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A mediatorless biosensor for putrescine using multiwalled carbon nanotubes

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Abstract

Poly(diallyldimethylammonium) chloride, having a capability of dispersing multiwalled carbon nanotubes (MWCNTs), permits the modification of electrode surfaces. Together with putrescine oxidase, a MWCNT modified glassy carbon electrode was constructed for the development of a mediatorless putrescine biosensor. Nanoscale "dendrites" of MWCNTs were reasoned to form a network, projecting outward from the electrode surface acting like bundled ultra-microelectrodes, thereby permitting access to the active site and facilitating direct electron transfer to the immobilized enzyme. Our biosensor was capable of efficiently monitoring the direct electroactivity of putrescine oxidase at the electrode surface. Direct electron transfer permits the detection of putrescine at negative potentials, circumventing the interference of endogenous ascorbic and uric acids, which often complicate the analysis of important compounds in plasma. Compared with the most common interfering species, such as spermine, spermidine, cadaverine, and histamine, a detection limit of 5 μM and a response 20 times greater were found for putrescine. Tests performed on plasma of cancerous mice demonstrated that the detection of putrescine could be carried out very quickly on mammalian plasma without previous purification.

Keywords: Direct electron transfer; Mouse plasma cancer; Multiwalled carbon nanotubes; Putrescine biosensor

Since the discovery of carbon nanotubes (CNTs),[1] significant efforts have been made to develop applications and establish the novel properties of this new class of materials [1]. The electrical conductivity and flexibility of the CNTs have suggested the very interesting possibility of using such materials to promote electron transfer reactions with enzymes [2]. Enzymes are large molecules with redox centers deeply embedded within their structures. Therefore, it is very difficult for the enzymes to exchange electrons directly with the electrode surface [3]. However, CNTs can form a network and project outward from the electrode, acting like bundled ultra-microelectrodes that permit access to the active sites of the enzymes, facilitating direct electron transfer (DET) [4]. CNTs may exist as single-walled CNTs (SWCNTs) or multiwalled CNTs (MWCNTs) [5]. SWCNTs usually exist as ropes or bundles, forming a dense complex structure; therefore, they are more resistant to wetting than are MWCNTs. SWCNTs may be metallic or semiconducting, whereas MWCNTs are essentially metallic [6]. If electrically contacted at the outer wall, current is conducted only through the outermost shell of MWCNTs [7].

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[1] *Abbreviations used:* CNT, carbon nanotube; DET, direct electron transfer; SWCNT, single-walled carbon nanotube; MWCNT, multiwalled carbon nanotube; PDDA, poly(diallyldimethylammonium) chloride; APTES, 3-aminopropyltriethoxy-silane; PuO, putrescine oxidase; CV, cyclic voltammetry; GCE, glassy carbon electrode; HPLC, high-performance liquid chromatography; FTIR, Fourier transform infrared spectroscopy; FAD, flavin adenine dinucleotide; MMTV, mouse mammary tumor virus.

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The advantages of CNTs have not been fully realized because of the difficulty of obtaining dispersed nanotubes. Although CNTs have been solubilized by functionalizing their sidewalls, this approach often damages their tubular structure. Surfactants have been used to disperse CNTs [8], but they might denature the enzyme or at least impair its activity. Such drawbacks make surfactant-modified CNTs less desirable for biosensing applications. The best solvents reported for effecting CNT dispersions are *N,N*-dimethylformamide and *N*-methylpyrrolidone [9]. Unfortunately, the dispersions aggregate on a time scale of days, and such solvents are not compatible with biomolecules. A few polymers [10,11] are known to disperse CNTs effectively, with a strong tendency to randomly wrap around CNT bundles unless they have a specific binding interaction [12]. CNTs have been dispersed in Nafion, a perfluorosulfonated polymer, to facilitate the modification of electrode surfaces toward the development of a glucose biosensor [13]. MWCNTs have been dispersed in 3-aminopropyltriethoxysilane to promote efficient electron transfer for glucose oxidase [3].

