Microchimica Acta
https://doi.org/10.1007/s00604-018-2863-x

ORIGINAL PAPER

Amperometric immunoassay for the obesity biomarker amylin using a screen printed carbon electrode functionalized with an electropolymerized carboxylated polypyrrole

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Received: 12 March 2018 / Accepted: 4 June 2018

Abstract

Amylin (the islet amyloid polypeptide) is a hormone related to adiposity, hunger and satiety. It is co-secreted with insulin from pancreatic B-cells. An amperometric immunosensor is presented here for the determination of amylin. It is making use of a screen printed carbon electrode (SPCE) functionalized with electropolymerized poly(pyrrole propionic acid) (pPPA) with abundant carboxyl groups that facilitate covalent binding of antibody against amylin. A competitive immunoassay was implemented using biotinylated amylin and streptavidin labeled with horse radish peroxidase (HRP-Strept) as the enzymatic tracer. The amperometric detection of H_2O_2 mediated by hydroquinone was employed as an electrochemical probe to monitor the affinity reaction. The variables involved in the preparation and function of the immunosensor were optimized and the electrodes were characterized by electrochemical impedance spectroscopy and cyclic voltammetry. The calibration graph for amylin, obtained by amperometry at −200 mV vs Ag pseudo-reference electrode, showed a range of linearity extending from 1.0 fg·mL^{-1} to 50 pg·mL^{-1}, with a detection limit of 0.92 fg·mL^{-1}. This is approximately 7000 times lower than the minimum detectable concentration reported for the ELISA immunoassays available for amylin. The assay has excellent reproducibility and good selectivity over potential interferents.

Keywords Screen-printed carbon electrodes · Electrochemical biosensor · Conducting polymer · Mix&Go™ · Urine · Serum

Introduction

Obesity, considered as being pandemic by the World Health Organization, is one of the most deadly diseases and causes more than 2.8 million deaths per year. The normal control of body weight and food intake by the brain relies upon the detection and integration of signals related with adiposity, hunger and satiety. Amylin (islet amyloid polypeptide, hIAPP or AMY) is a 37-residue peptide hormone co-secreted with insulin from pancreatic β-cells [1]. Human amylin is found as fibrillar deposits in pancreatic extracts of nearly all type-II diabetics. Both synthetic and endogenous AMY have significant cytotoxicity to islet cells. This effect is related to the death of such cells occurring in diabetes which results in the loss of blood glucose homeostasis and subsequent insulin dependence [2, 3]. Like insulin, plasma AMY levels are low during fasting and increase during meals and following glucose administration, the concentrations being directly proportional to body fat. AMY and insulin are normally co-secreted in a fixed molecular ratio between ten and one hundred insulin to AMY. However, obesity, diabetes mellitus, pancreatic cancer and certain pharmacological interventions tend to increase the amount of AMY relative to insulin [1]. Studies related to physiology of AMY [4, 5] revealed that individuals with type 1 diabetes are AMY deficient [6] whereas post-meal secretion of AMY seems to be defective in advanced type 2 diabetes [7]. Conversely, individuals who are insulin resistant and hyperinsulinemic have high concentrations of AMY in plasma [8]. The AMY levels in human serum oscillates around a few tens of pg·mL^{-1} [4, 9, 10].

Despite its importance, a scarce number of analytical methods for the determination of AMY have been described

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Published online: 09 June 2018

in the literature. ELISA kits using competitive strategies with biotinylated antigen are commercially available. The RayBiotech (RayBio®) [https://www.raybiotech.com/files/manual/EIA/EIA-AMY.pdf] kit exhibits a dynamic range from 1 to 1000 ng·mL^{-1} with a minimum detectable concentration of 0.62 ng·mL^{-1}. In this assay, Biotin-AMY competes with AMY for anti-AMY, then interacting with HRP-Strept. A similar assay from LifeSpan BioSciences Inc. [https://www.lsbio.com/elisakits/manualpdf/ls-f9686.pdf] using HRP-Avidin provides a linear range between 6.17 and 500 pg·mL^{-1}, with detectable concentrations typically less than 6.17 pg·mL^{-1}. A sandwich-type immunoassay with fluorometric detection was also reported for the detection of AMY in plasma with a dynamic range between 2 and 100 pmol·L^{-1} and a minimum detectable concentration of 0.5 pmol·L^{-1} [4].

