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Electrocatalytical properties of polymethylferrocenyl dendrimers and their applications in biosensing

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Abstract

The electrochemical characterization of polymethylferrocenyl dendrimers deposited onto a platinum electrode and their applications as hydrogen peroxide and glucose sensor are described. The redox dendrimers consist of flexible poly(propileneimine) dendrimer cores functionalised with octamethylferrocenyl units. Amperometric biosensors for glucose were prepared by immobilization of glucose oxidase onto these modified electrodes. The influence of the dendrimer generation and the thickness of the dendrimer layer, the effect of the substrate concentration, and the interferences and reproducibility on the response of the sensors were investigated.

Keywords: Polymethylferrocene; Dendrimers; Modified electrodes; Glucose oxidase; Amperometric biosensor

1. Introduction

In the last years, a new class of macromolecules, dendrimers, have been synthesized and described [1,2]. The dendrimers can be deposited onto several electrode surfaces by forming layers that exhibit high mechanical stability and can be functionalised without loss of material from the electrode surface. Moreover their highly branched structure and their large area becomes a useful material for enzyme immobilization and biosensors fabrication [3,4].

Dendritic macromolecules containing a controlled number of redox-active organometallic units at the core, within the branches or at the periphery of the dendritic structure are good candidates as catalyst systems. In the last years we have investigated redox-active organometallic silicon- and nitrogen-based dendritic and polymeric materials containing ferrocenyl and permethylferrocenyl groups [5–9].

While macromolecules containing ferrocenyl moieties are numerous, polymers constructed from polymethylferrocene monomers have been relatively less explored. However, organometallic compounds containing permethylcyclopentadienyl ligands are interesting since they often exhibit significantly different properties to those of their non-methylated analogues. As a result of the enhanced electron donating ability of permethylated cyclopentadienyl rings, polymethylferrocenyl derivatives exhibit lower redox potentials. In addition, it is well-known from studies of monomeric ferrocene mediators that modification of the cyclopentadienyl ring with methyl substituents increases the rate of the electron transfers [10,11]. These facts promise to be of importance when these compounds are used as mediators for biosensors in order to increase their efficiency and minimize interferences [12].

Amperometric determination of hydrogen peroxide is of great importance for analytical purposes [13]. Several ferrocene compounds have been reported as useful electrocatalysts for H_2O_2. The redox polymer poly(vinylferricinium) has been described as a suitable mediator for H_2O_2 electro-oxidation [14]. Also siloxane-based polymers containing electronically communicated ferrocenyl groups have shown to be used as efficient electrocatalysts for the electro-oxidation and/or the electro-reduction of hydrogen peroxide [15,16]. This effect of the ferrocene centre was attributed to its capability of electron mediation between H_2O_2 and the electrode.

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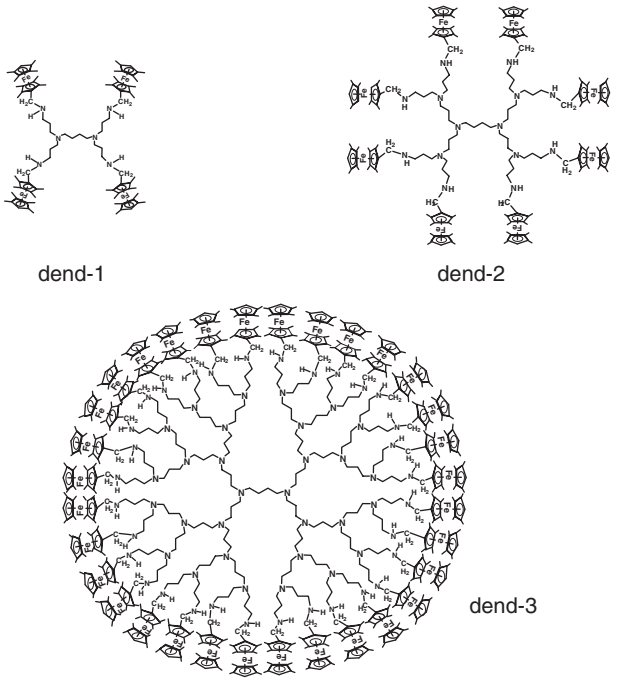


Scheme 1.

The use of electrodes for the amperometric detection of hydrogen peroxide is a suitable way of constructing oxidase-based enzyme sensors that has been widely developed [17,18]. For continuous measurements the activity of the enzymes should be regenerated after the catalytic reaction and therefore, enzyme electrodes based on monitoring direct electro-reduction, or electro-oxidation of H_2O_2 generated by the enzymatic reaction can be used when oxygen acts as the natural electron acceptor for restoring the active form of the enzymes. However, the major limitation of these techniques is the high operating potential required for detecting H_2O_2, which makes these devices susceptible to interfering substances. One of the methods aimed to minimize the interferences and improve the sensitivity of sensors is the modification of the electrode surface with catalysts which are able to decrease the overvoltage of the redox reaction of hydrogen peroxide [19,20]. The development of these amperometric enzyme biosensors has included several techniques for the immobilization of enzymes on chemically modified electrodes.

In this article we report the preparation and electrochemical and enzymatic characterization of amperometric enzyme electrodes based on glucose oxidase immobilized on Pt electrodes modified with octamethylferrocenyl dendrimers (Scheme 1), and their use for the determination of glucose under aerobic conditions.

