Electron Transfer between Glucose Oxidase and Electrodes via Redox Mediators Bound with Flexible Chains to the Enzyme Surface

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Abstract: Electrical communication between redox centers of glucose oxidase and vitreous carbon electrodes is established through binding to oligosaccharides, at the periphery of the enzyme, ferrocene functions pendant on flexible chains. Communication is effective when the chains are long (>10 bonds), but not when the chains are short (<5 bonds). When attached to long flexible chains, the peripherally bound relays penetrate the enzyme to a sufficient depth to reduce the electron-transfer distances between a redox center of the enzyme and the relay and between the relay and the electrode, thereby increasing the rate of electron transfer.

Introduction

The redox centers of many enzymes are electrically insulated by thick protein or glycoprotein shells, preventing direct electrical communication between the centers and electrodes. The rate of electron transfer[1] between a redox center of an enzyme and an electrode is controlled by (a) the distance between the redox center and the electrode, (b) the potential difference between the redox center and the electrode, and (c) the reorganization energy associated with the electron transfer.[2] For enzymes such as glucose oxidase, with buried redox centers, diffusing redox mediators including O_2/H_2O_2[3] and ferrocene/ferricinium derivatives[4] have been used to shuttle electrons between enzyme redox center and electrodes. Leakage of ferrocene/ferricinium mediators from thin-film enzyme electrodes leads to their deterioration.[5] Leakage can be avoided through the use of soluble diffusing high molecular weight redox mediators, such as ferrocene-derivatized bovine serum albumin[6] and ferrocene bound to high molecular weight poly(ethylene glycol)[7] that can be confined within membranes having sufficiently small pores.

Direct, i.e., not diffusionally mediated, electrical communication between a buried redox center of an enzyme and an electrode can be achieved through insoluble, electrode-attached redox polymers that penetrate the enzyme sufficiently deeply for electron exchange.[8] This route provides the significant advantage of eliminating the need for membrane containing the soluble macromolecular mediator. Yet another way to establish direct electrical communication between a buried redox center of an enzyme and an electrode is through covalently binding to the protein of the enzyme (well below its "periphery") electron relays.[9] For example, in glucose oxidase, a rather rigid glycoprotein with two identical polypeptide chains and a hydrodynamic radius of ~50 Å, the distances involved in electron transfer between the active sites and the electrode are shortened upon binding 12 or more ferrocenecarboxylic acid functions, through amide links, to the enzyme. Replacement of ferrocenecarboxylic acid by ferroceneacetic acid or ferrocenebutanoic acid enhances the kinetics of electron transfer.[9c,d] In the preparation of materials for affinity chromatography, redox-active species of enzymes, such as NAD^+/NADH, are bound to supports with long and flexible spacer chains. Such chains facilitate access of the active species to their specific binding sites.[10]

We report here the modification of glucose oxidase by covalently binding of ferrocene derivatives, via spacer chains of different lengths, to sugar residues on its outer surface. We show that the length of the spacer chain has a crucial influence on the electrooxidation of the enzyme, i.e., on electron transfer from the reduced active site of the enzyme, via the spacer chain attached ferrocenes, to electrodes. This process is rapid only when the spacer chain is sufficiently long to allow the ferrocene to penetrate the enzyme sufficiently to approach the redox center.

Experimental Section

Chemicals. Glucose oxidase type X (EC 1.1.3.4, from *Aspergillus niger*, 128 units mg^{-1}), sodium *m*-periodate, sodium boron hydride, 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH), 1,2-ethylenediamine, 1,3-diaminopropane, 1,6-diaminohexane, 1,8-diaminooctane, 1,10-diaminodecane, and diethylenetriamine were purchased from Sigma; ferrocene carboxaldehyde (98%) was obtained from Aldrich. (Aminoethyl)ferrocene was synthesized according to literature[11] and precipitated as the chloride salt. All other chemicals were of the best available grade and used without further purification. Unless otherwise noted, all experiments were performed at room temperature in a standard aqueous buffer solution containing 100 mM phosphate and 200 mM NaCl at pH 7.2.

