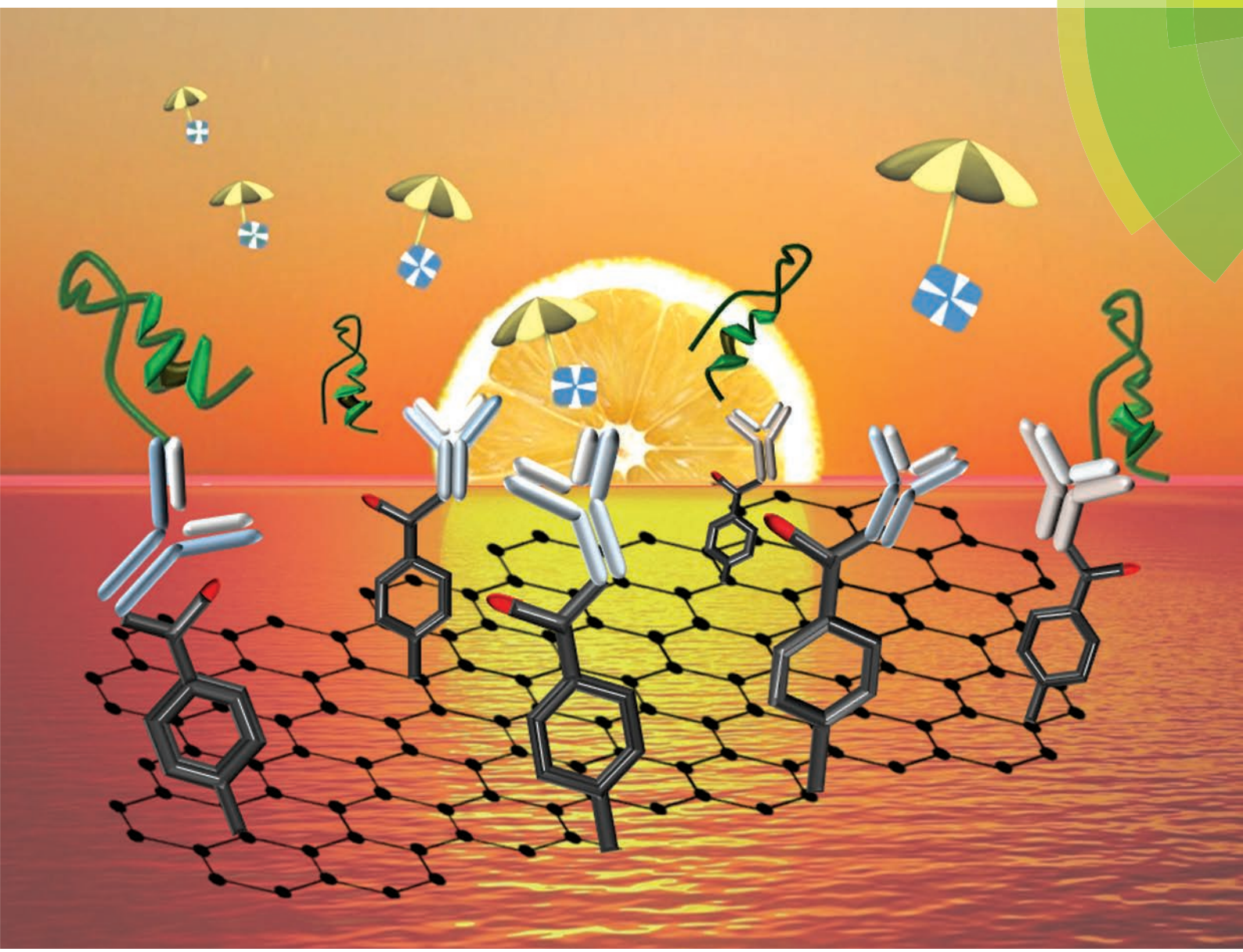
Volume 140 | Number 22 | 21 November 2015 | Pages 7453–7830

