4} and 10^{2} ng/mL, which is adequate for the determination of PYY in real samples, and a low detection limit of 0.01 pg/mL. The immunosensor exhibited also remarkably higher sensitivity, better precision and shorter assay time than those of available ELISA kits, and its applicability is demonstrated by analyzing human serum samples spiked with PYY at three concentration levels.

Acknowledgments

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Figures

Figure 1. Schematic display of the different steps involved in the construction of an electrochemical immunosensor for PYY involving grafting of 4-ABA diazonium salt onto rGO-modified GCE and covalent immobilization of anti-PYY.

Figure 2. Cyclic voltammograms (a) and Nyquist plots obtained by electrochemical impedance spectroscopy (b) at (1) bare GCE, (2) rGO/GCE, (3) HOOC-Phe-rGO/GCE, and (4) HOOC-Phe-rGO/GCE activated with EDC/NHSS, in 5 mM $Fe(CN)_6^{3-/4-}$ 0.1 M KCl solution.

Figure 3. Calibration plot for PYY (a) and differential pulse voltammograms (b) for (1) 0, (2) 0.01, (3) 1.0, and (4) 5.0 x 10^4 ng/mL PYY; (5) unspecific adsorption at the AP-

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Strept-Biotin-PYY-anti-PYY-Phe-rGO/GCE immunosensor. See text and Table S1 for the experimental conditions.

Figure 4. Effect of the presence of APN, GHRL, do-GHRL, INS, hGH and FSH on the differential pulse voltammetric responses obtained for 0 μg/mL PYY with the AP-Strept-Biotin-PYY-anti-PYY-Phe-rGO/GCE immunosensor.

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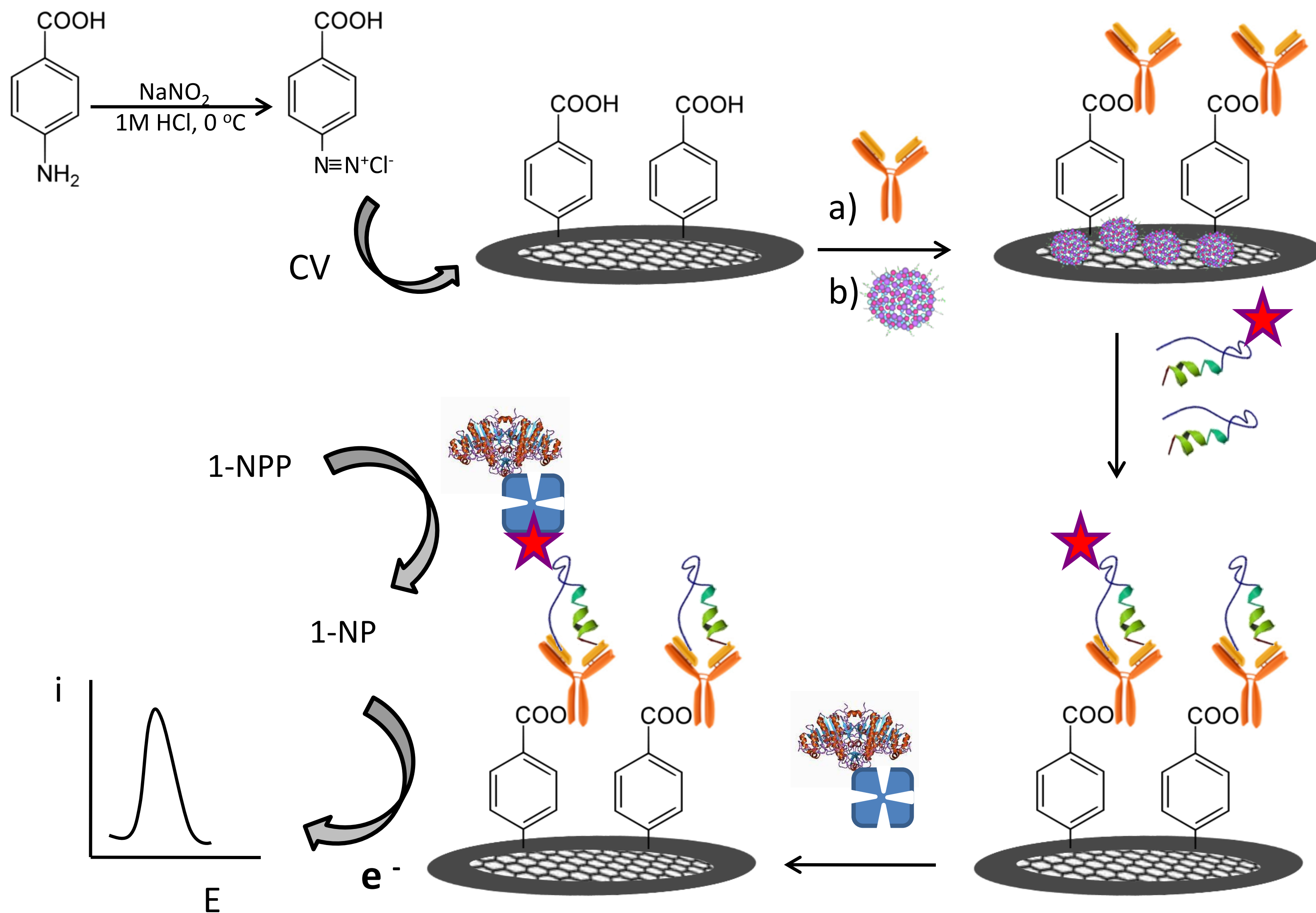