2. Experimental

2.1. Reagents

The synthesis of octamethylferrocenyl dendrimers with up to 32 redox centres is based on the reductive amination of octamethylformylferrocene with the different generations of diamino-based poly(propileneimine) dendrimers functionalised with terminal NH_2 groups. In the first step the amino functions of the dendrimer were condensed with the aldehyde. The subsequent reduction of the formed imino groups led to dendrimers with 4, 8 or 32 octamethylferrocenyl moieties per molecule. The structures were straightforwardly established on the basis of multinuclear (^1H and ^{13}C) NMR data, IR spectroscopy, and mass spectrometry (MALDI-TOF). The synthesis, complete characterization and electrochemical studies will be published elsewhere [21].

Hydrogen peroxide 30% was supplied from Fluka. Glucose oxidase (GOx) from *Aspergillus niger* (type VII, 185,000 μ/g), bovine serum albumin (BSA), glutaraldehyde (25 wt.% solution in water) and glucose were supplied from Sigma. Glucose solutions were allowed to reach mutarotational equilibrium at room temperature for 24 h before use. All other chemicals were analytical grade and were used without further purification. Ultrapure water was used for preparation of the buffers, standards and electrochemistry work.

2.2. Apparatus

Electrochemical measurements were performed using an Ecochemie BV Autolab PGSTAT 12. All experiments were carried out in a conventional three-electrode cell at 20–21 °C. A Pt disk of 3 mm diameter as working electrode, a Pt wire as auxiliary electrode, and a saturated Calomel reference electrode (SCE) were employed. In steady-state measurements, a Metrohm 628-10 rotating electrode was used. All amperometric measurements were performed in 0.1 M phosphate buffer with 0.1 M $NaClO_4$ (pH 7.0). In hydrogen peroxide determination, all solutions were deoxygenated by bubbling high-purity nitrogen for at least 15 min. The solutions for glucose measurements were saturated. The background current was allowed to decay to a steady value before aliquots of stock hydrogen peroxide or glucose solution were added.

2.3. Electrode preparation

The Pt disk electrode was polished using 0.1 μm alumina powder and rinsed with water in an ultrasonic bath. The electrode surface was then conditioned by cycling the potential between the limits for hydrogen and oxygen evolution in 0.5 M H_2SO_4 solution until well-defined cyclic voltammograms were obtained, and then rinsed with water.

The dendrimer modified electrodes were prepared by evaporation of 5 μl CH_2Cl_2 solution containing the appropriate amount (about 0.02 μmol Fc/ml) of the corresponding dendrimer (dend-1–3) in order to get the wanted coverage on top of the above prepared electrode surface and then allowing them to dry in air at room temperature. The surface coverage of electroactive octamethylferrocenyl sites in the films, Γ, was determined from the integrated charge of the cyclic voltammetric waves.

The biosensors were constructed by cross-linking using a bifunctional group. To immobilize the enzyme, 5 μl of 5 units GOx in pH 7.0 phosphate buffer containing 40 μg BSA was

applied to the modified electrode surface and then the electrodes were kept for 10 min in glutaraldehyde vapour at room temperature. The prepared enzyme electrodes were allowed to dry in air, and rinsed thoroughly with the buffer.

The dendrimer modified and enzyme electrodes were stored at room temperature and at 4 °C, respectively, when not in use.

3. Results and discussion

3.1. Electrode characterization

The electrochemical properties of the dendrimers have been investigated by cyclic voltammetry (CV) in homogeneous solution, as well as with the materials confined onto electrode surfaces [21]. For all generations of this family (dend-1–3) a reversible oxidation process is observed, which is clearly assigned to the simultaneous one-electron oxidation at the same potential of the peripheral octamethylferrocenyl units.

All dendrimer films deposited onto platinum electrodes exhibit in aqueous and non-aqueous solutions a similar electrochemical response (Fig. 1), which is reminiscent of that of the corresponding compounds in solution. One well-defined, reversible system is observed with a formal potential value of about 0.05 V (versus SCE). The voltammetric features unequivocally indicate the surface confined nature of the electroactive dendrimer films. The results found indicate that for sweep rates below about 500 mV s^{-1} these films exhibit rapid electron and charge transfer kinetics.

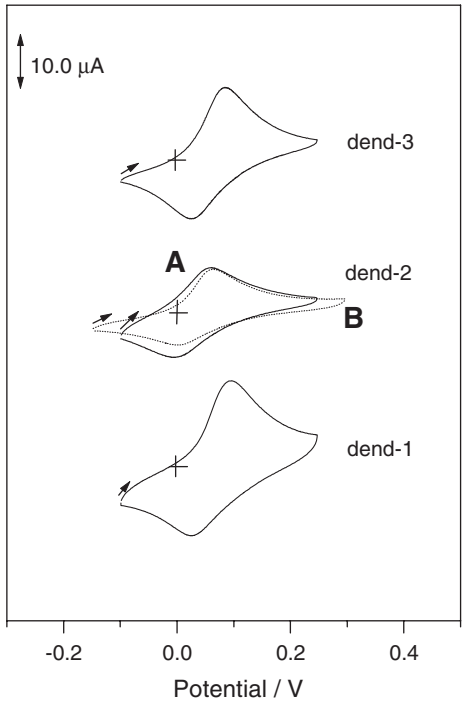


Fig. 1. Cyclic voltammograms of dend-1, dend-2 (curve A) and dend-3 deposited at platinum electrodes ($\Gamma = 1.7\times10^{-9}$, 1.0×10^{-9} and 1.6×10^{-9} mol ferrocene cm^{-2} thickness film, respectively) in deaerated CH_3CN/TBAH 0.1 M. Curve B corresponds to the dend-2 modified electrode in deaerated phosphate buffer (pH = 7.0)/$NaClO_4$ 0.1 M. Scan rate: 100 mV s^{-1}.

