Osmium Bipyridyl Redox Polymers Used in Enzyme Electrodes

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A review of the background of biosensors which utilise osmium complexes for monitoring the glucose levels in diabetic patients is presented. An electrochemical system, based on an osmium redox polymer, is described; the system is aimed at being a reliable, compact and miniature in vivo sensor for implantation into diabetic patients for continuous monitoring of their blood glucose levels. The sensor is about to begin trials on human volunteers.

Biosensors are extensively used in clinical diagnostic procedures (1–3). The research and development currently being undertaken in biosensors is aimed at achieving a faster response with more accurate results from smaller samples, so that analytes, such as glucose, lactate, enzymes and immunoproteins, can be monitored. The largest segment of the biosensor market is concerned with the care of diabetic patients.

It was reported recently that patients suffering from Type 1 diabetes who strictly controlled their diet and in addition measured their blood glucose levels four times a day experienced 60 per cent fewer diabetes-related complications, such as blindness, kidney failure, nerve damage, circulatory problems and heart disease (4). Type 1 diabetics produce very little of their own insulin and depend on insulin injections to regulate their glucose levels.

Unfortunately, most diabetics do not measure their blood sugar levels more than twice daily because of the cost, inconvenience and discomfort – such measurements require the withdrawal of blood and the injection of insulin. This suggests that there is a need for a miniature and easily implantable sensor which could monitor glucose intermittently or continuously, thus eliminating the need to withdraw blood. Such an intermittently or continuously operating glucose sensor could be coupled to a portable insulin pump through a microprocessor-controlled feedback loop (5). This would provide better control of glucose levels by reducing the magnitude of fluctuations caused by diet or physical activity. Such a sensor would require miniaturisation and improvements to the existing stability, selectivity and biocompatibility.

Electrochemical Biosensors

Electrochemical biosensors are now replacing optical ones in commonly used applications because they are of relatively simple construction, inexpensive and can be easily miniaturised. They are quite sensitive to amperometric currents in the picoampere range or charges in the nanocoulomb range. The dynamic range of an electrochemical sensor can be made as wide as is needed for overlapping the medically relevant range. Another advantage is that they can sense samples in light absorbing or turbid solutions, such as whole blood.

The wet/dry optical test strips which are used for home glucose measurements in whole blood often require that the test strip be wiped or blotted with a tissue, so that the opaque blood sample does not interfere with measuring the colour change. The reflectance colorimeter, into which part of the whole blood-treated test strip is inserted, becomes fouled by residual blood and thus requires periodic cleaning.

However, in electrochemical glucose measurements taken at home only the leads of the electrode need to contact the electronic measuring instrumentation. Thus, such assaying systems require less maintenance as residual

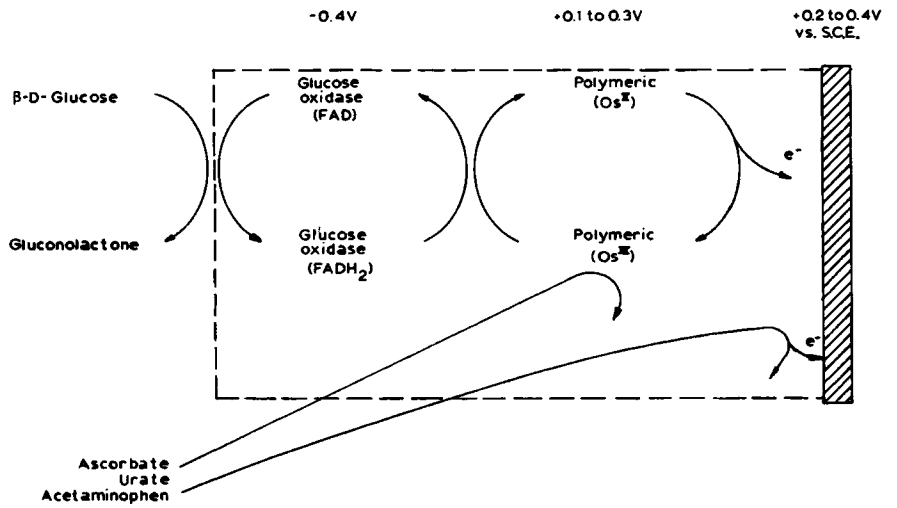

Fig. 1 Reaction cycles of "electrically wired" glucose oxidase, for an electrode coated with an osmium polymer complex and glucose oxidase where the electrons released during the oxidation of glucose move via the osmium complex to the electrode. Interferant oxidation pathways are also shown

whole blood is left on the test strip and cannot foul the instrument (6).

