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Amperometric Enzyme-Modified Electrodes Based on Tetrathiafulvalene Derivatives for the Determination of Glucose[1]

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ABSTRACT

A number of tetrathiafulvalene (TTF) derivatives have been synthesized and tested as electron transfer mediators in glucose oxidase-based amperometric biosensors. Using cyclic voltammetry and stationary potential experiments, it is shown that several of these derivatives can effectively mediate electron transfer from the reduced flavin adenine dinucleotide redox centers of glucose oxidase to a conventional carbon paste electrode. An insoluble polymeric electron relay system, based on the covalent attachment of TTF moieties to a highly flexible siloxane polymer, is also shown to facilitate a flow of electrons from the enzyme to the electrode. The resulting glucose biosensors function efficiently over a clinically relevant range of glucose concentrations.

INTRODUCTION

Amperometric glucose electrodes based on glucose oxidase undergo several chemical or electrochemical steps which produce a measurable current that is related to the glucose concentration. In the initial step, glucose converts the oxidized flavin adenine dinucleotide (FAD) center of the enzyme into its reduced form ($FADH_2$). Because these redox centers are essentially electrically insulated within the enzyme molecule, direct electron transfer to the surface of a conventional electrode does not occur to any measurable degree. The most common method [1–4] of indirectly measuring the amount of reduced glucose oxidase, and hence the amount of glucose present, relies on the natural enzymatic reaction:

$$\text{glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconolactone} + H_2O_2$$

where oxygen is the electron acceptor for glucose oxidase. The oxygen is reduced by the $FADH_2$ to hydrogen peroxide, which may then diffuse out of the enzyme and be detected electrochemically. The working potential of such a device is quite high (H_2O_2 is oxidized at approximately + 0.7 V vs. the saturated calomel electrode, or SCE), however, and the sensor is therefore highly sensitive to many common interfering electroactive species, such as uric acid and ascorbic acid; H_2O_2 is also known to have a detrimental effect on glucose oxidase activity. Alternatively, one could use the electrode to measure the change in oxygen concentration that occurs during the above reaction. In both of these measuring schemes, this type of sensor has the considerable disadvantage of being extremely sensitive to the ambient concentration of O_2.

In recent years systems have been developed which use a nonphysiological redox couple to shuttle electrons between the $FADH_2$ and the electrode by the following mechanism:

$$\text{glucose} + \text{GO(FAD)} \rightarrow \text{gluconolactone} + \text{GO(FADH}_2\text{)}$$

$$\text{GO(FADH}_2\text{)} + 2M_{Ox} \rightarrow \text{GO(FAD)} + 2M_{red} + 2H^+$$

$$2M_{red} \rightarrow 2M_{OX} + 2e^- \text{ (at the electrode).}$$

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[1]This paper is dedicated to Professor Herbert C. Brown on the occasion of his 80th birthday.

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In this scheme, GO(FAD) represents the oxidized form of glucose oxidase and GO($FADH_2$) refers to the reduced form. The mediating species M_{ox}/M_{red} is assumed to be a one-electron couple. Sensors based on derivatives of the ferrocene/ferricinium redox couple [5–7] and on quinone derivatives [9–11] have been studied. Recent work has also shown that tetrathiafulvalene (TTF) can serve as a good mediator with glucose oxidase [11–15], lactate oxidase [16], and choline oxidase [17]. In the present paper, we describe the synthesis of several TTF derivatives and their use as mediators in glucose oxidase-based amperometric biosensors. The monomeric derivatives studied in this work are shown in Figure 1.