In this work, the first electrochemical immunosensor for the determination of AMY is reported. A screen printed carbon electrode (SPCE) functionalized with electropolymerized poly(pyrrole propionic acid) (pPPA) was used. This transducer provided a high content of surface confined carboxyl groups suitable for direct covalent binding of anti-AMY antibody after activation with Mix&Go™, a polymer containing several metallic complexes selected for their efficiency to bind proteins [11, 12]. Particularly, in the case of antibodies, Mix&Go uses small molecule ligands to bind Fc domains as an anchoring point, these ligands mimicking binding domains from proteins A and G, and extending as two adjacent sidechains from a polymer backbone [13]. In addition, metal complexes integrated into the polymer chains enhance binding of Fc domains providing a higher stability. The polymer also forms strong multivalent interactions with electron donating groups such as carboxylate [11]. Accordingly, coupling of pPPA with the use of Mix&Go™ for antibody immobilization resulted in a successful strategy suitable to be used as a general route for preparing electrochemical immunosensors with potential application in point-of-care devices. A competitive immunoassay involving biotinylated AMY and streptavidin labelled with horse radish peroxidase (HRP-Strept) as the enzymatic tracer was implemented (Fig. 1). The amperometric detection of H_2O_2 mediated by hydroquinone (HQ) was employed to monitor the affinity reaction. The variables involved in the preparation and functioning of the immunosensor were optimized and the modified electrodes were characterized by electrochemical impedance spectroscopy and cyclic voltammetry. The calibration graph for AMY shows a range of linearity extending from 0.001 to 50 pg·mL^{-1}, with a detection limit of 0.92 fg·mL^{-1}. This value is approximately seven thousand times lower than the minimum detectable concentration reported for the available ELISA immunoassays for AMY. An excellent reproducibility of the measurements carried out with different immunosensors as well as an excellent selectivity against other hormones was shown. In addition, good results were obtained by application to the AMY analysis in urine and serum.

Experimental

Reagents and solutions

Human monoclonal anti-amylin (anti-AMY) from mouse, human amylin (AMY) and biotinylated human amylin (Biotin-AMY) were the reagents included in the RayBio® Human/Mouse/Rat Amylin Enzyme Immunoassay Kit (EIA-AMY) from RayBiotech, Inc. (www.raybiotech.com) Solutions of the antigen and biotinylated antigen were prepared in Assay Diluent B pH 7.5 from RayBiotech. Anti-AMY capture antibody was reconstituted in the same solvent and diluted up to the working concentration with 25 mM 2-(*N*-morpholine) ethanosulfonic acid (MES) buffer of pH 5.0 (Gerbu, www.gerbu.de). 1H-pyrrole-1-propionic acid monomer (PPA) (Sigma-Aldrich, www.sigmaaldrich.com, 97%) and KCl (Scharlau, www.scharlab.com, 99.5%) were also used. 0.1 M PPA solutions were prepared in deionized water containing 0.5 M KCl. Mix&Go™ was from Anteo Diagnostics (www.anteotech.com). Streptavidin labeled with horseradish peroxidase (HRP-Strept) was from Roche (www.custombiotech.roche.com). Solutions of hydrogen peroxide (Aldrich, 30% (*w*/w)) and hydroquinone (HQ, Sigma) were prepared in 0.05 M phosphate buffer of pH 6.0. 0.05 M potassium ferro- and ferricyanide solutions were prepared in 0.1 M phosphate buffered saline (PBS) of pH 7.4 containing 2.002 g NaCl (Labkem, 99%), 0.050 g KCl (Probus), 0.287 g NaH_2PO_4 (Scharlau, 98%) and 0.051 g KH_2PO_4 (Merck, 99%) in 250 mL of deionized water. 2% (*w*/*v*) solutions of bovine serum albumin (BSA) in the same buffer were used as blocking agent. Ascorbic acid (AA, Fluka), uric acid (UA, Sigma), bilirubin (BR, Aldrich), cholesterol (Chol, Sigma), glucose (Glu, Panreac, www.itwreagents.com) and peptide YY (PYY, Phoenix Pharmaceuticals, Inc., www.phoenixpeptide.com.) were tested as interfering compounds. Deionized water was from a Millipore Milli - Q purification system (18.2 MΩ·cm at 25 °C).

Lyophilized human serum from Sigma reconstituted in deionized water, spiked with 0.75 and 4.0 pg·mL^{-1} amylin and diluted with Assay Diluent B from RayBio® was analyzed. Liquichek urine Chemistry Control (Level 1, BioRad 63,221, www.bio-rad.com) containing uric acid, amylase, cortisol, creatinine, glucose, albumin, urea, calcium, potassium, sodium, chloride and phosphorous, spiked with 1.50 and 8.00 pg·mL^{-1} amylin and diluted with the Assay Diluent B from RayBio® was also analyzed.

