Heterogeneous Electron-Transfer Kinetics for Flavin Adenine Dinucleotide and Ferrocene through Alkanethiol Mixed Monolayers on Gold Electrodes

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The heterogeneous electron-transfer properties of flavin adenine dinucleotide (FAD) were studied at self-assembled monolayer (SAM)-modified gold electrodes and compared to ferrocene-modified SAMs as a reference system. A modified form of FAD, N^6-(2-aminoethyl)-FAD, was covalently immobilized onto a mixed self-assembled monolayer (SAMs) formed by two series of alkanethiols ($HS-(CH_2)_n-COOH$ with a diluent $HS-(CH_2)_n-CH_3$ where $n = 5$, 7, 10, or 15 and $HS-(CH_2)_n-COOH$ with a diluent $HS-(CH_2)_n-CH_2OH$ where $n = 5$, 7, 10, or 11). The electron-transfer (ET) rates followed an exponential decay with the increasing thickness of the SAM ($k_{et} = A \exp(-\beta n)$; n = number of bonds) with an attenuation factor (β) of 1.0 per bond for FAD with both diluents. Despite the similarity in β the apparent rate constant for electron transfer was 2 orders of magnitude greater with the alcohol-terminated diluent relative to the methyl-terminated SAM. In comparison the ferrocenyl SAMs also had β values of 1.0−1.2 per bond, depending on the composition of the diluent layer, with the apparent rate constant for electron transfer being significantly faster with the alcohol-terminated diluent layer. The reconstitution of apo-glucose oxidase (apo-GOx) onto a FAD-modified gold electrode was also carried out; however, no biocatalytic activity was observed. Based on the β value for the FAD-modified electrodes and the magnitude of the apparent rate of electron transfer, rate constants for direct electron transfer to glucose oxidase at carbon nanotube-modified electrodes and graphite electrodes suggest the enzyme has been partially denatured from its native configuration, thus bringing the redox-active center of the enzyme closer to the electrode and allowing appreciable ET to be observed.

Introduction

In bioelectronics, biological molecules are integrated with electronic elements to provide devices for biosensing,[1] creating electronic readouts of biomolecular function,[2] the assembly of nanocircuit elements, or the conversion of biocatalytic processes into electrical power.[3,4] A crucial step in all these applications is the transfer of electrons to and from a biological molecule. In many instances, however, the redox center of the protein of interest is embedded deep within the tertiary structure of the polypeptides,[5] which effectively insulates the redox-active site from the environment surrounding the protein. Taking the enzyme glucose oxidase as a model protein in this study, the closest approach of the redox-active center of glucose oxidase, namely flavin adenine dinucleotide (FAD), to the surface of the glycoprotein is 8 Å.[6] However, the minimal distance between the isoalloxazine ring of FAD, where the redox reaction occurs, and the surface of glycoprotein is 13 Å. Therefore, if the enzyme is to retain its natural conformation then 13 Å is the minimum distance an electron must tunnel if it is to be transferred between the biological molecule and an electrode. As the rate of electron transfer (ET) decays exponentially with tunneling distance, such long distances suggest only slow ET can be achieved.[7,8]

There have been a number of strategies for achieving efficient shuttling of electrons between an electrode and the redox-active center of our model enzyme glucose oxidase including using redox mediators,[9−12] electron relays[5,13,14] (and the extension of this idea to 'wired' enzyme electrodes using redox polymers[15,16] or SAM-based electrode constructs[17,18]), or using electrode materials where the ET becomes sufficiently rapid due to a favorable spatial relationship between the electrode and the redox-active center to observe appreciable electrochemistry via tunneling through the glycoprotein. In this way direct ET has been observed between glucose oxidase and SAM-modified electrodes,[19] pyrolytic graphite,[20] and nanotubes-based electrodes.[21,22] However, in all these cases of a favorable spatial relationship it is not clear whether the enzyme is still within its native conformation or that there has been some change in conformation to bring the redox-active center close enough to the electrode to allow appreciable electron tunneling to occur. The possibility of a conformational change in the enzyme exists because all the surfaces in which direct ET has occurred are highly charged and highly charged surfaces frequently cause protein denaturation.[23]

An important question therefore is the following: can appreciable tunneling currents be observed between FAD and an electrode when glucose oxidase is in its native configuration? That is, can appreciable ET to FAD be achieved over distances of 13 Å or more? Long-range ET between redox-active probes and an electrode has been studied extensively through self-assembled monolayers (SAMs) formed on a gold substrate with alkanethiols,[24−28] norbornylogous bridges,[29−31] conjugated molecules,[32,33] and DNA[34,35] to redox molecules such as ferrocene,[24−26,36−44] and ruthenium complex,[44,45] and to proteins such as cytochrome *c*,[46−48] azurin,[49,50] horseradish peroxidase,[51,52] or laccase.[53,54] Liu et al. [55] have summarized the variation of the tunneling coefficient and have shown a large range in values for the rate constants; yet the attenuation factor β shows remarkable consistency for saturated molecules, being 1.0 ± 0.2 Å^{-1}.[8,56,57] Studies show significant currents despite the ferrocene being more than 20 Å from the electrode surface,