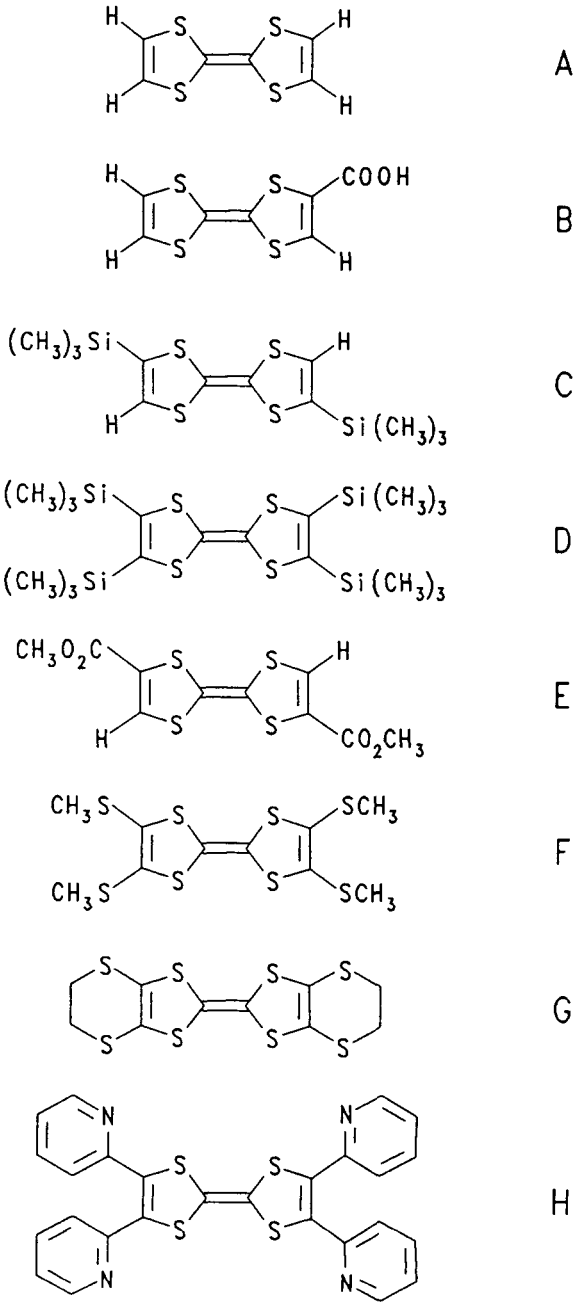


FIGURE 1 Monomeric TTF derivatives used as mediators in glucose oxidase based amperometric biosensors.

TTF undergoes two one-electron oxidation processes in aqueous solution, leading to the formation of TTF^+ and TTF^{2+} [18,19]. The first oxidation process is reversible, and it is this process which is involved in the electron transfer mediation process with glucose oxidase:

$$\text{glucose} + \text{GO(FAD)} \rightarrow \text{gluconolactone} + \text{GO}(FADH_2)$$

$$\text{GO}(FADH_2) + 2TTF^+ \rightarrow \text{GO(FAD)} + 2TTF + 2H^+$$

$$2TTF \rightarrow 2TTF^+ + 2e^- \text{ (at the electrode).}$$

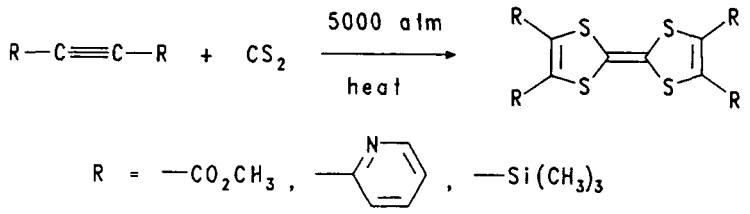
The oxidation of TTF occurs at lower potential values than ferrocene [5], so biosensors based on this mediator may be operated at a lower potential, where interference due to commonly present electroactive species, such as ascorbate and urate, may be minimized.

It is well known that sensors based on electron-shuttling redox couples suffer from the inherent drawback that the soluble, or partially soluble, mediating species can diffuse away from the electrode surface into the bulk solution, which precludes the use of these devices in some clinical applications and restricts their use in long-term *in situ* measurements (e.g., fermentation monitoring). With this in mind, several research groups have investigated systems where the mediating species is chemically bound in a manner which allows close contact between the FAD/$FADH_2$ centers of the enzyme and the mediator, yet prevents the latter from diffusing away from the electrode surface. For instance, systems have been developed where the mediating redox moieties are covalently attached to insoluble polymeric materials such as polypyrrole [20] poly(vinylpyridine) [21,22], and in our laboratories, polysiloxane [23–25] and poly(ethylene oxide) [26]. Such systems serve to "electrically wire" the enzyme, facilitating a flow of electrons from the enzyme to the electrode. In the present paper, we discuss the development of a new polymeric relay system based on the covalent attachment of TTF to a highly flexible, insoluble siloxane polymer. This polymer is shown in Figure 2.

