SUPPLEMENTARY MATERIAL

1. Characterization of ascorbic acid-reduced GO by UV/vis spectrophotometry

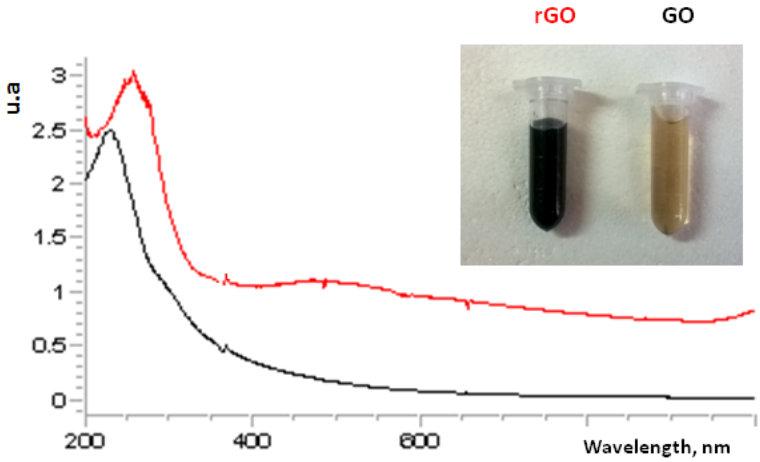

Figure S1. UV/visible spectra and pictures of unreduced GO (black) and ascorbic acid - reduced GO (red) aqueous suspensions. For more information, see Results and Discussion section in the main Manuscript.

2. Optimization of the variables affecting the preparation of modified electrodes.

a) Loading of rGO on the GCE surface

Optimization of this variable was carried out by depositing 10 µL of rGO suspensions with concentrations ranging between 0.25 and 1.0 mg/mL on the GCE. Then, different immunosensors were prepared by applying the procedure described in the Experimental section involving 4-ABA grafting and successive modification with 10 µL of 30 µg/mL anti-PYY (2 h incubation), 10 µL of 0.1 % BSA as blocking agent (1 h), 10 µL of 100 ng/mL Biotin-PYY (1 h) and, finally, 10 µL of 5 µg/mL AP-Strept (1 h). All incubation steps were performed at 37 ^{o}C. Once the immunosensors were prepared, differential pulse voltammograms were recorded according to the protocol described in the Experimental section. Figure S2a shows the specific and unspecific (in the absence of anti-PYY) responses obtained in the absence of target PYY. The immunosensor reached the maximal specific signal for a 0.5 mg/mL rGO concentration with a slight decrease for larger loadings which is probably due to a higher electron transfer resistance associated to large amounts of immobilized antibody. Conversely,

unspecific responses increased notably with rGO concentration which led us to select 0.5 mg/mL rGO as the concentration to get the largest specific/unspecific currents ratio.

b) ABA concentration

The effect of ABA concentration used for grafting at rGO/GCE was also evaluated. Figure S2b shows the responses of the immunosensors prepared similarly to that described in section (a) for three different ABA concentrations, 10, 20 and 30 mg/mL. Clearly, better signal-to-background current ration was obtained when using 20 mg/mL ABA indicating that an adequate number of carboxy phenyl radicals were grafted on the electrode surface allowing a suitable loading of the covalently immobilized antibody.

c) *Number of cycles and potential scan rate for the electrochemical grafting of ABA onto rGO/GCE*

Electrochemical grafting of carboxy phenyl radical onto rGO/GCE was carried out by immersing the modified electrode in the diazonium salt solution and performing successive cyclic voltammetric scans between 0 and -1.0 V vs. Ag/AgCl. The number of these scans was also optimized (Figure S2c). The largest specific-to-unspecific current ratio was obtained for 10 cycles. Probably, under these conditions, a sufficiently large loading of carboxyl moieties grafted onto the modified electrode surface was achieved then ensuring an appropriate covalent binding of antibodies. A higher number of voltammetric scans produced a decrease in the immunosensor responses probably as a consequence of the lower modified electrode conductivity due to an extensive grafting of carboxyl groups. Furthermore, the effect of the potential scan rate on the immunosensor responses was also tested. As it can be seen in Figure S2d, best results were achieved for v = 200 mV/s.