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which suggests that ET to FAD over similar distances should be achievable.[26]

Electrochemical studies of FAD have mostly been conducted by adsorption onto electrode materials such as graphite,[58] glassy carbon,[59] or mercury electrodes.[60] These studies show the electrochemistry is sensitive to the pH as well as the surface to which it adsorbs. For example, Gorton and Johansson[58] quote a heterogeneous rate constant of ET of 1 s^{-1} on graphite electrodes while on the same surface Verhagen and Hagen[59] report values around 200 s^{-1}. Willner et al.[61] have studied the ET of FAD when N^6-(2-aminoethyl)-FAD was covalently attached on the monolayers of 3,3′-dithiodipropionic acid−NHS ester and thioctic acid; high rate constants of 350 and 230 s^{-1} were obtained, respectively. Apart from this last study, there have been no other electrochemical studies of FAD attached directly to SAMs, although there have been reports of flavin mononucleotide[62,63] and riboflavin[64] attached to alkanethiol SAMs. Furthermore, the influence of distance on the electrochemistry has not yet been investigated with FAD.

The purpose of this paper is to investigate the influence of distance between the FAD and an electrode on the rate of ET and to ascertain whether appreciable electrochemistry can be observed through aliphatic hydrocarbons at distances equivalent to the separation of FAD from the enzyme exterior. The FAD analogue investigated was N^6-(2-aminoethyl)-FAD where a simple aminoethyl linker is attached to the amine on the adenine ring. The electrodes were prepared by first assembling a mixed self-assembled monolayer composed of a carboxylic acid-terminated alkanethiol and the equivalent length *n*-alkanethiol followed by attachment of the FAD to the carboxylic-acids-terminated species using carbodiimide coupling. As ferrocene attached to alkanethiols serves as a standard for ET through self-assembled monolayers, the FAD electrochemistry is compared with ferrocenemethylamine attached to the same alkanethiols.

Experimental Section

(1) Reagents and Materials. 3-Mercaptopropionic acid (MPA), 11-mercaptoundecanoic acid (MUA), 16-mercaptohexadecanoic acid (MHDA), and 1-hexadecanethiol (1-HDT) were purchased from Fluka (Sydney, Australia). 1-Propanethiol (1-PT), 1-octanethiol (1-OT), 1-undecanethiol (1-UT), mercaptododecanoic acid (MDA), mercaptohexanol (MLH)), mercaptooctanol (MLO), mercaptoundecanol MLU), and mercaptododecanol (MLD) were from Aldrich. 1-Hexanethiol (1-HT) was from Lancaster (England). 6-Mercaptohexanoic acid (MHA) and 8-mercaptooctanoic acid (MOA) were synthesized according to the method by Brevnov et al.[65] N^6-(2-Aminoethyl)-FAD was synthesized following the procedure of Bückmann.[66] Ferrocenemethylamine was synthesized using the procedure from Kraatz.[67] Apo-GOx was prepared following the method developed by Swoboda.[68] *N*-Hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 6-bromocuproic acid, and 8-bromooctanoic acid were from Fluka (Sydney, Australia). Flavin adenine dinucleotide (FAD), *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), and glucose oxidase (GOx) were from Sigma (Sydney, Australia). Ferrocenecarboxaldehyde and sodium cyanoborohydride were from Aldrich (Sydney, Australia). Ethyleneimine was from Alltech Associates (Aust.) Pty. Ltd. (Australia). K_2HPO_4, KH_2PO_4, KCl, hydrochloric acid, ethanol, $HClO_4$ (70% W/W), LiOH, NaOH, $KSCOCH_3$, methanol, ether, $NaSO_4$, and NaCl were all from Ajax Chemicals (Sydney Australia).

(2) Fabrication of Self-Assembled Gold Electrodes. Polycrystalline gold electrodes, prepared as described previously,[69] were polished to a mirror-like finish with 1.0 μm alumina, followed by 0.3 and 0.05 μm alumina slurry on microcloth pads (Buehler, Lake Bluff, IL). After removal of trace alumina from the surface by rinsing with water and brief cleaning in an ultrasonic bath with ethanol and then water, electrochemical cleaning in 0.05 M H_2SO_4 by cycling the electrodes between −0.3 and 1.5 V was carried out until a reproducible cyclic voltammogram was obtained. All mixed monolayers were prepared by immersing the gold electrodes in 1 mM mixed thiols solution (carboxylic-acid-terminated thiol and equal length thiol diluent with a ratio of 1:20) in ethanol for 24 h. The electrode was rinsed with copious amounts of ethanol and then water and dried under a stream of nitrogen prior to the next step.

(3) Covalent Coupling of FAD and Ferrocene on SAMs Modified Electrodes. Covalent attachment of N^6-(2-aminoethyl)-FAD and ferrocenemethylamine to carboxylic-acid-terminated SAMs was achieved by incubating the SAM-modified surfaces in an aqueous solution of 40 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 10 mM *N*-hydroxysuccinimide (NHS) for 1 h as described by Staros et al.[70] After the activation, the electrode was rinsed in water and incubated in 5 mM N^6-(2-aminoethyl)-FAD or 5 mM ferrocenemethylamine solution in HEPES buffer pH 7.3 for 24 h.