We describe here a simple approach to dispersing MWCNTs, using poly(diallyldimethylammonium) chloride (PDDA), without affecting their electrical properties. The fabrication of a PDDA-modified MWCNT biosensor, using putrescine oxidase, is presented together with its performance relating to DET between the enzyme and the MWCNT-modified electrode.

Putrescine detection is generally based on the detection of hydrogen peroxide ($FADH_2 + O_2 \leftrightarrow FAD + H_2O_2$) [14]. The DET of flavo enzymes is extremely difficult because the FAD moiety is deeply embedded within a protective protein shell [15]. However, MWCNTs have shown good electrochemical communication with proteins having a redox center close to their surfaces [16,17] as well as embedded within glycoproteins such as glucose oxidase [4,18,19]. With DET (Fig. 1), the $FADH_2$ is reoxidized to FAD more rapidly than when the $FADH_2$ reacts with oxygen to form hydrogen peroxide [3]. The DET provides faster reoxidation of the enzyme, which can then react more quickly with subsequent putrescine molecules, resulting in better sensitivity and selectivity.

Biogenic amines, mainly putrescine, cadaverine, and histamine, have been used for various analytical and clinical purposes—plant stress markers, bacterial vaginosis [20,21], and food spoilage [15,22]. Such amines have also been used as a freshness index [18] and as a cancer marker [23].

Fig. 1. Oxidation of the FAD PuO subunit via DET using a −0.45 V potential.

Materials and methods

Materials

MWCNTs (95% purity, diameter 10–20 nm, length 1–5 μm) were obtained from Nano-Lab (Brighton, MA, USA). Nafion perfluorinated ion exchange resin (0.5% in water and ethanol), 3-aminopropyltriethoxy-silane (APTES, diluted to 20% in water), PDDA (MW 200,000–350,000, diluted to 0.1% in water), cadaverine, histamine, spermine, spermidine, dansyl chloride (98%), NaOH, HCl, and methanol were obtained from Sigma–Aldrich (St. Louis, MO, USA). Putrescine oxidase (PuO) from *Micrococcus roseus* (PuO, 37 U/mg) was obtained from Toyobo (Osaka, Japan). Milli-Q (Millipore) A-10 gradient (18 MΩ cm) deionized water was used for the preparation. Cyclic voltammetry (CV) and amperometric measurements were carried out in an Electrochemical Analyzer Multi-Potentiostat (CH Instruments, Austin, TX, USA). Pt wire (Sigma–Aldrich, 99.9% purity, 1 mm diameter) was used as a counter electrode. The reference electrode (Ag/AgCl, 3 M NaCl) and glassy carbon electrode (GCE, 3 mm), used as the working electrode, were obtained from BAS (West Lafayette, IN, USA). The cation exchange resin (Bio-Rex 70, 100–200 mesh, sodium form) was purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Working electrode preparation

The GCEs were initially polished with wet fine Emery paper, followed by polishing for 5 min on soft paper with a 0.05-μm alumina slurry. After another 5 min of sonication, the electrodes were rinsed with distilled water and dried under nitrogen. A mixture of MWCNTs, PDDA, APTES, and PuO (1.5 μl) was deposited on the electrode surface. This mixture was prepared in a 1.5-ml vial by adding 27 μl of MWCNTs (1 mg/ml), suspended in a 0.1% solution of PDDA, and sonicated for 15 min. APTES (3 μl, 20%) and PuO (0.1 mg) were then admixed for a few seconds to facilitate the interaction of the different compounds. The use of APTES permits the adsorption of glucose oxidase on CNTs [24,25]. After overnight air-drying, 1.5 μl of 0.5% Nafion was deposited onto the modified electrode and dried (2–3 h). The surface of each modified working electrode contained approximately three enzyme units. When not being used, the modified electrodes were stored at 4 °C.