Electrodes and Equipment. Electrochemical measurements were performed with an EG&G Princeton Applied Research 175 universal programmer, a Model 173 potentiostat, and a Model 179 digital coulometer. The signal was recorded on a Kipp and Zonen Y-Y-Y′ recorder. Glassy carbon rods (Sigradur, 3-mm diameter) sealed with epoxy resin into glass

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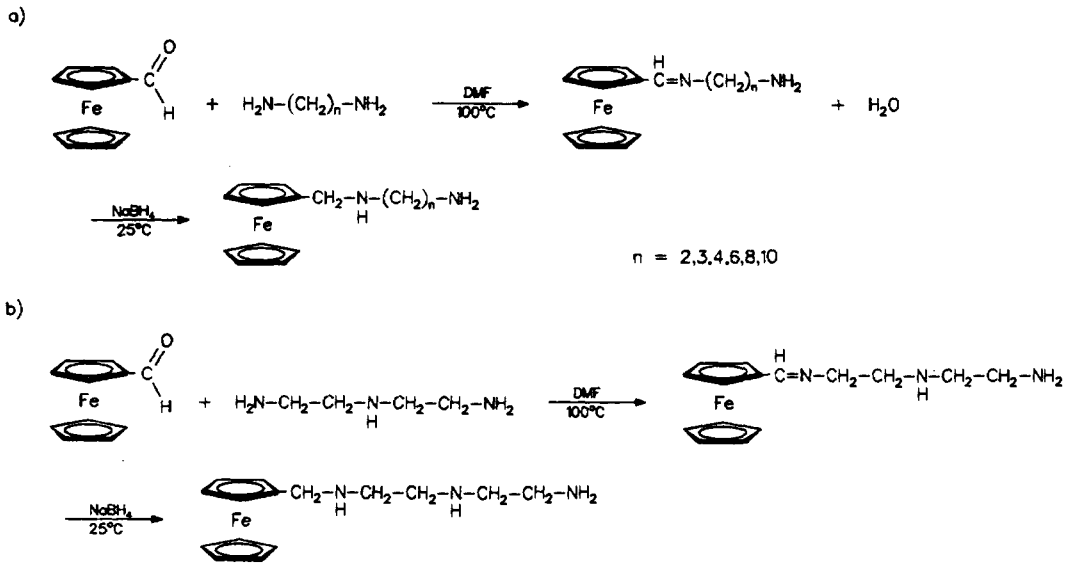

Figure 1. Synthesis of ferrocene amines with spacer chains separating the redox and amine functions.

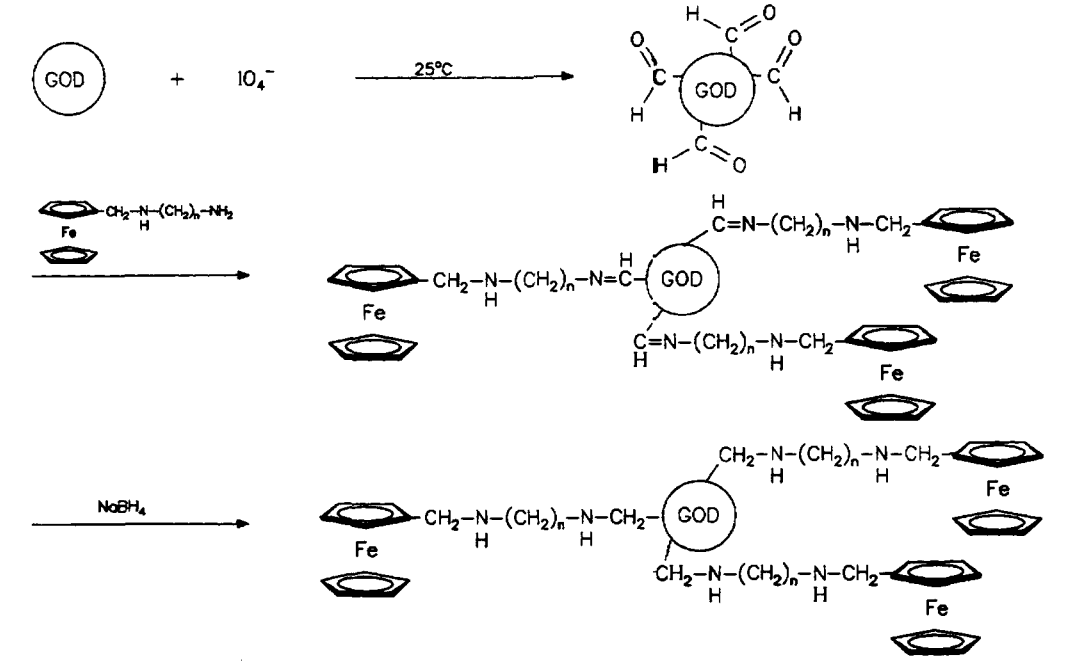

Figure 2. Preparation of glucose oxidase modified by peripherally bound ferrocenes.

were polished prior to use on a polishing cloth sequentially with alumina of decreasing particle size (1, 0.3, 0.5 μm), sonicated, rinsed with distilled water, and then dried in air. A single-compartment electrochemical cell was used with an aqueous KCl/saturated calomel (SCE) reference electrode and a platinum counter electrode. All potentials are referred to this reference electrode (+244 mV vs NHE).

