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Specific labelling of sulfhydryl-containing biomolecules with redox-active *N*-(ferrocenyl)iodoacetamide

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The synthesis, characterisation, X-ray crystal structure and electrochemical properties of a new redox-active labelling reagent, *N*-(ferrocenyl)iodoacetamide (**Fc-IAA**), are reported. This compound exhibits a reversible ferrocenium/ferrocene couple at *ca.* +0.345 V *vs.* SCE at a sweep rate of 100 mV s^{-1} in CH_3CN at 298 K, and two irreversible reduction waves at *ca.* −1.324 and −2.048 V, attributable to the reduction of the iodoacetamide group. Since the iodoacetamide moiety can react specifically with the sulfhydryl group, **Fc-IAA** has been coupled to various biomolecules including a sulfhydryl-modified oligonucleotide, cysteine, glutathione and sulfhydryl-modified bovine serum albumin. The electrochemical properties of the bioconjugates have also been investigated.

Introduction

Since the iodoacetamide moiety can undergo facile reaction with the sulfhydryl group to form a stable thioether,[1] it has been commonly used to modify the sulfhydryl groups of biomolecules to provide desirable physical properties to the biological substrates. There have been many reports on incorporating an iodoacetamide group into fluorescent dyes such as fluorescein,[2] pyrene,[3] rhodamine,[4] coumarin[5] and dansyl[6] derivatives to produce fluorescent labels for biomolecules. Many of these labels have also been employed as energy donors and acceptors to monitor biomolecular interactions by fluorescence resonance energy-transfer.[7] In addition, the cysteine residue of a DNA-binding protein has also been modified with a copper(I)-iodoacetamido-1,10-phenanthroline unit to give a site-specific artificial nuclease.[8] A spin label, *N*-(1-oxy-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide, has also been attached to an engineered human carbonic anhydrase to probe local structural changes upon unfolding and aggregation of the protein by electron paramagnetic resonance measurements.[9] Despite all these reports, to the best of our knowledge, the design of sulfhydryl-specific electrochemical biological labels using the iodoacetamide functional group has never been explored. In view of some reports in which active functional groups such as the succinimide ester[10] and maleimide[11] have been introduced to redox-active ferrocene to give site-specific electrochemical labels for biomolecules, we believe that a sulfhydryl-specific electrochemical label can be obtained by functionalising a redox-active moiety with iodoacetamide. Here we report the synthesis, characterisation, X-ray crystal structure and electrochemical properties of a new ferrocene-containing biological labelling reagent *N*-(ferrocenyl)iodoacetamide (**Fc-IAA**). This compound has been used to label various biomolecules such as a sulfhydryl-modified oligonucleotide, cysteine, glutathione and sulfhydryl-modified bovine serum albumin. The electrochemical behaviour of the bioconjugates has also been examined.

Experimental

Materials and reagents

All solvents were of analytical grade. Aminoferrocene was prepared from the reaction of ferroceneboronic acid and hydrazine monohydrate in the presence of palladium on charcoal in refluxing ethanol.[12] The thiolated oligonucleotide M13-R-comp-SH [5′-HS$(CH_2)_6$CATGGTCATAGCTGTT-3′] (Sigma-Genosys), cysteine (Sigma), reduced form of glutathione (Sigma), bovine serum albumin (Calbiochem), *N*-succinimidyl *S*-acetylthioacetate, SATA (Pierce), hydroxylamine hydrochloride (Pierce), glucose oxidase (Calbiochem) and glucose (Aldrich) were used as received. All buffer components were of molecular biology grade and used without further purification.

