Electronic Supplementary Information

A modular electrochemical peptide-based sensor for antibody detection

Mihaela Puiu[a], Andrea Idili[b], Danila Moscone[b], Francesco Ricci*[b] and Camelia Bala*[a,c]

[a]*Laboratory for Quality Control and Process Monitoring, University of Bucharest, 030018-Bucharest, Romania*
[b]*Dipartimento di Scienze e Tecnologie Chimiche, University of Rome Tor Vergata, Rome, Italy*
[c]*Department of Analytical Chemistry University of Bucharest, 030018-Bucharest, Romania; email: camelia.bala@g.unibuc.ro; tel./fax: +40214104888*

Experimental

Apparatus and Reagents

Voltammetric measurements (cyclic-, alternating current- and square wave: CV, ACV, SWV) were performed on Autolab PGSTAT100 apparatus (Eco Chemie BV, Utrecht, The Netherlands) using GPES 4.9 software in a standard 3 electrodes-electrochemical cell with a rod gold working electrode (99.9% 2 mm diameter BAS, West Lafayette, IN, USA), a platinum wire (CH Instruments) as the counter electrode and an Ag/AgCl reference electrode saturated with 3 M NaCl (BAS, West Lafayette, IN, USA.) Surface plasmon resonance (SPR) measurements for the peptide auto-assembly onto the gold surface were carried out using double channels AUTOLAB ESPRIT equipment (Eco Chemie BV, Utrecht, The Netherlands) operating at a constant temperature of 25^{o}C.

Reagent-grade chemicals: 6-mercapto -1-hexanol (6-MH), 1,6-hexandithiol (1,6-HDT), acetonitrile (AcN), N,N-dimethylformamide, 1-ethyl-3- (3-dimethylaminopropyl carbodiimide) hydrochloride (EDC), 4-dimethylaminopyridine (DMAP), absolute ethanol, formic acid, sodium phosphate monobasic, dibasic, sodium chloride, potassium chloride, sodium bicarbonate, potassium hydroxide, potassium hexacyanoferrate(III) and sodium dodecyl sulphate (SDS) were obtained from Sigma Aldrich. Alumina slurry (water suspension of Al_2O_3 of 0.3 and 0.5 μm particle size) was purchased from BAS. The acid piranha solution was a mixture 3:1 (v/v) of H_2SO_4 (purity > 95%, Fluka) and H_2O_2 (30% w/w, Merck). Adult bovine serum, sterile filtered, was also purchased from Sigma Aldrich. Monocarboxy Methylene Blue-Succinimidyl ester (3,7-Bis(N-(3-carboxypropyl)-N-methylamino)-phenothiazin-5-ium perchlorate) was purchased from EMP-BIOTECH. Support peptide sequence X-YAAAHAEAR-NH_2 (M.W = 1146.4) – acronym SP, where X is the α-lipoic acid, obtained from Abgent Europe, UK (with a mass spectroscopy –determined purity > 98%) was used without subsequent purification. Deamidated alpha2-gliadin peptide (DGP), 33 mer, (Acetyl-aa56-58, amide, Q65E) - N-and C-terminal protected, Ac-QLQPFPQPELPYPQPQLPYPQPQLPYPPQPQPF-NH_2, (M.W. = 3953.5), purity >95% was obtained from Zedira GmbH. Gliadin Peptide (14D5) mouse monoclonal antibody (IgG) (M.W. = 150,000) was obtained form ABBIOTEC, San Diego USA as 1 mg/mL IgG solution in phosphate buffer saline at pH 7.4 with 0.09% sodium azide. Ochratoxin A (4F3g2) mouse monoclonal antibody was obtained from Novus Biologicals, Cambridge United Kingdom as 0.1 mg lyophilized powder and was diluted with 10 mM phosphate-buffered saline (PBS) to a 1mg/mL concentration prior to use; 10 mM PBS with pH ranging from 5 to 7 solutions with 0.9% (w/v) NaCl were used as

association/dissociation buffers in the DGP/anti-DGP antibody interaction and washing steps. 0.1 M HCl was used as regeneration solution. All aqueous solutions were prepared with ultrapure water obtained with a Millipore Direct Q3 system.

