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Electrochemical biosensors on thin-film metals and conducting polymers

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ABSTRACT

Electrochemical biosensors using advanced thin-film technology employing 1,4-arenequinones substituted with at least two halogens in *para* positions as new agents for the immobilization of enzymes are described. For special applications, thin-film electrodes were combined with permeation-selective polypyrrole layers as sensory modifying devices.

INTRODUCTION

The interest in miniaturized biosensors in medicine, food and process technology has increased significantly in the last few years[1–7] and a wide variety of oxidase-based biosensors have been developed. However, various problems have to be solved in order to allow the construction of satisfactory sensors for practical use in large numbers. The stability of the immobilized biosystem is very important, *e.g.*, enzyme (even under dry storage conditions) and can be increased by stable and efficient coupling procedures. The selectivity of the electrochemical transducer and the reproducibility with respect to response and temperature dependence must not be neglected[8–12]. Thin-film technology is able to provide the high purity and reproducibility required of the electrode surface and the high spatial resolution of the electrode structure. Our aim was to obtain devices for the rapid and highly reproducible measurement of small samples.

For microdevices, stepwise derivatization starting from the metal electrode surface turned out to be the most practical procedure. To achieve this aim two different techniques were studied and compared with each other: covalent coupling procedures with halogeno-1,4-arenequinones[13–15] on flat ultra-thin metal films, and covalent

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immobilization of enzyme on electrochemically polymerized, porous, substituted polypyrroles as top layers on vapour-deposited metal films.

EXPERIMENTAL

Materials

Platinum, palladium, rhodium and titanium were purchased from Balzers (Vaduz, Liechtenstein). Silicon nitride was prepared by plasma-enhanced chemical vapour deposition from silane, nitrogen and ammonia, all from Matheson (Oevel, Belgium). The negative lift-off photoresist AZ 1350 was from Hoechst (Wiesbaden, F.R.G.). Buffer substances, iron(III) chloride, potassium dichromate, nitric acid, bromine, sodium sulphate, calcium chloride, tin(II) chloride, titanium(III) chloride, hexamethyldisilazane (10% solution in xylene), N-cyclohexyl-N′-[2-(N-methylmorpholino)ethyl]carbodiimide 4-toluenesulphonate, *p*-chloranil, tetrabutyl tetrafluoroborate, organic solvents (acetone, toluene, ethanol, acetonitrile, acetic acid, etc.) and triethylamine were from Merck (Darmstadt, F.R.G.). *p*-Fluoroanil and *p*-bromoanil were purchased from Lancaster Synthesis (White Lund, U.K.). Glucose oxidase (GOD) (E.C. 1.1.3.4), galactosidase (E.C. 3.2.1.23), naringinase (E.C. 3.2.1.21 and 3.2.1.40), alkaline phosphatase (E.C. 3.1.3.1) and glucose-6-phosphate were supplied by Sigma (Deisenhofen, F.R.G.). Nafion (10% solution in water) and aliphatic alcohols was purchased from Aldrich (Steinheim, F.R.G.). 3-Aminopropyltriethoxysilane, lithium perchloride and tetrabutyl hexafluorophosphate were from Fluka (Buchs, Switzerland). Pyrrole, obtained from Merck, was distilled twice and stored at −20°C in the dark.

Preparation of the sensor electrode

Mechanically stable sodium silicate glass sheets of thickness 100–300 μm were used as electrode carriers. After standard cleaning procedures with detergents, ultrasonication and organic solvents, metals were evaporated by an electron gun in a high-vacuum instrument (Balzers) and used to coat glass sheets with thin titanium layers up to a thickness of 50–100 nm as an adhesion layer. Platinum, rhodium or palladium layers up to a thickness of 100 nm, evaporated on top of the titanium film, act as electrochemical electrodes. Structuring of these thin films was performed by a lift-off technique with AZ 1350 photoresist. These layers were isulated by a 3-μm silicon nitride layer and structurized by plasma etching[16]. The platinum surface was cleaned by etching with an oxygen plasma (30 W) for 3 min and if necessary the surface could now be hydrophobized by dipping into a 10% solution of hexamethyldisilazane in xylene for 15 min.

