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Protease Amperometric Sensor

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An amperometric biosensor for the detection of trypsin was developed. The latter was based on a two-layer configuration, namely, a polymer−glucose oxidase inner layer and a gelatin outer layer. In the presence of glucose, the enzyme layer produces H_2O_2 and hence an amperometric signal due to H_2O_2 electrooxidation was generated by potentiostating the electrode at 0.6 V. The biosensor detects the change in the increase in the maximum current caused by the proteolytic digestion of gelatin, which covers the platinum electrodes, thereby facilitating a speedier access for the glucose substrate to the electrode modified with both poly(pyrrole−alkylammonium) and glucose oxidase molecules. Our biosensor detected low trypsin concentrations down to 42 pM with a response time of ∼10 min, making it a very sensitive device in the detection of lower trypsin levels with such future putative applications as the diagnosis of pancreatic diseases.

Any disorder in enzymatic expression can induce diseases. Among different classes of enzymes, proteases are known to participate in numerous physiological processes such as cell growth and differentiation, cell−cell communication, and cell death. One such protease, trypsin, is the most important digestive enzyme produced by the pancreas. Trypsinogen (the precursor for trypsin) is the only proenzyme that is specifically activated by a special brush border enzyme (enterokinase) within the duodenum where the conversion of trypsinogen to active trypsin is mediated by the release of its 8-amino acid activation peptide, trypsinogen activation peptide.[1] Trypsin is the only enzyme involved in the digestive enzyme activation cascade, which changes all of the other pancreatic proenzymes into their active forms within the intestine, and then initiating autodigestion. Finally, trypsin plays a key role in controlling pancreatic exocrine function. If free trypsin activity in the duodenum diminishes because of a meal, then a trypsin inhibitor, a trypsin-sensitive peptide, cholecystokinin (CCK) releasing factor, avoids hydrolysis, accumulates, and stimulates CCK release from specialized cells on the intestinal mucosa. The CCK then stimulates the pancreas to secrete more digestive enzymes until the meal is digested and basal levels of free enzyme activity return.[2]

Various types of pancreatitis such as chronic pancreatitis (inflammation), acute pancreatitis (inflammation) and pancreatic abscess (related to an infection) involve either irritation, inflammation, or infection of the pancreas. Trypsin and trypsinogen levels are increased with some types of pancreatic disease such as acute pancreatitis and cystic fibrosis. Unusually, low or normal levels may be seen in chronic pancreatitis.

Acute pancreatitis is often defined as abdominal pain associated with hyperamylasemia (or lipasemia) of greater than three times above normal.[3,4] Acute pancreatitis affects around 40/100 000 of the Western general population, and its attacks are classified as severe in 20−30% of the patients. This complex and ill-understood common acute abdominal disorder lacks specific therapy, and its clinical course and outcome are difficult to predict clinically at the time of admission. Although conservative measures are usually sufficient for the management of the large majority of cases of acute pancreatitis that prove mild, early aggressive intervention including enteral feeding, endoscopic retrograde cholangiopancreatography with sphincterotomy, broad spectrum antibiotics, and intensive care unit monitoring are imperative in specific cases of severe acute pancreatitis in order to reduce the accompanied high morbidity and mortality.[5] Unfortunately, the mortality rate of acute pancreatitis can vary from 2 to 16% and in severe cases from 7 to 47%.[6]

To diagnose a pancreatic disorder, multiple clinical tests[7] are performed that can require more than 48 h to achieve reliability.[8,9] Such tests use serum and urine samples where commonly high concentrations of immunoreactive anionic trypsinogen failed to

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predict severe attacks of acute pancreatitis.[10−14] By using radioimmunoassay tests, serum trypsin levels have been estimated for healthy patients (248 ± 94.9 μg/L), for patients with chronic renal failure (1100 ± 548 μg/L), and for patients with acute pancreatitis (1399 ± 618 μg/L).[15]

The degradation of gelatin film has been used as a semiquantitative screening test for pancreatic disorders. However, such a test cannot easily be calibrated to give an absolute measurement of trypsin concentration. Therefore, other sensitive tests such as affinity-based reactions, Bragg reflector devices that measure the change of temperature, pressure,[16] and humidity[17] on gelatin-based films were developed. Since all these techniques are time-consuming and require specific laboratory-based instrumentation, plus trained personal, a more rapid and precise tool is needed to overcome such drawbacks.

