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# An antibody-based amperometric biosensor for 20S proteasome activity and inhibitor screening†

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The 20S proteasome enzyme complex is involved in the proteolytic degradation of misfolded and oxidatively damaged proteins and is a focus of medical research for the development of compounds with pharmaceutical properties, which are active in cancer cells and/or neurodegenerative diseases. The present study aims to develop a biosensor for investigating the 20S proteasome activity and inhibition by means of electrochemical methods. The 20S proteasome is best immobilized at the electrode surface through bio-affinity interactions with antibodies that target different subunits on the 20S proteasome, enabling the investigation of the effect of an enzyme's orientation on biosensor response. The enzymatic activity is analyzed by fixed potential amperometry with the highest sensitivity of  $24 \mu\text{A cm}^{-2} \text{mM}^{-1}$  and a LOD of  $0.4 \mu\text{M}$ . The detection principle involves the oxidation of an electroactive probe that is released from the enzyme's substrates upon proteolysis. The most sensitive biosensor is then used to study the multicatalytic activity of the 20S proteasome, *i.e.* the caspase-, trypsin- and chymotrypsin-like activity, by analyzing the biosensor's sensitivity towards different substrates. The behavior of the immobilized 20S proteasome is investigated as a function of substrate concentration. The kinetic parameters are derived and compared with those obtained when the enzyme was free in solution, with  $K_{0.5}$  values being one to two orders of magnitude lower in the present case. Two 20S inhibitors, epoxomicin and bortezomib, are investigated by analyzing their influence on the 20S biosensor response. The proposed analytical method for proteasome activity and inhibitor screening has the main advantage of being cost-effective compared to the ones typically employed.

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## 1. Introduction

Reactive oxygen species (ROS) mediated oxidation of proteins leads to nonfunctional, malfunctioning and/or protein aggregates that can hinder many metabolic processes,<sup>1</sup> resulting in aging and age-related diseases such as sclerosis, Parkinson and Alzheimer as well as cancer.<sup>2</sup> To avoid accumulation of damaged proteins and therefore the associated diseases, organisms possess self-defense mechanisms that degrade these damaged proteins. There are two principal paths for protein degradation: the lysosomes and the proteasomes. Proteasomes are protein complexes named according to their sedimentation coefficient, *e.g.* 19S, 20S, 26S *etc.*<sup>3</sup> The ubiquitin-26S proteasome degradation pathway is the most known,<sup>4</sup> initially thought to be the primary way for protein proteasomal degradation,<sup>5</sup> the activity of which is also associated with the

rate of aging and thus human longevity.<sup>6,7</sup> The ubiquitin-26S proteasome degradation pathway requires poly-ubiquitination *via* ubiquitin-activating enzymes,<sup>5</sup> the target being shortly lived or degrades the protein to oligopeptides and one or two terminal 19S regulatory particle(s) that serve as a proteasome activator, by recognizing the polyubiquitinated substrates. It is now becoming clear that proteins can also be targeted for degradation by 26S without requiring ubiquitination and that degradation may occur in the core 20S alone.<sup>8,9</sup> The principal targets are proteins that contain unstructured regions due to oxidation, mutation, aging *etc.*

The 20S proteasome is composed of 28 subunits, arranged in 4 stacked rings of two  $7\alpha$  subunits and two  $7\beta$  subunits, which form a highly conserved cylindrical structure. The two outer rings contain  $\alpha$ -type subunits and their main function is to maintain a "gate" through which proteins enter the catalytic site/core.<sup>10</sup> The  $\beta$ -subunits form the two inner rings, of which  $\beta_1$ ,  $\beta_2$  and  $\beta_5$  are catalytically active. Upon analysis of the activity of yeast mutant proteasomes on small fluorogenic substrates, it was found that the subunit  $\beta_1$  was associated with caspase-like activity,  $\beta_2$  with trypsin-like activity and  $\beta_5$  with chymotrypsin-like activity.<sup>11,12</sup> Recently a regulatory mecha-

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nism that coordinates 20S proteasome-mediated degradation was published, concluding that the 20S degradation pathway is crucial when rapid elimination of proteins is required for maintaining cellular viability.<sup>13–15</sup>

Due to its complex role in regulating protein metabolism and because it is highly active in cancer cells, the proteasome has been receiving great interest in the development of new cancer therapies, which rely on the use of proteasome inhibitors.<sup>16,17</sup> Determination of proteasome activity is therefore crucial, enabling the study of the mechanism of action of such inhibitors. The methods developed for the determination of proteasome activity are fluorimetry<sup>18,19</sup> and biochemiluminescence,<sup>20</sup> which are based on fluorogenic<sup>21</sup> or biochemiluminescent<sup>21–23</sup> peptides respectively, the most used one being 7-amino-4-methylcoumarin (AMC). Taking advantage of AMC electroactivity, electrochemical methods have also been recently developed<sup>24,25</sup> based on the electrochemical detection of the AMC released after proteolysis, which is electrochemically oxidized at positive potentials, at the primary amine at position 7 of the AMC.<sup>25</sup> In these studies, the proteasome and the enzymatic substrates are free in the solution, and differential pulse voltammograms were recorded after incubation for increasing periods of time. Electrochemical biosensors for 20S proteasome have not been reported yet. However, detection of some proteases has been reported, but in these architectures peptides are often labelled with redox reporters and immobilized on the electrodes to be cleaved by proteases which are free in solution, in the presence and in the absence of inhibitors.<sup>26</sup> Immobilization of proteases at different nanoparticles for homogenous catalysis was also investigated for biotechnological applications,<sup>27,28</sup> with the exception of a microfluidic device for inhibitors screening,<sup>29</sup> or immobilization at the surface of a hydrogen ion-selective electrode for peptide determination.<sup>28</sup> In this context, it is envisioned that a biosensor in which an enzyme is immobilized at the electrode surface presents advantages for investigating the activity and inhibition of 20S proteasome allowing a faster and sensitive response at reduced costs (reduced enzyme usage since the biosensor can be used several times); such a biosensor is more appropriate for fast screening of inhibitors and compounds with potential pharmaceutical applications.