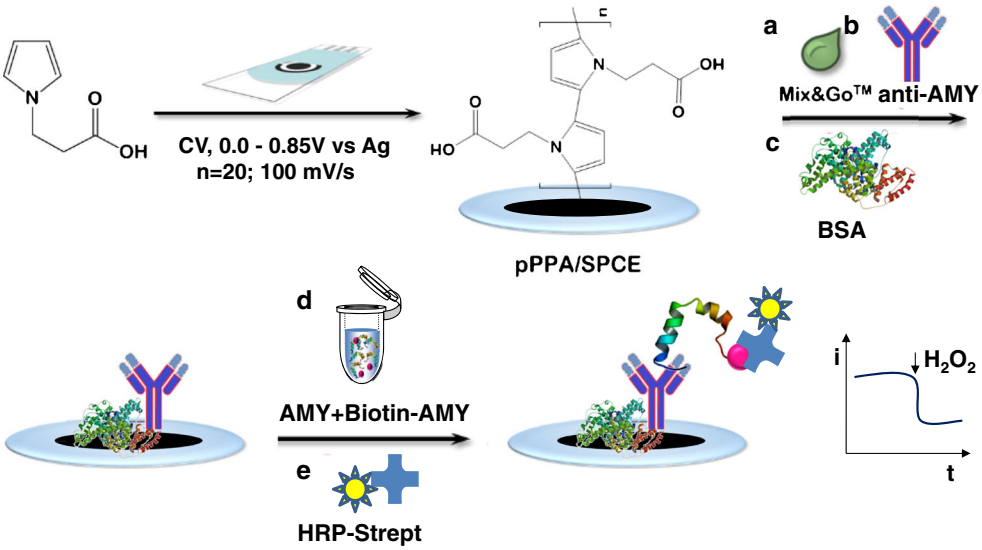

Fig. 1 Schematic display of the different steps involved in the construction of the amperometric immunosensor for AMY involving pPPA-modified SPCEs and covalent immobilization of anti-AMY: (**a**) Mix&Go™ addition; (**b**) covalent immobilization of anti-AMY antibody; (**c**) blocking step with BSA; (**d**) competitive immunoassay; (**e**) addition of HRP-Strept conjugate

Apparatus and electrodes

Amperometric measurements were performed using an INBEA potentiostat provided with the Ib Software. Voltammograms were registered using a μAutolab type III potentiostat controlled by the GPES 4.7 software (Ecochemie). EIS measurements were made using an Autolab type III controlled by FRA2 software (RcoChemie). Screen-printed carbon electrodes (SPCEs, 110DRP, ϕ 4 mm) from DropSens (Oviedo, Spain) were used as working electrodes. These electrodes are provided with a silver pseudo-reference electrode and a carbon counter electrode. pH measurements were made using a Crison Basic 20 + pH meter. An Elmasonic S-60 (Elma) ultrasonic bath, a Vortex (Velp Scientifica) shaker, and a centrifuge MPW-65R (MPW Med. Instruments) were also used. All experiments were performed at room temperature.

Procedures

Preparation of the electrochemical immunosensor

Electropolymerization of pPPA on the SPCE was accomplished by immersing the electrode into a 100 mM PPA monomer solution containing 0.5 M KCl and applying 20 successive voltammetric cycles between 0.0 and + 0.85 V at 100 mV s^{-1}. The resulting pPPA/SPCEs were immersed into 1 mM HQ solution in 0.1 M phosphate buffer of pH 7.4, and a control cyclic voltammogram was recorded between −0.2 and + 1.0 V (vs. the Ag pseudo-reference electrode of the SPCE) at 50 mV s^{-1}. Surface confined carboxyl groups were activated by adding 10 μL of Mix@Go™ on the pPPA/SPCEs and allowing incubation for 60 min at 25 °C. Thereafter, the electrodes were washed with the MES buffer of pH 5.0, and the capture antibody was covalently immobilized by dropping 5 μL of a 1/100 diluted anti-AMY solution prepared in 25 mM MES buffer of pH 5.0 and incubating during 60 min at 25 °C in a humid ambient chamber. After washing again with the same MES buffer, the un-reacted activated groups on the anti-AMY-pPPA/SPCEs were blocked by adding 7.5 μL of 2% BSA in 0.1 M phosphate buffer of pH 7.4, allowing incubation for 30 min, and washing again with the MES buffer. A competitive immunoassay was carried out by incubation of 5 μL of a solution containing 2.5 ng·mL^{-1} Biotin-AMY conjugate and the antigen, AMY, for 45 min. Then, 5 μL of a 1/500 HRP-Strept solution were added and, after 20 min incubation, the resulting HRP - Strept - Biotin - AMY - AMY- anti - AMY- pPPA / SPCE immunosensor was washed with 0.1 M phosphate buffer of pH 7.4 and kept in humid ambient until measurements were made.

Amperometric measurements

A volume of 45 μL of 0.05 M phosphate buffer of pH 6.0 and 1.0 mM hydroquinone (prepared just before the electrochemical measurement) was deposited onto the immunosensor and the current was recorded by applying a potential of −200 mV vs Ag pseudo-reference electrode. Once the background current was stabilized (after about 100 s) a 5-μL aliquot of a 50 mM hydrogen peroxide solution in 0.05 M PBS of pH 6.0 was added and the current was measured allowing 200 s for the enzymatic reaction to take place.

Determination of amylin

Analysis of spiked human serum and urine using the HRP-Strept-Biotin-AMY-AMY- anti-AMY-pPPA/SPCE immunosensor was performed after a simple sample treatment consisting of a five- or tenfold dilution, respectively, with the Assay Diluent B RayBio®.