(4) Electrochemical Measurements. All electrochemical measurements were performed with a BAS-l00B electrochemical analyzer (Bioanalytical System Inc. Lafayette, IL) and a conventional three-electrode system, comprising a gold working electrode, a platinum foil as the auxiliary electrode, and a Ag/AgCl 3.0 M NaCl electrode (from BAS) as reference. All potentials were quoted relative to the Ag/AgCl reference at room temperature. All cyclic voltammetry measurements were carried out in pH 7.0 phosphate buffer for FAD-modified electrodes and degassed with argon for approximately 30 min prior to data acquisition and were blanketed with argon atmosphere during the entire experimental period. The ferrocene-modified electrodes were measured in 1 M $HClO_4$ aqueous solution. The rates of ET were calculated using the method of Laviron,[71] which relies on the change in peak potential (ΔE_P) with scan rate (ν) to obtain k_{et}. The method requires E_P values that are not significantly influenced by ohmic lossest (iR_u) due to uncompensated solution resistance. As low resistance aqueous solutions were used in this study, using the method of Bowyer et al,[72] the ohmic loss in cases used to derive k_{et} was less than 3 mV. Therefore no iR_u compensation was employed.

Results and Discussion

(1) Electrochemistry of Ferrocene Modified Electrodes. Prior to the investigation of the distance dependence of the electrochemistry of FAD, we first calibrated our experimental setup by measuring the distance dependence of ET dynamics through gold−alkanethiol−ferrocene constructs since they have been widely studied in this system.[24−26,36−43] Unlike previous studies of ET through aliphatic hydrocarbons to ferrocene, the electrodes in this study were first modified with a self-assembled monolayer, followed by covalent attachment of the ferrocenemethylamine. The SAM used was a 1:20 mixture of the carboxylic-acid-terminated alkanethiol and either a methyl-terminated alkanethiol or an alcohol-terminated alkanethiol. The carboxylic acid was subsequently activated with EDC and NHS followed by the coupling of ferrocenemethylamine to give the final electrode constructs shown in Figure 1. The electrochemical performance of the ferrocenemethylamine-modified electrodes

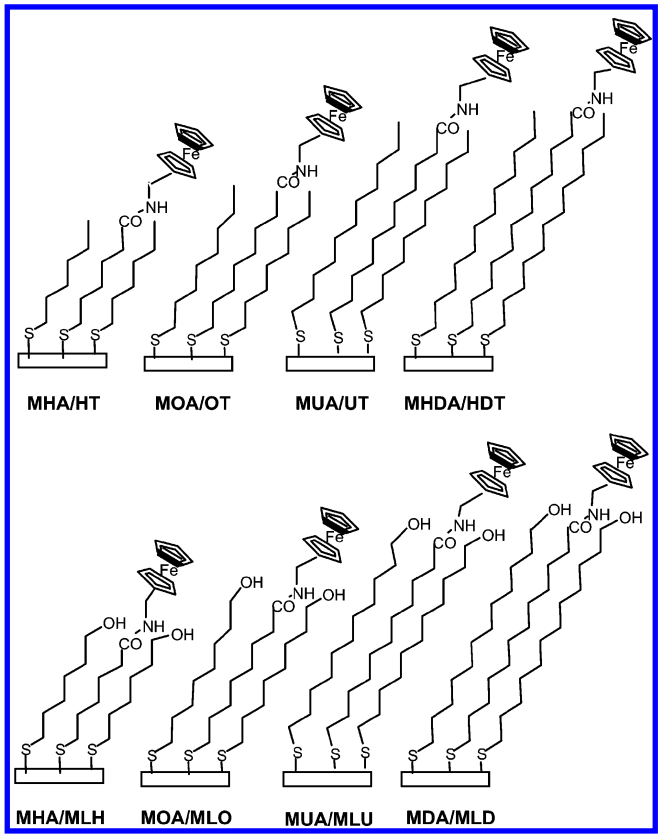


Figure 1. Schematic of ferrocenemethylamine immobilized covalently on mixed monolayers containing a methyl-terminated diluent to give MHA/MLH, MOA/MLO, MUA/MLA, and MDA/MLD and an alcohol-terminated diluent to give MHA/MCH, MOA/MCO, MUA/MCU, and MDA/MCD.

is summarized in Table 1. The SAMs formed with the methyl-terminated diluent will be discussed first followed by the SAMs where the alcohol-terminated diluent was employed.