The present study aims to evaluate the activity of the 20S proteasome immobilized at the electrode surface. Different immobilization procedures were tested including bio-affinity interactions with monoclonal and polyclonal antibodies. The influence of the antibody–proteasome interactions towards proteasome activity was evaluated, employing fixed potential amperometry at a potential required for the oxidation of the enzymatically released AMC. The same electrochemical procedure was used to evaluate the three different enzymatic activities of the proteasome using 6 substrates, 2 each for caspase-, trypsin- and chymotrypsin-like activity. The influence of two 20S inhibitors, the naturally occurring epoxomicin and the synthetic bortezomib, on the biosensor response was also evaluated.

## 2. Results and discussion

The activity of the proteasome 20S enzyme is measured through the occurrence of peak currents that appear upon oxidation of an electroactive probe released from the enzyme's substrates upon proteolytic reaction. In this study 20S proteasome substrates specific to each proteolytic activity (chymotrypsin, trypsin and caspase) were used. All substrates were marked with AMC as an electroactive marker. It has been previously shown that the release of AMC upon proteolysis produces an anodic peak characteristic of free AMC molecules ( $E_{pa} \sim +0.80$  V vs. Ag/AgCl at pH  $\sim 7.0$ )<sup>24,25</sup> (Fig. S1†) which does not occur when the molecule is bound to the substrate. No adsorption of either AMC or its oxidation products was observed (Fig. S1B†).

### 2.1. Electrochemical detection of AMC

The first step was to evaluate GCE sensitivity towards AMC using DPV and CA in order to find the best electroanalytical technique for biosensor development.

As observed in Fig. 1A, the peak intensity increased linearly upon injection of AMC in the concentration range of 5 to 25  $\mu$ M, with a saturation of the response for AMC concen-

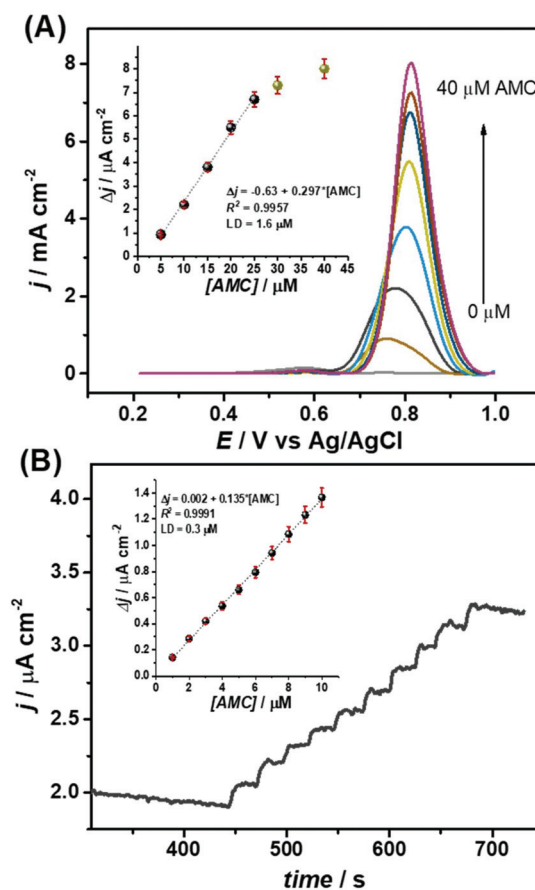


Fig. 1 Electrochemical detection of AMC. (A) DP voltammograms and (B) CA at +0.80 V, with the GCE in solutions of increasing concentrations of AMC in PAB pH 7.0; insets are the corresponding calibration plots.

tration above 30  $\mu\text{M}$ , due to AMC adsorption at the GCE surface. The sensitivity was of  $297 \mu\text{A cm}^{-2} \text{mM}^{-1}$  with a detection limit of 1.6  $\mu\text{M}$  calculated using the formula  $\text{LOD} = 3 \times \text{SD} \times (\text{sensitivity})^{-1}$ , where SD is the standard deviation determined by the linear fit of the linear part of the calibration plot.

Taking into account the oxidation potential of AMC observed in DPV, CA measurements were also performed at a bare GCE (Fig. 1B) and at the GCE modified with Ab $\beta$ 5 (Fig. S2†) in order to choose the best electrochemical method to evaluate the final biosensor activity. A typical CA response recorded at +0.80 V upon successive additions of AMC showed an anodic change in current following its injection due to oxidation reactions at the electrode surface. Even though the sensitivity of the sensor was lower,  $135 \mu\text{A cm}^{-2} \text{mM}^{-1}$ , when compared to DPV measurements, AMC was detected at lower concentrations, from 1 to 10  $\mu\text{M}$ , and the detection limit was considerably lower, at only 0.3  $\mu\text{M}$ . For this reason, CA was chosen for biosensor development.

Chronoamperometry was also performed at +0.70 and +0.60 V and the sensitivity decreases considerably, to less than 50%, compared to the one recorded at +0.80 V. Taking this into account, CA for monitoring the enzymatically released AMC from the peptide-AMC 20S substrates was performed at +0.80 V.

