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Received: January 14, 2004
Final version: May 7, 2004

Abstract
Ferrocene derivatives containing primary amines and maleimide groups were attached covalently onto N-hydroxsuccinimidyl (NHS)-terminated alkanethiol self-assembled monolayers (SAMs) and SAMs of alkanedithiol. The surface coverage and efficiencies of the two cross-linking reactions were evaluated with cyclic voltammetry. All the ferrocene derivatives attached onto the alkanethiol or alkanedithiol SAMs exhibit reversible redox waves. The surface coverage of the aminated ferrocene groups was compared to that of N-hydroxsuccinimidyl (NHS)-terminated alkanethiol SAM. The covalent attachment of β-ferrocenylethylamine onto a 11,11'-dithio-bis(succinimidyldodecanate) SAM yielded an efficiency as high as 63.1%. The cross-linking efficiency of this reaction was found to increase with the nucleophilicity of the amino groups. SAMs of longer alkyl chains favor the attachment of a greater number of ferrocene derivatives. As for the Michael-type electrophilic addition between the sulfhydryl groups of the alkanedithiol SAMs and the ferrocenyl maleimide, the cross-linking efficiencies were found to range from 6.5% to 25.7%, depending on the alkanedithiol chain length. The difference in the efficiencies between the two types of cross-linking reactions might be partially attributable to the steric hindrance imposed by the SAMs and the relative sizes of the functional groups.

Keywords: Self-assembled monolayers, Voltammetry, Cross-linking reactions, Aminated ferrocene, N-(2-Ethylferrocene)maleimide

1. Introduction
Self-assembly of alkanethiols or alkanedithiols at metals has been widely regarded as an attractive route for creating highly ordered and functional surfaces. Surfaces modified with self-assembled monolayers (SAMs) of alkanethiols or alkanedithiols have been employed as model systems for the study of interfacial processes [1, 2], such as inhibition of protein adsorption onto surfaces [3, 4], heterogeneous catalysis [5], electron transfer reactions [6–13], or electrochemically and/or photochemically triggered surface restructuring [14–16]. These surfaces have also been extensively utilized for the immobilization of various organic and biological species for the construction of chemosensors and biosensors [2]. In general, functional groups at the end of a SAM are cross-linked to the species of interest in solution through covalent bond formation. For example, the cross-linking reaction between a carboxyl group and an amine group to form an amide bond can be realized with the aid of carbodiimide and N-hydroxsuccinimide (NHS) or N-hydroxysulfosuccinimide (NHSS) [17–20]. Such a methodology has been applied to the immobilization of a wide range of biomolecules, including, but are not limited to, nucleic acids [21–23], enzymes, [17, 24, 25], and proteins and peptides [26–28], onto SAMs preformed at metal electrodes. As high as 90% of the carboxyl groups can be activated and stabilized [17]. An alkanethiol SAM terminated with NHS esters can result in the attachment of an even greater number of solution species [29].

Another useful cross-linking reaction is that between sulfhydryl and maleimide groups. The double bond of the maleimide group undergoes an alkylation reaction (Michael-type electrophilic addition) by forming a stable thioether bond with sulfhydryl groups. The reaction rate has been shown to be several orders of magnitude faster than that between the maleimide group with amino or hydroxyl groups [30]. Organic compounds such as biotin [31, 32], and biomolecules including proteins [33, 34], peptide [32, 35–37], DNA [30, 38], and even cells [32] have been attached.

