Biosensing With Coated-wire Electrodes Part 2.* Urea Sensors†

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Enzyme electrodes are described for the determination of urea based on an ammonium ion-selective coated-wire electrode. The electrodes consisted of homogeneous membranes of poly(vinyl chloride) (PVC) or silicone rubber containing both the electroactive species and immobilised enzyme. Those sensors in which PVC was utilised as the membrane proved to be the most useful and for a 0.2 mm thick membrane a super-Nernstian calibration was obtained with a mean slope of 61 ± 1 mV decade^{-1} under static conditions of measurement. The lifetime of the electrodes was approximately 10 d. The electrode could be used in a miniaturised, custom-designed flow cell for automated analysis. In this mode the slope of the calibration obtained was 67 ± 2 mV decade^{-1}.

Keywords: *Coated-wire electrode; urea sensor; ammonium; urease*

As discussed previously,[1] there has been considerable interest in enzyme electrodes in recent years. This interest has been greatest in the field of clinical analysis, where small sample sizes are often a limiting factor. As a result, steps have been taken to develop miniaturised sensors that require only small sample volumes.[2] With ion-selective electrodes the construction of small devices is complicated by the need for an internal reference solution. In this work the development of miniature urea coated-wire enzyme sensors has been investigated. Two urea sensors based on coated-wire electrodes have previously been reported,[3,4] but in both instances the base electrode was a pH sensor. The enzyme was immobilised on the pH sensor as a second layer, rather than consisting of a single homogeneous membrane. Joseph[3] reported a sensor based on an antimony metal wire electrode which gave a sub-Nernstian calibration with a slope of 40–45 mV decade^{-1} for a concentration range of 0.1–10 mM urea. The electrode had a response time of less than 1 min. Ianniello and Yacynych[4] developed a similar electrode, but this was based on an iridium oxide pH monitor.

In this work the base sensor is an ammonium ion-selective coated-wire electrode. The electrode consists of a polymer membrane into which a neutral carrier, nonactin, is embedded. It is therefore of similar design to the ion-selective electrode based sensor described by Guilbault and Nagy[5] in which the ammonium ions produced by the breakdown of urea are detected.

Experimental

Reagents

Urease (E.C. 3.5.1.5, 80 U mg^{-1}) purified from jack beans, nonactin (from *Streptomyces griseus*), glutaraldehyde (grade 1, 25% aqueous solution) and bovine albumin (98–99% pure) were obtained from Sigma (Poole, Dorset, UK) and were stored in a refrigerator at 4 °C. Poly(vinyl chloride) (PVC) (low relative molecular mass) and dioctyl phenylphosphonate were purchased from Aldrich (Gillingham, Dorset, UK); all other reagents were obtained from BDH (Poole, Dorset, UK) including the silicone rubber solution (Dow Corning 3140RTV). Urea standards were prepared from stock solutions in sodium phosphate buffer (0.1 M, pH 8) (AnalaR grade reagents).

Preparation of the Urea-selective Electrodes

The laboratory-manufactured electrodes consisted of a 4-mm chassis-mounted banana socket on to which a 4 mm length of platinum wire (1 mm o.d.) was soldered. The enzyme electrodes were prepared as homogeneous membranes including both the electroactive species and the enzyme. Three types of electrode were constructed (as summarised in Table 1). In the first two, the enzyme was physically immobilised by entrapment in the membrane material (either PVC, membranes 1 and 2, or silicone, membranes 3 and 4). The third electrode was constructed with PVC, but the enzyme was chemically immobilised by cross-linkage with glutaraldehyde (membrane 5). Membranes 1–4 were constructed in a similar way. The platinum wire was first dip-coated with a solution of either silicone rubber or PVC with dioctyl phenylphosphonate plasticiser (20% *m/V* PVC, 0.3% *m/V* plasticiser in cyclohexane). Although difficult to measure precisely, it is estimated that this resulted in a deposition of approximately 9 mg of material on to the platinum wire. After allowing the electrode to dry for 10 min, a mixture of the dry enzyme (3–5 mg) and nonactin (2 mg) was pressed into the membrane to produce a single layer in contact with the platinum wire. The electrodes

Table 1. Construction and composition of membranes for coated-wire urea sensors

	Membrane No.				
	1	2	3	4	5
Silicone rubber	—	—	Yes	Yes	—
PVC	Yes	Yes	—	—	Yes
Chemical immobilisation	—	—	—	—	Yes
Dry enzyme mass/mg	3	5	3	5	3

* For Part 1 see reference 1.

