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Voltammetric sensing of trypsin activity using gelatin as a substrate

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ABSTRACT

The relevance of proteases in many areas such as the industry, microbiology, and clinical analysis has prompted the development of different methods for protease detection and proteolytic activity determination. Electrochemical sensors and assays are valuable techniques for their short response time, simple fabrication, high sensitivity and possibility of miniaturization. Here, we describe the determination of the proteolytic activity of trypsin by using a gelatin-embedded electrochemical redox mediator the (4-((4-aminophenyl)imino)-2,6-dimethoxycyclohexa-2,5-dien-1-one). The peak current (i_p) was used as the analytical signal and measured by means of alternating current voltammetry (ACV). Upon exposure to different concentrations of trypsin, a decrease in the i_p expressed as the relative change was observed due to the release of the redox mediator in solution. A linear response range (5–80 μg/mL) was obtained, this trend was further corroborated using standard techniques such as the azo-casein, and the gel diffusion assay. A significant and negative correlation between these assays and the trypsin activity measured as the i_p relative change was observed.

1. Introduction

Proteases are enzymes that catalyse the hydrolysis of peptide bonds in proteins, both with a non-specific or a highly selective cleavage activity [1]. Proteases have a multifaceted role in biological systems and are involved in different biochemical, physiological, and regulatory processes, such as cell differentiation, cell cycle progression, cell death, DNA replication, immune response, tissue morphogenesis and remodelling just to mention a few [2]. Thus, it is not surprising that the human degradome is comprised of about 588 genes that code for proteases [3]. Since proteases are involved in multiple processes, alteration of the proteolytic activity is associated to a variety of pathologies, such as arthritis, cancer, neurogenerative disorders, inflammatory cardiovascular diseases among others [1,4]. Therefore, proteases are important biomarkers in clinical diagnosis and relevant drug targets [2].

Proteolytic activity is traditionally assessed in solution using universal or specific substrates modified with a chromophore or a fluorophore. The substrate hydrolysis by the protease leads to the release of peptides containing the chromogenic or fluorogenic group, whose color or fluorescence intensity, respectively, is used as an approximation of the proteolytic activity [5]. More informative techniques such as zymography differentiate the active and inactive forms of the enzyme by electrophoretic separation [6]. Enzyme-linked immunosorbent assays (ELISA) allow the assessment of the proteolytic activity of a specific target due to the selection with antibodies. Although these assays provide accurate information, they involve a multi-step process, require specialized personnel, instrumentation, and long processing times. Given the relevance of proteases, the development of simple, fast and sensitive assays using different technologies such as fluorescence resonance energy transfer (FRET), and electrochemical transduction methods has been afforded [7]. Among them, electrochemical systems are promising for their short response time, simple fabrication, high sensitivity, and possibility of miniaturization [8].

Systems using electrochemical transduction methods for protease detection and activity quantification are based on the selective binding (e.g. immune-based) or the proteolysis reaction (e.g. peptide or substrate-based), respectively. Immune-based systems use immobilized antibodies on different nanomaterials or magnetic beads [9–11], important issues concerning their cost and the binding ability of the immobilized biomolecules still arise, and importantly active and inactive protease forms are equally detected so they do not provide information about the proteolytic activity. Instead, the use of different substrates for proteases allows to directly assess the proteolytic activity, since substrate degradation is associated to a change in the

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electrochemical signal [8]. Modified peptides with a redox probe have been mainly described and proved to have a high specificity and stability, however, the modification of such peptide substrates is a complex process [12–17]. Alternatively, non-modified biomacromolecules like gelatin, have been also employed as substrates in the development of systems for the proteolytic assessment.

Gelatins the hydrolysis products of collagen, have been used to assess the proteolytic activity of trypsin. The described electrochemical systems are based on the use of additional immobilized enzymes such as glucose oxidase or urease covered by a gelatin film. The degradation of gelatin allows the immobilized enzymes to react with their respective substrates and the generated signal is proportional to the protease activity [18–20]. These systems have proved to be able to measure the proteolytic activity. However, their assembly and function are complex, requiring different reagents for the digestion process and the electrochemical measurement. As an alternative, other systems that employ a redox probe in solution and an electrode coated with a gelatin film, have been also developed. The gelatin film acts a kinetic barrier and its degradation by the protease facilitates the electron exchange [21,22]. Compared to the systems already described, the measurement process is less complicated, but still involving multistep processes, like enzyme incubation, rinsing and electrochemical signal recording. So, their application as rapid diagnostic tools, is compromised.

In this study, the development of a sensor measuring the proteolytic activity of trypsin is described. The ease of fabrication and simplicity of working mechanism are two important properties of this sensor, which requires no additional buffers or rinsing. Alternating Current voltammetry (ACV) was used as the transduction technique, because the selection of a fixed frequency in the range of 10–100 kHz permits to focus on a particular process occurring in the sample [23]. In this case, we were interested in monitoring the electron exchange of a redox mediator, eliminating parasitic currents such as the one associated to the charge and discharge of the double layer capacitance. An indoaniline derivative (4-((4-aminophenyl)imino)-2,6-dimethoxycyclohexa-2,5-dien-1-one) the IAd compound synthesized and previously described by Vivaldi et al. was used as the redox mediator [24]. The presence of an $–NH_2$ terminal allowed to bind the mediator to a surface using glutaraldehyde as the cross-linker. The sensor performance was compared to two conventional techniques (i.e. gel diffusion and azo-casein assay) for the assessment of proteolytic activity.