In this work, we synthesized several ferrocene (Fc) derivatives consisting of different functional groups and subsequently immobilized these molecules onto SAMs of alkanethiol or alkanedithiol of different chain lengths. The impetus behind this work partially stems from the fact that an accurate determination of the cross-linking efficiency should provide a guide for the construction of well-defined and effective chemosensors and biosensors. For example,
Thor and co-workers used aminoferrocene (FcNH2) to assess the possibility of attaching DNA molecules onto a thin film of poly[Ru(bpy)3]^2+ (bpy = 4-ethyl-4'-methyl-2,2'-bipyridine) [39]. Another noteworthy work is the successful utilization of N-(2-ethyl-ferrocene)maleimide (Fe−Mi) to label electroactive proteins in solution for subsequent voltammetric characterization [33, 34]. The presence of the Fe groups at the surface facilitates voltammetric measurements of the surface coverage of the attached species, providing an opportunity to evaluate the cross-linking reaction efficiency. In addition to the alkyl chain length, we have also examined other experimental factors, such as the preparative parameters for the SAM formation and those for the Fe derivative attachments, and considered the influence of the steric hindrance on the efficiency of the attachment. We show that voltammetry is a simple and sensitive technique for the evaluation of the cross-linking reactions. Moreover, our approach affords an avenue for varying the types of cross-linking reactions at the metal/solution interface to create different chemically modified surfaces, obviating the necessity of synthesizing certain chemical modifiers and conducting tedious separations of the reaction products in a homogeneous solution.

To demonstrate the applicability of these immobilization schemes for the detection of biomolecules, the reduced form of glutathione (GSH) was successfully attached to a SAM-modified electrode and measured by voltammetry.

2. Experimental

2.1. Reagents

N-hydroxysuccinimide (NHS) was from Acros Organics (Belgium). 11-Bromoundecanoic acid, (dimethylamino-methyl)-ferrocene, 1,3-propanedithiol (HSC,SH), 1,6-hexanedithiol (HSC,SH) and 1,9-nonanedithiol (HSC,SH) were from Sigma-Aldrich. 4-(Dimethylamino)-pyridine (Schuchardt, Hohenbrunn, Germany), 3,3'-dithiobis(succinimidylpropionoate) (C9-NHS, Sigma-Aldrich), reduced form of glutathione (GSH, Amresco) and aminoferrocene (FcNH2, Tokyo Kasei Kogyo Co., LTD) were also acquired commercially. 11,11'-Dithio-bis(succinimidylundecanoate) (C11-NHS), β-ferrocenylethylamine (FcCH2CH2NH2), and N-(2-ethyl-ferrocene)maleimide (Fe−Mi) were synthesized according to literature procedures [29, 33, 40, 41]. Column chromatography was performed using silica gel H60 to purify the intermediates and final products. 1H-NMR, transmission FT-IR, and thin-layer chromatography (TLC) were carried out to confirm the structures of the individual compounds. Other reagents involved in this work are all from commercial sources with analytical purity and used as received.

2.2. Immobilization of Fe Derivatives onto Preformed Self-Assembled Monolayers

SAMs terminated with two functional groups, viz., N-hydroxysuccinimidyland sulphydryl, were formed by immersing the gold electrodes into the respective solutions for various lengths of time. For the NHS-terminated SAMs, C9-NHS and C11-NHS were used to examine the effect of the alkanethiol chain length on the efficacy of the Fe derivative attachment. Upon soaking in a 2 mM ethyl acetate solution for 20−25 h, the electrodes were washed sequentially with ethyl acetate and water. The influence of the nucleophilicity of the aminated Fe derivatives on the cross-linking reactions was also evaluated. Specifically, an alkyl amine (β-ferrocenylethylamine, FeCH2CH2NH2) and an aromatic amine (aminoferrocene, FeNH2) were compared. This was accomplished by soaking the electrodes covered with NHS-terminated SAMs in an ethanol solution containing either 10 mM FeCH2CH2NH2 or 10 mM FeNH2 for 20−25 h. For cross-linking Fe−Mi onto a sulphydryl-terminated SAM, HSC,SH, HSC,SH, and HSC,SH SAMs were first produced by soaking the electrodes in ethanol solutions containing 1 mM of the respective alkanedithiol for 52 h, followed by transferring the SAM-modified electrodes to a 2 mM Fe−Mi ethanol solution.

Influence of immersion time on the electrochemical response of the Fe derivatives attached onto the termini of the preimmobilized SAMs was assessed. For the cross-linking reaction between aminated Fe derivatives and NHS-terminated SAMs, it was found that 25 h for the immobilization of the C9- or C11-NHS SAM and 20 h for the Fe derivative attachment were optimal, in terms of attaining appreciable voltammetric currents and well-defined redox waves. As for the concentration of the NHS-terminated alkanethiol used for the SAM formation, 2 mM was deemed to be suitable. For the cross-linking reaction between the Fe−Mi molecules and the sulphydryl groups at the end of the alkanedithiol SAM, the preparative parameters were chosen according to procedures developed by Gleria et al. [33, 34].