Procedures

Before peptide immobilization the gold electrodes were pre-treated and cleaned using the following procedures.

1) The mechanical pre-treatment procedure (M) consisted of manually polishing the gold electrodes to a mirror-finished with slurry alumina (1 and 0.05 μm) and polishing cloth during approximately 5 min. Finally the electrodes were rinsed with water and cleaned ultrasonically in water for 1 min to remove residual alumina particles that could be trapped at the surface [1].

2) The chemical pre-treatment procedure (C) consists of dipping the gold electrode into piranha solution for about 10 min. Then, the electrodes were rinsed with water and sonicated in water during 1 min.

3) The electrochemical pre-treatment (E) was performed by successive scans between gold redox potentials (-0.35 to 1.5 vs. Ag/AgCl) as it follows[2-4]:

- One oxidation step (2 V for 5 s) and a reduction step (-0.35 V for 10 s) in 0.5M H_2SO_4 on the gold electrode
- Oxidation and reduction scans under acidic conditions (in 0.5 M H_2SO_4) over the potential -0.35 to 1.5 V (20 scans at a scan rate of 4 $V \cdot s^{-1}$, followed by 4 scans at a scan rate of 0.1 $V \cdot s^{-1}$).
- Oxidation and reduction scans under acidic conditions (0.01 M KCl/0.1 M H_2SO_4) using four different potential ranges sequentially (all performed for 10 segments at a scan rate of 0.1 $V \cdot s^{-1}$):

(i) potential range from 0.2 to 0.75 V;
(ii) potential range from 0.2 to 1.0 V;
(iii) potential range from 0.2 to 1.25 V;
(iv) potential range from 0.2 to 1.5 V.

We finally used a combined pre-treatment procedure M+C+E: first the electrodes were mechanical polished followed by immersion in piranha solution and finally after rinsing with ultrapure water they were electrochemically treated - successive scans between the gold redox potentials, as depicted above; this combined treatment was used 1) to obtain an increased electrochemical surface area (ESA), 2) to activate the surface taking into account that the C pre-treatment of the gold surface was reported as the most efficient procedure for obtaining a densely-packed peptide SAM [5] and 3) because it provided the most reproducible results. After that, the electrode was rinsed with water and sonicated in water for 1 min.

Sensor fabrication

The E-PB sensor was fabricated according to the following steps:

(i) Preparation of a support peptide self-assembled monolayer (SAM). The cleaned electrode with the mentioned pre-treatment procedures was dried under a nitrogen stream and immersed in 100 μL SP solution with concentrations ranging from 0.5 μM to 1 mM in ACN with 1% formic acid for 18 h. SAMs were also obtained from binary mixtures of SP/1,6-HDT in molar ratios 0.5/300, 1/300 and 10/300 in ACN with 1% formic acid. After deposition, the mixed SAM modified electrode was rinsed with ACN to remove the physisorbed molecules, further with ultrapure water, gently dried by flushing nitrogen and immediately transferred into 1 mM 6-mercaptohexanol solution in 10 mM PBS for 2 h at room temperature (after performing stability tests with the MB and DGP modules grafted on the support layer we have chosen the optimum 0.5 μM SP/300 μM 1,6-HDT/1mM 6-MH support layer for the antibody binding assays). The excess 6-mercaptohexanol was rinsed off with ultrapure water for 1 min. SAM formation was monitored by CV using $K_3Fe(CN)_6$ 1 mM solution in 1 M KCl in the 0 to 0.6 V potential range, scan rate 0.1 V s^{-1}.

Another method used to monitor the formation of the support peptide SAM onto gold surface was SPR. All the SPR measurements were performed in non-flow conditions in a double channel (reference and sample) system. The observed change of the SPR signal was proportional with the amount of immobilised peptide. For the AUTOLAB instrument every 122 milidegrees (m^o) angle shift corresponds to 1ng·mm^{-2} of immobilized peptide. Prior to use, bare gold disks were completely soaked in a freshly prepared piranha solution (3:1 mixture of H_2SO_4 and H_2O_2) 3 times each for 10 s, and then the disks were thoroughly washed with ultrapure water and dried with nitrogen gas. 100 μL of 10μM SP solution in ACN (with 1% formic acid) was injected in the sample channel while ACN with 1% formic acid was injected in the reference channel. The increase of SPR signal due to the deposition of the support peptide SAM was followed for 40 minutes (enough for the disulphide functionalized peptide to bind the gold surface), and then the SPR cuvette was sealed with parafilm and was left in the mentioned solutions overnight. After 18h incubation the SP-coated gold disks were rinsed with ACN and ultrapure water for the removal of the unbound peptides. The final SPR signals were read in ACN in order to preserve the same conditions as during the incubation.