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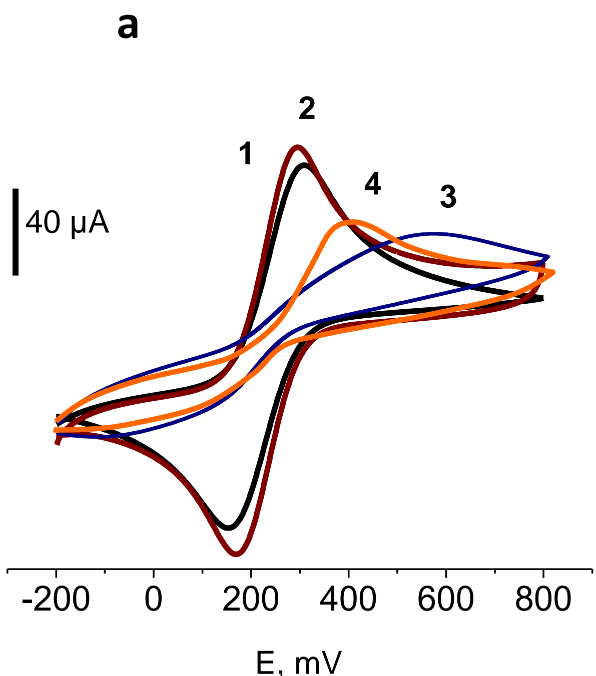

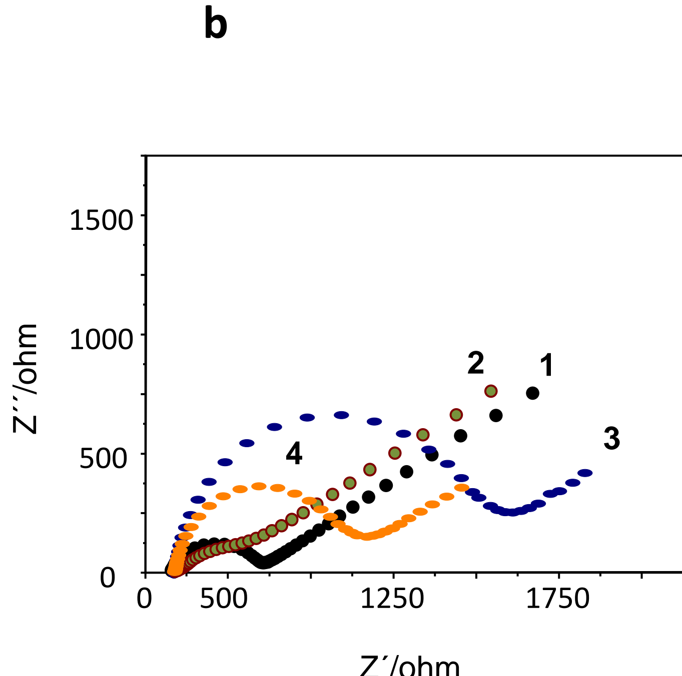

Fig 2

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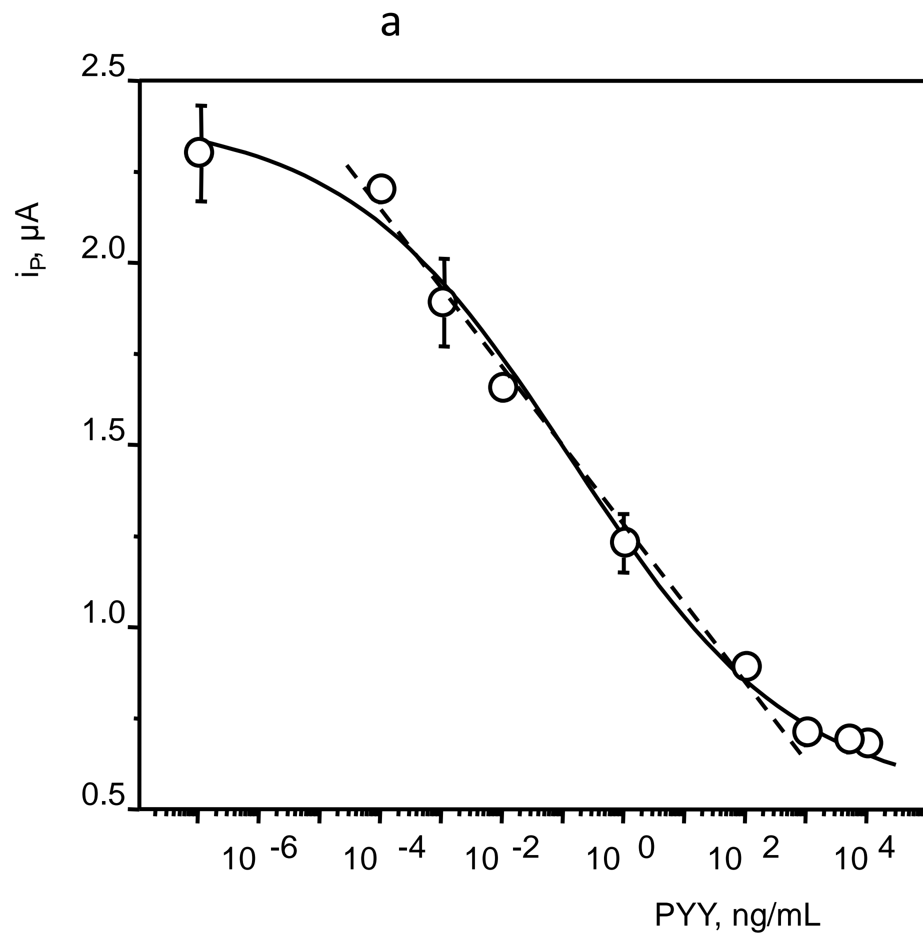