Using these procedures, three different electrode types were constructed. (i) A single 0.64 mm^2 Pt/Ti sandwich working electrode (Fig. 1a) was made in order to test chemical processes and instrumentation. (ii) A three-electrode miniaturized electrochemical cell (Fig. 1b) with an outer diameter of 200 μm was produced for further studies. The Ag/AgCl reference electrode was produced by evaporating and structuring a 1-μm thick silver film, which was subsequently chlorinated with 10 m*M* iron(III) chloride to obtain a thickness of 2 μm. (iii) A four-electrode electrochemical cell (Fig. 1c) with an outer diameter of 2500 μm and possessing two identical working electrodes was produced for differential measurements.

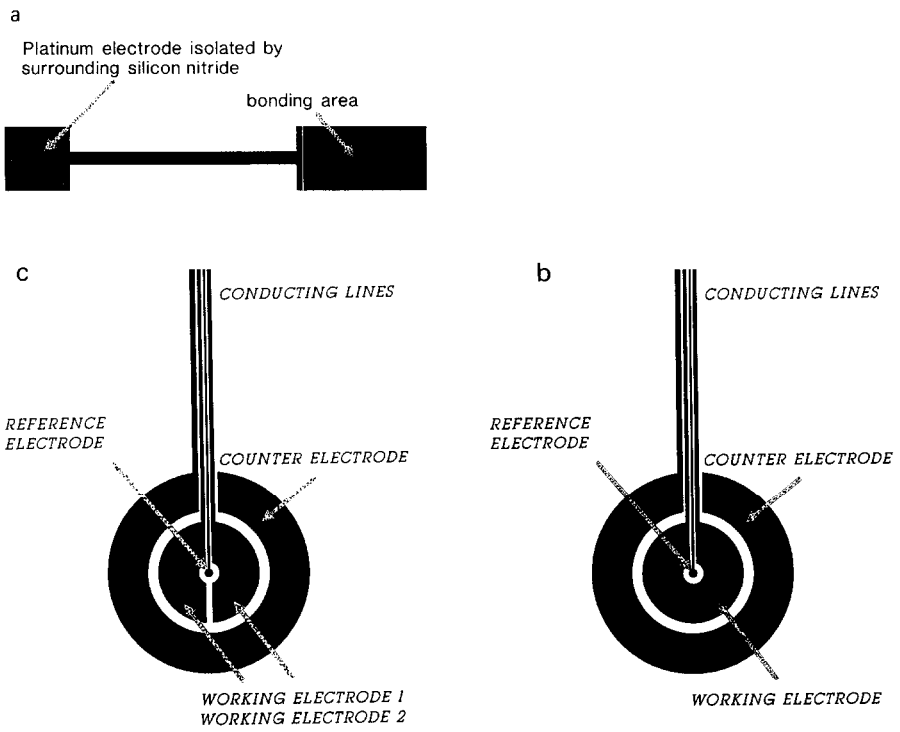


Fig. 1. Electrode types: (a) test electrode; (b) three-electrode type; (c) four-electrode type.

Preparation of the biosensors using 1,4-arenequinones[13–15,17] *(Fig. 2)*

Activation of the metal surfaces. Platinum electrodes were oxidized either in a 2.5% solution of potassium dichromate in 15% nitric acid at a potential of 2.5 V *vs.* Ag/AgCl for 2 min or by a coupled oxygen plasma. Palladium and rhodium electrodes were oxidized in a solution containing sodium nitrate instead of nitric acid. The oxidized electrodes were rinsed with water, followed by acetone, dried and imemdiately derivatized with silane.

Coupling with 3-aminopropyltriethoxysilane. The oxidized electrodes were dipped into a 10% solution of silane in toluene and kept at 60°C for 30 min. The silylated surface was cleaned with toluene and ethanol. An alternative procedure was spinning of a 1% solution of the silane in propanol–water (95:5, v/v) on the electrodes at 3000 rpm for 30 s and drying in an oven at 90°C for 3 min.

Reaction with p-chloranil (Fig. 3). The silylated electrodes were kept in a 1% solution of *p*-choranil in toluene at 40°C for 30 min. The electrodes were rinsed several times with toluene followed by acetone. The derivatized electrodes were stored dry in the dark.

Reaction with modified p-quinones (Fig. 4). In addition to *p*-chloranil, other derivatives such as *p*-fluoroanil, *p*-bromoanil and related quinones were used to activate the electrode surface for coupling of the enzyme.