2.3. Electrochemical Instruments and Procedures

Electrochemical experiments were conducted with a CHI 440 electrochemical workstation (CH Instruments, Austin, TX) in a conventional three-electrode cell. The working electrodes are polycrystalline gold disks with a diameter of 2-mm embedded in Kel-F rods. A platinum electrode and an Ag/AgCl electrode were used as the auxiliary and the reference electrodes, respectively. Prior to each measurement, the Au electrodes were polished with diamond pastes and alumina slurry down to 0.05 μm on a polishing cloth (Buehler, Lake Bluff, IL), followed by sonicating in water and ethanol. The electrodes were then rinsed with a copious amount of deionized water and dried under a stream of N2. The supporting electrolyte was a 0.1 M HClO4 solution. All solutions were prepared with deionized water treated with a
water purification system (Simplicity 185, Millipore Corp.). All electrochemical experiments were conducted under a nitrogen atmosphere at the ambient temperature.

2.4. Quartz Crystal Microbalance Measurements

9.995-MHz AT-cut crystals coated with gold films in a keyhole shape (QCM active area = 0.212 cm²) were acquired from ICM Technologies (Oklahoma City, OK). The gold-coated crystals were cleaned with a piranha solution and rinsed with deionized water. CAUTION: Piranha solution reacts violently with organic solvents and is a skin irritant. Extreme caution should be exercised when handling piranha solution. The frequency measurements were carried out with a CHI 440 Electrochemical Quartz Crystal Microbalance in air. Briefly, crystals modified with the NHS-terminated alkanethiols SAMs were dried under a stream of N₂ and their resonance frequencies were measured. Following attachment of the aminated ferrocene molecules, the crystals were dried again and the new frequencies were recorded. The difference in frequency was used to deduce the mass of the Fc derivatives.

3. Results and Discussion

The cross-linking reactions explored for attaching Fc derivatives onto preformed SAMs are shown in Scheme 1. In Scheme 1a, the amino groups on the Fc derivatives undergo nucleophilic substitutions with the NHS ester groups situated at the solution termini of the SAMs. Two aminated Fc derivatives (FcNH₂ and FcCH₂CH₂NH₂) were studied in an attempt to probe the relationship between the nucleophilicity of the amino groups and the surface coverage of the Fc groups on the final monolayers. In Scheme 1b, the Fc–Mi molecules undergo a Michael-type electrophilic addition reaction with the free sulfhydryl groups of an alkanedithiol SAM. The influence exerted by the chain length was evaluated by utilizing two different alkyl chains (C₉-NHS and C₇-NHS) for Scheme 1a and three different alkyl chains (HSC₃SH, HSC₆SH, and HSC₉SH) for Scheme 1b.

Curve a in Figure 1 is a cyclic voltammogram (CV) of FcCH₂CH₂NH₂ anchored onto a C₁₁-NHS SAM. To ensure that the signal was not originated from the nonspecifically adsorbed Fc derivative, we performed a control experiment in which a C₁₁-NHS SAM was exposed to a 10 mM Fc solution. The absence of any discernable peaks in curve b indicates that peaks of curve a are associated with Fc moieties that have been covalently attached through the amino groups on the Fc derivative. The anodic peak appears at 0.328 V (Eₚₐ) and the peak potential separation (ΔEₚ) is 0.027 V in curve a (Table 1). The plot of anodic peak currents (iₚ) against the scan rates between 0.01 and 0.5 V/s was linear with a r² value of 0.9998 (inset), suggesting that FcCH₂CH₂NH₂ was surface-confined [42]. All these characteristics are indicative of the facile electron transfer reactions between Fc moieties at the end of the SAM and the underlying Au electrode.