2. Materials and methods

2.1. Reagents and materials

Gelatin, 5% Nafion® 117 solution, glutaraldehyde 25% aqueous solution, trichloroacetic acid (TCA), Trizma base used for the preparation of Tris-HCl (pH 8; 50 mM) reaction buffer, bacteriological agar, sodium chloride, calcium chloride, potassium chloride, trypsin type I from bovine pancreas (10000 BAEE units/mg solid), Coomassie Brilliant Blue R-250, methanol, acetic acid, and azo-casein substrate were all purchased from Sigma Aldrich (Merck, Italy). Indoaniline derivative (4-((4-aminophenyl)imino)-2,6-dimethoxycyclohexa-2,5-dien-1-one), IAd compound was synthesized and previously described by Vivaldi et al. [24]. IAd was characterized by NMR (Bruker Advance DRX 400) [24] and ATR (Perkin Elmer Spectrum 100 spectrometer coupled with a MIRacle TM ATR accessory equipped with SeZn crystal) (Supplementary material).

Screen-printed gold electrodes on a ceramic substrate were purchased from Dropsens (SPGE, C220BT, Dropsens).

2.2. Sensing board

Electrodes consisted of a gold working (WE) (4 mm in diameter), a gold counter electrode (CE) and an Ag/AgCl pseudoreference electrode (RE). The RE was protected from degradation phenomena in basic environments by applying 0.3 μL of a 5% Nafion® solution.

Additionally, 3 μL of Ringer's solution (0.123 M NaCl, 1.5 mM $CaCl_2$, 5 mM KCl) added with 1% agar were also drop cast onto the pseudoRE to stabilize its potential by a gel layer keeping constant the local concentration of chloride ion.

2.3. Preparation of the electrode

Aqueous gelatin solutions (1 mg/mL) were prepared and dissolved at 50 °C in a water bath for about 15 min, and then cooled at room temperature. Stock solutions of 77 mM IAd and 1.3 M glutaraldehyde were prepared separately and then added to the gelatin solution to obtain a final concentration of 40 mM IAd and 0.6 M glutaraldehyde. Glutaraldehyde was used to anchor the IAd by its $–NH_2$ group and to increase the thermal and mechanical stability of the gelatin film [25,26]. Finally, 20 μL of the solution (equivalent to 20 μg of gelatin and 8 μg of IAd) were manually drop cast on the WE surface. Electrodes were then dried at room temperature for about 3 h.

The same volume of a gelatin solution only added with glutaraldehyde was drop cast on electrodes to be used as blank reference devices to compare to sensors.

3. Electrochemical measurements

3.1. System characterization

All measurements were performed at 37 °C in a natural convection oven (ICN 55, ArgoLab) in Tris-HCl. First the stability over time of the pseudo RE was evaluated by Open Circuit Potential (OCP) measurements against an external Ag/AgCl RE/NaCl 3 M (RE-3VT, ALS Co, Ltd), during 1 h in Tris-HCl and after 30 min of incubation with 200 μg/mL of trypsin. Electrochemical characterization of IAd embedded in the gelatin film and the blank, was performed by Cyclic voltammetry (CV), parameters were set as follows: window potential −0.6 to 0.2 V vs Ag/AgCl; E_{step} 0.01 V, scan rate 0.1 V/s. Additionally, a non-modified bare electrode was used to evaluate the electrochemical behaviour of IAd in solution in contrast to the embedded IAd by means of CV using the same amount of IAd (8 μg) in 3 mL of buffer solution, parameters were set as previously mentioned. Palmsens 4 potentiostat was used to perform the measurements with PSTrace 5.8 as controlling software.

3.2. System validation and trypsin activity evaluation

Trypsin type I from bovine pancreas (10000 BAEE units/mg solid) (Sigma - Aldrich) known to cleave gelatin at the C-terminal of lysine and arginine residues was used as a model protease. All digestion reactions were performed in Tris-HCl buffer at 37 °C, as the optimal pH range for trypsin activity is 7–9 [27]. Trypsin solutions were freshly prepared in Tris-HCl, and reactions were conducted in 900 μL Tris-HCl (pH 8; 50 mM) to which 100 μL of an enzyme solution were added to give a final concentration of 5, 8, 20, 50, 80, and 200 μg/mL. Alternating current voltammetry (ACV) was used as the transduction technique and set as follows: window potential −0.4 to 0 V; E_{step} 0.01 V; scan rate 0.05 V/s; 0.01 V AC voltage vs Ag/AgCl with a fixed frequency of 110 Hz. The peak current (i_p) value at the E_{redox} potential of the IAd was used as the analytical signal. Gelatin-Iad coated electrodes were immersed in the reaction solution (Tris-HCl) at 37 °C and the ACV response was continuously measured every 30 s until obtaining a stable i_p value. Upon enzyme addition, the i_p was continuously registered up to 30 min. The i_p change was expressed as the relative variation i_p Rel. var. (%) and calculated as follows (eq. (1)):

$$i_p\text{·Rel. var.}\,(\%) = \left(\left(i_{pf} - i_{p0}\right)/i_{p0}\right) \times 100 \quad (1)$$

where i_{p0} and i_{pf} are the i_p values before at t_0 and after (t_f) the enzyme addition, respectively. Three replicates per each enzyme concentration

were performed.

In an additional experiment, gelatin coated electrodes were incubated with 200 μg/mL of trypsin, after different time intervals 5, 15 and 30 min. The electrodes were gently rinsed and then transferred in another vial containing the reaction buffer with no enzyme. The ACV current was measured before the addition of the enzyme and after the transfer of the electrode in the fresh buffer.