Enzymes are employed in most amperometric biosensors because of their high selectivity; ideally, it would be better to avoid enzymes because they are inherently unstable. However, man-made molecular recognition systems do not yet offer the selectivity and sensitivity which can be achieved with enzymes (7). There are some redox enzymes, including glucose oxidase (GOX), which cannot exchange electrons directly with electrodes because their redox active sites are buried deep inside a glycoprotein shell. Many enzymes have buried active sites in order to prevent the indiscriminate exchange of electrons with other redox proteins and to assure selectivity. Recent crystal structure studies of GOX have shown that the active site is approximately 15 Å from the periphery of the enzyme (8, 9).

In order to transfer electrons between the redox active site and the electrodes, mediators (med) such as osmium bipyridyl complexes (10), ferricyanide (11), quinones (12), ferricinium derivatives (13, 14) and ruthenium complexes (15) are used, see following Equations (i) and (ii).

$$\text{Glucose} + \text{GOX-FAD} \rightarrow \text{Gluconolactone} + \text{GOX-FADH}_2 \quad \text{(i)}$$

$$\text{GOX-FADH}_2 + \text{med(ox)} \rightarrow \text{GOX-FAD} + \text{med(red)} \quad \text{(ii)}$$

GOX-FAD/GOX-$FADH_2$ represents the oxidised/reduced states of the flavin active site of GOX. For *in vivo* sensing, where leaching of the mediator is not acceptable, the electrons must be relayed to the electrode from the enzyme through an immobilised electron relay. Electron relays of low molecular weight, which can diffuse freely, allow the indiscriminate exchange of electrons between the redox protein enzymes and also short circuit the essential electrochemical pathways necessary for life. The herbicide Paraquat™ (N,N'-dimethylviologen) is an example of a toxic electron relay that short circuits physiological redox systems.

Osmium Polymer Mediated Biosensors

Osmium complexes, particularly bipyridines, terpyridines and phenanthrolines, have particularly high self-exchange rates – divalent and trivalent complexed osmium ions exchange

