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Development of a sandwich format, amperometric screen-printed uric acid biosensor for urine analysis

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ABSTRACT

A screen-printed carbon electrode (SPCE) incorporating the electrocatalyst cobalt phthalocyanine (CoPC), fabricated using a water-based ink formulation, has been investigated as the base transducer for a uric acid biosensor. A sandwich biosensor was fabricated by first depositing cellulose acetate (CA) onto this transducer (CoPC–SPCE), followed by uricase (UOX) and finally a polycarbonate (PC) membrane; this device is designated PC–UOX–CA–CoPC–SPCE. This biosensor was used in conjunction with chronoamperometry to optimize the conditions for the analysis of urine: temperature, 35 °C; buffer, pH 9.2; ionic strength, 50 mM; uricase, 0.6 U; incubation time, 180 s. The proposed biosensor was applied to urine from a healthy subject. The precision determined on unspiked urine ($n = 6$) was 5.82%. Urine was fortified with 0.225 mM UA, and the resulting precision and recovery were 4.21 and 97.3%, respectively. The linear working range of the biosensor was found to be 0.015 to 0.25 mM (the former represents the detection limit), and the sensitivity was calculated to be 2.10 μA/mM.

Uric acid (UA)[1] is an important nitrogen-containing compound commonly found in biological fluids such as blood serum and urine. It is a principal end product of purine metabolism in the human body. Hence, its level serves as a biomarker for the detection of disorders associated with purine metabolism [1]. In healthy humans, the concentration of UA in urinary excretion is in the range of 214 to 4400 μM, whereas that in serum is 240 to 520 μM [2,3]. Abnormal levels of UA are symptomatic of several diseases such as gout, hyperuricemia, Lesch–Nyhan syndrome, and cardiovascular and renal diseases [1–5]. Urine analysis is the standard method for the diagnosis of diseases such as gout, Lesch–Nyhan syndrome, and hyperuricemia and may differentiate disease states that are not revealed in blood analysis [6,7]. Therefore, there is the need to regularly monitor UA levels in urine to diagnose and avoid any complications associated with these diseases.

Numerous methods, including spectrophotometric, enzymatic, high-performance liquid chromatographic, microfluidic, capillary electrophoresis, chemiluminescence, fluorescence, and electrochemical techniques, have been employed for the analysis of UA [2,4,8,9]. However, the procedures involved in many of these methods are cumbersome and time-consuming, and they often lead to direct oxidation of some amounts of UA [9]. Among these methods, electrochemical measurements, particularly those using electrochemical biosensors, are most attractive for UA analysis because they are rapid, simple, economic, highly sensitive, and specific; enable direct real-time and on-line data analysis; and exclude preseparation procedures [2,10]. Previous electrochemical methods based on the oxidation of UA at carbon-based electrodes suffered from interference from other electroactive constituents such as ascorbic acid, which oxidizes at close to the same potential as UA on various types of electrodes [3]. Several different techniques have been used to modify electrode surfaces to solve the problem from interference, including enzyme immobilization, adsorption/medium exchange, electrochemical pretreatment, and polymerization [3,4,11]. Among these methods, UA biosensors based on uricase (UOX) generally have much higher selectivity and are able to achieve detection at neutral pH [11]. As is well known, good selectivity is based on the enzyme catalysis of the UA. The enzymatic reaction of UOX is shown below (Eq. (1)):

$$UA + O_2 + H_2O \xrightarrow{\text{Uricase}} \text{Allantoin} + H_2O_2 + CO_2 \quad (1)$$

The analytical method is usually based on amperometric monitoring of the catalytically produced H_2O_2 by way of a redox mediator [2]. Typically, the use of cobalt phthalocyanine (CoPC) as a mediator has enabled the detection of H_2O_2 at lower potentials

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[1] *Abbreviations used:* UA, uric acid; UOX, uricase; CoPC, cobalt phthalocyanine; CA, cellulose acetate; GLA, glutaraldehyde; PC, polycarbonate; CoPC–SPCE, screen-printed water-based carbon electrode incorporating CoPC; BSA, bovine serum albumin; AA, ascorbic acid; PA, paracetamol; CV, coefficient of variation.

