Supplementary material

Antibody-based amperometric biosensor for 20S proteasome activity and for inhibitors screening

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S1. Cyclic voltammetric response of AMC at GCE

The electrochemical behaviour of AMC was first investigated by cyclic voltammetry. The CV scans recorded in the solution of AMC showed a typical irreversible behaviour, with an oxidation peak at +0.80 V (**Fig. S1A**), and no other electrochemical signal.

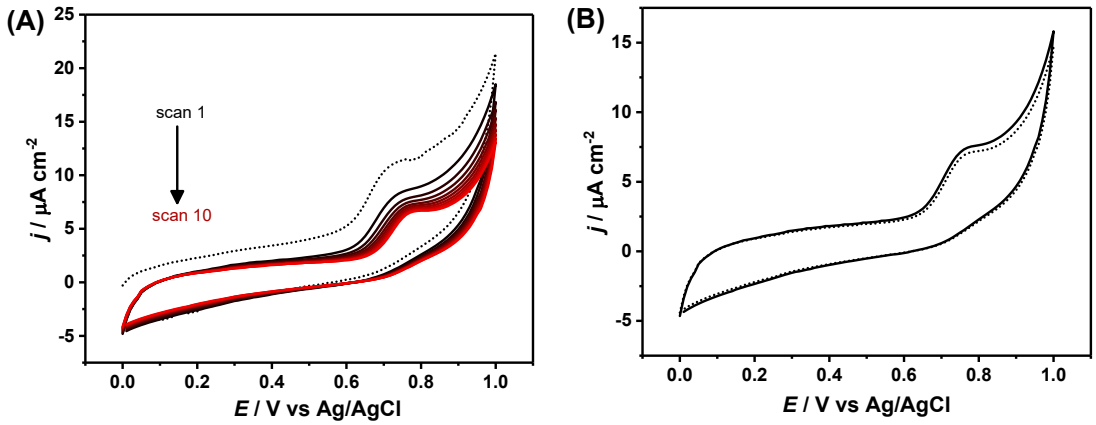

Fig. S1. CVs recorded at GCE in 40 µM AMC in PAB pH 7.0, at 50 mV s^{-1} A) for 10 scans and B) before (—) and after (•••) applying +0.8 V vs Ag/AgCl for 15 min.

10 successive CV were recorded at a bare GCE in a solution containing 20 µM AMC in PAB pH 7.0 before (—) and after (•••) applying +0.8 V vs Ag/AgCl for 15 min. There is a decreases in the peak intensity after the first scan (as also seen in Fig. S1 A), the following cycles maintaining almost unaltered the oxidation peak intensity values. Moreover, by comparing the scans recorded before and after the fixed potential amperometric measurement, only a slight decrease in peak current value is noticed (**Fig. S1B**) with this proving that they aren't any oxidation products of AMC adsorbed at the electrode surface.

S2. Chronoamperometric response of AMC at GCE/Ab_ β5

CA experiments at GCE/Ab_ β5 upon successive injections of AMC showed a longer response time of 25±3 s compared to that at bare GCE, since the AMC needs to diffuse trough the proteic layer (**Fig. S2**). However, a higher sensitivity and a slightly lower detection limit were achieved, probably due to a pre-concentration of AMC within the proteic layer

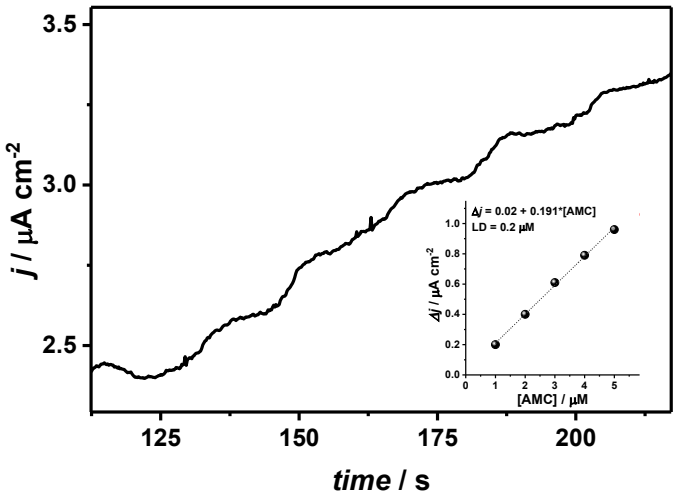

Fig. S2. CA responses of 1 µM AMC recorded at +0.80 V in PAB pH 7.0 at GCE/Ab_β5

S3. Chronoamperometric response of the 20S proteasome in solution and immobilized at the Ab_β5 antibody

Comparative chronoamperometry (CA) experiments were done with the proteasome in solution (5 μg mL^{-1}) and immobilized on Ab_β5, with the addition of the same concentration of Suc-LLVY-AMC, **Fig. S3**.