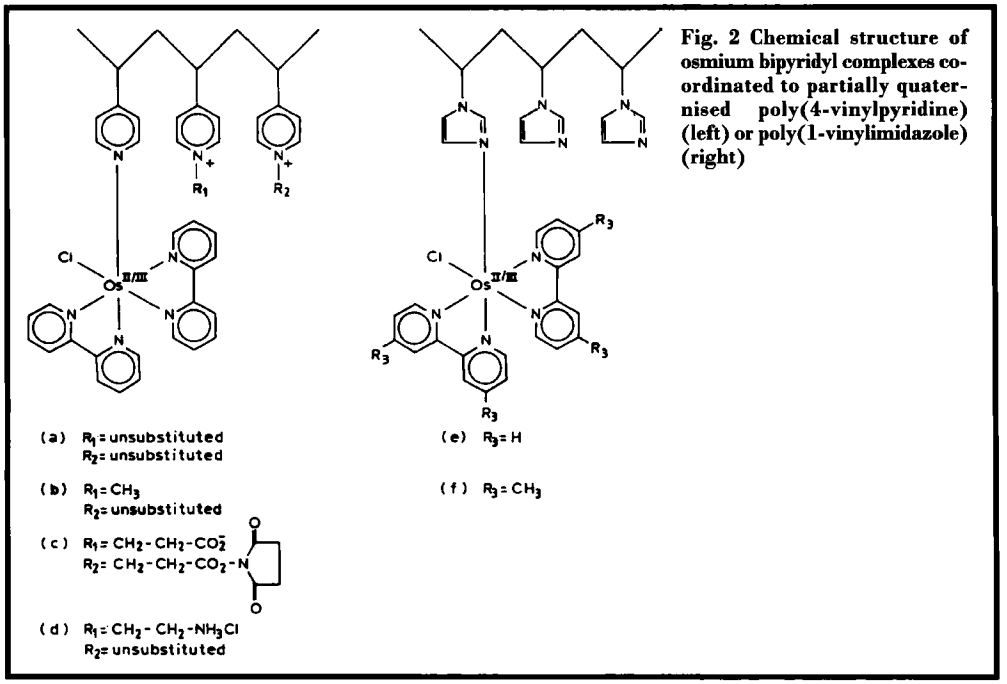

Fig. 2 Chemical structure of osmium bipyridyl complexes coordinated to partially quaternised poly(4-vinylpyridine) (left) or poly(1-vinylimidazole) (right)

electrons at high rates. We have therefore built reagentless sensors containing a redox enzyme and an osmium complexed polymer, where the polymer relayed electrons via the osmium centres between the enzymes and the electrodes. The osmium polymer electrically connects the redox centres of enzymes, such as GOX, by introducing electron relays close to the enzyme active site. The osmium redox sites serve as stepping stones, allowing electrons to hop from the electron-rich active site of the reduced enzyme to the first osmium relay, from the first relay to a second relay, and so on until finally from a relay to the electrode.

These "wires", which relay electrons, form electron conducting hydrogels upon crosslinking. The fluid (non-rigid) chains in these gels act to form electrostatic complexes between the enzyme molecules and their segments, and transfer electrons upon colliding with each other. The collisions shorten the path lengths that an electron must traverse on its way to an electrode. This path would be much longer if the electron proceeded exclusively along the tortuous route of a folded polymer chain.

Figure 1 shows schematically how a "wired" GOX sensor transduces the glucose concentration, that is flux, into an oxidation current. Electrodes such as gold, vitreous carbon, graphite, or tin oxide can be modified with additions of the osmium redox hydrogel containing GOX. GOX serves as the selective biological component (hence the term biosensor), while the osmium redox polymer serves as the electron relay, shuttling electrons from GOX to electrodes. Glucose is oxidised to gluconolactone in the reactive centre of the GOX molecule, (Equation (i)) and in the process it reduces GOX-FAD to GOX-$FADH_2$. Next, an $osmium^{III}$ redox site in close proximity to the enzyme accepts an electron from GOX-$FADH_2$, which effectively oxidises the enzyme back to its GOX-FAD state. The electrons associated with the $osmium^{II}$ redox site are shuttled via the redox polymer to the electrode through a series of self-exchange reactions, when the anode is held at a sufficiently positive potential. Ideally, the reactions occurring inside the film are very fast making the response time short, typically of a duration less than one second.

The structures of some osmium redox polymers or "wires" which have been studied in our laboratory are shown in Figure 2. The backbone of the polymer usually consists of poly(4-vinylpyridine) (PVP) or poly(1-vinylimidazole) (PVI), where some of the pyridine or imidazole groups are complexed with $Os(bpy)_2Cl$ to form polymers termed PVP-Os, Figure 2(a), and PVI-Os, Figure 2(e), respectively (3, 10, 16–26).

PVP-Os is only slightly soluble in water except at high osmium loadings. However, when some of the remaining uncomplexed pyridine groups are quaternised with various side chains, the solubility of PVP-Os in water is enhanced. If the side chains also introduce amines, see Figure 2(d), PVP-Os then becomes easy to crosslink.

Earlier Osmium Polymer Mediated Glucose Electrodes

The first glucose electrodes mediated by osmium polymers were prepared in 1989 by Degani and Heller (17). They synthesised a water soluble cationic redox polymer consisting of PVP complexed with $Os(bpy)_2Cl$ and partially N-methylated at some of the remaining uncomplexed pyridine groups, see Figure 2(b). The positively charged osmium polymer was adsorbed onto a graphite electrode by dip-coating in an osmium polymer solution. The electrode was then treated with the negatively charged GOX, forming an ionic complex.

