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Amperometric pH-sensing biosensors for urea, penicillin, and oxalacetate

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Abstract

The possibility of constructing a biosensor exploiting amperometric pH-sensing was investigated. The principle is based on the use of pH-sensitive redox-active probe molecules. The selected probe molecules applied in various forms, e.g. dissolved hematein, electrode bulk lauryl gallate, adsorbed methylene blue poly(*o*-phenylenediamine) film, were used for the construction of penicillin (with penicillinase), urea (with urease), and oxalacetate (with oxalacetate decarboxylase) biosensors. Platinum, gold and solid composite electrodes were used as transducers. The biosensors exhibited low detection limits, from 2 to 10 μM, linear responses up to 2 mM, insensitivity to a small variation in the ion concentrations, a good accuracy and storage stability. The present, new concept could extend the range of analytes detectable using the amperometric transduction technology, such as substrates of decarboxylases, amidohydrolases, esterases and other hydrolases.

Keywords: Amperometric pH-sensing; Biosensor; Urea; Penicillin; Oxalacetate

1. Introduction

In recent articles [1,2], prospects and progress in the area of biosensors were evaluated and it was seen that the amperometric biosensor as opposed to potentiometric, optical, piezoelectric, and thermal ones was the most successful of all and hence had a promising future. As a major part of amperometric biosensors represents enzyme electrodes employing oxidoreductases, a relatively limited number of analytes can be determined by them. On the other hand, many enzymes from the groups of hydrolases, lyases, oxidoreductases, ligases, and transferases are known to affect pH as a consequence of their biocatalytic action. So pH-sensing has a clear potential for utilisation in the area of biosensors.

The most frequent transducers in pH-sensing are potentiometric ones [3] that are represented by glass electrodes [4], ion-selective membrane or film electrodes [5,6], ion-selective field effect transistors [3,7] and two-terminal microsensors [8]. Various optical [9] and conductometric [10] pH-sensing tranducers were also developed. However, biosensors based on these transducer types exhibit some of these drawbacks: relatively high detection limits, rather sluggish response to solutions of low analyte concentration, vulnerability to the interferences, expensive or complicated fabrication and insufficient stability.

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Amperometric pH-sensing was also reported. Some designs are based on the pH-switchable permselectivity of specific membranes or films on the electrode surface for cationic and anionic redox-active probe molecules, such as ferricyanide and hexaammineruthenium(III) [11,12]. But these electrodes were not combined with enzymes, and moreover, a relatively low sensitivity can be expected in the neutral pH region, where most enzymes exhibit their optimum activity. Kirstein et al. [13] described a urea biosensor in which amperometric pH-sensing was realised by the pH-dependence of electrochemical oxidation of hydrazine in the Tafel region. Disadvantages lie in the low sensitivity (the linear range from 0.8 to 35 mM) and in the use of such a hazardous compound as hydrazine.

It is known that a large number of pH-sensitive compounds from the groups of quinones, hydroquinones, polyphenols, antioxidants, acido-basic indicators and organic dyes are also redox-active. Although it is difficult to suppose they can be used for direct amperometric pH measurement, they could serve as probe molecules in amperometric pH-sensing sensors, when only relative pH change monitoring is required.

In this work, we demonstrate the feasibility of the use of various pH-sensitive redox-active compounds (hematein, lauryl gallate, *o*-phenylenediamine, methylene blue (MB)) in combination with several types of electrodes (platinum, gold, solid composite) and pH affecting enzymes (penicillinase, urease, oxalacetate decarboxylase) for the construction of simple amperometric biosensors exhibiting acceptable sensitivity, selectivity and stability.

2. Experimental

2.1. Materials

Urease (3.5.1.5; from jack beans, type VII), penicillinase (3.5.2.6; from *Bacillus cereus*, type I), oxalacetate decarboxylase (4.1.1.3; from *Pseudomonas* sp.), *o*-phelylenediamine, *n*-eicosane and stearic acid were purchased from Sigma (St. Louis, MO). Lauryl gallate, benzylpenicilline (Penicillin G) sodium salt, hematein, 2-hexadecanone, and graphite powder were from Fluka (Buchs, Switzerland) and MB from Aldrich (Steinheim, Germany). Other analytical grade reagents were commercially available.