Results and discussion

Fig. 1 illustrates schematically the different steps involved in the preparation and functioning of the HRP - Strept - Biotin - AMY- AMY - anti - AMY - pPPA / SPCE immunosensor. Upon modification of the SPCE by electropolymerization of pPPA, Mix & Go™ was used (step a) for covalent immobilization of capture antibodies on the surface confined carboxyl groups of the pPPA/SPCE (step b). Then, after a blocking step of the remaining activated un-reacted sites with a BSA solution (step c), a competitive immunoassay with target AMY and Biotin-AMY was implemented (step d). Finally, HRP-Strept conjugate was added (step e) and the amperometric responses at −0.20 V (vs the Ag pseudo-reference electrode) upon addition of H_2O_2 in the presence of HQ were measured.

Optimization of the experimental variables

The different variables affecting the preparation of pPPA/SPCEs and the performance of the resulting HRP-Strept-Biotin-AMY-AMY-anti-AMY-pPPA/SPCE immunosensors were evaluated. The ratio between the currents measured for the blank (0.0 pg·mL^{-1} AMY) and for 1.25 pg·mL^{-1} AMY was taken as the selection criterion for each tested variable. The optimization of variables involved evaluation of: a) the strategy for covalent immobilization of anti-AMY; b) anti-AMY loading on pPPA/SPCEs; c) concentration of the blocking agent BSA; d) time for competition between AMY and Biotin-AMY; e) HRP-Strept loading on Biotin-AMY-AMY-anti-AMY-pPPA/SPCEs. The results of these studies are summarized in Table 1. Other variables such as the number of voltammetric cycles carried out during PPA electropolymerization on SPCEs or the concentration of PPA monomer were the same than those optimized previously [14]. Furthermore, the reduction currents from benzoquinone (BQ) generated in the HRP enzyme reaction with HQ at the SPCE were measured at a detection potential of −200 mV vs Ag pseudo-reference electrode, which was that previously selected for this catalytic system [15].

Fig. 2a compares the amperometric responses of the HRP-Strept-Biotin-AMY-AMY-anti-AMY-pPPA/SPCE immunosensor prepared by covalent immobilization of the capture antibody, anti-AMY, by using two different strategies, the usual procedure based on activation of carboxylic moieties existing on the pPPA-modified SPCE by EDC/NHSS chemistry, and the methodology using Mix&Go™ polymer. As it can be seen, this latter approach allowed an enhanced response for the solution containing no AMY) and, therefore, a larger difference vs. the amperometric current measured for 1.25 pg·mL^{-1} AMY, thus demonstrating the suitability of coupling the use of pPPA with Mix&Go™ for the immobilization of the capture antibody.

Fig. 2b shows the effect of the HRP-Strept loading onto the Biotin-AMY-AMY-anti-AMY-pPPA/SPCE on the amperometric response of different immuno-sensors prepared from pPPA/SPCEs modified with 10 μL Mix&Go™, 5 μL 1/100 diluted anti-AMY, 7.5 μL 2% BSA, 0 (dark grey) or 1.25 (white) pg·mL^{-1} AMY, 5 μL 1 ng·mL^{-1} Biotin-AMY, and HRP-Strept dilution over the 1/250–1/2000 range. Both the current measured in the absence of AMY and in the presence of 1.25 pg·mL^{-1} antigen decreased with the conjugate dilution. Larger current ratios were obtained for a 1/500 dilution which, accordingly was chosen for further work. Regarding the time for competition between AMY and Biotin-AMY for the binding sites of the antibody, Fig. 2c shows a progressive increase in the amperometric response of the immunosensor for solutions with no AMY along with an almost constant current measured in the presence of 1.25 pg·mL^{-1} AMY. Considering the larger signals ratio as well as the shorter time for analysis, 45 min was selected to allow competition to proceed.

The step by step preparation of the immunosensor was monitored by electrochemical impedance spectroscopy (EIS) and cyclic voltammetry. Figure 3a shows the Nyquist plots recorded in a 2 mM $[Fe(CN)_6]^{4-/3-}$ solution in 0.05 M phosphate buffer of pH 6.0. As expected, pPPA/SPCE (curve 2) exhibited a larger charge transfer resistance (R_{CT} = 7835 Ω) than the bare SPCE (curve 1) with R_{CT} = 1226 Ω, due to both the lower conductivity of the polymer and the electrostatic repulsion between the dissociated pPPA carboxilic groups on the electrode surface at the working pH and the negatively charged redox probe [16]. The further addition of Mix&Go provoked a remarkable decrease in the R_{CT} value up to 978 Ω (curve 3) probably due to charges neutralization. Successive increases in the R_{CT} values were observed when anti-AMY (curve 4) and BSA (curve 5) were incorporated on the electrode, with R_{CT} values of 1723 Ω and 2342 Ω respectively, due to the insulating nature of the biomolecules. Interestingly, the incorporation of the biotinylated antigen (Biotin-AMY) and the subsequent formation of the complex with the HRP-