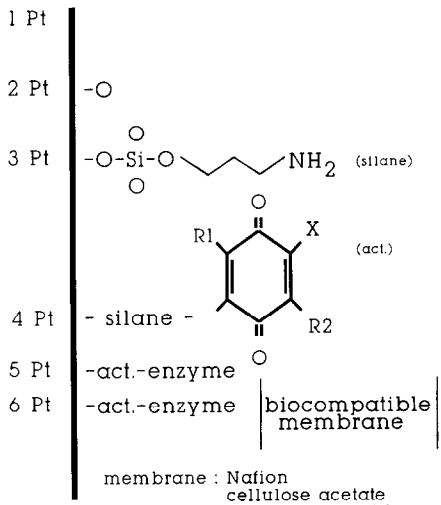


Fig. 2. Immobilization steps: 1 = untreated electrode; 2 = oxidized surface; 3 = derivatization with silane; 4 = activation; 5 = enzyme coupling; 6 = membrane coating.

Coupling of the enzymes. To couple glucose oxidase to the activated surface, the electrodes were immersed in a solution of 5 mg/ml glucose oxidase in 0.1 *M* phosphate buffer (pH 7.0) for 2 h. The electrodes were rinsed several times with 4 *M* phosphate-buffered saline to eliminate adsorbed protein. Immobilized apo-glucose oxidase (GOD freed from its FAD coenzyme)[18] was used for an apoenzyme reactivation assay and, likwise, *β*-galactosidase, naringinase and alkaline phosphatase (using glucose-6-phosphate as a substrate) in a GOD coupled assay.

Coating of the electrodes[19]. A polymeric top layer may be applied to prevent unwanted adsorption of most proteins and to minimize clotting effects in biological

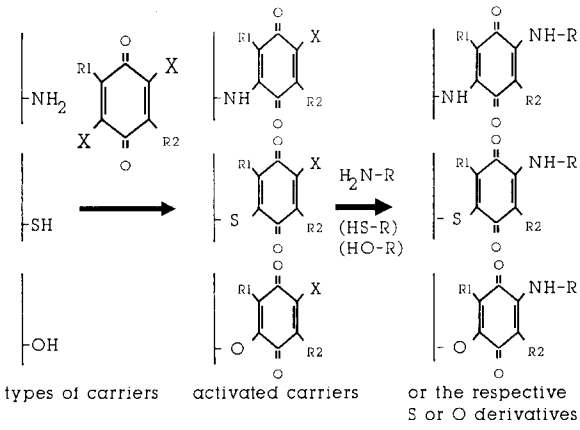
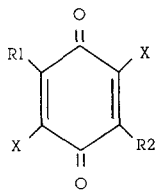


Fig. 3. Reaction scheme of immobilization with halogeno-1,4-arenequinones.



X = halogen
R1, R2 = halogen, R, COR, COOR,
C_1 - C_8 alkyl, COOH, CN, CNS, NO_2, N_3

Fig. 4. Halogeno-1,4-arenequinone reagents for enzyme coupling.

samples. Using Nafion (a perfluorinated and sulphonated polymer) or other polysulphonic acids a further ion sieving effect can suppress unwanted inferences of anionic compounds. A 1-ml volume of Nafion solution was buffered by the addition of 20 μl of triethylamine and 25 μl of acetic acid. The electrodes were either dipped into the solution or the solution was sprayed or spun onto the surface. The solvents were evaporated with water-saturated nitrogen at room temperature for 10–30 min.

Preparation of 3- and N-substituted pyrroles

The following pyrroles were studied: (1) 1-(Ω-carboxymethyl)pyrrole, (2) 1-(Ω-carboxyethyl)pyrrole, (3) 1-(Ω-carboxypropyl)pyrrole, (4) 1-(Ω-carboxypentyl)pyrrole, (5) 1-(Ω-carboxydecyl)pyrrole, (6) 1-(1,3-dicarboxypropyl)pyrrole, (7) 1-[(1-carboxy-3-methyl)butyl]pyrrole, (8) 2-(1-pyrrolo)acetylglycine, (9) 1-dodecylpyrrole, (10) 1-(4-carboxybenzyl)pyrrole, (11) 1-(4-nitrophenyl)pyrrole, (12) 1-(4-carboxyphenyl)-pyrrole, (13) 3-carboxymethyl-4-methylpyrrole, (14) 1-*o*-tosylpyrrole, (15) 1-*p*-tosyl-pyrrole, (16) 1-benzenesulphonylpyrrole, (17) 4-(3-pyrrolo)-4-hydroxybutyric acid, (18) 4-(3-pyrrolo)-4-ketobutyric acid, (19) 3-[(hydroxy-4-nitrophenyl)methyl]pyrrole and (20) 3-[(keto-4-nitrophenyl)methyl]pyrrole.