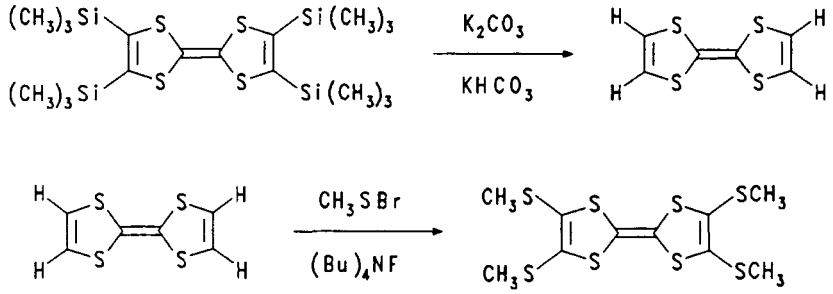
EXPERIMENTAL

Synthesis of TTF Monomers

The synthesis of TTF and its derivatives generally involves a multistep route, with the production of unstable intermediates. However, we have developed a single-step procedure for the synthesis of various substituted TTF derivatives via reaction of carbon disulfide with appropriate acetylene compounds



SCHEME 1



SCHEME 2

under high pressure (Scheme 1) [27–29]. Tetrakis(methylthio)tetrathiafulvalene (TMT-TTF) was prepared using Scheme 2 [29]. Bis(ethylene dithio) tetrathiafulvalene (BEDT-TF) was obtained from American Tokyo Kasei Inc. and used as received.

Synthesis of the TTF Polymer

The TTF containing siloxane polymer (I) was prepared via the following procedure (Scheme 3). First, 1-allyloxyethyl ether (*3a* in Scheme 3) was produced by a literature method [30], which involved treating 32.2 g of allyl alcohol in 300 mL anhydrous ether with 28.8 g of ethy vinyl ether, in the presence of toluenesulfonic acid at 0°C for 1 hour. After a further half hour of stirring, the ether solution was washed with aqueous sodium carbonate and water. After the solution was dried over magnesium sulfate, the ether was evaporated, and the obtained 1-allyloxyethyl ether (30 g) was distilled at 120–125°C. *IR (neat):* 3082.9; 2987.9 2873.0; 1648.3; 1390.7; 1338.7; 1100.3; 923.5 cm^{-1}. *NMR ($CDCl_3$):* δ 1.3 (m, 6H); 3.5(q, 2H); 4.1 (d, 2H); 4.75 (q, 1H); 5.1–6.3 (m, 3H).

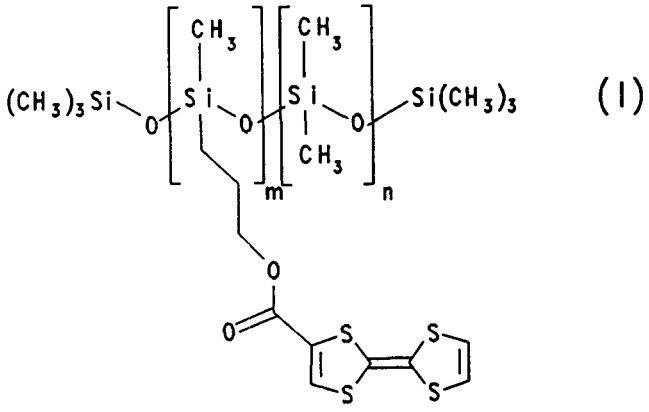
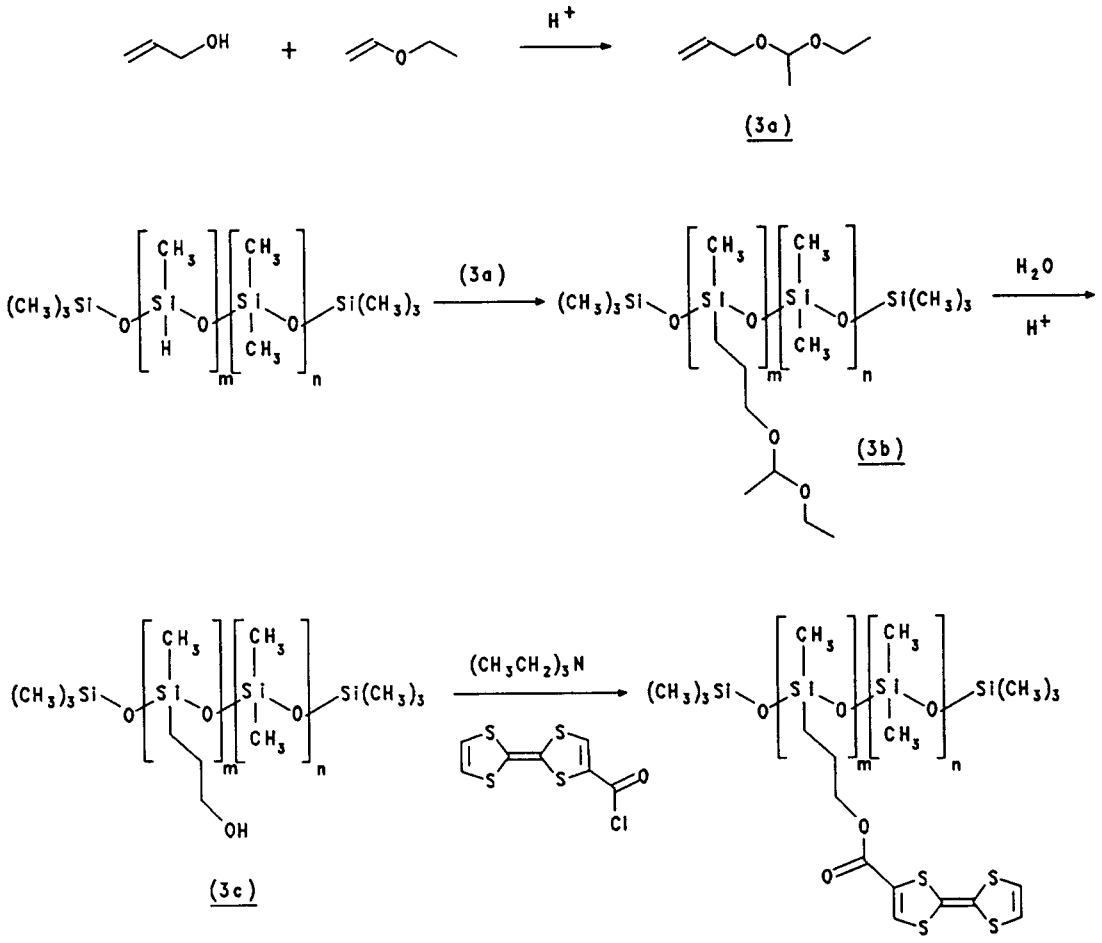


