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The influence of structure in the reaction of electrochemically generated ferrocenium derivatives with reduced glucose oxidase

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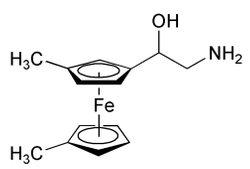
The synthesis and characterisation of a series of ferrocenylaminoalcohols is reported. 1,2-Aminoalcohol compounds were prepared from the respective ferrocene aldehydes *via* reaction with trimethylsilylcyanide followed by reduction with $LiAlH_4$. This series includes the ferrocene derivative 1,1′-dimethyl-3-(2-amino-1-hydroxyethyl)ferrocene **1**, which is used as a redox mediator to glucose oxidase in a commercial biosensor for determining blood glucose levels in diabetics. The aminoalcohol derivatives are included in a structure–activity study involving the electrochemical determination of the mediation rates of a range of systematically substituted ferrocenes with glucose oxidase. These mediation rates are correlated with structure.

Introduction

Glucose oxidase (GOx) is a commercially important flavo-enzyme in the food and medical diagnostic industries and as such has been the subject of many investigations. The enzyme, as isolated from *Aspergillus niger*, is a glycoprotein of molecular mass[1] *ca.* 160 kDa with a carbohydrate content[2] of 16% w/w. Two identical subunits have been identified[1] each of which contains 583 amino acid residues with three cysteines and eight potential sites for N-linked glycosylation.[3] In common with glucose oxidases from the genus *Penicillium*, the *Asp. niger* enzyme is characterised by a relatively high proportion of hydrophobic (Ala, Val, Leu, Phe, Gly, *etc.*) and dicarboxylic (Glu, Asp) amino acids.[4] As a result the protein is polyanionic; a charge of −58 at neutral pH has been estimated from the amino acid composition.[3] The carbohydrate moiety consists primarily of mannose (80%)[5] and is N- and O-glycosidically linked to aspargine and serine or threonine residues respectively.[6] Each subunit contains a tightly bound flavin adenine dinucleotide (FAD) cofactor which stabilises the quaternary structure[7] of the enzyme in a nearly spherical form of Stokes radius 43 Å (ref. 2). The *re* face of the flavin isoalloxazine ring is exposed for interaction with the glucose substrate.[8] The subunits are linked by two disulfide bonds[1,9] with the two remaining cysteine residues each being located in the flavin binding sites as inferred from studies with 4-thioflavin probes.[10]

The mechanism of action of the enzyme (on glucose) has been analysed in some detail[11,12] and has been found to follow the "ping-pong" mechanism[13] whereby the product, δ-gluconolactone, is released before binding with oxygen (and other electron acceptors). The enzyme is very specific for β-D-glucose; α-D-glucose, mannose and galactose are oxidized but at rates 100 times slower.[14] In contrast, GOx accepts numerous oxidants apart from O_2 as co-substrates which has been taken by some authors[15] as evidence for the presence of a second "non-selective co-substrate binding site." A study of the pH dependence of the enzyme reaction led to the proposal that a carboxylate group has a catalytic function within the active site.[12] The crystal structure of partially deglycosylated GOx from *Asp. niger* has been determined.[16] The active site is characterized by a deep pocket, at the bottom of which, lies the flavin ring system.

Direct electron transfer between an electrode and $FADH_2$, deeply buried within reduced GOx (redox potential: −0.41 V *versus* SCE at pH 7),[17] is prevented by the electrically insulating carbohydrate shell which also serves to stabilise the enzyme thermally.[18] Kulys and Cenas[19] have calculated that the cofactor lies at a depth of 12–14 Å (GOx from *Penicillium vitale*) which accounts for the poor voltammetric response of the enzyme. This is confirmed from the crystal structure of the *Asp. niger* enzyme where the minimum distance between the protein surface and the flavin is more than 13 Å (ref. 16). Electrical communication between the enzyme prosthetic group and an electrode can be established using rapidly diffusing redox mediators such as O_2,[20] quinones,[19,21] ferricenium derivatives,[22,23] octacyanotungstate,[24] ferricyanide,[25] hexacyanoruthenate,[26] Os bipyridine complexes,[27,28] Mn half sandwich complexes[29] and cyclometalated Ru(II) compounds.[30] In recent times, ferrocenes have come to the fore as mediators to GOx surpassing many other candidates in terms of a combination of efficiency, stability in the reduced form, pH independent redox potentials, ease of synthesis and substitutional versatility. In contrast to some higher molecular weight mediators, ferrocene is also of small enough size to be able to penetrate the active site of GOx.[31] Extensive work in our Laboratories and at the University of Oxford has culminated in the use of a ferrocene derivative, 1,1′-dimethyl-3-(2-amino-1-hydroxyethyl)ferrocene **1**, in enzyme electrodes[32] for the hand-held MediSense® ExacTech™ and Precision QID™ blood glucose meters for use by diabetics as an aid in the control of blood sugar levels.[33] Here, **1** mediates the transfer of electrons from reduced GOx, produced during oxidation of glucose by the enzyme, to an electrode where the corresponding current is measured by the meter.



(1)

Only limited studies have been previously carried out to investigate whether there are any relationships between the structure of a ferrocene mediator and its rate of interaction with the reduced enzyme. Early work,[23] which involved the analysis of kinetic data according to the method developed by Nicholson and Shain,[34] quickly demonstrated that the charge

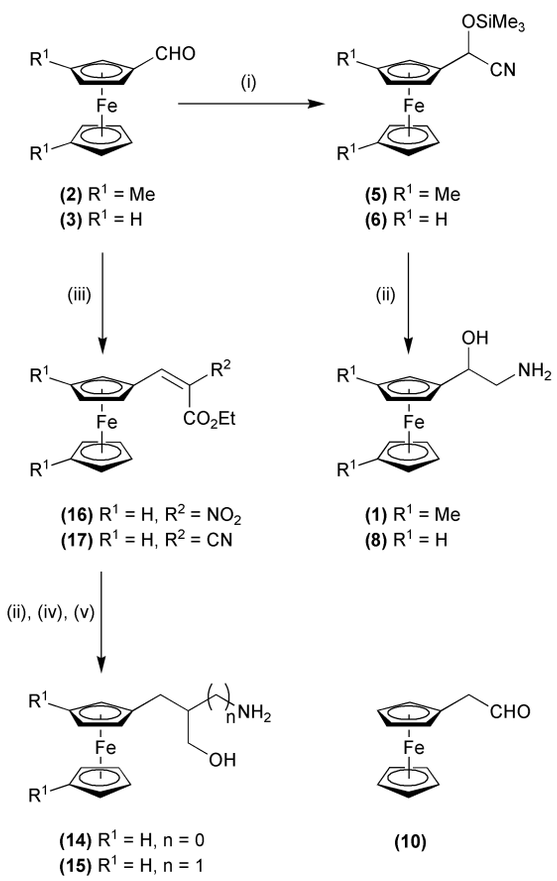
on the mediator is important; negatively charged ferrocenes are very poor mediators compared to neutral ones.[23] Beyond this fact, nothing more is known so that the selection of a ferrocene as a mediator remains very much a "black art." For example, it has been noted[30,35] that there is apparently no simple correlation between the mediation rate constant and the ferrocene redox potential.