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such as +0.4 V during UA analysis [11]. Accordingly, the development of a sensitive and selective UA biosensor is highly desirable. One possible route is to use selective membranes in conjunction with a redox mediator, thereby excluding the access of many interfering species to the electrode surface.

In the current study, various perm-selective membranes, namely cellulose acetate (CA) in acetone, glutaraldehyde (GLA) in 50 mM phosphate buffer solution. and polycarbonate (PC), were used in systematic studies to investigate the fabrication of a sandwich UA biosensor based on the use of a screen-printed water-based carbon electrode incorporating CoPC (CoPC–SPCE) (Fig. 1) that effectively excludes access of interfering electroactive species to the electrode surface. The electrochemical measuring technique is chronoamperometry, as required for a commercial device.

Materials and methods

Materials and reagents

All chemicals, including acetone, were of analytical grade and purchased from Sigma–Aldrich (UK). H_2O_2 solutions were prepared from 50% (w/w) pure water stock (17.46 M) and added to supporting electrolyte (50 mM phosphate buffer solution). Phosphate buffer (50 mM) was prepared by combining appropriate amounts of trisodium phosphate dodecahydrate, sodium dihydrogen orthophosphate dehydrate, and disodium hydrogen orthophosphate dehydrate to yield the desired pH. UOX (cat. no. U7128-100UN, from *Arthrobacter globiformis*), UA (cat. no. U2625, lot STBB1642A0), and CA (cat. no. 3782, lot 29F0024) were obtained from Sigma–Aldrich. PC membranes (cat. no. HAWP02500, lot R2JN75953) were obtained from Millipore (Ireland). GLA (50% solution, code G/0518/PB08, batch 0586613) was obtained from Fisher Scientific (UK). UA was dissolved in 50 ml of 50 mM NaOH following up to 15 min of sonication. CA was dissolved in acetone following up to 2 min of sonication.

Apparatus and measurements

All electrochemical measurements were conducted with a three-electrode system consisting of a CoPC–SPCE working electrode and an Ag/AgCl reference electrode (GEM product code C61003P7) screen-printed onto Valox (Cadillac Plastics, UK) and a separate Pt wire counter electrode. The area of the working electrode was defined with an insulating tape (RS Components, UK) to a 3 × 3-mm^2 area. The working and reference electrodes were connected to the potentiostat with gold clips, and the cell was completed with a separate Pt wire counter electrode. Solutions were stirred to ensure thorough mixing prior to chronoamperometry with a magnetic stirring disk and stirrer (Stuart Scientific, UK). An Autolab electrochemical analyzer with General Purpose Electrochemical System software (GPES 4.9) was used for the acquisition of data and experimental control, with a 10-ml electrochemical cell inside a water jacket for electrochemical measurements. All weighings were done using a Precisa (UK) precision balance 262SMA-FR device. Measurement and monitoring of pH was conducted with a Fisherbrand Hydrus 400 pH meter (Orion Research, USA). Sonications were performed with a Devon FS100 sonicating water bath (Ultrasonics, UK). The temperature was controlled with a thermostated water bath (Thermo Scientific HAAKE DC10-P5/U unit).