2.2. Apparatus

Cyclic voltammetric studies were carried out with the computerised electrochemical analyser AMEL 433/W (Milan, Italy). Chronoamperometric studies were performed with a potentiostat (AMEL 559) and a recorder (AMEL 868). A saturated calomel electrode (SCE) and a Pt spherical electrode were used as the reference and counter electrodes, respectively. PHM 85 precision pH-meter (Radiometer, Copenhagen, Denmark) was used for pH measurement.

2.3. Preparation of electrodes

Solid binding matrix (SBM)-based composite electrodes were prepared as described previously [14,15]. Briefly, 50 mg of graphite powder were added to 50 mg of molten *n*-eicosane at 45°C and mixed intensively. The molten mixture was used to fill a hole (2.0 mm in diameter, 3 mm in depth) at the end of an electrode body (a PVC tip, 2 mm i.d., 5 mm o.d., length 20 mm). After cooling at room temperature, the excess of solidified material was removed with the aid of sand paper. The surface was then polished on a sheet of paper. Electrical contact was ensured by a brass rod. The surface of the solid composite electrode was subsequently modified by the deposition of poly(*o*-phenylenediamine) (POPDA) (Composite 1) performed by electropolymerisation of the monomer [16]. A solid composite electrode containing lauryl gallate (Composite 2) was prepared in a similar manner. First, 10 mg of lauryl gallate were dissolved in 0.5 ml acetone, and after the addition of 45 mg of graphite, the mixture was stirred vigorously with a magnetic stirrer until the evaporation of the solvent. Acetone traces were allowed to evaporate in an oven at 45°C. The modified graphite was added to the molten mixture (60°C) containing 40 mg of 2-hexadecanone and 5 mg of stearic acid. The electrodes were prepared as described above, omitting the deposition of polymer.

The gold electrode (2 mm in diameter, AMEL) was carefully cleaned by polishing to a mirror finish with an alumina slurry of 1 μm and sonicated in pure water to remove the embedded alumina particles. Then, the

electrode was immersed in a 4 mM solution of MB for 30 min and rinsed thoroughly with distilled water. Platinum electrode (2 mm in diameter, AMEL) was used without any modification.

Enzyme solutions (1 μl; 4.4 U/μl of urease, 5 U/μl of penicillinase, or 5 U/μl of oxalacetate decarboxylase) were spread over the electrode surface. After drying at room temperature, the surface was covered with a dialysis membrane (Spectra/Por type 1, Spectrum Medical Industries, TX) fixed by an O-ring.

2.4. Measurements

All experiments were carried out in the batch-mode at 25°C in a jacketed reaction vessel equipped with magnetic stirring. For the pH-dependency curve measurements, the working electrode without enzyme and the reference electrode were immersed in 10 ml of 0.05 M phosphate solution (pH 10). After signal stabilisation at a selected constant operating potential, the current changes were monitored after each addition of the 2 M HCl solution. Simultaneously, the pH values were measured by the pH-meter.

For biosensor measurements, the working electrodes covered by enzymes and the reference electrode were immersed in 10 ml of a 1 mM phosphate buffer containing 0.1 M of electrolyte (NaCl). After current stabilisation, a standard solution of an analyte (penicillin, urea, or oxalacetate) was added, and current–time response curves were recorded. The height of the recorded amperometric wave was correlated to the concentration of the analyte in the measuring cell.

