Sensing With Chemically and Biologically Modified Carbon Electrodes

A Review

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Summary of Contents

Keywords: *Chemically and biologically modified carbon electrode; point-of-care testing; bioelectrochemistry; clinical and environmental biomolecule; review*

range of selectivity-enhancing techniques that may permit analyses in 'real' samples. The emphasis of such modification techniques will be on biologically tailoring sensory interfaces, although broader aspects, such as aprotic solution electrochemistry, biosensor evolution and their impact on the clinical market, are also discussed.

Background

In voltammetry and amperometry, the potential applied to a working electrode governs the oxidation state of a particular species at its surface, with the resulting charge-transfer process transducing quantitative data into a measurable current signal. As these methods monitor the current flowing through an electrode following a voltage perturbation, the working electrode may be considered to be the heart of the experiment. Consequently, much effort has been devoted to electrode fabrication and maintenance. The noble metals, *e.g.*, platinum,[1,2] nickel[3] and gold,[4] have been commonly employed in electroanalysis, the first finding the widest application, owing to its inertness and useful potential window.

In recent years, considerable attention has been focused on the production of carbon-based electroanalytical sensors, as carbon is a versatile and inexpensive electrode material. Such devices are amenable to chemical and biological 'engineering' with modifying agents such as polymeric membranes, enzymes and redox mediators, to impart the requisite selectivity for analyses in complex matrices.

This review is by no means exhaustive, but serves to illustrate the desirable characteristics and versatility of carbon as an electrode material for the fabrication of electrochemical sensors. The readers will also be introduced to the diverse

Carbon as an Electrode Substrate: Properties, Manufacture and Modification

Carbon, in many respects, is an ideal electrode substrate. Its attractive features include access to a wide anodic potential range, low electrical resistance and residual currents and a reproducible surface structure. It is morphologically diverse, existing in a variety of forms suitable for electrochemical applications, *e.g.*, carbon fibres,[5] glassy (vitreous) carbon,[6] graphite pastes[7] and composites[8] and carbon films.[9] Examples of the analytical utility of the last four electrode substrates are detailed in this review.

Graphite

Graphite has been used extensively in electroanalytical studies.[9–12] Although it occurs naturally in the Earth's crust (Sri Lanka and the Malagasy Republic possessing the largest deposits), the high ash content (5–20%) of this material limits its electrochemical utility. Instead, the use of a purer, artificial graphite is commonplace in electroanalytical experiments. This chemically cleaned graphite is generally prepared using a procedure developed by Acheson in 1896,[9] whereby petroleum coke is heated to remove volatiles, mixed with coal-tar pitch, consolidated into the desired form and then subjected to a two-step heat-treatment process. A temperature of 1000 °C is used in a non-oxidizing environment, to expel unwanted volatile constituents from the pitch. The resulting amorphous carbon is transformed into graphite by further heating between 2500 and 3000 °C. Pyrolytic graphite is another synthetic structure, formed by the thermal decomposition of carbonaceous gases, such as methane, at temperatures in excess of 1200 °C.[9]

Crystallographically, graphite consists of layers of carbon atoms arranged in hexagonal rings, stacked in a repeating anisotropic ABAB pattern; this is termed hexagonal graphite. A less common form is rhombohedral graphite, which consists of ABCABC repeating patterns. The lowest resistance of graphite (approximately 10^{-4} Ω cm) is in the direction parallel to the hexagonal carbon planes and electrical conduction through this plane confers its metal-like properties.[9]

Glassy (Vitreous) Carbon

Glassy carbon electrodes (GCEs) have been widely employed in voltammetric studies,[13–18] for electrochemical detection

following liquid chromatographic separations[13,19,20] and in flow injection (FI).[21–23] Glassy carbon is a solid isotropic material composed of thin convoluting microfibrils that interlock to form strong interfibrillar bonds. It is produced by the thermal degradation (approximately 1800 °C) of selected organic polymers.[9]

The most striking feature of glassy carbon is its extremely low gas permeability compared with that of synthetic graphites, which arises from an extensive closed void network (as opposed to the open spaces present in its artificial graphite counterparts), allowing only discontinuous gas permeation of the material. The peculiarities of its surface structure permit analyses in extreme environments, such as organic solvents. This is important in the area of organic-phase electrochemistry, which offers the possibility of detecting hitherto inaccessible analytes, for example those that are insoluble or partially soluble in aqueous media.[24] Other inherent benefits are that polar interferents are poorly soluble and that the absence of water may facilitate the fabrication and enhance the longevity of sensors.[25]

This exciting field of research continued to grow apace in the study of non-aqueous enzymology.[24–30] Although the observation that enzymes retain their catalytic activity in aprotic media was made in 1913,[31] there have until recently been few reports exploiting this finding. This is surprising, as workers have demonstrated accelerated electron kinetics between the active centre of enzymes and the electrode surface in the organic phase.[29] As most biocatalysts are encapsulated in a carbohydrate sheath, electron 'tunnelling' to the active site is tortuous.[32] It is postulated that organic solvents disrupt these polysaccharide moieties, improving access to the catalytic site.[33]

It is critical, however, that an appropriate solvent is used to maintain the integrity of the hydrated shell, which also engulfs enzymes and is fundamental to catalytic activity. The longevity of this essential water layer is dependent on the solvent system used. The selection of an appropriate solvent is based on the logarithmic partition coefficient (log P) of the test medium in an octanol–water two-phase system.[34] Solvents with log $P < 4$ are generally not suitable as they induce distortion of the enzyme–water interaction. Solvents with a log $P > 4$ are normally biocompatible, that is, their active site configuration is retained. There are some anomalies to this model but, despite these, this remains the best current guide to predicting biocatalytic activity in a given solvent system.

It transpires that hydrophobic water-immiscible solvents best suit these applications, and there are many to choose from. In contrast, water-miscible solvents tend to desorb the critical water layer, although this may be overcome by adding small amounts of water to satisfy the solvent's 'thirst'. Sakurai *et al.*[35] have shown that some enzymic reactions are characterized by a release of enzyme-bound water accompanying substrate complexation. Hydrophilic solvents are thought to render these transfer processes more energetically favourable, by enhancing water transfer from the prosthetic group to the solvent medium concurrently with enzyme–substrate binding.

Carbon-based Electrochemical Sensors

Carbon electrodes may be modified in a number of ways to improve their response characteristics to permit determinations in complex fluids, such as biological fluids. There are four principle enhancement techniques for voltammetric and amperometric electrodes, namely selective preconcentration, permselectivity, selective recognition and electrocatalysis.[36–40] The merits of these modification procedures and a selection of their applications are described in the following section.

Selective Preconcentration

The accumulation of electroactive analytes into or onto an electrode through partitioning, ion-exchange, complexation or simple adsorption processes has been the subject of several studies.[41–46] Selective preconcentration may be induced *via* the deliberate addition of modifying agents such as organic acids,[43] magnesium silicates,[44] zeolites[45] and organisms, *e.g.*, mosses.[46] Alternatively, accumulation may occur as a serendipitous consequence of electrode design, *e.g.*, the extractive properties of the mulling liquids used in carbon paste electrodes (CPEs).[43] In any event, these techniques allow sensitive determinations typically at the sub-micromolar level, *e.g.*, Ag^{I}, Cu^{II},[41] Au^{III}[42] and the anti-tumour drug daunorubicin.[43] Here, CPEs provide a suitable substrate for the surface adsorption of the four-ring hydronaphthacene nucleus of daunorubicin. Optimum conditions for analyses comprised an accumulation time (T_{acc}) of 2 min, an accumulation potential (E_{acc}) of −0.3 V and an acetate buffer adjusted to pH 4.4. The cathodic peak at −0.6 V, ascribed to the electroreduction of the quinone group to the hydroquinone moiety, was used for analytical purposes. The interfacial preconcentration and its subsequent measurement following medium exchange afforded a rapid and sensitive [limit of detection (LOD) approximately 10^{-8} mol dm^{-3}] method for determining this anticancer agent in urine-spiked samples.

Silver- and linuron-sensitive devices have also been developed based on the respective accumulating propensities of zeolite- and sepiolite-containing CPEs.[44,45] Zeolite-modified electrodes possess cation selectivities, whereas clay-containing CPEs possess adsorptive capacities towards certain organic compounds. The surface-attached silver or linuron was stripped into solution using the differential-pulse (DP) waveform and the resulting reduction peak at +0.22 and oxidation peak at +1.2 V were used for quantitative purposes.

A variety of prokaryotic and eukaryotic organisms are also known to accumulate metals selectively and the analytical utility of this behaviour has been investigated. Moss-modified CPEs provide a good example of this occurrence. Ramos *et al.*[46] used a CPE containing a *Sphagnum* species to preconcentrate Pb^{2+} selectively into the graphite matrix. DPV was used to strip the divalent cation into solution and the reduction peak at −0.65 V was used for lead determinations in natural and fresh waters.

Permselectivity

Modification of electrode surfaces with permselective membranes has proved to be an important consideration when designing biosensors. These membranes are polymeric in nature and may be deposited or laid down over electrodes, creating mesh-type, cross-linked or continuous-phase interfaces. They are used for a number of reasons, including restricting substrate access to the active site of enzymes, isolating the electrode from potential interferences and preventing surface fouling by macromolecules. The most commonly used are permselective films which function as anti-interference barriers on the basis of size- or charge-exclusion phenomenon. In principle, such physical modification prevents unwanted species interacting with the electrode while retaining its heterogeneous electron-transfer characteristics. Polyelectrolyte coatings, in contrast, selectively exclude certain compounds on the basis of charge. Perfluorosulfonate ionomers, such as Nafion, are typical examples. Nafion is a strongly acidic cation-exchange polymer and hence has a tendency to repel anionic species whilst allowing the passage of cations (in particular divalent hydrophobic cations) to the electrode surface. Consequently, the literature is replete with reports detailing its usefulness as an anti-interference barrier in electroanalysis.

Nitric oxide (NO) is an important bioregulatory molecule responsible for endothelium-derived relaxing factor (EDRF).[47] EDRF abnormalities have been incriminated in conditions such as atherosclerosis. At present, the technology does not exist to distinguish between NO and nitrite, so the release, distribution and reactivity of endogeneous NO cannot be assessed. A microsensor has been constructed from carbon fibres and chemically modified with a p-type semiconducting polymeric porphyrin and Nafion.[48] Operating in the amperometric mode, a linear response up to 300 μmol dm^{-3} and a detection limit of 10 nmol dm^{-3} were achieved. The principle of the assay is that Nafion is highly permeable to NO, whereas the unwanted diffusion of anions such as NO_2^- is prevented, so selectivity is attained on the basis of charge repulsion.

On a similar theme, miniaturized Nafion-based glucose sensors have been fabricated for *in vitro* and *in vivo* evaluation of sugar metabolism in dogs.[49] Again, potential interferents, such as ascorbic and uric acids, are negatively charged at physiological pHs and thus electrostatically repelled from the electrode surface.

Nafion has also been modified with sequestering agents to improve the response characteristics of electrodes. An elegant technique developed by Gao *et al.*[50] exploits the selective cobalt–2,2-bipyridyl-accumulating ability of a Nafion membrane. The water-soluble modifier 2,2-bipyridyl selectively binds to Co^{2+}, forming a cationic complex, which preferentially traverses the Nafion film. DPV yielded an anodic wave at +0.1 V, which was used for quantification.

The juxtaposition of size-exclusion membrane on electrodes has been the centre of much effort in the quest for enhanced selectivity. Cellulose acetate (CA) has been studied extensively for this purpose and has been shown to be one of the most promising permselective membranes.[51–54] The application of such a membranes formed the basis of the Yellow Springs Instrument sensor, which was the first commercial biosensor for the measurement of glucose in blood.[51] Selectivity was attained through a CA–glucose oxidase (GOD)-active membrane placed over a platinum H_2O_2-sensing anode, only the small H_2O_2 molecules liberated *via* the specific enzyme-substrate reaction can reach the electrode surface and elicit a signal. This configuration is widely regarded as being the reference method for the selective determination of glucose.

Extending this theme, Colton *et al.*[52] have investigated the possibility of altering the retardation profiles of a variety of cellulosic membranes. They examined the true diffusive permeabilities of commercial, modified commercial and laboratory-cast cellulose-based membranes using 15 solutes as probe species. The distinct pattern of solute transport through the films led them to believe that they could be adopted for electrochemical studies, *i.e.*, to tailor the response of the electrode to a particular analyte of interest.

More recently, Amine *et al.*[53] have described the casting of CA membranes directly over a GOD-harbouring platinum anode. The H_2O_2 liberated from the immobilized GOD layer was detected amperometrically at +1.1 V *versus* SCE. In the absence of the CA membrane, large contributory currents were obtained for ascorbic and uric acids and the dynamic range of the sensor was limited. A CA membrane deposited over the sensor assembly served to remove positive interference from the organic acids and to extend the linear range of the sensor. The exclusion of ascorbate at the sensory interface also prevented the vitamin from scavenging dioxygen, thus sustaining biocatalytic capability.