Gelatin degradation upon trypsin exposure was also characterized by impedance (window potential −0.4 V to 0 V, E_{step} 0.01 V, 0.01 V AC voltage vs Ag/AgCl at a fixed frequency 110 Hz). Trypsin (200 μg/mL) was used, and measurements performed in Tris-HCl buffer at 37 °C and three replicates were performed.

3.3. Coomassie Blue staining

Coomassie Blue staining was performed to evaluate the gelatin degradation on the WE surface resulting from the enzyme activity. Briefly, sensors were stained with 1× Coomassie Brilliant Blue R-250 (Sigma-Aldrich) staining solution (0.5% Coomassie Brilliant blue, 5% methanol, 10% acetic acid) for 1 h at room temperature and then destained in destaining solution (10% methanol, 5% acetic acid) for 1 h [28].

3.4. Gel diffusion assay

The proteolytic activity was measured by a radial diffusion assay based on the use of agarose-gelatin gels in which clear zones appear after staining as a result of gelatin degradation. The assay was performed as described by [29,30] with minor modifications. Briefly, 1 mm thick 2% agarose gels in Tris-HCl, containing 1 mg/mL gelatin were prepared and poured between two plexiglass plates. After solidification, one plate was slid away, and wells (ø= 3.5 mm diameter) were punched in the gel surface. Trypsin solutions at different concentrations at different concentrations (5, 8, 20, 50, 80 μg/mL) were prepared as above described and 8 μL were loaded into the wells and incubated for 6 h at 37 °C in a humid chamber. Upon incubation, gels were covered with filter paper and then slightly dried by heat, followed by immersion in 10% trichloroacetic acid (TCA) solution for 10 min to allow the hydrolyzed gelatin to diffuse from the agar. Finally, gels were stained with 1× Coomassie Brilliant Blue R-250 staining solution for 1 h at room temperature, then destained in the destaining solution overnight [28], and the diameter in millimeters of the clear zones around the wells was measured. Three replicates were performed, and Tris-HCl buffer alone was used as the negative control.

3.5. Azo-casein assay of proteolytic activity of trypsin

The proteolytic activity of trypsin was additionally assessed using azo-casein i.e. a derivate of casein conjugated to an azo-dye as the substrate. In this assay, the degradation of casein associated to the proteolytic activity of endopeptidases results in the release of a soluble dye that can be quantified in the solution by light absorption at 400 nm. We used the procedure described by Kessler et al [5] with minor modifications. Briefly, 375 μL of 4 mg/mL azo-casein in Tris- HCl and 125 μL of trypsin at different concentrations (5, 8, 20, 50, 80 μg/mL) in Tris-HCl were mixed and incubated at 37 °C for 1 h. Then, 250 μL of 10% (w/v) (TCA) were added to stop the reaction, and the undigested or partially digested azo-casein were allowed to precipitate at room temperature for 1 h, followed by centrifugation at 13000 rpm for 20 min (Universal Centrifuge Z326 K, Hermle Labortechnik). Finally, the supernatant was carefully collected, and absorbance measured at 400 nm (UV/Vis Jasco V-550). Three replicates per sample were evaluated and the reaction buffer alone was used as the blank.

3.6. Test on wound exudates

Exudate samples were collected at the Wound Healing Research Unit of the Department of Dermatology at University of Pisa and kept at −80 °C until use. The collection was performed in accordance with the Declaration of Helsinki and a written informed consent was obtained from donors. Four exudate samples were collected, then pooled and used for the experiments. As proposed by Tengrove et al. [31], trypsin was used as the reference protease standard, and the trypsin calibration curves obtained for trypsin activity measurement by ACV and by the azocasein assay were used to approximate the protease concentration in the wound exudate sample. The quantification of protease in exudate samples was assessed using the azocasein assay as described above and calculated from the trypsin standard curve.

Gelatin-IAd based sensors were immersed in Tris-HCl and the i_p value was allowed to stabilize at 37 °C. 20 μL of the wound exudate sample were spiked in 980 μL of reaction buffer, and AC voltammetry was performed as previously described to determine the protease concentration. Changes in i_p values were registered up to 30 min and expressed as the relative variation. Four replicates were performed.

4. Data analysis

Data were expressed as the mean ± standard deviation (SD). Differences over time in the pseudo-RE potential in presence of the buffer only (1 h of incubation) and after the addition with trypsin (30 min of incubation) were assessed by the Kruskal-Wallis test. Correlation analysis between the proteolytic activity measured by the developed assay, expressed as the relative variation of i_p, the gel diffusion and azo-casein assay was performed by the Pearson correlation test using the GraphPad Instat software (version 6.05 for Windows, La Jolla, CA, United States). The significance level was set at $p < 0.05$.

5. Results and discussion

5.1. Working principle

The sensor exploits a gelatin layer loaded with a redox mediator (IAd) bound to glutaraldehyde through its amine group [32]. Glutaraldehyde is known to promote the formation of cross-links in collagen by the reaction of the free amine groups of lysine and hydroxylysine, thus improving the thermal and mechanical stability of the gelatin film [26,33]. Under these conditions, IAd closely interacts with the electrode surface and so the electron transfer would take place thus originating a current peak in the ACV voltammogram at the E_{redox}. At this potential, the maximum current value (i_p) was observed and used as the analytical signal and its change upon exposure to different concentrations of trypsin was registered. We hypothesize that digestion of the gelatin substrate by a proteolytic enzyme releases the IAd in solution and decreases both the electron transfer and the ACV current peak (i_p) (Fig. 1), thus permitting to monitor the enzymatic activity.