Bio-Rex 70 column preparation

The Bio-Rex resin was used to extract the biogenic amines from the mouse plasma. After hydration with Tris–HCl buffer (pH 9) for at least 30 min (0.201 g/ml), the resin (0.2 ml) was transferred to a 1-ml syringe, forming the column. The 0.2-ml column was regenerated with

0.6 ml of 0.5 N NaOH using a flow rate below 1 ml/min cm^2. The column was then washed with Tris–HCl, pH 9, until the liquid eluted from the column attained a stable pH.

Mouse plasma putrescine purification

To facilitate the reaction between putrescine and PuO, 500 µl of the plasma was treated with HCl. Plasma aliquots (0.5 ml) were placed in a 1.5-ml vial with 0.5 ml concentrated HCl acid and were incubated at 100 °C for 5 h. To minimize evaporation, tiny holes were made through the vial cap with a syringe needle. Then 300 µl of the resulting plasma was used for bioanalysis, while the remaining sample (500 µl) was loaded onto the column and allowed to flow through the Bio-Rex 70 by gravity. The resin was washed with 350 µl of sodium carbonate (2 g/l, pH 9.3) to remove the nonpolyamine cation bound to the resin. The collected resin was centrifuged at 800*g* for 1 min to remove the supernatant.

Derivatization

Dansylation of the polyamines was performed as described by Gilbert and Gonzalez [26], with a minor modification. The polyamine-containing resin was suspended in 250 µl of deionized water by a few seconds of vortexing. The solution was admixed with 100 µl of sodium carbonate (285 g/l, pH 10.3), followed by 300 µl of dansyl chloride (25 g/l in acetone) [27]. This procedure permits UV detection of the derivatized polyamines at 220 nm. The vials were capped and heated at 70 °C for 20 min. During reaction, the vials were vortexed two or three times, keeping the temperature near 70 °C. After 20 min, a vial was cooled down before the addition of 850 µl of absolute methanol, followed by 20–30 s of vortexing. Methanol caused the precipitation of the majority of the carbonate. To complete the precipitation, the vials were maintained at −20 °C for at least 1 h. The samples were then centrifuged at high speed, in a table centrifuge, to collect the resin and the carbonate at the bottoms of the vials. The supernatants were transferred to other vials, and 50-µl samples were taken for high-performance liquid chromatography (HPLC) analysis.

Amperometric biodetection

Analysis was carried out in 10 ml of 0.05 mM (pH 7) phosphate buffer, except for the detection of putrescine from the mouse plasma (1 ml of phosphate buffer), so as to minimize dilution of the analyte.

HPLC detection

The HPLC separation of the dansylated polyamines was run on a 50-µl sample of the treated plasma. The sample was injected onto a 25 × 0.46-cm CSC Inertsil (150 Å/ODS2) column and was chromatographed at room temperature using water, acetonitrile, and methanol gradients (Table 1).

Table 1
HPLC elution program for putrescine

Time (min)	Flow (ml/min)	Percentage of total		
		A	B	C
0	1.2	50	30	20
4	1.2	50	30	20
6	1.2	44	34	22
26	1.2	0	60	40
30	1.2	0	100	0
32	1.2	50	30	20
45	1.2	50	30	20

FTIR spectroscopy

Fourier transform infrared spectroscopy (FTIR) was carried out using a Bio-Rad Excalibur series FTIR spectrometer (Randolph, MA, USA). FTIR samples were prepared by placing one or two drops of the different solutions onto pieces of undoped silicon transparent in the infrared spectroscopy. The samples were dried under mechanical vacuum (several tens of torrs). The spectra were obtained at a modulation frequency of 5 kHz and a spectra resolution of 8 cm^{-1} in the range 400–4000 cm^{-1}.

Results and discussion

MWCNT dispersion in PDDA

PDDA, even at low concentrations, disperses high concentrations of MWCNTs well. Approximately 0.1–1% PDDA is sufficient to disperse 1 g/l of MWCNTs. Such dispersions were stable for 5–6 days without any precipitation. The interaction between PDDA and MWCNTs is due to the π-orbital overlap of these two molecules; this π − π stacking interaction takes place between the π orbitals of MWCNTs and those of the vinyl groups present as contaminants in PDDA (Fig. 2B). Their interaction is responsible for the peak diminution at 1600 cm^{-1} in the FTIR spectrum of PDDA/MWCNT (Fig. 3). This interaction is how PDDA facilitates the preparation of stable dispersions of MWCNTs.