Synthesis of Ferrocene Derivatives. The ferrocene derivatives with different spacer lengths were synthesized as shown in Figure 1. A 4-fold excess of the appropriate diamine was heated in 100 mL of DMF to 100 °C, and 500 mg of ferrocenecarboxaldehyde dissolved in 50 mL of DMF was added dropwise within 1 h to prevent formation of the bridged diferrocene compound. After another hour an excess of sodium borohydride in water was dropped into the solution, and the reaction mixture was stirred for an additional hour at room temperature. The solvent mixture was rotavaporated to dryness and the residue extracted with dichloromethane and separated through a silica column (1.5 cm × 30 cm). A first fraction—the bridged diferrocene—was eluted with dichloromethane, the main fraction with dichloromethane/methanol 10:1. The solvent was evaporated to dryness, the residue dissolved in diethyl ether, and the hydrochloride precipitated by bubbling gaseous hydrochloric acid through the solution. All compounds show the expected ^1H NMR spectra.

Preparation of Ferrocene-Modified Glucose Oxidase. The oxidation of the enzyme-bound sugar residues was performed with sodium *m*-periodate according to established procedures.[12] The ferrocenes were attached to the aldehyde groups formed thus on the outer enzyme surface via Schiff bases, which were reduced with sodium borohydride subsequently (Figure 2). The modified enzyme was isolated from low molecular weight compounds and desalted by gel chromatography (Sephadex G25 equilibrated with water; column 2.5 cm × 20 cm). The volume was reduced by means of ultrafiltration through a membrane (Amicon PM30, MWCO 30000), and the modified enzyme was freeze-dried. To verify that the unreacted ferrocenes were not electrostatically bound to the enzyme, the freeze-dried product was redissolved and extracted with copious amounts of a solution containing 0.1 M phosphate and 0.1 M NaCl at pH 7.1 in an ultrafiltration cell. After refreeze-drying, the electrochemical characteristics of the modified enzyme were unchanged, confirming the absence of noncovalently bound ferrocenes. Determination of the amount of aldehyde groups at the enzyme surface was performed by a procedure of Sawicki et al.[13] The activity of the lyophilized enzymes was determined spectrophotometrically by the *o*-dianisidine/peroxidase assay.[14] The labeling of the enzyme with ferrocenes was

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Table I. Effect of the Spacer Chain Length on the Catalytic Current of Ferrocene-Modified Glucose Oxidase

no.	compound	bonds	i_{cat},[a] nA	$[Fc]_{rel}$[b]	$i_{cat}/[Fc]_{rel}$	rel enzyme activ $[O_2]$[c]
1	Enz-CH_2-NH-$(CH_2)_2$-NH-CH_2-Fc	7	200	1.50 ± 0.20	400 ± 160	0.27
2	Enz-CH_2-NH-$(CH_2)_3$-NH-CH_2-Fc	8	1010	1.00 ± 0.10	1010 ± 100	0.38
3	Enz-CH_2-NH-$(CH_2)_6$-NH-CH_2-Fc	11	1190	1.00 ± 0.10	1190 ± 120	0.45
4	Enz-CH_2-NH-$(CH_2)_8$-NH-CH_2-Fc	13	2800	1.00 ± 0.10	2800 ± 280	0.41
5	Enz-CH_2-NH-$(CH_2)_{10}$-NH-CH_2-Fc	15	2680	1.00 ± 0.10	2680 ± 270	0.49
6	Enz-CH_2-NH-$(CH_2)_2$-Fc	5	460	0.75 ± 0.25	600 ± 200	0.33
7	Enz-CH_2-NH-$[(CH_2)_2$-$NH]_2$-CH_2-Fc	10	3200	1.00 ± 0.10	3200 ± 320	0.36

[a] Catalytic glucose oxidation current on 3-mm-diameter glassy carbon electrodes at 0.35 V (SCE). [b] Coulometrically determined relative number of ferrocenes per enzyme. [c] Hydrogen peroxide rate of formation, measured relative to the native glucose oxidase rate.