The advent of more rapid methods for the kinetic analysis[35] of mediator–enzyme reactions has allowed us to determine the electron transfer rates of a relatively large number of systematically substituted ferrocenes with GOx. Our aim was to attempt to obtain a clearer picture of the factors that govern these rates, interpreting them in terms of the known structural features of GOx, with the ultimate intention of being able to design more efficient mediators to oxidoreductase enzymes.

Results and discussion

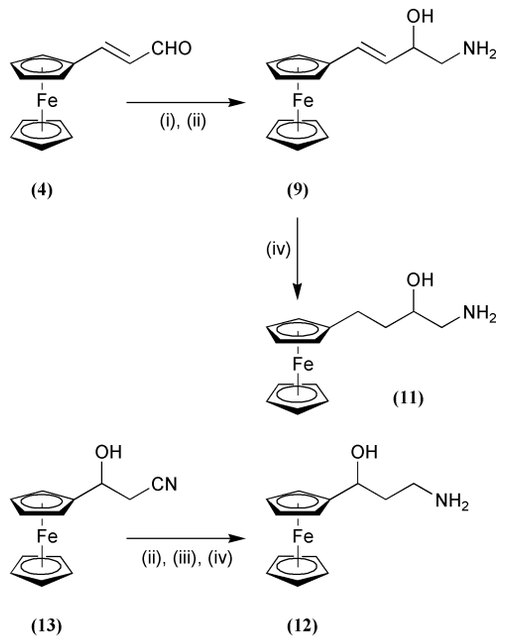
Synthesis of ferrocene derivatives

The ferrocene aldehydes **2** and **3** reacted smoothly, in the absence of solvent, with trimethylsilylcyanide (TMSCN) and a catalytic amount of ZnI_2 to produce the corresponding TMS–cyanohydrin derivatives **5** and **6** as dark, moisture-sensitive oils. This ZnI_2-catalysed cyanosilation reaction was adapted from a literature procedure[36] applied to organic aldehydes and ketones. The adducts **5** and **6** were not isolated but instead were dissolved in dry ether and reduced with $LiAlH_4$ to afford the 1,2-aminoalcohols **1** and **8** in moderate yields (Scheme 1). The crude reaction mixture in the preparation of **1** contained a mixture of diastereomers. Washing with Et_2O allowed the isolation of a single diastereomer of **1**. No product was isolable when the cyanosilation/reduction reaction sequence was performed on ferrocenylethanal **10** which is reported[37] to be air- and temperature-sensitive.



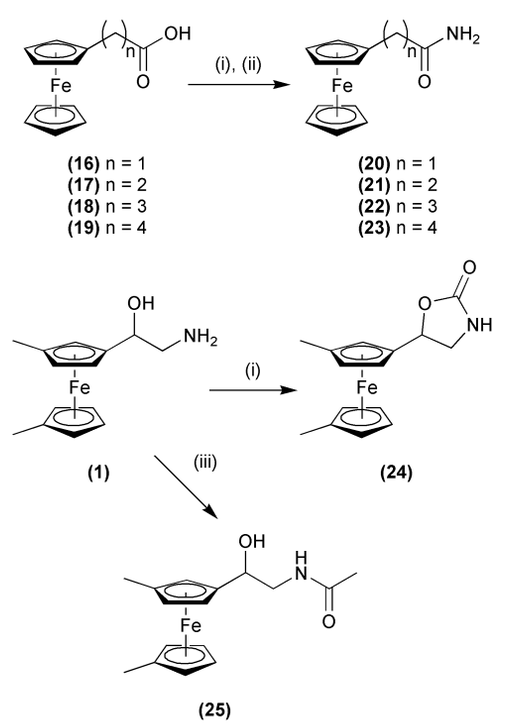
Scheme 1 Reagents: (i) TMSCN, ZnI_2 (cat.); (ii) $LiAlH_4$, Et_2O; (iii) $R^2CH_2CO_2Et$, base; (iv) CBz-Cl, Na_2CO_3; (v) H_2, Pd/C.

Similarly, the unsaturated 1,2-aminoalcohol **9** can be prepared from ferrocenylpropenal **4** *via* a TMS–cyanohydrin derivative. Catalytic hydrogenation of **9** provided the saturated derivative **11** in good yield (Scheme 2). The 1,3-aminoalcohol **12** was synthesized *via* the lithal reduction of the known[38] cyanoalcohol **13** (Scheme 2). The branched aminoalcohols **14** and **15** were obtained in a two step sequence from ferrocene carboxaldehyde **3** involving the reduction of the nitro- and cyano-acrylate esters **16** and **17** respectively (Scheme 1). The desired aminoalcohols **12**, **14** and **15** were separated from partially reduced, dehydrated and decarboxylated side-products by first converting them to their respective N-carboxybenzyl (CBz) derivatives followed by chromatography and finally cleavage of the protecting group by catalytic hydrogenation.[39]



Scheme 2 Reagents: (i) TMSCN, ZnI_2 (cat.); (ii) $LiAlH_4$, Et_2O; (iii) CBz-Cl, Na_2CO_3; (iv) H_2, Pd/C.

The primary amides **20**–**23** were prepared in a straightforward manner (Scheme 3), in a one-pot reaction, from the



Scheme 3 Reagents: (i) CDI, THF; (ii) aq. NH_3; (iii) AcCl, Et_3N, CH_2Cl_2.