Concurrent with the shift in the diagnostic market's emphasis from 'non-distributed' to 'distributed' or 'decentralized' clinical testing, disposable sensors are becoming the centre of much attention. Screen-printing methods appear to be the most economical and reliable means of producing 'single-shot' devices and those based on carbon as an electrode substrate are generally considered to be the most successful.[54–60] To date, printed sensor strips have been developed for many metabolites and drugs including paracetamol,[54] cholesterol,[55] glucose[56] and salicylic acid.[57] In particular, such an approach has facilitated the translation of biosensor technology to near-patient glucose testing. The device, invented by MediSense, is known as the ExacTech and is the first commercially available hand-held instrument that can convert chemical information, *i.e.*, the concentration of glucose, into a digital readout.[56] A carbon-based working electrode, containing GOD and a ferrocene derivative, is printed alongside an Ag–AgCl pseudo-reference electrode. The ferrocene molecules serve to mediate the flow of electrons from glucose, to the enzyme's active site, to the electrode. The whole cascade is initiated by the application of a drop of blood in the latest instruments and is completed in around 20 s.

Our group have used screen-printing technology to fabricate base sensors on a large-scale basis, the dimensions and configuration of which are depicted in Fig. 1. The square-ended working area (A) is subject to chemical and biological tailoring while the connecting strip (B) provides a means of linking the devices to a potentiostat. An insulating layer (C) enables the user to define the geometry of the working area and prevents solution 'creeping'.

The method of creating CA membranes in close association with the base transducer has been applied to screen-printed carbon electrodes (SPCEs) manufactured in our laboratory. Indeed, the combination of screen-printing and permselective technologies by our group has led to the development of a disposable, solid-phase sensor for the determination of paracetamol.[54] SPCEs were drop-coated with a CA solution of the correct composition to allow the specific measurement of paracetamol whilst screening out interference from a wide range of physiological biomolecules. Rapid ($t_{95} \leqslant 60$ s) and reproducible ($s_r = 6\%$, $n = 3$) responses were obtained over a wide functional range. Good correlation ($r = 0.995$) was obtained with a hospital enzyme–colorimetric assay kit purchased from Cambridge Life Sciences (CLS). Particularly propitious were the storage properties of the surface-modified strips as no biological recognition components were included in the sensor design. These screen-printed sensors provided a novel format for the rapid detection of this drug that would be clearly advantageous in a hospital emergency room.

We have used CA membranes in the fabrication of a screen-printed biosensor for cholesterol.[55] The membrane was used in this instance to entrap physically surface-adsorbed cholesterol oxidase at the base transducer. The polymeric matrix did not appear to induce any conformational changes in the enzyme's structure and, consequently, the biosensor's analytical efficiency was maintained.

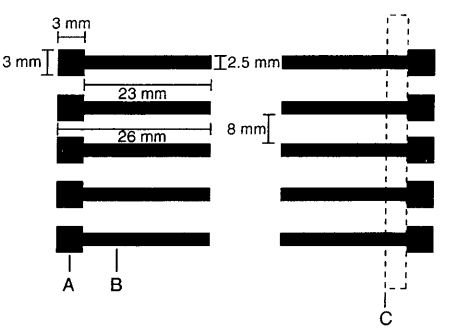

Fig. 1 Diagrammatic representation of the electrode arrangement following the screen-printing process. The carbon ink is forced through a stainless-steel screen, leaving the desired ink pattern deposited on an inert, PVC support. Points to note are the working area (A), connecting strip (B) and insulating layer (C).

Sternberg *et al.*[61] have introduced another dimension to the applications of CA membranes. They used CA as an immobilization support for GOD owing to its facile casting and permselective properties and biocompatability for an *in vivo* glucose sensor. The stability of CA over collagen membranes at 37 °C is a particularly attractive feature for the development of implantable sensors. Ordinarily there is a low accessibility of OH groups on CA, so bovine serium albumin (BSA) was used to increase the number of potential enzyme-linking sites. GOD was activated with *p*-benzoquinone and subsequently coupled to the activated CA–BSA membrane. The immobilization procedure was fairly reproducible and produced thin (5–20 μm) enzyme membranes exhibiting high surface activities (1–3 U cm^{-2}) that were stable over 1–3 months.

This approach has been simplified in our work directed towards the development of an amperometric sensor for uric acid.[62] The use of coupling agents (see above) has been precluded by the exploitation of simple, surface-adsorption processes. Uricase has been attached to an H_2O_2-selective, solvent-cast CA membrane and mounted over a cobalt phthalocyanine (CoPC) SPCE. The electrode-permselective membrane ensemble is depicted in Fig. 2. The sensor's dependable operation was pivotal on detecting the H_2O_2, enzymically generated from uric acid by the specific action of uricase, at a CoPC SPCE. H_2O_2, being a small solute, readily traverses a CA membrane, whereas larger molecules, including uric acid, are prevented from participating in the electrode reactions.

Films may also be deposited using electropolymerization.[63,64] This technique permits 'all-chemical' *in situ* membrane synthesis, and is therefore applicable to coating complex surfaces that are also in close proximity with one another. These criteria are essential for the development of miniaturized and multi-analyte sensors. The membranes are generally 'grown' from the oxidation of monomers (*e.g.*, diaminobenzene and pyrrole) and may be insoluble, conducting or insulating in nature. Such modification shows tremendous promise for the preparation of enzyme electrodes, as the catalyst may be simply entrapped in the deposited membrane. In particular, immobilization of enzymes within conducting polymers formed during the oxidation of the monomer is especially advantageous, facilitating the control of enzyme deposition and its spatial distribution, whilst maintaining low instrumentation costs. Additionally, electropolymerized films have been produced with permselective and anti-fouling properties, while minimizing the problems associated with long diffusion pathways.

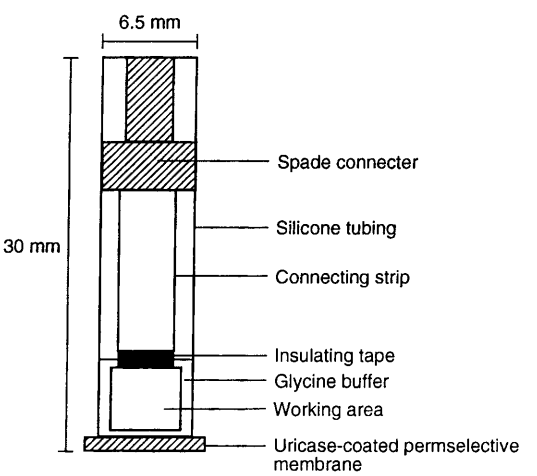

Fig. 2 Diagrammatic representation of the electrode–permselective membrane configuration used to isolate the electrode from potential interferents.

Heider *et al.*[63] have reported on the permselective properties of a poly(1,2-diaminobenzene) (DAB) electropolymerized insulating film. A reticulous vitreous carbon electrode was electrochemically platinized to catalyse the oxidation of H_2O_2, liberated from GOD entrapped in a DAB film. The DAB polymer coating provided a meshwork to immobilize the GOD, improving the enzyme's thermal stability, whilst functioning as a barrier to physiological interferents. When subjected to FI, no fouling was observed for 60 repetitive injections of serum.

GOD has also been immobilized on a GCE in an electropolymerized conducting polypyrrole film with a polymetallophthalocyanine (cobalt tetraaminophthalocyanine, CoTAPC) redox mediator.[64] In essence, GOD-generated H_2O_2 chemically reduces CoTAPC, which is then electrochemically re-oxidized at the GCE, permitting the application of a lower operating potential.

Selective Recognition

Enzymes

A prerequisite of analyses in biological fluids is a high degree of selectivity. This mandate may be achieved by modifying electrodes with molecular recognition elements, such as enzymes, antibodies, protein receptors or nucleic acids. (There is extensive published work on the use of enzymes and electrocatalysts in bioanalysis; as these applications are particularly germane to the authors' studies, some novel and exciting examples are expanded upon later in the paper.)

Enzyme electrodes have become a prominent area of research as a result of the pioneering work of Clark and Lyons[65] in 1962. Since the inception of this first-generation biosensor, a myriad of configurations have ensured and these generally confirm to the same basic principles allowing determinations by a number of routes:

(*a*) The current may be monitored following the enzymic degradation of the analyte, *e.g.*, Wring *et al.*[66] used *tert*-butyl hydroperoxide for the selective removal of glutathione. This technique allowed quantifications in suitably prepared biological samples.

(*b*) The enzymic liberation of a product (invariably H_2O_2)[67,68] or the disappearance of an electron acceptor, such as O_2,[69] may be followed.

(*c*) By converting an ordinarily electroinactive substance into an electrochemically detectable species, a new range of analytes may be investigated. Frew and Green[70] used salicylate hydroxylase to convert salicylate into catechol, which was electrooxidized at the expense of oxygen and NAD(P)H.

(*d*) The enzymic elimination of potentially interfering species present in biological matrices enhances the selectivity of the method, *e.g.*, ascorbic acid may be removed using L-ascorbic acid oxidase.[71,72]

Antibodies

Antibodies may be labelled with enzymes and the rate of product formation or substrate loss determined.[73] Alternatively, an antigen may be labelled with a group that renders it electroactive with the resulting complex undergoing redox processes in a potential range over which the unlabelled antigen is electroinactive.

A novel biosensor based on a competitive immunoassay and the reversible deactivation of an enzyme has been described. This is the subject of World Patent Application PCT 91/16630, assigned to Optical Systems Development Partners, California, USA.[74] One version of the sensor involves the specific

binding of the target species (x) to an immobilized enzyme (y). A second molecule (z), labelled with an enzyme-reactivating agent (*e.g.*, an apoenzyme prosthetic group), competes with x to bind to y. On addition of a sample containing x, x binds to y and z is thus free to diffuse to the enzyme, which is then reactivated, producing an electrical signal. In samples devoid of x, z binds to y and is unable to diffuse, and cannot replenish enzyme activity. This biosensor obviates the need for the extensive sample manipulation associated with conventional homogeneous and heterogeneous immunoassays.

Protein receptors

The notion that the dynamic, specific and sensitive responses of organisms could be incorporated into biosensor design was first proposed by Rechnitz[75] in 1975. Neurotransmitter proteins (*e.g.*, the nicotinic acetylcholine receptor) have been attached to electrode surfaces to measure certain neurostimulants.[76] There has been limited success owing to the complexity of the structures involved, their labile nature at room temperature and difficulties in extracting significant amounts for biosensor studies. These drawbacks have been largely circumvented by the implementation of entire chemosensing structures. The first 'receptrode' was constructed from the antennule of the blue crab *Callinectes sapidus* by Belli and Rechnitz[77] in 1986. The antennule served as a specific and sensitive recognition element and a biological transducer in an environment optimized by evolution. The prototype sensor was based on nerve signals from olfactory chemoreceptors at the sensory apex of the antennule. This arrangement allowed rapid detection (10^{-3} s) of amino acid solutions below 1.0×10^{-6} mol dm^{-3} and was linearly related to concentration over three orders of magnitude.

Electrocatalysis

Before one can comprehend the fundamentals of electrocatalysis, it is useful to introduce the concept of overpotential, particularly as the elimination of overpotential effects is a near-universal goal in the design and development of electrochemical sensors and biosensors.

For a perfectly reversible redox system, $E_{\frac{1}{2}} = E^{\circ}$. Any deviation from this ideal behaviour will result in a certain overpotential (η), where $\eta = E_{\frac{1}{2}} - E^{\circ}$; there will therefore be, an overpotential ($\eta_{effective}$) for a given voltage, which may arise from a combination of four sources that have been discussed elsewhere.[78] In brief, these consist of mass transport effects ($\eta_{mass\ transfer}$), reaction overpotentials ($\eta_{reaction}$), resistance effects (η_{ohmic}) and activation overpotentials ($\eta_{activation}$). In the studies described in this review, the last form of overpotential predominates as protic electrolytes and comparatively low-resistance working electrodes are used.

Most clinically important compounds evince sluggish and irreversible electron kinetics at traditional electrodes owing to the overpotential phenomena outlined above. Such slow exchange of electrons between the target species and the electrode surface may be accelerated by incorporating redox mediators (electrocatalysts), either by chemisorption or admixing with the electrode constituents, into the base sensor.[78]

Redox mediators are small, electroactive compounds that effectively shuttle electrons between the enzyme and the electrode. Fig. 3(*a*) shows a schematic diagram of the electrocatalytic charge-transfer process for a soluble molecule undergoing chemical oxidation by a mediator that is subsequently re-oxidized at the electrode surface. This mechanism is summarized as a chemical–electrochemical (CE) process; alternatively, the mediator itself may be electrochemically oxidized prior to its reaction with the substance [*e.g.*, ferrocene,[79] Fig. 3(*b*)], which is known as an electrochemical–chemical (EC) process.