3. Results and discussion

3.1. Principle of amperometric pH-sensing

To demonstrate the principle, we chose the quinone/hydroquinone redox couple, which exhibits pH-dependency (reaction (1)) [8]:

$$Q + 2e^- + 2H^+ \rightleftharpoons QH_2 \quad (1)$$

As can be seen from Fig. 1, the formal potential of Q/QH_2 is pH-dependent. This is exploited in potentiometric pH-sensing. On the other hand, at a suitable constant potential, the pH change of the system is

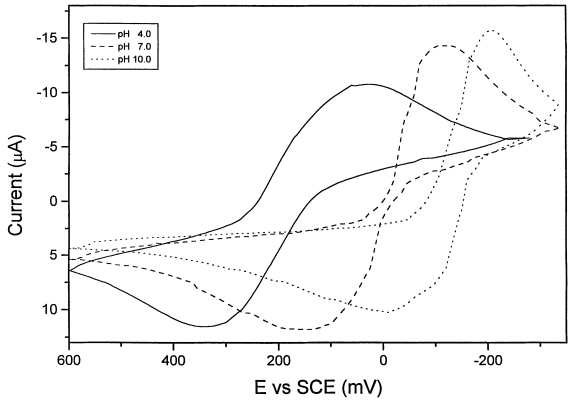

Fig. 1. Cyclic voltammograms of 2 mM hydroquinone on an *n*-eicosane–graphite composite electrode obtained at various pHs in a 0.1 M phosphate oxygen-free solution. Scan rate: 50 mV/s.

accompanied by a current change. Although it is difficult to exactly measure the pH through this mode, this principle can be utilised for the construction of amperometric pH-sensing biosensors, when only relative pH change monitoring is required. Thus, when the local pH on the electrode surface or in its proximity changes as a consequence of the biocatalytic reaction, the current flow resulting from the electrochemical reaction of the pH-sensitive redox compound also changes to an extent which is correlated to the substrate concentration [17]. In the literature, the relationship between chemical and electrochemical phenomena is described extensively, and the significance of experimental evidences discussed deeply in relation to the potential–pH diagrams for the case of phenols [18], gallates [19], poly-*o*-phenylamino derivatives [20], redox polymers [21], and phenothiazine derivatives [22,23].

From the practical point of view, a suitable pH-sensitive redox compound should have physical stability and should allow work at a potential near 0 mV to avoid electrochemical interference originating from real samples. Some examples of the probe molecules and their utilisation for the construction of pH-sensing biosensors of various designs are described below, considering that each redox-active pH-sensitive molecular probe responds to the pH variation induced by the enzymatic activity of any of the biocatalysts selected in the present investigation.

3.2. Penicillin biosensor with hematein

Hematein is a natural dye used in the selective staining of biological materials [24]. It is a stable water-soluble electroactive compound also employed as a pH indicator, turning from yellow at acidic pH to pinkish-violet at basic pH. From the cyclic voltammograms measured at various pH values (data not shown), the constant working potential of 0 mV (versus SCE) was selected for the following amperometric measurements. The dependence of current on pH was determined using a platinum electrode immersed in a 0.5 mM hematein solution and polarised to the working potential (Fig. 2a). Changing the pH of the buffer solution from 3.9 to 9.8, alternatively, six times an hour over 3 h of continuous work, the resulting current response was found to be reversible without affecting the magnitude of ΔI and the response time at steady state at either pH extreme.

The penicillinase biosensor was prepared simply by the application of penicillinase on the platinum electrode surface which was then covered by a dialysis membrane. Penicillinase catalyses the hydrolysis of penicillin, where H^+ is liberated from the penicilloic acid produced (reaction (2)):

$$\text{penicillin} + H_2O \rightarrow \text{penicilloate}^- + H^+ \qquad (2)$$

The calibration curve for penicillin G sodium salt at a starting pH of 8.0 is illustrated in Fig. 3 and the comprehensive results are summarised in Table 1. Repeated addition of 0.2 mM penicillin G to the cell for 3 h of continuous work, with the electrode being rinsed with distilled water after every eight additions of penicillin G, supplied a mean current of 20.5 nA with a relative standard deviation (R.S.D.) of 2.1%.