Table 1 Optimization of the different experimental variables involved in the functioning of the competitive immunosensor for the determination of AMY

Variable	Studied range	Selected value
Loading of anti-AMY, dilution	1/100–1/200	1/100
BSA concentration, %	1–5	2
Time for competition AMY-Biotin-AMY, min	15–60	45
HRP-Strept dilution	1/250–1/2000	1/500

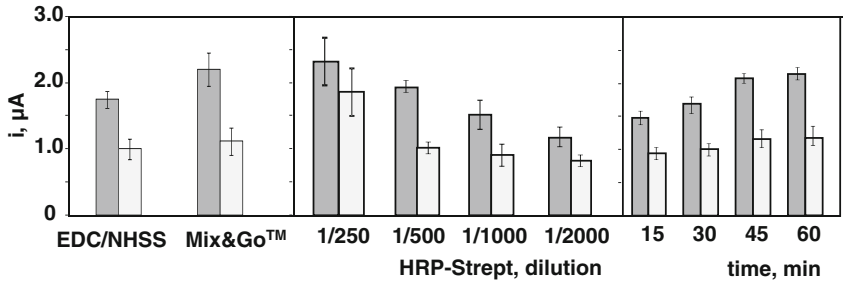

Fig. 2 Amperometric responses measured in the absence (dark grey) or in the presence of 1.25 pg·mL^{-1} AMY (white) as a function of the strategy used for covalent immobilization of anti-AMY (**a**), HRP-Strept dilution (**b**), and competition time between AMY and Biotin-AMY for the binding sites of antibody (**c**). 10 μL 100 mM EDC/NHSS, 30 min (**a**); 10 μL Mix&Go™, 60 min; 5 μL 1/100 diluted anti-AMY, 60 min; 7.5 μL 5% BSA, 30 min; 5 μL 1.25 AMY + 2.5 ng·mL^{-1} Biotin-AMY, 60 min (**a**,**b**); 1/500 diluted HRP-Strept, 20 min (**a**,**c**)

Strept conjugate (curves 6 and 7, respectively) produced Nyquist plots with a different shape similar to that of a conductive surface. This behavior can be explained by assuming the presence of positively charged biotin on the biotin-AMY-anti-AMY-pPPA/SPCE at the working pH (the pKa value of biotin is 4.51 [17]), thus leading to electrostatic attraction with the electrochemical probe and favoring the charge transfer. This effect seems to be sufficiently strong to avoid a noticeable increase in R_{CT} upon the incorporation of the streptavidin conjugate.

The behavior observed by EIS is corroborated by cyclic voltammetry (Fig. 3b). The voltammogram of the bare SPCE (curve 1) showed the characteristic oxidation and reduction peaks of the redox pair at potential values of +0.24 and + 0.16 V, respectively, with anodic and cathodic peak currents of 72 and 60 μA. The subsequent modification of SPCE with pPPA (curve 2) caused a decrease in the peak currents, and a larger peak potentials separation attributed to the low conductivity of the polymer and the electrostatic repulsion effect commented above. Incorporation of Mix&Go (curve 3) gave rise to a voltammetric behavior closer to that of the unmodified electrode due to minimization of charge repulsion. Voltammograms 4 and 5 corresponding to the successive incorporation of anti-AMY and Biotin-AMY, show slight changes towards a lesser reversible behavior as a consequence of the presence of insulating layers with increased thickness on the electrode surface. The behavior exhibited by voltammograms 6 and 7 is in agreement with that observed by EIS.