5.2. System characterization

The stability of the pseudoRE coated with Nafion and Ringer's agar solution was evaluated by OCP. A stable potential over the incubation time was observed (82 ± 3 mV at t_0), 82 ± 3 mV after 1 h of incubation in Tris-HCl and 81 ± 3 mV after 30 min. of incubation in Tris-HCl and trypsin. No significant difference was observed ($p = 0.67$).

In the traditional Ag/AgCl RE, the electrode potential follows from the Nernst equation:

$$E = E^0 - 2.303(RT/F)\log([Cl^-])$$

where E^0 is the electrode standard potential (V), R is the molar gas constant (J $K^{-1}mol^{-1}$), T is the absolute temperature (K), F is the

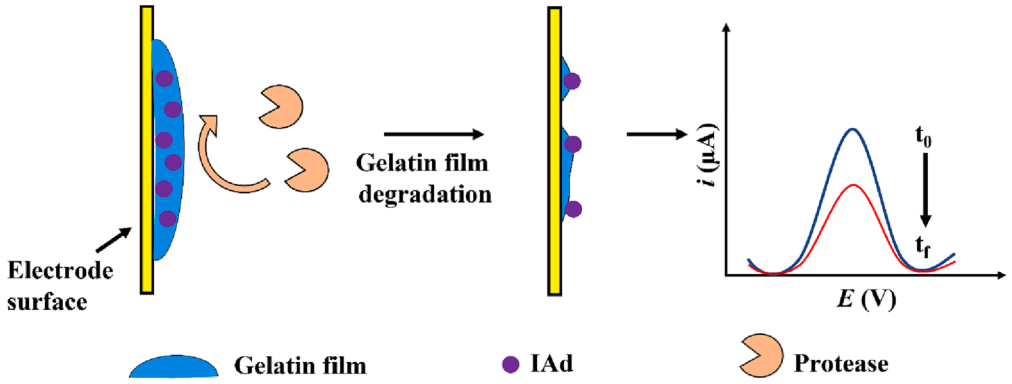

Fig. 1. Schematic representation of the sensing principle for the determination of the proteolytic activity.

Faraday constant (C mol^{-1}), and [Cl^-] is the concentration of free chloride ions (M). The potential of a RE is proportional to the [Cl^-], which is typically kept constant by surrounding the electrode with KCl [34]. In this work, the potential of the Ag/AgCl pseudo-RE was stabilized by the deposition of a Ringer's agar gel, which can help keeping a constant chloride ion concentration even when working with complex matrices.

The first electrochemical characterization of the system was performed by CV. The signal obtained from the blank reference device, i.e. the sensing board whose WE coating layer did not include IAd, was acquired and compared to the sensor signal (Fig. 2 A), The voltammogram of the blank (dotted line) presented no peaks, in contrast with the sensor signal (plain line), where two peaks were evident at −0.180 V (peak *a*) and −0.280 V (peak *b*) that corresponded to the oxidation and reduction of IAd during the reversible redox process. This indicated that the electrochemical sensor response was due to the presence of IAd in the gelatin film, which allowed the exchange of electrons with the WE.

Changes in the electrochemical behaviour of IAd upon binding to glutaraldehyde were also evaluated using CV by comparing the sensor and the IAd in solution voltammograms (Fig. 2 B). Oxidation and reduction peaks were observed at −0.22 V (peak *c*) and −0.25 V (peak