One of the most promising areas in the development of precise analytical techniques for the detection of various biological compounds is based on the development of biosensors. Such biosensors have been reported for protease (trypsin) detection. For instance, hologram trypsin sensors[18,19] and biosensors for collagenase detection[20,21] were developed. Unfortunately, these biosensors are not sufficiently sensitive for the lowest trypsin detection limits and have reached to date just below the nanomolar range.

The present paper reports on a successful quantitative test for a protease (trypsin) picomolar detection by measuring the current increase recorded from glucose oxidase (GOX)−poly(pyrrole−alkylammonium)-modified platinum electrodes coated with a gelatin film that was subjected to a proteolytic enzymatic action.

EXPERIMENTAL SECTION

Chemicals. Gelatin (type A, porcine skin, analytical grade, G-2500), trypsin (from bovine pancreas, 9380 units/mg, T-4665), GOX (from *Aspergillus niger*, type VII-S, 181 units/mg, G-7016), and glucose (G-7528) were obtained from Sigma. (11-Pyrrol-1-ylundecyl) triethylammonium tetrafluoroborate (Figure 1) was synthesized in the following manner according to a previously described protocol.[22] 11-Pyrrol-1-ylundecanol (2.6 g, 11 mmol) was reacted with tosyl chloride (2.85 g, 15 mmol) in anhydrous pyridine (3 mL). The mixture was stirred at 20 °C for 15 h, washed with water, and extracted with dichloromethane. After evaporation, the crude product was purified by chromatography (3.05 g, yield 74%). 11-Pyrrol-1-ylundecyl *p*-toluenesulfonate (1.5 g) was refluxed for 15 h at 90 °C in ethanol (15 mL) with an excess of triethylamine (11 mL). The solvent and excess of triethylamine were removed under vacuum. Tosylate anions were then replaced by tetrafluoroborate anions on an ion-exchange column (Amberlite IRA 93) leading to a brown oil (1.44 g, yield 65%). H^1 NMR (250 MHz/CD_3Cl_3): δ (ppm) 6.62 (s, 2H), 6.09 (s, 2H), 3.83 (t, 2H), 3.24 (m, 6H), 3.07 (m, 2H), 1.58 (m, 2H), 1.35−1.23 (m, 25H). $LiClO_4$ (194711000) was obtained from Acros Organics. The 8% (w/v) gelatin was dissolved in distilled water and heated to 50 °C, until it became completely molten. The gelatin solution was prepared anew for each experiment.

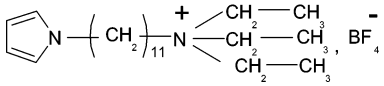

Figure 1. Structure of pyrrole−alkylammonium monomer.

Apparatus. Cyclic voltammogram and electropolymerization were performed with EG&G PAR, model 173 potentiostat equipped with a model 175 universal programmer, and a model 179 digital coulometer in conjunction with a Kipp and Zonen BD 91 XY/t recorder. An electrochemical three-electrode cell (Metrohm) was used. The amperometric measurements were performed using a Tacussel PRG-DL potentiostat in conjunction with a thermostated electrochemical cell at 20 °C. The working electrodes were platinum electrodes (i.d. = 5 mm) systematically polished with 2-μm diamond paste (Mecaprex Press PM). The reference electrode used was a saturated Ag−AgCl−KCl electrode (Ag/AgCl) while a Pt wire was used as a counter electrode.

Biosensor Preparation. The polymer−enzyme electrodes were prepared according to a two-step procedure previously reported by Cosnier.[23] The 6 mM pyrrole−alkylammonium monomer (brownish, oily suspension) was suspended in pure distilled water and sonicated for 3 h to facilitate a total monomer solubilization. The working electrodes (platinum disk) were modified at room temperature by spreading over their surface an aqueous mixture based on 15 μL of monomer solution and 200 μg of GOX and then dried under vacuum atmosphere for 15 min. The resulting "dry" modified electrodes were transferred into a cell containing an aqueous 0.1 M $LiClO_4$ solution. Electrochemical polymerization of the adsorbed coating (GOX + pyrrole monomer) was carried out by controlled potential electrolysis at 0.85 V versus Ag/AgCl. Further, the enzyme-modified electrodes were coated with 20 μL of 8% (w/v) gelatin solution and dried under vacuum atmosphere for 20 min.