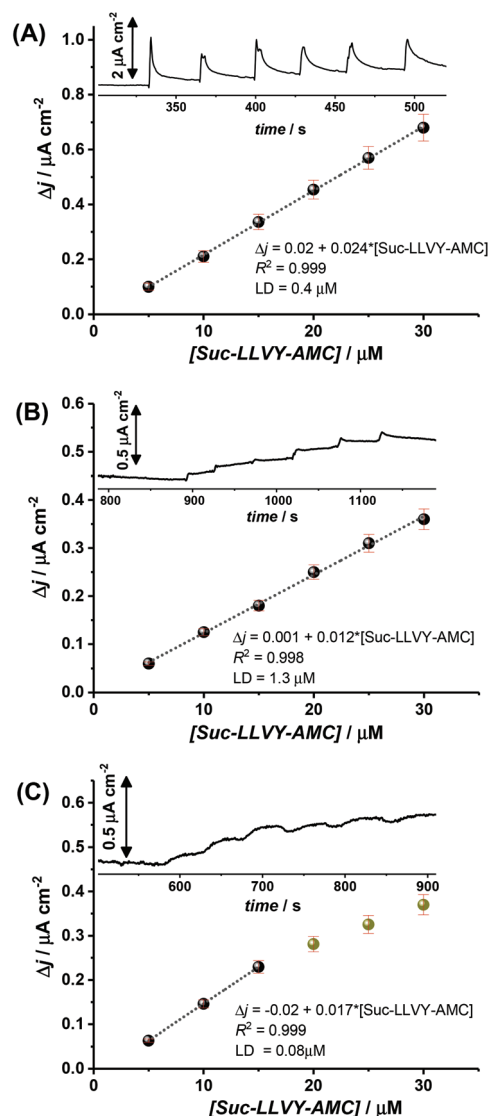
## 2.2. Evaluation of the activity of 20S proteasome immobilized at different antibodies

The architecture of the 20S biosensor based on the immobilization of 20 S on two specific antibodies Ab $\beta$ 5 and Ab $\alpha$  and on the nonspecific Ab $\text{core}$ , which are immobilized previously by cross-linking with GA is described in Scheme 1.

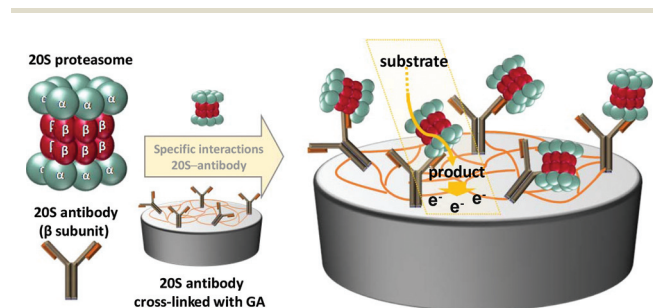
The enzymatic activity of the 20S was monitored using fixed potential amperometry at +0.80 V. The substrate used for this study, Suc-LLVY-AMC, was for the chymotrypsin-like activity, taking into account the high affinity of the 20S toward this substrate observed in previous investigations.<sup>24</sup> The biosensor containing the 20S immobilized by cross-linking with GA at the bare electrode did not show any electrochemical response upon substrate injection. This is due to an unfavourable orientation of the proteasome, since the cross-linking with GA

involves the participation of the terminal amino groups of the proteins, which may cause a restricted substrate access to the catalytic core. Therefore, only the biosensors based on the 20S-antibody specific interactions will be discussed further.

The chronoamperometric responses recorded at GCE/Ab $\beta$ 5-20S, GCE/Ab $\alpha$ -20S and GCE/Ab $\text{core}$ -20S upon successive injections of the Suc-LLVY-AMC substrate are displayed in the insets of Fig. 2A–C, where an anodic change in current is observed, in agreement with the released AMC oxidation at the GCE surface. As observed, for this concentration range, the current increases linearly with the substrate concentration, in agreement with the amount of free AMC at the biosensor surface.



**Fig. 2** Evaluation of the activity of 20S proteasome immobilized at different antibodies. CA response recorded at +0.80 V for successive injections of Suc-LLVY-AMC in PAB pH 7.0 at: (A) GCE/Ab $\beta$ 5-20S, (B) GCE/Ab $\alpha$ -20S and (C) GCE/Ab $\text{core}$ -20S; insets are the corresponding calibration plots.



**Scheme 1** Biosensor construction based on 20S antibody bio-affinity interaction, with the antibody immobilized at the GCE via cross-linking with GA.

The biosensor response time was  $6 \pm 1$  s, much lower than that of experiments carried out in incubated solutions where approximately  $30 \pm 3$  s was necessary for current stabilization (Fig. S3†). These experiments, together with GCE/Ab\_β5 upon injection of AMC, indicate that when proteasome is immobilized, the release of AMC takes place in a very close proximity to the electrode surface, which significantly reduces the response time of the sensor.

The sensitivities and the detection limit values were calculated using the calibration plots presented in Fig. 2A–C, with the analytical parameters presented in Table 1. As observed, the GCE/Ab\_β5-20S had the highest sensitivity of  $24 \mu\text{A cm}^{-2} \text{mM}^{-1}$ , almost twice that of the other two biosensors, while the LOD presented practically similar values for all biosensors. On the other hand, it may be observed that the sensitivity of the GCE/Ab\_core-20S biosensor was similar to that obtained for the GCE/Ab\_α-20S biosensor, which indicates a similar activity of the 20S immobilized on these antibodies. This is explained considering different orientations of the 20S proteasome at the electrode surface, as demonstrated by the DPV data presented in Fig. S4.†

The superior sensitivity of the GCE/Ab\_β5-20S biosensor indicates a conformation of 20S, which allows for easy access of the substrate to the interior and the catalytic core of the 20S proteasome.<sup>30</sup> Contrarily, when immobilized through Ab\_α and Ab-core interactions, the substrate access is hindered by the formation of bonds at the outer α rings of the proteasome, which represent the gate for the peptide to enter the catalytic core formed by the two 7β rings.