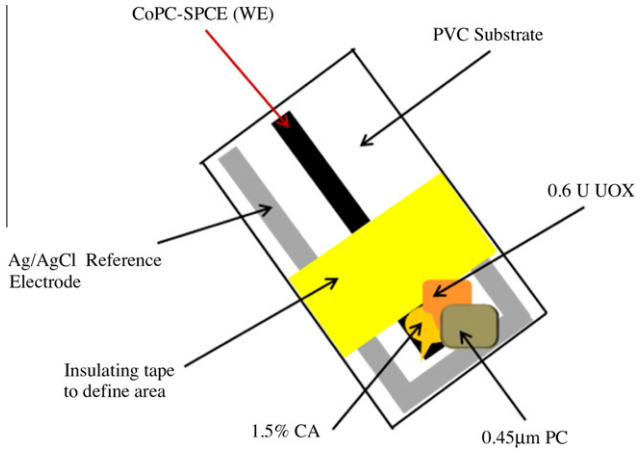

Fig.1. Diagram of proposed sandwich biosensor strip. The order of preparation was as follows: (i) screen print the CoPC–SPCE (working electrode, WE) and Ag/AgCl reference electrode; (ii) define both WE and reference electrode with insulating tape; (iii) drop coat 1.5% CA and allow to dry; (iv) drop coat enzyme and allow to dry; (v) position a 4 × 4-mm PC membrane over the enzyme layer and fix with adhesive. All studies were performed using a separate Pt wire counter electrode. PVC, polyvinyl chloride.

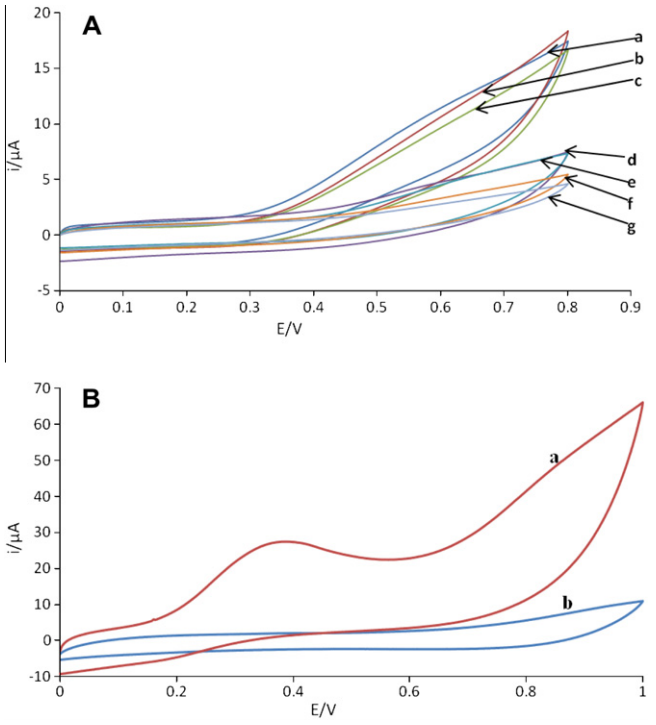

Fig.2. Cyclic voltammograms obtained with 50 mM phosphate buffer (pH 8.0) containing 50 mM NaCl for the following: (A) 2 mM H_2O_2 at 0% (a), 1% (b), 1.5% (c), 2% (d), 3% (e), 4% (f), and 5% (g) CA–CoPC–SPCE; (B) 2 mM UA at 0% (a) and 1.5% (b) CA–CoPC–SPCE. Scan rate: 20 mV s^{-1}.

Table 1
Optimal operating conditions for UA biosensor.

Parameter	Range studied	Optimal value
Incubation time (s)	60–180	180
Enzyme loading (U)	0.2–2.0	0.6
pH	5.0–10.0	9.2
Buffer strength (M)	0.025–0.450	0.05
Temperature (°C)	25–40	35

Fabrication and surface modification of CoPC–SPCEs

The base unmodified CoPC–SPCE transducer was prepared using a water-based ink (code C40511D8), and the sensors were screen-printed in groups of six onto Valox as described previously [12]. GLA and CA solutions of 0%, 1%, 1.5%, 2%, 3%, 4%, and 5% were prepared to coat the electrodes. GLA and CA were diluted in