In order to bind the members of the enzyme/polymer complex together covalently, diazonium salt derivatives of the polymer were reacted with tyrosine residues on GOX. This was accomplished by the co-polymerisation of 4-aminostyrene with 4-vinylpyridine followed by treatment with sodium nitrite. The diazonium salt formed an azo linkage with the tyrosine groups on GOX. However, the resulting polymer/enzyme complex formed was a poorly crosslinked structure, due to their being few tyrosine residues on GOX.

The next osmium redox polymers used for enzyme electrodes were chosen so that they would react with the more abundant lysyl residues on GOX. This enabled crosslinked 3-dimensional polymer networks to be formed on the electrode. A PVP-based osmium polymer quaternised with the reactive propionic ester of N-hydroxy succinimide, see Figure 2(c), was synthesised by Gregg and colleagues (19). They constructed sensors by mixing the osmium redox polymer with GOX and tetraethylenetetraamine to form rugged 3-dimensional crosslinked films. These films were unique in having both adequate electron diffusion coefficients and also in being permeable to water soluble substrates and products of enzymatic reactions.

However, the extent of the crosslinking formed by the reaction of the N-hydroxy succinimide ester and the enzyme amines was not reproducible, due to the N-hydroxy succinimide ester undergoing competing hydrolysis.

Subsequently, another PVP-based osmium polymer, derivatised with bromoethylamine, was synthesised, POs-EA, where EA represents ethylamine, see Figure 2(d), (10, 20). The polymer was quaternised with ethylamine functions

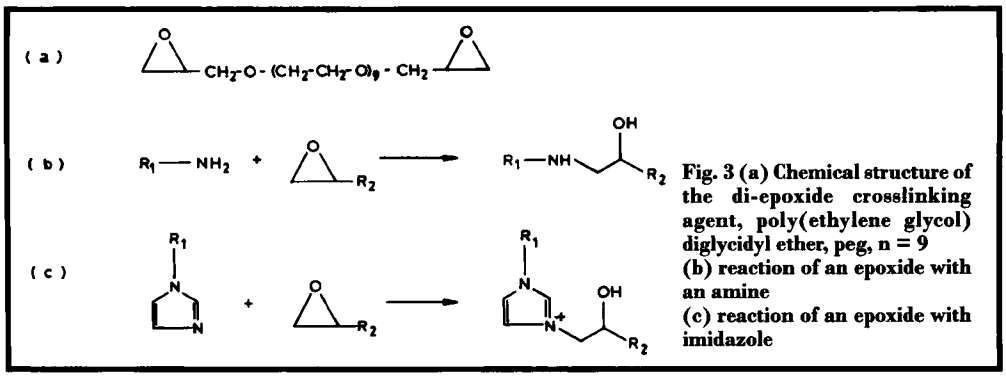

Fig. 3 (a) Chemical structure of the di-epoxide crosslinking agent, poly(ethylene glycol) diglycidyl ether, peg, n = 9
(b) reaction of an epoxide with an amine
(c) reaction of an epoxide with imidazole

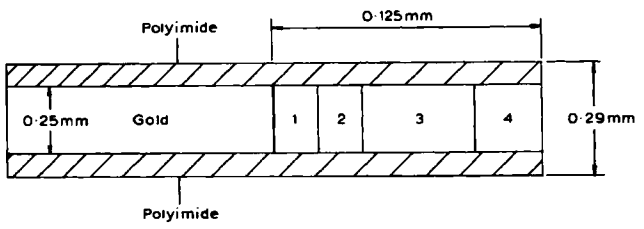

Fig. 4 Schematic drawing of a four-layer recessed miniature implantable glucose electrode. 1: sensing layer; 2: insulating layer; 3: interferant eliminating layer, which consists of horseradish peroxidase and lactate oxidase; 4: biocompatible layer of photocrosslinked tetraacrylated polyethylene oxide

to increase its water solubility, to allow for facile crosslinking and to promote favourable ionic interaction between the polymer and the enzyme. Pendant amine groups on the osmium redox polymer and on the lysyl residues of GOX were crosslinked with a commercially available long-chain water-soluble di-epoxide curing agent poly(ethylene glycol) diglycidyl ether (peg), see Figures 3(a) and 3(b).