Instruments and methods

^{1}H NMR spectra were recorded on a Varian Mercury 300 MHz NMR spectrometer at 298 K. ESI mass spectra were measured on a Perkin-Elmer Sciex API 365 mass spectrometer. IR spectra were recorded on a Perkin-Elmer 1600 series FT–IR spectrophotometer. Elemental analyses were performed on a Carlo Erba 1106 elemental analyser at the Institute of Chemistry, Chinese Academy of Sciences. Electronic absorption spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer. The electrochemical measurements were performed on a CH Instruments Electrochemical Workstation CHI750A. The electrochemical experiments were carried out at room temperature with a two-compartment glass cell with a working volume of 500 μl. A platinum gauze counter electrode was accommodated in the working electrode compartment. The working electrode was a glassy carbon electrode. A silver/silver chloride electrode and a silver/silver nitrate electrode were used as the reference electrode for aqueous and non-aqueous electrochemical measurements, respectively. The reference electrode compartment was connected to the working electrode compartment *via* a Luggin capillary. All potentials were referred to SCE.

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Synthesis

***N*-(Ferrocenyl)iodoacetamide (Fc-IAA).** Aminoferrocene (50 mg, 0.249 mmol) and iodoacetic anhydride (132 mg, 0.373 mmol) were stirred in CH_3CN (8 ml) for 36 hours at room temperature in the dark under an inert atmosphere of nitrogen. The solution was evaporated to dryness to give a brownish-yellow solid. The solid was then dissolved in CH_2Cl_2 and loaded onto a chromatographic column (alumina). The impurity that appeared as a pink band on the column was eluted with CH_2Cl_2–acetone (1 : 1 v/v) while the desired product (yellow band) was eluted with acetone. The solution was collected and evaporated to dryness. Recrystallisation from acetone–petroleum ether (bp 40–60 °C) gave **Fc-IAA** as brownish-yellow crystals. Yield = 35 mg (38%). ^{1}H NMR (300 MHz, acetone-d_6, 298 K, relative to TMS): δ 8.94 (s, 1H, NH), 4.65 (s, 2H, H_2 and H_5), 4.16 (s, 5H, $H_{1'}$–$H_{5'}$), 3.97 (s, 2H, H_3 and H_4), 3.77 (s, 2H, CH_2). IR (KBr)/cm^{-1}: 3264 (m, NH), 1646 (s, C=O), 1564 (s, NH). Positive-ion ESI-MS ion cluster at *m*/*z* 369 $\{M\}^+$. Anal. calc. for $C_{12}H_{12}NOIFe$: C, 39.06; H, 3.28; N, 3.80. Found: C, 39.33; H, 3.22; N, 3.84. UV/Vis [λ_{abs}/nm (ε/dm^3 mol^{-1} cm^{-1})]: CH_3CN, 278 (4560), 337 (740), 443 (265); CH_2Cl_2, 278 (5990), 337 (635), 443 (285).

Labelling of biomolecules

Labelling of M13-R-comp-SH with Fc-IAA. In a typical labelling reaction, **Fc-IAA** (1.0 mg, 2.7 μmol) in 20 μl anhydrous DMSO was added to M13-R-comp-SH (54 nmol) dissolved in 180 μl of 50 mM potassium phosphate buffer at pH 7.4. The mixture was stirred in the dark at room temperature for 24 hours. The solid residue was then removed by centrifugation. To the supernatant were added 50 μl of NaOAc (3.0 M, pH 5.2) and 1 ml of isopropanol. The labelled DNA **Fc-M13** was collected by centrifugation and the pellet was washed with 70% aqueous EtOH, twice with absolute EtOH, once with diethyl ether, and finally dried *in vacuo*. The labelled oligonucleotide was further purified by RP-HPLC using 50 mM potassium phosphate buffer at pH 7.4 as the mobile phase.

Labelling of cysteine with Fc-IAA. In a typical labelling reaction, **Fc-IAA** (1.4 mg, 3.8 μmol) in 20 μl anhydrous DMSO was added to cysteine (1.9 μmol) in 180 μl of 50 mM potassium phosphate buffer at pH 7.4. The mixture was stirred in the dark at room temperature for 24 hours. The solid residue was then removed by centrifugation. The excess labels were removed by extracting the supernatant with ethyl acetate (500 μl × 10). The conjugate **Fc-Cys** was further purified by RP-HPLC using 50 mM potassium phosphate buffer at pH 7.4 as the mobile phase. Positive-ion ESI-MS ion clusters at *m*/*z* 363 $\{M + H^+\}^+$, 401 $\{M + K^+\}^+$, 439 $(M - H^+ + 2K^+\}^+$.

