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Short communication

Penicillinase-based amperometric biosensor for penicillin G

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ABSTRACT

A biosensor for penicillin G was created by immobilizing penicillinase to a gold electrode by means of a cysteine self-assembled monolayer. The biosensor amperometrically monitored the catalytic hydrolysis of penicillin in a very sensible manner. Furthermore, it was successfully used to measure the Michaelis–Menten enzymatic constant and a low limit of detection of 4.5 nM was obtained.

1. Introduction

Penicillin G (benzylpenicillin) is a parenterally administered form of penicillin, a pioneer in β-lactam antibiotics. Although having a broad spectrum it is more effective against Gram negative microorganisms and though it is one of the oldest antibiotics available to MDs it is still one of the first choices in the treatment of several pathologies, like syphilis [1].

The efficacy of β-lactam is threatened by naturally occurring bacterial β-lactamases [2], enzymes that destroy β-lactam stopping them from destroying the bacteria's cell wall. Furthermore, β-lactamases with time become more effective in doing so. Thus, this leads to a real arms race by medical researchers where, along with a careful judgment on when to use antibiotics, two paths can be followed: whether to develop new antibiotics to which resistance have not been established or conjugate the therapeutical administration with β-lactamases' inhibitors like clavulanic acid.

When treating a new patient with a potentially serious bacterial infection, the clinician will start empirical antibiotic treatment, i.e., a choice of antibiotics thinking of the most probable infectious agent taking into account besides the patient's signs and symptoms all epidemiological data available including age, gender, previous health condition, among several others factors. Nevertheless, the clinician often starts growing bacterial cultures to establish resistances and susceptibilities of that microorganism.

Furthermore, the wide use of these antibiotics, particularly with veterinary purposes, has led to environmental contamination that, besides aggravating the emergence of antibiotic-resistant bacterial strains, has created many problems from disturbing ecosystems to polluting natural water sources among many other not fully understood issues.

Therefore any new tools to monitor the efficacy of β-lactamases and to determine β-lactams in all kinds of environmental samples are welcome. β-Lactamase catalyzes the hydrolysis of penicillin to penicillinoic acid. This changes the pH and can be measured in varied forms [3–5]. However, an amperometric approach to measure penicillin enzymatic consumption is scarcely found in literature. Nevertheless, one can find from the early work of Stred'anský et al. [6], a hematein based biosensor on a platinum electrode surface, and Pividori et al. [7], with a dot-blot genosensor, and more recently by Chen et al. [8], with co-immobilization of carbon nanotubes and hematein on a glassy carbon electrode, and Gamella et al. [9], using a screen-printed carbon electrode.

2. Material and methods

2.1. Reagents and equipment

All chemicals were purchased from Sigma-Aldrich.

The aqueous solutions were prepared using ultrapure water with resistivity not less than 18.2 MΩ cm at 298 K. The composition of the phosphate buffer solution (PBS) was the following: NaCl, 137 mmol L^{-1}; KCl, 2.7 mmol L^{-1}; $Na_2HPO_4 \cdot 12\ H_2O$, 10 mmol L^{-1}; KH_2PO_4, 2 mmol L^{-1}.

Class B penicillinase (EC 3.5.2.6) from *Bacillus cereus* was acquired from Sigma-Aldrich, with a mixture of β-lactamase I and β-lactamase II.

Electrochemical measurements were performed with a PGSTAT302N potentiostat from Metrohm. A 3 electrode system was used, whereas the

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working electrode was the gold based biosensor mentioned above, with a platinum counter-electrode and a KCl saturated calomel electrode (SCE) as the reference electrode.

2.2. Biosensor preparation

To adequately prepare the gold electrode to the electrode formation the following procedure was performed: cleaning in a *piranha* solution (3:1, H_2SO_4:H_2O_2) at 80 °C, for 1 min; chemical desorbing in 1 mmol L^{-1} sodium hydroxide by 25 cycles of CV, between −0.5 and −1.7 V, at a scan rate of 100 mV s^{-1}; the electrode was mechanically polished with alumina powder (1, 0.3 and 0.05 μm); finally, the gold electrode was electrochemically cleaned by performing 25 cycles of CV, between −0.2 and −1.5 V, at a scan rate of 100 mV s^{-1}, in a solution of 0.1 mol L^{-1} sulfuric acid.

The biosensor started to develop with the adsorption of cysteine, by applying to the electrode surface a solution of 10 mmol L^{-1} of this amino acid in PBS. Afterwards, an aqueous solution of 10 mmol L^{-1}, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and 20 mmol L^{-1}, *N*-hydroxylsuccinimide (NHS) was added for 2 h to activate the SAM. Then the co-factor $CoCl_2$ (1 mmol L^{-1}) and the enzyme penicillinase (0.15 mg mL^{-1}) were added for 2 h. At the end, the electrode was washed with PBS and water to remove the excess and dried with nitrogen (Fig. 1).

3. Results and discussion

Self-assembly monolayers (SAM) are single molecule layers that spontaneously adsorb onto a chosen surface [10,11]. Controlling the surface up to a monomolecular level has immense advantages, including in electroanalytical studies with varied objectives [12]. Enzymes can be attached to suitably study their interaction with substrates [13–15].