(ii) Labelling the SP-modified electrode with the MB-redox tag. The electrode modified with the support peptide layer was incubated with 100 μL of 200μM MB-NHS solution in 10 mM bicarbonate buffer pH 8.5 (containing 22% v/v DMF) for 30 minutes**.** After this time, the electrode was rinsed first with bicarbonate buffer, then with 1 M NaCl for removing the MB molecules

physisorbed on SAM. The binding of the MB-NHS ester to the peptide SAM was monitored through SWV and ACV measurements with the potential ranging from 0 to -0.55 V vs. Ag/AgCl in 10 mM PBS. The increase of the peak current corresponding to surface confided MB was observed at $E_{p(reduction)}$ around -0.250V. SWV parameters were as follows: pulse amplitude of 25 mV and frequency of 50 Hz. The ACV parameters in the same potential range were: frequency 50 Hz, step potential 10 mV and step amplitude of 25 Vrms (root mean square). In order to optimize the ACV signal as well to calculate the MB-labelled PP density the frequency will be varied from 5 to 100 Hz.

(iii) Grafting of the DGP recognition block onto MB-SP-modified electrode. The DGP block has both N- and $C_{-terminus}$ blocked and has no free amino groups available for the covalent binding to the carboxyl groups of glutamate residues of the SP SAM so, the covalent binding was achieved through the phenolic -OH of the tyrosine residues from the DGP via Steglich esterification[6]. The activation of the terminal carboxylic groups of the linker was achieved by immersing the electrode in 100 μL of a 1:1 mixture of 0.4 M EDC and 0.1 M DMAP in 100 mM HCl (pH 4.5) into the cuvette for 30 min; the DGP was immobilized onto activated surface by immersing the electrode in 100 μL of 0.063 mM DGP in 10 mM PBS buffer, pH ranging from 5 to 7 for 2 hours. Blank experiments with DGP were performed also in the absence of EDC/DMAP surface activation.

The above procedures were followed by washing with 100mM HCl in order to remove the unbound reagents and stable intermediates. After 30 minutes equilibration in 10mM PBS the DGP coupling was monitored through SWV measurements. The storage conditions for the DGP-MB-SP- modified electrode were: a) in 10mM PBS, pH 5, 6 and 7 b) in 10 mM HCl, all at 4^{o}C, in the dark. The DGP removal was performed by immersing the sensor's surface in a 10mM bicarbonate buffer pH 8.5 for 30 minutes. After washing with ultapure water and 100 mM HCl the sensors was ready for a new functionalization with the DGP block. The removal of the peptide SAM was performed electrochemically using linear sweep voltammetry (LSV) in the -0.35 to -1.5 V potential range corresponding to the reductive desorption of the sulphur-containing molecules from the electrode surface at a scan rate of 0.1 $V \cdot s^{-1}$ in 0.5 M KOH solution. These measurements will be used to also calculate the overall support peptide surface density and MB-labelling degree of the peptide SAM.

Binding assays

Prior to use the DGP-MB-SP modified sensor was left to equilibrate in 10 mM PBS, pH 6 solution for 30 minutes. Once the background signal was stable, anti-DGP was added to reach concentrations ranging from 0.033 to 1.092μg/mL (0.22 – 6.7 nM). The MB signalling in SWV was recorded after a stabilization time of 40 minutes, at room temperature. The possible interferences to the SWV signal were checked with BSA and anti Ochratoxin A monoclonal antibody in the same

concentration range and conditions as the anti DGP antibody. Serum samples were diluted with PBS and the pH was adjusted to 6.