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were stored in buffer solution at 4 °C. The chemically immobilised sensors were prepared by first coating the platinum wire with PVC and the active species. After 10 min the sensor was dipped into the stirred glutaraldehyde - bovine albumin solution for several hours according to the method of Al-Hitti *et al.*[6]

Instrumentation and Measuring Procedure

Measurements were made with the same equipment and under the same conditions as described previously[1]; however, automated analysis was also used for the urea study. The simple single-channel flow injection (FI) manifold consisted of a peristaltic pump (Gilson Minipuls 2), a six-port rotary injection valve with an adjustable sample loop (Omnifit, Anachem) and a flow cell. All connections were made with PTFE tubing (0.5 mm i.d.) and polypropylene flangeless connections (Omnifit).

The flow cell was constructed in-house from a single block of Perspex which was drilled to house a coated-wire electrode, a reference electrode and fittings for the inlet and outlet (Fig. 1). The cell was designed with a small internal volume to prevent peak broadening and this volume decreased to approximately 8 μl when the electrode was introduced. The flowing stream entered from the bottom of the cell and flowed past the enzyme electrode, with the reference electrode downstream. A flow-rate of 0.5 ml min^{-1} was used throughout for reasons discussed under Operation of the FI manifold.

Results and Discussion

All results for the slopes of the calibration graphs are quoted at the 95% confidence level.

Optimisation of Base Electrode

Before preparing the enzyme electrode it was essential to check the operation of the ammonium ion-selective coated-wire electrode. The electrode that was tested had a membrane containing nonactin, a neutral carrier. Solutions of ammonium chloride in sodium phosphate buffer in the range 0.1 mM–1 M were prepared. The electrode was found to have a near-Nernstian response over a concentration range of 1 mM–1 M with a mean slope of 57 ± 1 mV $decade^{-1}$ under static measurement conditions and a mean slope of 58 ± 4 mV $decade^{-1}$ under FI conditions. The electrode had response and recovery times of 1 and 2 min, respectively and the lifetime of the electrode was greater than 3 weeks.