Labelling of glutathione with Fc-IAA. The preparation was similar to that for **Fc-Cys** except that glutathione (1.9 μmol) was used instead of cysteine. The conjugate **Fc-GSH** was purified by RP-HPLC using 50 mM potassium phosphate buffer at pH 7.4 as the mobile phase. Negative-ion ESI-MS ion cluster at *m*/*z* 547 $\{M - H^+\}^-$.

Labelling of bovine serum albumin with Fc-IAA. Bovine serum albumin (BSA) was first modified with *N*-succinimidyl *S*-acetylthioacetate (SATA) to increase the number of free sulfhydryl moieties. SATA (0.1 mg, 432 nmol) in 20 μl anhydrous DMSO was added to BSA (1.1 mg, 17 nmol) in 180 μl of 50 mM potassium phosphate buffer at pH 7.4. The solution was stirred at room temperature for 30 minutes. Then it was extensively dialysed against 50 mM potassium phosphate buffer to remove excess SATA molecules. To the reaction mixture was then added hydroxylamine hydrochloride (1.0 mg, 14.4 μmol) and the solution was stirred at room temperature for 2 hours. The solution was then extensively dialysed against 50 mM potassium phosphate buffer. To the solution was added **Fc-IAA** (1.0 mg, 2.7 μmol) dissolved in 20 μl anhydrous DMSO. The reaction mixture was stirred at room temperature for 24 hours. The solid residue was then removed by centrifugation. The excess labels were removed by extracting the supernatant with ethyl acetate (500 μl × 10). The conjugate **Fc-BSA** was further purified by size-exclusion HPLC using 50 mM potassium phosphate buffer at pH 7.4 as the mobile phase.

X-Ray crystal structure determination

Crystal data for Fc-IAA. $C_{12}H_{12}FeINO$, M = 368.98, monoclinic, $P2_1/c$, a = 14.041(3), b = 9.534(2), c = 9.553(2) Å, β = 106.47(3)°, U = 1226.4(4) Å^3, Z = 4, D_c = 1.998 g cm^{-3}, μ(Mo-Kα) = 3.724 mm^{-1}, F(000) = 712, T = 301 K. A crystal of dimensions 0.8 × 0.2 × 0.1 mm mounted in a glass capillary with mother liquor was used for data collection on a MAR diffractometer with a 300 mm image plate detector using graphite monochromated Mo-Kα radiation (λ = 0.71073 Å). The images were interpreted and intensities integrated using the program DENZO.[13] The structure was solved by direct methods (SIR-97 program).[14] All atoms were located according to direct methods and the successive least-squares Fourier cycles. The structure was refined by full-matrix least-squares (SHELXL-97).[15] 5233 reflections were measured and 1934 independent reflections (R_{int} equal to 0.0311) were used in the full-matrix least-squares refinement against F^2. One crystallographic asymmetric unit consists of one formula unit. In the final stage of least-squares refinement, all non-hydrogen atoms were refined anisotropically. H(1) was located in the difference map and refined isotropically. Other H atoms were generated by the program SHELXL-97. The positions of H atoms were calculated based on riding mode with thermal parameters equal to 1.2 times that of the associated C atoms, and participated in the calculation of final R-indices. Convergence [$(\Delta/\sigma)_{max}$ = −0.001, av. 0.001] for 149 variable parameters by full-matrix least-squares refinement on F^2 reached to R_1 = 0.0344 and wR_2 = 0.0768.

CCDC reference number 176563.

See http://www.rsc.org/suppdata/dt/b1/b111529d/ for crystallographic data in CIF or other electronic format.