Gravimetric measurements recorded at an Au quartz crystal modified previously with Ab\_β5 upon immersion in a  $1 \text{ mg mL}^{-1}$  20S solution and after repeated washing cycles indicated a total mass of  $1.7 \mu\text{g}$  20S adsorbed on the Ab\_β5 layer. In this way, the molar ratio Ab\_β5/20S was 3:2 (for this calculation relative molar weights of 150 and 750 kDa were considered for Ab\_β5 and 20S proteasome, respectively). The frequency of the crystal Au/Ab\_β5/20S did not change when immersed in water for a period of 120 min, meaning that the proteasome immobilized is stable and does not leak from the antibody film.

### 2.3. Repeatability, reproducibility, selectivity and operational/storage stability of the GCE/Ab\_β5-20S biosensor

The repeatability of the GCE/Ab\_β5-20S was evaluated by measuring the response to  $10 \mu\text{M}$  Suc-LLVY-AMC for 5 consecutive injections (5 different measurements, allowing the

baseline to be stabilized) and an R.S.D. of 5.4% was calculated.

Three different GCE/Ab\_β5-20S biosensors were fabricated following the procedure described in section 3, and the R.S.D. values were calculated taking into account the sensitivities of the biosensors for a 5-point calibration plot, with the obtained value of 5.5%.

Control experiments were carried out using BSA, non-electroactive short chain peptides not marked with AMC (polylysine and poly-histidine), with no signal being recorded at the biosensor.

One biosensor was tested over a period of 6 weeks. The storage was at  $4^\circ\text{C}$ , and the biosensor was kept in PAB pH 7.0 when not used. The evaluation of its sensitivity was done each week by recording a 5-point calibration plot, and it was observed that the sensitivity decreased linearly up to one month to 65% of its initial value, retaining 43% of the initial sensitivity after the whole period of 6 weeks of testing.

Another biosensor was stored at  $4^\circ\text{C}$  in PAB pH 7.0 and tested after 2 months with no use in between, with the sensitivity being 80% of the initial value after this period of time.

### 2.4. Kinetics and evaluation of caspase-, trypsin- and chymotrypsin-like activity of the immobilized 20S proteasome with the GCE/Ab\_β5-20S biosensor

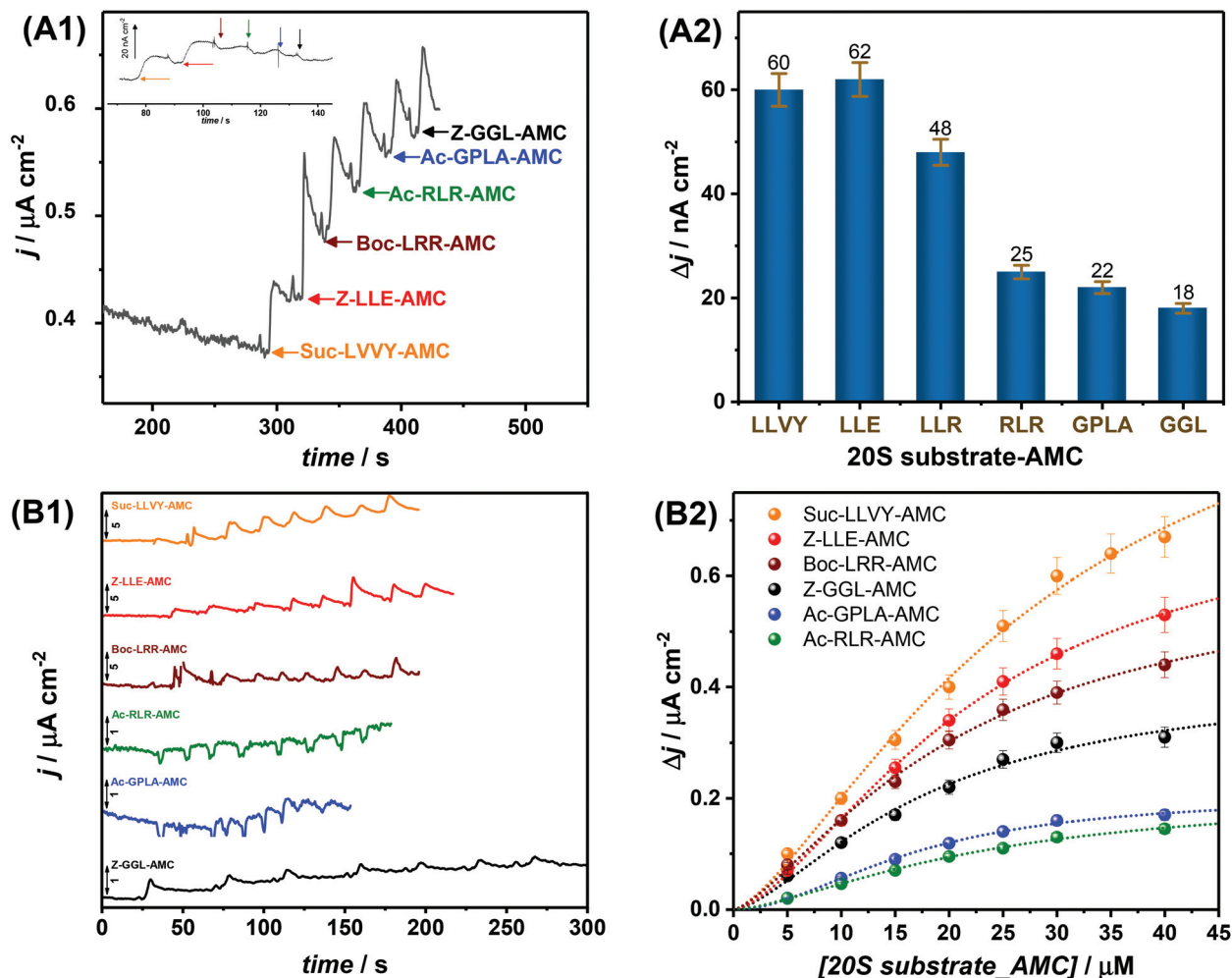
Due to the superior performances as described above, the biosensor GCE/Ab\_β5-20S was used to investigate the three different proteasome enzymatic activities, by using 2 different substrates each for caspase -, trypsin- and chymotrypsin-like activity. The substrates were Z-LLE-AMC, Ac-GPLA-AMC (caspase), Boc-LRR-AMC, Ac-RLR-AMC (trypsin) and Suc-LLVY-AMC, Z-GGL-AMC (chymotrypsin).