To probe the relationship between the nucleophilicity of the amino groups on the Fc derivatives and the efficiency of the cross-linking reaction, we overlaid CVs of FcCH₂CH₂NH₂ (curve a) and FcNH₂ (curve b) in Figure 2. The comparison between these two CVs and the voltammetric parameters listed in Table 1 show that the attachment of FcNH₂ produced a broader and less intense redox wave with Eₚ values shifted slightly in the anodic direction. The decrease in the peak current can be attributed to the reduction of the cross-linking efficiency caused by the weaker nucleophilicity of the amino group in FcNH₂. In FcNH₂, the lone pair electrons on the nitrogen atom are delocalized through conjugation with the π orbitals on the Fc cyclopentadiene (C₅) ring, while the NH₂ group in FcCH₂CH₂NH₂ merely serves as a relatively weak electron-withdrawing group. Moreover, the amino group in FcCH₂CH₂NH₂ is further away from the somewhat bulky C₅ ring,
reducing steric hindrance at the SAM/solution interface. It is worth noting that the peak intensity and the shape of curve b are actually slightly better than those observed by Thorp and co-workers who immobilized FcNH₂ onto a carboxyl-containing poly[Ru(vbpy)₃²⁺/vba] film-modified electrode.[39] The coupling efficiency between NHS esters and FcNH₂ is expected to be better than that between COOH groups and FcNH₂ with the aid of carbodiimide and N-hydrosuccinimide. It is also clear that molecules that contain aniline groups of a stronger nucleophilicity and are easily accessed should be more suitable for voltammetric verification of immobilization routes involving activated carboxyl groups at a surface.

We then estimated the cross-linking efficiencies of the aminated Fc derivatives attached onto the alkanethiol SAM by comparing the surface coverage (Γ) of curves a and b in Figure 2 to that of a NHS-terminated SAMs. Using a quartz crystal microbalance (QCM), the amount of NHS adsorbed (Δm) can be deduced with the Sauerbrey equation, Δm = \(- (N_p / \rho_q) \Delta f\), where Δf is the difference between the resonant frequency of a bare crystal (f₀) and that of a crystal coated with a NHS-terminated SAM, N is the frequency constant for quartz, and \(\rho_q\) is the quartz density (2.648 g cm⁻³). The Γ value of the C₁₁-NHS SAM was estimated to be 6.80 × 10⁻¹⁰ mol/cm². Γ values of FcNH₂ and FcCH₂CH₂NH₂ on the other hand, can be obtained based on the equation Γ = \(Q/nFA\), where Q is the area of the Fc oxidation peak, n is the moles of electrons involved in the Fc oxidation (n = 1), F is the Faraday’s constant, and A is the area of the electrode (A = 0.0314 cm²). Thus, by taking the ratios of the Γ values of the aminated ferrocenes (Table 1) over those of NHS-terminated SAMs, the efficiencies in cross-linking FcNH₂ or FcCH₂CH₂NH₂ onto the two types of NHS-terminated SAMs can be estimated. As can be seen from the cross-linking efficiencies in Table 1 and the larger CV wave of FcCH₂CH₂NH₂ at a C₁₁-NHS SAM with respect to that at an electrode modified with a C₃-NHS SAM (Figure 3), the longer NHS-terminated alkanethiol SAM results in a greater attachment of a given aminated Fc molecules. This is understandable, given the well-known fact that longer alkanethiols tend to form more compact and ordered SAMs.[43] Such a contention is also supported by a recent work by Dordi et al. about the chain length effect on the hydrolysis of NHS ester-terminated SAMs.[44] Interestingly, the more compact C₁₁-NHS film does not appear to impose a greater steric hindrance to FcCH₂CH₂NH₂. Again the extension of the amino group further away from the Fc group could have facilitated the cross-linking process. This observation has an interesting implication to the interpre-

![Fig. 1. A CV of FcCH₂CH₂NH₂ attached onto a C₁₁-NHS SAM acquired in a 0.1 M HClO₄ solution (a) and a CV collected at a C₁₁-NHS SAM that had been exposed to a 10 mM Fc ethanol solution (b). The scan rate employed was 0.1 V/s and the arrow indicates the scan direction. The inset shows the plot of anodic peak current of FcCH₂CH₂NH₂ against the scan rate.](image)

![Fig. 2. An overlay of CVs of FcCH₂CH₂NH₂ (a, dotted line curve) and FcNH₂ (b) attached to C₁₁-NHS SAMs. The scan rate employed was 0.1 V/s and the arrow indicates the scan direction.](image)

Table 1. Voltammetric parameters and surface coverage values of aminated Fc derivatives and the corresponding cross-linking efficiencies at NHS-terminated SAMs.