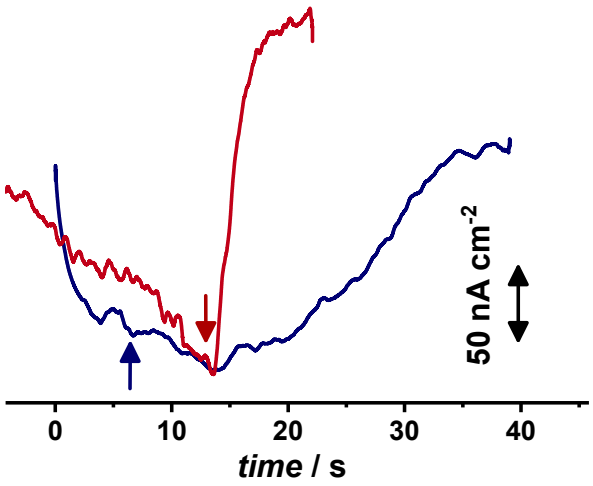

Fig. S3. CA responses of 15 μM Suc-LLVY-AMC recorded at +0.80 V in PAB pH 7.0 at (red curve) the GCE/Ab_β-20S biosensor and (blue curve) 20S proteasome free in solution.

It can be seen that when the proteasome is in solution (blue curve), there is an incubation time required for the proteolysis to occur in order to release the AMC from the peptide chain (see the blue arrow that indicates the injection time), resulting in a delay in the increase in the current up to approximately 10 s. On the contrary, when the proteasome is immobilized on the Ab_β antibody (red curve), the current increases immediately after the injection of the substrate, indicating a much easier accessibility of the AMC to the electrode surface, since in this case it needs to diffuse only through the thin proteic layer to reach the electrode. For the same reason, the response time (the time required to reach 90% of Δj) is much lower for the detection of Suc-LLVY-AMC at GCE/Ab_β-20S. The results indicate that the immobilization of 20S on Ab_β does not compromise its enzymatic activity, leading to a biosensor with good sensitivity and fast response time.

S4. Voltammetric characterization of the 20S proteasome interaction with Ab_β, Ab_α and Ab_core

The DPV profile of 20S proteasome can give indications on the conformational changes that occur upon its immobilization, by correlating the oxidation peaks to the amino acids exposed and which are susceptible to electrochemical oxidation [1].

DPV measurements were performed in solutions of 20S in PAB. The voltammogram revealed two oxidation peaks, the first at +0.65 V and the second at +1.00 V , corresponding to the oxidation of tyrosine/tryptophan (Tyr/Trp) and histidine (His) residues, respectively [2], **Fig. S4A**.

The second measurement was performed at a GCE with 20S immobilized by cross-linking with GA, GCE/(BSA+GA)-20S (see *Section 2.3*). The DPV in these conditions revealed a new oxidation peak at +0.45 V attributed to cysteine (Cys) residues oxidation, while the peak at +0.65 V showed higher current when compared to the one observed when 20S in solution. In order to identify the contribution of BSA, a DPV was recorded at the GCE/(BSA+GA), **Fig.**

S4A-inset, and same oxidation peaks appeared at +0.45 and +0.65 V. The differences in peak currents are indicative of 20S interaction and immobilization on the (BSA+GA) proteic layer. Moreover, the fact that the peak at +0.65 V with GCE/(BSA+GA)-20S was higher than that observed for 20S in solution can be attributed to the fact the BSA adds to the Tyr/Trp existent in the 20S. When immobilized on Ab_β, **Fig. S4A**, the first peak (at +0.65 V in solution) maintain the potential value and increases even more, while the second peak (at +1.00 V in solution) is shifted towards less positive potentials.