Initially, the change in current upon the injection of one concentration of each substrate in the same CA experiment was analyzed (Fig. 3A). The biosensor exhibited highest responses for the substrates Suc-LLVY-AMC, Z-LLE-AMC and Boc-LRR-AMC, while for the substrates Ac-RLR-AMC, Ac-GPLA-AMC and Z-GGL-AMC, the responses were less than half of the responses for the former set of substrates. In order to investigate the effect of saturation or any type of cooperative interaction, the substrates were injected also in different orders (not shown), but no difference in the biosensor response was observed.

The kinetic behavior of the immobilized 20S proteasome was also investigated. Usually, enzyme kinetics is different when immobilized or free in solution, mainly due to conformational changes occurring during the immobilization procedure. Therefore, the kinetic constants  $K_M$  and  $j_{\text{max}}$  (which corresponds to  $v_{\text{max}}$  in electrochemistry) are susceptible to alteration by such processes, since the conformational changes may hinder the access to its active site. Moreover, molecular diffusion in the proximity of the immobilized enzyme is different than that in bulk solution, leading to apparent kinetic parameter values (determined experimentally) different from the intrinsic values.<sup>31</sup> Therefore, the kinetics of

**Table 1** Analytical parameters of 20S biosensors based on the three different antibodies, Ab\_β5, Ab\_α, and Ab\_core, evaluated using the substrate Suc-LLVY-AMC

| 20S biosensor   | Sensitivity ( $\mu\text{A cm}^{-2} \text{mM}^{-1}$ ) | RSD (% , $n = 3$ ) | LOD ( $\mu\text{M}$ ) |
|-----------------|------------------------------------------------------|--------------------|-----------------------|
| GCE/Ab_β5-20S   | $24 \pm 0.9$                                         | 3.8                | 0.4                   |
| GCE/Ab_α-20S    | $12.1 \pm 0.7$                                       | 6.0                | 1.3                   |
| GCE/Ab_core-20S | $16.6 \pm 1.0$                                       | 6.2                | 0.1                   |



**Fig. 3** Kinetics and evaluation of caspase-, trypsin- and chymotrypsin-like activity of the immobilized 20S proteasome. CA responses recorded at +0.80 V in PAB pH 7.0 at GCE/Ab $_{\beta 5}$ -20S biosensor for (A1) consecutive injections of substrates, with each injection corresponding to one substrate (inset is for GCE/Ab $_{\beta 5}$ ) and (B1) one substrate for successive injections. (A2) Corresponding  $\Delta j$  derived from A1 for each substrate. (B2) Calibration plots from B1.

the 20S immobilized by Ab $_{\beta 5}$  was analyzed and compared to that obtained previously when free in solution.<sup>24</sup>

CA measurements were performed with the GCE/Ab $_{\beta 5}$ -20S biosensor for consecutive injections of each substrate (Fig. 3B1). The graphs of the variation of current recorded after the injection of each substrate vs. substrate concentration are shown in Fig. 3B2 (all substrates were analyzed using the same biosensor, with 30 minutes of rest time in between measurements when the biosensor was kept immersed in the PAB pH 7.0 solution). The plots present typical shapes in which the currents reach constant values for high substrate concentration due to the saturation of enzyme catalytic center with substrate molecules.

The sensitivity of the biosensor towards each substrate was analyzed by linear fitting of the first data points (Fig. 3B2). The biosensor displayed different sensitivities towards the 6 different substrates (Table 2), with highest sensitivities being recorded for the substrates of chymotrypsin-like activity: Suc-LLVY-AMC and Z-GGL-AMC, with values of 24 and 11  $\mu\text{A cm}^{-2}$

$\text{mM}^{-1}$ . The sensitivities for Z-LLE-AMC and Boc-LRR-AMC for caspase- and trypsin-like activity respectively were 17 and 14  $\mu\text{A cm}^{-2} \text{mM}^{-1}$ , but much lower for the substrates Ac-RLR-AMC and Ac-GPLA-AMC, targeting the same enzyme activities, which were 0.6 and 1.2  $\mu\text{A cm}^{-2} \text{mM}^{-1}$ . Lowest detection limits were calculated for substrates towards which the biosensor exhibited highest sensitivity values and were in the order Suc-LLVY-AMC, Z-LLE-AMC and Boc-LRR-AMC, with LOD values of 0.6, 0.7 and 0.9  $\mu\text{M}$ , respectively.