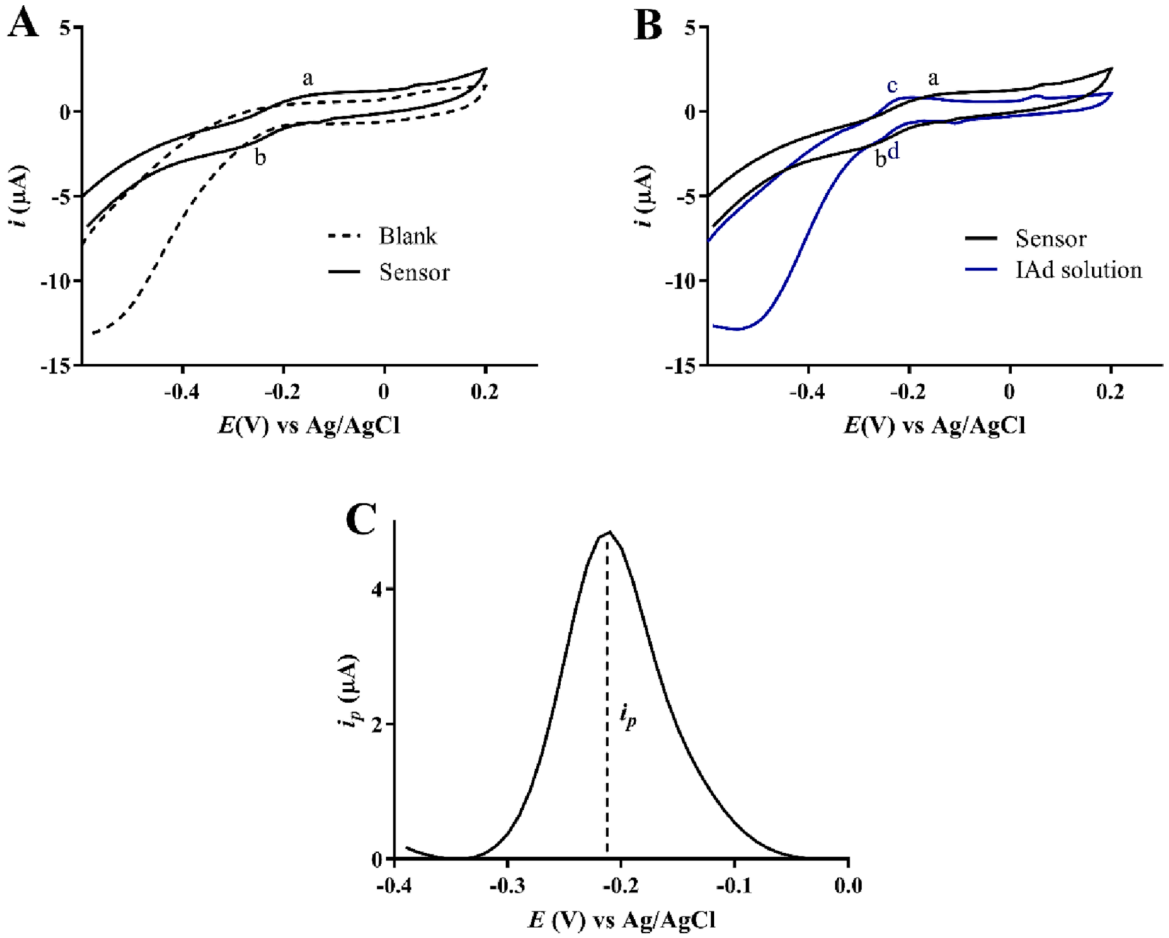

Fig. 2. A) CVs of the blank reference (dotted line) versus sensor device (continuous line) gold electrode in Tris-HCl. Bound IAd oxidation (a) and reduction (b) peaks; B) CVs of bulk IAd (blue line) versus gelatin embedded IAd (black line) in Tris-HCl. Bulk IAd oxidation (c) and reduction (d) peaks and C) ACV voltammogram of IAd before gelatin hydrolysis showing the i_p current peak.

d), respectively, in the IAd solution voltammogram (blue line), with a peak-to-peak separation (ΔE) of about 30 mV, in accordance with the ΔE previously reported by Vivaldi and et al. for a two-electron and two-proton redox reaction of IAd [24]. In contrast, the ΔE of the IAd embedded in the sensing layer (100 mV) was higher than the one calculated for the IAd in solution. The increase in the ΔE observed for the embedded IAd may be associated to a slower reaction kinetics due to its encapsulation in the gelatin film. Although it is evident that the redox probe can interact with the electrode surface, its access could be slightly hindered by the presence of gelatin. Previous studies reporting the use of gelatin modified electrodes referred to gelatin as a kinetic barrier for the electron transfer between the redox probe in solution and the electrode, which resulted in an increase in the ΔE, compromising as well the reversibility of the system [35].

Vivaldi et al described the use of IAd as a pH sensitive molecule, in a study where the E_{redox} position was used for pH determination [24]. In this case, the E_{redox} calculated as the mean of the anodic and cathodic peak potential at pH 8 (Tris-HCl 50 mM) was −0.23 V. At this potential, the i_p current value measured by ACV had its maximum value and for this reason it was used in this work as the analytical signal (Fig. 2C).

5.3. Sensor response to trypsin

The universal proteolytic activity was measured using trypsin, this enzyme is commonly used as a reference standard protease in commercial assays. Prior to enzyme exposure, electrodes were immersed in the reaction buffer and incubated at 37 °C until a stable i_p signal was obtained. Upon stabilization, a known concentration of trypsin solution