<table>
<thead>
<tr>
<th>Reactions</th>
<th>(E_{pa}) (V)</th>
<th>(E_{pc}) (V)</th>
<th>(\Delta E_p) (V)</th>
<th>(i_{pa}) (µA)</th>
<th>(\Gamma) (nmol/cm²)</th>
<th>Cross-linking efficiency(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₁₁-NHS (FcCH₂CH₂NH₂)</td>
<td>0.328</td>
<td>0.301</td>
<td>0.027</td>
<td>1.10</td>
<td>0.429 ± 0.014</td>
<td>63.1</td>
</tr>
<tr>
<td>C₁₁-NHS (FcNH₂)</td>
<td>0.400</td>
<td>0.335</td>
<td>0.065</td>
<td>0.32</td>
<td>0.190 ± 0.013</td>
<td>27.9</td>
</tr>
<tr>
<td>C₁₁-NHS (FcCH₂CH₂NH₂)</td>
<td>0.334</td>
<td>0.299</td>
<td>0.035</td>
<td>0.95</td>
<td>0.326 ± 0.020</td>
<td>56.3</td>
</tr>
</tbody>
</table>
tation about the effect of the alkanedithiol chain length on the second type of cross-linking reaction described below.

The second cross-linking reaction we studied concerns the attachment of a maleimide-containing Fc derivative onto alkanedithiol SAMs of different chain lengths (Scheme 1b). Two points can be made upon examining the CVs overlaid in Figure 4. First, reversible CV waves were observed for all three cases with \( E_{pa} \) values being around 0.3 V. The control experiment (curve d) was conducted after a 1,9-nonanedithiol SAM was exposed to an ethanol solution containing 2 mM ferrocene. Again the absence of any appreciable wave confirms that peaks in curves a–c are the result of the cross-linking reaction between Fe–Mi and the sulfhydryl groups. Similar to Scheme 1a, the \( i_{pa} \) values of the peaks in Figure 4 are also proportional to the scan rates (data not shown), suggesting that the Fe–Mi molecules are firmly affixed onto the surface. Second, the \( \Gamma \) values of Fe–Mi at the three alkanedithiol SAMs (Table 2) are all smaller than those of aminated Fc derivatives attached to the electrode surface. This trend suggests that Scheme 1b is less efficient than Scheme 1a. The maximum \( \Gamma \) value of a typical alkanedithiol SAM is about \( 2.8 \times 10^{16} \) mol/cm\(^2\) [45]. Compared to this value, the cross-linking reaction efficiencies were estimated to be 25.7%, 18.4%, and 6.5%, respectively. Similarly to Scheme 1a, the longer alkanedithiol favors the attachment of a greater number Fe–Mi molecules. We should note that a possible reason for the relatively low efficiencies could be due to the attachment of the alkanedithiol to the electrode with the “looped” structure. In other words, both the sulfhydryl groups of the alkanedithiol molecules formed covalent bonds with the gold surface. But the presence of such a structure should not be predominant since X-ray photoelectron spectroscopy measurements [46, 47] and other works [46–52] have shown that most alkanedithiols tend to tether onto the gold surface with one thiol end group.

The amenability of the two cross-linking schemes for biological sensing described herein is explored in a preliminary study, using the reduced form of glutathione (GSH) as a test species. GSH, a tripeptide containing a cysteine residue, does not exhibit well-defined voltammetric behavior at a solid electrode (only at an Hg electrode [53]). We first attached GSH onto a C\(_3\)-NHSS SAM-modified Au electrode via the amide bond formation (Scheme 1a). This is followed by tagging the cysteine sulfhydryl group with Fe–Mi. The resultant CV is displayed in Figure 5, with \( E_{pa} = 0.420 \) V and \( \Delta E_p = 0.107 \) V. Clearly, both anodic and cathodic waves are well-resolved. Thus, the simple cross-linking reactions engender redox activity to biomolecules that are otherwise difficult for voltammetric determinations. We are currently exploring the use of these reaction schemes for sensitive electrochemical studies of proteins immobilized onto surfaces.

