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A Comparison of Five Bioconjugatable Ferrocenes for Labeling of Biomolecules†

Dongbiao Ge and Rastislav Levicky*

Dept. of Chemical & Biological Engineering, Polytechnic Institute of NYU, 6 MetroTech Center, Brooklyn, NY 11201, USA. Fax: 001-718-260- 3125; Tel: 001-718-260-3682

Abstract

Five electroactive ferrocene tags for labelling of biomolecules are contrasted with regard to conjugation reactivity with amine and thiol moieties, stability to loss of electrochemical activity, and impact of molecular structure on the redox potential of the free and DNA-conjugated forms.

Ferrocene¹ (Fc) has found broad applications due to its chemical stability, its amenability to derivatization, and its electrochemically reversible behavior in which ferrocene is converted to the oxidized ferricinium (Fc⁺) state, $\text{Fc} \leftrightarrow \text{Fc}^+ + e^-$, in which the iron center converts from Fe(II) to Fe(III) while the cyclopentadienyl rings maintain a formal charge of -1 . As extensively reviewed elsewhere,^{2–5} bio-applications of ferrocene derivatives have spanned biomaterials, drug development, and bionanotechnology. In diagnostic applications, ferrocene compounds can serve as electroactive tags that have enabled electrochemical characterization of nucleic acids,^{6–12} proteins,^{13, 14} and peptides.¹⁵ Such labeling affords precise, real-time measurement of surface coverage of labeled molecules that, through use of tags with different formal potentials, can be extended to simultaneous monitoring of more than one surface species.^{16, 17} Motivated by these applications of bioconjugatable ferrocenes, the present communication compares five conveniently prepared labels, including two new ones, with respect to bioconjugation reactivity, electrochemical characteristics, and stability at elevated temperatures.

The *N*-hydroxysuccinimide (NHS) esters of *N*-ferrocenylformylglycine (FcFG-NHS), ferrocene carboxylic acid (FcCA-NHS), and ferrocene acetic acid (FcAA-NHS) are selectively reactive toward amines, while *N*-ferrocenylacrylamide (FA0) and *N*-(2-ferrocene-ethyl) maleimide (FEM) are reactive toward sulfhydryl groups (Table 1). These reactivities are compatible with common bioconjugation protocols for nucleic acids through amine and sulfhydryl modifications, and for peptides and proteins through terminal amines or reactive amino acid side chains. FcCA-NHS and FcAA-NHS were synthesized following the procedure for FcCA-NHS in reference ¹⁷, while FEM was prepared as in reference ⁸. Preparation of the two new labels, FA0 and FcFG-NHS, is described in the Supplementary Information, sections S.1–S.3. Synthesis of FA0 was motivated by a desire to compare its redox and bioconjugation behavior to FEM, the other sulfhydryl-reactive tag. FcFG NHS with its longer linker arm was designed to improve conjugation yields over those of FcCA-NHS, while preserving thermostability.

Ferrocene tags were conjugated to the 5' end of 18mer DNA “targets” of sequence 5' CAC TTG CAG AAT TTA AAA 3' using a 100-fold excess of tag to DNA and 16 h reaction

† Electronic Supplementary Information (ESI) available: FA0 and FcFGNHS synthesis and characterization including NMR spectra, conjugation protocols, preparation of DNA-modified electrodes. See DOI: 10.1039/b000000x/

rlevicky@poly.edu.

times, as detailed in Supplementary Information section S.4 and Table S1. Conjugation yields were calculated by dividing the HPLC peak area of the labeled oligonucleotide by the total of labeled and unlabeled peaks. For these conditions, as shown in Table 1, NHS-ester tags resulted in 70 % to 80 % modification when the NHS-ester was sufficiently far from the ferrocene moiety, as in FcFG-NHS and FcAA-NHS. The labeling efficiency was significantly decreased, to 30 %, for FcCA-NHS in which the NHS-ester and the ferrocene are immediately adjacent, an effect attributed to steric hindrance from the near by ferrocene. For the sulfhydryl-reactive tags, FA0 and FEM, FEM was more reactive with a yield of 90 % compared to 40 % for FA0. The improved yield for FEM is consistent with the dual reactivity of the maleimide ring toward thiols.