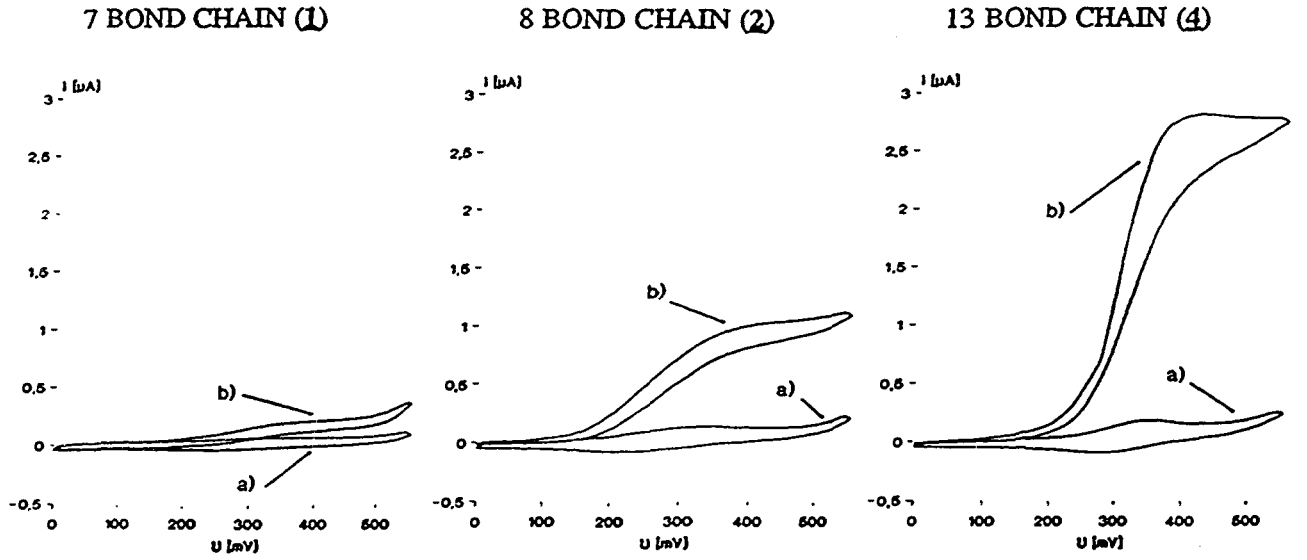

Figure 3. Effect of the chain length connecting peripherally bound ferrocene to glucose oxidase on the electrocatalytic glucose oxidation current. Curves a represent oxidation currents in the absence of glucose; curves b represent currents at 40 mM glucose. All solutions contain 2 mg mL^{-1} of one of the modified enzymes, 0.1 M phosphate buffer (pH 7.2), and 200 units/mL^{-1} catalase; 3-mm-diameter glassy carbon disks; all potentials vs SCE; scan rate 10 mV s^{-1}.

evaluated by atomic absorption spectroscopy and by coulometry.

Results and Discussion

Synthesis of Ferrocene-Labeled Glucose Oxidase. Glucose oxidase (EC 1.1.3.4 from *Aspergillus niger*) is a dimer glycoprotein with a molecular mass of 186 000 daltons. The oligosaccharide chains, which form a hydrophilic periphery, represent ~12% of its weight. Oxidation of these with periodate[12] has been used to provide peripheral aldehyde groups for the immobilization of glycoenzymes to polymeric supports[15] or to electrode surfaces.[16] Analogously, we have now applied this method to bind ferrocene derivatives with different spacer lengths to the surface of glucose oxidase. The periodate oxidation of glucose oxidase was investigated with respect to the number of aldehyde functions obtained and the decrease of enzymatic activity during the reaction. As expected, the aldehyde concentration increased when the reaction times were longer and the enzymatic activity decreased. Optimal results were obtained at a reaction time of 1 h and a periodate concentration of >20 mM, the conditions of our experiments. The number of aldehyde groups, introduced upon oxidation with 20 mM sodium periodate, was determined spectrophotometrically after its reaction with 3-methyl-2-benzothiazolinone hydrazone hydrochloride, following a procedure of Sawicki et al.[13] Assuming that the extinction coefficient reported for the hydrazones of aldehydes formed from mannitol (ϵ = 95 000 L mol^{-1} cm^{-1}) is similar to that of the hydrazones of the oxidized enzyme, we estimate 6.4 aldehyde groups per enzyme molecule.[17] However, because polysaccharides do not react as completely as monosaccharides with this hydrazone, and because the extinction coefficient for the aldehydes derived from mannitol is higher than that of other sugars, this estimate may be low. The functionalized enzyme used for the covalent binding of the different ferrocene compounds showed an activity of 66 units mg^{-1}.