In order to investigate the selectivity of the biosensor with regard to inorganic interferences, amperometric measurements were performed using a 5 mM concentration of interfering ions. The results reveal that KCl, $NaNO_3$, $MgCl_2$, $FeCl_3$, $CaCl_2$ and $(NH_4)_2SO_4$ did not give a response on the penicillin biosensor, which could be practical in the measurement of real samples. The amperometric penicillin biosensor presented here exhibits a better detection limit in comparison with the reported potentiometric [25] (100 μM) and conductometric [26] (50 μM) ones. Other advantages are a shorter response time and a negligible drift of the base line. The biosensor was used to determine the penicillin G content in pharmaceutical preparations (tablets and emulsions of commercially available products, Farmitalia, Italy). The results agreed well with those obtained by the standard HPLC method (with an R.S.D. of −5.3%, n=5).

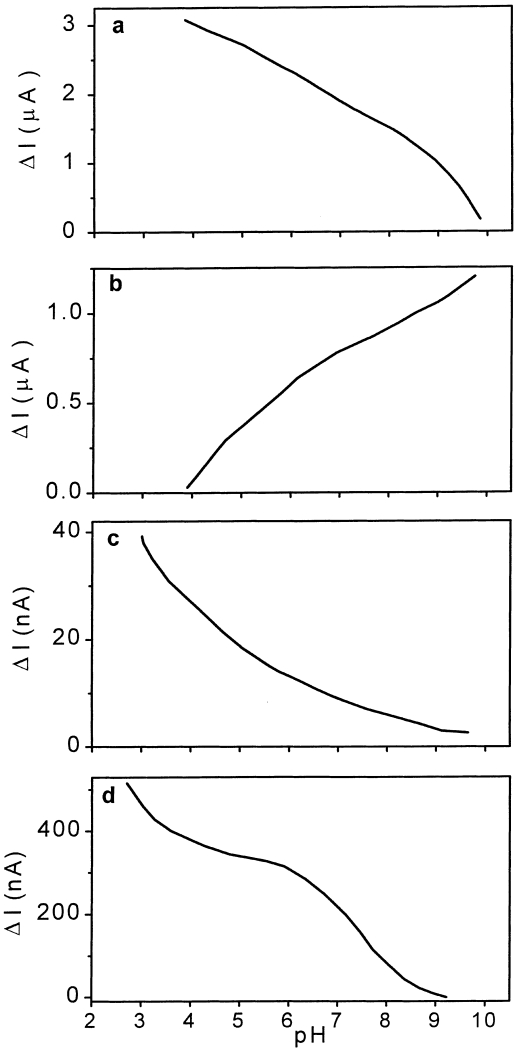

Fig. 2. Current responses of each electrode/redox active pH-sensitive molecular probe system in a 1 mM phosphate buffer containing 0.1 M NaCl at different pH values: (a) platinum electrode immersed in 0.5 mM hematein solution obtained at a working potential of 0 mV vs. SCE; (b) graphite composite electrode modified with lauryl gallate at a working potential of +200 mV vs. SCE; (c) gold electrode modified with MB at a working potential of −100 mV vs. SCE; (d) graphite composite electrode modified with POPDA at a working potential of −600 mV vs. SCE.