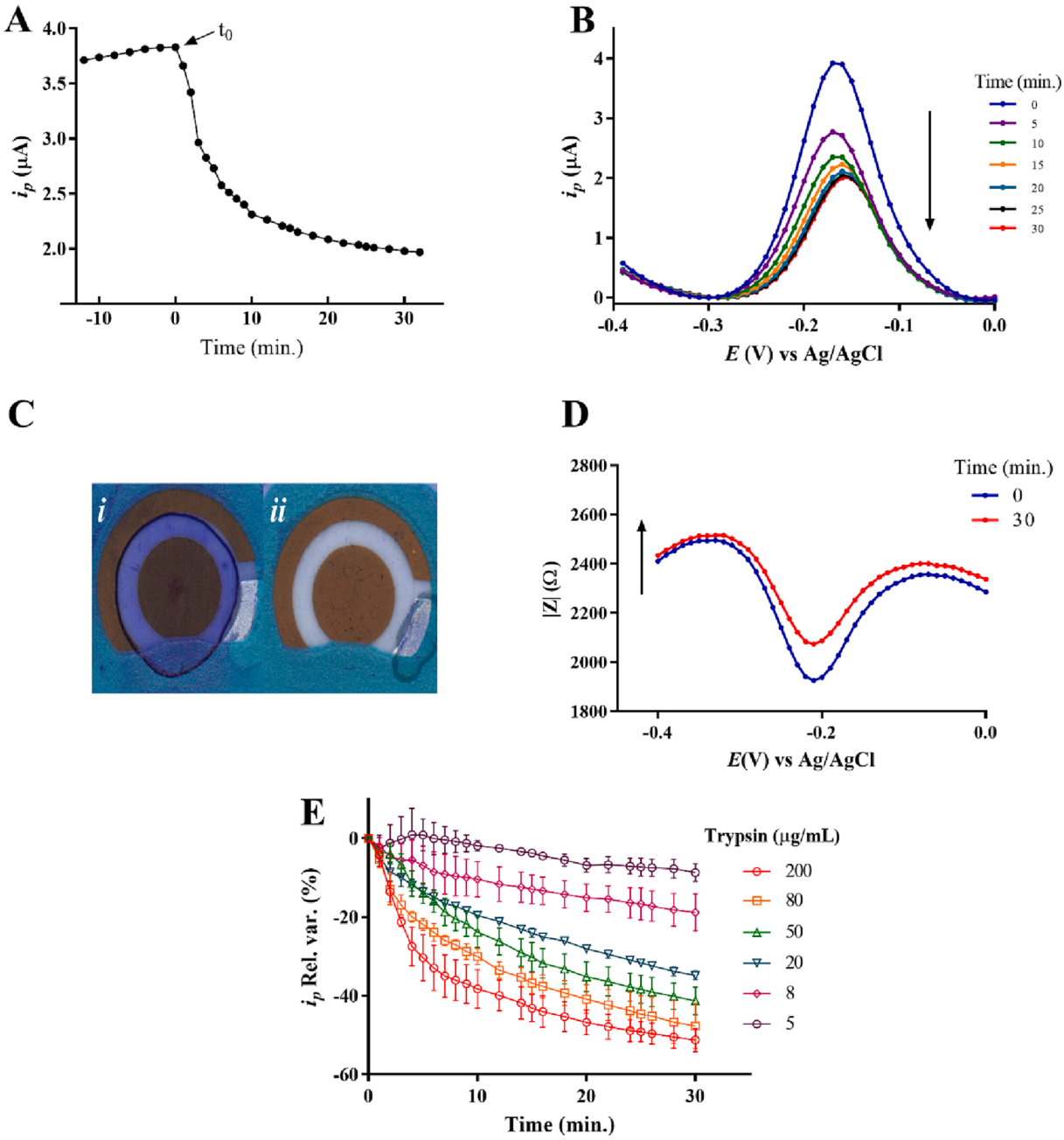

Fig. 3. (A) AC voltammetry peak current (i_p) vs incubation time, upon exposure to 200 μg/mL trypsin: t_0 indicates the last measurement before enzyme addition; (B) decrease of the ACV peak (i_p) over time; (C) Coomassie blue staining i) negative control and ii) electrode exposed to 200 μg/mL of trypsin for 30 min; (D) Impedance module variation upon exposure to 200 μg/mL trypsin and (E) Relative variation (%) of i_p at different trypsin concentrations vs reaction time.

was added and changes of the i_p signal were registered over time (Fig. 3A). The registered signal relies on the diffusion of the redox probe from the WE upon gelatin degradation, so the rate of gelatin degradation varies according to the protease concentration. In this study, the electrode characterization was first performed with a trypsin concentration of 200 μg/mL. The exposure to such concentration of trypsin led to a gradual decrease of the i_p value which suggests the release of the IAd in solution due to the proteolytic activity of trypsin on the gelatin film. The i_p signal decreased with the incubation time and a plateau was reached after 30 min of incubation (Fig. 3A-B), which may denote the time needed for a nearly complete degradation of the gelatin film under the test conditions. Indeed, the Coomassie blue staining of the gelatin film following 30 min of incubation with 200 μg/mL of trypsin (Fig. 3C) effectively showed the removal of gelatin in contrast to a negative control (buffer only). Based on these results a 30-minute incubation time was used as the reaction time in subsequent tests.

Further experiments showed that incubation with 200 μg/mL trypsin and subsequent ACV measurement in fresh buffer without the enzyme, after electrode rinsing, resulted in the decrease of the i_p value, being this value higher at prolonged incubation times (Fig. S2). These results indicate the validity of the working principle proposed for this sensor. Since ACV measurement after gelatin degradation was performed in a buffer with no enzyme, the registered signal current would only account for the IAd still present in the gelatin film.

EIS was used to further confirm that the proteolytic activity resulted in the release of IAd and so to the decrease of the amount available for the electron transfer. The addition of 200 μg/mL of trypsin and the subsequent removal of the gelatin film produced an increase of the impedance module $|Z|$ at the E_{redox} of about 10 ± 3 % after 30 min of incubation (Fig. 3D). In accordance with the results obtained by ACV, such behaviour could be associated to a decrease of the number of IAd molecules available for the electron exchange. This is consistent with what can be found in literature, where in a number of papers from different authors, the proteolytic activity resulting in the release of a redox tag from peptide-based sensors or the release of an enzyme embedded in gelatin taking part in the electron transfer process, have been associated to the decrease of the current signal due to a diminished electron transfer with the electrode [15–17,20].

Different concentrations of trypsin (5, 8, 20, 50, 80, and 200 μg/mL) were evaluated and associated to a relative decrease of the i_p over time (Fig. 3E). A more evident i_p decrease was observed at high concentrations of trypsin, which may be associated to a faster proteolysis of the gelatin film. For instance, a decrease of 30 % ± 6 was observed after 5 min of incubation in the presence of 200 μg/mL, whereas a 0.8 % ± 4 variation was detected at the lowest concentration (5 μg/mL). Indeed, at this concentration level a subtle increase of the relative variation of i_p was first observed before the decrease. It is thought that the perturbation of the signal associated with the addition of the enzyme at such a low concentration may be higher than the signal associated to the enzyme's proteolytic activity.