Analyst

www.rsc.org/analyst



ISSN 0003-2654

ROYAL SOCIETY OF CHEMISTRY

PAPER

P. Yáñez-Sedeño *et al.*

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

Analyst

PAPER

View Article Online
View Journal | View Issue

Cite this: *Analyst*, 2015, **140**, 7527

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

S. Guerrero, G. Martínez-García, V. Serafín, L. Agüí, P. Yáñez-Sedeño* and J. M. Pingarrón

The first electrochemical immunosensor for the determination of peptide YY is reported in this paper. A novel electrochemical platform, prepared by the electrochemical grafting of the diazonium salt of 4-aminobenzoic acid onto a reduced graphene oxide-modified glassy carbon electrode, was used, on which the covalent immobilization of specific anti-PYY antibodies was accomplished. The HOOC-Phe-rGO/GCEs were characterized using 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^{-1} was found. This range is adequate for the determination of this protein in real samples, since the expected concentration in human serum is around 100 pg mL^{-1}. The limit of detection was 0.01 pg mL^{-1} of PYY. The immunosensor exhibited good reproducibility of the PYY measurements, excellent storage stability and selectivity, as well as a shorter assay time than those 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.

Received 13th June 2015,
Accepted 30th July 2015

DOI: 10.1039/c5an01185j

www.rsc.org/analyst

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 into the circulatory system in response to food.[1,2] There are two endogenous forms of the hormone: PYY_{1-36} and PYY_{3-36}, released PYY_{1-36} is rapidly metabolized by dipeptidyl peptidase-IV to active PYY_{3-36} through the removal of the two *N* terminal amino acids from the full length 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 the 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 the relationship between PYY and body weight,[5] it is well known that PYY reduces food intake by acting on the arcuate nucleus in the hypothalamus, possibly by inhibiting neuropeptide Y neurons and stimulating POMC expressing neurons *via* the Y2 receptors.[8] This behavior has led to PYY becoming a therapeutic target for reducing hunger and calorie intake.[1]

Despite its importance, methods for determining PYY are restricted to RIA or ELISA immunoassays. A variety of commercial ELISA 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 in concentration ranges from 0.1–1 pg mL^{-1} to 100–1000 pg mL^{-1}, with minimum detectable concentrations of 0.5 pg mL^{-1} to approximately 3 pg mL^{-1}. The times required for these assays are around 2.5–3.5 h.

The scientific and technological advances shown by graphene in recent years are enormous. Due to its remarkable physical properties, this material has largely proven to be extremely versatile and suitable for electroanalytical applications.[9] The use of graphene in the preparation of electrochemical biosensors commonly requires modifying an electrode surface with colloidal suspensions of this material. Graphene suspensions are prepared from graphene oxide (GO) using chemical

Department of Analytical Chemistry, Faculty of Chemistry, University Complutense of Madrid, 28040-Madrid, Spain. E-mail: yseo@quim.ucm.es

†Electronic supplementary information (ESI) available. See DOI: 10.1039/c5an01185j

methods which typically involve the reduction of dispersed single-layer GO sheets to form stable rGO suspensions. In this paper we have introduced a green alternative for rGO preparation using the natural antioxidant ascorbic acid as a reducing agent, which demonstrated good efficiency for such a purpose.[10] Glassy carbon electrodes were then modified with the as prepared rGO and the resulting rGO/GCEs were used as platforms for the development of the first electrochemical immunosensor for PYY. The protocol for the immobilization of immunoreagents on the rGO/GCEs involved the grafting of free radicals onto the electrode surface.[11,12] Specifically, the diazonium salt of 4-amino benzoic acid (4-ABA) was electrochemically reduced at the electrode surface, resulting in the covalent attachment of 4-carboxy phenyl to the rGO/GCE.[13] Then, anti-PYY antibodies were covalently immobilized onto the modified electrode, and a competitive immunoassay involving PYY and biotinylated PPY (Biotin-PYY) was performed. The determination of PYY was carried out by differential pulse voltammetry using alkaline phosphatase-labeled streptavidin (AP-Strept) and 1-naphthyl phosphate (1-NPP) as the enzyme substrate. AP catalyzes the hydrolysis of 1-NPP to 1-naphtol and the electrochemical oxidation of this compound on the electrode surface is measured by DPP.

