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Novel Monolabeled Redox Biomaterials: Amino-Ended Poly(ethylene oxide) Ferrocenes and their Biotin-Conjugates

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Abstract: Treatment of ferrocenoyl chloride with symmetrical poly(oxyalkylene) diamine **1** in excess affords the monofonctionalized derivative **2** in moderate to good yields. This one-step procedure provides a convenient entry to novel monolabeled redox precursors such as the biotin derivative **3** for more elaborate architectures.

Ferrocene derivatives obtained by covalent attachement to simple poly(ethylene oxide) (PEO)s-based chains are of interest as they promise to combine the distinctive characteristics of the PEO chains with the well-known redox properties of the ferrocene/ferricinium system. PEO-modified ferrocenes exhibit enhanced aqueous-solubility and increased size over classical ferrocene derivatives and have proven useful as non-leaking homogeneous redox mediators in membrane biosensors and reactors.[1] In recent reports, ferrocene labeled PEOs have been used as successful models for investigations of charge transport and electron dynamics within polymer films,[2] demonstrating that important properties including permeability and viscosity may be adjusted by the PEO chain length.

PEO ferrocenes bearing at the far end of the chain a reactive functionality have not yet been reported. However, availability of ferrocene reagents with the well-defined PEO structural features would be of fundamental importance for derivatization of supports or macromolecule surfaces. For example, PEO ferrocenes endowed with the high flexibility and hydrophilicity of the PEO tether should provide efficient electron relays when incorporated into polymeric materials,[3] linked to enzymes,[4] or other specific locations in bioelectrochemical assemblies.[5] Here is reported for the first time the preparation and characterization of a simple series of PEO amide substituted ferrocenes with a reactive primary amine group at the end of the polymer chain. Taking advantage of the well-known chemical versatility of the amino end group on PEOs,[6] these novel heterofunctional PEO derivatives may be coupled to various supports via stable linkages using the mild active ester chemistry. Therefore, they are expected to be useful biosynthetic intermediates for redox-labeling of biologically relevant molecules including enzymes, functional groups, and ligands. To adress one of these issues, we incorporated biotin that would offer a ready approach to labeling avidin-containing surfaces.

The synthesis involves the direct condensation of one of the primary amino end group of commercially available PEO diamine linkers with the readily available ferrocenoyl chloride.[7] This straightforward reaction was

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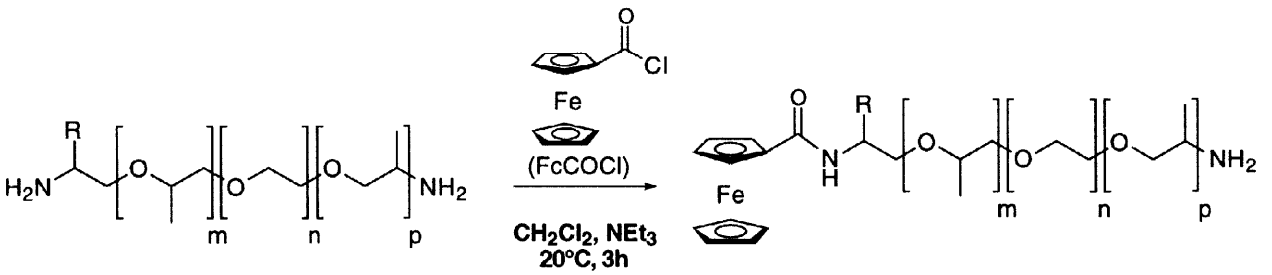
first performed with $H_2NCH_2CH_2$-$(OCH_2CH_2)_2$-NH_2 (**1a**) of fixed length to optimize chemistry and facilitate analytical characterization when using the two random copolymers Jeffamines ED-X,[8] varying in MW, PEO backbone (**1b**, **1c**; avg. MW: X: 600, 2001) (Scheme 1).