Table 1 Electrochemical and kinetic data for ferrocene derivatives

Compound	No.	$E_{1/2}$/mV	$10^{-5}k_M$/M^{-1} s^{-1}	Synthesis ref.
$FcCO_2H$	**27**	+275	1.1	[b]
$FcCH_2CO_2H$	**16**	+120	0.3	40
$FcCH{:}CHCO_2H$	**28**	+220	1.1	41
$Fc(CH_2)_3CO_2H$	**18**	+90	0.3	42
$Fc(CH_2)_4CO_2H$	**19**	+80	0.3	42
$FcCH_2CH(NH_2)CO_2H$	**29**	+200	3.6	40
$FcCH_2SCH_2CH(NH_2)CO_2H$	**30**	+200	2.5	43
$FcCH_2CONH_2$	**20**	+210	3.1	[a]
$Fc(CH_2)_2CONH_2$	**21**	+170	1.8	[a]
$Fc(CH_2)_3CONH_2$	**22**	+105	[c]	[a]
$Fc(CH_2)_4CONH_2$	**23**	+105	2.5	[a]
FcOH	**31**	−200	0.3	44
$FcCH_2OH$	**32**	+185	8.2	45
$Fc(CH_2)_2OH$	**33**	+120	2.8	42
FcCH(Me)OH	**34**	+175	3.0	46
$FcCH_2O(CH_2)_2OH$	**35**	+205	5.7	47
1,1′-$Fc(CH_2OH)_2$	**36**	+230	5.5	48
1,2-$Fc(CH_2OH)_2$	**37**	+230	5.8	49
$FcCH_2O$-glucose	**38**	+210	2.5	50
$FcNH_2$	**39**	−95	2.5	51
$FcCH_2NH_2$	**40**	+295	6.2	52
$Fc(CH_2)_2NH_2$	**41**	+185	5.0	53
$Fc(CH_2)_3NH_2$	**42**	+135	4.1	54
1,1′-$Me_2FcCH_2NH_2$	**43**	+190	2.3	55
$FcCH_2NMe_2$	**44**	+315	5.2	[b]
(*R*)-FcCH(Me)NMe_2	**45**	+340	3.7	[b]
(*S*)-FcCH(Me)NMe_2	**46**	+340	3.6	[b]
1,2-$Me_3SiFcCH_2NMe_2$	**47**	+340	1.4	56
$FcCH_2NMe_3^+X^-$	**48**	+390	4.8 [d] 4.1 [e]	57
$FcCH_2NH(CH_2)_2NH_2$	**49**	+300	3.6	58
1,1′-$Me_2FcCH(OH)CH_2NH_2$	**1**	+145	4.3 [f]	[a]
		+145	5.0 [g]	[a]
$FcCH(OH)CH_2NH_2$	**8**	+240	6.9	[a]
$FcCH{:}CHCH(OH)CH_2NH_2$	**9**	+200	6.9	[a]
$Fc(CH_2)_2CH(OH)CH_2NH_2$	**11**	+125	5.9	[a]
$FcCH_2CH(NH_2)CH_2OH$	**14**	+205	4.8	[a]
$FcCH_2CH(CH_2NH_2)CH_2OH$	**15**	+210	3.9	[a]
$FcCH_2NH(CH_2)_2OH$	**50**	+305	5.4	59
1,1′-Me_2FcCHOCONHCH$_2$	**24**	+145	4.8	[a]
$FcCH(OH)(CH_2)_2NH_2$	**12**	+200	7.6	[a]
1,1′-$Me_2FcCH(OH)CH_2NHAc$	**25**	+90	1.5	[a]
$FcB(OH)_3$	**51**	+110	1.4	[b]
$FcC_6H_4OPO_3Na_2$	**52**	+180	2.2	60

[a] This paper. [b] Aldrich. [c] Not included in the kinetic study due to a poor elemental analysis. [d] $X^- = ClO_4^-$. [e] $X^- = BF_4^-$. [f] Ether-soluble diastereomer. [g] Ether-insoluble diastereomer.

known carboxylic acids **16**–**19** *via* the reaction of ammonia with the unisolated imidazolides produced from carbonyl diimidazole (CDI). We found this to be a useful route to **20**–**23** rather than that involving the intermediate preparation of acid chlorides using PCl_3. Ferrocenylacetic acid **16** was noted to be particularly sensitive to this acidic reagent resulting in low yields of the amide **20**. CDI is also of use in the preparation of the oxazalidinone **24** from the amino alcohol **1**, which could also be converted to the N-acetyl derivative **25**.

All the remaining ferrocene derivatives used in this study are known compounds and were synthesized according to the literature procedures cited in Table 1 or obtained from commercial sources. Where possible, these derivatives were selected to conform to the formula $R^1{}_2Fc–(CH_2)_n–R^2$ [where R^1 = H, Me; n = 0–4; R^2 = OH, NH_2, NMe_2, $CONH_2$, CO_2H, $CH(OH)CH_2NH_2$] such that their rates of interaction with GOx could be related to structure in a systematic manner.

Aqueous electrochemistry

All ferrocene derivatives, if sufficiently water-soluble, were investigated by cyclic voltammetry in phosphate buffered saline solution containing glucose. Degassing of the solutions with nitrogen was important for the sensitive hydroxyferrocene **31** and aminoferrocene **39**. The majority of derivatives displayed voltammograms consistent with reversible one electron transfer, *i.e.*, $\Delta E_p \approx 60$ mV. The half wave potentials $E_{1/2}$ for the ferrocene complexes studied are collected in Table 1.

Relatively few electrochemistry studies of ferrocenes have been performed in aqueous media compared to the large number conducted in organic solvents. This is largely due to the poor solubility of many ferrocene derivatives in water. Aqueous solubility of at least 0.5 mM was required for ferrocenes to be included in the present investigation. Of the neutral compounds, those containing hydroxy, amino and amido functional groups were sufficiently soluble. In general, the aqueous solubility of ferrocene derivatives containing these groups was found to lie in the order NH_2 > OH > $CONH_2$. As expected, increasing the length of a methylene chain in $Fc–(CH_2)_n–R$ and introducing methyl ring or alkyl side-chain substituents results in reduced aqueous solubility.

The half wave potentials of the ferrocene derivatives varied according to the electronic effects of the substituents and their distance from the cyclopentadienyl rings. Thus, the size of the effect for various functional groups lies in the order NMe_3^+ > NMe_2 > NH_2 > $CONH_2$ > OH > CO_2H. Increasing the length of a methylene chain in $Fc–(CH_2)_n–R$ diminishes the electronic effect of a substituent R. Little difference in the half wave

potentials is observed when the value of n reaches 3 and 4. The effect of a C–C double bond in increasing the redox potential *via* conjugation is clearly seen by comparison of the $E_{1/2}$ values for the aminoalcohols **9** and **11** (Table 1). Again, the effect of conjugation is seen in the low $E_{1/2}$ values for FcOH and $FcNH_2$. A single ring methyl group reduces the redox potential by approximately 50 mV. This effect is additive. In contrast to ring methyl groups, a trimethylsilyl substituent had the effect of slightly increasing the redox potential.