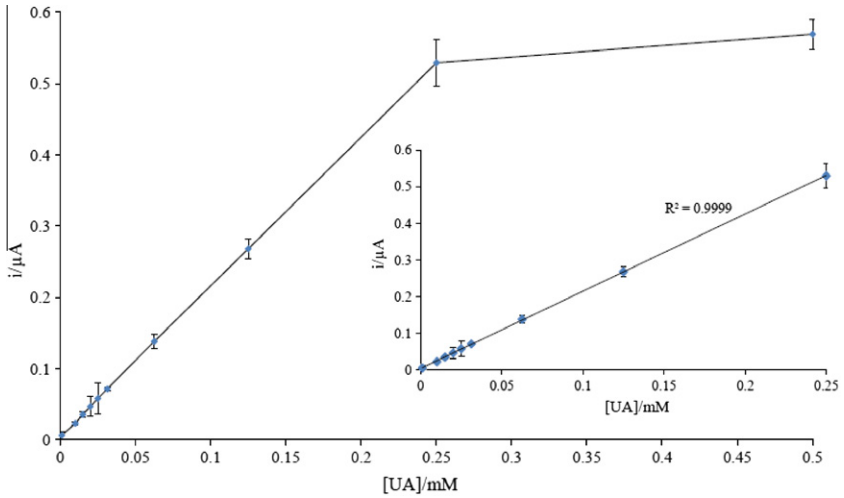

Fig.3. Plots of chronoamperometric current responses (at 180 s) versus concentration of UA in the presence of 50 mM phosphate buffer (pH 9.2) containing 50 mM NaCl. Sensitivity = 2.1 μA mM^{-1}; CV = 4.3%; each data point is the mean of three replicates.

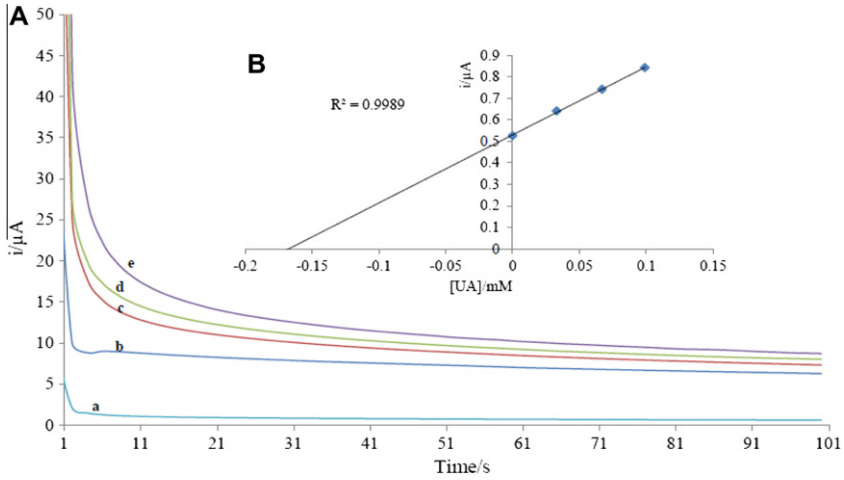

Fig.4. (A) Chronoamperograms obtained with the dummy (BSA) biosensor and urine sample (a) and with the biosensor plus spiked urine sample (b), spiked sample plus 0.033 mM UA (c), spiked sample plus 0.067 mM UA (d), and spiked sample plus 0.099 mM UA (e). (B) Multiple standard addition plot (R^2 = 0.9989). The dummy biosensor signal was subtracted from the biosensor signals to obtain plot B.

50 mM phosphate buffer solution and acetone, respectively. The CoPC–SPCEs were modified by drop-coating 5 μl of various concentrations of either GLA or CA solution directly onto the exposed working electrode with an area of 9.0 mm^2. Once dry, the electrodes were coated with 5 μl of UOX solution containing 0.6 U, and the electrodes were left to dry overnight. A PC membrane (0.45 μm) was then placed over the active working area and secured using insulating tape. The same procedure was used to obtain dummy biosensors by using the same mass of bovine serum albumin (BSA) as of the enzyme, and any responses resulting from these devices were substracted from the biosensor response. An illustration of the proposed sandwich biosensor strip is shown in Fig. 1.

Once prepared, biosensors were dried in a vacuum desiccator containing silica gel and stored in the refrigerator until ready for use.

Permselectivity studies

Cyclic voltammograms were obtained with 50 mM phosphate buffer solution (pH 8.0) containing 50 mM NaCl and either 2 mM H_2O_2 or 2 mM UA in the contacting solution to assess the permselectivity of the GLA–CoPC–SPCEs and CA–CoPC–SPCEs toward these molecules. The scan rate was 20 mV s^{-1}.