1 (excess) **JeffX** **2** **FcCO-JeffX**

1	JeffX	R	n	m+p
1a	Jeff148	H	2	0
1b	JeffED-600	CH_3	~9	~2.4
1c	JeffED-2001	CH_3	~41	~2.3

2	R	n	m+p	MW
2a	H	2	0	360
2b	CH_3	~9	~3.6	900
2c	CH_3	~42	~2.3	2250

Scheme 1

In order to minimize the attachment of two molecules of ferrocene to the linker, excess **1** was treated for 3 h in dichloromethane with ferrocenoyl chloride and triethylamine. Cyclic voltammetry in acidic acetonitrile ascertained complete disappearance of the starting FcCOCl to give solely ferrocene amide-type derivative(s) (see below). The desired ferrocene product FcCO-JeffX (**2**) was easily separated from unlabeled Jeffamine by silicagel column chromatography (TLC, ninhydrin detection).[9] All purified **2a,b** and **c** (typical yield 50-55%) exhibit exceptional stability on storage and in most organic solvents, in contrast with simple ferrocenes linked to short spacer alkyl amines. The average MW for copolymers **2b** and **2c** were calculated using the integrals for the methyl protons and the PEO backbone with that of the upmost downfield ferrocene multiplet (2H, 4.74 ppm).[10] Results are given in Scheme 1 and evidence that for **2c** (MW ~2250) the average copolymer composition as well as the average PEO content of the Jeffamine precursor used are preserved during the purification steps. For **2b** (MW ~900), the random hydrophobic PO units are slightly increased in the copolymer chain while the average hydrophilic PEO backbone size is maintained.

Table 1. Cyclic Voltammetry Data. v = 0.1V s^{-1}

Compound (0.5mM)	$E°$ [a]	D (cm^2 s^{-1})[b]	$E°$ [a]
	CH_3CN[c]		acidic CH_3CN[c,d]
Ferrocene	0.40	2.4 x 10^{-5} [e]	0.40
FcCOCl	---	---	0.82
2a	0.57	2.2 x 10^{-5}	0.68
2b	0.55	6.5 x 10^{-6}	0.70
2c	0.55	2.9 x 10^{-6}	0.70

[a] In V vs SCE. [b] Calculated from peak current. [c] ca. 50 mM H_20 + $n$$Bu_4PF_6$. [d] +10mM HBF_4. [e] From Ref 2b.

Cyclic voltammetry in the ferrocene series **2a-c** exhibits in CH_3CN a reversible wave with a standard potential close to 0.55V as expected for simple amide-linked ferrocenes,[11] plus the irreversible oxidation wave of the terminal primary amine group (ca. 1.15V). The diffusion coefficients D, (Table 1), show a decrease with increasing chain length and are consistent with reported data for methyl end-capped PEO ferrocenes in this medium.[2] It is worth noticing that in acidic CH_3CN, an unexpected proton-induced one-wave anodic shift of large magnitude (≥ 0.11V) is observed for these ammonium-terminated (ferrocene/ferricinium) couples.