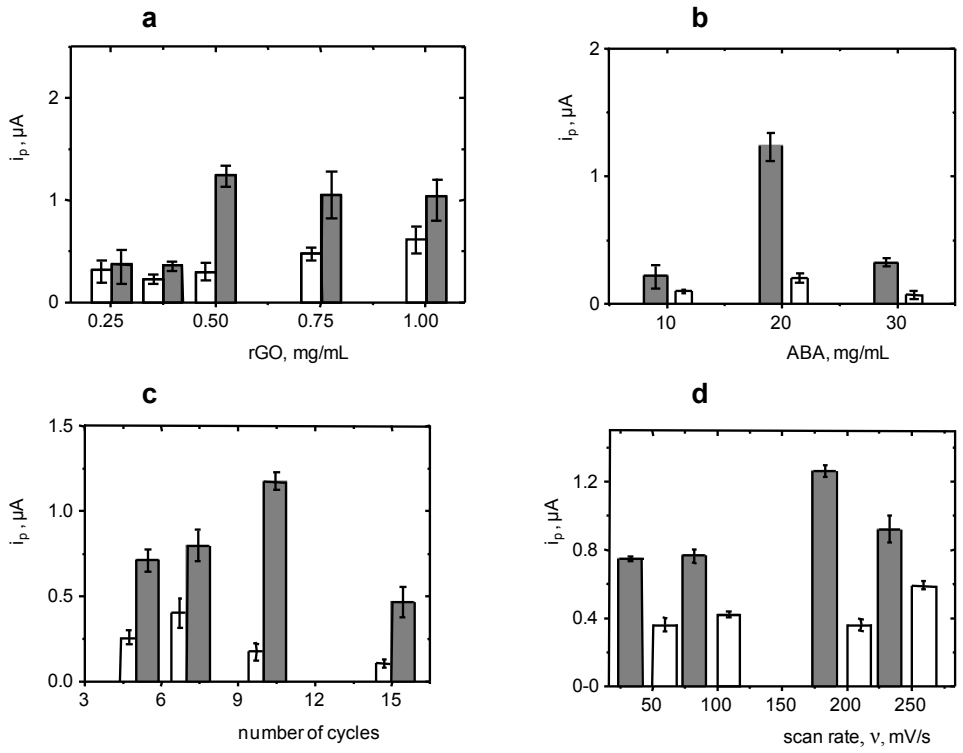

Figure S2. Effect of: the loading of rGO onto GCE (a), ABA concentration (b), the number of cycles (c) and the potential scan rate for the electrochemical grafting of ABA onto rGO/GCE electrodes (d), on the electrochemical responses of AP-Strept-Biotin-PYY-anti-PYY-Phe-rGO/GCE immunosensor in the absence of target PYY. See the text and the Experimental section in the main manuscript for more information.

3. Optimization of the experimental variables involved in the immunosensor preparation

a) Loading of anti-PYY and incubation time

The effect of the amount of immobilized antibody onto HOOC-Phe-rGO/GCE on the immunosensor voltammetric response was tested using different anti-PYY concentrations in the 5 to 30 µg/mL range. All other immunoreagent concentrations and incubation times are the same that those specified in paragraph 2a. Figure S3a shows as the specific responses increased with the antibody concentration up to 20 µg/mL then levelling off for larger loadings. The small unspecific responses exhibited negligible change over the whole concentration range. Accordingly, 20 µg/mL anti-PYY was selected for further work. Regarding the antibody incubation time (Figure 2b), the largest specific-to-unspecific current ratio was obtained for 60 min, this value being selected for the preparation of the anti-PYY-Phe-rGO/GCE conjugates.