Throughout all steps of the biosensor development cyclic voltammetry of a ferrocene probe was performed to establish the effectiveness of each stage. As can be observed in Fig. 2, when the cysteine layer is added the current decreases to less than a tenth. Then it decreases a bit more with the addition of the enzyme. A minimal change is observed when the analyte is added. This clearly shows that the cysteine monolayer inhibits the electronic transfer to the ferrocene in solution. Penicillinase further augments such an effect, however penicillin G does not.

In this case, the penicillinase used was a metallo-β-lactamase that catalyzed the hydrolysis of penicillin to penicillinoic acid. In a mediated

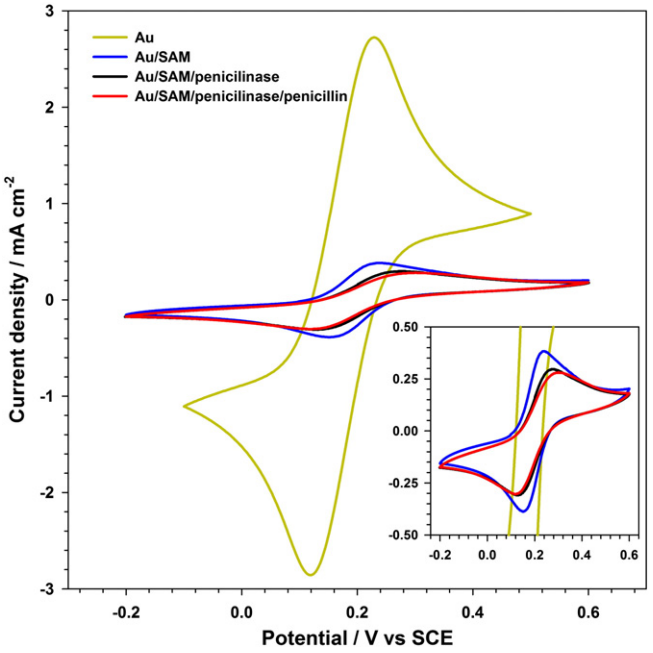

Fig. 2. Cyclic voltammetries of a ferrocene solution, 2 mmol L^{-1}, in KNO_3, 1 mol L^{-1}, with different electrode surfaces: bare gold; gold and cysteine SAM; gold, cysteine SAM with penicillinase; and gold, cysteine SAM with penicillinase and the analyte penicillin. Inlay: detail of lower currents.

reaction by cobalt where protons are liberated, this reaction could be measured by cronoamperometry [6], as shown in Fig. 3. Cronoamperometry was performed at −550 mV vs. SCE (potential optimization not shown), and the measurement was performed without any pH redox probe like hematein.

The Michaelis–Menten equation was a major breakthrough that helped explain the hyperbolic behavior found in studies of enzymatic rates with variable substrate concentrations. At low substrate concentrations there is a linear correlation between substrate concentration ([*S*]) and initial velocity (V_0) with a slope that is at maximum velocity (V_{max}) by the Michaelis–Menten constant (K_M)—first order kinetics:

$$V_0 = \frac{V_{max}[S]}{K_M + [S]}. \quad (1)$$

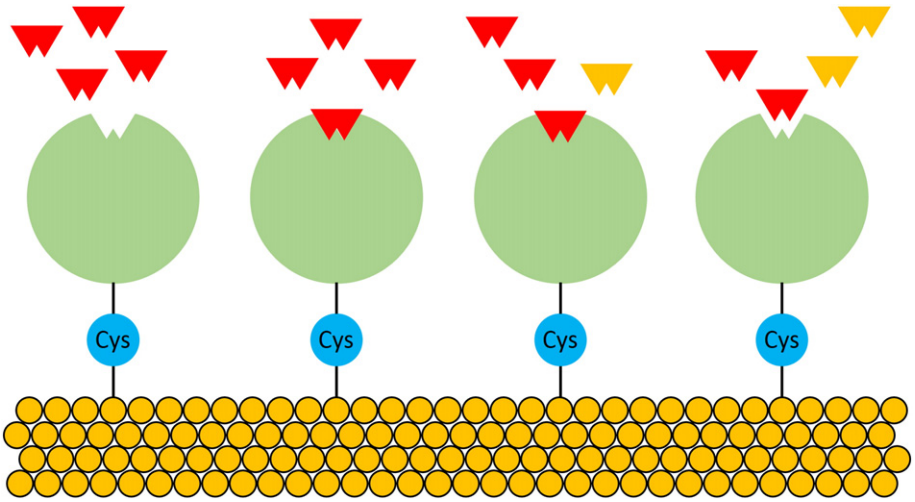

Fig. 1. Schematics of the experimental configuration, β-lactamases are connected to the gold electrode surface by cysteine molecules. Penicillin G molecules in solution react with the β-lactamases (molecules are not drawn to scale).