RESULTS AND DISCUSSION

Electrochemical Characterization of Poly(pyrrole−alkylammonium)−GOX-Modified Electrodes. As reported previously,[23] an original strategy of enzyme entrapment into electrogenerated polymers involved the immobilization of amphiphilic

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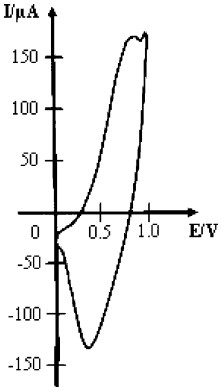

Figure 2. Cyclic voltammogram of an electrode modified by a poly(pyrrole−alkylammonium) film containing 200 μg of GOX molecules in H_2O + 0.1 M $LiClO_4$; scan rate, 0.1V·s^{-1}.

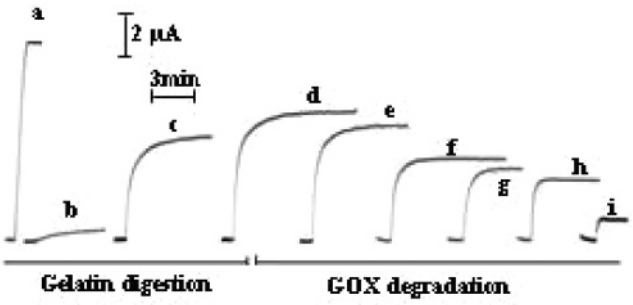

Figure 3. Evolution of the biosensor response to glucose injection (2 mM) as a function of gelatin degradation by trypsin (300 μg/mL) over time. (a) Before gelatin coating; (b) after gelatin coating; there after, different times soaking the biosensor in various trypsin solutions: (c) 40 s; (d) 60 s; (e) 80 s; (f) 230 s; (g) 5 min; (h) 10 min; (i) 30 min. The biosensor was potentiostated at 0.6 V in stirred 0.1 M PBS (pH 7).

monomers and enzymes together, by their adsorption on the electrode surface before the electropolymerization. Taking advantage of the poor solubility and strong adsorption properties of (11-pyrrol-1-ylundecyl) triethylammonium tetrafluoroborate in water, this amphiphilic pyrrole derivative was dispersed by ultrasonication and mixed with GOX enzymes, and the resulting mixture was adsorbed on a platinum electrode. After the transfer of the modified electrodes into an aqueous 0.1 M $LiClO_4$ solution, free of monomer, the oxidative electropolymerization of the adsorbed coating at 0.85 V provides the entrapment of GOX molecules in the "in situ" generated polypyrrole film. The electrochemical characterization of the resulting electrodes with poly(pyrrole−alkylammonium) film containing 200 μg of GOX molecules was investigated by cyclic voltammetry in 0.1 M $LiClO_4$ aqueous solution. The cyclic voltammogram presents in the positive region, a reversible peak system at 0.56 V reflecting the well-known electroactivity of the polypyrrolic skeleton (Figure 2). This $E_{1/2}$ value is in good agreement with those reported for conducting functionalized polypyrroles, corroborating thus with the formation of a polypyrrole film from the adsorbed biocoating. The apparent surface coverage of polymeric film ($\Gamma = 5.88 \times 10^{-8}$ mol·cm^{-2}) was determined from the charge recorded under the polypyrrole oxidation wave, leading to an electropolymerization yield of 41%.