Pyrroles 1–12 were prepared according to the method of Giuliano *et al.*[20], 13 according to the method of van Leusen *et al.*[21], 14–16 by a new synthesis based on the procedure of Giuliano *et al.*[20] and 17–20 by a new synthesis based on the procedure of Rokach *et al.*[22] followed by 3-alkylation.

Preparation of biosensors using substituted polypyrroles

Cleaning of the electrode. Platinum thin-film electrodes were cleaned by ultrasonication in distilled water, rinsed with acetone and dried carefully under dust-free conditions. The electrodes were cycled five times in acetonitrile–2.5% lithium perchlorate between −500 and 1800 mV (100 mV/s) *versus* Ag/AgCl using a three-electrode configuration of an electrochemical cell. A potentiostat interfaced by a 14-bit analog-to-digital, digital-to-analog converter to an AT personal computer was employed for generating the required voltages.

Coating with polymerized substituted homopolypyrroles[23]. Pyrroles 1–20 were used as monomers for the coating of platinum electrodes with thin polymeric layers. A 0.5% solution of these compounds in acetonitrile containing 2.5% of $LiClO_4$, NR_4BF_4 or NR_4PF_6 (R = CH_3, C_2H_5, C_4H_9) was dried over sodium sulphate or

calcium chloride for several hours. The clear solution was transferred to a glass cell and bubbled with argon for 10 min. The electrode immersed in the deaerated solution was then cycled 10–30 times between −300 and +1600 mV (100 mV/s). If the substituted pyrrole was polymerizable the formation of a thin, brown polymeric layer was observed.

Coating with polymerized heteropolypyrroles. It is necessary to make a copolymer having a high content of unsubstituted polypyrrole in order to obtain thick and stable substituted polypurrole films. As the polymerization speed of the substituted pyrroles is significantly lower than that of unsubstituted pyrrole it is nevertheless necessary to have a 10:1 excess of the substituted monomer. The electrolyte of the above-mentioned composition was complemented with an additional 0.05% of pyrrole. The electrode was cycled up to ten times between −300 and +1600 mV (100 mV/s). A black polymer layer of varying thickness was obtained.

Coupling of the enzymes. A variety of methods were employed to activate the terminal carboxylic acid groups. The use of water-soluble carbodiimides may be recommended for the best results. The polypyrrole-coated electrodes were incubated with a saturated solution of N-cyclohexyl-N′-[2-(N-methylmorpholino)ethyl]carbodiimide 4-toluenesulphonate for 60 min at 25°C without shaking. The electrodes were rinsed several times with water and immediately reacted with a 5 mg/ml enzyme solution in 0.1 *M* phosphate buffer (pH 7.0) for 2 h. Substituted polypyrrole films having nitro groups may be reduced using a solution of 15% titanium(III) chloride or 1% tin(II) chloride in 10% hydrochloric acid for 30 min forming a film with pendant amino groups. After thorough rinsing with dilute hydrochloric acid, distilled water and ethanol, these electrodes can be dried. Now 1,4-arenequinones can be employed to couple the enzyme using the same activating and immobilizing procedure as mentioned above.

The sensors thus obtained were rinsed with phosphate buffer and water and stored at 4°C. In addition to the enzymes mentioned above, ADH was used to study immobilized dehydrogenases.

RESULTS

Our aim was to construct biosensors that can be produced in large numbers with high reproducibility, stability and selectivity for commercial use. Given the well defined structure of the microelectrodes, we developed and studied two different strategies: covalent coupling on thin-film metals with a new immobilization technique and covalent coupling on modified polypyrrole layers.

Covalent immobilization with 1,4-arenequinones (Figs. 3 and 4)

For optimum results a defined covalent coupled monolayer should be achieved avoiding enzyme membranes or cross-linked gels using bifunctional reagents, both having characteristic disadvantages due to the varying thickness of the enzyme layer or mechanical and chemical instability.