The methyl-terminated diluent layer exposes a low energy hydrophobic surface to the aqueous solution in which the electrochemistry is performed. As ferrocene is hydrophobic it would be expected to interact with the methyl-terminated SAM.[73] There are some general trends that can be observed from the table of data (although in some cases the MHDA does not conform to the trends observed). For example, regardless of the length of the alkanethiol chain, approximately the same amount of ferrocene is electrochemically accessible and (apart from the MHDA) the area under the anodic and cathodic peaks approaches 1. The surface coverage of ferrocene molecules was between 1.03 and 1.75 $\times$ 10^{-11} mol cm^{-2}. The theoretical maximum surface coverage for an alkanethiol monolayer is 7.6 $\times$ 10^{-10} mol cm^{-2} [74] and hence only 1−2% of the alkanethiol monolayer has a ferrocene attached. A bond is not expected between the methyl-terminated SAM and the ferrocene but with a 1:20 ratio of carboxyl-terminated species this low surface coverage indicates that less than 50% of the carboxyl species has a ferrocene attached. Assuming a homogeneous distribution of attached ferrocene molecules the calculated surface coverage range equates to between 10 and 16 nm^2 per ferrocene. Such a large area between ferrocenes suggests there is little chance of the ferrocene moieties interacting with each other. It should be noted that some phase separation has been observed in SAMs containing two similar components[75,76] although there is no evidence from previous electron-transfer studies using ferrocene that any phase separation that may be occurring affects the quality of the electrochemical rate data obtained.

The voltammetry suggests that fabricating the ferrocenyl-SAMs by forming the monolayer and then attaching the redox moiety does result in the ferrocene existing in a range of environments. Both the greater than ideal fwhm[77] (ideally 90.6/n mV) and a nonzero ΔE_p [29,78] (ideally $\Delta E_p = 0$ at slow scan rates but typically 30 mV for the ferrocene SAMs in this study) have been attributed to a redox species in a monolayer being in a range of environments with a range of $E^{\circ\prime}$. Creager and Rowe[73] have postulated previously that with methyl-terminated SAMs the ferrocene group nestles on the top of the SAM such that it is partially solvated by the contacting aqueous solution and partially solvated by the monolayer, thus creating a range of environments in which the ferrocene is found. To create the free volume for the ferrocene group to nestle requires some disorder and it was proposed that minor defects in the SAM, including gauche defects in the carbon chains,[79] provide the free volume.

A consequence of the nonideal behavior exhibited by the methyl-terminated ferrocene SAMs is that a simple electron-transfer reaction is not observed. The observed increase in the standard electrode potential, $E^{\circ\prime}$, for ferrocene with the length of the alkanethiol, is clear evidence that the electron transfer is not conforming to a simple mechanism. The increase in $E^{\circ\prime}$ is due to the oxidation process getting progressively more difficult. As the oxidation process involves ion transfer to balance the charge of the ferrocinium ion it is possible that access of ions to the hydrophobic ferrocene is becoming more difficult with SAM length. The implication of this nonideal electron transfer behavior is the rate constants derived are apparent rate constants, k_{app}.

Apparent rate constants for ET, shown in Table 1, for SAMs of different lengths were calculated using the Laviron method.[71] The ET rate constants are significantly lower than that reported previously for ferrocene with methyl-terminated diluent layers.[25,26,40,41,80] Encouragingly, despite these slow k_{app} an exponential decay in the rate with increasing alkanethiol chain length (Figure 2) was observed with a slope of 1.2 per bond for the electron-tunneling coefficient (1.1 $Å^{-1}$). A value of 1.2 per bond for β is within the range of previous reports.[41,57,80]

Changing the diluent layer to an alcohol-terminated alkanethiol, which has been suggested to diminish the solvation of the ferrocene by the SAM and creating a more aqueous-like environment around the ferrocene.[73] The alcohol-terminated diluent layer appears to provide an environment for simple electron transfer to occur as $E^{\circ\prime}$ for ferrocene was less sensitive to the length of the SAM. The broad E_{fwhm} and ΔE_p do however indicate that the ferrocene moieties are not all in the same environment. The apparent rate constants with the alcohol-terminated diluent are approximately double those with the methyl-terminated SAM but the β value is similar (1.0 per bond or 0.9 $Å^{-1}$). The values of k_{app} are still lower than those observed previously with alcohol-terminated SAMs,[26,80] which may be a consequence of the different link between the ferrocene and the alkanethiol compared with other studies.[81] This suggests the rate constants quoted in relation to FAD SAMs will represent a lower limit. The change in magnitude of the apparent rate constant does however highlight the importance of the environment in which the redox center exists on the electron-transfer properties, which is important for redox centers in enzymes.

(2) Basic Electrochemistry of FAD. The electrochemistry of FAD has been studied using glassy carbon,[58,59,64] platinum,[58,59,64] gold,[58,59,64] and mercury[60] as electrodes. The reduction of the isoalloxazine ring is shown in Scheme 1 where the redox reaction involves two single-electron exchanges with the

TABLE 1: A Few Parameters Measured at a Scan Rate of 100 mV s^{-1} for Ferrocene Attached on the Mixed Monolayers of MHA/HT, MOA/OT, MUA/UT, MHDA/ HDT and MHA/MLH, MOA/MLO, MUA/MLU, and MDA/MLD with a Dilution Ratio of 1:20 (The Number of Measurements is Three for All the Parameters)