### 4. Conclusions

This work demonstrates that Fe derivatives can be utilized as model compounds to gauge the efficiency of the cross-linking reaction between the surface-confined NHS ester

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**Table 2. Voltammetric parameters and surface coverage values of ferrocene maleimide attached onto alkanedithiol SAMs of different chain lengths.**

<table>
<thead>
<tr>
<th>Reactions</th>
<th>( E_{pc} (V) )</th>
<th>( E_{pa} (V) )</th>
<th>( \Delta E_p (V) )</th>
<th>( i_{pa} (\mu A) )</th>
<th>( \Gamma ) (nmol/cm(^2))</th>
<th>Cross-linking efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSC3SH</td>
<td>0.309</td>
<td>0.280</td>
<td>0.029</td>
<td>0.046</td>
<td>0.018 ± 0.002</td>
<td>6.5</td>
</tr>
<tr>
<td>HSC6SH</td>
<td>0.327</td>
<td>0.305</td>
<td>0.022</td>
<td>0.129</td>
<td>0.051 ± 0.005</td>
<td>18.4</td>
</tr>
<tr>
<td>HSC9SH</td>
<td>0.335</td>
<td>0.317</td>
<td>0.018</td>
<td>0.170</td>
<td>0.072 ± 0.010</td>
<td>25.7</td>
</tr>
</tbody>
</table>

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**Fig. 3.** CVs of FcCH\(_2\)CH\(_2\)NH\(_2\) attached onto a C\(_3\)-NHSS SAM (a) and a C\(_{11}\)-NHSS SAM (b). The scan rate was 0.1 V/s and the arrow indicates the scan direction.

**Fig. 4.** CVs of Fe–Mi anchored onto SAMs of 1,9-nonanedithiol (a), 1,6-hexanedithiol (b), and 1,3-propanedithiol (c) in a 0.1 M HClO\(_4\) solution. Curve d is a CV acquired at a 1,9-nonanethiol SAM that had been exposed to a 2 mM ferrocene ethanol solution and subsequently transferred to a 0.1 M HClO\(_4\) solution. The scan rate was 0.1 V/s and the arrow indicates the scan direction.
and a primary amine-containing species in solution or that between a surface-bound sulphydryl moiety and a solution species comprising a maleimide group. Experimental procedures (i.e., soaking time and concentrations of the individual compounds used in this study) for the formation of compact NHS or alkanedithiol SAMs and the subsequent attachment of the Fc derivatives were optimized. For immobilizing aminated Fc derivatives, we found that the nucleophilicity of the amino groups has a profound influence on the final surface coverage of the Fc derivative. Our study also revealed that the organization of the preimmobilized SAMs affects the extent of Fc derivative attachment, with SAMs of a longer alkyl chain leading to a greater surface coverage. We also developed a new route for modifying electrodes with electroactive Fc groups onto a surface by performing a Michael-type electrophilic addition reaction between the sulphydryl groups on an alkane-dithiol SAM and Fc–Mi in solution. While the efficiency of this reaction was not as high as that associated with the NHS ester-terminated SAM, well-defined reversible voltammetric waves with appreciable faradaic currents were observed. The difference in the efficiencies between the two types of cross-linking reactions might be partially attributable to the steric hindrance imposed by the SAMs and the relative sizes of the functional groups.

The voltammetric determination of Fc derivatives composed of different functional groups is simple, sensitive, and straightforward. The investigation of the various steric and chemical factors at the SAM/solution interfaces serves as a guide for screening an appropriate route for linking a specific analyte molecule onto the sensing surface. The attachment of Fc–Mi to surface-confined species containing sulphydryl groups imparts electroactivity to the surface, making voltammetric techniques amenable to the detection of electroinactive species in the sample solution.

5. Acknowledgements

Partial support of this work by grants from the Chinese Academy of Sciences (One Hundred Persons Award) and the National Natural Science Foundation of China (Outstanding Young Scientist Award No. 20225517) is gratefully appreciated.

6. References