Regeneration of the sensor's surface

The optimum regeneration procedure of the DGP-MB-SP modified sensor consisted in the immersion of the electrode in 100 mM HCl only, followed by washing with ultrapure water prior the next SVV experiment; a 15-20 minutes immersion in 100 mM HCl gave a 96% recovery of the initial peak current for the binding assays where the concentrations of anti-DGP antibody were up to 0.3 μg/mL. Similar results were obtained after 3-4 h immersion of the electrode in 100mM HCl for the experiments where anti-DGP antibody concentrations ranged within 0.3 – 1 μg/mL

Estimation of the support peptide surface density from LSV measurements

Three successive reduction scans were performed in strong basic medium for each electrode modified with 0.5, 1, 10, 100 and 1000 μM SP in order to remove the MB-SP SAM from the gold surface. Blank experiments with the electrode modified with peptide SAM only (without MB-labelling) for checking eventual contributions of the MB moiety to the peak current corresponding to the reduction of the lipoic acid residue from the peptide SAM. If a reduction peak associated to the Au–S bond in the peptide SAM is observed around -0.975 V, this is due to the reductive desorption of lipoic acid from gold surfaces. This result confirms the binding of the disulphide group on the gold electrode. The successive LSV scans showed a marked decrease of the Au–S peak intensity, denoting extensive peptide SAM desorption induced by the applied reductive potential. This finding suggests that the peptide SAM is not stable under an applied potential, possibly as a result of weak lateral interactions between the short peptide chains [2]. The electrochemical reduction of the gold-bound thiolated layer in an alkaline solution occurs according the equation:

$$Au(s) - S_{\lambda}R + \lambda e^{-} \rightarrow Au(s) + R(S^{-})_{\lambda}$$

where $\lambda = 2$ for the disulphide group of the lipoic acid moiety and represents the charge transferred between the sulphur atom and the surface.

The area under the reduction peak observed at -0.975 V from the first scan (fig.SI3) was further used to calculate the SP surface density using the equation:

$$\Gamma = \frac{Q}{\lambda FA}$$

where Q is the charge required for the reduction of the Au–S bond, F is the Faraday constant (96485 C· mol^{-1}) and A the geometric area of the gold electrode