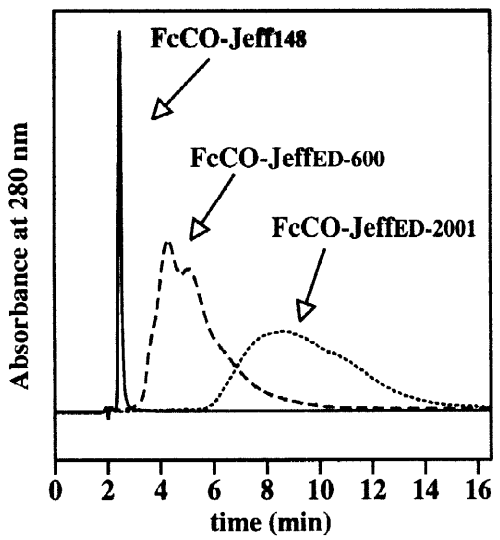
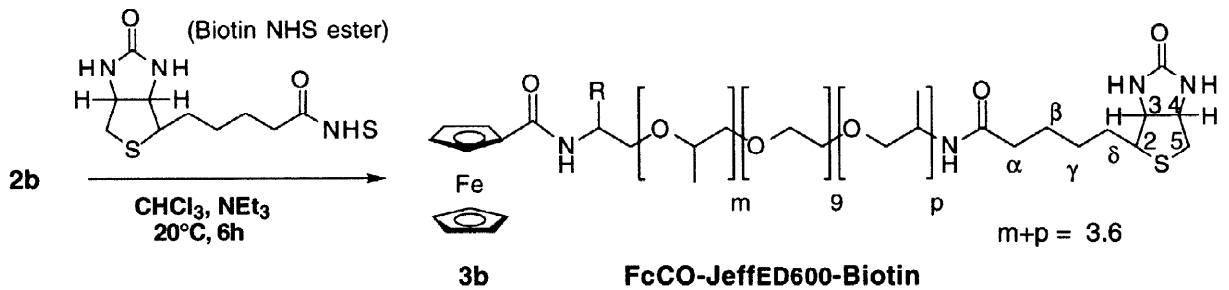


Fig.1. HPLC Elution Profiles of Purified Ferrocene Derivatives **2** on Waters 150 x 3.9 mm μ-Bondapack C-18 column in a linear gradient (eluent: 15mM KH_2PO_4 + 5mM tetraethyl ammonium hydrogenosulfate pH 3.1) from 66 to 72 % methanol in 20 min.

RP-HPLC was employed to ensure that the ferrocenes FcCO-JeffX were free from contamination of bridged bis-ferrocenes and also served as a qualitative tool for verifying the consistency of the product from lot-to-lot. Under selected RP-HPLC conditions, unmodified PEO diamines **1** are not retained. The coupling of a ferrocene group to the PEO chains increases the hydrophobicity of the molecule. As shown in Figure 1, the elution time of PEO-modified ferrocene **2** increases with increasing chain length of the PEOs. Thus, the hydrophobicity of the PEO derivatized ferrocene can be changed gradually over a wide range using **1** with an appropriate molecular weight distribution. Compared to **2** that carries only one terminal ferrocene, the elution profile of the more hydrophobic bridged bis-ferrocene is shifted toward later elution times (data not shown).

To show the immediate synthetic utility of analytically pure amino-ended PEO ferrocenes, the free terminal amine of **2b** was treated with commercially available biotin N-hydroxysuccinimide ester in chloroform and triethylamine.[12] The isolated biotin conjugate **3b** was obtained in 85% yield with a high degree of purity, as determined by NMR and cyclic voltammetry.[13]



Scheme 2

Preliminary studies aiming at evaluating the reactivity of **3** as a redox labeled biotin ligand in systems utilizing the avidin/biotin technology are encouraging. Treatment of avidin (MW ~68000) with biotinylated ferrocene **3b** (MW ~1140) in phosphate buffer pH 8.0 caused a ready reaction that could be monitored by cyclic voltammetry and quantified using the HABA standard test. When introducing **3b** in a 4-fold excess, examination of the reaction mixture after filtration (size membrane exclusion, molecular cut-off 30000 D) showed no free ferrocene, demonstrating that biotin derivative **3b** could be anchored to the four binding sites of avidin.