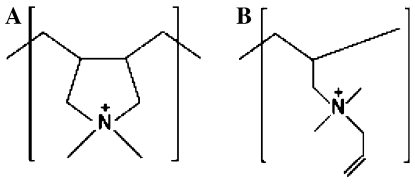

Fig. 2. (A) PDDA repeat unit and (B) PDDA contaminant.

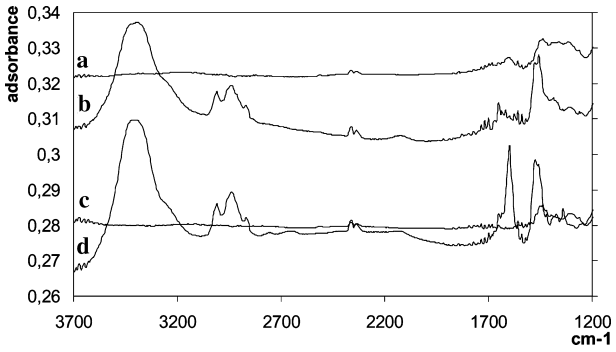

Fig. 3. FTIR spectra of (a) MWCNT – water suspension, (b) MWCNT + PDDA, (c) silicon blank, and (d) PDDA.

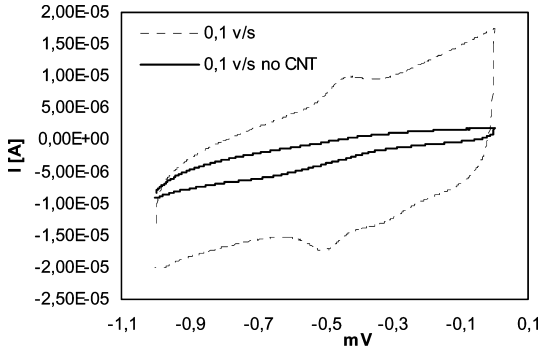

Fig. 4. DET of PuO at −0.45 V (vs. Ag/AgCl) at a 0.05-V/s scan rate in a 0.1 mM phosphate buffer. The dotted lines are the modified electrode, and the solid lines are the unmodified electrode.

Direct electron transfer

CNTs are able to create an electron bridge between the GCE surface and the flavin adenine dinucleotide (FAD) group that is deeply embedded in the center of the enzyme.

Due to their large surface/volume ratio, only a small amount of CNTs is sufficient for a considerable increase in electrode surface area. As shown in Fig. 4, the presence of MWCNTs on the electrode facilitates the DET of the FAD subunit, with an oxidation peak near −0.45 mV and a reduction peak near −0.49 mV. The DET between the enzyme and the CNT is confirmed in Fig. 5, which shows a linear relationship between the current and the scan rate. Each peak present on the CV curve corresponds to the oxidation or reduction of a FAD subunit. By recycling the reduced and oxidized form ($FADH_2 \leftrightarrow FAD + 2H^+ + 2e^-$), the enzyme provides an increase of electrons circulating throughout the modified working electrode, responsible for the increased current. Good contact between the enzyme and electrode ensures increased biosensor sensitivity [28].

Putrescine detection

Putrescine detection by DET permits the use of a lower potential of −0.45 V, which reduces the possibility of interference caused by the presence of small quantities of hydrogen peroxide ($H_2N–(CH_2)_4–NH_2 + O_2 + H_2O \rightarrow H_2N–(CH_2)_3–CHO + NH_3 + H_2O_2$). Indeed, no noticeable difference was noted in the amount of putrescine detected when the detection cell was bubbled with nitrogen and hydrogen peroxide below 2 mM at −0.45 V. This is an important finding because the conventional scheme for detection of this biogenic amine is carried out at 700–800 mV, which also detects electroactive interferences such as ascorbic acid, uric acid, and acetaminophen. A lower potential (50 mV) can be realized only by coupling PuO with horseradish peroxidase and in the presence of artificial mediators such as ferrocene and its derivatives [29].