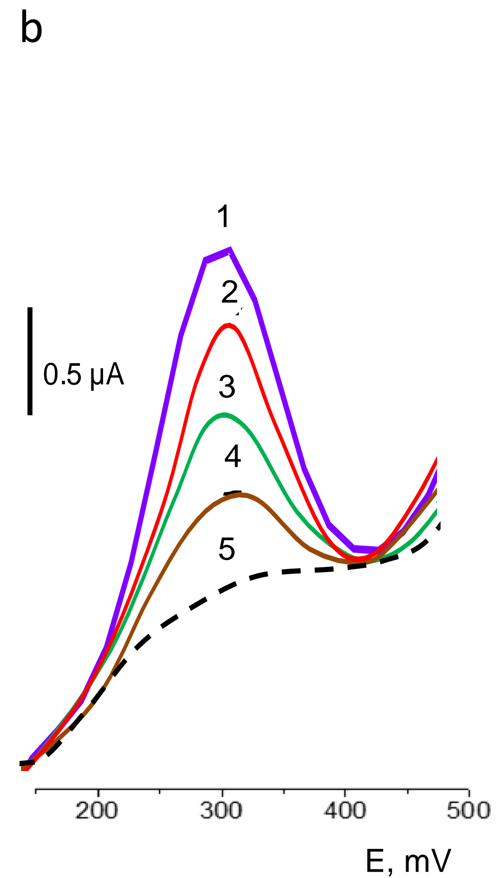

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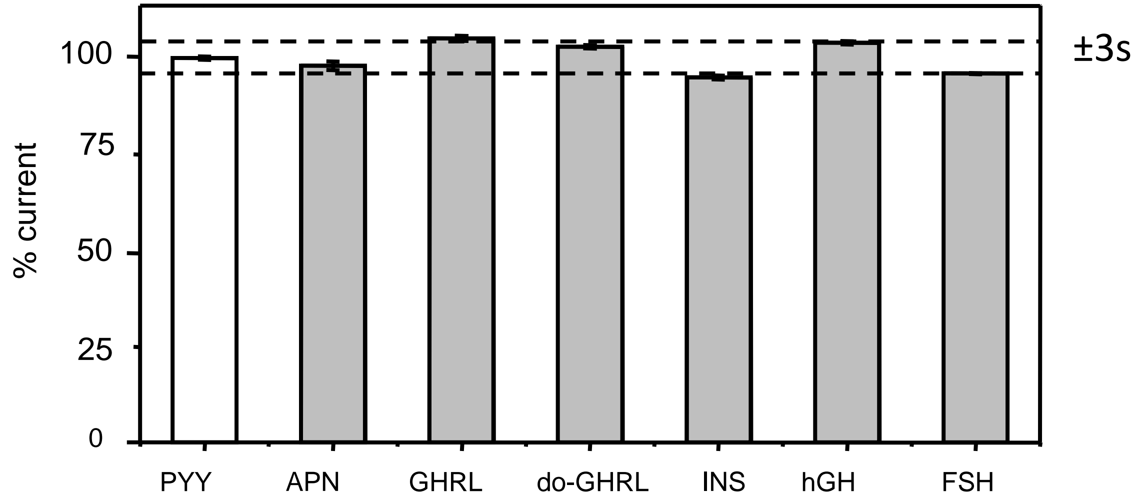

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Table 1. Determination of PYY in spiked serum samples with the AP-Strept-Biotin-PYY-anti-PYY-Phe-rGO/GCE immunosensor

Sample	PYY, pg/mL	PYY found, pg/mL	Mean PYY*, pg/mL	Mean recovery,%
1	35	35; 33; 35; 36	35 ± 1	99 ± 3
2	3.5	3.55; 3.67; 3.58; 3.67	3.62 ± 0.06	102 ± 2
3	0.35	0.342; 0.358; 0.348; 0.345	0.348 ± 0.007	99 ± 2

*mean value ± $ts/\sqrt{n}$

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