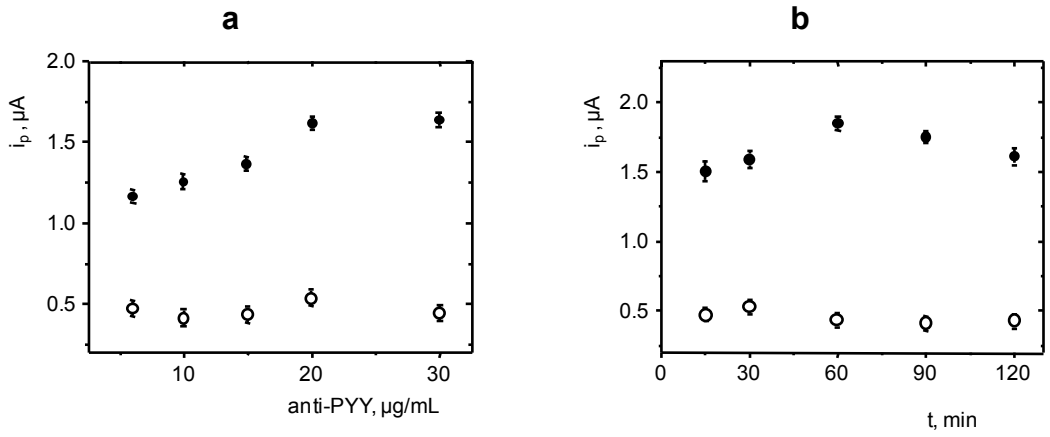

Figure S3. Effect of: the anti-PYY loading (a) and the time for incubation of anti-PYY onto Phe-rGO/GCE (b) on the electrochemical responses of the AP-Strept-Biotin-PYY-anti-PYY-Phe-rGO/GCE immunosensor in the absence of target PYY. See the text and the Experimental section in the main manuscript for more information.

b) Optimization of the blocking step

In order to minimize unspecific adsorptions of immunoreagents on the electrode surface, various blocking strategies were evaluated. Although previous optimization studies were made using 0.1 % BSA as the blocking agent, other suitable reagents for this purpose (ethanolamine (ETA) and casein in addition to BSA) were tested. The protocol consisted of adding 10 µL of a 0.1% blocking solution onto the anti-PYY-Phe-rGO/GCE allowing incubation for a pre-established time of 1 h at 37 ^{o}C. The results are shown in Figure S4a and revealed that both specific and unspecific responses were much larger when ETA was used, probably as a consequence of the instability of the electrode coating with this compound. The best specific-to-unspecific responses ratio was achieved using casein and, therefore, it was selected as the blocking agent for further work. Its concentration and time for incubation were also checked. Figure S4b shows as the largest specific-to-unspecific ratio was found for a 0.2% casein concentration. Moreover, 60 min incubation allowed a specific response to be obtained more than 5 times larger than that corresponding to nonspecific interactions (Figure S4c).