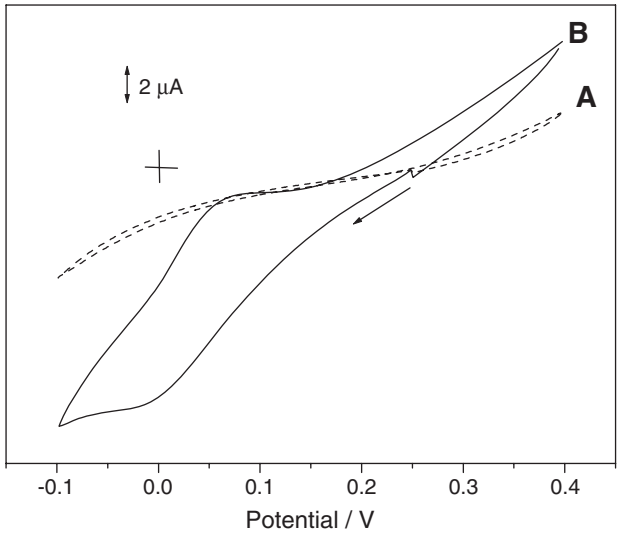


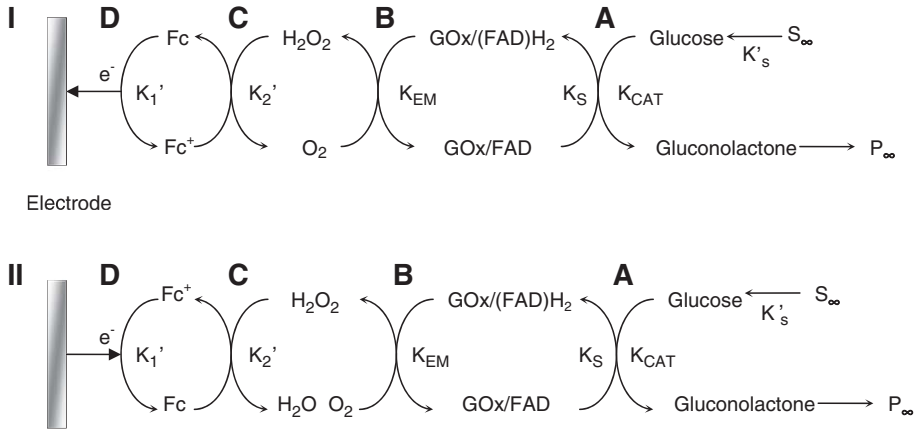
Fig. 2. Cyclic voltammograms of (A) a platinum bare and (B) a dend-2 ($\Gamma = 1.07\times10^{-9}$ mol ferrocene cm^{-2} thickness film) coated platinum electrode in deaerated phosphate buffer (pH = 7.0) solution, in the presence of hydrogen peroxide 5 mM. Scan rate 50 mV s^{-1}.

The immobilized dendrimer cannot be undergone to potentials above 400 mV (versus SCE) because the oxidation of amine groups takes place and the dendrimer gets damaged irreversibly [22].

3.2. Response to hydrogen peroxide

The electrochemical catalytic reduction of H_2O_2 by dendrimer films was examined by cyclic voltammetry. Fig. 2 displays cyclic voltammograms for 5 mM hydrogen peroxide in aqueous phosphate buffer at bare and dend-2-modified-platinum electrodes. Similar results were obtained with all dendrimers. It can be seen that the cathodic peak corresponding to the ferricinium groups in the electrodeposited film (see also Fig. 1) is enhanced in the presence of hydrogen peroxide, whereas no anodic peak is observed. This behaviour indicates that the dendrimer acts as mediator for the electrocatalytic reduction of H_2O_2 from about +0.05 V. Actually, the process might be explained by the oxidation of the ferrocenyl moieties in the dendrimer immobilized on the electrode by hydrogen peroxide and re-reduction of the ferricinium groups at suitable applied potentials [23].

The voltammogram B in Fig. 2 also shows that the oxidation wave of H_2O_2 at the modified electrode is cathodically shifted and the current values are substantially higher than those obtained with the uncoated surface. This wave appears located at a more positive potential than that corresponding to the ferrocene system. In this case, the standard potentials of both species are close and the oxidation of hydrogen peroxide needs an overvoltage to occur. The CV shows that the overvoltage is substantially smaller than the overvoltage needed when a Pt bare electrode is used. This behaviour with the modified electrode can be explained by the electrocatalytic effect of the



Scheme 2.

dendrimer deposited on the Pt surface. The dendrimer decreases the activation energy, E_a, of the electrochemical oxidation of H_2O_2 in comparison with a Pt bare electrode. The dendrimer-modified electrode offers a higher oxidation response over the +0.25 to +0.40 V range, due to its enhanced electrocatalytic activity. It has been already mentioned that the use of working potentials above +0.45 V produces irreversible changes in the electrochemical behaviour of the electrode (see above).