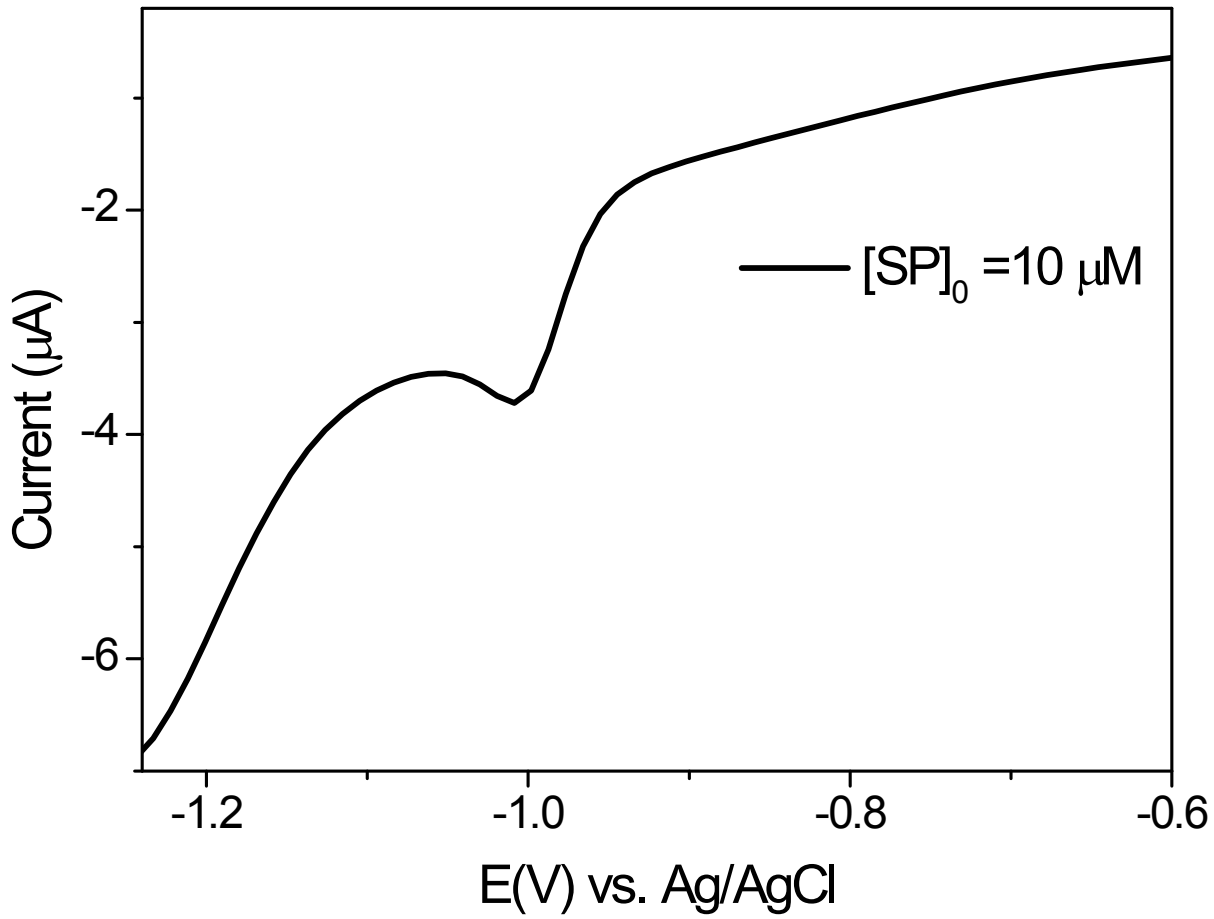

Fig.SI1 Linear sweep voltammetric curve recorded in 0.5 M KOH for the gold modified electrode by MB-SP SAM (scan rate 100 mV/s)

Estimation of the support peptide surface density from SPR measurements

We performed control SPR experiments to estimate the surface density of the support peptide for a gold surface incubated with 10 μM support peptide solution in ACN using a double channel (sample and reference) non-flow system; we monitored the increase of the SPR signal in the sample channel, where the support peptide was injected towards the increase of the SPR signal where the solvent only was injected; the SPR signal corresponding to the reference channel was then subtracted from the SPR signal given by the sample channel. The equilibrium value of the subtracted SPR signal (R_{max}) was used to calculate the support peptide density [7]; this value was then compared with the one obtained from LSV experiments for the same concentration of incubated support peptide solution (10μM).

$$\Gamma = \frac{\Delta R}{122 \times \text{M.W}} \cdot 10^{-7}\ \text{moles/cm}^2$$

where ΔR =243 m^o (the average value from 3 experiments) , M.W. $_{\text{support peptide}}$ = 1146.4.