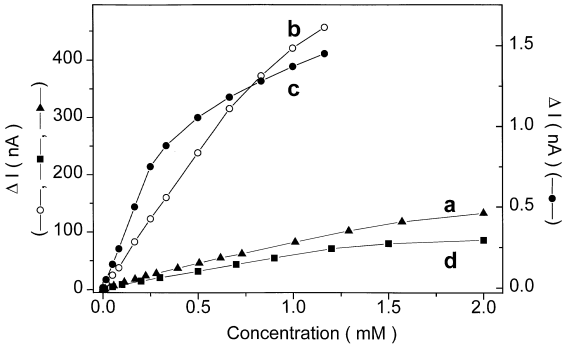

Fig. 3. Dependencies of current changes on penicillin G (a), urea (b, c) and oxalacetate (d) concentrations of the biosensors studied. Conditions: a 0.1 M phosphate buffer containing 0.1 M NaCl. (a) Platinum electrode with penicillinase immersed in a 0.5 mM hematein solution at a working potential of 0 mV vs. SCE, starting pH 8.0; (b) graphite composite electrode modified with lauryl gallate (Composite 2) and urease at a working potential of +200 mV vs. SCE, starting pH 7.0; (c) gold electrode modified with MB and urease at a working potential of −100 mV vs. SCE, starting potential 7.5; (d) graphite composite electrode modified with POPDA (Composite 1) and oxalacetate decarboxylase at a working potential of −600 mV vs. SCE, starting pH 7.5.

3.3. Urea biosensor with lauryl gallate

In the previous section, an example of a biosensor based on a water-soluble pH-sensitive redox probe molecule was shown. On the other hand, the use of a water-insoluble pH-sensitive redox compound, such as lauryl gallate, allows the construction of a bulk-modified graphite composite sensor. The pH-dependence of current (Fig. 2b) was determined with the bulk-modified solid composite electrode (Composite 2) at a working potential of +200 mV (versus SCE). On switching the buffer pH from 3.9 to 9.8, alternatively, six times an hour over 3 h of continuous work, the resulting current response was found to be reversible.

The urea biosensor was prepared by spreading urease on the electrode surface. Urease catalyses urea hydrolysis, where H^+ ions are consumed (reaction (3)):

$$NH_2CONH_2 + 2H_2O + H^+ \rightarrow HCO_3^- + 2NH_4^+ \quad (3)$$

The calibration curve for urea at a starting pH of 7.0 is illustrated in Fig. 3 and the comprehensive results are summarised in Table 1. The curvature of the calibration plot of high substrate concentrations can be explained by a decrease in the enzymatic reaction rate owing to local pH values exceeding the optimum pH. Repeated addition of 0.2 mM urea to the cell for 3 h of continuous work, with the electrode being rinsed with distilled water after every eight additions of urea, supplied a mean current of 96.5 nA with an R.S.D. of 3.0%.

The response was not affected by variation in the concentration of ions in the order of millimoles, with the exception of Fe^{3+}, that showed a negative effect (1 mM $FeCl_3$ leads to a 32% inhibition of the current response of 0.2 mM urea) probably caused by the formation of iron(III)–gallate complexes [27]. Ammonium ions did not interfere, which could be advantageous in the measurement of real samples. An important drawback was a long time of signal stabilisation before the measurement, which was 20 min, approximately four times higher compared to the stabilisation time of the others biosensors characterised in the present paper, and depending on the electrode

Table 1
Characteristics of the amperometric pH-sensing biosensors

Electrode	Redox-active pH-sensitive molecular probe	Analyte	Detection limit (S/N=5) (μM)	Linearity range (mM)	Upper limit of dynamic range (mM)	Sensitivity (μA/M cm)	Response time (min)	Remaining sensitivity after 1 month of storage (%)[a]
Platinum	Hematein	Penicillin G	4	0.004–2	10	2.92	2–3	100
Composite 2	Lauryl gallate	Urea	2	0.002–0.75	4	15.2	3–5	100
Gold	Methylene blue (MB)	Urea	10	0.01–0.25	0.5	0.11	0.25–0.5	80
Composite 1	POPDA	Oxalacetate	10	0.05–1.2	3	2.01	3–4	90

[a] When the biosensor was stored in the dry state at 4°C and under deoxygenated atmosphere.

components. The urea biosensor presented here showed a better detection limit in comparison with the reported potentiometric one [6] (0.1 mM) and one that was comparable to the amperometric urea biosensors reported by Cho and Huang [28] (0.5 μM) based on the specific sensitivity of polyaniline-perfluorosulfonated ionomers deposited on the glassy carbon electrode to ammonium ions, and those reported by Guilbault and Seo [29] (5 μM) based on the glassy carbon electrode with co-immobilised L-glutamate dehydrogenase and urease. The biosensor was used to determine urea content (13.4 g/l) in the urine of healthy persons. The results agreed well with those obtained by the standard spectroscopic Berthelot method (with an R.S.D. of +4.4%, n=5).