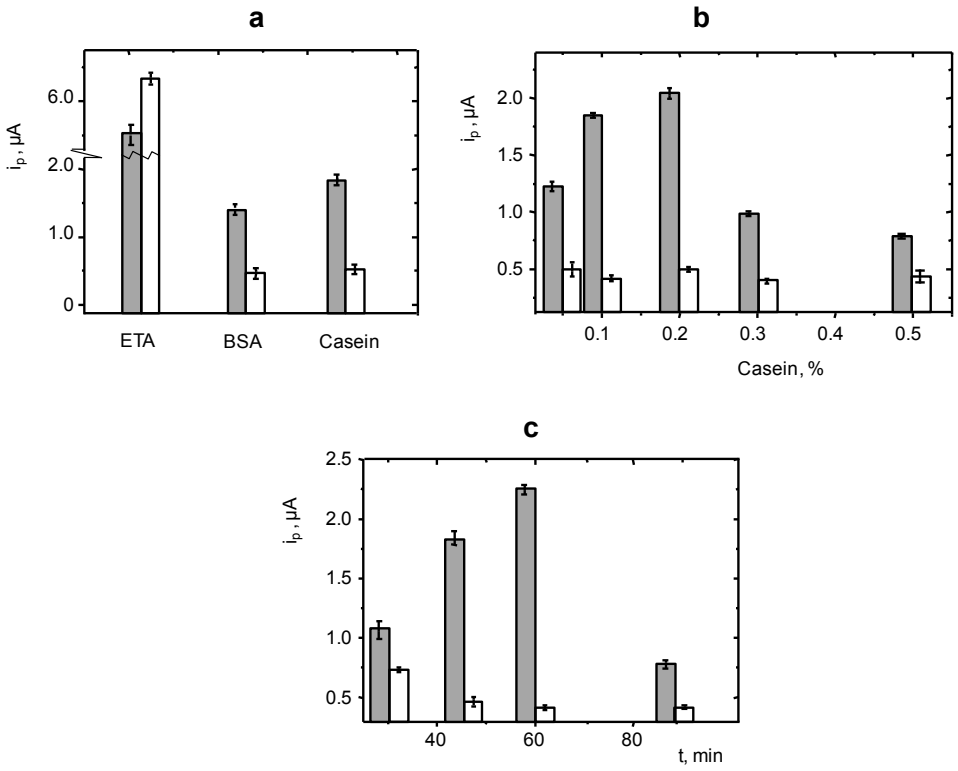

Figure S4. Effect of: the type of blocking agent used (a), the concentration of casein (b) and the time for incubation of casein (c) onto anti-PYY-Phe-rGO/GCE, in the electrochemical responses of AP-Strept-Biotin-PYY-anti-PYY-Phe-rGO/GCE immunosensor in the absence of target PYY. See the text and the Experimental section in the main manuscript for more information.

c) Loading of Biotin-PYY and incubation time

Different AP-Strept-Biotin-PYY-anti-PYY-Phe-rGO/GCE immunosensors were prepared by incubating anti-PYY-Phe-rGO/GCEs in Biotin-PYY solutions with concentrations ranging between 50 and 400 ng/mL. The measured DPV peak current values increased sharply with the Biotin-PYY concentration from 50 to 100 ng/mL and then leveled off, whereas the unspecific currents practically did not vary over the whole tested concentrations range (Figure S5a). Accordingly, 100 ng/mL was selected for further work. In addition, 30 min was shown to be a sufficient incubation time in the Biotin-PYY solution (Figure S5b).

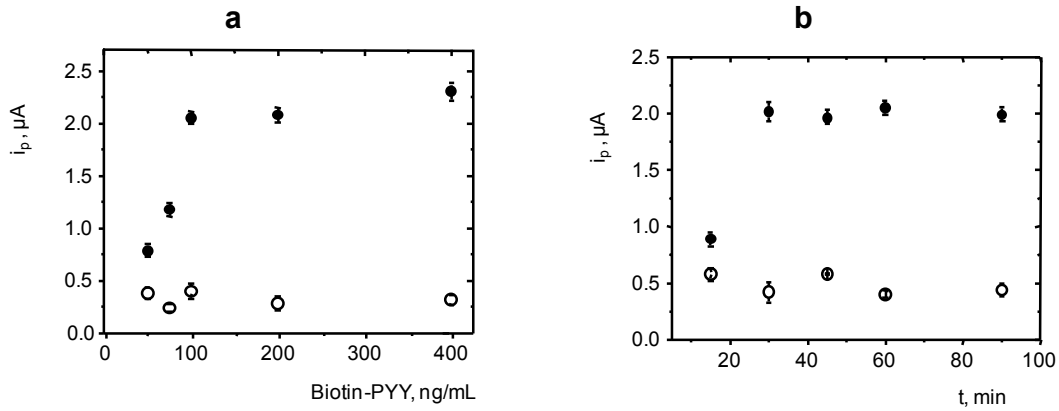

Figure S5. Effect of: the loading of Biotin-PYY (a), and the time for incubation of Biotin-PYY onto anti-PYY-Phe-rGO/GCE (b), on the electrochemical responses of AP-Strept-Biotin-PYY-anti-PYY-Phe-rGO/GCE immunosensors in the absence of target PYY. See the text and the Experimental section in the main manuscript for more information.

d) Loading of AP-Strept and incubation time.

The effect of the AP-streptavidin loading was checked by constructing calibration plots for PYY over the 10^{-6} (or 10^{-7}) to 10^{3} ng/mL concentration range, with AP-Strept-Biotin-PYY-anti-PYY-Phe-rGO/GCE immunosensors prepared with 7.0, 5.0 and 2.5 µg/mL AP-Strept. As it can be seen in Figure S6a, the largest used AP-Strept loading did not provide a useful calibration graph probably due to saturation of the bioelectrode and a decrease in conductivity. Although 2.5 µg/mL AP-Strept allowed responses varying with PYY concentration in a wide range, the largest slope value for the linear portion of the calibration plot was obtained using 5.0 µg/mL AP-Strept and then this value was selected for further work. Regarding the time for incubation with of this labeled protein, Figure S5b shows that 30 min was an appropriate time to obtain a good specific-to-unspecific current ratio.