Crosslinking POs-EA and GOX with peg formed reproducible hydrogels and resulted in enzyme electrodes that produced electro-oxidation currents proportional to glucose concentrations. The POs-EA-GOX-peg system is effective for "wiring" not only GOX, but also other enzymes, such as lactate oxidase (22, 26), glycerophosphate oxidase (22), glucose dehydrogenase (25), horseradish peroxidase (24), glutamate oxidase (27), sarcosine oxidase (27), cellobiose oxidase (28), and many others.

Miniature Glucose Electrodes Implanted Subcutaneously

Glucose electrodes constructed with osmium redox polymers are easily miniaturised for *in vivo* use because they can carry high current densities. Typically miniature glucose electrodes with response times of less than 1 second have been reported, these being supported on a 7 μm sized carbon fibre (21). Additionally, microelectrodes with this construction have displayed a 10-fold increase in current density of 10^{-2} A/cm^2 and a reduction in oxygen sensitivity has been observed (21).

These advantageous properties are caused by radial electron transfer through the osmium polymer on a microelectrode as compared with planar electron transfer in the macroelectrode. For *in vivo* subcutaneous sensing, 290 μm diameter GOX electrodes coated with osmium redox polymer have already been developed. These contain no components that can leach into the body and require no additional reagents.

In order to provide selectivity, stability and biocompatibility for our miniature implantable glucose electrode, we employed a four layer sandwich structure in a recessed gold electrode, see Figure 4, (29).

By using a recessed electrode, the sensing layer is protected from mechanical damage and the transport of glucose is reduced, which thus increases the linear range of the sensor. Ascorbate (vitamin C), urate, and acetaminophen (Tylenol™) and other interferants which are commonly found in serum can be non-selectively oxidised at the osmium redox sites and at the electrode, see Figure 1. In order to prevent such interference, the interferants were eliminated by oxidation in a crosslinked layer of horseradish peroxidase and lactate oxidase between the sensing layer and the body tissue (23).

Lactate and oxygen are also common components of serum, and the reaction between them is catalysed by lactate oxidase to produce pyruvate and hydrogen peroxide, see Equation (iii). The hydrogen peroxide which is generated oxidises the horseradish peroxidase which "burns up" the interfering hydrogen donors, HA, such as ascorbate, urate, and acetaminophen, see Equation (iv).

In this way the crosslinked layer of horseradish peroxidase and lactate oxidase reactively eliminates the flux of interferants from both the