Fig. 6 shows the increase in current generated by successive additions of different concentrations of putrescine into the biosensor. A linear relationship between the current increase and the putrescine concentration was established by a calibration curve. This curve demonstrates a detection limit of 5 μM, a linear range of 5–200 μM, and a slope of 2 nA/μM, with a correlation coefficient $R^2 = 0.996$. Thus, the linear range of the biosensor is applicable for putrescine detection in human plasma—2 to 50 μM for a cancer patient and 0.7–2.0 μM for a healthy person. Our biosensor shows results superior to those of the biosensor developed by Nagy and Nagy [15], who found a slope of 0.57 nA/μM.

The biosensor performance was also evaluated by comparing the response obtained using 1 mM putrescine with that obtained using 1 mM of some common electroactive interferents. Spermidine, spermine, and cadaverine are biogenic amines produced by the same metabolic process responsible for putrescine synthesis. All are involved in tissue regeneration and cell proliferation, making them good cancer markers [30]. Previous results demonstrated that putrescine has a more constant progression in some types of cancer, making its use a better choice for following tumor evolution [31]. Lyophilized human plasma was used to prepare all of the solutions so as to reproduce conditions similar to actual plasma biodetection. The responses given by the interfering species were, in every case, less than 6.8% of the response of the same amount of putrescine. Those results show that a putrescine biosensor, using DET, is more selective than the first generation of biosensors based on the detection of H_2O_2, which gave responses to cadaverine and spermidine of 83 and 71% the response given by the putrescine [14]. This test clearly shows the higher biosensor selectivity toward putrescine in comparison with the most common interferents. The biosensor sensitivity was identical regardless of whether a putrescine standard prepared in 0.05 mM phosphate buffer or a putrescine solution prepared in lyophilized human plasma was used, suggesting that the absence of plasma compounds interferents.

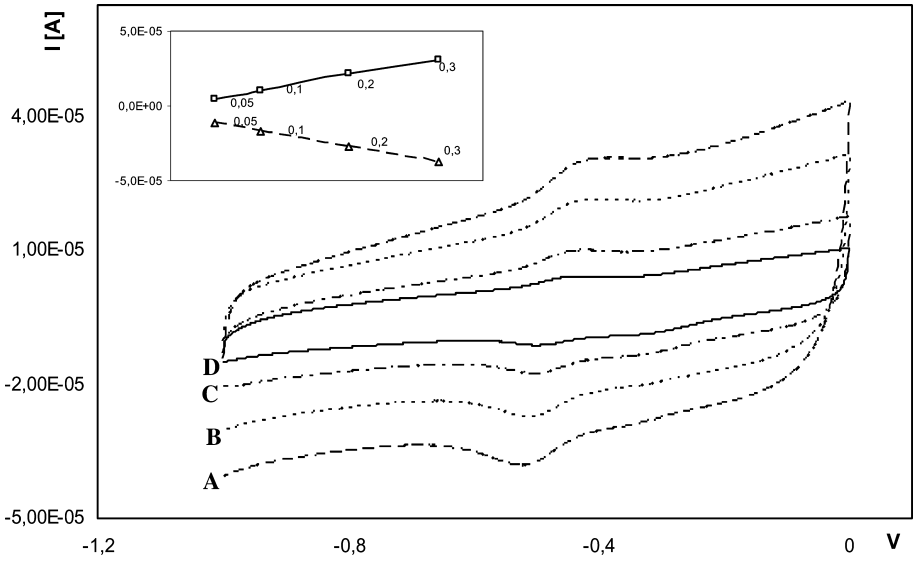

Fig. 5. DET of PuO at −0.45 (vs. Ag/AgCl), confirmed by multiple scan rate CVs (A: 50 mV/s; B: 100 mV/s; C: 200 mV/s; D: 300 mV/s) for enzyme immobilized on PDDA/APTES/Nafion/MWCNT-modified GCEs in 0.05 M phosphate buffer. The appearance of the oxidation and reduction peaks and their linear relationship with the scan rate (inset) confirm the DET.