Fc-thiols	E^0 (mV)	ΔE (mV)	E_{fwhm} (mV)	Γ (mol cm^{-2})	Γ_a/Γ_c	k_{app} (s^{-1})
			Methyl-Terminated Diluent			
MHA/HT	237 ± 15	59 ± 8.5	171 ± 10	$1.75 \pm 0.45 \times 10^{-11}$	0.93 ± 0.07	2450 ± 495
MOA/OT	248 ± 18	75 ± 8	217 ± 19	$1.46 \pm 0.26 \times 10^{-11}$	0.89 ± 0.10	285 ± 106
MUA/UT	289 ± 19	174 ± 15	240 ± 25	$1.03 \pm 0.31 \times 10^{-11}$	1.07 ± 0.11	5.2 ± 1.3
MHDA/HDT	377 ± 27	472 ± 72	298 ± 38	$1.06 \pm 0.24 \times 10^{-11}$	0.42 ± 0.14	0.022 ± 0.01
			Alcohol-Terminated Diluent			
MHA/MLH	275 ± 14	53 ± 3	212 ± 7	$1.92 \pm 0.10 \times 10^{-11}$	0.54 ± 0.05	4140 ± 550
MOA/MLO	276 ± 24	80 ± 7	218 ± 13	$1.17 \pm 0.22 \times 10^{-11}$	0.46 ± 0.04	490 ± 100
MUA/MLU	284 ± 7	82 ± 3	227 ± 21	$1.75 \pm 0.45 \times 10^{-11}$	0.35 ± 0.05	43 ± 11
MDA/MLD	314 ± 11	77 ± 4	211 ± 6	$0.74 \pm 0.14 \times 10^{-11}$	0.71 ± 0.12	8.9 ± 1.4

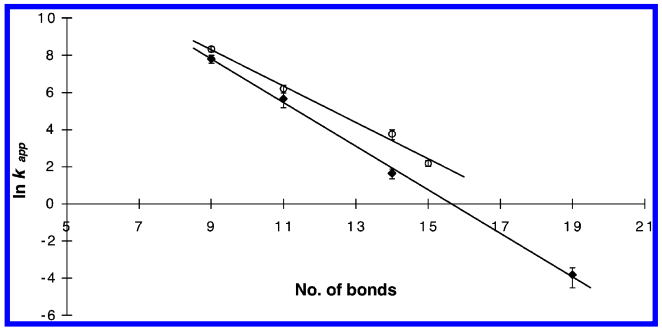
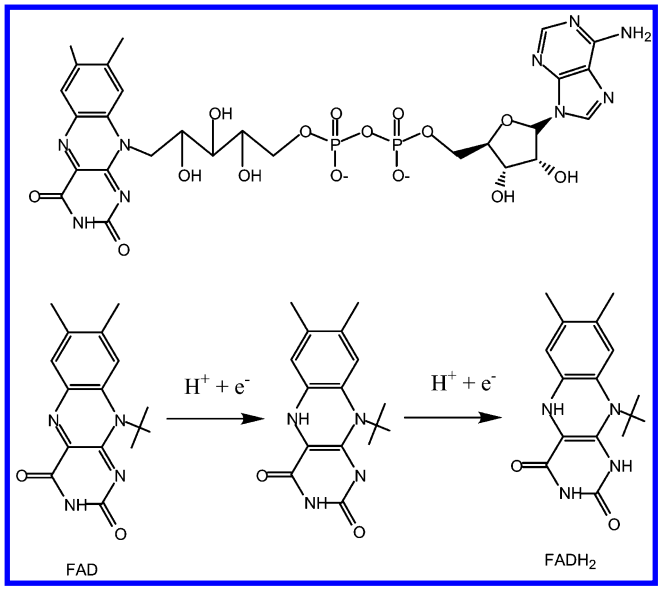


Figure 2. The logarithm of electron-transfer rates against the number of bonds between ferrocene and electrodes for ferrocene coupled onto a mixed monolayer containing a methyl-terminated diluent (diamonds) and alcohol-terminated diluent (circles). In each case the dilution ratio was 1:20 for carboxyl-terminated alkanethiol to diluent. For the methyl-terminated SAM the β value was calculated to be 1.2 per bond (1.1 Å) while for the alcohol-terminated SAM β was 1.0 per bond (0.9 Å).

SCHEME 1. The Scheme of the Structure of FAD and Its Redox Reaction



gain of a proton in each step.[82,83] First, FAD accepts a proton and one electron to its unsaturated nitrogen to form a semiquinoid free radical, and then another proton and electron can access another unsaturated nitrogen to form $FADH_2$. Hence the formal redox potential is pH sensitive,[58,59,64,84] shifting more negative with an increase in pH.[58] Typically, at solid electrodes the electrochemistry in aqueous solution proceeds via the FAD first adsorbing onto the electrode surface followed by oxidation and reduction. In this study the equivalent electrodes to the