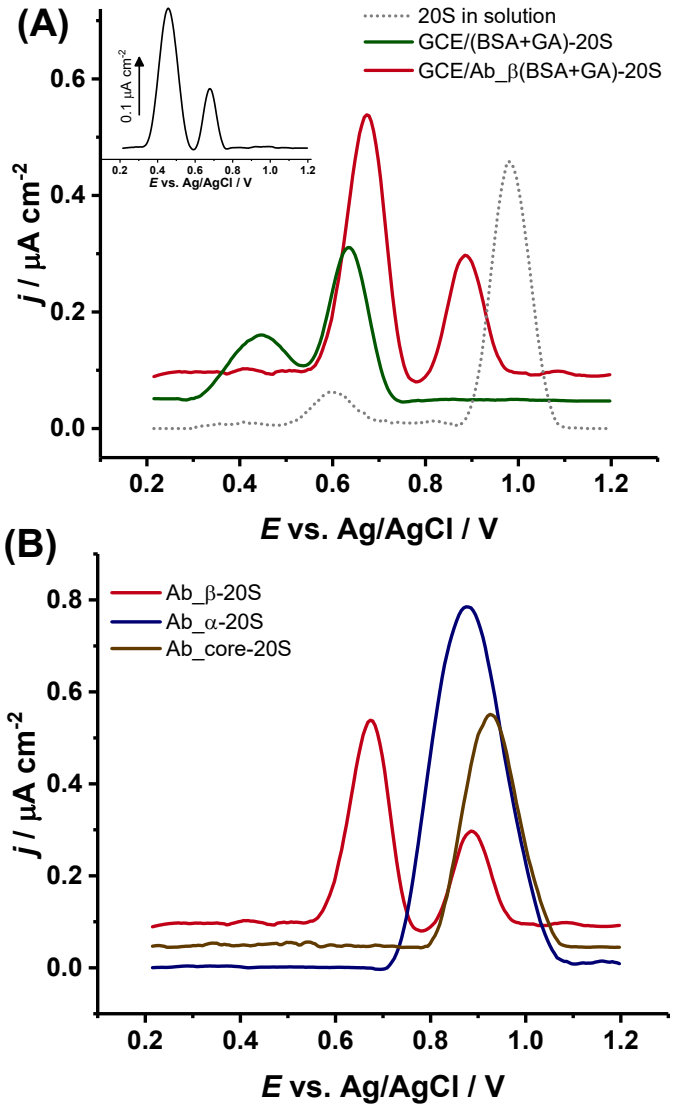

Fig. S4. DP voltammograms recorded in PAB for:
A) 20S proteasome solution (5 μg mL^{-1}) with the clean GCE (dotted curve) and the 20S proteasome immobilized by cross-linking (green) and specific 20S- Ab_β interactions (red); in the inset is the DPV recorded at a GCE modified with BSA+GA.
B) 20S proteasome immobilized to Ab_β (red), Ab_α (blue curve) and Ab_core (brown).

The 20S was immobilized by employing three different antibodies. Two specific antibodies: the Ab_β, which targets the specific subunit β5 constituent of the two inner cylinders that are responsible for the catalytic activity, and the Ab_α, triggering the terminal α1-7 subunits, which are situated at the extremity of the 20S and represents the entrance route for substrates not

involved in the peptide cleavage [3]. One less specific antibody that recognizes the core subunits, Ab_core was used as well.

DPV profiles in buffer of the obtained biosensors were different, **Fig. S4B**. As already mentioned, the GCE/Ab_β-20S presented two oxidation peaks at +0.65 V (Tyr/Trp) and +0.90 V (His). Contrary, the biosensors GCE/Ab_α-20S and GCE/Ab_core showed only one higher peak at +0.90 (His).

Considering that the biosensors were all constructed following the same procedure, and that the structure/sequence of antibodies does not vary much between the different types then, from statistical point of view, the DPV signals of the GCE/Ab_(BSA+GA) will be similar. It results that the differences observed after attachment of the proteasome are due to this specific biomolecule. On the other hand, non-specific adsorption of the proteasome at the modifying layer may also occur. However, it is considered that the voltammetric differences in **Fig. S4B** indicate different orientations of the 20S, with similarities between GCE/Ab_α-20S and GCE/Ab_core-20S. Comparing the DPV profiles of the latter biosensors with the one recorded when 20S was in solution, it can be observed that they also share the profile, with the peak at +0.45 V (Cys) missing and the one attributed to His at lower potential, due to close proximity of 20S to the electrode surface.

S5. Comparison of the enzyme kinetic parameters for 20S proteasome in solution phase and immobilized at the electrode surface in the present biosensor architecture

Table S1. Comparison of the $K_{0.5}/j_{max}$ for 20S proteasome in solution phase for references 24 and 25 (in the main manuscript) and immobilized at the electrode surface in the GCE/Ab_β-20S biosensor configuration

20S activity	***substrate***	***20S immobilized***	***20S free in solution phase***	j_{max} ***in solution phase / nA***
Caspase	Z-LLE-AMC	44.5	833	0.6
	Ac-GPLA-AMC	84.0	n.d.	n.d.
Trypsin	Boc-LRR-AMC	34.5	210	0.95
	Ac-RLR-AMC	31.9	143	1.1
Chymotrypsin	Suc-LLVY-AMC	23.8	73	1.73
	Z-GGL-AMC	104.0	82	1.51

S3. References

[1]O.M. Popa, V.C. Diculescu, Direct electrochemical oxidation of Abelson tyrosine-protein kinase 1 and evaluation of its interaction with synthetic substrate, ATP and inhibitors, J. Electroanal. Chem., 742 (2015) 54-61. doi:10.1016/j.jelechem.2015.01.035.

[2]T.A. Enache, A.M. Oliveira-Brett, Peptide methionine sulfoxide reductase A (MsrA): Direct electrochemical oxidation on carbon electrodes, Bioelectrochemistry. 89 (2013) 11–18. doi:10.1016/J.BIOELECHEM.2012.08.004.

[3]E. Kish-Trier, C.P. Hill, Structural biology of the proteasome, Annu Rev Biophys. 42 (2013) 29–49. doi:10.1146/annurev-biophys-083012-130417.