For all mediated reactions, the driving force is dependent on the $E^{\circ\prime}$ values of the substrate ($E_S^{\circ\prime}$) and electrocatalyst ($E_M^{\circ\prime}$) and for an EC sequence this is described by the equations:

$$E_M^{\circ\prime}: \text{mediator}_{reduced} \xrightarrow{K_S, M} \text{mediator}_{oxidized} + n_S e^- \quad (1)$$

$$E_S^{\circ\prime}: \text{substrate}_{reduced} \xrightarrow{K_S, S} \text{substrate}_{oxidized} + n_S e^- \quad (2)$$

A common feature of EC and CE processes is the catalytic regeneration mechanisms that allow mediator replenishment, thus affording further interactions with the substrate. The rate constants (K_S) for the mediator and substrate dictate the ease of their respective oxidations and are therefore also important in this context. As most electrocatalysts are used in tandem with enzymes, these will be expanded upon later in the review.

Much of our research has been directed along these lines. The effectiveness of cobalt phthalocyanine (CoPC) as a redox mediator for many compounds has sustained its incorporation into our SPCEs. Much research[80–85] has shown that the central metal exhibits favourable catalytic activity towards various thiol-containing substances. Using this methodology, disposable, amperometric sensors for thiocholine[81,83] and glutathione[80,82] have been produced. Thiocholine sensing is used for the indirect measurement of organophosphorus pesticides (OPs) in the aqueous environment. The detection system is based on the following rationale. Acetylcholineesterase (ACE) catalyses the deacetylation of acetylthiocholine iodide to generate thiocholine stoichiometrically (see Fig. 4), which is monitored amperometrically at a CoPC SPCE. The thiocholine is electrocatalytically oxidized according to the sequence of homogeneous and heterogeneous charge-transfer events displayed in Fig. 5. In brief, the enzymically liberated thiocholine chemically reduces Co^{2+} to Co^{+}, which is electrochemically re-oxidized at +0.1 V (*versus* SCE). This facilitates the determination of the thiol at a substantially lower operating potential, which enhances the response and operational characteristics of the sensor. Organophosphorous pesticides irreversibly phosphorylate the serine residues of ACE's catalytic site, reducing their activity, with a parallel attenuation in the anodic current for the electrocatalytic oxidation of thiocholine. Using the disposable sensors, pesticides such as paraoxon and dichlorvos may be detected at levels down to 10^{-8} mol dm^{-3}.

Carbon-based Biosensors

Biosensor technology is an expanding field in the quest for innovative approaches to bioanalysis.[86–88] A biosensor is an

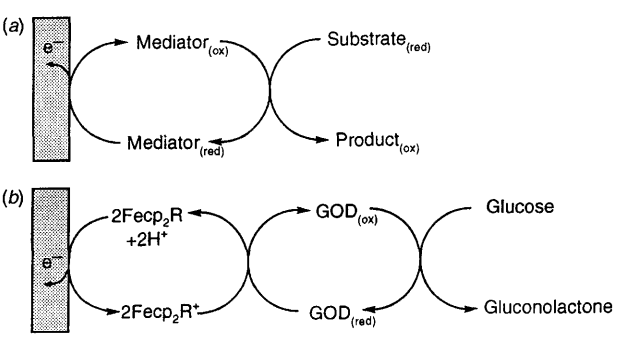

Fig. 3 (*a*) Course of electron-transfer events from a reduced biomolecule to an oxidized mediator. (*b*) Schematic diagram of the ferrocene-mediated oxidation of reduced GOD used for the amperometric determination of glucose.

analytical device based on the union of a biological component and an appropriate physico-chemical transducer (Fig. 6). The biocomponent is immobilized, generally in intimate physical contact or integrated with the transducing element, and may catalyse chemical reactions (purified enzymes, microbes, organelles and tissue slices) or bind specifically to the analyte (antibodies or receptors).[86] The various sources of biological material used in biosensor construction, and how they are categorized, *e.g.*, metabolic, catalytic or affinity, are depicted in Fig. 7.

Although only small amounts of the biorecognition element are generally required, they have to satisfy several analytical requirements. They must exhibit a high degree of specificity, be stable under different temperature, pH, ionic strength and operating conditions, retain their biological activity in the immobilized state and produce no undesirable sample contamination effects.

Biosensors rely on conformational biomolecule changes and/or physical changes in the immobilization medium, *e.g.*, charge, thickness, temperature or optical parameters (colour or fluorescence), induced by analyte–bioligand interactions.[86,88] The most widely applied are those relying on the measurement of current following the application of a fixed voltage, *i.e.*, amperometry. The transduced signal can be viewed as a secondary one, in that it follows the unique recognition event between the bioactive and target species. The utilization of this indirect assay means that chemically similar compounds can be measured by their biospecific reaction with the immobilized biosensing element. Other biosensor attractions lie in their low cost, simple operation and amenability to miniaturization. These characteristics are typically realized by the implementation of enzymes, which allow the determination of an extensive range of analytes. Enzymes that catalyse redox reactions are eminent candidates as electrode-modifying agents, as intrinsic features of their activity are their reusability, a high degree of specificity and electron-transfer events. Consequently, such enzymic reactions can be harnessed to engender a device with highly desirable properties. The plethora of functional groups present on the surface of carbon electrodes[9] and the facile incorporation of modifying agents into the carbon matrix[54–60] predicate the usefulness of this material in this regard.

Without doubt, the major impetus for the advancement in sensor technology stems from health care requirements, which demand instant, in-house tests for a gamut of metabolites and pharmaceutical compounds. Such rapid, on-site assays would be clearly beneficial, providing a biochemical snapshot of the local metabolic state.[59] A recent report by Cave[89] has highlighted the merits of decentralized pathology tests. A pilot study, involving 170 patients over a 6 week period, showed that a practice-based service provided easier and more convenient management of routine assays, immediate results (particularly useful with acute conditions), fewer follow-up consultations, less telephone calls and more convincing patient reassurance. Clearly, if analyses can be performed by untrained staff, in a rapid, economic and reproducible manner, then the routine introduction of biosensors into the hospital and commercial environment cannot be too distant.

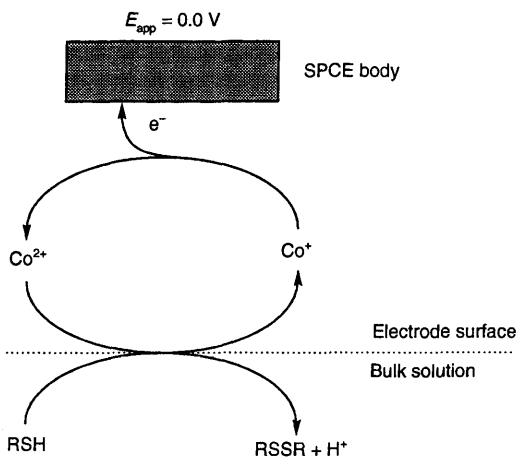

Fig. 4 Sequence of events involved in the electrocatalytic oxidation of thiocholine (RSH) at CoPC SPCEs: the basis of the disposable, amperometric biosensor.

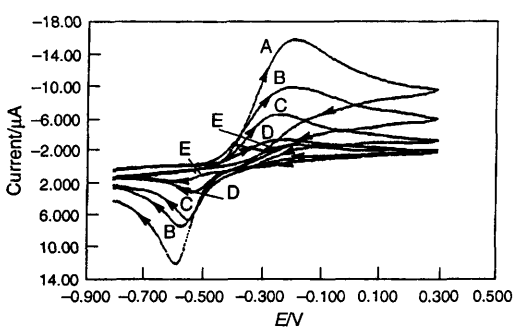

Fig. 5 Cyclic voltammograms recorded at CoPC SPCEs for concentrations: A, 2.9×10^{-3}; B, 1.5×10^{-3}; C, 1.0×10^{-3}; D, 5.2×10^{-4}; and E, 2.3×10^{-3} mol dm^{-3} thiocholine in 0.05 mol dm^{-3} phosphate buffer (pH 8.0).

Enzyme-coupled Mediation

A variety of electron mediators have been used in conjunction with enzymes for biosensor construction. Arguably the most

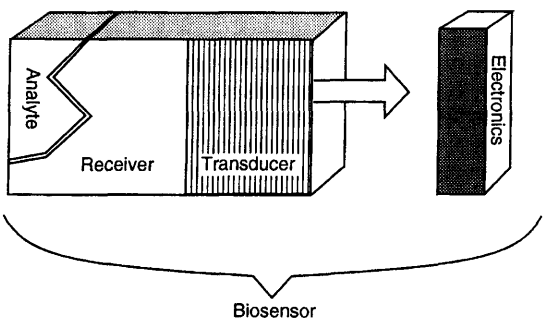

Fig. 6 Schematic diagram of the integral components of a typical biosensor. (Adapted from Scheller *et al.*[86])

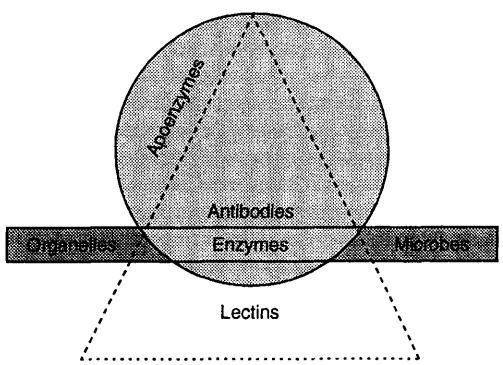

Fig. 7 Sources of biological elements used in biosensor design and their mode of action. The rectangle represents metabolic/enzymic, the circle catalytic and the triangle binding-based sensing systems.

successful biosensors are those based on oxidase or dehydrogenase-mediated reactions and, accordingly, the final section of this review focuses upon such systems.

Oxidases

Cass *et al.*[79] have shown the utility of the ferrocene–ferricinium ion couple as an effective mediator between GOD and a graphite electrode, marking the inception of enzyme-coupled mediation. A graphite foil base electrode was surface modified with 1,1′-dimethylferrocene (DMFc) dissolved in toluene. The sensor was further modified by attaching GOD to the oxidized graphite surface with a carbodiimide covalent linkage. The amperometric enzyme electrode functioned at 160 mV *versus* SCE at pH 7.0 and 25 °C according to the following scheme (where cp_2 represents a cyclopentadienyl ring):

$$\text{glucose}_{(red)} + \text{GOD}_{(ox)} \rightarrow \delta\text{-gluconolactone} + \text{GOD}_{(red)} \quad (3)$$

$$\text{GOD}_{(red)} + 2\text{Fecp}_2\text{R}^+ \rightarrow \text{GOD}_{(ox)} + 2\text{Fecp}_2\text{R} + 2\text{H}^+ \quad (4)$$

$$2\text{Fecp}_2\text{R} \underset{}{\overset{+\,160\text{ mV } \textit{versus}\text{ SCE}}{\rightleftharpoons}} 2\text{Fecp}_2\text{R}^+ + 2\text{e}^- \quad (5)$$

On the whole, the low operating potential (60 mV more positive than $E^{\circ\prime}$ for DMFc) used to reoxidize the reduced form of the mediator obviated the need for a permselective membrane to enhance selectivity, although a response was obtained for physiological levels of ascorbate. The system exhibited minimal O_2 dependence and functioned in a rapid and reproducible manner. Problems associated with the greater solubility of the ferricinium ion induced workers to seek alternative mediators that could be incorporated into electrode designs, forming a more stable arrangement.

Heiduschka and Scheller[90] have created a sensor surface with a high density of binding sites for the attachment of GOD. In brief, the procedure involved coating a GCE with platinum microparticles by a single linear sweep to −3.0 V. A poly(nitrophenol) film was formed by cyclic sweeps between 0.1 and 1.1 V. The resulting NO_2 groups were then electrochemically reduced to amino species *via* two linear sweeps form −0.5 to −1.4 V and subsequently treated with a 2.5% solution of glutaraldehyde. GOD was covalently linked to the abundant aldehyde moieties, producing a biosensor that allowed rapid glucose analyses over a clinically acceptable range.

Tsionsky *et al.*[91] have described a novel class of composite electrodes for biosensing applications made of sol–gel-derived carbon–silica materials, which are robust, porous and have a renewable external surface. As they are modifiable and may be produced in a variety of dimensions and geometric configurations, they are particularly suitable for biosensor construction. Recent studies have shown them to be extremely conductive and electrochemically well behaved. By incorporating GOD into the sol–gel matrix, it was shown that the biocatalytic catalytic activity is still maintained. The biosensor evinced a response to the GOD-generated H_2O_2 that was linearly related to glucose concentrations in excess of 10 mmol dm^{-3}.

Beh *et al.*[92] have also produced stable sensors by modifying the substrate binding material. They used a cellulose acetate to bind ferrocene-doped CPEs and a GOD-active nylon membrane positioned over the CPE to complete the assembly. FI was used to determine glucose *via* the H_2O_2 enzymolysis, amperometrically at 160 mV (*versus* Ag–AgCl). Sensor features of note were their pronounced stability (24 months), minimal susceptibility to ascorbate interference and wide linear range (0.01–70 × 10^{-3} mol dm^{-3}).