FIGURE 2 The TTF-containing siloxane polymer used as electron transfer relay system in glucose oxidase based amperometric biosensors. This is a random block copolymer with the indicated m:n ratio being approximately 1:2.

Methyl[3-(1-ethoxyethoxy)propyl]dimethylsiloxane polymer (*3b* in Scheme 3) was prepared by reacting 4.16 g methylhydro,dimethylsiloxane polymer in 30 ml anhydrous THF with 4.5 g of *3a*, in the presence of hydrogen hexachloroplatinate (200 mg) ai 60°C for 5 hours. After removal of the solvent, the desired polymer *3b* was obtained. *IR (neat):* 2964.7; 2872.8; 1446.1; 1381.6; 1309.2; 1260.8; 1026.1; 913.5; 803.6 cm^{-1}. *NMR ($CDCl_3$):* δ 0.1 (s, 15H); 0.3–0.65 (m, 2H); 1.25 (m, 6H); 1.6 (m, 2H); 3.5 (m, 4H); 4.65 (q, 1H). Methyl(3-hydroxypropyl)dimethylsiloxane polymer (*3c* in Scheme 3) was prepared by dissolving polymer *3b* in 10 mL THF and 20 mL methanol and adding 0.2 mL concentrated HCl. The mixture was heated at 60°C for 1 hour with charcoal. After concentrating the filtrate, the residue was taken up in ether, washed with water, and dried. After evaporation of the solvent, the colorless, viscous polymer *3c* was obtained. *IR (neat):* 3345.8; 2962.6; 2877.4; 1412.2; 1260.9; 1026.3; 801.3 cm^{-1}). *NMR ($CDCl_3$):* δ 0.1 (s, 15H); 0.3–0.7 (m, 2H); 1.6 (m, 2H); 3.5 (t, 2H).

Tetrathiafulvalene carboxylic acid (400 mg, 1.6 mM), prepared using a procedure similar to that in the literature [31], in a mixed solvent of 8 mL acetonitrile and 20 mL benzene, was treated with 325



SCHEME 3

mg (2.6 mM) oxalyl chloride in 5 mL benzene, in the presence of 0.02 mL DMF under nitrogen for 40 minutes. After removal of the solvent, the residue was dissolved in 40 mL benzene and filtered. 266 mg of polymer *3c* in 8 mL THF and 200 mg triethylamine was added to the filtrate, which was then stirred at room temperature for 24 hours. The solution changed from deep purple to deep red-orange. After the addition of ether, the solution was washed with dilute sodium carbonate and water, and then dried to give the gummy polymer product (I). *IR (neat):* 3074.5; 2961.6; 1711.1; 1539.2; 1260.6; 1069.5; 802.3 cm^{-1}.