Electrochemical sensors using gelatin as the substrate for the determination of the protease activity have been reported in different studies [20–22,36]. In presence of a gelatin film, the electrochemical signal is associated to the electron exchange between the electrode and a probe present in solution. The measurement using such system is a multistep process since it requires an incubation step with the enzyme, a rinsing step and then the transfer in a solution containing the redox probe [21,22]. Although this strategy has proved to efficiently respond to the enzyme proteolytic activity, its application as a diagnostic tool is compromised by the requirement of different steps for the measurement. Other studies have reported the combination of the gelatin film with an enzyme, so the recorded signal depends on the reaction catalyzed by the embedded enzyme once it is released in solution [20]. However, in these systems it should be considered that enzymes may lose their activity during the entrapment process. The reported systems, mainly use disk electrodes, which compromise their portability. In our study we present a sensor based on the use of screen-printed gold electrodes with a gelatin film containing a redox probe that allow to evaluate the protease activity in a single step.

5.4. Sensor calibration

The relative variation of i_p with respect to the logarithm of trypsin concentration showed a linear trend in the range of (5–80 μg/mL) after a 30-minute incubation time (Fig. 4A). The largest percentage decrease of i_p, about 48 ± 6 was observed with 80 μg/mL of trypsin, while a decrease of 9 ± 2 % was found at 5 μg/mL trypsin concentration. A linear regression curve was used to fit the data.

The proteolytic activity of trypsin at different concentrations was parallelly assessed using two different quantitative methods, the gel diffusion, and the azo-casein assay. Using these assays, at increasing concentrations of the enzyme, larger diameters of the clear zones (mm) (Fig. 4B and Fig. S3) and higher A.U at 400 nm (Fig. 4D) values, were observed. For both these assays a linear trend was observed in the selected logarithmic concentration range (5–80 μg/mL). Importantly, the sensor response expressed as the i_p relative variation significantly and negatively correlated to the two contrasting methods, the gel diffusion ($r = -0.998$, $p < 0.001$) (Fig. 4C) and azo-casein assay ($r = -0.987$, $p < 0.005$) (Fig. 4E) in the aforementioned range. This finding demonstrates the validity of this sensor, with a real advantage in the assay-turnaround time and the ease of use, if compared to azo-casein and gel diffusion assay.

Using the azocasein assay, concentrations lower than 5 μg/mL (0.1–2 μg/mL) were also tested to widen the calibration range at low protease levels and a linear response was observed (Fig. S4).

5.5. Sensor response to wound exudate

Exudate samples from chronic wounds were used to assess the ability of the sensor to estimate the proteolytic activity in a complex matrix. Protease concentration in these samples was approximated from the calibration curve generated using trypsin as a standard. 20 μL of untreated wound exudate samples were added to a final volume of 1 mL of reaction buffer and the signal change was registered for 30 min.

Exudate samples were pooled, and four replicate measurements were performed. From the i_p response, the concentration of proteases in the sample was estimated to be about 191 ± 88 μg/mL (considering the dilution factor). From the azocasein assay, the estimated concentration of proteases was about 29 ± 8 μg/mL. So, the wound exudate protease concentration approximated from the i_p response was seven-fold higher than the one estimated by the azocasein assay.

Trypsin is commonly used as a reference standard protease [31]. In this study, the calibration curves generated for trypsin were used for the determination of the proteases in the wound exudate sample. As with trypsin, the i_p value decreased upon the addition of the exudate sample. The concentration of active proteolytic enzymes in the sample was determined and compared to the values obtained using the azocasein assay, being the estimated concentration seven-fold higher than the one approximated with the azocasein assay. Exudate from chronic wounds may contain elevated levels of different proteases such as Human Neutrophil Elastase (NHE) and Matrix Metalloproteinases (MMPs) [37], with diverse activities. Additionally, proteases within chronic wounds may have different affinities towards the two substrates here used (i.e. azocasein and gelatin). For instance, in chronic wound samples besides MMP's, other serine proteases can degrade azocasein, instead MMP-2 and MMP-9 are the most predominant enzymes known to degrade the gelatin [31]. Moreover, the presence of microbial species able to release proteolytic enzymes, like *Pseudomonas aeruginosa* may also contribute to the disparities observed in the measurement of proteolytic activity [38]. However, these interfering effects do not affect the self-consistent measurement of the proposed device. In fact, as shown in the previous sections the analysis is well correlated with the reference measurements.