Marcinkeviciene and Kulys[93] have developed an amperometric screen-printed biosensor for glucose based on GOD, peroxidase and hexacyanoferrate(II) entrapped in a hydroxyethylcellulose–graphite matrix. Enzymically generated H_2O_2 oxidizes hexacyanoferrate(II) to hexacyanoferrate(III), which is subsequently electrochemically reduced at 0.0 V. The reaction scheme is described by the equations

$$\text{glucose} + \text{O}_2 \xrightarrow{\text{GOD}} \delta\text{-gluconolactone} + \text{H}_2\text{O}_2 \quad (6)$$

$$\text{H}_2\text{O}_2 + 2\text{Fe(CN)}_6^{4-} + 2\text{H}^+ \xrightarrow[\text{peroxidase}]{\text{horseradish}} 2\text{H}_2\text{O} + 2\text{Fe(CN)}_6^{3-} \quad (7)$$

$$2\text{Fe(CN)}_6^{3-} + \text{e}^- \xrightarrow{0.0\text{ V } \textit{versus}\text{ Ag–AgCl}} 2\text{Fe(CN)}_6^{4-} \quad (8)$$

The biosensor's response was linear up to a 25 × 10^{-3} mol dm^{-3} concentration of glucose, yielded 90% of the steady-state cathodic current between 50 and 60 s and was insensitive to ascorbate at 40 × 10^{-6} mol dm^{-3} final concentrations.

Moore *et al.*[94] presented the first report of an electrocatalytic reaction between several flavoprotein oxidases (glucose, lactose and sarcosine oxidases) and the oxidized form of paracetamol. This phenomenon was exploited to permit the chemical amplification of the electrooxidation of the drug with a concomitant improvement in biosensor efficiency. Paracetamol is electrolytically oxidized in a heterogeneous $2e^-$, $2H^+$ step to *N*-acetyl-*p*-aminoquinoneimine (NPAQI), which is fairly labile and hydrolyses to benzoquinoneimine.[95,96] In the presence of a reduced flavin enzyme and its primary substrate, however, NPAQI serves as an alternative electron acceptor to O_2 at a substantially greater rate than it hydrolyses. Paracetamol is thus chemically regenerated and available for a second heterogeneous electrochemical oxidation. If the substrate is in excess it maintains the initial concentration of reduced oxidase and a kinetically pseudo-first-order redox cycle exists between enzyme, mediator and transducer.[97] Electron flow in the catalytic loop is controlled by paracetamol's redox state. A further benefit is that the response increases without an accompanying co-amplification of the background signal.

Substituting various groups on the cyclopentadienyl rings of ferrocene has proved to be an effective route to circumvent mediator leaching.[98–101] One of the earliest examples was reported by Hale *et al.*,[98] who used a ferrocene-modified siloxane polymer to 'anchor' the mediator in a CPE matrix. The flexible structure of the polymer afforded an effective electron acceptor for flavoprotein enzymes. Another strategy for preventing mediator dissolution involves modifying GOD with covalently linked ferrocenyl derivatives.[102]

Recently, Zhao and Luong[103] have advanced a technique for extending the operational life of a biosensor by minimizing mediator leakage. Tetrathiafulvalene (TTF) was dissolved in silicone oil (methylphenyl polysiloxane) and embedded in a graphite disc electrode. The porous nature of the graphite afforded an ideal environment for the successful incorporation of the mediator into the electrode. Next, the electrodes were coated with flavoprotein enzymes, followed by a layer of glutaraldehyde (glut)–bovine serum albumin (BSA). The electrode–enzyme–glut–BSA ensemble was finally covered with a dialysis membrane. The biosensor was used to determine phenylalanine (L-amino acid oxidase), hypoxanthine (hypoxanthine oxidase) and glucose (glucose oxidase), according to which particular flavoenzyme was immobilized on the electrode surface. Selectivity was attained by the ability of TTF to re-oxidize the reduced prosthetic group of each enzyme at +150 mV (*versus* Ag–AgCl). When operated in the amperometric mode, rapid, reproducible and linear responses were obtained for each of the oxidase substrates studied. A typical glucose biosensor was shown to be stable for 2 months when stored at 4 °C, enabling more than 500 reliable assays to be undertaken.

A host of electrocatalysts have also been used in conjunction with oxidases to reduce the overvoltage of H_2O_2 oxidations, (*e.g.*, CoPC,[54,55,104] platinization procedures[105] and metal oxide films[106]) and to render certain electrodes insensitive to variations in partial pressures of dioxygen.[107,108]

We have developed a reagentless, disposable, screen-printed biosensor for uric acid based on this principle.[109] The system represented a further 'fine-tuning' of a previously designed sensor for measuring this clinically significant purine.[57] By producing a CA film in intimate association with the CoPC SPCE surface and subsequently coating it with uricase, the enzymically liberated H_2O_2 was detected amperometrically. The biosensor's response was rapid, reliable and linearly related to urate concentration over a wide dynamic range. A typical amperometric calibration recorded with a single biosensor strip is depicted in Fig. 8. A summary of the homogeneous and heterogeneous electron-transfer events is displayed in Fig. 9. Briefly, uricase liberates H_2O_2 concurrent with the enzyme-catalysed oxidation of uric acid. H_2O_2, being a small solute, readily traverses the CA membrane and chemically reduces Co^{2+} to Co^{+}, which is then replenished at the SPCE surface by electrochemical re-oxidation. We have elucidated the mechanisms involved in the homogeneous and heterogeneous electron-transfer processes by voltammetric and X-ray photoelectron spectroscopic techniques.[110]

Second-generation biosensors possess certain drawbacks, such as mediator-leaching and dependence on oxygen concentration, that may limit their analytical utility. An approach to circumvent such drawbacks involves the synthesis of novel sensory interfaces adept at exchanging electrons directly with the prosthetic groups of enzymes.[11,36,111–115]

This is by no means an easy feat, as through evolution enzymes have been bestowed with protective mechanisms that inhibit indiscriminate electron transfer with surrounding redox macromolecules. Many enzymes are typically enshrouded in a glycoprotein sheath and their catalytic centres

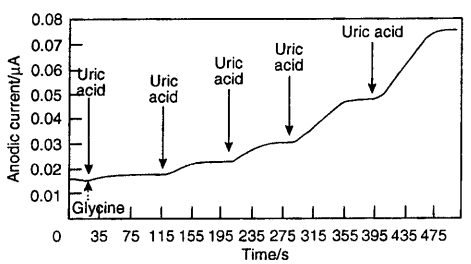

Fig. 8 Amperometric uric acid calibrations performed using a disposable, screen-printed, carbon-based biosensor. The arrows indicate the incremental additions of uric acid over the range 1.3×10^{-6}–4.87×10^{-4} mol dm^{-3}. Applied voltage, +0.4 V; supporting electrolyte, 0.1 mol dm^{-3} glycine buffer (pH 9.2); temperature, 31 °C; and time base, 0.2 mm s^{-1}.

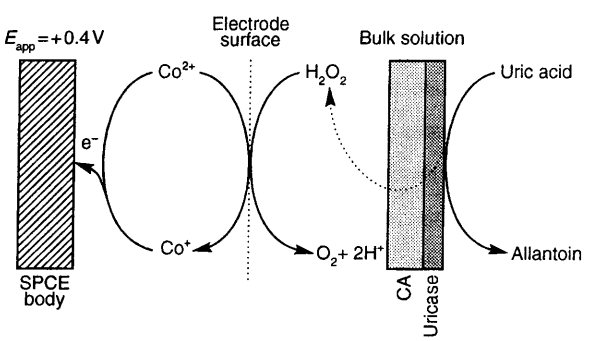

Fig. 9 Sequence of intra- and extra-membrane events involved in the determination of uric acid using the screen-printed biosensor.

are buried deep within the macromolecular structure, both of which prevent electron tunnelling. Workers have attached various ferrocene derivatives, including ferrocenemonocarboxylic acid[111] and ferroceneacetic acid,[112] onto GOD and TTF onto LOD.[113] In all instances, electron 'hot-wiring' was observed in rapidly responding and acceptably stable biosensors.

Maidan and Heller[116] have reported on an exciting expansion of this thesis. They combined an electrically wired glucose-sensing carbon electrode and an interferent-eliminating layer with a highly propitious outcome. GOD was connected to a vitreous carbon electrode *via* an osmium–polyvinylpyridine backbone with part of the pyridine quaternized with ethylamine. H_2O_2 liberated from the glucose–GOD interaction was detected at +0.4 V *versus* SCE (pH 7.2). Contributing currents from the osmium-catalysed reduction of peroxide were averted by using an operating voltage of +0.5 V (thus maintaining the osmium in the non-catalytic trivalent state), for less complex fluids, and an electrically insulated barrier was created over the biolayer, for analyses in biological fluids.

The basis of the super-selective biosensor was that potentially interfering compounds such as paracetamol, ascorbate and urate were removed by an immobilized layer of horseradish peroxidase (HRP) and lactate oxidase (LOX) over the glucose-detecting zone. LOX produced H_2O_2 from lactate, present in the sample, which in the presence of HRP effected the pre-oxidation of interferents according to

$$H_2O_2 + \text{interferent}_{\text{reduced}} \xrightarrow{\text{HRP}} 2H_2O + \text{interferent}_{\text{oxidized}} \quad (9)$$

Peroxidases are classical catalysts for the oxidation of hydrogen-donating species (interferent$_{\text{reduced}}$), thus problem substances, such as ascorbate, are removed of their reducing propensity as they pass on their electrons to HRP. Conversely, glucose and lactate are not oxidized in this way and thus participate in this novel scheme as described above.

Apart from improving electron kinetics, these highly evolved biosensors should prove to be powerful tools in understanding the redox chemistry of enzymes, *e.g.*, permitting the direct electrochemical interrogation of the cytochrome-based, respiratory electron-transport chain. Such third-generation biosensors also have implications in neurochemical studies. A vinyl polymer, containing nitroaromatic groups, has been produced to which dopamine has been covalently attached *via* an amide linkage. This polymer was subsequently coated onto an electrode and the whole assembly was immersed in solution. The nitroaromatic centres are readily reduced, yielding an increased negative charge which effects the electrochemical dissolution of the amide bond, thus releasing the neurotransmitter into solution. Such a translation of electrical information into a highly specific chemical signal resembles the firing of a neurone, *i.e.*, effectively mimics the propagation of a nerve impulse.[36]

Dehydrogenases

Dehydrogenases are a class of oxido-reductase enzymes dependent on the cofactors β-nicotinamide adenine dinucleotide (NAD^+) or β-nicotinamide adenine dinucleotide phosphate ($NADP^+$). Phenoxazines, phenathiazines and quinones are reputedly the most efficient mediators for re-oxidizing the reduced forms of these cofactors. However, a diverse range of alternatives have been studied, including conducting salts[117] and ferrocenes.[118] Ordinarily, analyses based on the NAD^+–NADH-dependent dehydrogenases are hampered by side and abortive reactions[119] and overpotential effects.[120–122] Elegant electrode designs have, however, been developed to solve

these problems, creating novel ways to exploit these enzymes. A typical reaction scheme for a typical dehydrogenase-catalysed process is

$$\text{substrate} + \text{NAD}^+ \underset{\text{dehydrogenase}}{\overset{K_{eq}}{\rightleftharpoons}} \text{product} + \text{NADH} + \text{H}^+ \quad (10)$$

As the formal potential for the NAD$^+$–NADH pair (−560 mV *versus* SCE, pH 7, 25 °C) and the equilibrium constant (K_{eq}) for the enzyme-catalysed reaction are low, the forward chemical reaction is suppressed, causing non-rectilinear calibrations.[121]

A number of strategies have been proposed to address these drawbacks,[123,124] the simplest and arguably the most effective of which employ redox mediators. These bridge the catalytic exchange of electrons between the NAD$^+$–NADH couple and the electrode surface, so the reaction is dictated by the $E^{\circ\prime}$ of the mediator. Other alternatives include trapping an excess amount of NAD$^+$ at the electrode surface,[123] enzyme amplification[124] and electrooxidizing NADH as soon as it is formed.[120]

Dominguez and co-workers[125,126] have devised a reagentless amperometric biosensor for ethanol by co-immobilizing alcohol dehydrogenase (ADH) and NAD$^+$ in a polyaromatic phenathiazine mediator-modified CPE. Toluidine Blue O (TBO) was dissolved in dimethylformide (DMF) and yielded a characteristic $E^{\circ\prime}$ of −285 mV (pH 7.0 *versus* SCE) when adsorbed on graphite particles, too low to evoke a high reaction rate with NADH. By reacting the primary amine functionality in position 3 of TBO with naphthoyl chloride, the $E^{\circ\prime}$ shifted to −165 mV with a concomitant enhancement in the efficacy of NADH mediation. The components were maintained in close physical proximity by dissolving ADH and NAD$^+$ in polyethylenimine (PEI), which was mixed vigorously with the TBO–graphite powder. When dry, paraffin oil was added and mulled until a uniform paste was obtained.