As the rate of electron transfer decays exponentially with the distance of the involved redox centers, a significant influence of the spacer length between enzyme surface and relay on the electron-transfer properties of the modified enzyme in question was expected. To evaluate the effect of chain length on the effectiveness of electron transfer to electrodes, we prepared the series of ferrocene-derivatized enzymes shown in Table I (compounds **1–7**). The amino-functionalized ferrocene derivatives have been synthesized through the reaction sequence shown in Figure 1 and purified by column chromatography. Following IO_4^- oxidation of the oligosaccharide residues on the enzyme, the resulting aldehyde groups were reacted with ferrocene amines, to form Schiff bases. These were reduced with $NaBH_4$ to the secondary amines (Figure 2). Binding of amino spacer modified ferrocene derivatives to the surface of the functionalized glucose oxidase did not lead to a further decrease of enzymatic activity (see Table I).

Electrochemical Investigations of Ferrocene-Modified Glucose Oxidase. The results of the electrochemical measurements are summarized in Figure 3 and Table I. The cyclic voltammograms shown in Figure 3 were run at 2 mg mL^{-1} concentration of the

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Table II. Catalytic Current of Partially Deactivated Ferrocene-Modified Enzymes

no.	compound	bonds	i_{cat},[a] nA	i'_{cat} (deactiv),[b] nA	i''_{cat} (deactiv + native enz),[c] nA
1	Enz-CH_2-NH-$(CH_2)_2$-NH-CH_2-Fc	7	200	120	170
4	Enz-CH_2-NH-$(CH_2)_8$-NH-CH_2-Fc	13	2800	350	470

[a] Catalytic current for modified enzyme from Table I. [b] Catalytic current for modified, then partially deactivated enzyme. [c] Catalytic current of (b) after addition of an equal amount (1 mg mL^{-1}) of native glucose oxidase.

ferrocene-modified enzymes **1**–**7** in 0.1 M phosphate buffer (pH 7.2). The three-electrode cells were equipped with a glassy carbon (3-mm diameter) working electrode, a platinum wire counter electrode, and a KCl-saturated calomel reference electrode. Catalase was added to the solutions (200 units mL^{-1}) to decompose any hydrogen peroxide that might be formed in the presence of residual oxygen. Curve 1 of Figure 3 shows the cyclic voltammograms of a solution of compound **1** in buffer (a) without glucose and (b) with 40 mM glucose. Curves 2 and 3 show the cyclic voltammograms observed under identical conditions for compounds **2** and **4**, respectively. The limiting currents, normalized for the amount of attached ferrocene, increase with chain length (Table I). Notable enhancement of the catalytic current is observed in compound **7**, where i = 6.5 μA, i.e., the current density reaches 90 $\mu A\ cm^{-2}$.