Results and discussion

The synthesis of substituted ferrocene molecules has been well documented. However, to the best of our knowledge, the attachment of an iodoacetamide moiety to ferrocene has not been reported. The redox-active label **Fc-IAA** was synthesised by the reaction of aminoferrocene with iodoacetic anhydride in CH_3CN at room temperature. The compound was characterised by ^{1}H NMR, ESI-MS, IR and UV-VIS and gave satisfactory elemental analyses. The X-ray crystal structure of **Fc-IAA** has also been studied.

Single crystals of **Fc-IAA** were obtained by layering an acetone solution of the compound with petroleum ether. The perspective drawing of **Fc-IAA** is shown in Fig. 1. Selected bond distances and angles are listed in Table 1. The two cyclopentadienyl rings are essentially co-planar, with a tilt angle of *ca.* 2.2°. These rings adopt an eclipsed conformation (average torsional angle = 4.1°). The Fe–C bond lengths range from 2.022(6) to 2.045(6) Å and are similar to those of related ferrocene derivatives.[16] The tilt angle between the cyclopentadienyl ring formed by C(3) to C(7) and the acetamide moiety [N(1)–C(1)–O(1)–C(2)] is *ca.* 24.9° while the torsional angle along N(1)–C(1)–C(2)–I(1) is *ca.* 105.6°.

The electrochemical properties of **Fc-IAA** have been studied by d.c. cyclic voltammetry. The electrochemical data are listed in Table 2. At a sweep rate, v, of 100 mV s^{-1}, the compound exhibits a reversible ferrocenium/ferrocene couple at *ca.* +0.345 V *vs.* SCE in CH_3CN at 298 K, and two irreversible reduction

Table 1 Selected bond distances (Å) and bond angles (°) for **Fc-IAA**

C(1)–C(2)	1.494(7)	C(1)–N(1)	1.348(6)
C(1)–O(1)	1.234(5)	C(2)–I(1)	2.152(5)
C(3)–N(1)	1.400(6)	Fe(1)–C(3)	2.043(5)
Fe(1)–C(4)	2.045(6)	Fe(1)–C(5)	2.039(5)
Fe(1)–C(6)	2.039(5)	Fe(1)–C(7)	2.039(5)
Fe(1)–C(8)	2.025(5)	Fe(1)–C(9)	2.024(5)
Fe(1)–C(10)	2.039(6)	Fe(1)–C(11)	2.039(7)
Fe(1)–C(12)	2.022(6)		
I(1)–C(2)–C(1)	107.8(4)	C(2)–C(1)–N(1)	115.6(4)
C(2)–C(1)–O(1)	121.9(4)	O(1)–C(1)–N(1)	122.5(4)
C(1)–N(1)–C(3)	125.8(4)	N(1)–C(3)–C(4)	127.2(4)
N(1)–C(3)–C(7)	124.9(4)		

Table 2 Electrochemical data of **Fc-IAA** in CH_3CN (0.1 mol dm^{-3} $^nBu_4PF_6$) at 298 K (glassy carbon working electrode, electrode area = 7 mm^2)

v/V s^{-1}	E_{pa}/V	E_{pc}/V	$E_{1/2}$/V	ΔE/mV
0.01	+0.377	+0.306	+0.342	71
		−1.286		
		−2.003		
0.02	+0.381	+0.309	+0.345	72
		−1.293		
		−2.056		
0.05	+0.370	+0.314	+0.342	56
		−1.293		
		−1.995		
0.1	+0.383	+0.307	+0.345	76
		−1.324		
		−2.048		
0.2	+0.366	+0.314	+0.340	52
		−1.316		
		−2.056		
0.5	+0.369	+0.299	+0.334	70
		−1.324		
		−2.116		
1.0	+0.402	+0.276	+0.339	126
		−1.362		
		−2.184		