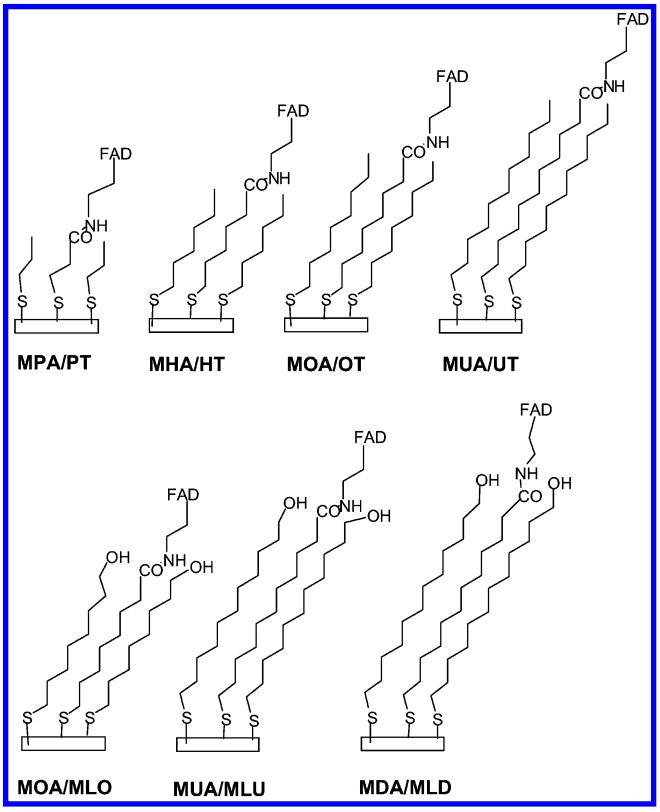


Figure 3. Schematic of N^6-(2-aminoethyl)-FAD immobilized covalently on mixed monolayers containing a methyl terminated diluent to give MPA/PT, MHA/HT, MOA/OT and MUA/UT and an alcohol terminated diluent to give MOA/MLO, MUA/MLU and MDA/MLD.

ferrocene-modified electrodes are fabricated using N^6-(2-aminoethyl)-FAD to give the electrode constructs shown in Figure 3.

The electrochemistry of an MPA/PT-modified gold electrode before and after attachment of FAD is shown in Figure 4. Prior to the coupling of FAD there is no electrochemistry in the region of 0 to −0.650 V versus Ag/AgCl. After coupling there is pronounced electrochemistry, due to the reduction and oxidation of FAD, with $E^{\circ\prime}$ of −0.417 V and ΔE of 15 mV at a scan rate of 100 mV s^{-1}. The peak current is linear with scan rate, which is consistent with a surface immobilized redox-active species. The electrochemistry is similar to that observed for FAD adsorbed onto gold[17,58,64] or carbon electrodes.[20,58,64] There is no evidence of the separation of the two one-electron processes as observed for FMN by Rotello et al.[85] In contrast to the observation with ferrocene with methyl-terminated SAMs, the $E^{\circ\prime}$ is almost constant with the length of the alkanethiol chain, with the exception of a −16 mV decrease with the mercaptoundecanoic acid (see Table 2). The MUA/UT system with

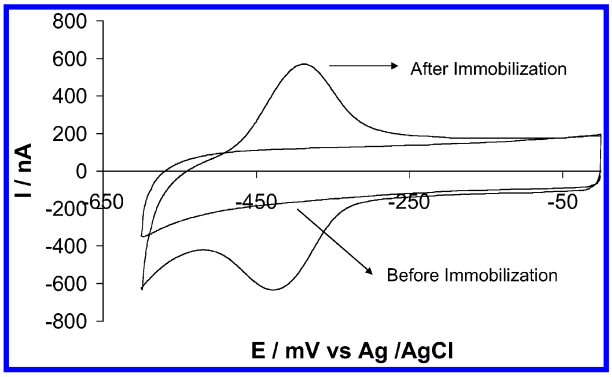


Figure 4. Cyclic voltammograms before and after the coupling of N^6-(2-aminoethyl)-FAD onto mixed monolayers of MPA/PT on a gold electrode.

FAD shows a different performance to the other FAD based SAMs with regard to not only $E^{\circ\prime}$ but also E_{fwhm} and Γ_a/Γ_c suggesting the FAD experiences a greater range of environments with the longer SAMs.

The fwhm of the reduction peak was 77 mV which, in common with the ferrocene results, is greater than the ideal (in this case 45.3 mV for a two-electron process) but consistent with the range of 75−150 mV for the fwhm of adsorbed FAD reported previously.[58,64] The broad peaks have previously been attributed to the reduction process occurring by two overlapping, but poorly resolved, single-electron reductions as distinct from a single two-electron reduction.[64] The ferrocene data suggest part of the peak broadening can also be attributed to some variability in the environment to which the FAD is found. Evidence for different environments contributing to the peak broadening comes from the increase in E_{fwhm} as the thickness of the alkanethiol layer increases (Table 2). Note, however, that the insensitivity of $E^{\circ\prime}$ on the thickness of the alkanethiol layer shows that the increase in peak width is not associated with any decrease in accessibility of ions during the reduction process and that a simple electron transfer mechanism is operating. The surface coverage of FAD molecules is significantly lower than that observed for ferrocene. Hence, as with ferrocene, the low surface coverage suggests there will not be any significant interactions between the FAD molecules.