Covalent coupling of glucose oxidase, naringinase, β-galactosidase and alkaline phosphatase was carried out employing an improved technique according to our recently desorbed method[13,14] adapted from the chemistry of quinones. 1,4-Arenequinones substituted with at least two halogens or pseudohalogens in *para* positions

are highly reactive as regards nucleophilic substitution. Amino and thiol groups react with the quinone compound, replacing two *para*-halo groups of the reagent. A related method with a different reaction mechanism is the immobilization of proteins by a redox coupling reaction with *p*-benzoquinone[24,25].

When using immobilized amines or thiol groups on the electrode surface, the quinone reagent reacts at one halogen position, eliminating hydrohalogenic acid. Thus the halogen atom in a *para* position is highly activated by mesomeric and inductive effects. Electrode material activated in this way may be stored dry and in the dark for at least 6 months.

To couple the enzyme, the activated electrodes should be dipped in a buffered solution of the enzyme for 1–12 h followed by a washing procedure (see Experimental) to eliminate adsorbed protein. The activated *para*-halogen atom is now capable of reacting with the amino and mercapto groups of proteins, peptides and coenzymes.

The influence of the X substituents (reactive halogen) (see Fig. 4) on the coupling efficiency of the immobilization of enzymes had been characterized: on varying the reactive halogen from F to Cl and Br no alteration in the surface enzyme coating of the electrodes could be observed. Changing the reactive halogen group from F to Cl and Br altered the reactivity, resulting in an increased coupling time. Using tetrahalogen compounds (*p*-fluoroanil, *p*-chloroanil and *p*-bromoanil) highly reproducible results were obtained[13,14].

As reactive groups of proteins, peptides and ligands being able to couple to the activated support, ε-amino groups of lysine or α-amino groups of amino acids as well as thiol groups of cysteine were characterized by the use of model compounds. Very stable bonds were formed, immobilizing proteins and ligands, by this technique. This immobilization technique was also adapted to porous glass and silica supports, polyacrylhydrazide, Enzacryl AA and SH, partially hydrolysed nylon membranes and aminolysed polyesters for the construction of multi-enzyme sensors.

The same method permitted the consecutive coupling of glucose oxidase as defined multi-layers to the same electrode, causing an increase in the electrode response.

Characterization of a glucose sensor based on this immobilization technique

The change in response as a function of voltage, substrate concentration and

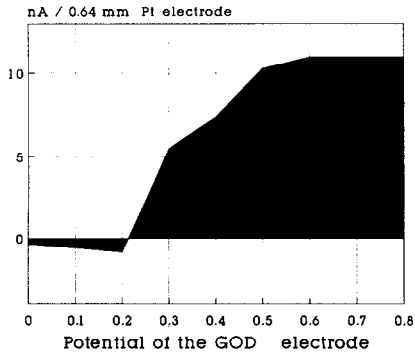


Fig. 5. Voltage (V) dependence of the electrode response (*vs.* Ag/AgCl). Shaded area: response 0.1 *M* phosphate buffer (pH 7.0); glucose = 820 mg/l (physiological concentration).

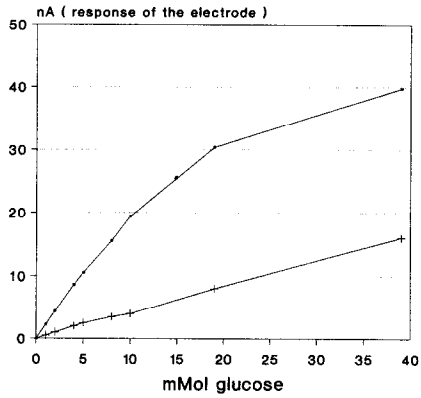


Fig. 6. Calibration graph for the Pt thin-film metal glucose oxidase electrode (0.64 mm^2). Immobilized with tetrachloro-1,4-benzoquinone. 0.1 *M* phosphate buffer (pH 7.0). • = Without Nafion; + = with Nafion.

time was studied in order to characterize the electrode (Figs. 5–7). Under optimum conditions the electrodes working at a potential of 500 mV *vs.* Ag/AgCl gave a linear response up to 12 m*M* without any further diffusion-limiting membrane. By covering the electrode with a dip-coated Nafion membrane, the linear range for glucose was extended to 50 m*M*.