Experimental

Reagents and solutions

Peptide YY (PYY) (3-36) (human) purified IgG antibodies (antiPYY), peptide YY (PYY) (3-36) (human), and biotinylated-PYY (Biotin-PYY) (3-36) (human) were purchased from Phoenix Pharmaceuticals, Inc. The graphene oxide (NIT.GO.M.140.10) was from Nanoinnova Technologies. Alkaline phosphatase labelled-streptavidin (AP-Strept), 1-naphthyl phosphate (1-NPP), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), and *N*-hydroxysulfo-succinimide (NHSS) were from Sigma. 4-Aminobenzoic acid (ABA) was from Acros. Ethanolamine (ETA, Aldrich), bovine serum albumin (BSA) from Gerbu, and casein from Thermo Scientific were used as blocking agents. A 0.1 M phosphate buffer solution (PBS) of pH 7.4 was prepared from sodium di-hydrogen phosphate and di-sodium hydrogen phosphate (Scharlau). A 50 mM tris (tris (hydroxymethyl)amino-methane, Sigma) buffer solution containing 10 mM of $MgCl_2$ (Panreac) at pH 9.6 (Trizma) was also used. All reagent solutions were prepared in 0.1 M PBS except the 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, and adiponectin (APN, Abnova), ghrelin (GHRL) and des-acyl-ghrelin (da-GHRL, Anaspec), were tested as potential 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 accompanied by BAS 100/W software for the electrochemical analysis. A three electrode (BAS VC-210-mL) glass electrochemical cell was used. Modified 3 mm diameter CHI 104 glassy carbon electrodes from CH Instruments were used as the 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 of a 1 mg mL^{-1} GO aqueous dispersion was sonicated for 120 min and then centrifuged at 10 000 g for 10 min. The precipitate was discarded and the supernatant was treated with a 25% NH_3 solution to achieve a pH of 9–10. Then, the reduction of GO was performed by adding solid ascorbic acid up to a 2 mM final concentration and letting it react at 100 °C for 15 min. The resulting rGO dispersion was left in the dark at room temperature. The product was replenished every week, although it was stable for at least two weeks.

Preparation of the immunosensors. Fig. 1 shows the steps involved in the modification of the electrodes and preparation of the immunosensors. Firstly, the diazonium salt was prepared by adding dropwise a 2 mM $NaNO_2$ aqueous solution to a 1 mg mL^{-1} ABA solution prepared with 1 M HCl and cooled with ice (38 mL of $NaNO_2$ for each 200 mL of ABA). The reaction was allowed to proceed for 10 min under stirring. Separately, glassy carbon electrodes were polished with 0.3 μM alumina slurries for 1 min, sonicated for 30 s in water and dried in air. Then, 10 μL of a 0.5 mg mL^{-1} rGO suspension were deposited onto the electrode surface and, after drying at room temperature, the rGO/GCEs were immersed in 450 μL of the diazonium salt. Ten successive voltammetric cycles from 0 to −1.0 V *vs.* Ag/AgCl (ν = 200 mV s^{-1}) 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.

The activation of the carboxylic groups was achieved by dropping 10 μL of an EDC/NHSS (0.1 M each) aqueous solution onto the HOOC-Phe-rGO/GCEs and leaving them to react for 1 h in the dark. After rinsing them with water and methanol and allowing them to dry, 10 μL of a 20 μg mL^{-1} anti-PYY solution was casted onto the electrode and left to stand for 1 h at 37 °C. Then, 20 μL of a 0.2% casein blocking solution was deposited onto the electrode and incubation was allowed for 1 h at 37 °C. A competitive immunoassay was performed by spotting 10 μL of a mixed solution of PYY (or the sample) and 100 ng mL^{-1} of Biotin-PYY onto the anti-PYY-Phe-rGO/GCE, then allowing incubation for 30 min at 37 °C. Thereafter, 10 μL of 5 μg mL^{-1} AP-Strept was added to the Biotin-PYY-anti-PYY-Phe-rGO/GCE followed by incubation for 30 min at 37 °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 was added. After a delay time of 5 min to allow the enzyme reaction to take place, differential pulse voltammograms were