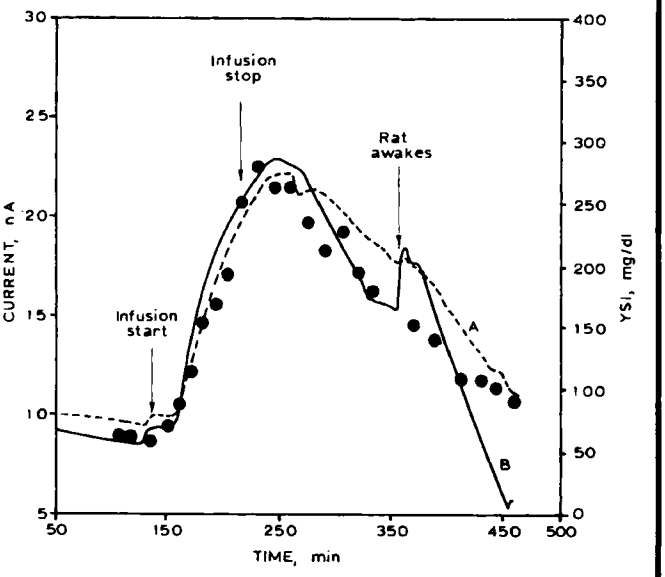

Fig. 5 Current output of two subcutaneously implanted sensors, tracking the blood glucose levels before, during and after an intraperitoneal infusion of glucose. The solid circles represent the blood glucose levels measured on samples withdrawn from the tail vein of the rat using an independent glucose reference assay (Yellow Springs Instrument, YSI, glucose analyser).
(A) shows the output of the electrode implanted subcutaneously in the chest
(B) represents the output of the electrode implanted subcutaneously in the intrascapular area of the rat

osmium polymer "wired" sensing region and the electrode. Because lactate oxidase and horseradish peroxidase can themselves be "wired" with the osmium redox polymer, an insulating layer is placed between the osmium polymer and the interferant pre-oxidising layer.

$$\text{Lactate} + O_2 \xrightarrow{\textit{lactate oxidase}} \text{Pyruvate} + H_2O_2 \quad \text{(iii)}$$

$$2\,HA + H_2O_2 \xrightarrow{\textit{horseradish peroxidase}} 2A + 2H_2O \quad \text{(iv)}$$

The outermost layer of the *in vivo* sensor, which contacts the body, consists of a biocompatible polymer layer (photocrosslinked tetraacrylated polyethylene oxide) which prevents adsorption of protein (30).

Because these sensors are thus not affected by interferants, their output in the absence of glucose is nil, so that their calibration line passes through the origin. This means they require only a one point calibration *in vivo*.

In these sensors, at body temperature of 37°C, a glucose concentration change of 1 mM causes a change in the current of 3.5 nA. Current increases linearly with glucose concentration, up to a concentration of 20 mM. The sensors show only a ± 5 per cent variance in sensitivity throughout 72 hours of being in continuous use.

In vivo glucose monitoring with two electrodes implanted subcutaneously in the thorax and between the scapulae of a rat, is shown in Figure 5. An electrocardiograph electrode was used as both counter and reference electrodes. Current was monitored before and after intraperitoneal glucose infusion for 1 hour. Glucose concentrations in the blood were determined periodically from blood withdrawn from the tail using an independent reference assay. The glucose concentration correlated well with the current of the two subcutaneous sensors.

Increased Selectivity on Utilising a Polymer of Lower Redox Potential

In order to eliminate the necessity of a four layer sandwich electrode a simpler method was devised to reduce the level of oxidation of the interferants. An osmium polymer of sufficiently low redox potential which allows the sensor to operate at potentials where the oxidation of interferant is slow, both on the electrode and in the redox hydrogel of the sensing layer, has been used (31).

This low potential osmium redox polymer has a PVI backbone partially complexed to

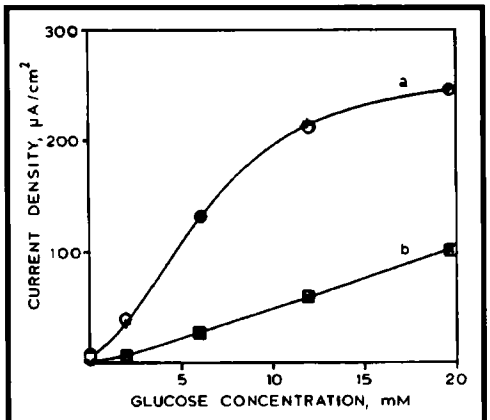

Fig. 6 Dependence of the steady-state current density on glucose concentration in the presence of 50 μM ascorbate, 0.4 mM urate, and 1 mM acetaminophen and without interferants present:
(a) no external membrane, with interferants (circles), without interferants (+);
(b) Nafion membrane, with interferants (squares), without interferants (×). The flow cell operates at 1.4 ml/min in air

$Os(dimethylbpy)_2Cl$, termed PVI_{15}-dme Os, see Figure 2(f). The electron donating methyl substituents on the bipyridyl rings shifted the redox potential of the polymer to 95 mV vs. S.C.E., which is 185 mV more reducing than POs-EA. At the operating potential of the resulting enzyme electrode, ~ 200 mV vs. S.C.E., the electric currents which result from the electro-oxidation of interferants are small in comparison to the glucose electro-oxidation current.