Electrochemical Methods

Graphite powder (product no. 50870) and paraffin oil (product no. 76235) were obtained from Fluka (Ronkonkoma, NY). Glucose oxidase (EC 1.1.3.4, type VII, 129 units/mg) was obtained from Sigma (St. Louis, MO). Glucose (Sigma) solutions were prepared by dissolving appropriate amounts in 0.1 M phosphate/0.1 M KCl buffer (pH 7.0); the glucose was allowed to reach mutarotational equilibrium before use (*ca.* 24 hours). All other chemicals were reagent grade and were used as received.

The modified carbon paste for the sensors was made by thoroughly mixing 50 mg of graphite powder with a measured amount of the TTF derivative; in the present work, the molar amount of mediator was the same for all electrodes (1 μmole of mediator per 50 mg of graphite powder). 5 mg of glucose oxidase and 10 μL of paraffin oil were then added, and the resulting mixture was blended into a paste. We have previously shown [23–26], as have others [32], that glucose oxidase retains its activity when incorporated into a carbon paste matrix. The paste was packed into a 1.0 ml plastic syringe (7.0 mm outer diameter; 1.8 mm inner diameter) which had previously been partially filled with unmodified carbon paste, leaving approximately a 2 mm deep well at the base of the syringe. The electrodes were polished by rubbing gently on a piece of weighing paper, which produced a flat shiny surface with an area of approximately 0.025 cm^2. Electrical contact was achieved by inserting a silver wire into the top of the carbon paste.

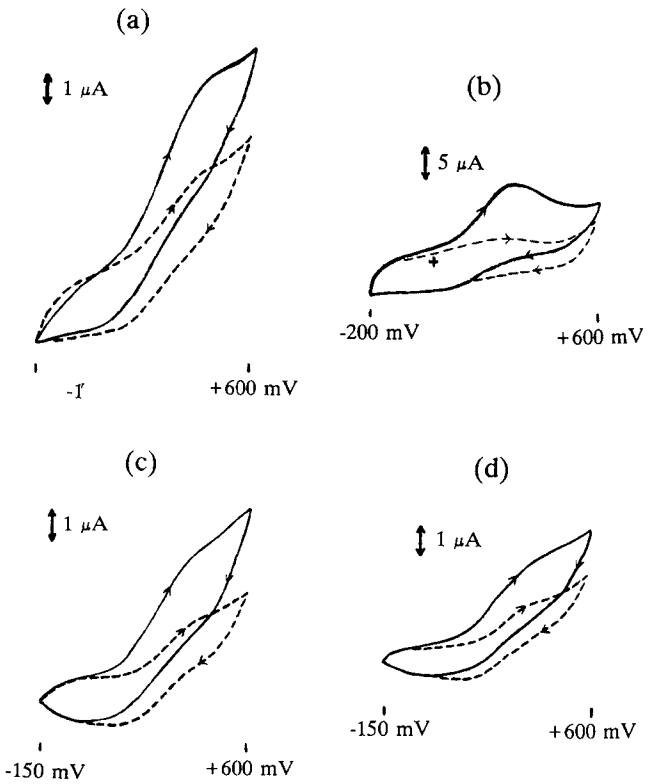


FIGURE 3 Cyclic voltammograms for the TTF (A-D) / glucose oxidase / carbon paste electrodes (scan rate: 10 mV/s) in pH 7.0 phosphate buffer (with 0.1 M KCl) solution with no glucose present (dashed line) and in the presence of 100 mM glucose (solid line): (a) mediator A, (b) mediator B, (c) mediator C, (d) mediator D.

Cyclic voltammetry and constant potential measurements were performed using a Princeton Applied Research Potentiostat (Model 173), a Universal Programmer (Model 175), and a Western Graphtec X-Y-T recorder (Model WX2400). All experiments were carried out in a conventional electrochemical cell containing pH 7.0 phosphate (0.1 M) buffer with 0. 1 M KCl at 23(±2)°C. All experimental solutions were thoroughly deoxygenated by bubbling N_2 through the solution for at least 10 minutes; in the constant potential experiments, a gentle flow of N_2 was also used to facilitate stirring. Glucose samples injected into the cell were also thoroughly deoxygenated. In addition to the modified carbon paste working electrode, a saturated calomel reference electrode (SCE) and a platinum wire auxiliary electrode were employed. In the constant potential experiments, the background current was allowed to decay to a constant value before samples of a stock glucose solution were added to the buffer solution. A constant background current was attained approximately 10–20 minutes after application of the potential.