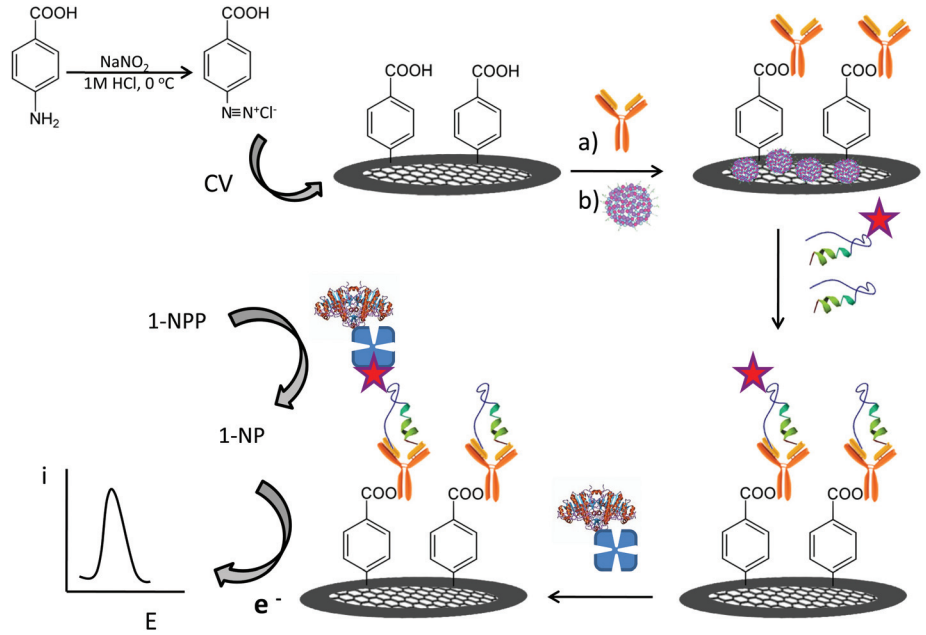


Fig. 1 Schematic display of the different steps involved in the construction of an electrochemical immunosensor for PYY involving the grafting of 4-ABA diazonium salt onto a rGO-modified GCE and the covalent immobilization of anti-PYY.

recorded over the −0.15 to + 0.70 V range to obtain the electroanalytical signals, using ΔE = 50 mV and ν = 20 mV s^{-1}.

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^{-1} 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 a calibration plot constructed using the PYY standard solutions.

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, onto a tailor-made electrode surface modified with rGO in order to take advantage of the well known properties of this nanomaterial (high conductivity and large specific surface area), which enhances the performance of electrochemical biosensors. Moreover, the electrode modification involved a green route using ascorbic acid as the 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 easy optimization of the experimental conditions for the reduction reaction to get the optimal electrocatalytic properties.[15,16]

The as obtained rGO was characterized by UV-vis spectrophotometry. Fig. S1 in the ESI† shows two peaks at 230 and 300 nm corresponding to the $\pi \rightarrow \pi^*$ 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 disappears and a red-shift of the $\pi \rightarrow \pi^*$ peak to 241 nm occurs. This peak position has been used as a convenient probe of the reduction degree achieved using different reducing reagents.[10] Furthermore, the aqueous ascorbic-reduced GO suspension was clearly different from that of the unreduced GO (inset in Fig. S1†), and exhibited long-term stability without observing precipitation for several weeks. These results demonstrate the efficiency of the method used for the reduction of GO.

Preparation of the modified electrodes

The preparation of the modified electrodes required optimization of (a) the loading of rGO onto the GCE surface, (b) the ABA concentration and (c) the number of cycles and potential scan rate used during the electrochemical grafting of the rGO/