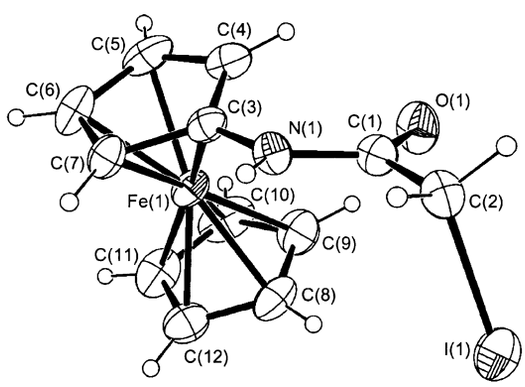

Fig. 1 Perspective drawing of **Fc-IAA** with the atomic numbering scheme. Thermal ellipsoids are shown at the 30% probability level.

waves at *ca.* −1.324 and −2.048 V. These two irreversible waves are attributable to the reduction of the iodoacetamide group. Concerning the ferrocenium/ferrocene redox couple, the currents increase linearly with the square root of the sweep rates, suggestive of a diffusion-controlled process. The diffusion coefficient of **Fc-IAA** in CH_3CN is estimated to be *ca.* 8.1×10^{-6} cm^2 s^{-1}, which is smaller than that of ferrocene (2.4×10^{-5} cm^2 s^{-1}).[17]

Since the iodoacetamide group is well known to undergo facile substitution reaction with the sulfhydryl group, **Fc-IAA** has been used to label a sulfhydryl-modified oligonucleotide M13-R-comp-SH. The modified oligonucleotide is very soluble in aqueous buffer solution and its electrochemical properties have been studied. **Fc-M13** displays a reversible ferrocenium/ferrocene couple at *ca.* +0.016 V *vs.* SCE (v = 0.02 V s^{-1}) in 50 mM potassium phosphate buffer at pH 7.4. The linear relationship between the currents and the square roots of the sweep rates indicates the diffusion-controlled nature of the electron transfer.

Table 3 Electrochemical data of **Fc-Cys**, **Fc-GSH** and **Fc-BSA** in 50 mM potassium phosphate buffer at pH 7.4 and 298 K (sweep rate = 0.02 V s^{-1}, glassy carbon working electrode, electrode area = 7 mm^2)

Conjugate	E_{pa}/V	E_{pc}/V	$E_{1/2}$/V	ΔE/mV
Fc-Cys	+0.100	+0.034	+0.067	66
Fc-GSH	+0.091	+0.021	+0.056	70
Fc-BSA	+0.108	+0.023	+0.066	85

Fc-IAA has also been conjugated to other sulfhydryl-containing biological substrates such as cysteine and glutathione to give the conjugates **Fc-Cys** and **Fc-GSH**, respectively. Meanwhile, bovine serum albumin modified with additional free sulfhydryl groups by SATA and hydroxylamine[1] has also been covalently labelled with **Fc-IAA** to give **Fc-BSA**. These three coupled products were purified by extractions with ethyl acetate, followed by RP-HPLC for **Fc-Cys** and **Fc-GSH**, and size-exclusion HPLC for **Fc-BSA**. The successful conjugation for **Fc-Cys** and **Fc-GSH** were confirmed by electrospray ionisation mass spectrometry, and the stoichiometries (ferrocene : biomolecule) were found to be 1 : 1 in both cases. A control labelling experiment in which alanine was used instead of cysteine or glutathione did not give any ferrocene-containing bio-products, suggesting that the amine groups of the biomolecules were not reactive towards the iodoacetamide moiety of **Fc-IAA** under our reaction conditions. Based on the electronic absorbance spectral data, the ferrocene-to-BSA ratio for the bioconjugate **Fc-BSA** is estimated to be *ca.* 40 : 1. All three conjugates **Fc-Cys**, **Fc-GSH** and **Fc-BSA** display reversible electrochemistry in potassium phosphate buffer at pH 7.4. The $E_{1/2}$ values vary from +0.056 to +0.067 V *vs.* SCE (Table 3). The currents of all three bioconjugates increase linearly with the square roots of the sweep rates, suggesting that the electron-transfer processes are diffusion-controlled.