Cyclic voltammetry (CV) of unconjugated tags was performed in 0.3 mmol L⁻¹ solutions of the tag in 1 mol L⁻¹ pH 7.2 sodium phosphate buffer (SPB). The buffer was deoxygenated by nitrogen bubbling for 20 min at room temperature and a nitrogen blanket was maintained at all times. A polycrystalline gold working electrode and an Ag/AgCl/3 mol L⁻¹ NaCl reference (0.209 V vs NHE at 25 °C) were used. All potentials are quoted relative to the reference electrode. Fig. 1A shows examples of CV data measured at 65 °C for the free (i.e. unconjugated) FA0 label.

The following observations were true for all solutions of free tags: (i) difference between anodic and cathodic peak potentials was close to 60 mV, indicating electrochemically reversible behavior; (ii) only slight degradation in electroactivity (peak currents) was observed at 65 °C over the 4 h duration investigated. Formal potentials E^0 of the unconjugated tags were calculated from the average of anodic and cathodic peak positions, and are listed in Table 1.

E^0 are known to be affected by sidegroup electron withdrawing properties; e.g. more strongly withdrawing substituents, through induction and/or electron delocalization, tend to hinder oxidation and increase E^0 .¹⁸ If present, charge on the substituent can also facilitate or hinder oxidation depending on whether its interaction with the positive ferricinium state is stabilizing or not. Moreover, complexation of the iron atom by groups such as sidechain carbonyls can impact the redox thermodynamics and influence E^0 .¹⁹

For the NHS-esters, E^0 potentials follow expected order of electron-withdrawing strength, with the α ester of FcCA-NHS ($E^0=0.57$) the highest followed by the α amide of FcFG-NHS ($E^0=0.42$) and, lastly, by FcAA-NHS ($E^0=0.17$) in which the methylene unit α to ferrocene strongly diminishes electron-withdrawal. The low value of $E^0=0.18$ for FA0, in which an amide connects to the cyclopentadienyl ring through the nitrogen instead of the carbonyl as in FcFG-NHS, is attributed to the weaker electron withdrawing power of the nitrogen-ligated arrangement. This trend is anticipated by Hammett σ_p constants for comparable compounds; e.g. σ_p for substituent $-(C=O)NHCH_3$ is 0.36 but is only 0.00 for $-NH(C=O)CH_3$.²⁰ As previously postulated for acetamido ferrocenes,¹⁹ the low E^0 of FA0 may also reflect preferential interaction of the Fe(III) in ferricinium with the sidegroup carbonyl.¹⁹

While ferrocene itself is stable, ferricinium is susceptible to degradation through reaction with various species^{21–23} including, in aqueous environments, the everpresent ⁻OH; therefore, any route leading to conversion of ferrocene into ferricinium can cause tag decomposition. The common mechanism of ferricinium generation was recently proposed to involve protonation of ferrocene followed by oxidation involving transfer of a hydrogen atom to molecular oxygen,²⁴ leading to an overall $4Fc + 4H^+ + O_2 \rightarrow 4Fc^+ + 2H_2O$ stoichiometry.^{25, 26} Thus, if each generated Fc⁺ degrades, then four Fc are expected to be lost per O₂ consumed. If some Fc⁺ is reduced back to Fc before it can degrade, then the

stoichiometry would be less than 4:1. Using sealed containers with known initial ratios of O₂ and ferrocene acetic acid (FcAA) in SPB, we found 3.5 ± 0.2 FcAA consumed per O₂. FcAA and O₂ concentrations were monitored using peak CV currents and an O₂ meter, respectively. We suspect that in SPB the usual fate of degraded ferricinium is complexation of the Fe atom to iron phosphate, and neutralization of released cyclopentadienyl anions to cyclopentadiene by abstraction of a proton from one of the phosphate species.

CV analysis of tag-DNA conjugates was performed by hybridizing them to films of immobilized, complementary 20mer DNA “probe” strands and characterizing the hybridized layers. Probe coverage was controlled at $\sim 5 \times 10^{12}$ strands cm⁻², with mercaptopropanol used as surface passivant (Supplementary Information, section S.6). Fig. 1B shows several CV traces for hybridized FA0-DNA conjugates after pretreatment at 65 °C for different times, as discussed further below. The area of the ferrocene peak, which is proportional to the number of active tags at the surface, was determined using a computer utility.¹⁷ The separation, ΔE_p , between anodic and cathodic peaks was ~ 5 mV in all cases, consistent with reversible electron transfer from immobilized species.²⁷ As for the free tags, E^0 were calculated from the average of anodic and cathodic peak potentials.