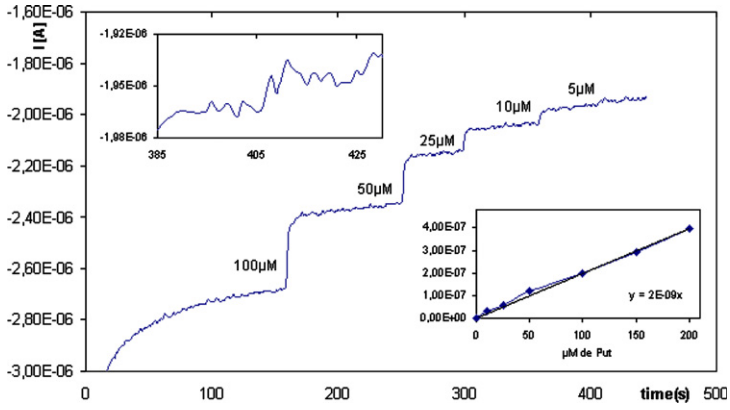

Fig. 6. Amperometric biodetection of 100, 50, 25, 10, and 5 µM of putrescine on a PDDA/APTES/Nafion/MWCNT/PuO-modified GCE in 0.05 M phosphate buffer, pH 7. The top inset is an enlargement of the detection of 5 µM, which appears to be the lower detection limit with a signal:noise ratio of 3:1. The bottom inset is the calibration curve for putrescine biosensor detection, showing a proportional increase of 2 nA per µM of putrescine detected. Note that the *y* axis of the bottom inset is −I [A] to facilitate reading.

Mouse plasma putrescine detection

The detection of putrescine in mouse plasma was carried out to evaluate the applicability of the biosensor. Two types of genetically modified mice were used. The first was a mouse mammary tumor virus (MMTV) type that had no sign of a tumor (mouse D). Plasma (1 ml) was withdrawn from this mouse to perform the test. The second was an NDL1-2 type with an ErbB-2 mutated gene. This type of mouse is predisposed to rapidly develop mammary tumors. A plasma aliquot of 0.8 ml was extracted from three different mice (mice A, B, and C) to evaluate the presence of plasma putrescine. The detection was carried out on the four mice at three different steps during the experiment. The first was on the plasma without any treatment. The second was made on neutralized plasma immediately after acid hydrolysis, used to liberate the putrescine from the plasma complex that might interfere with the detection. The third was carried out after the dansyl chloride derivatization, using the same solution as for HPLC detection. Among these three tests, only the untreated plasma of mice A, B, and C (ErbB-2 mice) provoked a response to the biosensor. Fig. 7 shows the current jump given by the putrescine detection of plasmas from three sick mice (A, B, and C) and from one healthy control mouse (D). On the base of calibration results and dilution factors, the putrescine concentrations were 65, 55, and 50 µM in samples A, B, and C, respectively.

HPLC was performed on the dansyl chloride-derivatized plasma to compare the results obtained by the