Many ferrocene derivatives are able to act as electrochemical mediators for redox-active proteins such as glucose oxidase for catalytic glucose oxidation.[18] Since the ferrocenium/ferrocene reduction potentials of **Fc-M13**, **Fc-Cys**, **Fc-GSH** and **Fc-BSA** described in this work are higher than that of glucose oxidase,[19] we have investigated the possibility of using these conjugates as redox-mediators for this enzyme. As an example, a mixture of **Fc-GSH** (0.4 mM) and glucose (32.5 mM) in degassed 50 mM potassium phosphate buffer shows a reversible couple at *ca.* +0.056 V *vs.* SCE [Fig. 2, trace (a)], which is indistinguishable to

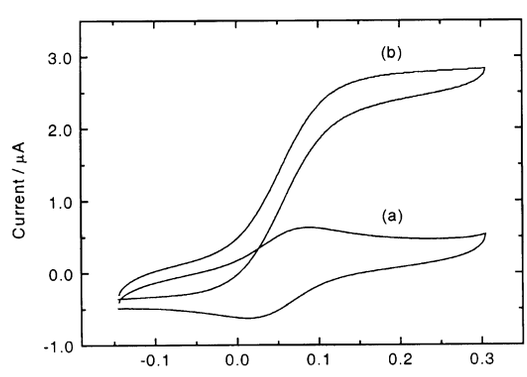

Fig. 2 Cyclic voltammograms of a mixture of **Fc-GSH** (0.4 mM) and glucose (32.5 mM) in degassed 50 mM potassium phosphate buffer (sweep rate = 0.02 V s^{-1}) in the (a) absence, and (b) presence of glucose oxidase (40 μM). A glassy carbon working electrode (area = 7 mm^2) was used.

that of the conjugate in the absence of glucose. Addition of glucose oxidase (40 μM) into this solution gives rise to a catalytic current [Fig. 2, trace (b)], indicating that the oxidised form of **Fc-GSH** can oxidise the reduced form of glucose oxidase, resulting in the catalytic conversion of glucose to gluconolactone. Similar observations of catalytic currents have also been found for **Fc-Cys**. Potentiostatically controlled steady-state current measurements of degassed buffer solutions containing the ferrocene-conjugates (0.4 mM) and glucose oxidase (7.0 μM) reveal linear current responses up to *ca.* 10 and 5 mM glucose for **Fc-Cys** and **Fc-GSH**, respectively. However, under the same experimental conditions, no catalytic responses have been noticed for **Fc-M13** and **Fc-BSA**. Since the redox-potentials of all four conjugates are similar, it is likely the large molecular size of **Fc-M13** and **Fc-BSA** inhibits the approach of these conjugates to the active site of glucose oxidase, rendering it impossible to function as mediators for this enzyme.

Conclusion

We have synthesised and characterised a new ferrocene derivative **Fc-IAA** that contains an iodoacetamide moiety. The X-ray crystal structure of the compound has also been investigated. The compound shows a reversible ferrocenium/ferrocene couple at *ca.* +0.345 V *vs.* SCE in CH_3CN at 298 K, and two irreversible reduction waves at *ca.* −1.324 and −2.048 V, attributable to reductions of the iodoacetamide moiety. Since the iodoacetamide group can undergo a facile substitution reaction with the sulfhydryl moiety, **Fc-IAA** has been conjugated with a sulfhydryl-modified oligonucleotide, cysteine, glutathione and sulfhydryl-modified bovine serum albumin. All the conjugates show reversible ferrocene electrochemistry in aqueous buffer solutions. The possibility of using these ferrocene-containing conjugates as redox-mediators for glucose oxidase has also been examined. Utilisation of bioconjugates labelled with **Fc-IAA** in various bioanalytical applications will be investigated.

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