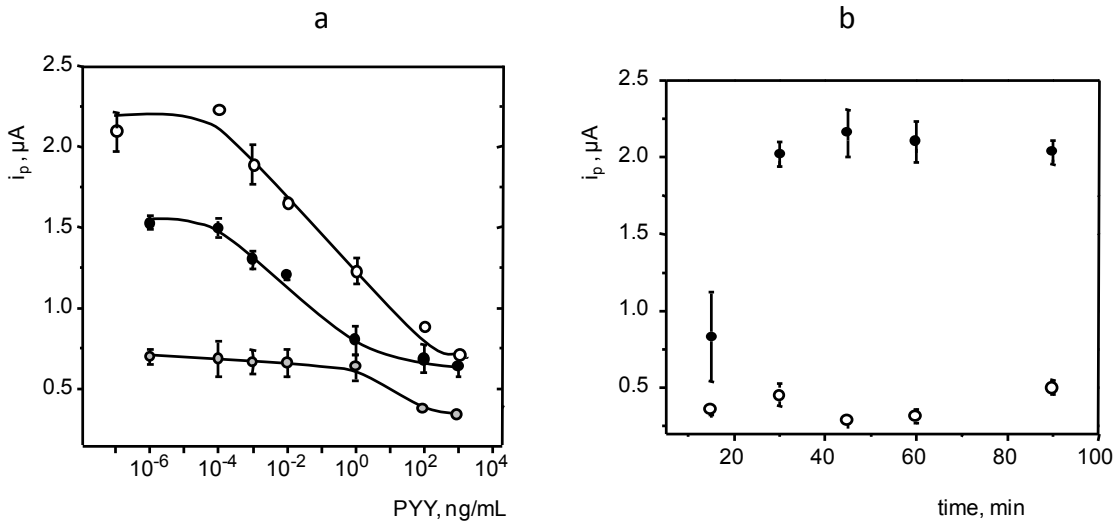

Figure S6. Effect on the electrochemical responses of AP-Strept-Biotin-PYY-anti-PYY-Phe-rGO/GCE immunosensors in the absence of target PYY of: the 5.0 (white dots) 2.5 (black), and 7.0 (grey) µg/mL AP-Strept loading (a), and the time for incubation of AP-Strept onto Biotin-PYY-anti-PYY-Phe-rGO/GCE (b). See the text and the Experimental section in the main manuscript for more information.

e) Concentration of 1-NPP and time for the enzyme reaction to proceed

The influence of AP substrate concentration on the differential pulse voltammetric responses measured with the AP-Strept–Biotin-PYY-anti-PYY-Phe-rGO/GCE immunosensor was also studied. Figure S7a shows as the largest peak current value was obtained for 5 mM 1-NPP, which represents a relatively high concentration then ensuring that the enzyme reaction rate depended only on the enzyme concentration. Moreover, Figure S7b shows that 5 min were sufficient to allow the reaction catalyzed by AP enzyme to be proceeded.