For each curve in Fig. 3B2, non-linear regression was applied to the experimental data points using the adapted Hill equation, eqn (1):

$$j = \frac{j_{\max} \times [\text{substrate}]^n}{K_{0.5}^n + [\text{substrate}]^n} \quad (1)$$

where  $n$  is the Hill coefficient and  $K_{0.5}$  is the substrate concentration at which  $j = j_{\max}/2$ . As a particular case, for  $n = 1$  it resembles the Michaelis-Menten equation. The data obtained

**Table 2** Analytical and kinetic parameters of the GCE/Ab- $\beta$ 5-20S biosensor towards various 20S substrates for the evaluation of caspase-, trypsin- and chymotrypsin-like 20S activity

| 20S activity | Substrate    | Sensitivity ( $\mu\text{A cm}^{-2} \text{ mM}^{-1}$ ) | LOD ( $\mu\text{M}$ ) | $\Delta j_{\text{max}}$ ( $\mu\text{A cm}^{-2}$ ) | $K_{0.5}$ ( $\mu\text{M}$ ) | $n$ |
|--------------|--------------|-------------------------------------------------------|-----------------------|---------------------------------------------------|-----------------------------|-----|
| Caspase      | Z-LLE-AMC    | $17 \pm 0.6$                                          | 0.7                   | 0.4                                               | 17.8                        | 1.5 |
|              | Ac-GPLA-AMC  | $1.2 \pm 0.06$                                        | 6.0                   | 0.2                                               | 16.8                        | 1.9 |
| Trypsin      | Boc-LRR-AMC  | $14 \pm 0.6$                                          | 0.9                   | 0.6                                               | 20.7                        | 1.4 |
|              | Ac-RLR-AMC   | $0.6 \pm 0.03$                                        | 7.1                   | 0.7                                               | 22.3                        | 1.6 |
| Chymotrypsin | Suc-LLVY-AMC | $24 \pm 0.9$                                          | 0.4                   | 1.2                                               | 28.5                        | 1.4 |
|              | Z-GGL-AMC    | $11 \pm 0.4$                                          | 1.2                   | 0.3                                               | 31.2                        | 1.4 |

after fitting the experimental data points are given in Table 2. As previously observed, yet in incubated solutions (both enzyme and substrate in solution),<sup>18,19</sup> for the Suc-LLVY-AMC substrate's chymotrypsin-like activity, the enzyme follows the closest Michaelis-Menten mechanism<sup>24</sup> with the highest velocity, evaluated here as  $j_{\text{max}}$ . Contrary to previous reports<sup>24,25</sup> this substrate showed the highest  $K_{0.5}$  value; however, all substrates presented  $K_{0.5}$  values that are one to two orders of magnitude lower than that observed when the enzyme is in solution. The values of  $K_{0.5}/\Delta j_{\text{max}}$  were compared to those obtained when proteasome is free in solution<sup>24,25</sup> (Table S1†). It was observed that when proteasome is immobilized, the values are lower but still in the same order of magnitude. This is explained considering that the immobilization of proteasomes on the electrode surface allows the release of the electroactive reaction product AMC in its spatial proximity and thus to a faster analytical signal. Contrarily, in incubated solution, the AMC reaction product needs to diffuse through the solution phase toward the electrode surface in order to react and give rise to the analytical signal.

The current response of the biosensor was transformed into concentration values based on the AMC calibration curve. Also considering the response time of the biosensor and the gravimetric analysis, the immobilized 20S proteasome specific activity with the  $10 \mu\text{M}$  Suc-LLVY-AMC substrate was calculated to be  $141 \text{ nmol min}^{-1} \text{ mg}^{-1}$ .

The specific activity of the 20S proteasome with the Suc-LLVY-AMC substrate determined with the proposed biosensor was compared with other methods including electrochemical techniques. It can be observed that the value obtained in the present work is the highest for the lower concentration of the substrate (Table 3).

**Table 3** Comparison of 20S activity against other methods of evaluation

| Method | Type | [Suc-LLVY-AMC] ( $\mu\text{M}$ ) | Specific activity ( $\text{nmol min}^{-1} \text{ mg}^{-1}$ ) | Ref.      |
|--------|------|----------------------------------|--------------------------------------------------------------|-----------|
| FS     | HM   | n.a.                             | 4                                                            | 32        |
| FS     | HM   | 40                               | 22                                                           | 33        |
| FS     | HM   | 75                               | 55.2                                                         | 33        |
| EC     | HM   | 100                              | 16.50                                                        | 24        |
| EC     | HT   | 10                               | 141                                                          | This work |

FS: fluorescence spectroscopy; EC: electrochemistry; HM: homogenous; HT: heterogeneous.

## 2.6. Inhibitor screening

Since most proteasome inhibitors especially block the chymotrypsin-like activity of the proteasome,<sup>16,34</sup> and considering that GCE/Ab- $\beta$ 5-20S exhibited the highest sensitivity towards the substrate Suc-LLVY-AMC, this substrate was further used to evaluate the influence of the two proteasome inhibitors on the biosensor activity, employing fixed potential chronoamperometry. For this study, the enzyme kinetics previously described was compared to the ones experimentally recorded in the presence of the natural inhibitor epoxomicin and of the synthetic bortezomib.

Epoxomicin is a potent naturally occurring proteasome inhibitor that disables the enzyme activity by mimicking the peptide substrates that bind covalently to some residues in the catalytic subunit of the enzyme. It was shown that it primarily inhibits the chymotrypsin-like activity of the proteasome, and is less efficient in inhibiting the trypsin- and caspase-like activities.<sup>24,34</sup>

The activity of the 20S proteasome at the biosensor surface in the presence of epoxomicin was evaluated by comparing its sensitivity value calculated from the CA experiment at  $+0.80 \text{ V}$  as shown in Fig. 4A, before and after the injection of  $5 \mu\text{M}$  of epoxomicin. Before the addition of Suc-LLVY-AMC, the biosensor was kept in the epoxomicin solution for 25 minutes.