Electron-Transfer Model. A peripherally attached redox mediator may accept electrons through either an intramolecular or an intermolecular process (Figure 4), acting in the latter as a conventional diffusing mediator. For example, mediation by ferrocene-modified albumin has been reported.[6] The dominance of the intramolecular electron-transfer process in the case of enzymes with long chains was established through the following experiment. Enzymes **1** and **4** were partially deactivated by 6 M urea (4 h, 25°) and then separated from the urea by gel-permeation chromatography. Their catalytic currents i' (Table II) were measured at an enzyme concentration of 1 mg mL^{-1} under conditions identical with those for i_{cat} in Table I. Then 1 mg mL^{-1} native glucose oxidase was added, and the catalytic current (i''_{cat}, Table II) was determined. If the process were entirely intermolecular, i''_{cat} would have been equal to or greater than i_{cat}, because the concentration of the electron-transfer mediator is unchanged and both the concentration and relative catalytic activity of the enzyme are increased (note in Table I that **1** and **4** retain, respectively, 0.27 and 0.45 of the native enzyme's activity). If the process were entirely intramolecular, addition of native enzyme would not have changed the catalytic current seen with the deactivated enzyme (i'_{cat}, Table II). Measurement of the catalytic current in the presence of deactivated **1** and **4** with native enzyme added shows that in the case of **1**, where the chain is short, the current approaches i_{cat} for the enzyme prior to deactivation, i.e., that the process of electron transfer either has a substantial intermolecular component or is entirely intermolecular. For compound **4**, made with long chains, i''_{cat}, the current observed with the partially deactivated enzyme plus native enzyme (470 nA), remains much below the 2800-nA catalytic current of the enzyme prior to its partial deactivation and is only marginally higher than the 350-nA current of the partially deactivated enzyme (Table II). This indicates that when the spacer chain is long the process is dominantly intramolecular. We thus conclude that the increase in catalytic currents with increase in chain length (Table I and Figure 3) originates in enhanced intramolecular electron transfer from the enzyme's redox centers to the chain-attached relay and, via the relay, to the electrode. Our observations do not allow us to define the extent of electron transfer by a dynamic process, where the chain-pendant mediator swings "in" and "out" of the enzyme, and a static process, where the relay is reasonably stationary, i.e., is bound by hydrophobic or electrostatic interaction to a specific region in the protein.

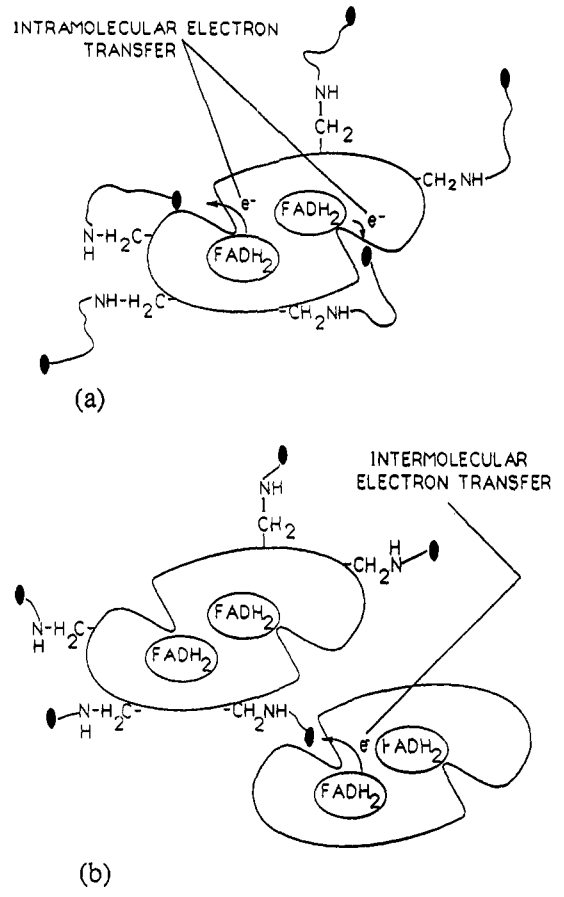

Figure 4. (a) Intramolecular and (b) intermolecular electron transfer via chain-attached mediators.

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Registry No. **1**, 130859-06-2; **2**, 130859-07-3; **3**, 130859-08-4; **4**, 130859-09-5; **5**, 130859-10-8; **6**, 130859-11-9; **7**, 130859-12-0; FCCH=$N(CH_2)_nNH_2$ (n = 2), 130859-13-1; FcCH=$N(CH_2)_nNH_2$ (n = 3), 130859-14-2; FcCH=$N(CH_2)_nNH_2$ (n = 4), 130859-15-3; FcCH=$N(CH_2)_nNH_2$ (n = 6), 130859-16-4; FcCH=$N(CH_2)_nNH_2$ (n = 8), 130859-17-5; FcCH=$N(CH_2)_nNH_2$ (n = 10), 130859-18-6; FcCH=$NCH_2CH_2NHCH_2CH_2NH_2$, 130859-19-7; 1,2-ethylenediamine, 107-15-3; 1,3-diaminopropane, 109-76-2; 1,6-diaminohexane, 124-09-4; 1,8-diaminooctane, 373-44-4; 1,10-diaminodecane, 646-25-3; diethylenetriamine, 111-40-0; ferrocenecarboxaldehyde, 12093-10-6; glucose oxidase, 9001-37-0.