Since the aim of this work is to develop a biosensor for the determination of hydrogen peroxide, both direct and produced by an enzymatic reaction (Scheme 2) when oxygen is the natural mediator, it is necessary to evaluate the oxygen response of the dendrimer modified electrode. In order to know the influence of the possible interference of oxygen, the electrochemical reduction of oxygen and hydrogen peroxide at the dend-2-modified electrode was studied by rotated disk voltammetry (Fig. 3). As it can be seen, the reduction of oxygen at a bare Pt electrode occurs at much more negative potentials than at a dendrimer modified electrode which is clearly indicative of an appreciable electrocatalytic effect over the reduction of oxygen too. However, the reduction wave of H_2O_2 appears at a more positive potential than that corresponding to the reduction of dissolved oxygen. A similar behaviour has been previously reported for the electrochemical reduction of H_2O_2 at TiO_2 and Prussian Blue modified electrodes [24,25].

Therefore, although dendrimers are efficient electrocatalysts for the reduction of both hydrogen peroxide and oxygen, the linear voltammograms show that the electrochemical reduction of H_2O_2 generated in the enzymatic reaction can be successfully used for direct measurement of glucose by applying operating potentials in the range from 200 to 100 mV (versus SCE).

Fig. 4 shows the response of the dendrimer electrodes to hydrogen peroxide measured as a steady-state, anodic and cathodic, current in phosphate buffer solution. Due to limitations of the applied potential in the subsequent cathodic glucose

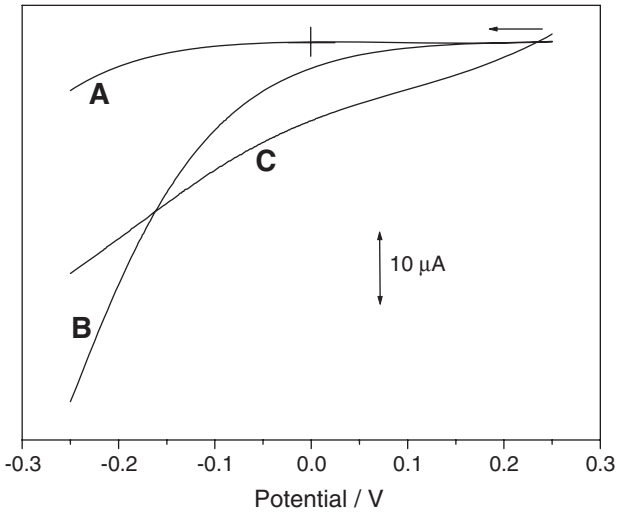


Fig. 3. Linear voltammograms with a rotated electrode (500 rpm) of (A) a platinum bare, (B) a dend-2 ($\Gamma = 1.6 \times 10^{-10}$ mol ferrocene cm^{-2} thickness film) coated platinum electrode in O_2 saturated phosphate buffer (pH = 7.0) solution, and (C) a dend-2 ($\Gamma = 1.6 \times 10^{-10}$ mol ferrocene cm^{-2} thickness film) coated platinum electrode in deaerated phosphate buffer (pH = 7.0) solution with 5 mM H_2O_2. Scan rate: 5 mV s^{-1}.

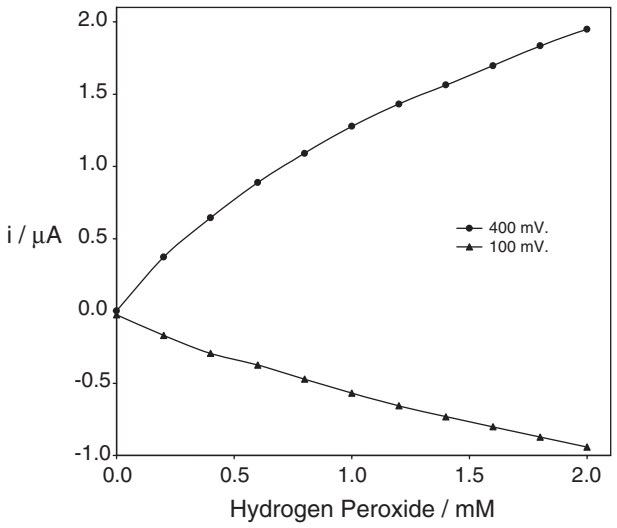


Fig. 4. Anodic and cathodic hydrogen peroxide calibration plots of a dend-2 modified platinum electrode ($\Gamma = 1.8 \times 10^{-9}$ mol cm^{-2} thickness film) in 0.1 M deaerated phosphate buffer (pH 7.0). Each curve is the mean result for five electrodes.

measurements, the chosen cathodic work potential was 100 mV (versus SCE).

The non-linearity observed between the electrode response and the hydrogen peroxide concentration in the calibration plots for both anodic and cathodic operations indicates some kinetic limitations on the overall reaction rate [20,26].

Since only processes C, D (Scheme 2) and the diffusion of hydrogen peroxide into or out of the dendrimer membrane can occur in this case, one or more of them will be responsible of kinetic limitations.

3.3. Response to glucose

As expected from the previous peroxide studies, the electrocatalytic properties of the dendrimers also lead to a significant improvement in the sensitivity of oxidase-based biosensors in aerobic operations. Fig. 5 shows cyclic voltammograms of a dendrimer (dend-2) enzyme electrode in the absence and presence of 5mM of glucose taken at 2 mV s^{-1} in oxygen saturated phosphate buffer (pH 7.0). As that observed in the hydrogen peroxide response of the dendrimer-modified electrodes, the enzyme-dendrimer electrodes exhibit both anodic and cathodic activity, which should be described, respectively by the mechanisms I and II shown in Scheme 2 [27].