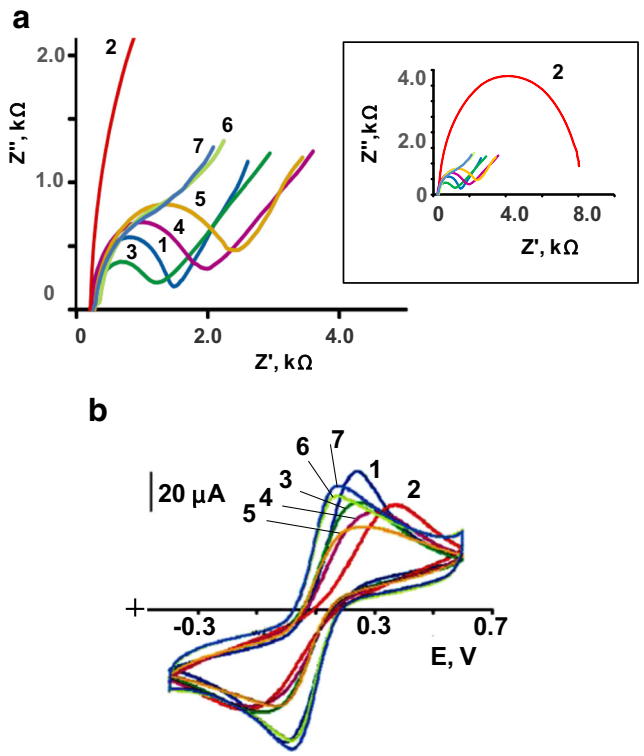

Fig. 3 Nyquist plots (**a**) and cyclic voltammograms (**b**) recorded in 2 mM $[Fe(CN)_6]^{3-/4-}$ in 0.05 M phosphate buffer of pH 3.0: SPCE (1); pPPA/SPCE (2); Mix&Go-pPPA/SPCE (3); anti-AMY-pPPA/SPCE (4); BSA/anti-AMY-pPPA/SPCE (5); Biotin-AMY-anti-AMY-pPPA/SPCE (6); HRP-Strept-Biotin-AMY-anti-AMY-pPPA/SPCE (7)

Analytical figures of merit

A calibration plot for AMY was constructed with the HRP-Strept-Biotin-AMY-anti-AMY-pPPA/SPCE immunosensor under the optimized working conditions at a potential of −200 mV vs Ag pseudo-reference electrode, over the 10^{-5} - 10^{4} pg·mL^{-1} range (Fig. 4). Current vs. AMY concentration curve was fitted by non-linear regression using the Sigma-Plot data analysis software. The adjusted equation ($R^2 = 0.994$) was:

$$i = i_{min} + \frac{i_{max} - i_{min}}{1 + \left(\frac{x}{EC_{50}}\right)^{-h}}$$

where i_{max} and i_{min} were the maximum and minimal current values of the calibration graph, 2.07 ± 0.05 μA and 0.26 ± 0.6 μA, respectively. The EC_{50} value, which is the AMY concentration corresponding to a 50 % competition, was 0.44 ± 0.1 pg·mL^{-1}, and the Hill slope at the inflection point of the sigmoid curve was h = −0.35 ± 0.04. The range of linearity ($R^2 = 0.99$) extended between 1.0 fg·mL^{-1} and 50 pg·mL^{-1}. This range is suitable for the determination of

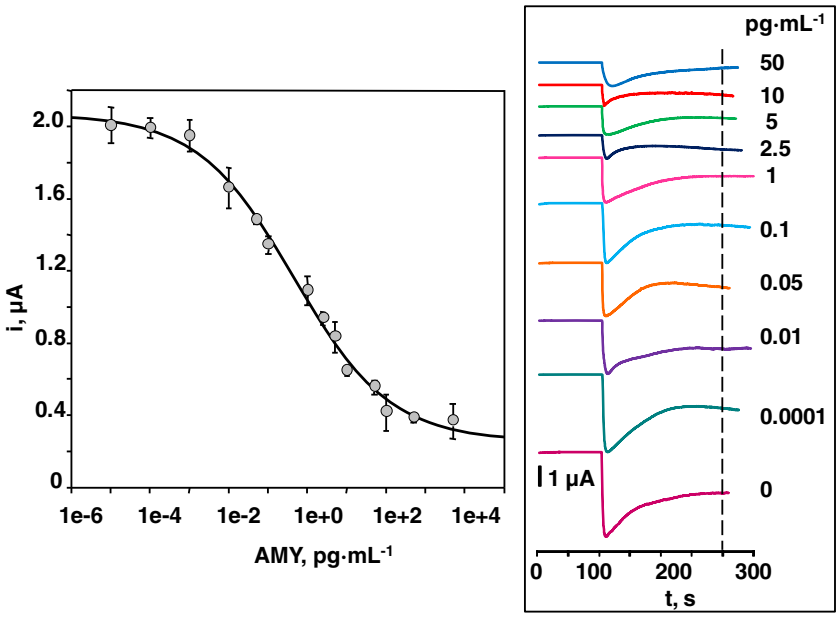

Fig. 4 Calibration plot for AMY and amperograms at HRP-Strept-Biotin-AMY-AMY-anti-AMY-pPPA/SPCE. Dotted line indicates the time of measurement. Working potential: −200 mV vs Ag pseudo.reference electrode. See the text for the other conditions

AMY in human serum since, as it was indicated in the Introduction section, serum concentration of AMY ranges around few tens of pg·mL^{-1} [4, 9, 10]. The limit of detection, 0.92 fg·mL^{-1}, was calculated from the equation:

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

where s is the standard deviation ($n = 10$) of the zero value (the amperometric current measured in the absence of AMY), ± 0.12 μA. It is important to remark that this LOD value is much lower than that claimed as the minimum detectable concentration of AMY for commercial ELISA kits using similar immunoreagents: 0.62 ng·mL^{-1} [https://www.raybiotech.com/files/manual/EIA/EIA-AMY.pdf] and 6.17 pg·mL^{-1} [https:// www.lsbio.com/elisakits/manualpdf/ls-f9686.pdf]. It is also worth to mention that the linear range extends over almost five orders of magnitude which is much wider than those claimed for the ELISA methods with dynamic ranges from 1 to 1000 ng·mL^{-1} [https://www.raybiotech.com/files/manual/EIA/EIA-AMY.pdf] and 6.17 to 500 pg·mL^{-1} [https://www.lsbio.com/elisakits/manualpdf/ls-f9686.pdf]. The high sensitivity achieved with this immunosensor is attributed to the presence of the polymer used as modifier of the electrode surface. The deposited porous conducting polymer allows large amperometric currents to be measured for the detection of the electroactive product, hydrogen peroxide mediated by the hydroquinone system. In addition, the polymer provides a high concentration of carboxyl groups confined on the electrode surface thus allowing the immobilization of a large capture antibody loading which, in turn, allows a high analyte concentration to compete with the biotinylated antigen for antibody binding. Therefore, the amount of HRP-Strept conjugate becomes high for very low amylin concentrations allowing large current signals as well as large current differences for different concentrations of antigen. Moreover, the time lasted for the assay is remarkably longer with the RayBiotech ELISA kit extending over 3 h 15 min and similar to the ELISA kit from LifeSpan BioSciences Inc. (the time needed with the immunosensor is 1 h 50 min counting in all cases since the immobilization of the capture antibody). In addition, the simplicity and ease of use, together with the possibility for developing multiplexed detection make the immunosensor highly attractive compared to ELISA conventional methodologies.

The reproducibility of the amperometric measurements for both 0 and 0.1 pg·mL^{-1} AMY was tested with five different immunosensors prepared on the same day. The relative standard deviation (RSD) values were 5.9 and 5.5%, respectively.

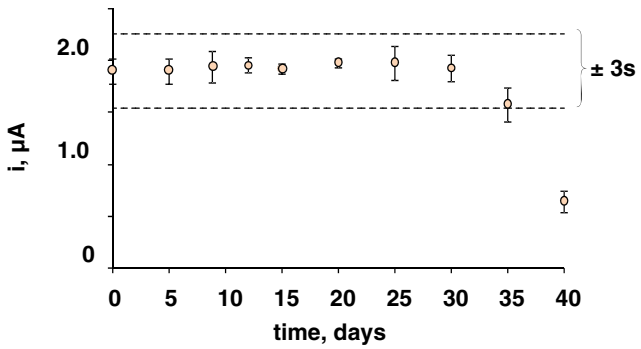

Fig. 5 Control chart constructed to check the storage stability of BSA/anti-AMY-pPPA/SPCE bioelectrodes upon storage at −20 °C in dry ambient. The central value was set as the average amperometric current for ten measurements of different solutions in absence of AMY. Upper and lower limits of control were set as three times the standard deviation of these measurements

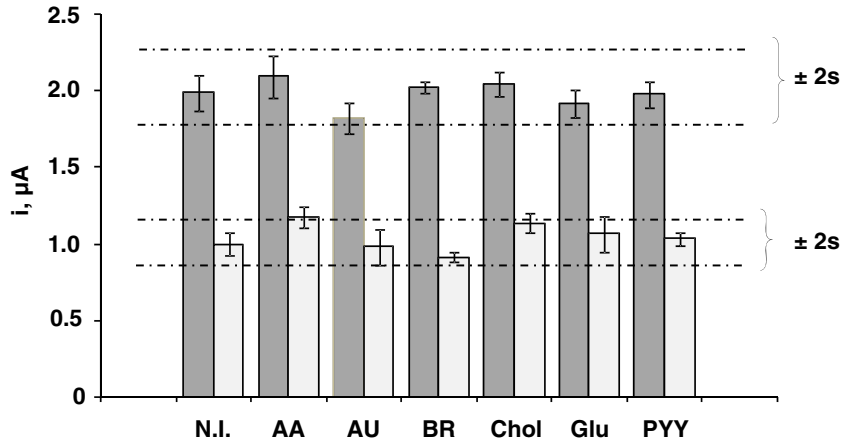

Fig. 6 Effect of the presence of 370 µg/mL ascorbic acid (AA), 50 µg/mL uric acid (UA), 3.4 µg/mL bilirubin (BR), 20 µg/mL cholesterol (Chol), 1.3 mg/mL glucose (Glu), and 100 pg·mL^{-1} peptide YY (PYY) on the amperometric responses measured with the HRP-Strept-Biotin-AMY-AMY-anti-AMY-pPPA/SPCE immunosensor for 0.0 (dark grey) or 1.0 (light grey) pg·mL^{-1} AMY. See the text for more information

Furthermore, RSD values of 6.8 and 7.4% were calculated from measurements made with five different immunosensors prepared in different days. The storage stability of BSA/anti-AMY-pPPA/SPCEs was checked by preparing different bioelectrodes on the same day and stored at −20 °C under dry ambient. Then measurements were made with the HRP-Strept-Biotin-AMY-anti-AMY-pPPA/SPCE immunosensor in the absence of AMY on different days according to the procedure described in Sections 2.3.1 and 2.3.2. The control chart constructed by setting ±3 s as control limits, where s was the standard deviation of the measurements ($n = 10$) carried out the first day of the assay (Fig. 5) shows that the immunosensor responses remained inside the control limits for 35 days, thus demonstrating a very good storage stability of the prepared bioelectrode.