Kinetic studies with glucose oxidase

Fig. 1 displays the typical effect on the cyclic voltammogram of

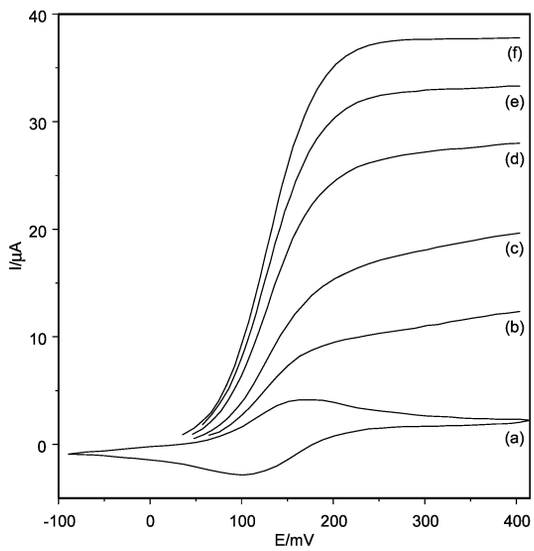


Fig. 1 Cyclic voltammograms (at 10 mV s^{-1}) of **1** (0.76 mM) in a 0.15 M phosphate/0.2 M NaCl buffer (pH 7.0) at a gold electrode (area 0.159 cm^2), containing (a) 0; (b) 5; (c) 15; (d) 30; (e) 50 and (f) 75 μl of stock GOx solution, in the presence of 0.1 M glucose. Reverse voltammetric sweeps are not shown for (b)–(f).

adding increasing amounts of GOx to an aqueous solution of a ferrocene. The mediation rate constants k_M derived from the corresponding sets of voltammograms for all ferrocenes in the present study are collected in Table 1.

A cursory look at the kinetic data in Table 1 indicates that there is no clear relationship of k_M with $E_{1/2}$. This has been noted previously[30,35] and appears to be because $E_{1/2}$ is one of a number of factors which affect k_M. Fig. 2 is an attempt to deconvolute some of these factors by displaying k_M as a function of both $E_{1/2}$ and structure.

From Fig. 2, the guide lines (a) to (d) show that k_M for monosubstituted ferrocene derivatives, containing various functional groups, lies in the order: $CH(OH)CH_2NH_2 > NH_2 > CONH_2 > CO_2H$. The mediation rate constants for two ferrocene amino acid compounds **29** and **30** are intermediate between the lines (b) and (d) for amines and carboxylic acids respectively. The guide line (e) for the ferrocene alcohol derivatives **31** to **33** intersects the lines (a) and (b) for aminoalcohols and amines respectively in Fig. 2.

Some dependence of k_M on $E_{1/2}$ for monosubstituted ferrocenes containing the same functional group is indicated by the lines (a) to (e). Two types of dependence are observed, a strong one for the alcohols (e) and carboxylic acids (d) but a weak one for aminoalcohols (a), amines (b) and amides (c). We have no explanation for this difference in behaviour although the two types are distinguished by the terminal side-chain group, namely OH and NH_2. Here, it is also noted that it is not possible to alter the redox potential of a ferrocene without changing its structure and this may affect k_M. For the monosubstituted ferrocenes grouped by lines (a) to (e), $E_{1/2}$ was varied by changing the length of the linker arm between the Cp ring

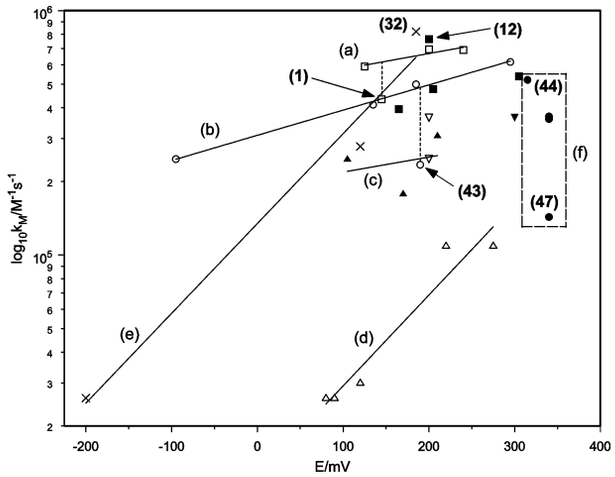


Fig. 2 Plots of rate constants (k_M) for the reaction of ferrocenium derivatives, containing various functional groups [OH (×), NH_2 (○), NMe_2 (●), $CONH_2$ (▲), CO_2H (△), $CH(OH)CH_2NH_2$ (□), other aminoalcohols (■), $CH_2CH(NH_2)CO_2H$ (▽), $CH_2NH(CH_2)_2NH_2$ (▼)] with GOx in the presence of glucose. Guide lines/boxes are drawn to group ferrocenes of similar structure; (a) Fc–X–$CH(OH)CH_2NH_2$ (**8**, **9**, **11**), (b) Fc–X–NH_2 (**39**, **40**, **41**, **42**), (c) Fc–X–$CONH_2$ (**20**, **21**, **23**), (d) Fc–X–CO_2H (**16**, **18**, **19**, **27**, **28**), (e) Fc–X–OH (**31**, **32**, **33**), (f) R–Fc–X–NMe_2 (**44**, **45**, **46**, **47**), where X = an alkyl chain linker group. Refer to Table 1 for numbering system.

and functional group. 1,1′-Dimethyl Cp ring substitution was also an effective route to reduce the redox potentials of monosubstituted ferrocenes by 100 mV. However, such trisubstituted ferrocenes proved to have much lower rate constants than expected, even accounting for their lower redox potentials. This is clearly illustrated in Fig. 2 by the 1,1′-dimethyl-substituted ferrocenes **1** and **43** which lie significantly below the appropriate guide lines (a) and (b) for 1,2-aminoalcohols and amines respectively. Similarly, trimethylsilyl ring substitution in **47** results in a much lower k_M than its unsubstituted counterpart $FcCH_2NMe_2$ **44** [box (f), Fig. 2] while $E_{1/2}$ is largely unaffected.

The influence of steric factors in the interaction of ferrocenium derivatives with reduced GOx seems clear from the above observations on the effect of Cp ring substitution. This is in agreement with the postulation of Alzari *et al.*[61] that steric hindrance during the approach to the flavin prosthetic group in the active site of GOx slows down electron transfer. From a computer simulation, Alvarez-Icaza *et al.*[31] reported that a Cp ring of a ferrocene molecule could achieve van der Waals contact with the active site (Fig. 3). It is envisaged that additional substituents in the leading Cp ring may impede optimal orientation for electron transfer close to the flavin. For an unsymmetrical trisubstituted ferrocene such as **1**, it is not clear whether the disubstituted ring containing methyl and 1,2-aminoalcohol substituents would preferably be leading or trailing during the approach to the active site. Indeed, this may depend on whether there is any specific interaction of a ferrocene side-chain functional group with an amino acid residue in the active site. In the case of the ferrocene glucopyanoside derivative **38**, it is presumed that the glucose unit will lead and interact the active site but this will only serve to prevent close approach of the ferrocene moiety with the flavin. Consequently, a relatively low k_M is recorded for **38**.