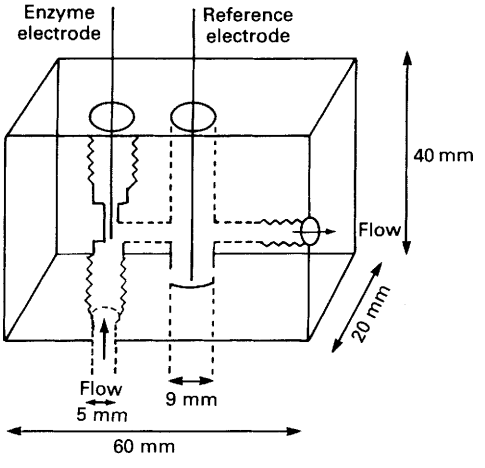

Fig. 1. Diagram of flow cell construction

Optimisation of Parameters Affecting the Performance of the Enzyme Electrode

Methods of immobilisation

Three types of membrane were investigated (Table 1). The PVC and silicone rubber electrodes were prepared with two different membrane thicknesses (approximately 0.3 and 0.4 mm). The membrane size was measured by closing a micrometer until visual contact was observed. The characteristics of the five different membranes are presented in Table 2. As can be seen, the 0.3-mm PVC membrane containing 3 mg of urease gave the best analytical performance. The electrode had a near-Nernstian response with a mean slope of 61 ± 1 mV $decade^{-1}$ under static measurement conditions and a mean slope of 67 ± 2 mV $decade^{-1}$ for the FI-based experiments. It also had an acceptable response time of 2–10 min and a lifetime of 10 d. The results obtained for the chemically immobilised electrode were poor by comparison. The sensitivity of the electrode was reduced, giving a sub-Nernstian calibration (mean slope 18 ± 5 mV $decade^{-1}$). This reduction in sensitivity suggested that either the immobilisation process had not been successful and the urease had been leached out of the electrode, or that chemical immobilisation had occurred and reduced the activity of the membrane. The lifetime of the electrode was reduced to 4 d, suggesting that a leaching process had occurred. Further studies will be necessary to develop a successful chemical immobilisation technique for homogeneous coated-wire sensors. As an alternative the enzyme layer could be added to a second layer; however, previous work showed reduced sensitivity for dual-layer electrodes[1] and increased demands on construction. The silicone rubber based membrane electrodes also showed reduced sensitivity (Table 2). The thickness of the membrane affects the performance of the electrode. A thicker electrode can hold more enzyme, but the response time of the electrode will lengthen. The electrodes developed here had relatively thick membranes and reduced sensitivity was observed as the membrane thickness increased even though the enzyme concentration had increased. This was most probably due to a significant reduction in ion mobility as a result of increased enzyme concentration in the membrane. The thickness of the membrane was partially controlled by the mass of enzyme incorporated. For both the PVC and silicone rubber electrodes the sensitivity of the electrode decreased with increased membrane thickness and in both instances the response time also increased. For all further work the sensor with physically immobilised enzyme and a PVC membrane approximately 0.3 mm thick (3 mg of enzyme added) was utilised.

Characterisation of the Sensor

The effect of sample temperature on the response of the electrode was studied. An approximately linear relationship was found between e.m.f. and temperature in the range 0–38 °C for a 100 mM solution of urea (least-squares fit: y =

Table 2. Characterisation of coated-wire urea sensors

	Membrane No.				
	1	2	3	4	5
Linear range/M	10^{-3}–3 × 10^{-1}	10^{-3}–3 × 10^{-1}	10^{-2}–1	10^{-2}–1	10^{-3}–3 × 10^{-1}
Slope/mV $decade^{-1}$	61 ± 1 67 ± 2*	35 ± 1	47 ± 17	19.9 ± 14	18 ± 5
Response time/min	2–10	3–15	2–20	3–15	2–10
Recovery time/min	5–20	7–25	5–20	7–25	5–20
Lifetime/d	10	—	7	—	4

* Flow conditions.

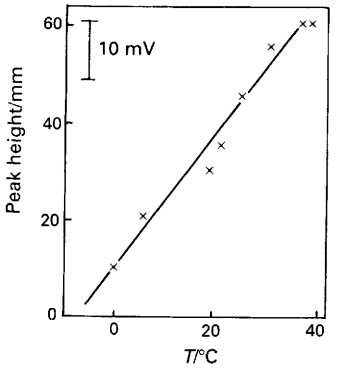

Fig. 2. Temperature profile for the urea sensor

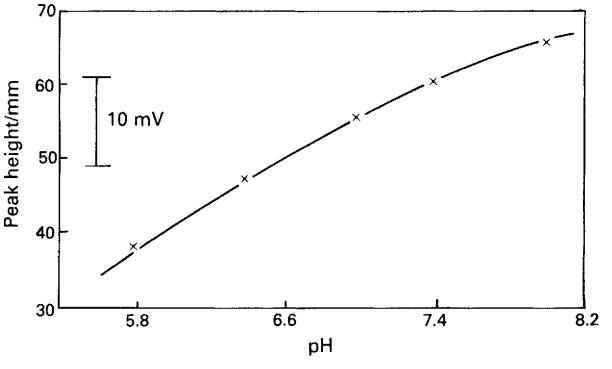

Fig. 3. Effect of buffer pH on urea coated-wire electrode response (0.1 M phosphate buffer)

$1.3x + 9.6$, correlation coefficient 0.9860). The temperature profile for the urea sensor is shown in Fig. 2. When used in the batch mode, the response time at 95% steady state was found to be 2–10 min, 10 min being necessary only for very high concentrations. The recovery time was similarly 5–20 min. Fig. 3 shows the effect of the pH of the phosphate buffer (0.1 M) on the electrode response over the pH range 5.8–8.0. As can be seen the change in e.m.f. is gradual in this region, thus having no great effect on the enzyme activity; at pH values greater than 8 the sensitivity decreased rapidly. The selectivity of the electrode depends on the selectivity of the ammonium electrode and the enzyme reaction. As the enzyme reaction has inherent selectivity, the selectivity of the ammonium electrode was the limiting factor, with univalent cations posing the greatest problem. Using the mixed solution method, K^+ was found to have a selectivity ratio of 0.2 at a 10 mM concentration.