Amperometry was used to investigate the permselectivity properties of the complete biosensor (PC–UOX–CA–CoPC–SPCE) to 2 mM ascorbic acid (AA) and paracetamol (PA).

Chronoamperometric studies with the proposed biosensor

Chronoamperometry was used to determine the effect of: temperature (25–40 °C), pH (5.0–10.0), ionic strength (0.025–0.45 mM), and incubation time (60–180 s) of the UA biosensor. The measurements were obtained by stepping from an open circuit to +0.5 V versus Ag/AgCl. The performance characteristics of the UA biosensor were deduced from calibration studies. These were performed with 50 mM phosphate buffer solution containing 50 mM NaCl over the concentration range of 0.001 to 0.500 mM UA.

Application of biosensor to urine analysis

Chronoamperometry, in conjunction with the method of multiple standard additions, was used to determine the concentration of UA in unspiked urine samples (obtained by adding 25 ml of original urine collected to 25 ml of 50 mM phosphate buffer, pH 9.2). Aliquots (10 ml) were subjected to chronoamperometry to determine

Table 2
UA concentrations in spiked and unspiked urine samples.

Repeat number	Endogenous ([UA], mM)	
1	0.117	
2	0.124	
3	0.110	
4	0.110	
5	0.108	
6	0.107	
Mean	0.113	
Standard deviation	0.007	
CV (%)	5.82	
Repeat number	Amount added ([UA], mM)	Amount found ([UA], mM)
1	0.225	0.324
2	0.225	0.313
3	0.225	0.345
4	0.225	0.325
5	0.225	0.350
6	0.225	0.335
Mean		0.332
Standard deviation		0.014
CV (%)		4.21
$\%\text{Recovery} = \frac{0.332-0.113}{0.225} \times 100 = 97.3$		

Note. Endogenous concentrations of UA (upper section) found using the amperometric biosensor and concentrations found (lower section) following fortification with 0.225 mM UA are shown. The mean recovery was calculated from the means of endogenous UA (0.113 mM) and recovered UA (0.332 mM); this was found to be 97.3%, with a CV of 4.21%.

the original UA concentration. To do this, 10-μl aliquots of standard UA solution containing 35 mM were added to the voltammetric cell. Oxidation currents were measured 50 s after applying a potential of +0.5 V, and the results were used to construct standard addition plots. The intersection of the extrapolated line on the abscissa ($y = 0$) was used to determine the concentration of the endogenous urine–UA. This procedure was repeated using urine fortified with 0.225 mM UA ($n = 6$). The means of both of these experimentally derived values of UA concentrations (endogenous urine–UA and UA recovered from fortified urine) were used to calculate the mean UA recovery.

Results and discussion

Principle of operation of the proposed biosensor

In the proposed system, there are three important processes occurring: an enzymatic reaction that involves the conversion of UA to allantoin (Eq. (1)); a chemical reduction of the oxidized form of CoPC (Co^{2+}) by H_2O_2 to produce the reduced form of CoPC (Co^{+}) (Eq. (2)); and the electrochemical reoxidation of Co^{+} to Co^{2+}, which involves the transfer of electrons to the SPCE (Eq. (3)). Clearly, the measurement of UA based on H_2O_2 detection has the advantage that the latter will pass through very small pore size membranes that would exclude interferences present in urine. Therefore, as a consequence, UA that would give a direct oxidation signal would also be excluded and not interfere:

$$2Co^{2+} + H_2O_2 \rightarrow 2Co^{+} + O_2 + 2H^{+} \quad (2)$$

$$Co^{+} \rightarrow Co^{2+} + e^{-} \quad (3)$$

Optimization studies

Initial cyclic voltammetric studies were performed to elucidate the optimal CA loading required to prevent UA from diffusing to the CoPC–SPCE surface while allowing H_2O_2 to freely diffuse to the electrode surface. Line c in Fig. 2A shows that with membranes obtained with 1.5% or less CA, the currents for H_2O_2 were minimally reduced, whereas complete loss of UA signal occurred with the membrane fabricated with 1.5% CA (Fig. 2B, line b). Therefore, 1.5% CA was employed in the fabrication of all further biosensors.