Hydroxymethyl ferrocene **32** was found to have the largest mediation rate constant of some fifty ferrocene derivatives included in the present study. It is notable that the 1-methyl side-chain substituent in the related FcCH(Me)OH **34** has the effect of reducing k_M significantly. Conversely, $FcCH(C_2H_4NH_2)OH$ **12** has a k_M value only slightly lower than that of **32**, indicating the effect is not purely one of steric hindrance. A reduction in rate is also seen for $FcCH(Me)NMe_2$ **45**/**46** compared to $FcCH_2NMe_2$ **44**. Furthermore, addition of

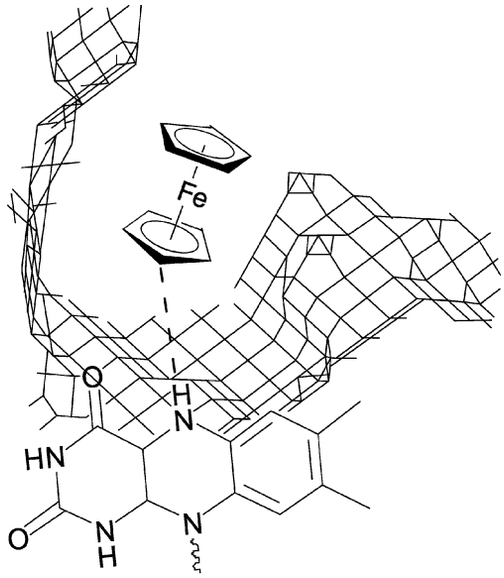


Fig. 3 Schematic diagram of ferrocenium ion near to the reduced cofactor $FADH_2$ in the active site of GOx (based on Fig. 1 of ref. 31).

an extra methylene group to the side-chain of **32** to give 2-hydroxyethyl ferrocene **33** results in a large drop in k_M. These observations suggest side-chain functional groups are likely to be involved in positioning ferrocenium derivatives in the active site of GOx for electron transfer. Such interactions are not so specific as to result in chiral discrimination in the case of (*R*)- and (*S*)-$FcCH(Me)NMe_2$, **45** and **46** respectively, where the chiral centre resides in the side-chain. This result is in agreement with an earlier kinetic study[61] of these enantiomeric ferrocenes with GOx. However, there is evidence of a small discrimination between the two racemic diastereomers of **1** where there is the combination of planar chirality for a 1,1′,3-trisubstituted ferrocene and a side-chain chiral centre. Significant discrimination has been reported in the oxidation of planar chiral ferrocene enantiomers by cytochrome c peroxidase.[62]

In summary, this study of over fifty systematically substituted ferrocene derivatives has shown that the rate of mediation with glucose oxidase depends on a number of conflicting factors. Conclusions from earlier studies on the influence of functional groups were confirmed. In addition, redox potential is a factor but it was difficult to separate it from the effect of structure since $E_{1/2}$ was varied by changing the side-chain length and introducing methyl substituents onto the Cp rings of ferrocenes. In particular, the latter method of altering $E_{1/2}$ had a large negative effect on k_M; this was attributed to steric effects impeding orientation of the ferrocene in the enzyme active site. Specific interaction of side-chain functional groups with active site residues was implicated from the large negative effects on k_M caused by relatively minor structural changes to the side-chain. The 1,2-aminoalcohol derivative **1**, having a good compromise of a relatively low $E_{1/2}$, high k_M and reasonable aqueous solubility, was selected for use in a commercial glucose biosensor.

Experimental

All manipulations and reactions were carried out using standard Schlenk-line techniques under an atmosphere of nitrogen. Predried solvents (Sure Seal grade; Aldrich) were used for reactions. Yields are unoptimised, the aim being only to obtain a small sample of the desired ferrocene derivative for kinetic studies with GOx. All reagents and starting materials for reactions were purchased from Aldrich except for 1,1′-dimethylferrocene (Strem) and TMSCN (Fluka) and were used as supplied. 1,1′-Dimethylferrocene carboxaldehyde **2** was prepared according to a modification of the literature procedure.[63] Glucose oxidase (EC 1.1.3.4 from *Aspergillus niger*) was supplied by Rhodia. The commercial dilute enzyme was concentrated, using tangential flow filtration (30 kDa size exclusion membrane, Millipore), to a protein level of *ca.* 250 mg ml^{-1} in 0.2 M BES buffer pH 7.5. A stock solution of the enzyme (*ca.* 15 μM) was then prepared by diluting the concentrate 100-fold with buffer solution (see below; no glucose). The concentration of GOx is expressed in terms of catalytically active FAD determined spectrophotometrically at 450 nm. A molar extinction coefficient of 1.31×10^4 M^{-1} cm^{-1} was used.[64] The buffer solution of 0.15 M phosphate (with 0.2 M NaCl as supporting electrolyte), pH 7.0, containing 0.1 M D-glucose was prepared using AnalaR reagents from Merck in deionised water. The solution was stored overnight at 4 °C prior to use to allow equilibration of the α- and β-anomers.

Proton NMR spectra were recorded on Brüker AM 500 (external NMR service, University of Oxford) and Hitachi R-24B instruments. Spectra were referenced internally to either the residual solvent resonance or $SiMe_4$. Microanalyses were performed by C.H.N. Analysis Ltd., Leicester and Butterworth Laboratories, Teddington. Mass spectra (electron impact) were obtained by the external mass spectrometry service at the University of Oxford. Cyclic voltammetry experiments were carried out with a two-chamber glass cell of working volume 0.5 cm^3. The cell contained a 1 cm^2 platinum gauze counter electrode and a KCl-saturated calomel electrode as reference. All potentials are referred to SCE. The working electrode was a 4.5 mm diameter gold disk. An Oxford Electrodes potentiostat was employed.