Since GOX catalyzes the aerobic oxidation of glucose with the concomitant production of H_2O_2, the analytical performances of the enzyme electrode for the determination of glucose were investigated. The amperometric detection of glucose was assayed in 0.1 M phosphate buffer (pH 7) by holding the modified electrodes at 0.6 V in order to oxidize the enzymatically generated H_2O_2 at the platinum underlying electrode. The calibration curve was linear with the changing glucose concentration and reached a plateau for glucose concentration above 22 mM. The sensitivity of the biosensor (determined as the slope of the initial linear part of the calibration curve) and its maximum current density at saturating glucose condition were 23 mA·M^{-1}·cm^{-2} and 102 mA·cm^{-2}, respectively. To quantify the effect of an additional gelatin layer on the performance of the glucose biosensor, the amperometric response of the enzyme electrode was recorded for 2 mM glucose. This glucose concentration was chosen since it corresponds to the linear part of the calibration curve where the biosensor response is directly proportional to the substrate concentration. As expected, the formation of an outer layer of gelatin generated high steric hindrances toward the glucose diffusion to the polymer−GOX layer since the current response decreased to 5% of its initial value (Figure 3a). As a consequence, it was expected that the detection of the protease activity could be carried out via an increase in amperometric current due to the degradation of the gelatin layer by the proteolytic digestion.

Detection of Protease Activity. The gelatin-coated enzyme electrodes were immersed in a trypsin solution (300 μg·mL^{-1}) for different timed intervals and then transferred into 0.1 M PBS to record the amperometric current response to glucose (2 mM). Before current measurements were registered, the gelatin−biosensor treated with trypsin solution was rinsed several times with PBS solution. The gelatin−GOX biosensor current response was initially recorded after 20 s of the proteolytic digestion treatment began, followed by sets of the current measurements up to 30 min (Figure 3). The change in current intensity as a function of time of trypsin incubation was compared to the initial current response of the gelatin uncoated biosensor. As expected, the removal of the gelatin layer with time induced initially an increase in amperometric current that reached, after 1 min of trypsin incubation, 64% of the current value observed for the gelatin uncoated enzyme electrode. After this time, the enzymatic digestion of gelatin was continued up to 30 min, inducing a progressive current decrease down to 8% from the initial current response. This clearly indicates a "toxic" effect of the trypsin on the entrapped GOX enzyme molecules within the poly(pyrrole−alkylammonium) films. Such an effect could be explained by the cleaving of the GOX molecule at lysine and arginine residues by trypsin action. To validate the role of trypsin on the immobilized GOX, a control experiment was carried out with an uncoated enzyme electrode incubated with trypsin solution (300 μg·mL^{-1}) for 30 min. As expected, a marked decrease in current response to glucose (95%) was observed demonstrating the possibility for trypsin to degrade the GOX activity although these molecules were entrapped in a polymer film. It should be noted that the polypyrrole film exhibited long linear chains that are more permeable than a cross-linked structure.[24]

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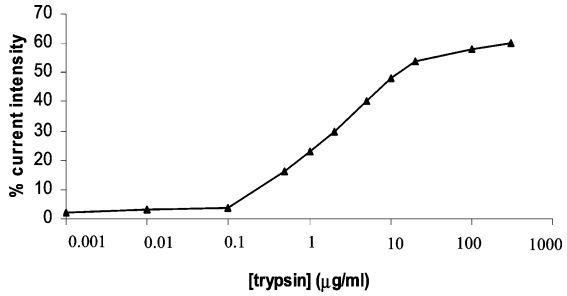

Figure 4. Increase in current response of gelatin−GOX biosensors to glucose (2 mM) in the presence of various trypsin concentrations after 1 min of soaking in protease solutions. The increase was reported as a ratio of the measured biosensor current response versus the initial one in the absence of gelatin.

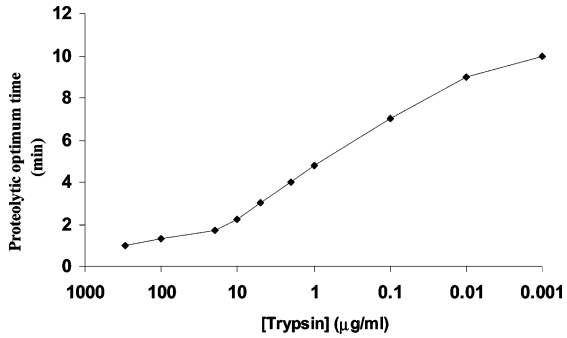

Figure 5. Influence of trypsin concentration on the time of gelatin degradation necessary to obtain the maximum current response of the biosensor to glucose (2 mM).