One problem, common to most coated-wire electrode sensors, is that of repeatable construction. The coated-wire urea sensors were found to be no exception. It was necessary to check the operation of each sensor constructed prior to application within the FI apparatus. Generally, the electrode either operated in a super-Nernstian fashion as described here, or failed catastrophically, indicating that the construction method possibly led to destruction of the membrane - metal interface with the failed electrodes. This inconsistency in the performance of one electrode relative to another may be due to poor reproducibility in the coating technique. A further consideration is the possible disturbance caused by the pressing of dry reagents on to the PVC/silicone membrane, which may cause the rubber membrane to become detached from the platinum wire. The failure rate in this work was about 20%. There is clearly a need for further development in the construction method if these devices are to be fabricated in large numbers without resort to time consuming calibration prior to use.

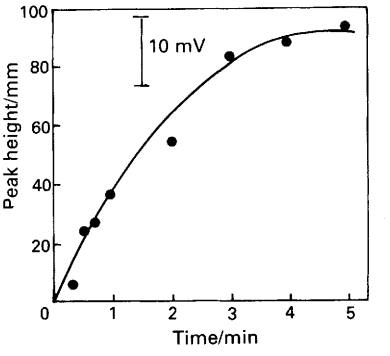

Fig. 4. Stopped-flow effect on urea coated-wire electrode

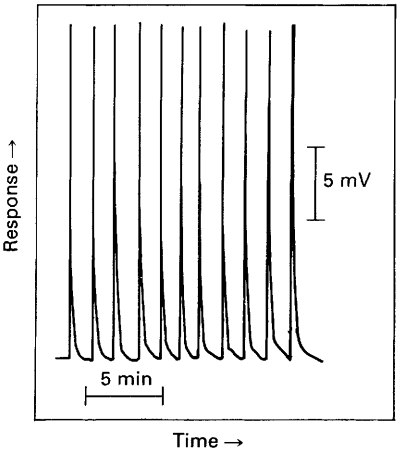

Fig. 5. Reproducibility of the urea sensor at 100 mM

Operation of the FI Manifold

In designing the FI manifold the sample volume injected, the tube length required between the injector and flow cell and the flow-rate had to be considered. A sample volume of 50 μl was selected as convenient in experimental terms with respect to the type of injection unit employed. Although it would be possible to modify the injection valve in order to accommodate a larger sample volume, 50 μl was considered a reasonable compromise between maximising detection sensitivity and avoiding system saturation. The tube length was kept as short as practicable (20 cm) to prevent dispersion of the sample. The carrier buffer flow-rate was 0.5 ml min^{-1}. This low flow-rate was chosen to accommodate the slow response time of the electrode, particularly at higher concentrations. Unfortunately, the peristaltic pump did not operate reliably below this flow-rate and, therefore, to gain higher sensitivity it was decided to use a stopped-flow technique. This involves switching off the pump for a set time when the sample reaches the detector. To ascertain the optimum time for the determination of high concentrations of urea, the change in electrode response with stop time was recorded for a 100 mM solution (Fig. 4). This concentration was chosen because it was relatively high and, therefore, the electrode response time would be greater. A time of 4 min was selected as the optimum. At shorter times the response changed rapidly; at longer times the rate of change decreased. Although this method is time consuming, it would not cause a problem for a totally automated system and would be a much faster method of analysis than using the sensor in the batch mode. In the FI mode (without stopped flow) the urea sensor showed good reproducibility on a between-day basis with a relative standard deviation of 0.6% for ten samples at the 100 mM level (Fig. 5).

Conclusions

This work has demonstrated the operation of a urea coated-wire electrode. As described previously for the glucose coated-wire electrode[1] the simplicity of construction, particularly with the homogeneous membrane containing both enzyme and ion sensor, offers the possibility of mass producing low-cost disposable spot-test sensors. This type of urea coated-wire electrode compares favourably, particularly in terms of sensitivity, with those constructed in two layers and based on pH measurement.[3,4] The sensors are ideal as detectors for FI and for clinical applications because of their small size and inherent robustness; however, further work is necessary to ensure repeatable inter-electrode performance and improved shelf lifetime.

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NOTE—Reference 1 is to Part 1 of this series.

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