Dominuguez *et al.*[126] postulated that predominately electrostatic interactions between the polycationic PEI and the anionic cofactor, enzyme and graphite residues stabilized the modifying agents, thus preventing their leakage. This configuration explains the improved stability of the biosensor, characterized by a 90% retention of the activity for ethanol over a day's continual usage.

A reagentless, disposable, amperometric screen-printed biosensor for lactic acid has been fabricated using dehydrogenase enzyme technology.[127,128] The device exploits the size-exclusion properties of CA membranes, the selectivity of lactic acid dehydrogenase (LDH) and the electrocatalytic propensity of Meldola's Blue (MB) towards NADH.[128] The hydrodynamic voltammogram in Fig. 10 clearly shows that the oxidation of NADH is more favourable at an SPCE chemically modified with MB than at its unmodified counterpart. Indeed, the MB SPCE permits the oxidation of NADH at 0.0 V, a potential at which electroactive interferents are unlikely to impinge upon analyses. By judicious selection of the CA composition, CA may be used to contain LDH and NAD at the MB-doped SPCE surface. Thus, any leaching of the essential immobilized surface components is minimized. The CA film also serves to eliminate macromolecular electrode fouling species. Although the passage of interfering biomolecules such as uric acid and paracetamol (acetaminophen) is merely retarded in the CA matrix, as they are not electrooxidizable at 0.0 V (*versus* SCE), they do not contribute to the current obtained. The principle of the lactate biosensor is shown in Fig. 11.

The longevity of the mediator is an essential consideration in biosensor design, as it is mediator leaching that generally limits their reusability and operational lifetime. Athey *et al.*[129] have recently described an electrode modification technique that not only allows analyses to be carried out using low working potentials, but also produces robust and highly stable sensors. Platinized activated carbon electrodes (PACEs) are fabricated by adsorbing colloidal platinum on carbon of high surface area at a concentration of 10% m/m. The platinized carbon is then mixed with an equal mass of colloidal poly(tetrafluoroethylene) (PTFE) and bonded to conductive carbon paper by sintering at 330 °C. The PACEs allowed reproducible and precise oxidations of NADH at +150 mV (*versus* Ag–AgCl), with a typical linear range from 2×10^{-6} to 10×10^{-3} mol dm^{-3}. The system was based on a homogeneous amperometric immunoassay and applied to the determination of the bronchiodilator theophylline in whole blood.

Persson *et al.*[130] have briefly reviewed the various formats available for the measurement of dehydrogenase substrates. The sensors were operated in the region of 0 mV *versus* SCE, and enabled the typically unfavourable equilibrium of a dehydrogenase-catalysed reaction to bias the product side. These sensors are reagentless in that NAD$^+$ need not be added to the sample. Minimization of component loss and enhanced interferent rejection were provided by poly(ester sulfonic acid) cation-exchange membranes (Eastman AQ 29D).[130]

A more complicated strategy, devised by Vreeke *et al.*,[131] involves the electrical connection of HRP redox sites to

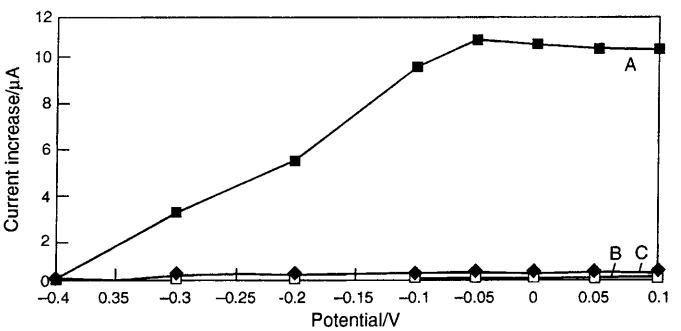

Fig. 10 Hydrodynamic voltammograms recorded using SPCEs. A, Catalytic oxidation of 0.64×10^{-3} mol dm^{-3} NADH at a 5% MB-containing sensor, B and C, response in plain 0.05 mol dm^{-3} phosphate buffer (pH 7.0) at MB-doped and unmodified electrodes, respectively.

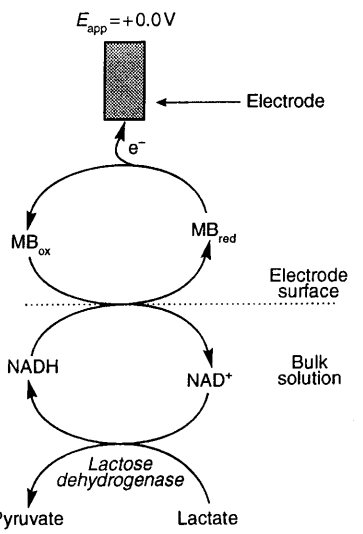

Fig. 11 Principle of lactate measurements at a chemically modified, screen-printed biosensor.

carbon electrodes through a three-dimensional electron relaying network. Briefly, NAD(P)H transfers $2e^-$ and $2H^+$ to a dissolved quinoid, which subsequently transfers $2e^-$ and $2H^+$ to molecular O_2. This reduction quantitatively produces H_2O_2 and the oxidized form of the quinoid. The H_2O_2 then oxidizes HRP, which serves as an electron acceptor for an Os^{2+} polymer, converting it to Os^{3+}, which is then electrochemically reduced. Given the low applied potential required to drive the reaction, this system should be highly selective. Such novel electrical wiring provides a versatile approach to bioanalysis and highlights the multifarious aspects of sensory interface modification.

Conclusions

The objective of this review has been to illustrate the benefits of sensing with carbon electrodes. We have also set out to provide an insight into the diverse modification techniques available for tailoring the performance of sensors for analyses in more complex matrices. At present there are no universal modification strategies, although this is gradually changing with ever-increasingly sophisticated surface-altering methodologies. Consequently, the future holds promise for 'generic' sensors, for example those based on oxidase systems, detecting H_2O_2, or on dehydrogenase enzymes, monitoring the reduced cofactor. These should permit considerable advances

Table 1 Beneficial instant assays in patient diagnosis

Analyte	Alternative methods	Clinical disorder	Notes	Ref.
Glucose	Aldehyde group of β-D-glucose is oxidized by GOD to give gluconic acid and H_2O_2, the latter compound is then broken down to H_2O and O_2 by HRP and if an oxygen acceptor is present, is converted into a coloured species. Trinder's reagents (phosphotungstate containing phenol and *p*-aminophenazone) are considered to be the most effective	Diabetes mellitus	Ascorbate and drug interference	132
	Spectrofluorimetric and chemiluminescence methods also available to detect GOD-liberated H_2O_2		Very sensitive, continuous-flow measurement over a wide dynamic range	
	YSI is the reference method for hospital glucose determinations		Paracetamol interference All unsuitable for bedside monitoring	51
Cholesterol	Old methods based on the ability of cholesterol to be converted into coloured substances in strong acid solvents possessing dehydrolysing, oxidizing and sulfonating properties. Liebermann–Burchard and Salkowski reactions produce green and red compounds, respectively	Myocardial infarction Artheriosclerosis Hypertension	Lengthy analyses Specificity problems	133
	Enzymic assays developed by Richmond used *Nocardia erythropolis* cholesterol oxidase to produce cholest-4-en-3-one and H_2O_2. The ketone was extracted in 2-propanol and read at 240 nm spectrophotometrically. Alternatively, H_2O_2 may be detected using Trinder's reagents		Enhanced selectivity Only measures free cholesterol	134
	Hydrolysis of ester cholesterol by ethanolic KOH or cholesterol esterase permits the determination of total cholesterol. Again, H_2O_2 is the target species		Total cholesterol but still selectivity problems	135
Lactic acid	NAD^+-dependent LDH converts lactate into pyruvate under alkaline conditions and the NADH formed is detected spectrophotometrically at 340 nm	Hypoxia	Simple and specific but needs cumbersome instrumentation; incongruous in an emergency room	133, 136, 137
Paracetamol	Weiner has reviewed methods for paracetamol detection. Spectrophotometric and colorometric techniques detect dye or nitrous acid generated *p*-aminophenol	Hepatotoxicity	An antidote has to be administered within 12 h to be effective, so speed is a mandate. Thus salicylate interference and pre-treatment steps compromise the analytical utility of these approaches	138
	GLC and HPLC methods with cation- or anion-exchange or reversed-phase chromatography have also been reported			
	Immunological assays		Address selectivity difficulties but require long incubation times	139, 140
	Price *et al.* have evaluated a rapid enzymic kit (available from CLS). Aryl acylamidase specifically cleaves the amide bond of acylated aromatic amines yielding acetate and *p*-aminophenol, which reacts with *o*-cresol in the presence of ammonia to give a blue indophenol dye		Shortens analysis times to 30 min	141
Uric acid	Exploit reducing properties or adsorption at 293 nm separation steps serve to remove potential interferents: (*i*) precipitation as the copper, silver or magnesium salts, (*ii*) ion-exchange chromatography and (*iii*) tests on a protein-free filtrate	Gouty arthritis	Poor specificity Complicated and long analyses	142 143–145

continued—

Table 1—*continued*

Analyte	Alternative methods	Clinical disorder	Notes	Ref.
Creatinine	Typically based on the Jaffé reaction, whereby a red compound is formed in alkaline picrate solutions. The chromogen is detected spectrophotometrically at 490 nm	Renal dysfunction	Severe pH and temperature dependence Up to 20% of non-creatinine chromogens in blood and 5% in urine are also detected	133
	A *Pseudomonas*-derived creatinine amidohydrolase is used in conjunction with the Jaffé reaction in a differential spectrophotometric mode		Improved selectivity	146, 147
	Reversed-phase HPLC with 'on-linc' Jaffé reaction on the column effluent has also been reported		Good performance but inconvenient for bedside tests	
	Lim *et al*. attempted to produce a definitive creatinine assay. HPLC was followed by conversion of *o*-triflu oroacetylcreatinine and its quantification by GC–MS		Unsuitable for near-patient tests	148
Urea	Urease-generated ammonia is detected colorimetrically by the formation of a blue indophenol compound in hypochlorite and phenol solutions (Berthelot reaction)	Renal failure	Obeys Beer's law but susceptible to atmospheric ammonia	149
	Urease coated on a poly(propylene) or Teflon membrane covering an ammonium potentiometric electrode has also been used		Easily contaminated and too expensive to be produced in the requisite single-shot format for clinical testing	150
	Azostix (Ames) uses a urease-and bromothymol-impregnated paper strip for the analysis of urea. A drop of blood is applied to the test area (protected from macromolecular fouling by a semi-permeable membrane), urea diffuses into the urease domain where it is converted into ammonia producing a concomitant colour change. Yellow, green or green–blue colours develop depending on the concentration of the analyte		Suitable for decentralized analysis but requires some pre-treatment (fluoro and ammonium salts need to be removed)	133
Salicylic acid	Trinder's colorimetric test is the most popular in the UK. Fe^{3+} ions complex with the phenolic group of the drug, producing a purple compound	Hepatotoxicity	Poor sensitivity and cross-reactivity	151
	Chromatographic and spectrofluorimetric detection systems are also available		Do not conform to requirements: rapid, accurate portable testing regimes	152–155
3-Hydroxy-butyrate	Rothera's test, based on the production of a red compound with salicylaldehyde	Diabetes mellitus causes increased fat metabolism, thus ketones accumulate in the blood	Semi-quantitative and unspecific. Ketone ratios are 3-hydroxybutyrate 78%, acetoacetate 20%, acetone 2%. Permits differentiation between diabetic and non-diabetic coma. Not amenable to near-patient testing	133 156 157, 158
	Gallows and Watkins devised a spectrofluorimetric enzymic assay using D-3-hydroxydehydrogenase to catalyse the interconversion of the two acids. Hydrazine was included to remove acetoacetate as it was formed. UV/VIS spectrophotometry was used to measure the decrease in absorbance at 340 nm due to the accompanying oxidation of NADH to NAD^+			
Oxalic acid	Titrimetric, olorimetric, chromatographic, isotachophoretic and enzymic methods have been described. Those using oxalate decarboxylase or oxalate oxidase are the most successful. The two systems are geared to detect enzymically generated formate (for the decarboxylase) or H_2O_2 (for the oxidase). The latter, when linked to a peroxidase–chromophore reaction, is potentially more sensitive. Ascorbate removal by acidifying the sample or treating with L-ascorbate oxidase is advantageous	Increased colonic oxalate absorption is associated with various gastrointestinal disorders, particularly fat malabsorption producing hyperoxaluria and thus urinary calculi	Ascorbate competitively inhibits oxalate enzymes and is also converted into oxalate in alkaline urine Relatively long analyses, which are also hampered by other excipient species, such as nitrate	56, 159 160
Aspartate amino transferase (AST)	2-Oxoglutarate is converted by AST into oxaloacetate, which is then converted into malate by malate dehydrogenase at the expense of NADH. The decrease in absorbance is monitored spectrophotometrically at 340 nm	AST increases 3–4 h after myocardial infarction and is also an indicator of parenchymal liver damage and muscular dystrophy	AST transfers an amino group from an α-amino acid to an α-oxo acid using pyridoxal-5′-phosphate as a cofactor. Although plentiful in most cells, it is present in low levels in red cells. Thus, AST's increased activity is related to an increased level of cofactor, which in turn is related to cellular damage. A rapid, disposable detection system is required	133 161

continued—

Table 1—*continued*

Analyte	Alternative methods	Clinical disorder	Notes	Ref.
Creatinine kinase (CK)	CK catalyses the phosphorylation of creatinine by adenosine triphosphate (ATP) in muscle cells and brain tissues. The rate of formation of ATP is measured using the increase in absorbance at 340 nm according to: $\text{creatine phosphate} + \text{ADP} \xrightarrow{\text{CK}} \text{ATP} + \text{creatine}$ $\text{ATP} + \text{glucose} \xrightarrow{\text{hexokinase}} \text{ADP} + \text{glucose-6-phosphate (G6P)}$ $\text{G6P} + \text{NADP}^+ \xrightarrow{\text{G6P dehydrogenase}} \text{6-phospho-gluconate} + \text{NADP}^+ + \text{H}^+$	Acute myocardial infarction and skeletal disorders	Single-shot and speedy assay suitable for an emergency room. Would be particularly useful when ECG findings are equivocal	133
	A peroxidase–Trinder coupled method is also available		Inconvenient in an emergency	162
α-Amylase	Amylase and glucosidase produce *p*-nitrophenol, which is detected spectrophotometrically at 405 nm.	Acute and chronic pancreatitis	Slow and susceptible to interference	59
	Amylase effects the disintegration of starch to smaller, soluble oligosaccharides and dextrins with much reduced light-scattering properties		Semiquantitative	133