The anodic electrochemical behaviour of the GOx-dendrimer electrode is influenced by the presence of glucose in solution and the addition of glucose leads to a substantial enhancement of the oxidation current.

Moreover, the cathodic measurement of hydrogen peroxide produced from the regeneration of the enzyme by the oxygen mediation is also possible but the range of potentials will be limited by the interference caused by the reduction of the oxygen. As cited above, this interference can be avoided by using applied potential values higher than 100 mV (versus SCE).

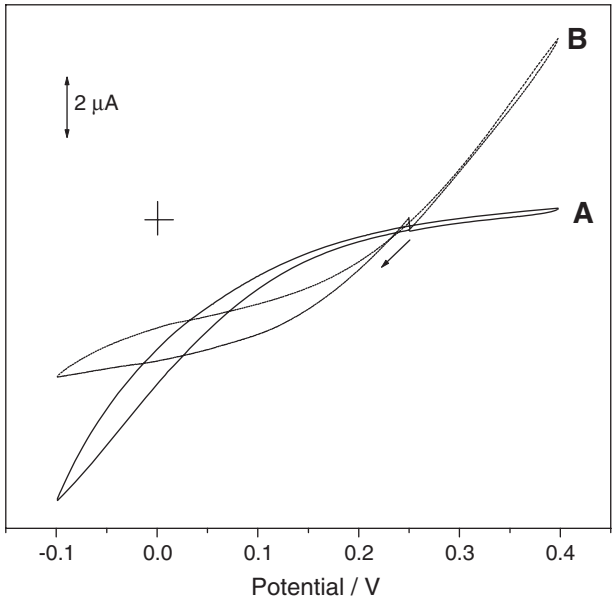


Fig. 5. Cyclic voltammograms of a platinum disk electrode modified with a film of dend-2-GOx ($\Gamma = 7.0 \times 10^{-10}$ mol ferrocene cm^{-2} thickness film) in O_2 saturated phosphate buffer (pH = 7.0) in absence (A) and presence (B) of glucose 5 mM. Scan rate 2 mV s^{-1}.

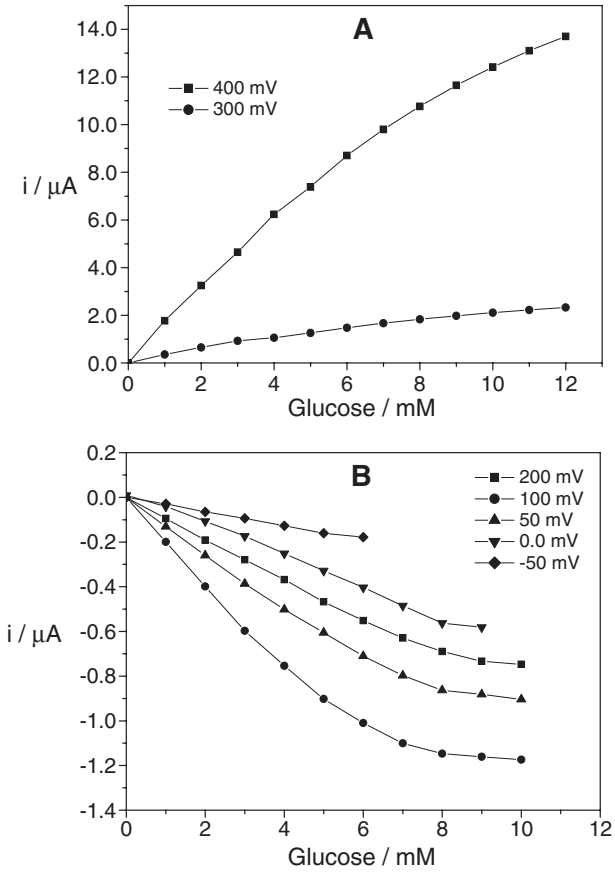


Fig. 6. Glucose calibration plots of the dend-2-GOx sensors ($\Gamma \approx 3 \times 10^{-9}$ mol ferrocene cm^{-2} thickness films). Steady state anodic (A) and cathodic (B) currents measured as a function of the applied potential in O_2 saturated phosphate buffer (pH = 7.0). Each value is the mean result for five electrodes.

The amperometric response of the dendrimer-GOx modified electrodes as a function of glucose concentration was determined at several applied potentials for all dendritic mediators. The maximal current values were obtained at pH 7.0, though the current response is essentially independent of the pH over the physiologically relevant range, i.e. pH 6.0–9.0. The effect of the polymer film thickness on the response signal of the sensors has been studied. Dendrimer films with a coverage of about 10^{-9} mol cm^{-2} (mol of ferrocene sites cm^{-2}) exhibited the best response.

As expected from the voltammetric profiles, improvements in the sensitivity were observed for the anodic detection of glucose at 300 and 400 mV with dend-2-sensor (Fig. 6A) that yielded sensitivities of 0.36 and 1.77 μA mM^{-1}, respectively. Therefore the best sensitivity is exhibited at the highest potential used, +0.4 V. In the same way, Fig. 6B shows the cathodic responses of the same enzyme-electrode to glucose concentration depending on the operation potential. The obtained results are in good agreement with the previous assays with hydrogen peroxide. As it can be observed, the best and higher working potential to be used is 100 mV (versus SCE), and lower

potentials cause a lower response because the interference of oxygen becomes more significant. Consequently, for all generations of this family (dend-1–3) the results obtained show that the sensitivity of the biosensor is dependent on the applied potential.