Biosensor Performances for the Determination of Trypsin. To emphasize the high sensitivity of the gelatin−GOX biosensors to the protease (trypsin) digestion, the effect of various concentrations of trypsin (0.001 up to 300 μg/mL) in 0.1 M PBS on the biosensor sensitivity was examined. Figure 4 shows a drastic increase in amperometric current response to glucose (2 mM) for concentrations ranging from 0.1 to 300 μg/mL. Although small current differences are noticed between 1 and 100 ng, this procedure cannot be used for the determination of extremely low trypsin concentrations. To improve the biosensor sensitivity toward protease, a proteolytic optimum time (POT) was defined. The POT corresponds to the incubation time in trypsin necessary to obtain the maximum current response, the latter being the best compromise between the gelatin digestion and the GOX deactivation. Figure 5 depicts the evolution of POT as a function of trypsin concentration. As expected, the lower the trypsin concentration used, the longer the POT is. Therefore, the proteolytic optimum time was found to range between 1 and 10 min, when using 300 μg/mL trypsin and 1ng/mL trypsin, respectively, for the gelatin degradation. As a consequence, the determination of low trypsin concentration was investigated by increasing the incubation time up to 10 min. For each trypsin concentration, three freshly prepared gelatin−GOX biosensors were used and their current response was recorded and averaged. A linear increase of the current response with the logarithm of trypsin concentration was observed between 1 and 250 ng/mL (Figure 6). The detection limit of trypsin (1 ng/mL or 42 pM) is markedly more sensitive than those reported for other biosensors, namely, 25 nM and

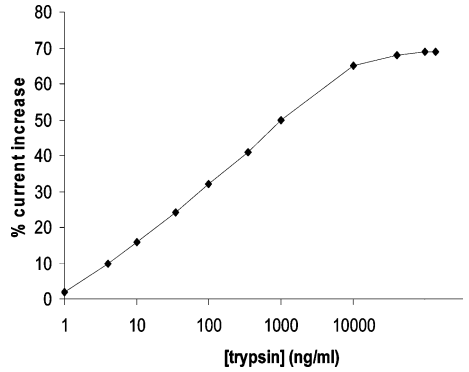

Figure 6. Protease (trypsin) calibration curve illustrated by the increase in current response of gelatin−GOX biosensors to glucose (2 mM) in the presence of various trypsin concentrations after 10 min of soaking in protease solutions. Experimental conditions are as in Figure 4.

40 ng/mL.[18,19] In addition, these biosensors require large response times (20 and 60 min) whereas our gelatin−GOX electrodes exhibited a rapid response time ranging from 1 min for 300 μg/mL trypsin and up to 10 min for 1 ng/mL trypsin. Furthermore, non-biosensor-based protease assays such as a ion-selective electrode detection,[25] a Coomassie-stained gelatin agarose gel assay,[26] a solid-phase protein cleavage assay,[27] turbidity,[28] and radiometry[15] showed low limit of detection values at 1−10 μM protease,[25] 10 pg of trypsin but the assay plates were incubated for 14 h within the protease solution before reading,[26] 10 ng/mL protease,[27] 5 μg/mL trypsin,[28] and 248 ng/mL trypsin,[15] respectively.

It should be noted that the repeatability of the amperometric response of one gelatin−GOX electrode after a proteolytic process in the presence of 300 μL/mL trypsin for 60 s was examined at a glucose concentration of 2 mM. The RSD was 3.2% for four successive current measurements. Moreover, the reproducibility of the analytical response obtained from different modified electrodes constructed by the same procedure was also investigated. Eleven protease biosensors were tested independently for the detection of a specific protease (trypsin) concentration (300 μL/mL) for a precise digestion time (60 s). A mean current response of 5.7 μA to glucose (2 mM) was recorded with an RSD of 4.8% ($n = 11$). This demonstrates an efficient and reproducible detection of the proteolytic digestion process, even though the procedures used for enzyme immobilization and gelatin deposition were done manually.

CONCLUSIONS

We describe herein an original and promising concept of protease detection based on the suppression of a biosensor response by steric hindrances and its reactivation by enzymatic digestion of the blocking membrane. It is expected that this extremely sensitive biosensor will be helpful for the easy and rapid monitoring of patient health status.

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