Table 2 Predicted and emerging instant diagnostic tests

Parameter	Comments	Requirement	Ref.
pH	During open-heart surgery and transplantation, the heart is stopped and frequently cooled to 7 °C with an attendant drop in pH. If this falls too great it is difficult to revive the cardiac system	A miniature, biocompatible disposable sensor for remote sensing of the myocardium. Conventional pH probes are prohibitively expensive for disposable purposes, fragile and susceptible to macromolecular fouling (*e.g.*, proteinaceous materials such as with fibrin)	163
Leutinizing hormone (LH)	Women with difficulties in conceiving need to monitor their reproductive cycles, more specifically their ovulation times. Ovulation is signalled by a surge in LH, which can be detected in the blood or urine	Disposable test strip that is not prone to cross-reactions	164
High- and low-density lipoproteins (HDL, LDL)	Provide a clearer and more readily interpretable means of assessing the state of lipid metabolism. More accurate measure of susceptibility to heart and related diseases	Convenient and speedy test that may be undertaken at the practitioner's desk	165
Nitrogen oxide (NO)	A vital signalling compound involved in a myriad of bioregulatory processes. Abnormal levels of NO have been linked to various diseases, including artherosclerosis	Ultramicroelectrode for *in situ* monitoring of NO. Also needs to be interference free and to function over a wide dynamic range	47, 48
Thyroxine (T4)	Elucidation of the biosynthesis, secretion and transport of T4 is important in control of the hypothalamic–pituitary–thyroid axis. The introduction of specific antibodies to T4 in the early 1970s improved measurement regimes markedly, but there is room for improvement	Typical methods are still slow, require long incubation periods and complicated multi-step procedures. A solid-phase, one-shot strip for the rapid determination of T4 would be eminently desirable	166, 177
Thyroid-stimulating hormone (TSH)	Production of thyroid hormones is controlled mainly by the glycoprotein hormone TSH, which is secreted by thyrotrophs located in the anterior pituitary gland. Thus measurement of TSH confirms congenital hypothyroidism after an initial screening test detects low T4 levels	Simple, rapid and reliable single-shot assay	163
Glycosylated 'fast-fraction' haemoglobin	Normal adults have 90% of haemoglobin as haemoglobin A (HbA). Glucose combines reversibly with the α-amino groups of HbA's valine residues at the *N*-terminus of the β-globin chains to form an aldimine (Schiff base) intermediate. Such glycosylation proceeds slowly during the 120 d lifespan of an erythrocyte and thus is a measure of blood glucose concentration over the preceding weeks. HbA evinces altered electrophoretic mobility and ion-exchange chromatography uniquely due to the glycosylation at the *N*-terminus of the β-globin chain	Disposable biosensor for more effective diabetic control	166, 168

Table 3 Possible and current applications of carbon-based sensors in clinical analysis

Parameter	Principle	Benefits	Notes	Ref.
Glucose	Carbon paste–GOD electrodes containing rhodinized microparticles to catalyse the reduction of GOD-evolved H_2O_2 from glucose which is measured amperometrically at −0.1 V (*versus* Ag–AgCl)	Super-selective, owing to low working voltage, facile and reliable fabrication (s_r = 5%), with rapid (t_{95} = 15 s) and linear responses	Unsuitable for mass production; needs to be translated to a disposable device, *i.e.*, screen-printing or micromachining methods	169
	First report of a CoPC SPCE and Ag–AgCl two-electrode disposable strip. The metallophthalocyanine-containing working electrode is tailored to the amperometric detection of glucose (at +0.4 V) through a CA–GOD bilayer produced contiguously over the sensor assembly	Single-use, generic biosensor that can be fabricated at low cost and on a large-scale basis	Response characteristics require further tuning	170
	MediSense's ExacTech sensor based on a carbon ink modified with GOD and a ferrocene derivative. Fe^{3+} ions mediate the flow of electrons exchanged during the substrate–enzyme interaction. The device is operated in the chronamperometric mode at + 0.3 V (*versus* Ag–AgCl)	Throw-away strips incorporated into a hand-held instrument. Excellent analytical performance; results are obtained in 30 s	Not generic, geared for a narrow analyte range (substrates for flavoenzymes). Fe^{2+} ions are more soluble and thus tend to leach from the electrode surface	56*
	The Japanese company Kyoto Dai-ichi Kagaku (KDK) have also produced a disposable device for diabetalogical purposes. In this instance hexacyanoferrate(III) ions serve to regenerate GOD's reduced prosthetic group	Suitable for in-house pathology testing	Methodology is not translatable to all of the required systems	171*
Lactic acid	TTF and LOX are absorbed on a disposable carbon foil. The conducting salt regenerates the active site of the oxidase, which becomes reduced during its interaction with lactate, and is subsequently amperometrically re-oxidized at 160 mV *versus* SCE	Good selectivity and amenability to mass production. Lack of O_2 dependence. Extremely fast responses at low lactate levels	Restricted analyte range. Narrow linear range limits application as lactate concentrations may reach 20 mmol dm^{-3} during physical exertion	113
	A modification of the glucocard designed by KDK incorporates LOX and hexacyanoferrate(III). The Fe^{3+} ions receive an electron from reduced LOX, producing Fe^{2+} ions, which are chronoamperometrically oxidized at + 0.5 V	Rapid and convenient method permitting point-of-care assays	Excessive overvoltage compromises the selectivity of the method. Also not a universal system	172*
Uric acid	SPCEs doped with CoPC and coated with CA and uricase. Oxidase-generated H_2O_2 preferentially traversed the permselective membrane, whereupon it was electrocatalytically oxidized by CoPC at + 0.4 V *versus* SCE	Simple and convenient test that may be adopted to measure many other oxidase substrates	Paracetamol interference may be reduced by modifying the casting conditions for CA	109
	A glassy carbon–bienzyme configuration has also been proposed. HRP was immobilized onto a GCE by a glutaraldehyde–bovine serum albumin cross-linking method. Hexacyanoferrate(II) was included to mediate the reaction between HRP and uricase-liberated H_2O_2. The hexacyanoferrate(III) ions formed were electroreduced at 0 V (*versus* Ag–AgCl)	Low interference	Complicated reaction scheme but does lend itself to the construction of generic biosensors	173
Cholesterol	Graphite electrodes modified with tetrathiafulvalene, HRP and ChOX. Oxidase-evolved H_2O_2 chemically oxidizes the conducting salt, in the presence of HRP, and is then electrolysed at 0.02 V *versus* Ag–AgCl	Good selectivity	Complex fabrication and reaction sequence. Free cholesterol only	174
	Cholesterol esterase liberates cholesterol from lipoprotein complexes. This rationale is combined with ChOX, HRP and ferrocene to produce a biosensor for total cholesterol. Ferrocene interacts with oxidized HRP, which is reduced by ChOX-generated H_2O_2 with the concomitant production of the ferricinium ion, which is electrochemically reduced	Total sterol detected with favourable performance characteristics	Suitable for point-of-care testing	175*
3-Hydroxy-butyrate	3-Hydroxybutyrate dehydrogenase, NAD^+ and 4-methyl-*o*-quinone (4-MQ) were incorporated into a disposable carbon electrode with a view to monitoring this important ketone body. The dehydrogenase-catalysed reaction generated acetoacetate and NADH. The reduced cofactor was then catalytically oxidized by 4-MQ, regenerating NAD^+ and *o*-benzoquinone, which was chronocoulometrically determined at + 0.3 V (*versus* Ag–AgCl)	Simple, rapid and inexpensive test for diabetologists	Susceptible to interference from readily oxidizable species such as ascorbic and uric acids and paracetamol. Further sensory tailoring is under way to address this drawback	176†

continued—

Table 3—*continued*

Parameter	Principle	Benefits	Notes	Ref.
Paracetamol	CA-modified SPCEs have been used for the direct electrochemical oxidation of the drug at +0.4 V *versus* SCE. Potential interferents were excluded on the basis of size. The amperometric method was in reasonable agreement (r = 0.995) with a CLS colorimetric enzymic assay	Simple, rapid quantifications on a single-use strip. Good stability as no biorecognition agent was employed	Design may be improved by coating aryl acyl amidase on to the outside of the CA film and detecting *p*-aminophenol, which is oxidized more readily than the parent compound	54
	Aryl acyl amidase in solution was used to convert paracetamol into *p*-aminophenol, which was monitored chronoamperometrically, after 30 s, at +0.4 V (*versus* SCE) using a GCE	Improved selectivity. Measures free plasma paracetamol (the portion that actually imparts its hepatological effect)	Studies are under way to replace the solid, renewable GCE with a carbon strip and to render the devices reagentless	177 178†
	The amidohydrolase enzyme has also been used by Bramwell *et al.* to generate *p*-aminophenol from paracetamol, which is monitored amperometrically at +0.25 V (*versus* Ag–AgCl) using a disposable carbon-based electrode. A glutathione-impregnated paper strip was placed over the sensor assembly to eliminate interference from thiols, *i.e.*, antidotes are based on thiols such as *N*-acetylcysteine.	Available in a single-shot format. Simple, reagentless and convenient	Some interference from excipient substances (such as ascorbate)	175†
Salicylic acid	Salicylic acid hydroxylase converts the electroinactive analgesic into an electroactive catechol in oxygenic solutions. Catechol formation was directly proportional to the concentration of salicylate in the sample and was followed chronocoulometrically at +0.3 V using carbon working and Ag–AgCl reference electrodes printed alongside one another	Dry test strips do not require the addition of further reagents. A drop of blood effected the reconstitution of dry agents	Analytical utility was limited by a narrow dynamic range. This was addressed by using an analogue of the drug, benzoate, which uncoupled hydroxylase activity from cofactor oxidation, thus modifying its affinity for salicylate	179†
Creatinine kinase (CK)	CK catalyses the dephosphorylation of creatine phosphate at the expense of adenosine diphosphate (ADP) to produce creatinine and adenosine triphosphate (ATP). When coupled to hexokinase, an enzyme that converts glucose and ATP into G6P and ADP, a glucose biosensor in conjunction with the CK–hexokinase scheme monitors the decrease in current arising from ferrocene reoxidizing $FADH_2$ molecules. The rate of decrease is related to the rate of formation of G6P, which in turn is directly proportional to CK activity	Wide accessible analyte range	Facilitates the detection of a range of metabolites and enzymes. The sequence uses several labile biomolecules, thus complicating fabrication procedures	59
α-Amylase	The enzyme α-glucosidase is used as a coupling enzyme for α-amylase in the enzymic generation of *p*-nitrophenol from *p*-nitrophenyl oligosaccharides. The analytical signal is derived from the electrochemical oxidation of *p*-nitrophenol	Amperometric detection of a hitherto inaccessible enzyme	System requires further tuning to permit quantifications in physiological fluids	176
Oxalic acid	Glazier and Rechnitz have modified a CPE with an oxidase-rich beet stem tissue and the H_2O_2 liberated was detected amperometrically at +0.9 V *versus* SCE in succinic acid–EDTA buffer (pH 4)	Low-cost, sensitive and rapidly responding biosensor. Shows concept for oxalate test	Reproducibility and selectivity problems can be addressed by using isolated enzymes. Ascorbate interferes directly and through enzyme inhibition	180

* Commercially available sensors.
† Near-commercially available sensors.

in biosensor development, forging paths to multi-parameter testing.