Similar studies were performed with GLA; however, this did not prevent UA from reaching the underlying CoPC–SPCE, resulting in unwanted oxidation signals. Clearly, the pore sizes of the GLA membranes were not sufficiently small to act as an exclusion barrier. In contrast, it is probable that the hydrophobic nature of CA aids in the exclusion process, and its application in acetone facilitates strong binding to the electrode surface. The permselectivity of the complete biosensor (PC–UOX–CA–CoPC–SPCE) was investigated by the addition of AA and PA using the biosensor in the amperometric mode. No signals were produced by either of these at a concentration of 2 mM (see Fig. A in supplementary material).

The effects of temperature (see Table A in supplementary material), enzyme loading, pH, ionic strength, and incubation time on the performance of the biosensor were also thoroughly investigated by chronoamperometry. The resulting optimal values are shown in Table 1 and were used in all further studies. Although the maximum sensitivity occurs at a temperature of 35 °C (2.10 μA/mM), good sensitivity (1.66 μA/mM) was achieved at 25 °C, indicating suitability for operation at room temperature when applicable.

Calibration studies with biosensor

Calibration studies were performed with the fully developed UA biosensor using the optimized conditions. First, chronoamperometry was performed with 3 ml of 50 mM phosphate buffer (pH 9.2) containing 50 mM NaCl only and then containing UA over the range of 0.001 to 0.500 mM. A fresh biosensor was used for each measurement, and this was done in triplicate for each concentration. The resulting plot (Fig. 3) shows typical Michaelis–Menten behavior, and the biosensor exhibited a linear response to 0.25 mM. The limit of detection was approximately 0.015 mM, which was considered as satisfactory for urine analysis.

It should be mentioned that we examined the stability of the PC–UOX–CA–CoPC–SPCE biosensors after storage in a desiccator at 4 °C. The response did not decrease for a standard 0.2-mM solution of uric acid over the 7-day study period, indicating that there was no deterioration in enzyme activity (see Fig. B in supplementary material).

Previous biosensors have been reported for UA measurements; however, those based on amperometry in stirred solution do not study and/or exclude all common interfering species [13,14] and/or have an inferior linear range [14] (0.0025–0.085 mM) compared with our chronoamperometric biosensor (0.015–0.25 mM). The only reported chronoamperometric UA biosensor was reported to suffer from a poor detection limit (i.e., 0.07 mM) [15] compared with 0.015 mM for our device.

Analytical application of the biosensor

Chronoamperometry, in conjunction with the method of multiple standard additions (Fig. 4), was used to determine the recovery for urine spiked with 0.225 mM UA. Six replicate urine samples were analyzed. The determination was performed on 10-ml aliquots of diluted urine, and a fresh biosensor was used for each measurement.

Table 2 shows the results obtained. They show a value of 97.3% for the mean recovery of UA, with coefficients of variation (CVs) of 5.82 and 4.21% for the unspiked and spiked urine samples, respectively.

Conclusions

We have demonstrated that the proposed sandwich format UA biosensor, when used in conjunction with chronoamperometry for urine analysis, gave reliable results. To the best of our knowledge, this is the simplest reported UA biosensor that is operated in the chronoamperometric mode and both prevents direct oxidation of uric acid and solves problems from potential interferences in urine. It should be mentioned that these biosensors were fabricated in-house using screen-printing technology that will allows mass production at low cost, particularly because a water-based carbon ink was used. Disposable glucose biosensor electrodes for home-based monitoring by diabetics retail for less than 1 euro (€1) per test, and because they are based on similar technology, the UA biosensors would be expected to retail at a similar cost if manufactured on a commercial scale and used for single measurements only. In this study, we used fresh biosensors for each measurement, and this produced reliable results for urine analysis; consequently, this would be readily applicable to clinical studies. It is also possible to reuse the same biosensor for multiple measurements without loss of any significant sensitivity. This may be a further cost-saving option suitable for automated flow injection analysis [16].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2012.05.027.

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