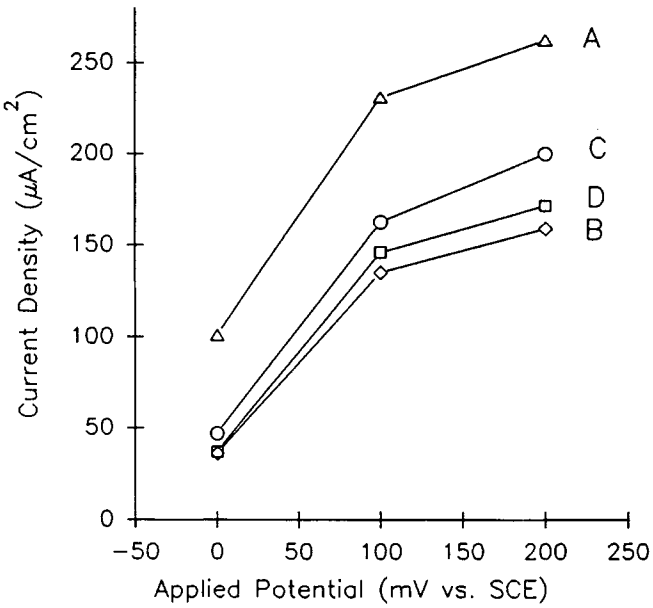


FIGURE 4 Steady-state current response to 31.5 mM glucose for the TTF (A-D) / glucose oxidase / carbon paste electrodes at several applied potentials. Each point is the mean result for three electrodes. The mediators are indicated next to each curve.

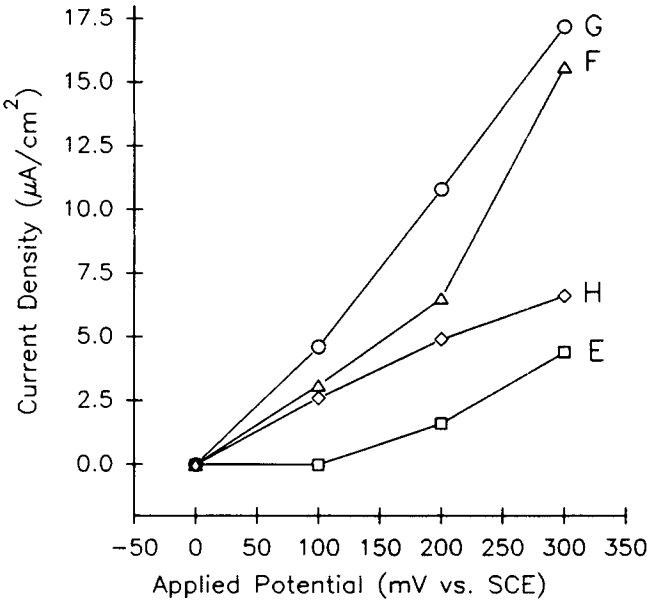


FIGURE 5 Steady-state current response to 31.5 mM glucose for the TTF (E-H) / glucose oxidase / carbon paste electrodes at several applied potentials. Each point is the mean result for three electrodes. The mediators are indicated next to each curve.

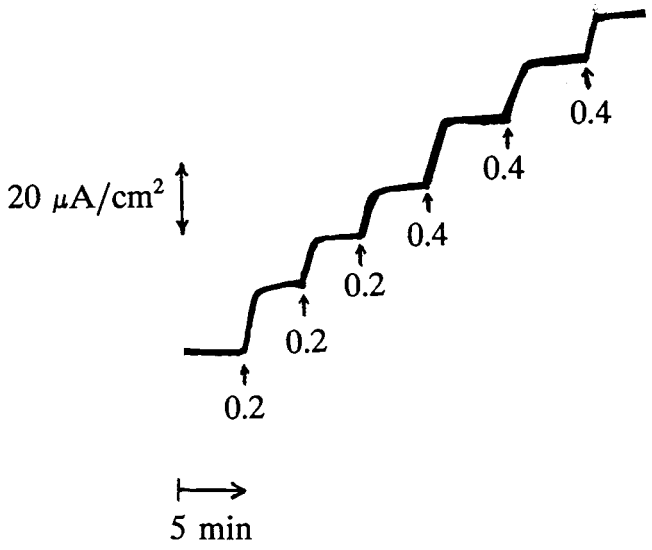


FIGURE 6 Typical response traces for the TTF-COOH (B) / glucose oxidase / carbon paste electrodes upon addition of glucose at E = 200 mV vs. SCE. Indicated under the response traces are the amounts (in ml) of 0.1 M glucose injected into the test solution (initial volume: 10 ml).

