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Multiplexed electrochemical immunosensing of obesity-related hormones at grafted graphene-modified electrodes

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ABSTRACT

An electrochemical immunosensor was prepared for the simultaneous determination of two hormones, ghrelin (GHRL) and peptide YY (PYY), which play important roles in the regulation of hunger and saciety. Dual screen-printed carbon electrodes modified with reduced graphene oxide (rGO) were used as scaffolds for the immobilization of the corresponding capture antibodies. Grafting of the diazonium salt of 4-aminobenzoic acid (4-ABA) on the modified electrode surfaces allowed covalent immobilization of antibodies. Competitive immunoassays were employed and the affinity reactions were monitored by differential pulse voltammetry upon addition of 1-naphthyl phosphate. Under the optimized working conditions, linear current vs. log [hormone] plots extending between 10^{-3} and 100 ng/mL GHRL ($r^2 = 0.990$), and 10^{-4} and 10 ng/mL PYY ($r^2 = 0.992$) were obtained. These ranges are adequate for the determination of both hormones at clinical levels in serum and saliva. An excellent analytical performance in terms of detection limit, reproducibility of the measurements, storage stability and selectivity against other proteins was achieved. The usefulness of the approach was demonstrated by its application to spiked human serum and saliva.

1. Introduction

Among the various hormones involved in the complex regulation of hunger and saciety, ghrelin and peptide YY are noteworthy since they play important roles at the levels of hypothalamus and peripheral circulation [1–3]. Ghrelin (growth hormone-releasing peptide, GHRL) is a peptide hormone containing 28 amino acids secreted in the stomach that acts in the gastrointestinal tract as a stimulant of motor activity, acid secretion and gastric contractility [4]. GHRL levels have shown to be increased before meals to concentrations sufficient to stimulate appetite, and then decreasing [5]. Peptide YY (peptide tyrosine tyrosine, PYY) consisted of 36-amino-acid peptide with a carboxy-terminal tyrosine amide synthesized by endocrine cells in the lower intestine. It acts as a regulator of pancreatic and gastrointestinal functions [6], and plays an important role upon saciety by limiting meal size and overall calorie intake [7,8]. Conversely to GHRL, peaks of increased PYY concentrations appear after meals [9]. From a clinical point of view, the levels of these hormones have been found to be elevated in anorexic patients, while lower levels appear in obese subjects [10].

GHRL concentration in plasma shows great variability depending on the individual and also on the method used for its determination. Values of several units [11] or tenths of ng/mL [12,13] have been found in healthy individuals. In addition, GHRL levels undergo postprandial falls and preprandial rises in circulating levels with peaks around 700 pg/mL in breakfast, lunch and dinner [13]. Regarding PYY, concentration levels in plasma are around 40–70 pg/mL [2]. In this case, variability in circulating levels is lower, with postprandial peaks reaching values around a hundred pg/mL [9].

It is also important to highlight that both hormones can be determined in saliva, this making this fluid, whose extraction is not invasive, a convenient and alternative sample for diagnosis. Salivary concentrations of 183–190 pg/mL GHRL [13] or 15–75 pg/mL PYY [14] have been reported in the literature.

Despite their clinical relevance, methods for determining these hormones are scarce. Various colorimetric ELISA kits for GHRL using competitive or sandwich-type assays with anti-GHRL, biotinylated immunoreagents, and streptavidin labeled with peroxidase are commercially available. Usually, calibration plots show dynamic concentrations ranging from 0.01 (or 0.1) to 100 (or 1000) ng/mL, with minimum detectable concentrations between 0.05 and 1 ng/mL. The assay time varies from 1 h 30 min to 4 h counting from the moment when the antibody was immobilized.

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Moreover, ELISA methods based on similar immunoassay schemes for PYY are also available. Competitive configurations involving specific PYY antibodies or biotinylated PYY binding, as well as HRP-labeled avidin or streptavidin conjugates, are the most common. These assays allow the determination of PYY in concentration ranges from 0.1–1 pg/mL to 100–1000 pg/ mL, with minimum detectable concentrations varying from 0.5 pg/mL to approximately 3 pg/mL. The times required for these assays are around 2.5–3.5 h. A scarce number of biosensors for these hormones have been reported in the literature. An example is the colorimetric microarray detection system for GHRL using aptamers technology developed by Mascini et al. [15], with a linear range between 0.2 and 245.5 ng/mL and a detection limit of 0.2 ng/mL,. The same authors proposed also an electrochemical aptasensor involving measurement of the decrease in the guanine oxidation signal in the presence of GHRL with a linear range from 14 to 100 ng/mL and a detection limit of 8 ng/mL [16]. Recently, our group reported the preparation of an electrochemical magnetic immunosensor for GHRL involving anti-GHRL immobilization onto Protein G-magnetic beads and competitive immunoassay using biotinylated GHRL and alkaline phosphatase-streptavidin conjugate. Differential pulse voltammetry of 1-naphthol formed upon addition of 1-naphthyl phosphate allowed the determination of the hormone to be performed in a linear range between 10^{-3} and 10^{3} ng/mL, and a limit of detection of 7 pg/mL [17].