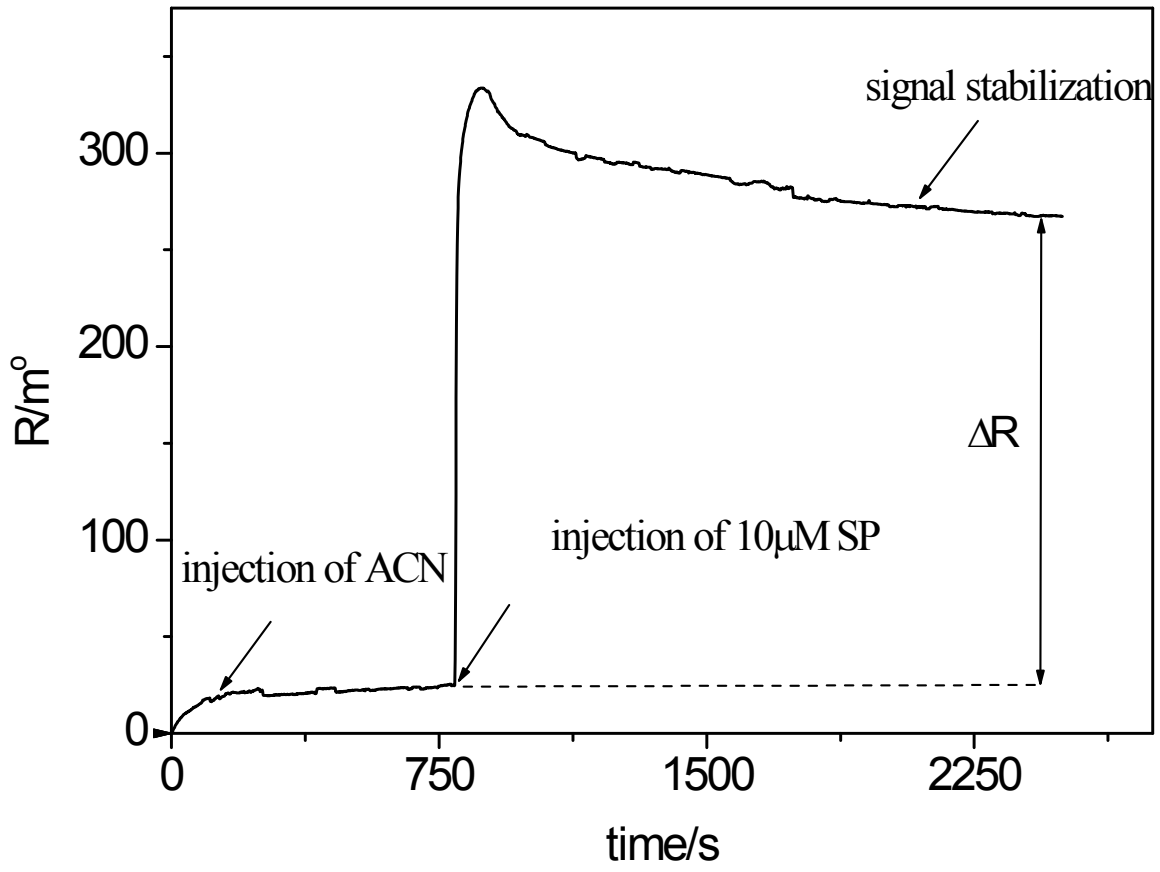

Fig. SI2 Typical SPR sensogram of peptide binding onto gold surface monitored within 40 minutes

Monitoring the insulating properties of the mixed support peptide/thiols layer from CV measurements