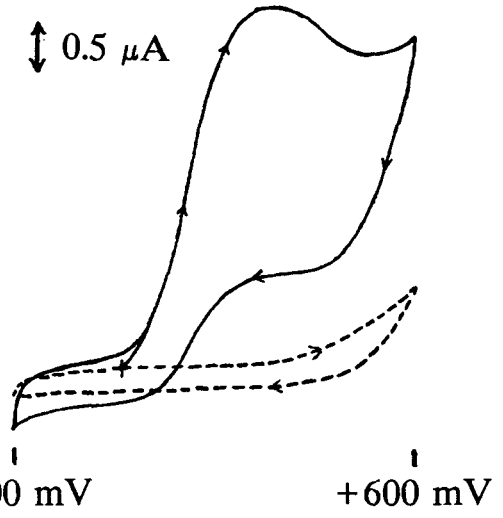


FIGURE 8 Cyclic voltammograms for the TTF siloxane polymer (I) / glucose oxidase / carbon paste electrode (scan rate: 10 mV/s) in pH 7.0 phosphate buffer (with 0.1 M KCl) solution with no glucose present (dashed line) and in the presence of 100 mM glucose (solid line).

RESULTS AND DISCUSSION

Figure 3 shows typical voltammetric results for carbon paste electrodes containing glucose oxidase and the TTF derivatives A–D as electron transfer mediators. With no glucose present, the voltammograms display anodic and cathodic waves due to the oxidation and reduction of the TTF and TTF^+ species, respectively. Upon addition of glucose, the voltammetry changes dramatically, with a large increase in the oxidation current and no increase in the reduction current. The fact that the reduction current does not increase along with the oxidation current is indicative of the enzyme-dependent catalytic reduction of the TTF^+ produced at oxidizing potential values. Upon comparison of the voltammograms with and without glucose present, it is apparent that these molecules can act as efficient electron transfer mediators between the $FAD/FADH_2$ centers of glucose oxidase and a carbon paste electrode. Electrodes containing glucose oxidase and the other mediators in Figure 1 (mediators E–H) do not display these voltammetric characteristics upon addition of glucose. Their voltammetric peaks are too small to be measured, and there is no change upon addition of glucose, which indicates that they are not efficient electron transfer mediators. In addition, voltammograms made with carbon paste electrodes containing only glucose oxidase with no mediator do not display the catalytic behavior shown in Figure 3.

This mediation is shown conclusively in stationary potential measurements using electrodes modified with both glucose oxidase and the TTF mediators. Figures 4 and 5 show the steady-state response to 31.5 mM glucose at several applied potentials for electrodes containing each of the electron transfer mediators. No response to glucose was observed in

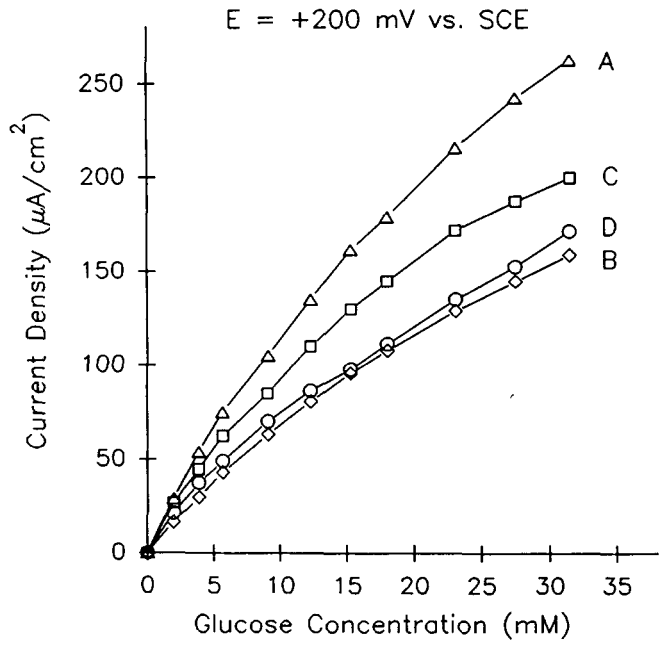


FIGURE 7 Glucose calibration curves for the TTF (A-D) / glucose oxidase / carbon paste electrodes at E = 200 mV vs. SCE. Each point is the mean result for three electrodes. The mediators are indicated next to each curve.