Selectivity

The selectivity of the prepared immunosensor was tested by measuring the amperometric responses for 0.0 and 1.0 pg·mL^{-1} AMY in the absence and in the presence of potentially interfering compounds and non-target proteins. The species tested and their respective concentration level were: ascorbic acid (AA, 370 µg/mL), uric acid (UA, 50 µg·mL^{-1}), bilirubin, (BR, 3.4 µg·mL^{-1}), cholesterol (Chol, 20 µg·mL^{-1}), glucose (Glu, 1.3 mg·mL^{-1}), and peptide YY (PYY, 100 pg·mL^{-1}). These concentrations corresponded approximately to the normal physiological level of the tested compound in human serum. Figure 6 shows as no significant different currents were measured in all cases because all mean values of the measured steady state currents were within the ±2 x standard deviation range of the current measured in the absence of interferent.

Determination of AMY in urine and serum

The usefulness of the immunosensor was tested by analyzing AMY in urine and serum. The possibility of the existence of matrix effect was evaluated by measuring samples spiked with AMY in the range from 0.001 to 1 pg·mL^{-1} (urine) and from 0.001 to 10 pg·mL^{-1} (serum) diluted with different volumes of Assay Diluent B. The results (not shown) revealed that a 1/10 dilution for urine and 1/5 for serum were enough to avoid the matrix effect observed with the undiluted samples. The calibration plots for diluted urine and serum samples were very similar to that of standard AMY solutions with linear ranges between 0.001 and 10 pg·mL^{-1} and slope values of −327 ± 22 (urine) and − 336 ± 12 (serum) µA per decade of concentration. The comparison of these slopes with the slope value for the linear range of calibration for AMY standard solutions, 307 ± 8 µA per decade of concentration, was performed by application of the Student t test for $\alpha = 0.05$ and $n = 12$, obtaining $t_{exp} = 0.832$ and 2.005 for urine and serum, respectively, which are lower than the tabulated value, $t_{tab} = 2.179$. Therefore, the determination of AMY in urine and serum can be performed directly by interpolation of the amperometric current measured with the immunosensor for an aliquot of the diluted sample into the calibration plot constructed with standard solutions. Table 2 summarizes the results in the

Table 2 Determination of amylin in urine and serum samples

Sample	[AMY], pg·mL^{-1}	[AMY] found, pg·mL^{-1}*	Recovery, %
Urine	0.75	0.75 ± 0.05	100 ± 7
	4.0	4.1 ± 0.2	102 ± 4
Serum	1.5	1.4 ± 0.1	94 ± 7
	8.0	8.0 ± 0.4	100 ± 6

*$t_{tab} \cdot s / \sqrt{n}$; $n = 8$

analysis of urine spiked with AMY at 0.75 and 4.0 pg·mL^{-1} concentration levels, and serum spiked at 1.5 and 8.0 pg·mL^{-1} levels. Recoveries ranged between 93 and 102%, thus demonstrating the usefulness of the immunosensor for the analysis of AMY at low concentration levels in urine and serum with minimal sample treatment.

Conclusions

The first electrochemical immunosensor for the determination of the hormone related to adiposity amylin is reported in this work. The disposable immunosensor design implies SPCE functionalized with electropolymerized poly(pyrrole propionic acid) thus providing a high loading of surface confined carboxyl groups suitable for direct covalent binding of anti-AMY antibody upon activation with Mix&Go™ polymer. This design demonstrates to be a successful strategy to be used as a general route for preparing electrochemical immunosensors. The competitive immunoassay involving biotinylated AMY and streptavidin labelled with HRP as the enzymatic tracer was able to provide a calibration graph for AMY with a range of linearity extending from 1.0 fg·mL^{-1} to 50 pg·mL^{-1}, and a detection limit of 0.92 fg·mL^{-1}. It is important to mention that this value is around 7000 times lower than the minimum detectable concentration reported for the commercial ELISA immunoassays for this target hormone. In addition, the immunosensor exhibits an excellent selectivity and shows applicability for the analysis of AMY at low concentration levels in urine and serum with minimal sample treatment and in a shorter assay time that that required for ELISA kits.

Acknowledgements The financial support of projects CTQ2015-70023-R (Spanish Ministry of Economy and Competitivity Research Projects), and S2013/MT-3029 (NANOAVANSENS Program from the Comunidad de Madrid) are gratefully acknowledged.

Compliance with ethical standards The authors declare that they have no competing interest.

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