(3) Rate of ET between FAD and the Electrode. Table 2 shows that as the thickness of the SAM separating the FAD from the electrode increases the separation between the oxidation and reduction peak also increases. An example of the variation in the position of the anodic and cathodic peaks relative to the formal electrode potential versus log(scan rate) is shown in Figure 5. The symmetry of Figure 5, and the slopes of the linear regions for the anodic and cathodic processes, indicates the transfer coefficient, α, is 0.5. The apparent rate constant of ET, k_{app}, for each electrode construct is shown in Table 2 and the variation in the natural log of this concentration with the length of the alkanethiol chain for both the methyl- and alcohol-terminated SAMs is shown in Figure 6. The excellent linearity of this plot allows the determination of the β of 1.0 per bond (0.9 $Å^{-1}$) in both cases, which is within the range expected for tunneling through a saturated aliphatic hydrocarbon.[57] Note the apparent rate constants for ET are more than 2 orders of magnitude greater for the alcohol-terminated diluent over the methyl-terminated diluent, again highlighting the importance of the environment of the redox center of the enzyme on the rate of ET.

An important implication of the rate of ET data and its variation with environment and distance is the information it gives with regard to tunneling between FAD embedded within a protein and an underlying electrode. In the case of glucose oxidase the closest distance between the isoalloxazine ring of FAD and the protein surface is 13 Å.[6] However, with the significant difference in apparent rate constants for the electrode constructs with the two different diluent layers, the question arises as to which better represents the environment of FAD in the protein, although clearly neither is a model of a protein environment. The pteridine ring of the flavin of FAD is held rigidly in place in the enzyme by hydrophobic contacts and adjacent to the redox center in the active site is a hydrophobic surface defined by amino acids Tyr 68, Phe 414, and Trp 426[86] to which the hydrophobic parts of glucose are thought to bind.[87] Hence the redox-active center is a reasonably hydrophobic environment. Of the two SAM constructs the methyl-terminated diluent is more protein-like with a hydrophobic surface to which the FAD can adsorb rather than the more water-like environment conferred by the alcohol-terminated diluent.[73]

Focusing on the rate data from the FAD SAMs prepared with the methyl-terminated diluent, the longest of the alkanethiol linkers in this study for which an appreciable rate of ET was measured was MUA with an apparent rate constant of 0.09 s^{-1}. The distance over which an electron must tunnel to the electrode from N^6-(2-aminoethyl)-FAD varies between about 12 and 19 Å depending on whether the isoalloxazine ring is lying flat on the alkanethiol monolayer or projects out from the interface, respectively. Therefore, considering the β value of 0.9 $Å^{-1}$, and assuming that the rate of ET through the protein backbone is similar to an aliphatic hydrocarbon, then rate constants for ET to glucose oxidase should be of the order of 0.1−0.0002 s^{-1}. Note however, that although the position of the FAD lying on the SAM or projecting out into solution has not been unambiguously determined, the increase in peak fwhm with the length of the SAM provides strong evidence that the FAD is lying on, or partially penetrated into, the hydrophobic SAM. Hence an apparent rate constant for glucose oxidase close to 0.0002 s^{-1} is more plausible.

In previous work by our group on the attachment of glucose oxidase to MPA SAMs, where the isoalloxazine ring of FAD embedded in the protein is 17 Å or greater, depending on the enzyme orientation relative to the electrode surface, no direct ET was observed. This absence of direct ET may simply represent poor electronic coupling between the FAD and the electrode or that the rate is too slow for appreciable electrochemistry to be observed. To improve the electronic coupling, FAD was attached to an MUA-modified electrode and apoglucose oxidase was reconstituted around the surface-immobilized FAD, this construct provides a direct bond between the FAD and the electrode. Again such an electrode construct showed no evidence of direct ET, an observation in agreement with previous work by the Willner group.[17] In contrast, Jiang et al.[19] have attached native glucose oxidase to an MPA-modified gold electrode activated using EDC and *N*-hydroxysulfosuccinimide. The result is an electrode construct equivalent to what we fabricated with glucose oxidase attached to MPA. Direct ET was observed with a rate constant of 0.026 s^{-1}, which is within the expected range of the simple calculation above.

Rate constants for direct ET have also be observed for glucose oxidase adsorbed onto other highly negatively charged carbon surfaces such as graphite[20] and carbon nanotubes.[21,22] In all these cases a consistent rate constant of ET of approximately 1.6 s^{-1} was reported. Based on our ET rates, such a value of k_{et} is consistent with the distance between the FAD and the electrode after the adsorption of GOx onto the electrode of about 8.5 Å. Such a distance is significantly smaller than the minimum 13

TABLE 2: A Few Parameters Measured at a Scan Rate of 100 mVs^{-1} for FAD Attached on the Mixed Monolayers of MPA/PT, MHA/HT, MOA/OT, MUA/UT and MOA/MLO, MUA/MLU, and MDA/MLD with a Dilution Ratio of 1:20 (The Number of Measurement is Three for All the Parameters)