Syntheses of ferrocene derivatives

1,1′-Dimethylferrocene carboxaldehyde (2). A stirred solution of *N*-methylformanilide (NMF) (51.4 g, 0.38 mol) in CH_2Cl_2 (50 cm^3) was treated with $POCl_3$ (58.2 g, 0.38 mol) in a dropwise fashion over a period of 1 h. The resulting pale orange mixture was stirred for a further 1 h at room temperature. To this mixture was added dropwise, a solution of 1,1′-dimethylferrocene (40.0 g, 0.19 mol) in CH_2Cl_2 (200 ml). The resulting purple solution was refluxed for 18 h to afford a dark red–brown mixture which was allowed to cool to room temperature. The reaction mixture was then cooled in an ice bath and treated slowly with aqueous sodium acetate (15% w/w, 400 ml). After stirring for 3 h, the organic layer was separated and washed successively with brine containing 1 M HCl, $NaHCO_3$ solution and brine. The crude product solution, after drying ($MgSO_4$), was added to neutral Al_2O_3 (150 g) before evaporation of the solvent. The dry powder was then Soxhlet-extracted with hexane. Finally removal of the solvent *in vacuo* yielded crude **2** (35.0 g) as a dark red oil. δ_H (60 MHz; $CDCl_3$) (3-isomer) 9.8 (1 H, s, CHO), 2.0 (3 H, s, Me) and 1.85 (3 H, s, Me); (2-isomer) 10.0 (1 H, s, CHO), 2.2 (3 H, s, Me) and 1.95 (3 H, s, Me); (both isomers) 4.1–4.7 (3 H, m, MeC_5H_3CHO), 4.0 (4 H, s and m, MeC_5H_4). The product is >70% pure (^1H NMR) with NMF being the major contaminant and is a 7 : 1 mixture of the 3- and 2-isomers. If required, further purification of small samples and separation of the isomers can be achieved by column chromatography (SiO_2, Et_2O–hexane) otherwise the crude product was used in reactions.

1,1′-Dimethyl-3-(2-amino-1-hydroxyethyl)ferrocene (1). Crude **2** (55.0 g, 0.227 mol) was placed in a flask wrapped in aluminium foil. A catalytic quantity of zinc iodide (150 mg) was added followed by TMSCN (25.0 g, 0.252 mol) in a dropwise manner over 45 min. The red solution was stirred at room temperature for 16 h. The resulting TMS-cyanohydrin adduct **5** was diluted with Et_2O (600 ml) and added dropwise to a suspension of $LiAlH_4$ (10.5 g, 0.276 mol) in Et_2O (1000 ml). A yellow suspension formed which was stirred at room tem-

perature for 3 h. The excess of $LiAlH_4$ was quenched by careful addition of H_2O followed by 1 M NaOH (30 ml). At this stage, the product precipitates (together with inorganic salts) and is collected by filtration through Celite and washed with Et_2O. The ethereal filtrate (containing NMF and product 2-isomers together with an ether-soluble diastereomer product) was discarded while the precipitates were extracted with hot $CHCl_3$ (500 ml) and the resulting yellow extract dried ($MgSO_4$). Partial removal of the solvent under vacuum gave a thick slurry which was diluted with Et_2O (1000 ml). Filtration and air-drying afforded yellow microcrystals of a single ether-insoluble diastereomer of **1** (15.5 g, 25%). Mp 147–149 °C (dec.) (Found: C, 61.6; H, 7.0; N, 5.2%; M^+ 273. $C_{14}H_{19}FeNO$ requires C, 61.6; H, 7.0; N, 5.1%; M 273); ν_{max}/cm^{-1} (NH_2) 3360m, 3285m (KBr); δ_H (500 MHz; $CDCl_3$) 4.27 (1 H, br m, C*H*OH), 4.05 (2 H, m, ring CH), 3.97 (5 H, m, ring CH), 2.91 (1 H, m, CH_2), 2.71 (1 H, m, CH_2), 1.99 (3 H, s, Me) and 1.96 (3 H, s, Me). The ether-soluble diastereomer of **1** is isolated by purification of the ether extracts (normally discarded). Evaporation of the combined extracts gave a brown solid. This crude material was initially purified *via* the CBz derivative to afford a mixture containing 70% of the desired diastereomer. Extraction of the mixture with hexane yielded a yellow solid which was recrystallised from boiling hexane to provide a sample of the ether-soluble diastereomer, mp 89–91 °C (dec.), which was shown to be free of the ether-insoluble diastereomer from the 500 MHz ^1H NMR spectrum of its oxazolidinone derivative (see below).

(2-Amino-1-hydroxyethyl)ferrocene (8). Prepared from **3** (10.0 g, 46.7 mmol) according to the above procedure using TMSCN and $LiAlH_4$. The aminoalcohol **8** is isolated as yellow crystals (2.8 g, 24%). Mp 159–161 °C (dec.) (Found: C, 58.8; H, 5.7; N, 5.6%; M^+ 245. $C_{12}H_{15}FeNO$ requires C, 58.8; H, 6.2; N, 5.7%; M 245); δ_H (500 MHz; $CDCl_3$) 4.36 [1 H, dd, *J*(HH) 4 Hz, *J*(HH) 7.5 Hz, CH], 4.21 (5 H, s, C_5H_5), 4.19 (4 H, m, C_5H_4), 2.96 [1 H, dd, *J*(HH) 4 Hz, *J*(HH) 14 Hz, CH_2] and 2.79 [1 H, dd, *J*(HH) 7.5 Hz, *J*(HH) 14 Hz, CH_2].

(3-Hydroxy-4-aminobut-1-enyl)ferrocene (9). Prepared from 3-ferrocenylpropenal[65] **4** (10 g, 41.7 mmol) according to the above procedure using TMSCN and $LiAlH_4$. After recrystallisation from hot $(n\text{-}Bu)_2O$, the aminoalcohol **9** is obtained as orange crystals (6.44 g, 57%). Mp 130–131 °C (Found: C, 62.0; H, 6.3; N, 5.1%. $C_{14}H_{17}FeNO$ requires C, 62.0; H, 6.3; N, 5.2%); δ_H (500 MHz; $CDCl_3$) 6.39 [1 H, d, *J*(HH) 15.7 Hz, vinyl CH], 5.78 [1 H, dd, *J*(HH) 5.5 Hz, *J*(HH) 15.7 Hz, vinyl CH], 4.33 (2 H, br s, C_5H_4), 4.21 (2 H, br s, C_5H_4), 4.10 (5 H, s, C_5H_5), 2.88 [1 H, dd, *J*(HH) 5.9 Hz, *J*(HH) 11.5 Hz, CH_2], 2.70 [1 H, dd, *J*(HH) 5.9 Hz, *J*(HH) 11.5 Hz, CH_2] and 1.87 (3 H, br, NH_2 and OH).