Electrochemical grafting consisting of covalent modification of carbon surfaces by aryl radicals generated from the electrochemical reduction of diazonium salts [18] has demonstrated to be an excellent strategy for immobilization of biomolecules [19–21]. Our group has reported various electrochemical immunosensors designs using such strategy by means of 4-aminobenzoic acid (4-ABA) grafted onto screen-printed electrodes [22–24]. More recently, reduced graphene oxide (rGO)/glassy carbon modified electrodes were also used as electrochemical platforms for grafting with 4-ABA to develop a voltammetric immunosensor for PYY [25]. Following a competitive immunoassay configuration, a calibration plot for PYY with a linear range extending between 10^{-4} and 10^{2} ng/mL, and a limit of detection of 0.01 pg/ mL PYY were obtained.

In this paper, we report the preparation of electrochemical immunosensors for the simultaneous determination of GHRL and PYY which satisfies the requirements of sensitivity, selectivity and reproducibility needed for clinical applications. Screen-printed electrodes modified with reduced graphene oxide (rGO) were used as scaffolds for the immobilization of capture antibodies. The diazonium salt of 4-ABA was electrochemically grafted at the electrode surfaces, resulting in the covalent attachment of 4-carboxy phenyl moiety to the rGO/SPCE. Then, the immunoreagents were covalently immobilized onto the modified electrodes, and competitive immunoassays involving the hormones and the respective biotinylated antigens were performed. Differential pulse voltammetry upon 1-naphtyl phosphate addition was employed to monitor the affinity reactions. The usefulness of the approach was demonstrated by its application to spiked human serum and saliva.

2. Experimental

2.1. Apparatus and electrodes

Electrochemical measurements with dual electrodes were carried out using a 1030 B Multi-potentiostat from CH Instruments provided with a multiplexed data adquisition circuitry (8 channels). A BAS 100B potentiostat provided with a BAS C2EF-1080 cell stand was also used for the electrochemical measurements with glassy carbon electrodes. Dual screen-printed electrodes (C 1110 DropSens) consisted of two elliptic carbon working electrodes with surface area of 5.6 mm^2, a carbon counter electrode and a silver pseudo-reference electrode. Modified 3-mm diameter CHI104 glassy carbon electrodes from CH Instruments, Ag/AgCl/

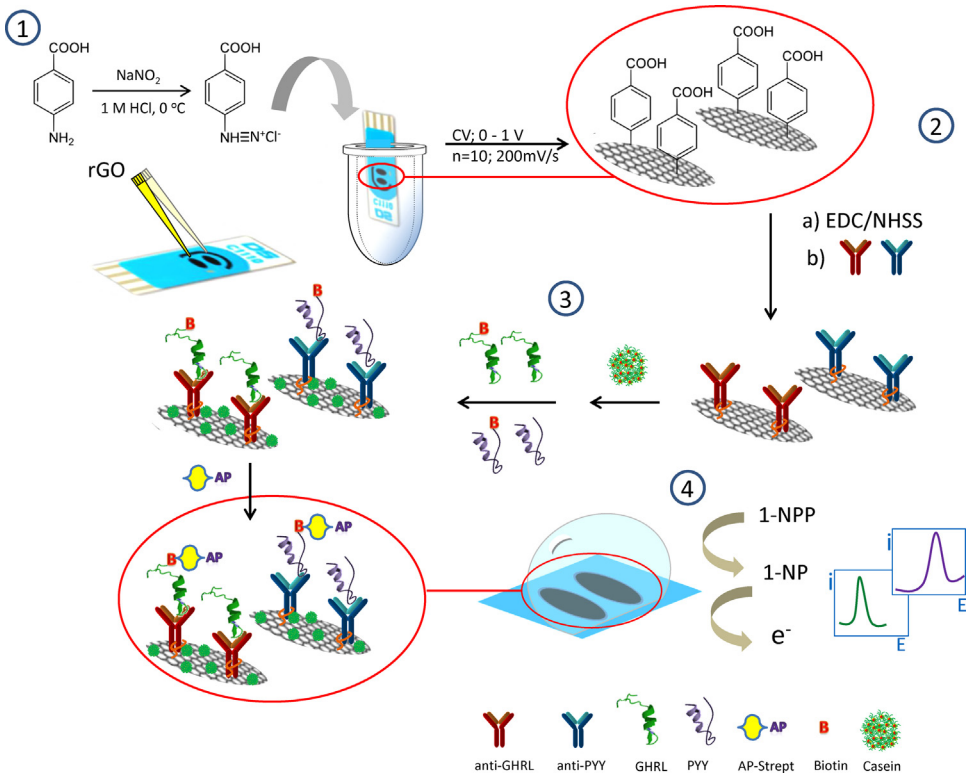

Fig. 1. Schematic display of the different steps involved in the preparation and funcioning of the dual GHRL and PYY immunosensors.