In contrast to the PVP-Os, the PVI_{15}-dmeOs polymers are already water soluble and do not require quaternisation with bromoethylamine. The uncomplexed imidazole groups can be easily crosslinked with water-soluble di-epoxides, see Figure 3(c). Since no ethylamine groups need to be added, the PVI_{15}-dmeOs is simpler to synthesise than POs-EA.

A steady-state flow cell calibration curve under air in the presence and absence of the interferants 0.4 mM urate, 50 μM ascorbate and 1 mM acetaminophen is shown in Figure 6, for an electrode prepared with a crosslinked layer of PVI_{15}-dmeOs and GOX. In order to extend the linear range up to 30 mM of glucose, a layer of Nafion overcoating was employed. This layer limits the flux of the substrate.

The normal concentration ranges in human serum for urate and ascorbate are between 0.18 and 0.42 mM and 0.23 to 85 μM, respectively (32). The upper level for the acetaminophen concentration in human serum is between 0.1 to 0.2 mM when the maximum therapeutic dose of 1000 mg of acetaminophen is administered (33).

Thus the PVI_{15}-dmeOs electrodes can measure glucose selectively in the presence of multiple interferants. The Nafion coating did not increase the selectivity of the sensor, but served only as a mass-transfer limiting layer to increase the linear range of the sensor.

Dehydrogenase-Based Enzyme Electrode

There are several hundred dehydrogenase enzymes that use NAD(P)H, (coenzyme nicotinamide adenine dinucleotide phosphate, an electron acceptor for many dehydrogenases; its reduced form NAD(P)H donates electrons to the electron transport chain) which have been isolated, and with them it is possible to sense about a thousand substrates.

However, the oxidation of NAD(P)H by electrochemical techniques has proved to be difficult because it requires the concerted transfer of 2 electrons and a proton (34). NAD(P)H can be irreversibly oxidised at the electrode surface at high overpotentials. There are however 2-electron mediators derived from phenoxazines and phenazines that electrocatalyse the oxidation of NAD(P)H at low potentials (35–37).

A complex but nonetheless fast and efficient method for the detection of NAD(P)H has been developed by Vreeke and co-workers in our laboratory, and is shown in Figure 7. NAD(P)H was reacted with oxygen in a homogeneous 2-electron transfer reaction which was catalysed by the 5-methyl-phenazonium ion (PMS), to produce hydrogen peroxide and $NAD(P)^+$. The hydrogen peroxide was then detected by its electroreduction to water with osmium polymer

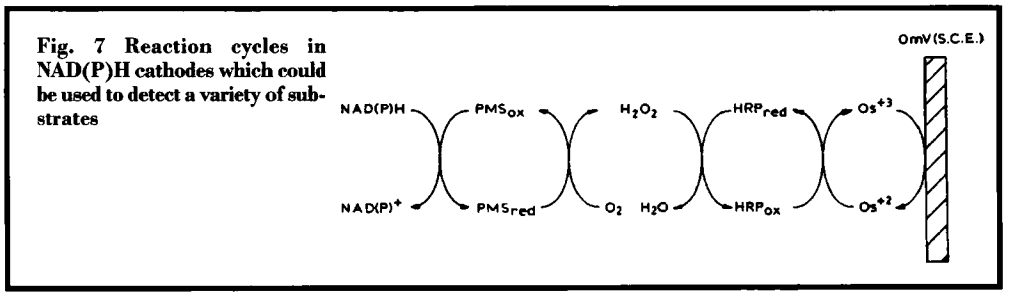

Fig. 7 Reaction cycles in NAD(P)H cathodes which could be used to detect a variety of substrates

"wired" horseradish peroxidase. Thus the NAD(P)H concentration was measured as a reducing current.