As observed in the inset of Fig. 4A, the response of the biosensor in the presence of the inhibitor is significantly low, with a decrease in sensitivity by a factor of 2.5. The values of  $j_{\text{max}}$  decreased from  $1.16$  to  $0.4 \mu\text{A cm}^{-2}$ , and  $K_{0.5}$  remained practically constant ( $29.3 \mu\text{M}$  in the absence and  $31.4 \mu\text{M}$  in the presence of the inhibitor). The biosensor response to the substrate decreased upon increasing its incubation time from 2 to 10 minutes in epoxomicin solution, with this being an indicative of irreversible inhibition.<sup>12</sup>

For lower concentrations of epoxomicin, no influence on biosensor activity was observed, unlike what was reported when 20S was free in solution, when only  $1 \text{ nM}$  of the inhibitor blocked almost completely its chymotrypsin-like enzymatic activity.<sup>24</sup>

Bortezomib is a boronic acid dipeptide, with the boron atom being involved in its specific bonding to the catalytic site of the proteasome, which in return mainly slows down its chymotrypsin-like activity.<sup>35</sup> Bortezomib is electroactive and undergoes oxidation at approximately  $+0.80 \text{ V}$  in  $\text{pH } 7.0$ .<sup>36</sup> Bortezomib oxidation was confirmed by an increase in the

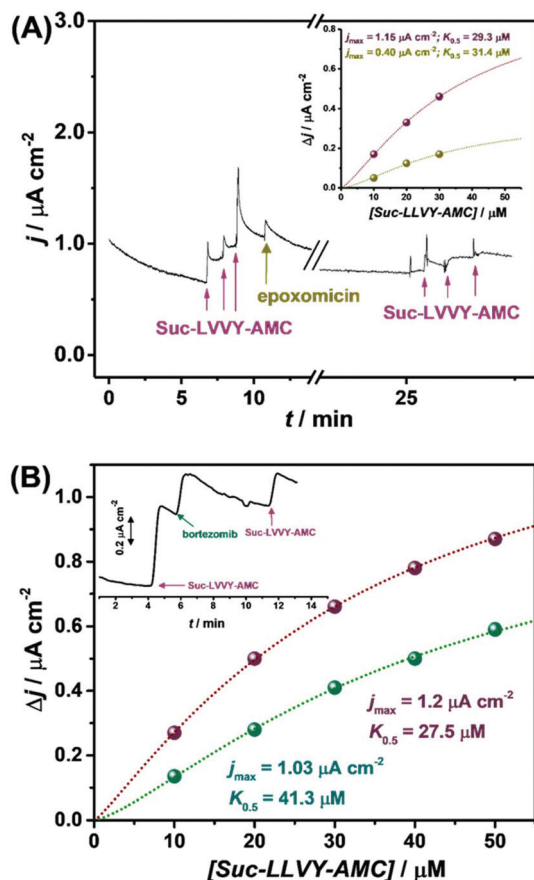


Fig. 4 Inhibitor screening. CA at +0.80 V in PAB pH 7.0 upon successive injections of 10  $\mu\text{M}$  of Suc-LLVY-AMC in the absence and in the presence of 5  $\mu\text{M}$  inhibitors in solution: (A) epoxomicin and (B) bortezomib.

anodic current upon its injection in the CA experiments at +0.80 V (Fig. 4B inset).

The influence of bortezomib on the immobilized 20S activity was evaluated by CA at +0.80 V, by comparing its response to Suc-LLVY-AMC in the absence and in the presence of 5  $\mu\text{M}$  bortezomib. The second injection of LLVY, in a solution containing bortezomib, was done after increasing the biosensor incubation time with the inhibitor from 1 minute to 30 minutes. The results revealed that the incubation time of the GCE/Ab $_{\beta 5}$ -20S biosensor with bortezomib did not have any influence on its inhibitory effect, the proteasome activity decreasing in all cases to half of the initial activity (see Fig. 4B).

The enzyme kinetics of the 20S in the presence of bortezomib was investigated as well, and results revealed an increase in  $K_{0.5}$  values from 27.3 to 41.3  $\mu\text{M}$  and a small decrease of the maximum current from 1.20 to 1.03  $\mu\text{A cm}^{-2}$ . The results were in agreement with the effect of the reversible and competitive inhibition,<sup>37</sup> the type of inhibition that bortezomib induces,<sup>38</sup> when  $v_{\text{max}}$  is eventually reached even in the presence of the bortezomib, but more substrate concentration is needed to reach it.

## 3. Experimental

### 3.1. Reagents and solutions

All reagents were of analytical grade and were used without further purification. Millipore Milli-Q nanopure water (resistivity  $\geq 18 \text{ M}\Omega \text{ cm}$ ) was used for the preparation of all solutions.

Proteasome 20S human, proteasome 20S  $\alpha$  subunit (human) monoclonal antibody (Ab $_{\alpha}$ ), proteasome 20S  $\beta 5$  subunit (human) monoclonal antibody (Ab $_{\beta 5}$ ), proteasome 20S core subunit polyclonal antibody (Ab $_{\text{core}}$ ) and proteasome substrates – *N*-succinyl(Suc)-LLVY-AMC of chymotrypsin, acetate(Ac)-RLR-AMC and *tert*-butyloxycarbonyl(Boc)-LRR-AMC of trypsin, and carbobenzoxy(Z)-LLE-AMC and acetate(Ac)-GPLA-AMC of caspase activity – were from Enzo Life Sciences. The substrate Z-GGL-AMC of chymotrypsin and 7-amino-4-methylcoumarin (AMC), poly-lysine and poly-histidine, bovine serum albumin and glutaraldehyde were from Sigma-Aldrich. The proteasome 20S inhibitors epoxomicin and bortezomib were from Millennium Pharmaceuticals and Enzo Life Sciences respectively.