Immobilization on substituted polypyrrole layers

A number of glucose sensors have already been constructed involving the immobilization of glucose oxidase by copolymerization with pyrrole and adsorption on polypyrrole surfaces. Free and immobilized redox-mediating compounds such as ferrocene or dimethylferrocene have been employed to enhance the redox transfer from FAD to the polypyrrole layer[26–28]. However, senors based on this principle have many disadvantages: low operational and storage stability; stability and linearity of response only in deaerated solutions; and low selectivity of the polypyrrole electrochemistry. By comparing the properties and limits of the above-mentioned electrodes, two fundamental advantages of electrodes covered by substituted polypyrrole layers having no redox activity were established: about a 20-fold increase in response per unit area due to the porous surface and significant permeation control by the polymeric

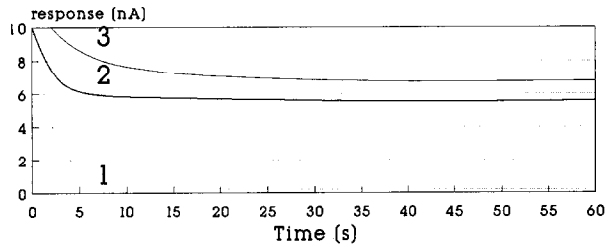


Fig. 7. Time dependence of electrode response. 1 = 0.1 *M* phosphate buffer (pH 7.0); 2 = 5 m*M* glucose buffer, stirred; 3 = 5 m*M* glucose buffer, unstirred.

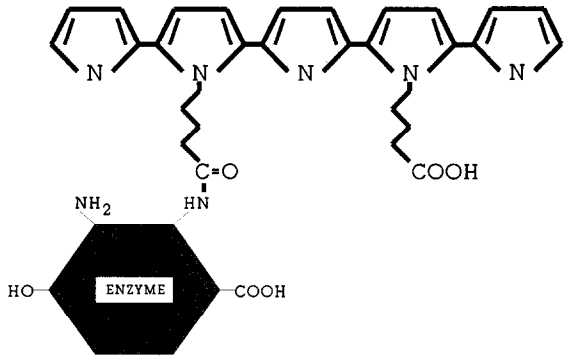


Fig. 8. Immobilization of enzymes to polypyrrole heteropolymers.

layer for interfering electroactive substances resulting in a distinct increase in selectivity (*e.g.*, 50–200-fold supression of ascorbic acid in glucose monitoring).

Optimum results for polypyrrole sensors described in this paper could only be obtained by synthesizing modified polypyrrole layers without significant conductivity and redox activity. For these electrodes, the electrocatalytic reaction should take place at the metal surface, otherwise high background currents and low selectivity will be obtained. Thus, the substituted polypyrrole layer acts as a highly effective barrier for interfering redox active compounds such as ascorbic acid, bioactive amines, sulphydryl-containing peptides and proteins, etc., but having a high permeability for, *e.g.*, hydrogen peroxide.

Various N- and 3-substituted pyrrole monomers were synthesized (see Experimental) in order to prepare layers posessing these properties. The synthesized pyrrole

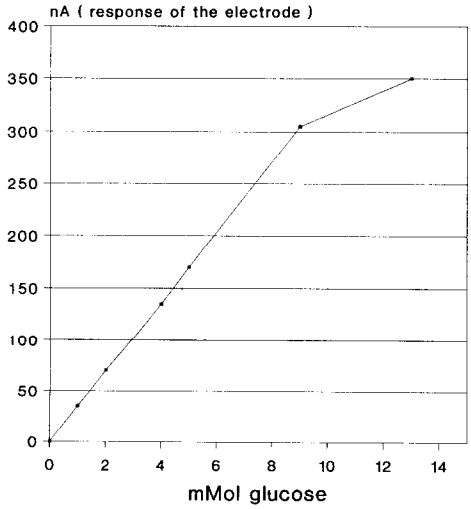


Fig. 9. Calibration graph for an N-alkylpolypryrrole glucose oxidase electrode (0.64 mm^2). 0.1 *M* phosphate buffer (pH 7.0).