Conclusion

"Wired" enzyme biosensors have become a useful analytical tool due to the simple construction, fast response and feasibility for miniaturisation. Using crosslinked osmium polymer-based redox hydrogels, a wide array of oxidoreductase enzymes has been "wired" and several analytes can be assayed. Because "wired" enzyme electrodes require no additional reagents and are easily miniaturised, they can be implanted in the body for continuous *in vivo* monitoring of analytes, for example glucose. The technical hurdles involved with *in vivo* implantation are now being overcome and experimental use in human volunteers has begun.

Acknowledgment

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Ligand-Enhanced Biphasic Rhodium Catalyst System

Heterogeneous catalysis has a distinct advantage over catalysis in homogeneous media, in that the catalysts are easily separated from the products. Homogeneous systems with two liquid phases were developed for soluble catalysts in order to overcome this separation problem, but the catalysed reaction relies on the transfer of organic substrates into an aqueous layer, and as a result the reaction rates are much lower than those for conventional homogeneous catalysis. Two-phase, or biphasic, catalysis is used commercially in the Ruhrchemie-Rhône Poulenc process for the hydroformylation of propylene to butyraldehyde using a water-soluble rhodium complex catalyst containing triphenylphosphine trisulphonate (TPPTS).

This is an interesting variation of the hydroformylation process developed by Johnson Matthey/Davy McKee/Union Carbide in the 1970s, which uses a single phase organic reaction medium. In 1986, it was reported that 5.9 million tonnes of oxo alcohols were produced annually using homogeneous catalysis; the largest scale use of such catalysts.

However, work is now reported which highlights an interesting and useful variation of the approaches that can be used in biphasic catalysis to improve the performance of the catalyst, ("Enhancement of Interfacial Catalysis in a Biphasic System Using Catalyst-Binding Ligands", R. V. Chaudhari, B. M. Bhanage, R. M. Deshpande and H. Delmas, *Nature*, 1995, **373**, (6514), 501–503).

The authors describe research which shows that catalysis at the interface between phases in a biphasic aqueous/organic system can be enhanced by using a 'promoter ligand'. This ligand is soluble in the organic phase and insoluble in the aqueous phase, but will bind to the water-soluble organometallic catalyst, thus functioning as an enabling agent. Using this technique, the catalyst concentration is increased at the interface between the phases and promotes a significant enhancement in the reaction rate.

The ligand, which is in addition to those in the rhodium TPPTS catalyst, is insoluble in the aqueous (catalyst) phase, but has a strong affinity for the metal complex catalyst. Since the catalytic complex is insoluble in the organic medium, the reaction of the ligand, such as triphenylphosphine, with the $[Rh(COD)Cl]_2$/TPPTS (I) catalyst, (where COD is 1,5-*cyclo*-octadiene) will essentially occur at the liquid-liquid interface, and enhance the concentration of catalytic species there. Thus, they can access the reactants present in the organic phase in much higher concentrations than when the triphenylphosphine is not present. This can result in a dramatic increase in the rate of the biphasic catalytic reaction.

The authors demonstrate this new approach using the hydroformylation of 1-octene in the presence of the rhodium catalyst system (I). Here the hydroformylation proceeds at a turnover frequency in the range 10 to 50 times faster when triphenylphosphine is added.

Recent work described here and elsewhere indicates that modifications of the homogeneous technique could increase and broaden its potential. Papers which support this view include one by K. T. Wan and M. E. Davis (*Nature*, 1994, **370**, (6489), 449–450) (with comments by J. M. Brown and S. G. Davies (*ibid.*, 418–419)) where heterogeneous analogues of homogeneous catalysts are reported, involving interfacial transfer and a catalyst supported on glass microbeads. The potential for high enantiomeric excess in stereospecific synthesis is demonstrated by combining advantages of homogeneous and heterogeneous catalyst systems. These papers are an important development from earlier work on phase transfer catalysis, and with the new approaches are likely to lead in due course to more commercial applications for soluble catalyst systems based upon the platinum group metals. D.T.T.