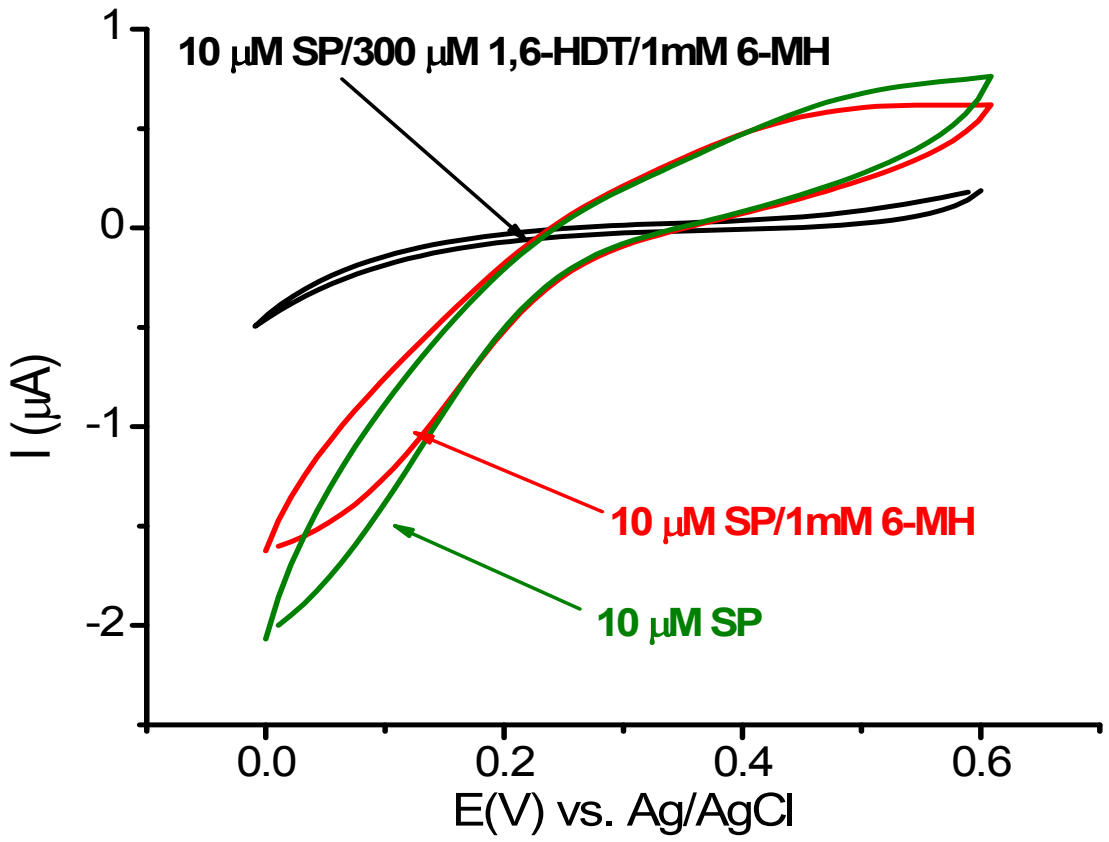

Fig. SI3 Prevention of the redox active species discharge due to mixed peptide SAM formation: CVs in 1mM K_3FeCN_6 in 1M KCl, obtained with support peptide modified electrodes for several mixtures SP/HDT/6-MH (scan rate 100mV/s)

Evidence of the deamidated gliadin peptide binding to the mixed support peptide/thiols layer from SWV measurements

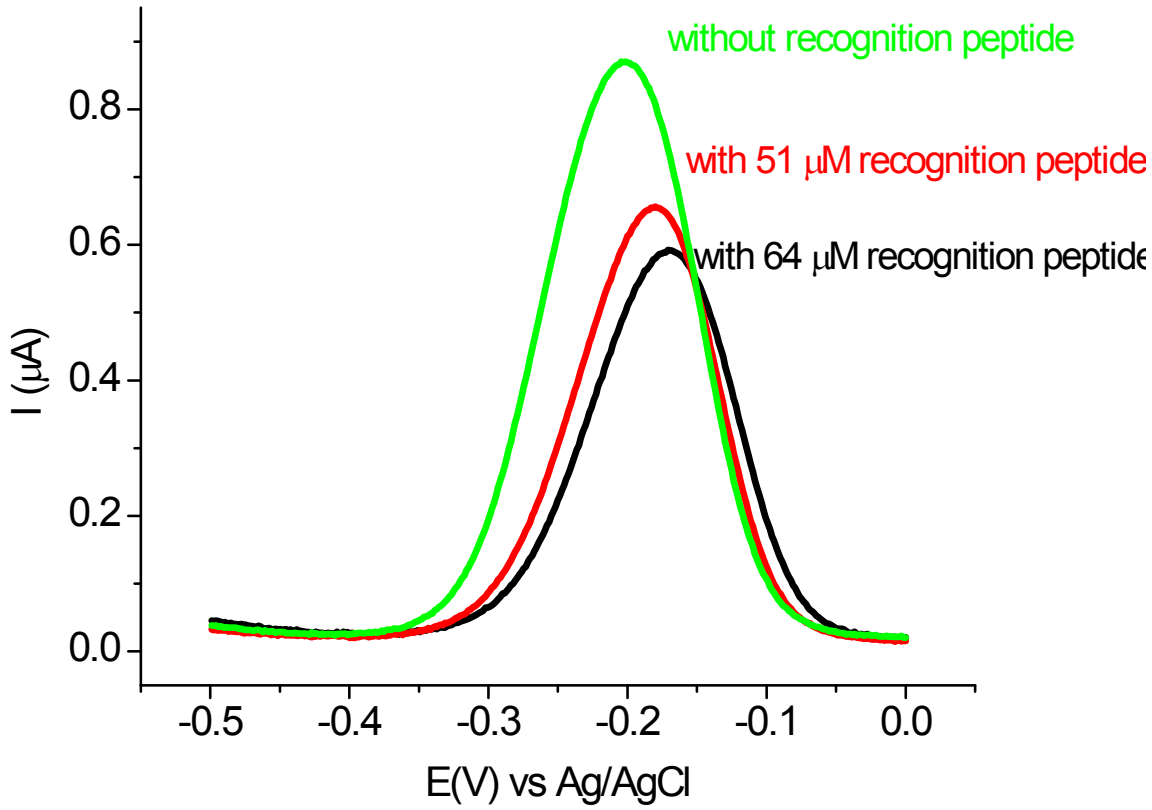

Fig. SI4 Decrease of SWV signal at DGP covalent binding to the support peptide in 10mM PBS pH = 6