In order to evaluate the analytical performance of the enzyme electrodes, calibration curves were also obtained for electrodes containing similar ferrocenyl surface coverage values. Fig. 7 shows the steady-state currents for electrodes modified with the three different dendrimers as a function of glucose concentration obtained at 400 (A) and 100 mV (B). The calibration curves show that at 100 mV the bioelectrocatalytic signals and the sensitivity of sensors containing dendrimers of different generation as mediators, increased in the order dend-1 < dend-2 < dend-3; however, at 400 mV the electrochemical responses for the three mediators are nearly the same.

In all cases the bioelectrocatalytic signals reached a steady-state value within 5–10 s after addition of the glucose samples and the steady-state currents were maintained with no fluctuation.

The analytical characteristics of the sensors applied to the anodic and cathodic determination of hydrogen peroxide and glucose are listed in Table 1.

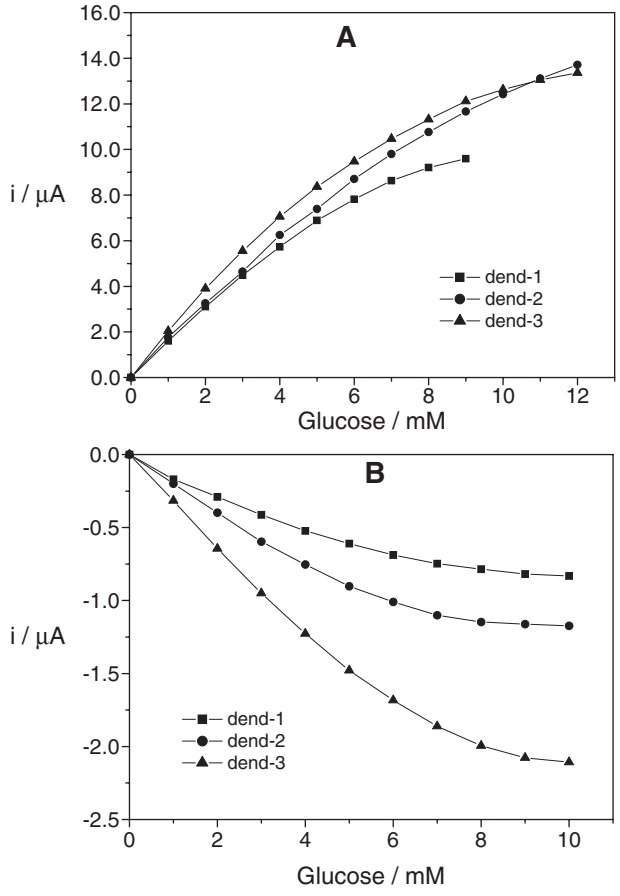


Fig. 7. Glucose calibration plots of the dend-1-3 sensors ($\Gamma \approx 3\times10^{-9}$ mol ferrocene cm^{-2} thickness films). Steady state anodic (A) and cathodic (B) currents measured at 400 mV (versus SCE) applied potential in O_2 saturated phosphate buffer (pH = 7.0). Each value is the mean result of five electrodes.

Table 1

Glucose determinations

	Linear range (mM)		Sensitivity (μA/mM)		Detection limit (μM)		K'_M(mM)	
	400 mV	100 mV	400 mV	100 mV	400 mV	100 mV	400 mV	100 mV
dend-1	3.0	2.0	1.61	0.17	0.8	43	14.3	2.0
dend-2	4.5	3.0	1.77	0.20	2.7	39	16.3	1.8
dend-3	3.0	3.0	2.04	0.32	1.2	15	15.2	3.0

Hydrogen peroxide determinations

	Sensitivity (μA/mM)		Detection limit (μM)	
	400 mV	100 mV	400 mV	100 mV
dend-2	1.87	0.72	0.96	3.61

3.4. Kinetic study

Some years ago Savinell et al. [27] developed a mathematical model describing the steady state amperometric operation of an enzyme electrode in anaerobic anodic glucose measurements, which allows to identify the rate-limiting step, or steps, by means of kinetic simplified equations. In order to study the rate-limiting process for the dendrimer-sensors, we have adapted the Savinell's model to our sensing system, by including a new term relative to the presence of oxygen as natural mediator for GOx into the reaction schemes involved in an enzyme electrode (Scheme 2). Also, the model has been applied for cathodic operation (Scheme 2 II). The equations obtained are very similar to the Savinell's model and allow to obtain the same diagnostics from the double reciprocal Lineweaver–Burke plot. Therefore, the adapted model allows us to identify the rate-limiting step in the aerobic as well as anaerobic operations, for both cathodic and anodic modes.

The three cases described by Savinell and the equations obtained by us for each case are.