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8 Jeffamine is a registered trademark of Texaco. Jeffamines ED-X **1b-c** have been recently analyzed by laser desorption Fourier transform mass spectrometry: Nuwaysir, L. M.; Wilkins, C.L.; Simonsick, W.J. Jr. *J. Am. Soc. Mass Spectrom.* **1990**, *1*, 66-71. The average numbers of propyleneoxide PO and ethylene oxide EO units, m + p, and n, were quantified in this work by ^{1}H NMR based on the vendor's MW values.
9. A typical procedure was as follows: Jeffamine **1** (~ 7 mmol) was dissolved under argon in dried CH_2Cl_2 (50 mL) containing NEt_3 (7.0 mmol). A solution of ferrocenoyl chloride (1.6 mmol) in CH_2Cl_2 (13 mL) was added dropwise into the solution over 50 min. The resulting mixture was maintained under argon and stirred for 2 h at 20°C. After solvent removal, the thick slurry was carefully chromatographed on silica gel using a gradient of $CHCl_3$/MeOH (from 9/1 to 8/2). The main yellow band containing the desired product **2** was collected and evaporated to dryness. A second chromatography was performed on the product using $CHCl_3$/MeOH (85/15) as eluant. The homogeneous fractions containing **2** were combined, dried over $MgSO_4$, evaporated, and dried in *vacuo*.
10. Typical NMR assignments for selected examples. ^{1}H NMR (200MHz, $CDCl_3$) FcCO-Jeff148 (**2a**): δ_H 6.42 (1H, br s, NHCO), 4.74 (t, 2H, J = 1.9 Hz), 4.37 (t, 2H, J = 1.9 Hz), and 4.24 (s, 5H) (all FcH), 3.69-3.60 (s + t, 8H, OCH_2CH_2O + CH_2 α and β to NHCO), 3.54 (t, 2H, J = 5.2 Hz, CH_2 β to NH_2), 2.92 (t, 2H, J = 5.2 Hz, CH_2 α to NH_2), 1.69 (br s, 2H, NH_2). FcCO-JeffED-2001 (**2c**): δ_H 6.55-6.30 (1H, m, NHCO), 4.74 (m, 2H), 4.32 (m, 2H), and 4.19 (s, 5H) (all FcH), 3.90-3.55 (s + m, ~178H, PEO backbone + (m+p+1) (H and CH_2)), 1.38-1.08 (m, ~9.9H, (m+p+1) CH_3).
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12. Biotin NHS ester (0.093 mmol) was added to a solution of **2b** (MW ~900, 92 mg, ~0.102 mmol) in 10 mL of $CHCl_3$ containing 170 µL of NEt_3 (0.122 mmol). The mixture was shielded from light and stirred for 6 h at room temperature. At this point, TLC ($CHCl_3$/MeOH: 85/15; 4-dimethylamino-cinnamaldehyde detection) indicated complete consumption of the biotinylating reagent (Rf = 0.46) to give the desired biotinylated product **3b** (Rf = 0.52). The solution was concentrated under reduced pressure and applied as a thin band to a preparative TLC plate. Elution was followed by extraction from the silica with $CHCl_3$/MeOH (9/1); filtration, evaporation and drying in *vacuo* gave **3b** as an orange solid (MW ~1140, 90 mg; 85%).
13. Selected analytical data for **3b**: E° aq.(pH8.0) = 0.40 V/SCE. δ_H 6.94-6.44 (3H, m, NHCO), 5.79 (1H, br s, NHCO), 4.73 (2H, m, FcH), 4.52 (1H, m, H_3), 4.09-4.05 (s + m, 3H, FcH and H_4), 4.19 (m, 5H, FcH), 3.70-3.55 (s + br. m, ~50H, PEO backbone + (m+p+1) (H + CH_2)), 3.50-3.25 (m, ~11H), 3.12 (app. q, 1H, J ~7 Hz, H_2), 2.90 (dd, 1H, $J_{5a,4}$= 4.6 Hz, $J_{5a,5b}$ = 12.5 Hz, H_{5a}), 2.72 (d, 1H, $J_{5b,5a}$ = 12.5 Hz, H_{5b}), 2.16 (t, 2H, J ~7 Hz, $CH_{2\alpha}$), 1.68 (4H, m, $CH_{2\beta}$+$CH_{2\delta}$), 1.42 (2H, m, $CH_{2\gamma}$), 1.28-1.09 (m, ~13.8H, (m+p+1) CH_3).