FAD-thiols	E^0 (mV)	ΔE (mV)	E_{fwhm} (mV)	Γ (mol cm^{-2})	Γ_a/Γ_c	k_{app} (s^{-1})
			Methyl-Terminated Diluent			
MPA/PT	-417 ± 7	15 ± 4	77 ± 10	$6.3 \pm 1.5 \times 10^{-12}$	1.08 ± 0.16	274 ± 90
MHA/HT	-417 ± 9	38 ± 5	113 ± 9	$4.8 \pm 1.7 \times 10^{-12}$	1.22 ± 0.09	13 ± 3.5
MOA/OT	-415 ± 12	85 ± 3	114 ± 12	$4.5 \pm 2.2 \times 10^{-12}$	1.08 ± 0.13	2 ± 0.6
MUA/UT	-433 ± 16	291 ± 34	210 ± 22	$1.2 \pm 0.3 \times 10^{-12}$	1.93 ± 0.24	0.09 ± 0.03
			Alcohol-Terminated Diluent			
MOA/MLO	-440 ± 3	15 ± 3	83 ± 3	$2.0 \pm 0.1 \times 10^{-12}$	1.27 ± 0.14	372 ± 4
MUA/MLU	-429 ± 3	34 ± 1	102 ± 3	$3.1 \pm 0.8 \times 10^{-12}$	1.34 ± 0.12	30 ± 7
MDA/MLD	436	88	118	3.8×10^{-12}	2.39	4.8

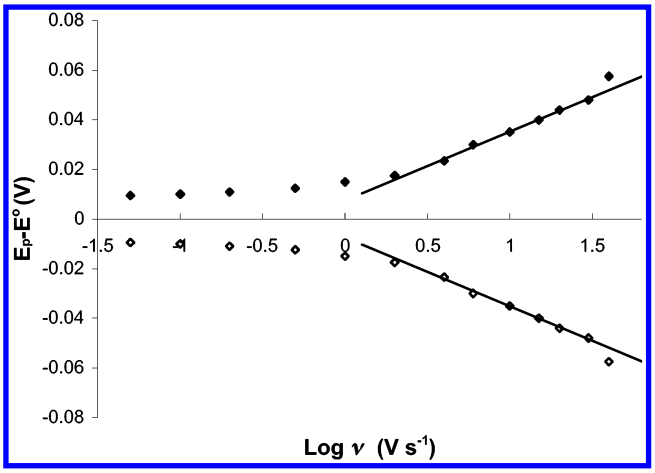


Figure 5. The plot of $E_p - E°$ versus the logarithm of scan rates for N^6-(2-aminoethyl)-FAD attached on the mixed monolayer of MPA/PT (1:20).

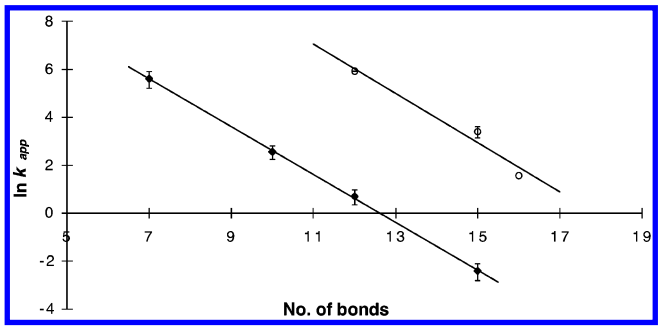


Figure 6. The logarithm of electron-transfer rates against the number of bonds between FAD and electrodes for FAD coupled onto a mixed monolayer containing a methyl-terminated diluent (diamonds) and alcohol-terminated diluent (circles). In each case the dilution ratio was 1:20 for carboxyl-terminated alkanethiol to diluent. In both cases the β value was calculated to be 1.0 per bond (0.9 Å).

Å distance between the FAD and the glycoprotein surface. Thus it seems that either the carbon electrode is penetrating closer to the active site of the enzyme or the enzyme partially unfolds, bringing the redox-active center closer to the electrode, on those highly charged surfaces. Of these two alternatives, with carbon-nanotubes-modified electrodes, the close approach of individual nanometer-size electrodes to the active center is easy to envisage but not with flat pyrolytic graphite electrodes. The alternative of the enzyme being partially unfolded but with retained activity is also possible as Degani and Heller[13,14] have shown that partially denatured glucose oxidase is still catalytically active. The adsorption of glucose oxidase on charged surfaces has been shown by scanning tunneling microscopy to cause some denaturing of the enzyme.[20,88] Thus these values for the rate constant of ET on carbon based electrodes are realistic but infer some change in the structure of glucose oxidase.

Conclusions

Ferrocene and FAD have been successfully attached on a series of mixed alkanethiol monolayers on gold electrodes by imide covalent coupling. Apparent rate constants for ET were obtained from different monolayers with different lengths. The distance dependence of the ET rates shows that electron tunneling is the dominant process for the ET through the SAMs to the electrode with the electron tunneling coefficients, β, being consistent with other previous studies.[8,56,57] When FAD was attached onto an alkanethiol SAM more than 13 Å long, which is of sufficient length to allow direct ET to the redox-active center of glucose oxidase, the ET rate dropped to a very small value ($k_{app} = 0.09$ s^{-1}). Such small values indicate direct ET to glucose oxidase achieved with nanotube and carbon electrodes is the result of some partial denaturing of the enzyme to bring the redox-active center closer to the electrode surface.

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