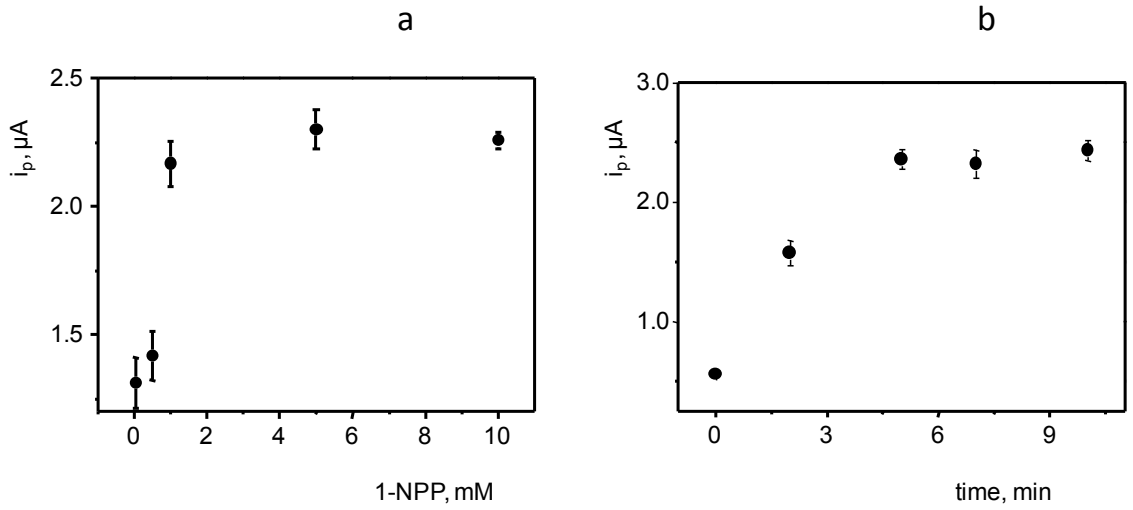

Figure S7. Effect on the electrochemical responses of AP-Strept-Biotin-PYY-anti-PYY-Phe-rGO/GCE immunosensors in the absence of target PYY of: the concentration of 1-NPP (a), and the time for the enzyme reaction to proceed (b). See the text and Experimental section in the main manuscript for more information.

Table S1. Optimization of the experimental variables affecting the preparation of grafted ABA-rGO/GCE and the performance of the AP-Strept-Biotin-PYY-anti-PYY-Phe-rGO/GCE immunosensors

Variable	Tested range	Selected value
rGO, mg/mL	0.25 - 1.0	0.5
ABA, mg/mL	10 - 30	20
Number of CV cycles	5 - 15	10
Potential scan rate, mV/s	50 - 250	200
anti-PYY, µg/mL	5 - 30	20
Incubation time for anti-PYY, min	15 - 120	60
Blocking agent type	Ethanolamine, BSA, casein	casein
Casein, % (w/v) in PBS	0.05 - 0.5	0.2
Incubation time for blocking, min	30 - 90	60
Biotin-PYY, ng/mL	50 - 400	100
Incubation time for Biotin-PYY, min	15 - 90	30
AP-Strept, µg/mL	2.5 - 7	5
Incubation time for AP-Strept,	15 - 90	30

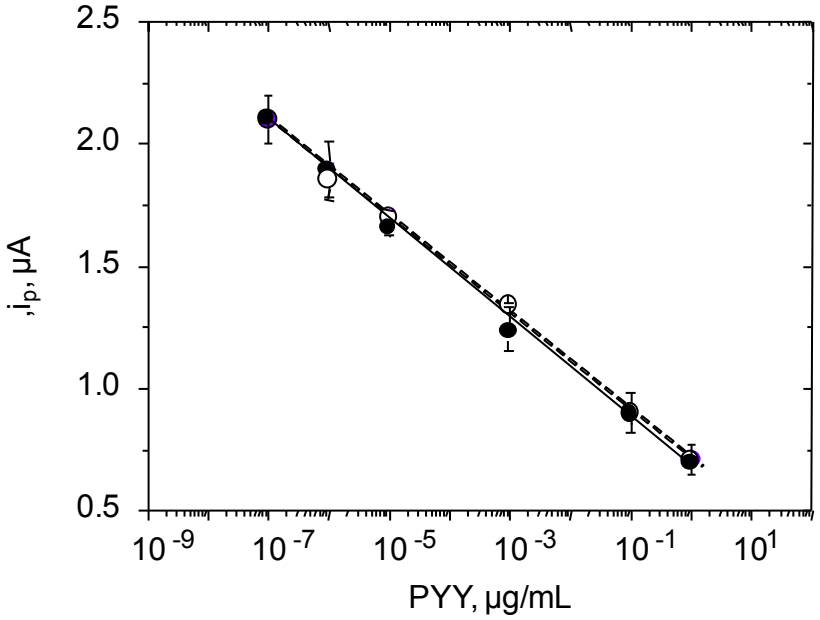

Figure S8. Calibration plots for PYY at the AP-Strept-Biotin-PYY-anti-PYY-Phe-rGO/GCE immunosensor: (—•—) standard PYY solutions; (**- - o - -**) serum samples.