Stock solutions of substrates and inhibitors were prepared in DMSO. Stock solutions of the 20S proteasome in proteasome assay buffer were prepared and kept at +4  $^{\circ}\text{C}$  until further utilization. Solutions of different concentrations of substrates were obtained by dilution in the proteasome assay buffer. The proteasome assay buffer (PAB) pH = 7.5 contained 50 mM Tris/HCl + 25 mM KCl + 10 mM NaCl + 1 mM  $\text{MgCl}_2$  + 100  $\mu\text{M}$  SDS all from Sigma-Aldrich.

### 3.2. Instrumentation

The electrochemical measurements were performed in a three electrode configuration, with a silver/silver chloride (Ag/AgCl) electrode as the reference electrode, a platinum wire as the counter electrode and the glassy carbon (GCE, with a geometric area of  $0.00785 \text{ cm}^2$ ) as the working electrode, immersed in a 3 mL electrochemical cell. All the electrochemical measurements were carried out with a MultiAutolab M101 potentiostat/galvanostat running on NOVA 2.1.4 software (Metrohm-Autolab, Utrecht, the Netherlands).

Cyclic voltammetry (CV) was performed with a step potential of 2 mV and a scan rate of  $100 \text{ mV s}^{-1}$ .

Differential pulse voltammetry (DPV) was performed with a pulse amplitude of 50 mV, pulse width of 100 ms, interval time of 1 s and scan rate of  $5 \text{ mV s}^{-1}$ .

For chronoamperometry (CA), the potential applied to the biosensor was fixed at +0.80 V vs. Ag/AgCl and the current was sampled every 0.5 s. The CA measurements were performed in an electrochemical cell containing 100  $\mu\text{L}$  solution, under continuous stirring (at approx. 300 rpm) while injection in the electrolyte solution in the proximity of the working electrode.

The GCE surface was cleaned first by mechanical polishing using a diamond spray, particle size 3  $\mu\text{m}$  (Kemet, UK). Then, a chemical cleaning was performed by recording several cyclic voltammograms in buffer until a reproducible baseline was obtained.

The quartz crystal microbalance Model QCM922 was obtained from Princeton Applied Research, with a Au quartz crystal with 9 MHz central frequency.

### 3.3. Preparation of the biosensors

The GCE was first modified with 0.5  $\mu\text{L}$  of 1  $\text{mg mL}^{-1}$  antibody (Ab $_{\beta 5}$ , Ab $_{\alpha}$  or Ab $_{\text{core}}$ ). To ensure the stability of the protein antibody layer, BSA 4% and GA 2.5% were mixed together with an antibody of choice. BSA was chosen due to its high terminal amino group content, to avoid the reaction of GA with the chosen antibody, which will eventually cause a denaturation of its native structure, essentially to be retained for further interactions with the proteasome. The antibody film was left to dry in air for 3 h and then immersed in a 1  $\text{mg mL}^{-1}$  proteasome 20S solution for 3 h. Finally, the electrode was washed in order to remove the loosely bound molecules and dried in order to obtain the GCE/Ab $_{\beta 5}$ -20S, GCE/Ab $_{\alpha}$ -20S or GCE/Ab $_{\text{core}}$ -20S biosensors. Biosensors containing a GCE modified with only a BSA + GA layer immersed in proteasome solutions, designated GCE/(BSA + GA)-20S, were also prepared, following the same procedure and skipping the antibody addition. When not used, the biosensors were kept at 4  $^{\circ}\text{C}$  immersed in PAB pH 7.0 solution.

## 4. Conclusions

A new amperometric 20S proteasome biosensor was developed based on the 20S immobilized on the surface of a GCE through antibody–20S specific interactions. Two monoclonal antibodies specific to  $\alpha$  and  $\beta 5$  subunits of the 20S proteasome, and one polyclonal antibody with a lower specificity, led to different orientations/configurations of the immobilized 20S proteasome and consequently different analytical properties. The activity of the proteasome 20S enzyme is measured by the occurrence of peak currents that appear upon oxidation of an electroactive probe, the 7-amino-4-methylcoumarin (AMC), that was released from the enzyme's substrates upon proteolytic reaction. The highest sensitivity was for the monoclonal antibodies specific to the  $\beta 5$  subunits of the 20S proteasome. The caspase-, trypsin- and chymotrypsin-like activity of the immobilized 20S proteasome was investigated with the biosensor based on  $\beta 5$  subunits, employing different substrates, two for each activity: Z-LLE-AMC, Ac-GPLA-AMC, Boc-LRR-AMC, Ac-RLR-AMC, Suc-LLVY-AMC, Z-GGL-AMC. The highest sensitivity was recorded for the substrates of chymotrypsin-like activity, Suc-LLVY-AMC. Non-linear regression was applied to the enzyme kinetic curves for the analysis of the kinetic parameters of the immobilized 20S proteasome. The effect of the inhibitors epoxomicin and bortezomib led to a decrease in biosensor sensitivity down to less than 50% of the initial value. The proposed biosensor represents a fast and economic technique for assessing the 20S proteasome activity and for sensitive screening of compounds with inhibitory activity in the micromolar range and potential medical applications.

## Author contributions

Madalina M. Barsan was involved in the conceptualization, data curation, formal analysis, investigation and writing of the original draft. Victor C. Diculescu was responsible for the funding acquisition and project administration, conceptualization and writing of the original draft.

## Conflicts of interest

There are no conflicts to declare.

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