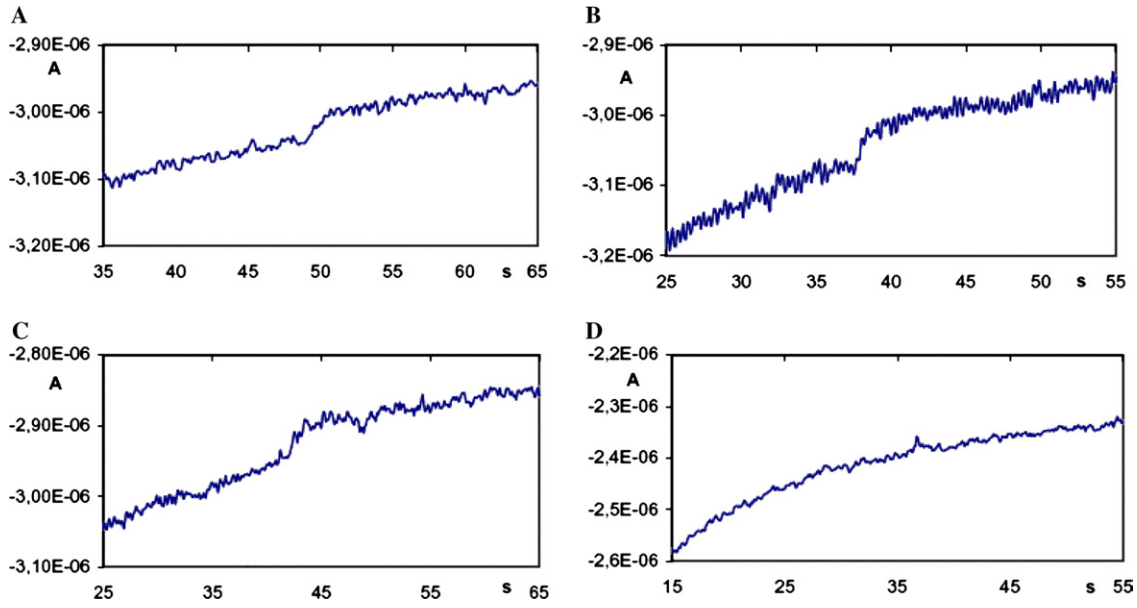

Fig. 7. Detection of putrescine in three plasma samples (A, B, and C) from mice predisposed to develop tumors and in a healthy mouse (D). The level of putrescine in the acid-treated plasma was possibly below the detection limit of the biosensor. The absence of the signal response to the derivatized plasma was not completely unexpected given that PuO is no longer capable of catalyzing the putrescine and the dansyl chloride complex.

biosensor. Dansyl chloride is known to react with amine groups and is generally used to label polyamides so as to permit UV detection at 220 nm [32]. On comparing the putrescine biodetection results with the concentrations obtained by HPLC, those obtained by the biosensor are clearly higher (Table 2). This is partly explained by the injection of a large amount of plasma (300 µl) into only 1 ml of phosphate buffer. As shown in Fig. 7D (injection of healthy plasma into phosphate buffer), the injection of 300 µl of liquid into 1 ml of phosphate buffer (at 36 s) creates a current disturbance. A second factor that might explain this high concentration of putrescine comes from the purification process of the plasma on the Bio-Rex column. During the transfer of the resin containing the putrescine into a 1.5-ml vial under a nitrogen flow, some resin may have remained on the glass fiber plug that held the resin in place during the washing steps. Some resin may also have remained on the side of the syringe wall during the transfer process. The loss of some resin during those manipulations may have reduced the final concentration detected by HPLC analysis.

Table 2
Putrescine concentration comparison of HPLC analysis and biosensor biodetection

Sample	Putrescine concentration given by the HPLC (µM)	Putrescine concentration given by the biosensor (µM)
A	28	65
B	25	55
C	20	50
D	0	0

Note. A, B, and C are plasma samples from mice predisposed to develop tumors, and D is the plasma from a healthy mouse.

Conclusion

An efficient mediatorless biosensor for putrescine has been constructed using PuO and MWCNTs, with PDDA being used to disperse the MWCNTs. The DET of redox enzymes or proteins at solid-state electrodes creates a new class of reagentless biosensors for various biosensing applications and offers an excellent platform for the fundamental understanding of biological redox reactions. The detection limits of the putrescine biosensor, and its selectivity with regard to potential interfering species such as spermine, spermidine, cadaverine, and histamine, have shown good results in artificial human plasma. Tests made on mouse plasma indicate that putrescine detection is also possible in mammalian plasma, using a very simple procedure in comparison with the standard HPLC method. Indeed, putrescine biodetection does not require any purification of the plasma sample or derivatization of the analyte.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2004.10.027.

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