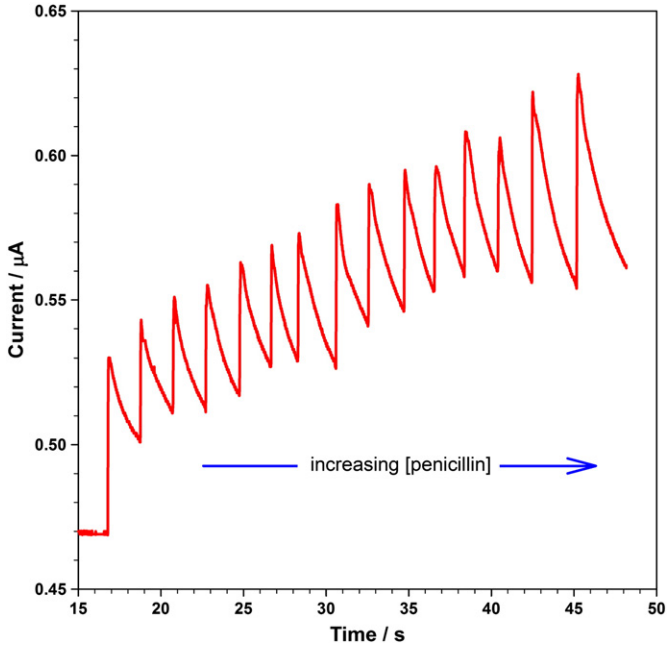

Fig. 3. Example of a cronoamperometry. Current was measured while subsequently increasing concentrations of penicillin were injected to the system (each concentration had an increment of 20 µmol L^{-1}).

At high substrate concentration, there is a saturation of the enzyme's active site and the initial velocity tends to its maximum values—zero order kinetics. Complete deduction of this equation, as well as its limitations, can be easily found in literature [16–19], they mainly derive from the concept enzyme–substrate complex and its steady-state assumption [20].

The Michaelis–Menten equation can be easily transformed into its linearized form, normally known as the double-reciprocal plot [21]. This simplifies the calculus of the variables K_M and V_{max}.

$$\frac{1}{V_0} = \frac{K_M}{V_{max}[S]} + \frac{1}{V_{max}}. \quad (2)$$

The cronoamperometric data could be directly transformed into velocity of consumption [15], and Fig. 4 could be plotted.

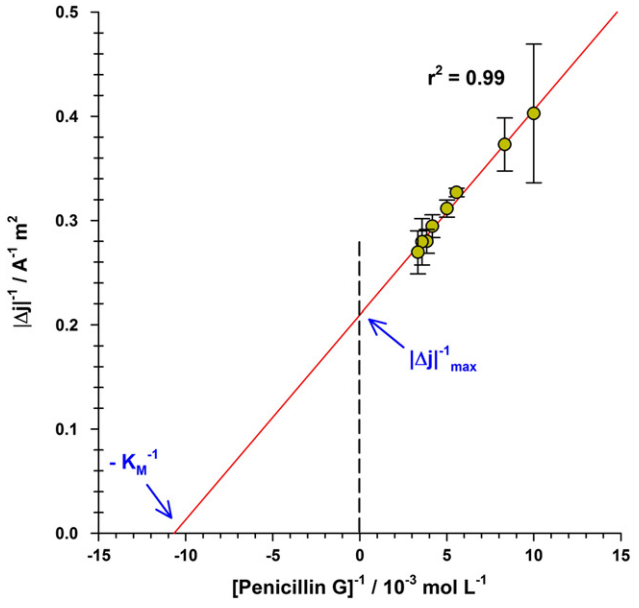

Fig. 4. Double-reciprocal plot (also known as Lineweaver–Burk plot) used to calculate the K_M.

The obtained value for K_M (apparent Michaelis–Menten constant) was 0.094 ± 0.005 mmol L^{-1}. Considering that literature values [22] for β-lactamase I are 0.060 (pH 7.0, and immobilized) [23] and 0.065 mmol L^{-1} (pH 7.0) [24] and for β-lactamase II, 3.3 mmol L^{-1} (pH 7.0) [25], and that K_M is sensible to small changes in temperature, pH, ionic strength among others, the obtained value is rather reasonable.

The figures of merit of the developed methodology were obtained by a calibration curve of standards, n = 5 and done in triplicate in a concentration range 10–50 nmol L^{-1}. A good linear correlation between the analytical response and penicillin concentration was obtained (r^2 = 0.993) as well as low limits of detection (LOD = 4.5 nmol L^{-1}, same as 1.5 µg L^{-1}) and quantification (LOQ = 15 nmol L^{-1}, same as 5.0 µg L^{-1}). LOD and LOQ were calculated as three and ten times the standard deviation of the intercept/slope. The obtained LOD was as low as the lowest values that can be found in the literature [9].

4. Conclusions

β-Lactamases were successfully attached to a gold electrode by means of a cysteine SAM, being possible to obtain in this way the K_M of the consumption of penicillin G. This experimental setup cannot only be further used to study the efficiency of β-lactamases' inhibitors and also resistances towards β-lactam antibiotics, but can also be used for analytical purposes since a low LOD was obtained.

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