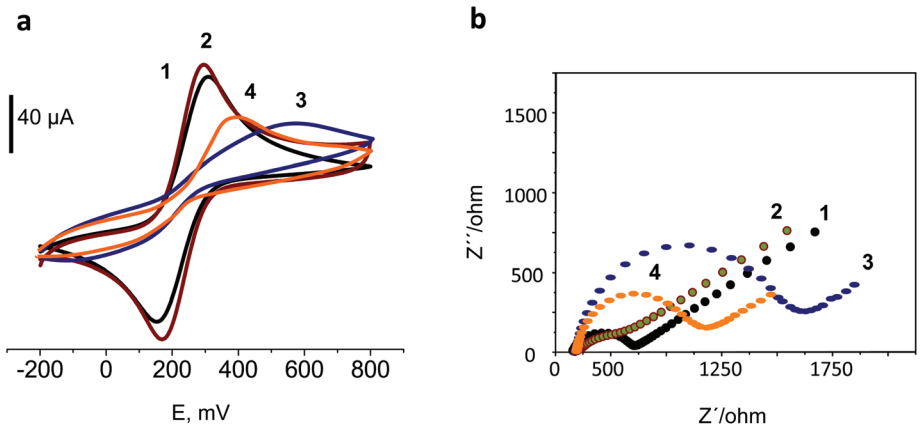


Fig. 2 Cyclic voltammograms (a) and Nyquist plots obtained by electrochemical impedance spectroscopy (b) for the (1) bare GCE, (2) rGO/GCE, (3) HOOC-Phe-rGO/GCE and (4) HOOC-Phe-rGO/GCE activated with EDC/NHSS, in 5 mM $Fe(CN)_6^{3-/4-}$ 0.1 M KCl solution.

GCE. Detailed information on the optimization of these steps can be found in the ESI.†

Once prepared, the rGO/GCEs were characterized by cyclic voltammetry and electrochemical impedance spectroscopy (EIS) using 5 mM $Fe(CN)_6^{3-/4-}$ as the redox probe in 0.1 M KCl. Fig. 2a displays the typical cyclic voltammograms recorded from the (1) bare GCE, (2) rGO/GCE, (3) HOOC-Phe-rGO/GCE, and (4) HOOC-Phe-rGO/GCE activated with EDC/NHSS. As can be seen, slightly larger voltammetric peaks and a smaller electron transfer resistance (Fig. 2b) were observed for the rGO/GCE with respect to the unmodified GCE, which could be attributed to both the larger conductivity and/or porous diffusion effects of the functionalized graphene electrodes.[18] Conversely, electrochemical grafting resulted in a poorer voltammetric behavior and 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 the carboxyl moieties were activated using the EDC/NHSS reagents, a semicircle with a shorter diameter appeared as a consequence of the neutralization of the negative charges on the carboxylate groups (curve 4). Equivalent behavior can also be observed by cyclic voltammetry. These results confirm the successful modification of the electrode surface through electrochemical grafting with the 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) the loading of anti-PYY and the incubation time, (b) the blocking step, (c) the loading of biotin-PYY and the incubation time, (d) the loading of AP-Strept and the incubation time and (e) the concentration of 1-NPP and the time for the enzyme reaction to proceed. Detailed information on the results obtained in these studies can be found in the ESI† and are summarized in Table S1.†

Analytical characteristics of the immunosensor for PYY

Once all the working conditions were optimized, a calibration plot of 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 the competitive immunoassay (Fig. 3a). The i_p *vs.* PYY concentration curve was fitted by a non-linear regression using Sigma Plot data analysis software. The error bars displayed were calculated from measurements obtained from three different immunosensors and the concentrations of PYY tested ranged between 10^{-7} and 10^4 ng mL^{-1}. The corresponding equation is:

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

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 the PYY concentration at fifty per cent competition, was 0.08 ng mL^{-1}, and the Hill slope was h = −0.24 ± 0.04. A linear range (r = 0.995) between 10^{-4} and 10^2 ng mL^{-1} of PYY was observed. This range is adequate for the clinical determination of PYY when taking into account the expected concentrations in human serum (around 100 pg mL^{-1}).[3,19] The limit of detection was calculated to be 0.01 pg mL^{-1} of 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, of the i_p values measured for solutions (n = 10) containing no PYY (zero value).

Some of the recorded DP voltammetric traces are displayed in Fig. 3b. The reproducibilities of the peak current measurements for solutions containing no PYY and 1.0 ng mL^{-1} of PYY, were tested using different immunosensors on the same