Conjugation did not alter the sidegroup immediately proximal to the ferrocene in FcFG, FA0, and FEM; accordingly, E^0 after conjugation are comparable to those of the free tags (Table 1). For FcCA-DNA, conjugation introduced an amide to the α position as confirmed by its E^0 falling to that of FcFG-DNA, which also bears an α amide. Interestingly, E^0 for FcAA-DNA increased relative to the free tag; the source of this increase is unknown but possibly reflects changes in coordination of the β carbonyl to the iron atom.

Susceptibility of tag-DNA conjugates to degradation in SPB was investigated at 65 °C, corresponding to fairly aggressive assay conditions. Nitrogen blanketing and a 20 min pre-deoxygenation limited the O₂ concentration to about 10 $\mu\text{mol L}^{-1}$, or approximately a 300 % excess over the amount needed for complete degradation. After a designated time at 65 °C, solutions were rapidly cooled to room temperature followed by hybridization to the probe films until the ferrocene peak no longer increased (~ 40 min), signaling end of hybridization. Because each sample was hybridized under the same conditions (Supplementary Information, section S.7) comparable extents of hybridization were expected; thus, if a decrease was observed in tag CV peaks it was attributed to label decomposition, not to a decrease in hybridized amount.

The results of these studies, shown in Fig. 2 and in the last column of Table 1, reveal that the conjugated tags degraded with widely variable rates. The most resistant conjugates, FcFG and FcCA, possessed the highest formal potentials (Table 1). That high E^0 correlates with resistance to degradation by an oxidative mechanism is not surprising. However, the pronounced loss of FcAA activity argues against E^0 being the sole criterion of stability, since FcAA is less resistant than the more easily electrochemically oxidized FEM and FA0. In this regard, FcAA degradation may be assisted by acidity of its α methylene group, expected to be particularly acidic because of resonance stabilization of the resultant enolate anion by the metallocene ring. Presence of acidic hydrogens in immediate proximity to the ferrocene may facilitate its protonation and ensuing oxidation, and/or enable a rapid degradation pathway for ferricinium via nucleophilicity of the enolate. Degradation at room temperature was much slower; for example, for FA0 90% activity remained after 4 h under otherwise identical conditions. Incidentally, the apparently good stability of unconjugated tags when kept at 65 °C for up to 4 h (e.g. Fig. 1A) is explained by O₂ acting as a limiting reactant, since the O₂ present was sufficient to only degrade about 10% of the tags.

Degradation of ferricinium has been implicated in production of hydroxyl radicals²⁴ which in turn can trigger radical-mediated scission of DNA.^{28–30} This raises the question of whether HO· produced during degradation of the tags, attached at the strand termini, could lead to significant DNA cleavage. To address this question control experiments were performed using MALDI TOF mass spectrometry which revealed that the DNA strands remained intact; i.e. that tag degradation did not produce appreciable DNA scission (Supplementary Information, section S.8).

From among the five electroactive tags considered, FcFG-NHS provides the best combination of stability and conjugation yield. Its relatively high formal potential is also attractive to multiple-tag schemes for simultaneous monitoring of two or more species, which requires sufficient separation in E^0 values. For “two-color” electrochemical detection FA0 is a good second tag, also because of its ease of preparation (Supplementary Information, section S.2), but a need remains for additional water soluble, stable, bioconjugatable tags with well separated E^0 potentials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) CV traces for solutions of FA0 in SPB as a function of storage time at 65 °C. Scan rate: 100 mV s⁻¹. (B) CV traces of FA0-DNA conjugates, pretreated in SPB at 65 °C for the indicated time, then hybridized to surface-bound complementary strands. Scan rate: 20 V s⁻¹.

Figure 2.
Remnant electroactivity for ferrocene-DNA conjugates as a function of storage time at 65 °C.

Table 1

Label properties

Molecular structure	conj. yield	E^0 (tag)	E^0 (conj.)	Electroactivity of conjugate (4 h 65°C)
FcFG-NHS	70%	0.42V	0.43V	88%
FcAA-NHS	80%	0.17V	0.22V	0%
FcCA-NHS	30%	0.57V	0.42V	88%
FA0	40%	0.18V	0.16V	16%
FEM	90%	0.21V	0.20V	7%