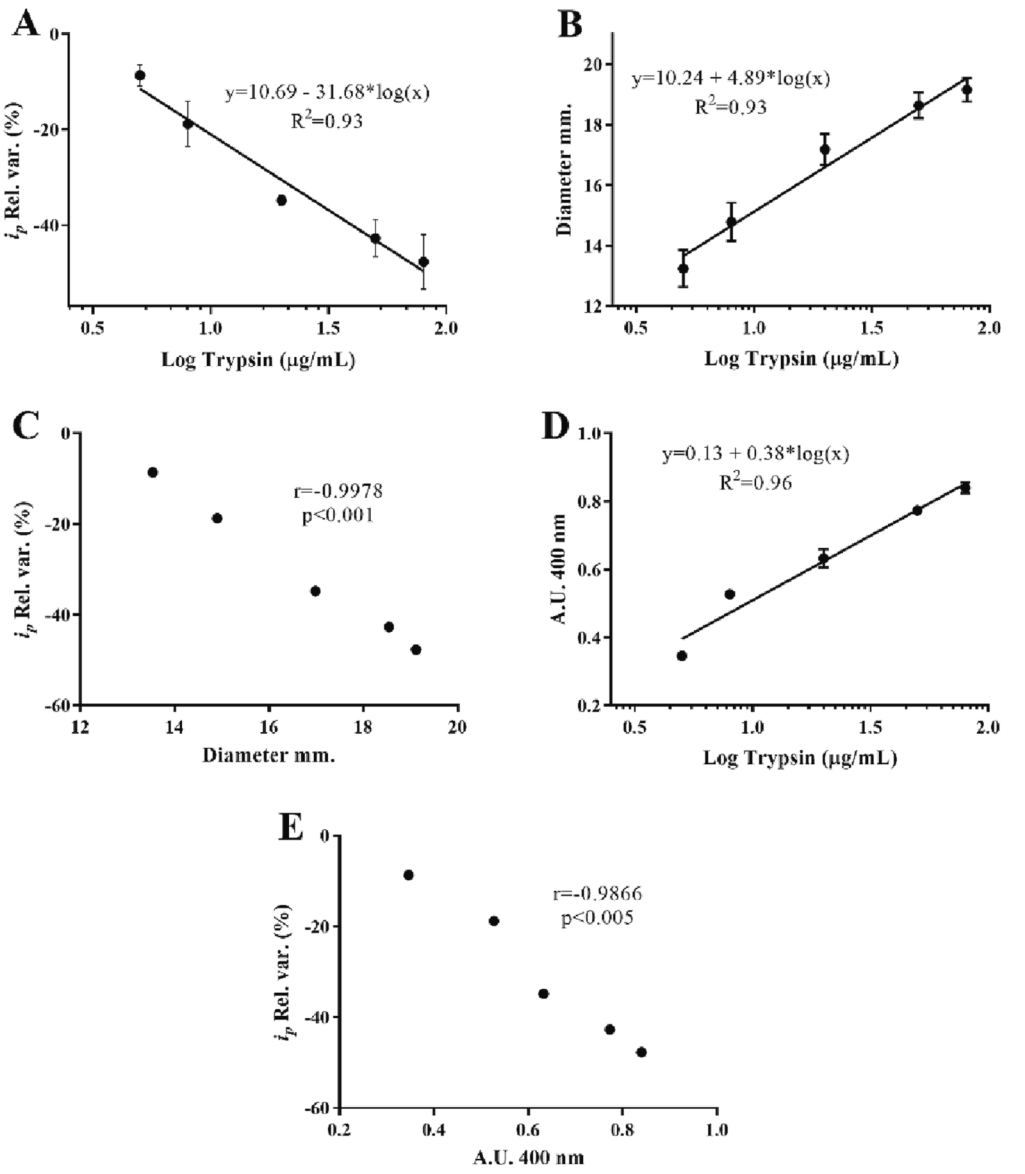

Fig. 4. (A) Calibration curve showing the relative decrease of i_p upon exposure to different concentrations (5–80 µg/mL) of trypsin measured by ACV; (B) Calibration curve of the proteolytic activity of trypsin measured by the gel diffusion assay; (C) Correlation between the i_p relative variation response and the diameter of clear for the gel diffusion assay; (D) Calibration curve of the proteolytic activity of trypsin measured by the azo-casein assay and (E) Correlation between the i_p relative variation response and the A.U at 400 nm for the azo-casein assay.

Future studies should assess the wound exudate protease activity using the sensor's substrate, i.e. gelatin, and zymography may be a suitable technique for this purpose [39].

6. Conclusions

In this work the development of an electrochemical sensor based on the use of ACV is described for the determination of the proteolytic activity of trypsin using gelatin as a substrate on which the redox mediator IAd was directly immobilized. AC voltammetry was used as the transduction technique and the percentage i_p signal variation was recorded upon exposure to different concentrations of trypsin. The decrease in the i_p was associated to the release of the embedded IAd probe in solution due to the degradation of the gelatin film by trypsin, making the electron transfer process more difficult. A complementary experiment confirmed the association of the degradation of the gelatin film with the IAd release and consequent i_p decrease. The use of fresh buffer without the enzyme and ACV measurement of electrodes previously exposed to trypsin showed a decrease in the i_p signal.

A linear regression curve was observed in response to different concentrations of trypsin in the range (5–80 µg/mL) with a reaction time of 30 min, a working range ideal for different applications, such as the measurement of high serum trypsin levels (5–20 µg/mL) in acute pancreas diseases [40]. The i_p variation upon gelatin film degradation was significantly and negatively correlated to the proteolytic activity results obtained by the gel diffusion and azo-casein assay. Although in this study 30 min of incubation was chosen as the optimal reaction time, higher time reactions might increase the i_p signal variation and in this case lower concentrations could also be assessed. This assay may represent a reliable and fast alternative for the determination of the proteolytic activity as well as a method for the screening of protease inhibitors.

The sensor was also tested in wound exudate samples. If compared to the azocasein assay, a seven-fold higher protease concentration in exudate samples was estimated using the sensor. We speculate that factors like the different nature of substrates and the different enzymatic activities may explain the difference. Notwithstanding this, the system may become a promising tool to assess proteolytic activity in complex samples, as no pretreatment procedures (i.e. precipitation, purification) are needed.

CRediT authorship contribution statement

Noemi Poma: Investigation, Writing – original draft, Visualization,

Conceptualization. **Federico Vivaldi:** Investigation, Methodology, Conceptualization, Writing – review & editing. **Andrea Bonini:** Investigation, Methodology, Writing – review & editing. **Denise Biagini:** Investigation, Methodology, Writing – review & editing. **Daria Bottai:** Writing – review & editing, Resources. **Arianna Tavanti:** Conceptualization, Writing – review & editing, Resources. **Fabio Di Francesco:** Supervision, Conceptualization, Methodology, Funding acquisition, Writing – review & editing, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2023.108631.

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