3.4. Urea biosensor with MB adsorbed on a gold electrode

MB is a phenothiazine dye used frequently as a mediator in amperometry [30]. Its electrochemical properties depend on pH [31]. As MB exhibits a strong interaction with gold [23], the modified electrode was prepared by a simple adsorption of MB on the surface of a gold electrode. The dependence of current on pH measured with the MB-modified gold electrode at the constant potential of −100 mV (versus SCE) is illustrated in Fig. 2c. Switching the buffer pH from 3.9 to 9.8, alternatively, six times an hour over 3 h of continuous work, the resulting current response was found to be reversible.

The urea biosensor was prepared simply by the application of urease on the MB-modified gold electrode surface which was covered by a dialysis membrane. The calibration curve for urea at a starting pH of 7.5 is illustrated in Fig. 3 and the comprehensive results are summarised in Table 1. The sensitivity was much lower than that found for the lauryl gallate based urea sensor, but the response times were very short, in the range of 15–30 s. Repeated addition of 0.2 mM urea to the cell for 3 h of continuous work, with the electrode being rinsed with distilled water after every eight additions of urea, supplied a mean current of 597 pA with an R.S.D. of 3.1%. The biosensor can tolerate interfering ions at concentrations of 5 mM.

The biosensor was used to determine urea content (13.4 g/l) in the urine of healthy persons. The results agreed well with those obtained by the standard spectroscopic Berthelot method (with an RSD of −8.8%, n=5).

3.5. Oxalacetate biosensor with POPDA film

Electropolymerised *o*-phenylenediamine is used as a redox mediator in amperometric biosensors [16] and its electrochemical properties are pH-dependent (Fig. 2d). Switching the buffer pH from 3.9 to 9.8, alternatively, six times an hour over 3 h of continuous work, the resulting current response at the constant potential of −600 mV (versus SCE) was found to be reversible.

The oxalacetate biosensor was prepared by spreading oxalacetate decarboxylase on the surface of the Composite 1 electrode. The enzyme catalyses oxalacetate decarboxylation, where H^+ ions are liberated (reaction (4)):

$$\text{oxalacetate} + H_2O \rightarrow \text{pyruvate} + HCO_3^- + H^+ \quad (4)$$

The calibration curve for oxalacetate at a starting pH of 7.5 is illustrated in Fig. 3 and the comprehensive results are summarised in Table 1. Repeated addition of 0.2 mM oxalacetate to the cell for 3 h of continuous work, with the electrode being rinsed with distilled water after every eight additions of analyte supplied a mean current of 14.9 nA with an R.S.D. of 3.7%. Interfering ions can be tolerated in concentrations of 5 mM.

The oxalacetate biosensor presented here is comparable to the classical amperometric one utilising co-immobilised oxalacetate decarboxylase and pyruvate oxidase [32] which was linear in the range from 0.015 to 0.6 mM with a detection limit of 10 μM and which had a lower stability that was caused by the fragility of pyruvate oxidase.

4. Conclusions

The concept of an amperometric pH-sensing biosensor based on the use of pH-sensitive redox probe molecules is presented. Using this concept, biosensors for urea, penicillin and oxalacetate were prepared and evaluated. The good sensitivity, accuracy and stability together with the ease of preparation and use could endow them with potential prospects, in particular, when using SBM-based transducers suitable for

low-cost mass production [14,15]. In fact, the existence and availability of a large number of enzymes affecting pH as a consequence of their biocatalytic action, e.g. amidohydrolases, esterases, decarboxylases etc., allow the construction of biosensors for the determination of their substrates. However, attention should be paid to the selection of working conditions, in particular, initial pH, operating potential, buffer type and capacity. Moreover, further improvement could be expected with a more systematic search for the pH-sensitive redox probe molecules.

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