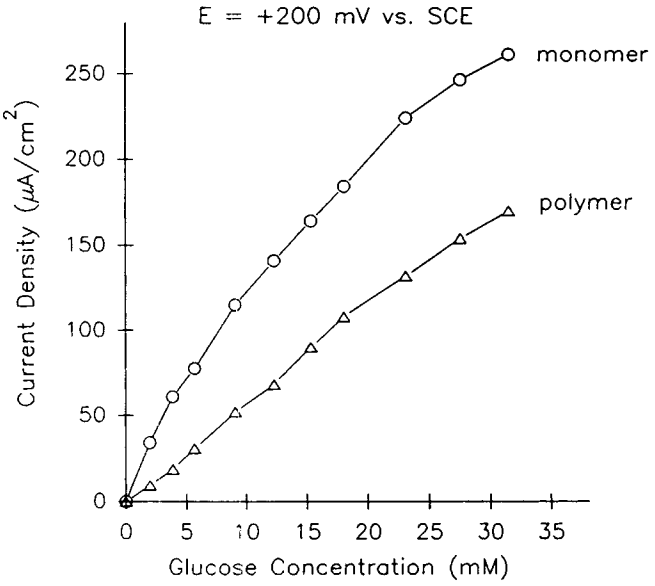


FIGURE 9 Glucose calibration curve for the TTF polymer (I) / glucose oxidase / carbon paste electrodes at E = 200 mV vs. SCE. Each point is the mean result for three electrodes. For comparison, the results using mediator A (monomeric TTF) are also shown.

the absence of either the mediators or glucose oxidase. As expected from the cyclic voltammetry results above, sensors containing mediators A–D display much larger responses to glucose than those containing mediators E-H.

A typical glucose response trace (current density vs. time) is shown in Figure 6 for a carbon paste electrode containing glucose oxidase and mediator B. This trace clearly shows the good sensitivity of the sensors to clinically relevant glucose concentrations. Electrodes containing mediators A, C, and D produced similar response curves. The time required to reach 95% of the steady-state current was typically less than 1 minute; this is somewhat slower than the response of sensors based on mediators such as ferrocene [6]. Typical glucose calibration curves are shown in Figure 7 for sensors containing each of the mediators A–D. From these results, it is apparent that the unsubstituted mediator A is more efficient than the substituted species; this is perhaps due to the greater diffusional mobility of the smaller molecule A compared with the bulky derivatized species. On the other hand, the fact that species C and D function much more efficiently as mediators than species E–H suggests that the trimethylsilyl groups may be displaced during the course of the electrochemical experiment. Attempts to detect trace amounts of unsubstituted TTF in the solutions were unsuccessful, but the possibility remains that mediators C and D are not adequately stable.

Figure 8 shows typical voltammetric results for carbon paste electrodes containing glucose oxidase and the TTF siloxane polymer (I) as an insoluble electron transfer relay system. In this case, with no glucose present, the voltammetric waves due to the oxidation and reduction of the TTF and TTF^+ species are not visible. Upon addition of glucose, a large increase in the oxidation current is found, with only a small increase in the reduction current. As in the case of the monomeric mediators, the fact that the reduction current does not increase along with the oxidation current is indicative of the enzyme-dependent catalytic reduction of the TTF^+ produced at oxidizing potential values. Upon comparison of the voltammograms with and without glucose present, it is apparent that this new polymer can act as an efficient electron transfer relay system between the $FAD/FADH_2$ centers of glucose oxidase and a carbon paste electrode. A glucose calibration curve is shown in Figure 9 for carbon paste electrodes containing glucose oxidase and the TF siloxane polymer (I). The sensor works quite well, with a response comparable to those measured previously using ferrocene containing siloxane polymers as electron relay systems [23–25].

This study demonstrates the feasibility of using various tetrathiafulvalene derivatives, both monomeric and polymeric, as electron transfer mediators in flavoenzyme-based amperometric sensors. In the case of the monomeric species, the addition of bulky substituents to the periphery of the molecule appears to hinder its ability to mediate electron transfer from reduced glucose oxidase to an electrode. As found previously for ferrocene-based systems [23–25], the highly flexible siloxane polymer allows the covalently attached TTF to achieve an intimate contact with the redox centers of glucose oxidase, and to thus function as an efficient electron transfer mediator. Further study of these systems, including long-term stability tests, are presently underway.

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