(3-Hydroxy-4-aminobutyl)ferrocene (11). A solution of **9** (2 g, 7.34 mmol) in EtOAc–MeOH (3 : 1) was stirred with 100 mg of 10% Pd/C under an atmosphere of H_2 for 16 h. Removal of the catalyst by filtration provided a yellow solution which was evaporated to dryness. The oily residue was triturated with Et_2O–hexane to afford the saturated aminoalcohol **11** as a yellow powder (1.5 g, 75%). Mp 66–67 °C (Found: C, 61.1; H, 6.5; N, 5.0%. $C_{14}H_{19}FeNO$ requires C, 61.6; H, 7.0; N, 5.2%); δ_H (500 MHz; $CDCl_3$) 4.11 (5 H, s, C_5H_5), 4.06 (4H, br m, C_5H_4), 3.57 (1H, br m, CHO), 2.90 (1H, br m, CH_2N), 2.63 (1H, br m, CH_2N), 2.55 (1H, br m, CH_2), 2.42 (1H, br m, CH_2) and 1.66 (2H, br m, CH_2).

General procedure for the purification of aminoalcohol derivatives 12, 14 and 15. Benzyl chloroformate (1.1 equiv.) was added dropwise to a stirred solution of the aminoalcohol (1 equiv.) in CH_2Cl_2, containing solid Na_2CO_3 or $NaHCO_3$. After 1 h, the reaction mixture was filtered and evaporated to dryness. The crude N-CBz derivative was purified by column chromatography (SiO_2, Et_2O). Finally, the N-CBz compound was converted back to the original aminoalcohol by catalytic hydrogenation (1 atm) over 10% Pd/C in EtOAc–MeOH (3 : 1) solution for 16 h. The catalyst was removed by filtration and the solvent evaporated to yield the pure aminoalcohol.

*(1-Hydroxy-3-aminopropyl)ferrocene (**12**).* Solid cyanoalcohol[38] **13** (9.0 g, 35.3 mmol) was added in small portions to a suspension of $LiAlH_4$ (1.61 g, 42.4 mmol) in Et_2O (250 cm^3) and the resulting mixture was stirred at room temperature for 3 h. The reaction mixture was worked-up according to the procedure described above for the aminoalcohol (**1**). This led to the isolation of crude **12**, which was purified *via* its CBz derivative to yield a yellow–orange powder (3.93 g, 43%). Mp 111–112 °C (Found: C, 60.3; H, 6.6; N, 5.5%. $C_{13}H_{17}FeNO$ requires C, 60.3; H, 6.6; N, 5.4%); δ_H (500 MHz; $CDCl_3$) 4.60 [1 H, dd, *J*(HH) 2.8 Hz, *J*(HH) 8.6 Hz, CH], 4.26 (1 H, m), 4.18 (5 H, s, C_5H_5), 4.14 (3 H, m), 2.92 (2 H, br m, CH_2NH_2), 1.84 (1 H, m, CH_2) and 1.73 (1 H, m, CH_2).

*(2-Amino-3-hydroxypropyl)ferrocene (**14**).* Prepared from nitro acrylate[66] **16** (1.6 g, 4.8 mmol) according to the above procedure for **12** using $LiAlH_4$ in Et_2O. The product was purified, *via* the CBz derivative, to afford **14** as orange crystals (0.69 g, 56%). Mp 76–77 °C (Found: C, 60.2; H, 6.6; N, 5.5%. $C_{13}H_{17}FeNO$ requires C, 60.3; H, 6.6; N, 5.4%); δ_H (500 MHz; $CDCl_3$) 4.11 (9 H, br m, C_5H_5 + C_5H_4), 3.57 [1 H, dd, *J*(HH) 9.7 Hz, *J*(HH) 8.6 Hz, CH_2OH], 3.31 [1 H, dd, *J*(HH) 7.2 Hz, *J*(HH) 9.7 Hz, CH_2OH], 2.85 (1 H, br m, CH), 2.55 [1 H, dd, *J*(HH) 4.8 Hz, *J*(HH) 14.0 Hz, CH_2] and 2.35 [1 H, dd, *J*(HH) 7.9 Hz, *J*(HH) 14.0 Hz, CH_2].

*[(2-Hydroxymethyl)-3-aminopropyl]ferrocene (**15**).* Prepared from cyano acrylate[67] **17** (1.2 g, 3.9 mmol) according to the above procedure for **12** using $LiAlH_4$ in Et_2O. The product was purified, *via* the CBz derivative, to afford **15** as a yellow powder (0.50 g, 47%). Mp 90–92 °C (Found: C, 61.1; H, 6.5; N, 5.0%. $C_{14}H_{19}FeNO$ requires C, 61.6; H, 7.0; N, 5.2%); δ_H (500 MHz; $CDCl_3$) 4.09 (9 H, m, C_5H_5 + C_5H_4), 3.76 [1 H, dd, *J*(HH) 3.0 Hz, *J*(HH) 10.6 Hz, CH_2OH], 3.60 [1 H, dd, *J*(HH) 7.8 Hz, *J*(HH) 10.6 Hz, CH_2OH], 3.01 [1 H, dd, *J*(HH) 3.0 Hz, *J*(HH) 12.1 Hz, CH_2NH_2], 2.68 [1 H, dd, *J*(HH) 8.7 Hz, *J*(HH) 12.1 Hz, CH_2NH_2], 2.37 [1 H, dd, *J*(HH) 6.9 Hz, *J*(HH) 14.2 Hz, FcCH_2OH], 2.29 [1 H, dd, *J*(HH) 7.1 Hz, *J*(HH) 14.2 Hz, FcCH_2OH] and 1.68 (m, 1 H, CH).

General procedure for the preparation of ferrocene amides 20–23. A solution of a ferrocene carboxylic acid **16**–**19** (1 equiv.) and CDI (1.1 equiv.) in dry THF was refluxed for 1 h. After cooling to room temperature, gaseous ammonia was then bubbled slowly through the solution for 1 h. The solvent was then evaporated and the residue chromatographed (SiO_2, Et_2O) to afford the amides **20**–**23** as yellow powders in 70–80% yield, allowing for recovered starting material.

*2-Ferrocenylacetamide (**20**).* Mp 168–169 °C (dec.) (Found: C, 59.4; H, 5.5; N, 6.0%. $C_{12}H_{13}FeNO$ requires C, 59.3; H, 5.4; N, 5.8%); δ_H (60 MHz; $CDCl_3$) 5.6 (2 H, br s, $CONH_2$), 4.2 (4 H, br s, C_5H_4), 4.15 (5 H, s, C_5H_5) and 3.3 (2 H, s, CH_2).