The health care market is, however, averse to change and for biosensors to become established they must displace existing methods of analysis. Compounds that are currently believed to be good indicators of disease and routine methods for their determination are shown in Table 1. Generally, the analysis times are extended by long incubation/reaction periods, sample pre-treatment or column conditioning and require expensive and dedicated laboratory equipment. The need to add extra reagents at the time of use is also a disadvantage for bedside monitoring, especially in acute cases such as drug intoxication and myocardial infarction where fast tests are a mandate.

Table 2 shows additional substances that clinicians perceive to require instant tests if the technology were available. In the development of new methods, factors such as minimal operator expertise and sample manipulation must be considered. Solid-phase, single-use sensors address many of these issues. Table 3 shows various analytes accessible with sensors from published works, the number of commercial or near-commercial devices on the market and the principle of their operation. Whereas the number of potential analytes is large (and constantly adjusting to meet the challenges laid down by diagnostic companies), the actual number of commercial products is comparatively small. No doubt the fabrication optimization, lengthly analytical trials and financial investment (MediSense's glucose meter cost around US$50 million

to develop) hinder such progress. However, when these barriers are surmounted the realization of disposable biosensors will be brought to fruition. Given the decentralized nature of the testing regime, they are destined to have a pronounced impact on the diagnostic market, and carbon as an electrode substrate is likely to be a strong contender in this context.[181]

The authors thank the University of the West of England (UWE) for financial support. Gwent Electronic Materials (GEM) are thanked for material assistance. Colleagues at UWE, particularly Caroline Jarvis, Steven Sprules and Ian Hartley, are thanked for their help and interest in this work.

References

1 Håkanson, H., Kyrölainen, M., and Mattiason, M., *Biosens. Bioelectron.*, 1993, **8**, 213.
2 Blaedel, W. J., and Engstrom, R. C., *Anal. Chem.*, 1980, **52**, 1691.
3 Willner, I., and Riklin, A., *Anal. Chem.*, 1994, **66**, 1535.
4 Silber, A., Bräuche, C., and Hampp, N., *Sens. Actuators*, 1994, **18–19**, 235.
5 Csöregi, E., Gorton, L., and Marko-Varga, G., *Anal. Chim. Acta*, 1993, **273**, 59.
6 Ruiz, B. L., Dempsey, E., Hua, C., Smyth, M. R., and Wang, J., *Anal. Chim. Acta*, 1993, **273**, 425.
7 Adams, R. N., *Anal. Chem.*, 1958, **30**, 1576.
8 Céspedes, F., Fàbregas, M. J., Bartrolí, J., and Alegrats, S., *Anal. Chim. Acta*, 1993, **273**, 409.
9 Kinoshita, K., *Carbon—Electrochemical and Physiochemical Properties*, Wiley-Interscience, New York, 1988.
10 Ikeda, T., Miki, K., Fushimi, F., and Senda, M., *Agric. Biol. Chem.*, 1987, **51**, 747.
11 Koopal, C. G., Bos, A. A., and Nolte, R. J., *Sens. Actuators*, 1994, **18–19**, 166.
12 Martorell, D., Céspedes, F., Martinez-Fàbregas, E., and Alegret, E., *Anal. Chim. Acta*, 1994, **290**, 343.
13 Zhou, Z. X., and Wang, E. K., *Electroanalysis*, 1994, **6**, 29.
14 Kronkvist, K., Wallentin, K., and Johansson, G., *Anal. Chim. Acta*, 1994, **290**, 335.
15 Jin, L. T., Ye, J. S., Tong, W., and Fang, Y. Z., *Mikrochim. Acta*, 1993, **112**, 71.
16 Sagar, K. A., Smyth, M. R., and Munden, R., *J. Pharm. Biomed. Anal.*, 1993, **11**, 533.
17 Shiraishi, H., and Takahashi, R., *Anal. Sci.*, 1994, **10**, 133.
18 Wang, J., Dempsey, E., Eremenko, A., and Smyth, M. R., *Anal. Chim. Acta*, 1993, **279**, 203.
19 Martinez, C. R., Gonzalo, R. E., Garcia, G. F., and Méndez, H. J., *J. Chromatogr.*, 1993, **644**, 49.
20 Goicolea, M. A., Debalugea, Z. G., Portela, M. J., and Barrio, R., *Analyst*, 1994, **119**, 269.
21 Ikeda, S., and Satake, H., *Bunseki Kagaku*, 1994, **43**, 247.
22 Chi, Q. J., and Dong, S. J., *Anal. Chim. Acta*, 1993, **278**, 17.
23 Fernandez, C., Chico, E., Yanezsedeno, P., Pingarron, J. M., and Polo, L. M., *Analyst*, 1992, **117**, 1919.
24 Deetz, J. S., and Rozzel, J. D., in *Biocatalysts for Industry*, ed. Dordick, J. S., Plenum Press, New York, 1991, ch. 9. pp. 181–191.
25 Wang, J., Dempsey, E., Eremenko, A., and Smyth, M. R., *Anal. Chim. Acta*, 1993, **279**, 203.
26 Burke, P. A., Smith, S. O., Bachovchin, W. W., and Kilbanov, A. M., *J. Am. Chem. Soc.*, 1989, **111**, 8290.
27 Saini, S., and Turner, A. P. F., *Biochem. Soc. Trans.*, 1991, **19**, 28.
28 Adeyoju, O., Iwuoha, E. I., and Smyth, M. R., *Talanta*, 1994, **41**, 1603.
29 Iwuoha, E. I., and Smyth, M. R., *Analyst*, 1994, **119**, 265.
30 Mionetto, N., Marty, J. L., and Karube, I., *Biosens. Bioelectron.*, 1994, **9**, 463.
31 Bouquelot, E., and Bridel, M., *Ann. Chim. Phys.*, 1913, **29**, 145.
32 Degani, Y., and Heller, A., *J. Am. Chem. Soc.*, 1988, **110**, 2615.
33 Dics, J. M., Cardosi, M. F., Turner, A. P. F., and Karube, I., *Electroanalysis*, 1993, **5**, 1.
34 Laane, C., Boeren, S., Vos, K., and Veeger, C., *Biotechnol. Bioeng.*, 1987, **30**, 81.
35 Sakurai, J. Margolin, A. L., Russell, A. J., and Kilbanov, A. M., *J. Am. Chem. Soc.*, 1988, **110**, 7236.
36 Faulkner, L. R., *Chem. Eng. News*, 1984, 28.
37 Murray, R. W., *Acc. Chem. Res.*, 1990, **23**, 128.
38 Xie, B., Khayyami, M., Nwosu, T., Larsson, P. O., and Danielson, B., *Analyst*, 1993, **118**, 845.
39 Wilson, R., and Turner, A. P. F., *Biosens. Bioelectron.*, 1992, **7**, 165.
40 Heller, A., *Acc. Chem. Res.*, 1990, **23**, 128.
41 Rivas, G. A., and Ortiz, P. I., *Anal. Lett.*, 1994, **27**, 751.
42 Turyan, I., and Mandler, D., *Anal. Chem.*, 1993, **65**, 2089.
43 Wang, J., *Analyst*, 1987, **112**, 1303.
44 Hernandez, P., Vicente, J., Gonzalez, M., and Hernandez, L., *Talanta*, 1990, **37**, 789.
45 Rolinson, D. R., Nowak, R. J., Welsh, T. A., and Murray, C. G., *Talanta*, 1991, **38**, 27.
46 Ramos, J. A., Bermejo, E., Zapardiel, A., and Perez, J. A., *Anal. Chim. Acta*, 1993, **273**, 219.
47 Malinski, T., and Taha, Z., *Nature (London)*, 1992, **358**, 676.
48 Moussy, F., Harrison, D. J., and Rajotte, R. V., *Int. J. Artif. Organs*, 1994, **2**, 88.
49 Wang, J., Tuzhi, P., and Golden, T., *Anal. Chim. Acta*, 1987, **194**, 129.
50 Gao, Z., Wang, G., Li, P., and Zhao, Z., *Anal. Chem.*, 1991, **63**, 953.
51 Dray, D. N., Keyes, M. H., and Watson, B., *Anal. Chem.*, 1977, **49**, 1067A.
52 Colton, C. K., Smith, K. A., Merril, E. W., and Farrell, P. C., *J. Biomed. Mater. Res.*, 1971, **5**, 459.
53 Amine, A., Kauffmann, J. M., Patriarche, G. J., and Christian, G. D., *Talanta*, 1993, **40**, 1157.
54 Gilmartin, M. A. T., and Hart, J. P., *Analyst*, 1994, **119**, 2431.
55 Gilmartin, M. A. T., and Hart, J. P., *Analyst*, 1994, **119**, 2331.
56 Matthews, D. R., Brown, E., Watson, A., Holman, R. R., Steemson, J., Hughs, S., and Scott, D., *Lancet*, 1987, **1**, 778.
57 Batchelor, M. J., Green, M. J., and Sketch, C. L., *Anal. Chim. Acta*, 1989, **221**, 289.
58 Cardosi, M. F., and Birch, S. W., *Anal. Chim. Acta*, 1993, **276**, 69.
59 Hall, E. A. H., *Biosensors*, Open University Press, Milton Keynes, 1990.
60 Wring, S. A., and Hart, J. P., *Electroanalysis*, 1994, **6**, 617.
61 Sternberg, R., Bindra, D. S., Wilson, G. S., and Thévenot, D. R., *Anal. Chem.*, 1988, **60**, 2781.
62 Gilmartin, M. A. T., Hart, J. P., and Birch, B. J., *Analyst*, 1994, **119**, 247.
63 Heider, G. H., Sasso, S. V., Huang, K.-M., Yacynych, A. M., and Wieck, H. J., *Anal. Chem.*, 1990, **62**, 1106.
64 Sun, Z., and Tachikawa, H., *Anal. Chem.*, 1992, **64**, 1112.
65 Clark, L. C., and Lyons, C., *Ann. N.Y. Acad. Sci.*, 1962, **102**, 29.
66 Wring, S. A., Hart, J. P., Bracey, L., and Birch, B. J., *Anal. Chim. Acta*, 1990, **231**, 203.
67 Guilbault, G. G., and Lubrano, G. J., *Anal. Chim. Acta*, 1973, **64**, 439.
68 Trevenot, D. R., Sternberg, R., Coulet, P. R., Laurent, J., and Gautheron, C. D., *Anal. Chem.*, 1979, **51**, 96.
69 Durand, P., David, A., and Thomas, D., *Biochim. Biophys. Acta*, 1978, **527**, 277.
70 Frew, J. E., and Green, M. J., *Anal. Proc.*, 1988, **25**, 276.
71 Gilmartin, M. A. T., Hart, J. P., and Birch, B. J., *Analyst*, 1992, **117**, 1299.
72 Rennerberg, R., Scheller, F., Riedel, K., Litschko, E., and Richter, M., *Anal. Lett.*, 1983, **16** (B12), 877.
73 Frew, J. E., and Hill, H. A. O., *Anal. Chem.*, 1987, **59**, 933A.
74 Terpstra, M., *Biosens. Bioelectron.*, 1992, **7**, 157.
75 Rechnitz, G. A., *Chem. Eng. News*, 1975, **53**, 29.
76 Wijesuriya, D. C., and Rechnitz, G. A., *Biosens. Bioelectron.*, 1993, **8**, 155.
77 Belli, S. L., and Rechnitz, G. A., *Anal. Lett.*, 1986, **19**, 403.
78 Wring, S. A., and Hart, J. P., *Analyst*, 1992, **117**, 1281.
79 Cass, A. E. G., Davis, G., Francis, G. D., Hill, H. A. O., Aston, W. J., Higgins, J., Plotkin, E. J., Scott, L. D. L., and Turner, A. P. F., *Anal. Chem.*, 1984, **56**, 667.