Case I, is when neither the diffusion nor the electrolysis process controls the overall rate of the enzyme electrode; and therefore a combination of enzyme catalysis and the electron mediation reactions limits the overall reaction rate. In the steady-state, the total flux j (in mol cm^{-2} s^{-1}) of such an enzyme electrode system, rearranged in the double reciprocal form gives:

$$\frac{1}{j} = \frac{1}{S_\infty}\frac{K_S}{\mathrm{GOx}L_k K_{CAT}} + \frac{K'_2\mathrm{Fc}L_k K_{CAT} + K_{EM}(ML_k K'_2\mathrm{Fc}-j)}{\mathrm{GOx}K_{EM}L_k K_{CAT}(ML_k K'_2\mathrm{Fc}-j)} = \frac{1}{S_\infty}\frac{1}{K'_{app}} + \frac{1}{K'_{app}K'_M}$$

were S_∞ is the concentration of substrate outside the membrane, GOx and Fc are the total concentrations of immobilized enzyme and ferrocene sites, M is the total concentration of peroxide-oxygen mediator ($M = H_2O_2 + O_2$), and L_k is the thin layer immediately adjacent to the electrode surface, where a uniform distribution of substrate concentration is assumed. The rest of constants are described in Scheme 2. In accordance with Savinell's model, the double reciprocal plot must be a straight

line with the slope equal to inverse of the apparent rate constant, K'_{app}, and the intercept equal to inverse of the product of apparent rate constant and apparent Michaelis–Menten constant, K'_M. The K'_{app} and K'_M equations are absolutely concordant with the mathematical model by Savinell:

$$K'_{app} = \frac{GOx L_k K_{CAT}}{K_S} \qquad K'_M = \frac{K_S}{1 + \frac{K_{CAT}}{K_{EM}[O_2]}}.$$

Case II, is when the rate of electrolysis is the slowest (e.g. the applied potential is not high enough):

$$\frac{1}{j} = \frac{1}{S_\infty}\frac{K_S}{GOx L_k K_{CAT}} + \frac{1}{GOx L_k K_{CAT}} + \frac{K_{CAT}}{GOx L_k K_{CAT} K_{EM}\left(M - \frac{K'_1 j}{K'_2 L_k (Fc K'_1 - j)}\right)}$$

$$\frac{1}{j} - \frac{E}{M - \frac{K'_1 j}{K'_2 L_K (Fc K'_1 - j)}} = \frac{1}{S_\infty} B + A$$

the shape of Lineweaver–Burke plot for this case is a straight line in the region where $1/j$ is much larger than $E/(M - K'_1 j/K'_2 L_k(Fc K'_1 - j))$. This last term becomes inevitable in the region where j is large, and then the plot will exhibit an upward concave curve. In the same way as Savinell's model, the apparent rate constant K'_{app} of the overall reaction is unchanged but K'_M is modified and smaller than that in the previous case:

$$K'_M = \frac{K_S}{1 + \frac{K_{CAT}}{K_{EM}\left(M - \frac{K'_1 j}{K'_2 L_k (K'_1 Fc - j)}\right)}}$$

Case III, is when the substrate diffusion becomes the slowest:

$$\frac{1}{j} = \frac{1}{S_\infty}\left[\frac{1}{K'_{app}} - \frac{Q_j}{K'_s}\right] + Q\ ;$$

$$Q = \frac{1}{K'_{app}} \frac{K_{EM}(M L_k K'_2 - j) + K'_2 Fc L_k K_{CAT}}{K_S K_{EM}(M L_k K'_2 Fc - j)}$$

where K'_S is the diffusional rate constant and

$$K'_{app} = \frac{GOx\, L_k K_{CAT}}{K_S}\left(1 - \frac{j}{K'_S S_\infty}\right);$$

$$K'_M = \frac{K_S}{\left(1 + \frac{K'_2 Fc L_k K_{CAT}}{K_{EM}(M L_k K'_2 Fc - j)}\right)\left(1 - \frac{j}{K'_S S_\infty}\right)}$$

It is significant that now K'_{app} is reduced compared with previous cases, but, in accordance with the Savinell's mathematic model, the linear range is greatly enhanced by a factor of $1/(1 - j/K'_S S_\infty)$. In this case the double reciprocal plot of $1/j$ versus $1/S_\infty$ will be a straight line whereas $1/j$ is large and equal to $1/K'_{app}$. When $1/j$ decreases the slope begins to increase and the plot concaves down.

In order to apply this mathematical model to the dendrimer-sensors, the Lineweaver–Burke plots for the steady-state response of the various sensors built with the three dendrimers under all studied conditions have been plotted. Surprisingly, all of them are non-lineal and concave up in the high glucose concentration range, corresponding to Case II, except for an applied potential of 400 mV, the plot of which is strictly lineal and corresponds to Case I.

Really it stands to reason that the rate of electrolysis is slow at the applied potentials in cathodic determinations and 300 mV in anodic mode, because they are not high enough. However the cathodic potentials are imposed by the limitations described before.

The surprising fact is that the diffusion throw the dendrimer-enzyme film is not significant for any applied potential and the diffusional rate is not the rate-determining step in any case. The dendrimer films are non-porous, as it can be seen in the micrograph of Fig. 8; however it seems that the dendrimer films are thin enough to allow the fast flux of substrate. The potential of 400 mV is high enough to consider that the rate of electrolysis is not rate-limiting, but in agreement with the model, neither the electrolysis process nor the diffusion controls the overall rate of the enzyme electrode, and a combination of enzyme catalysis (process A in Scheme 2) and mediation reactions (processes B and C in Scheme 2) limits the overall reaction rate.