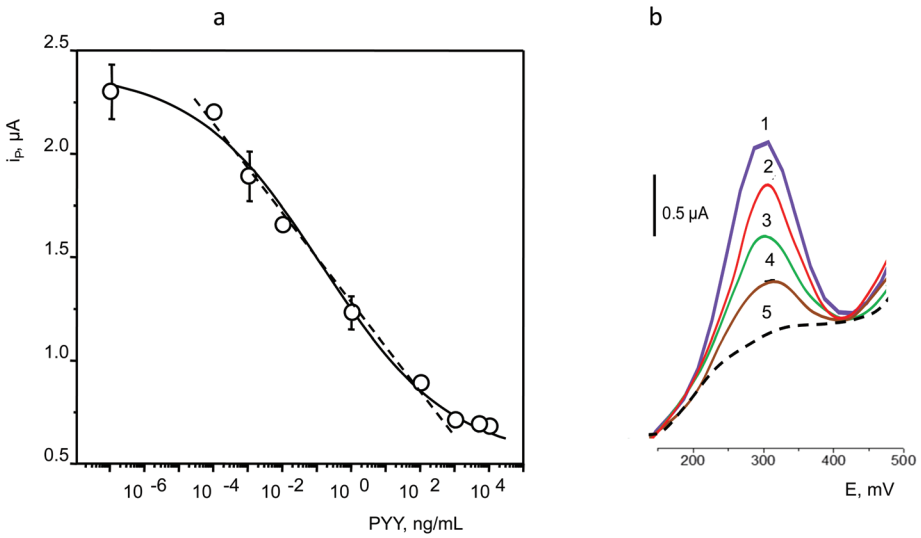


Fig. 3 Calibration plot for PYY (a) and differential pulse voltammograms (b) for (1) 0, (2) 0.01, (3) 1.0, (4) 5.0×10^4 ng mL^{-1} of PYY and (5) unspecific adsorption at the AP-Strept-Biotin-PYY-anti-PYY-Phe-rGO/GCE immunosensor. See text and Table S1† for the experimental conditions.

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^{-1} of PYY when the measurements were carried out on different days. These results reveal the good reproducibility achieved for 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 allows us to point out the following advantages. Firstly, the assay time is considerably shorter. In fact, once the antibody was immobilized, measurements could be made in two hours in contrast to colorimetric kits which take longer than three hours. Examples are the Enzyme Immunoassay Kit from RayBio® or the Abnova KA1686 for human, mouse or rat PYY that require 3 h 45 min. Furthermore, the accessible dynamic range with these kits ranges from 1 to 1000 pg mL^{-1}, which 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^{-1}, 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. The 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 irradiation.

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 the high stability of this configuration, since the measured peak currents remained within the control limits set at ±3 × the standard deviation of the responses obtained on the first day of the study for at least 12 days (no longer periods of storage time were tested).

Selectivity of the immunosensor

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. The selectivity evaluation was accomplished by comparing the immunosensor response to 0 μg mL^{-1} of PYY with those measured in the presence of each tested compound at a concentration of 1 μg mL^{-1}. Fig. 4 clearly shows that no significant differences in the measured responses 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^{-1}. As described in the Experimental section, the samples were analyzed without any treatment except for

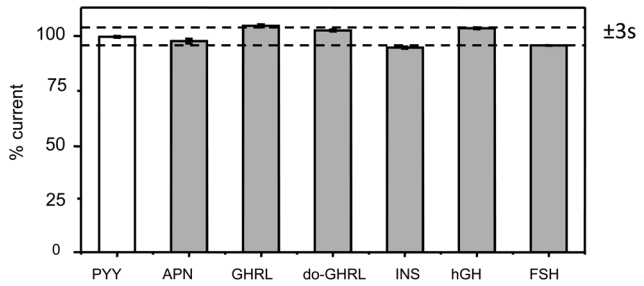


Fig. 4 Effects of the presence of APN, GHRL, do-GHRL, INS, hGH and FSH on the differential pulse voltammetric responses obtained for 0 μg mL^{-1} of PYY with the AP-Strept-Biotin-PYY-anti-PYY-Phe-rGO/GCE immunosensor.

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^{-1}	PYY found, pg mL^{-1}	Mean PYY[a], pg mL^{-1}	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

[a] Mean value ± $ts/\sqrt{n}$.

dilution. In order to evaluate potential matrix effects, a calibration plot for PYY in serum was constructed by appropriate dilution. Fig. S8† shows a comparison between the calibration plots obtained 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 the PYY standards, 0.198 ± 0.002 μA per decade of concentration, showed that t_{exp}, 0.853, was lower than the tabulated value of 2.365, for $n = 8$, at a 0.05 significance level, indicating that no significant difference existed between the slope values. Accordingly, significant matrix effects could be discarded and the determination of PYY in human serum could be carried out simply by the interpolation of the amperometric measurements from the samples into the calibration plot constructed from the standards. Table 1 summarizes the results obtained. It can be observed that satisfactory recoveries were obtained for four replicates and for all the tested concentration levels, with recoveries ranging between 99 ± 3 and 102 ± 3%.