80 Wring, S. A., Hart, J. P., and Birch, B. J., *Analyst*, 1991, **116**, 123.
81 Hart, J. P., and Hatley, I., *Analyst*, 1994, **119**, 259.
82 Wring, S. A., and Hart, J. P., *Analyst*, 1992, **117**, 1283.
83 Hartley, I. C., and Hart, J. P., *Anal. Proc.*, 1994, **31**, 333.
84 Halbert, M. K., and Baldwin, R. P., *Anal. Chem.*, 1985, **57**, 591.
85 Zagal, J., Fierro, C., and Rozas, R., *J. Electroanal. Chem.*, 1981, **119**, 403.
86 Scheller, F., Schubert, F., Pfeiffer, D., Hintsche, R., Dransfeld, I., Renneberg, R., Wollenberger, U., Riedal, K., Pavlova, M., Kühn, M., Müller, H.-G., Tan, P.-M., Hoffman, W., and Moritz, W., *Analyst*, 1989, **114**, 653.
87 Gibson, T. D., Hulbert, J. N., Parker, S. M., Woodward, J. R., and Higgins, I., *Biosens. Bioelectron.*, 1992, **7**, 701.
88 Icaza, M.-A., and Bilitewski, U., *Anal. Chem.*, 1993, **65**, 525A.
89 Cave, S., *Fundholding*, 1994, 14.
90 Heiduschka, P., and Scheller, F. W., *Biosens. Bioelectron.*, 1994, **9**, 7.
91 Tsionsky, M., Gun, G., Glezer, V., and Lev, O., *Anal. Chem.*, 1994, **66**, 1747.
92 Beh, S. K., Moody, G. J., and Thomas, J. D. R., *Analyst*, 1991, **116**, 459.
93 Marcinkeviciene, J., and Kulys, J., *Biosens. Bioelectron.*, 1993, **8**, 209.
94 Moore, T. J., Nam, G. G., Pipes, L. C., and Coury, L. A., Jr., *Anal. Chem.*, 1994, **66**, 3158.
95 Van Benschoten, J. J., Lewis, J. Y., and Heineman, W. R., *J. Chem. Educ.*, 1983, **60**, 772.
96 Miner, D. J., Rice, J. R., Riggin, R. M., and Kissinger, P. T., *Anal. Chem.*, 1981, **53**, 2258.
97 Coury, L. A., Oliver, B. N., Egekeze, J. O., Sosnoff, C. S., Brumfield, J. C., Buck, R. P., and Murray, R. W., *Anal. Chem.*, 1990, **62**, 452.
98 Hale, P. D., Inagaki, T., Karan, H. I., Okamoto, Y., and Skotheim, T. A., *J. Am. Chem. Soc.*, 1989, **111**, 3482.
99 Ikeda, S., and Oyama, N., *Anal. Chem.*, 1993, **65**, 1910.
100 Schuhmann, W., *Biosens. Bioelectron.*, 1993, **8**, 191.
101 Razumas, V., Kulys, J., Knichel, M., Wiemhöfer, H.-D., and Göpel, W., *Electroanalysis*, 1993, **5**, 399.
102 Heller, A., *Acc. Chem. Res.*, 1990, **23**, 128.
103 Zhao, S., and Luong, J. H. T., *Biosens. Bioelectron.*, 1993, **8**, 438.
104 Wang, J., Wu, L.-H., Li, R., and Sanchez, J., *Anal. Chim. Acta*, 1990, **228**, 251.
105 Gorton, L., *Anal. Chim. Acta*, 1985, **178**, 247.
106 Taha, Z., and Wang, J., *Electroanalysis*, 1991, **3**, 215.
107 Wang, D. L., and Heller, A., *Anal. Chem.*, 1993, **65**, 1069.
108 Staskeviciene, S. L., Cenas, N. K., and Kulys, J. J., *Anal. Chim. Acta*, 1991, **243**, 167.
109 Gilmartin, M. A. T., and Hart, J. P., *Analyst*, 1994, **119**, 853.
110 Gilmartin, M. A. T., Ewen, R., Hart, J. P., and Honeyborne, C. L., *Electroanalysis*, in the press.
111 Heller, A., *Acc. Chem. Res.*, 1990, **23**, 128.
112 Bartlett, P. N., Whitaker, R. G., Green, M. J., and Frew, J., *J. Chem. Soc., Chem. Commun.*, 1987, 1603.
113 Palleschi, G., and Turner, A. P. F., *Anal. Chim. Acta*, 1990, **243**, 459.
114 Ikeda, T., Matsushita, F., and Senda, M., *Biosens. Bioelectron.*, 1991, **6**, 299.
115 Todoriki, S., Ikeda, T., Senda, M., and Wilson, G. S., *Agric. Biol. Chem.*, 1989, **53**, 3055.
116 Maidan, R., and Heller, A., *Anal. Chem.*, 1992, **64**, 2889.
117 Chang, H.-C., Ueno, A., Yamada, H., Matsue, T., and Uchida, I., *Analyst*, 1991, **116**, 793.
118 Jaegfeldt, H., Kuwana, T., and Johansson, G., *J. Am. Chem. Soc.*, 1983, **105**, 1805.
119 Chenault, H. K., and Whitesides, G. M., *Appl. Biochem. Biotechnol.*, 1987, **14**, 147.
120 Wring, S. A., and Hart, J. P., *Analyst*, 1992, **117**, 1215.
121 Gorton, L., Persson, B., Hale, P. D., Bouguslavsky, L. I., Karan, H. I., Lee, H. S., Skotheim, T., Lan, H. I., and Okamoto, Y., in *Biosensors and Chemical Sensors*, ed., Edelman, P. J., and Wang, J., ACS Symposium Series, No. 487, 1992, ch. 6., pp. 56–83.
122 Bernt, E., and Gutman, I., in *Methods in Enzymatic Analysis*, ed. Bergmeyer, H. U., Verlag Chemie, Weinheim, and Academic Press, New York, 2nd edn., 1974, pp. 1499–1502.
123 Dominguez, E., Marko-Varga, G., Lan, H. L., Okamoto, Y., Hale, P. D., Skotheim, T. A., Hahn-Hägerda, B., and Gorton, L., *Ann. N.Y. Acad. Sci.*, 1992, **672**, 230.
124 Olsson, B., Marko-Varga, G., Gorton, L., Applequist, R., and Johansson, G., *Anal. Chim. Acta*, 1988, **206**, 49.
125 Dominguez, E., Lan, H. L., Okamoto, Y., Hale, P. D., Skotheim, T. A., Gorton, L., and Hahn-Hägerda, B., *Biosens. Bioelectron.*, 1993, **8**, 229.
126 Dominguez, E., Lan, H. L., Okamoto, Y., Hale, P. D., Skotheim, T. A., and Gorton, L., *Biosens. Bioelectron.*, 1993, **8**, 167.
127 Sprules, S. D., Hart, J. P., Pittson, R., and Wring, S. A., *Analyst*, 1994, **119**, 253.
128 Sprules, S. D., Hart, J. P., Pittson, R., and Wring, S. A., *Anal. Chim. Acta*, in the press.
129 Athey, D., McNeil, C. J., Bailey, W. R., Hager, H. J., Mullen, W. H., and Russell, L. J., *Biosens. Bioelectron.*, 1993, **8**, 415.
130 Persson, B., Lan, H. L., Gorton, L., Okamoto, Y., Hale, P. D., Boguslavsky, L. I., and Skotheim, T., *Biosens. Bioelectron*, 1993, **8**, 81.
131 Vreeke, M., Maidan, R., and Heller, A., *Anal. Chem.*, 1992, **64**, 3084.
132 Giorgio, D., in *Clinical Chemistry Principles and Practice*, ed. Henry, R. J., Cannon, D. C., and Winkleman, J. W., Harper and Row, New York, 2nd edn, 1974, p. 504.
133 Gowenlock, A. H., *Varley's Clinical Biochemistry*, Heinemann Medical Books, London, 6th edn., 1988.
134 Zeatris, A., and Zak, B., *Anal. Chem.*, 1969, **29**, 143.
135 Allain, C. C., *Clin. Chem. (Winston-Salem, N.C.)*, 1974, **20**, 470.
136 Barker, J. B., and Summerson, W. H., *J. Biol. Chem.*, 1941, **138**, 535.
137 Stern, H. J., *Ann. Clin. Biochem.*, 1994, **31**, 410.
138 Weiner, K., *Ann. Clin. Biochem.*, 1978, **15**, 187.
139 Campbell, R. S., and Price, C. P., *J. Clin. Chem. Biochem.*, 1986, **24**, 155.
140 Hammond, P. M., Scawen, M. D., Atkinson, T., Campbell, R. S., and Price, C. P., *Anal. Biochem.*, 1984, **143**, 157.
141 Price, C. P., Hammond, P. M., and Scawen, M. D., *Clin. Chem. (Winston-Salem, N.C.)*, 1983, **29**, 358.
142 Folin, O., and Denis, W. A., *J. Biol. Chem.*, 1912–13, **13**, 469.
143 Bittner, D., Halls, S., and McCleary, M., *Am. J. Clin. Pathol.*, 1963, **40**, 423.
144 Folin, O., *J. Biol. Chem.*, 1930, **86**, 179.
145 Carrol, J. J., Coburn, H., and Douglas, R., *Clin. Chem. (Winston-Salem, N.C.)*, 1971, **17**, 158.
146 Miller, B. F., and Dubos, R., *J. Biol. Chem.*, 1937, **121**, 457.
147 Masson, P., Ohlsson, P., and Björkhem, I., *Clin. Chem. (Winston-Salem, N.C.)*, 1981, **27**, 18.
148 Lim, C. K., Richmond, W., Robinson, D. P., and Brown, S. S., *J. Chromatogr.*, 1978, **145**, 41.
149 Chaney, A. L., and Marbach, E. P., *Clin. Chem. (Winston-Salem, N.C.)*, 1962, **8**, 130.
150 Guilbault, G. G., and Mascini, M., *Anal. Chem.*, 1977, **49**, 795.
151 Trinder, P., *Biochem. J.*, 1954, **57**, 301.
152 Walter, L. J., Briggs, D. F., and Coutts, R. T., *J. Pharm. Sci.*, 1974, **63**, 1755.
153 Caterson, R. R., Duggin, G. C., Horrath, J. S., and Tiller, D. J., *Aust. J. Pharm. Sci.*, 1978, **7**, 111.
154 Bjerre, S., and Porter, C. J., *Clin. Chem. (Winston-Salem, N.C.)*, 1965, **11**, 137.
155 Schater, D., and Manis, J. G., *J. Clin. Invest.*, 1958, **37**, 800.
156 Gallows, S., and Watkins, P. J., *Clin. Chim. Acta*, 1968, **19**, 511.
157 Laker, M. F., *Adv. Clin. Chem.*, 1983, **23**, 259.
158 Rofe, A. M., Pholenz, S. M., Bais, R., and Conyers, R. A. J., *Clin. Chem. (Winston-Salem, N.C.)*, 1985, **31**, 1574.
159 Mazzani, B. C., Teubner, J. I. C., and Ryall, R. L., *Clin. Chem. (Winston-Salem, N.C.)*, 1984, **30**, 1339.
160 Chalmers, A. H., Cowley, D. M., and McWhinney, B. C., *Clin. Chem. (Winston-Salem, N.C.)*, 1985, **31**, 1703.
161 Karman, A., *J. Clin. Invest.*, 1955, **33**, 131.
162 Wimmer, M. C., Artiss, J. D., and Zak, B., *Clin. Chem. (Winston-Salem, N.C.)*, 1985, **31**, 1616.
163 Owen, V. M., *Biosens. Bioelectron.*, 1994, **9**, 29.
164 Howles, C. M., and Macnamee, M. C., *Br. Med. Bull.*, 1990, **49**, 616.

165 Albers, J. J., Marcovina, S. M., and Kennedy, H., *Clin. Chem. (Winston-Salem, N.C.)*, 1992, **58**, 658.
166 Hilton, S., *Br. J. Gen. Pract.*, 1990, **40**, 32.
167 Chopra, I. J., Nelson, J. C., Solomon, D. H., and Beall, G. N., *J. Clin. Endocrinol. Metab.*, 1971, **32**, 299.
168 Broods, A. P., Nairn, I. M., and Baird, J. D., *Br. Med. J.*, 1980, **281**, 702.
169 Wang, J., Liu, J., Cheng, L., and Lu, F., *Anal. Chem.*, 1994, **66**, 3600.
170 Gilmartin, M. A. T., Patton, D. T., and Hart, J. P., *Analyst*, in the press.
171 Davis, B. D., *Clin. Chem. (Winston-Salem, N.C.)*, 1992, **38**, 2093.
172 Shimojo, N., Naka, K., Ucnoyama, H., Hamamoto, K., Yoshoika, K., and Okuda, K., *Clin. Chem. (Winston-Salem, N.C.)*, 1993, **37**, 2312.
173 Kulys, J., and Schmid, R. D., *Biosens. Bioelectron.*, 1991, **6**, 43.
174 Ball, M. R., Frew, J. E., Green, M. J., and Hill, H. A. O., *Proc. Electrochem. Soc.*, 1986, **86**, 7.
175 Bramwell, H., Cass, A. E. G., Gibbs, P. N. B., and Green, M. S., *Analyst*, 1990, **115**, 185.
176 Batchelor, M. J., Williams, S. C., and Green, M. J., *J. Electroanal. Chem.*, 1988, **246**, 307.
177 Vaughan, P. A., Scott, L. D. L., and McAleer, J. R., *Anal. Chim. Acta*, 1991, **248**, 361.
178 Jones, A. F., McAleer, J. F., Braithwaite, R. A., Scott, L. D., Brown, S. S., and Vale, S. A., *Lancet*, 1990, **335**, 793.
179 Frew, J. E., Bayliff, S. W., Gibbs, P. N. B., and Green, M. J., *Anal. Chim. Acta*, 1989, **224**, 39.
180 Glazier, S. A., and Rechnitz, G. A., *Anal. Lett.*, 1989, **22**, 2929.
181 Gilmartin, M. A. T., *PhD Thesis*, University of the West of England, Bristol, 1994.

Paper 4/03704I
Received June 20, 1994
Accepted November 28, 1994