The behaviour of the sensors at 400 mV lead us to revise the cause of the non-linearity observed in the calibration curves for the determination of H_2O_2 at the same potential. The possible processes involved in the hydrogen peroxide determination, once the diffusive limitation has been ruled out, are the electrolysis of ferrocene and the redox reaction between hydrogen peroxide and ferrocene (or ferricinium) [20,26]. Since the electrolysis rate is fast enough at 400 mV, it is clear that the oxidation or reduction of hydrogen peroxide by the ferrocene or ferricinium units is the rate-limiting process in the high substrate concentration range. Obviously, the enzyme sensors contain cross-linked GOx (by means of BSA and glutaraldehyde) and their films are thicker than single dendrimer films, therefore the glucose calibration curves show the diffusional effect for low glucose

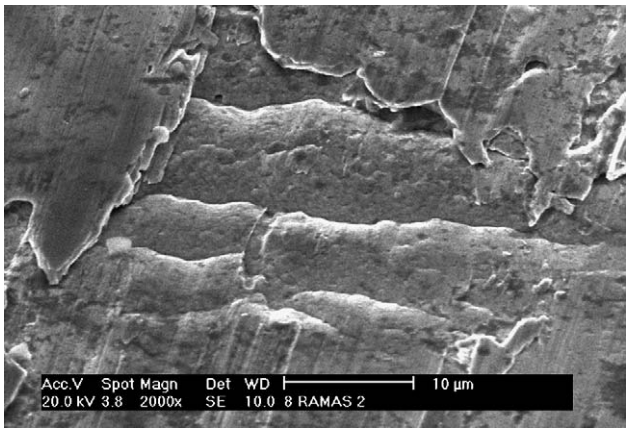


Fig. 8. SEM micrographs of a platinum wire electrode modified with a film of dend-2.

concentrations and have a wider linear range which must not extend beyond the K'_M values.

The apparent Michaelis–Menten constants, K'_M, were determined from the Lineweaver–Burke plots for the sensors based on three dendrimers for applied potentials 400 and 100 mV and they are shown in Table 1. These values are consistent with the predictions of the mathematic model expounded above, since for measurements at 100 mV (Case II) K'_M is smaller than that in the previous case (400 mV, Case I) and intrinsically smaller than the Michaelis constant in homogeneous enzyme kinetics ($K_S \approx 33$ mM) [27].

On the other hand, when the applied potential is not high enough and the electrolysis rate is the rate-determining step (in cathodic mode or with low oxidation potentials), the dendrimer generation demonstrates to play a significant role in the electrocatalytic activity (see Fig. 7B). The increase of flexibility of the dendrimer backbone, which is a function of the length of the dendrimer branches, together with the shorter separation between ferrocenyl neighbours, increase the electron transfer efficiency between ferrocene moieties and cause a higher catalytic response of the sensors based on the higher generation dendrimers [28].

3.5. Interferences

A major concern in the development of the peroxide biosensors is the elimination of the signal due to electrochemical interfering compounds present in real matrices. At 250 mV (versus SCE) applied potential, ascorbic acid (AA), the principal interfering agent in biological samples, produced a substantial increase in the steady-state response of sensors due to the electrooxidation of ascorbate ions. The sensor response was unaffected by the presence of normal physiological ascorbic acid concentrations only for operating potential values between 0 and −0.5 V. In fact, the signal due to the addition of 0.1 mM AA to a 1 mM hydrogen peroxide solution was unappreciable whereas the applied potential is kept into this range. However, the AA interference is appreciable when other working potential values are used, although it can be eliminated by using an additional Nafion layer [29].

With relation to oxygen interference, as cited already above, it does not constitute a problem for the chosen working potentials.

3.6. Reproducibility and stability

The reproducibility of the biosensors was examined in at 5.0 mM glucose solution (O_2 saturated 0.1 M phosphate buffer with 0.1 M $NaClO_4$; pH = 7.0). The relative standard deviation was 1.8% ($n = 5$). The electrode-to-electrode reproducibility was also examined between five different electrodes in above solutions, and the relative standard deviation was calculated to be 6.1%. The stability of the biosensor was evaluated by intermittent measurements of its response to 5 mM glucose. The response remained unchanged during a period of six days. Only a 25% decrease of their initial glucose response was observed for an electrode stored at 4 °C in air for 7 weeks.

4. Conclusions

Electrodes modified with polymethylferrocenyl dendrimers have shown to promote the redox reactions of hydrogen peroxide. As it was expected the polymethylated ferrocene dendrimeric mediators allow to use lower working potentials than those employed with non-methylated ferrocene compounds. The application of these modified electrodes in the amperometric determination of hydrogen peroxide has been demonstrated. A simple, versatile and easy handling process has been developed to prepare enzyme electrodes for sensor applications. Glucose oxidase has been immobilized, by cross-linking using BSA and glutaraldehyde, into the organometallic dendrimer film confined on a Pt electrode surface. The results obtained indicate that both anodic and cathodic operation modes may be used for glucose determinations with the dendrimer modified electrodes which act as electrocatalysts in either oxidation or reduction of hydrogen peroxide arisen in the enzyme catalyzed reaction. The sensors behaviour is affected by structural characteristics of the dendrimers when low working potentials are used.

The biosensors developed in this work respond quickly to the substrate with a good linear response region and offered sensitivity, detection limits and reproducibility comparable or even better than other ferrocene-mediated glucose sensors reported.

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