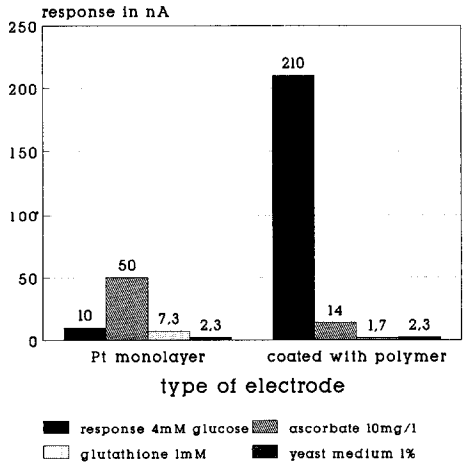


Fig. 10. Selectivity of the electrodes (0.64 mm^2). Comparison of a Pt–GOD monolayer and a Pt–polymer–GOD monolayer electrode. 0.1 *M* phosphate buffer (pH 7.0). Temperature, 25°C; potential, 500 mV.

derivatives were tested for their ability to form polymers. Polymeric films could be obtained with pyrroles 1–5, 8–11, 13 and 17–20. Homopolymers, heteropolymers and sandwich types of these polymer layers were prepared. The various layer types led to different properties of the resulting enzyme sensors. Satisfactory homopolymer layers could only be formed with pyrrole derivatives having no bulky side-groups directly attached to the pyrrole ring (*e.g.*, arenes). In synthesizing heteropolymers it was vital to consider that the compound with the more positive polymerization potential will be incorporated into the polymer to a much lower extent than its concentration in the polymerizing solution would normally allow.

It is necessary to introduce functional groups which are stable against oxidation under polymerizing conditions in order to obtain a modified polypyrrole layer which can be used for covalent coupling of enzymes. COOH and NO_2 groups have proved to be most suitable for this purpose. Not too hydrophobic polypyrrole layers containing carboxy groups turned out to give the best results (Fig. 8).

Characterization of electrodes

Typical properties of this type of sensor can be exemplified with glucose oxidase. The change in response as a function of substrate concentration and selectivity was studied in order to characterize the electrode (Figs. 9 and 10). The change in response as a function of voltage has the same shape as shown in Fig. 5, but the data must be multiplied by a factor of 20. Under optimum conditions the electrodes working at a potential of 500 mV *vs.* Ag/AgCl gave a linear response up to 9 m*M* without any further diffusion-limiting membrane. By covering the electrode with a polymer membrane, the linear range for glucose was extended to 50 m*M*.

CONCLUSION

The comparison of the two electrode types using immobilized glucose oxidase as

an example shows the advantages and limitations of the two sensor types, as follows.

A 0.64 mm^2 Pt–halogenoarenequinone immobilized glucose oxidase sensor has a response of 8–12 nA with 5 m*M* glucose–phosphate-buffered saline (pH 7.0) at 25°C. Porous polypyrrole immobilized glucose oxidase sensors have a 10–50-fold greater response per unit sensor surface area owing to a significant increase in the inner surface area.

The temperature dependence of the signal is 5%/°C and independent of the electrode type.

The base current is 0.2 nA per 0.64 mm^2 for the monolayer type and 1.5–3 nA/mm^2 for the polymer-coated type.

The storage stability is independent of the electrode type, showing a decrease in response per year of *ca.* 38% if stored in aqueous 4 *M* sodium chloride at 4°C.

With both electrode types the response time is in the range of a few seconds, increasing if the electrode is covered by a diffusion-limiting membrane.

The response of polymer-coated electrodes is nearly independent of fluctuations in the test solution in the vicinity of the sensors owing to the coverage of the enzyme layer by the polymer. Monolayer electrodes, being sensitive to these fluctuations, can be protected by a diffusion-limiting membrane (*e.g.*, Nafion), which supresses this effect.

The reproducibility of the immobilizing surface is optimum for thin-film metal electrodes; on the other hand, reproducibility is difficult to achieve when immobilizing on the internal surface of a polymer layer. Hence the reproducibility of the sensor response is significantly better on coated thin-film metal electrodes than on polymer-coated types.

To avoid unwanted adsorption layers on top of the enzyme film reversibly decreasing the electrode response in the range 10–40%, both electrode types should be covered with a hydrophilic membrane (*e.g.*, Nafion).

The non-specific response of interfering redox-active substances (*e.g.*, ascorbic acid, reduced glutathione, phenacetin) can be supressed by either using the four-electrode structure and employing differential measurement or using the polypyrrole type of electrodes exhibiting permeation-selective properties.

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