Optimization of the support layer composition according to the efficiency of the target antibody binding

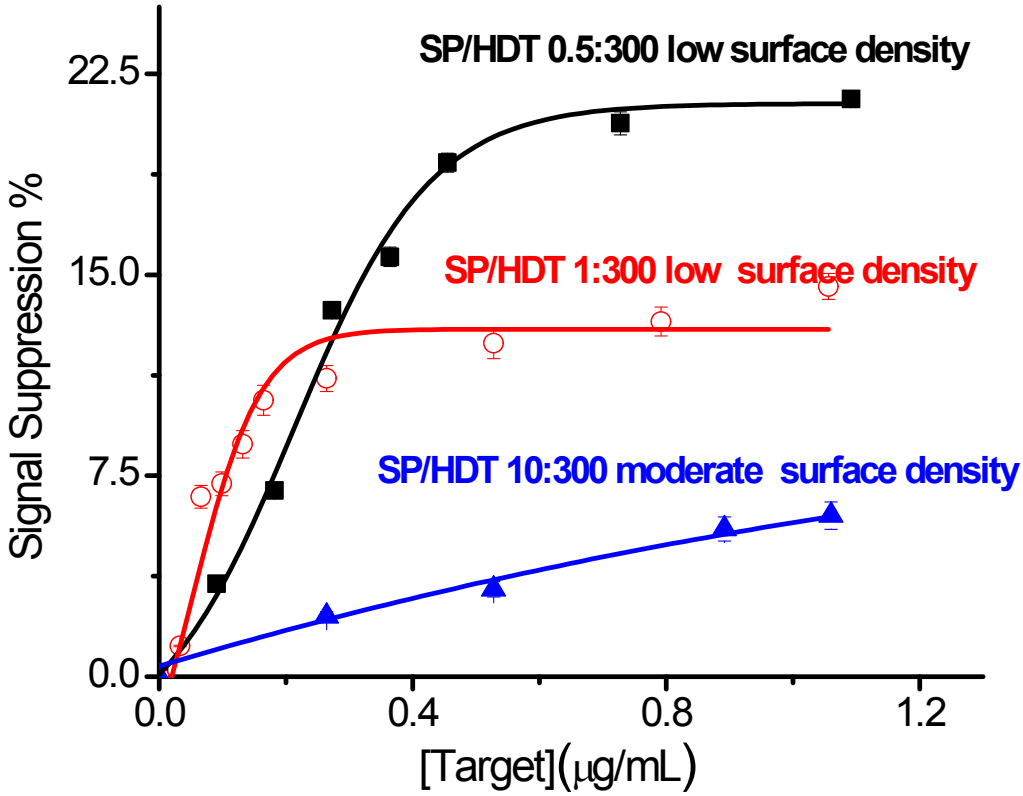

Fig. SI5 Anti-DGP binding curves in 10 mM PBS, pH = 6 obtained with the modified DGP-MB-SP electrode for three compositions of the support layer; of note, the value of the molar ratio support peptide/1,6-HDT plays a crucial role in the efficiency of antibody binding

References

1. Y. Xiao, R. Y. Lai and K. W. Plaxco, *Nat. Protocols*, 2007, **2**, 2875-2880.
2. E. Gatto, A. Porchetta, M. Scarselli, M. De Crescenzi, F. Formaggio, C. Toniolo and M. Venanzi, *Langmuir*, 2012, **28**, 2817-2826.
3. S. Okamoto, T. Morita and S. Kimura, *Langmuir*, 2009, **25**, 3297-3304.
4. E. Gatto, L. Stella, F. Formaggio, C. Toniolo, L. Lorenzelli and M. Venanzi, *Journal of Peptide Science*, 2008, **14**, 184-191.
5. R. F. Carvalhal, R. Sanches Freire and L. T. Kubota, *Electroanalysis*, 2005, **17**, 1251-1259.
6. B. Neises and W. Steglich, *Angewandte Chemie International Edition in English*, 1978, **17**, 522-524.
7. M. Puiu, O. Istrate, L. Rotariu and C. Bala, *Analytical Biochemistry*, 2012, 421, 587-594.