Conclusions

The first electrochemical immunosensor for the determination of the anorexigen PYY is described in this work. The immunosensor design involved the use of modified electrodes as scaffolds to covalently immobilize capture antibodies and a 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^{-1}, which is adequate for the determination of PYY in real samples, and a low detection limit of 0.01 pg mL^{-1}. The immunosensor also exhibited remarkably higher sensitivity, better precision and a shorter assay time than those of available ELISA kits, and its applicability was demonstrated by analyzing human serum samples spiked with PYY at three concentration levels.

Acknowledgements

The financial support of the Spanish Ministerio de Economía y Competitividad, Research Project CTQ2012-35041, and the NANOAVANSENS Program from Comunidad de Madrid (S2013/MT-3029) are gratefully acknowledged.

References

1 F. Colon-Gonzalez, G. W. Kim, J. E. Lin, M. A. Valentino and S. A. Waldman, *Mol. Aspects Med.*, 2013, **34**, 71.
2 T. E. Adrian, G. L. Ferri, A. J. Bacarese-Hamilton, H. S. Fuessi, J. M. Polak and S. R. Bloom, *Gastroenterology*, 1985, **89**, 1070.
3 R. Mentlein, P. Dahms, D. Grandt and R. Krüger, *Regul. Pept.*, 1993, **49**, 133.
4 D. Renshaw and R. L. Batterham, *Curr. Drug Targets*, 2005, **6**, 171.
5 J. A. Cooper, *Nutr. Res. Rev.*, 2014, **27**, 186.
6 R. L. Batterham, M. A. Cohen, S. M. Ellis, C. W. Le Roux, D. J. Withers, G. S. Frost, M. A. Ghatei and S. R. Bloom, *N. Engl. J. Med.*, 2003, **349**, 941.
7 D. Boey, L. Heilbronn, A. Sainsbury, R. Laybutt, A. Kriketos, H. Herzog and L. V. Campbell, *Neuropeptides*, 2006, **40**, 317.
8 R. L. Batterham, M. A. Cowley, C. J. Small, H. Herzog, M. A. Cohen, C. L. Dakin, A. M. Wren, A. E. Brynes, M. J. Low, M. A. Ghatei, R. D. Cone and S. R. Bloom, *Nature*, 2002, **418**, 650.
9 M. Pumera, A. Ambrosi, A. Bonanni, E. L. K. Chng and H. L. Poh, *TrAC, Trends Anal. Chem.*, 2014, **29**, 954.
10 M. J. Fernández-Merino, L. Guardia, J. I. Paredes, S. Villar-Rodil, P. Solís-Fernández, A. Martínez-Alonso and J. M. D. Tascón, *J. Phys. Chem. C*, 2010, **114**, 6426.
11 G. Liu, E. Luais and J. J. Gooding, *Langmuir*, 2011, **27**, 4176.
12 M. Moreno-Guzmán, I. Ojeda, R. Villalonga, A. González-Cortés, P. Yáñez-Sedeño and J. M. Pingarrón, *Biosens. Bioelectron.*, 2012, **35**, 82.
13 J. Liu, L. Cheng, B. Liu and S. Dong, *Langmuir*, 2000, **16**, 7471.

14 R. J. W. E. Lahaye, H. K. Jeong, C. Y. Park and Y. H. Lee, *Phys. Rev. B: Condens. Matter*, 2009, **79**, 125435.
15 T. W. Lu, R. B. Zhang, C. Y. Hu, F. Chen, S. W. Duo and Q. H. Hu, *Phys. Chem. Chem. Phys.*, 2013, **15**, 12963.
16 J. Liu, C. Hu, J. Xu, F. Jiang and F. Chen, *Mater. Lett.*, 2014, **134**, 134.
17 P. Sharma, G. Darabdhara, T. M. Reddy, A. Borah, P. Bezboruah, P. Gogoi, N. Hussain, P. Sengupta and M. R. Das, *Catal. Commun.*, 2013, **40**, 139.
18 C. Punckt, M. A. Pope and I. A. Aksay, *J. Phys. Chem. C*, 2013, **117**, 16076.
19 F. Cahill, Y. Ji, D. Wadden, P. Amini, E. Randell, S. Vasdev, W. Gulliver and G. Sun, *PLoS One*, 2014, **9**, e95235.