*3-Ferrocenylpropionamide (**21**).* Mp 100–101 °C (Found: C, 60.4; H, 6.1; N, 5.2%. $C_{13}H_{15}FeNO$ requires C, 60.7; H, 5.9; N, 5.5%).

*4-Ferrocenylbutyramide (**22**).* Mp 80–81 °C (Found: C, 62.9; H, 6.8; N, 4.6%. $C_{14}H_{17}FeNO$ requires C, 62.1; H, 6.3; N, 5.2%); δ_H (60 MHz; $CDCl_3$) 5.6 (2 H, br s, $CONH_2$), 4.1 (5H, s, C_5H_5), 4.05 (4H, br s, C_5H_4) and 1.7–2.6 (6H, complex m, $3CH_2$).

*5-Ferrocenylpentanoic acid amide (**23**).* Mp 83–84 °C (Found: C, 63.3; H, 6.9; N, 5.0%. $C_{15}H_{19}FeNO$ requires C, 63.2; H, 6.7; N, 4.9%); δ_H (60 MHz; $CDCl_3$) 5.5 (2 H, br s, $CONH_2$), 4.1 (5 H, s, C_5H_5), 4.05 (4 H, br s, C_5H_4), 2.3 (4 H, m, $2CH_2$) and 1.6 (4 H, m, $2CH_2$).

5-(3,1′-Dimethylferrocenyl)oxazolidin-2-one (24). A solution of the ether-insoluble diastereomer of **1** (1.0 g, 3.66 mmol) and

CDI (0.65 g, 4.01 mmol) in dry THF (150 ml) was stirred for 16 h. After removal of the solvent, the residue was chromatographed (SiO_2, Et_2O) to afford **24** as yellow crystals (0.34 g, 31%). Mp 137–138 °C (Found: C, 60.4; H, 5.7; N, 4.6%; M^+ 299. $C_{15}H_{17}FeNO_2$ requires C, 60.2; H, 5.7; N, 4.7%; M 299); δ_H (500 MHz; $CDCl_3$) 5.388 [1 H, dd, *J*(HH) 7.7 Hz, *J*(HH) 8.5 Hz, FcC*H*O], 5.19 (1 H, br s, NH), 3.98 (7 H, complex m, ring CH), 3.866 [1 H, dd, *J*(HH) 8.5 Hz, *J*(HH) 8.5 Hz, CH_2], 3.632 [1 H, dd, *J*(HH) 8.5 Hz, *J*(HH) 7.7 Hz, CH_2], 1.977 (3 H, s, Me) and 1.971 (1 H, s, Me). The ether-soluble diastereomer of **1** is similarly converted to an oxazolidinone, mp 156–157 °C (Found: C, 60.5; H, 5.8; N, 4.8%. $C_{15}H_{17}FeNO_2$ requires C, 60.2; H, 5.7; N, 4.7%); δ_H (500 MHz; $CDCl_3$) 5.391 [1 H, dd, *J*(HH) 7.7 Hz, *J*(HH) 8.5 Hz, FcC*H*O], 5.19 (1 H, br s, NH), 3.98 (7 H, complex m, ring CH), 3.858 [1 H, dd, *J*(HH) 8.5 Hz, *J*(HH) 8.5 Hz, CH_2], 3.611 [1 H, dd, *J*(HH) 8.5 Hz, *J*(HH) 7.7 Hz, CH_2], 1.983 (3 H, s, Me) and 1.976 (1 H, s, Me).

1,1′-Dimethyl-3-(2-acetamido-1-hydroxyethyl)ferrocene (25). Acetyl chloride (0.30 g, 3.82 mmol) was added to a CH_2Cl_2 (100 ml) solution of the single diastereomer of **1** (1.0 g, 3.66 mmol) containing triethylamine (0.37 g, 3.66 mmol). After stirring for 1 h, the solvent was evaporated and the residue chromatographed (SiO_2, Et_2O) to yield **25** as yellow crystals (0.61 g, 53%). Mp 97–98 °C (Found: C, 61.1; H, 6.7; N, 4.4%. $C_{16}H_{21}FeNO_2$ requires C, 61.0; H, 6.7; N, 4.4%); δ_H (60 MHz; $CDCl_3$) 6.8 (1 H, br s, NH), 4.45 (1 H, q, C*H*OH), 3.95 (7 H, complex m, ring CH), 3.6 (1 H, q, CH_2), 3.3 (1 H, q, CH_2) and 1.95 (9 H, m, 2 ring CH_3 + CH_3CO).

Kinetic measurements and analysis

Deoxygenated solutions (0.5–1.0 mM) of ferrocenes were freshly prepared in the above buffer solution containing a high level (0.1 M) of D-glucose such that the kinetics of GOx were saturated. Cyclic voltammograms of the resulting ferrocene solutions (0.5 ml) were recorded, at a scan rate of 10 mV s^{-1}, in the absence of enzyme and then upon each successive addition of 5, 10, 15, 20 and 25 μl of the stock GOx solution (*ca.* 15 μM). Before each measurement, the working electrode was thoroughly polished with 0.3 μm alumina, sonicated and then rinsed with deionised water. The electrode must be polished efficiently in order to obtain reproducible results. A series of catalytic waves were thus obtained from which i_{cat} could be measured for each level of enzyme. The kinetic experiments for all ferrocene derivatives were carried out under identical experimental conditions using **1** as a control.

Analysis of the data was carried out according to Case I of the model for homogeneous mediation developed by Bartlett *et al.*[35] and applied by others.[68] Thus, mediator rate constants (k_M) were determined from the slope of the linear plot of catalytic current i_{cat} *versus* the square root of the enzyme concentration e_Σ according to eqn. (1).

$$i_{cat} = nFAD_M^{1/2}k_M^{1/2}m_0e_\Sigma^{1/2} \quad (1)$$

where A is the surface area of the electrode (*ca.* 0.159 cm^2), D_M is the mediator diffusion coefficient (assumed to be approximately constant and equal to 3×10^{-6} cm^2 s^{-1}, the literature[22] value for a range of ferrocene derivatives), m_0 is the mediator concentration and n is assumed to have a value of 2† since the oxidation of GOx from fully reduced to fully oxidized forms is a two-electron process. Values of k_M for various ferrocene derivatives were normalised to that for **1** to allow for slight day to day variations in experimental conditions such as enzyme activity. The aim of this was to provide accurate relative comparisons of k_M for use in the structure–activity study.

† A referee has suggested that n should take a value of 1 since ferrocene is the diffusing species and is a one-electron oxidant. Readers should be aware that this will affect the absolute k_M values given in Table 1 but not the relative rates. Consequently, the overall conclusions derived from the structure–activity study in this article are unchanged.

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