

Full Paper

Voltammetric Determination of Surface-Confined Biomolecules with *N*-(2-Ethyl-ferrocene)maleimide

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Abstract

A thiol-specific electroactive cross-linker, *N*-(2-ethyl-ferrocene)maleimide (Fc-Mi), has been used to tag surface-confined peptides containing cysteine residues or oligodeoxynucleotides (ODNs) whose 3' ends have been modified with thiol groups. The peptides studied herein include both the oxidized and reduced forms of glutathione and a hexapeptide. Cyclic voltammograms (CVs) of the Fc-Mi groups attached to the surfaces were used to quantify the total number of cysteine residues that are tagged and/or can undergo facile electron transfer reactions with the underlying electrodes. A quartz crystal microbalance was used in conjunction with CV to estimate the total number of cysteine groups labeled by Fc-Mi per peptide molecule. By comparing to mass spectrometric studies, it is confirmed that not all of the Fc-Mi linked to the cysteine groups can participate in the electron transfer reactions. The methodology is further extended to the determination of ODN samples in a sandwich assay wherein the thiol linker on the 3' end can be tagged with Fc-Mi. The analytical performance was evaluated through determinations of a complementary ODN target and targets with varying numbers of mismatching bases. ODN samples as low as 10 fmol can be detected. Such a low detection level is remarkable considering that no signal amplification scheme is involved in the current method. The approach is shown to be sequence- and/or structure-specific and does not require sophisticated instrumentation and complex experimental procedure.

Keywords: Redox Labels, Voltammetry, Quartz Crystal Microbalance, *N*-(2-Ethyl-ferrocene)maleimide, DNA Hybridization

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1. Introduction

Sulfhydryl groups can modulate the functions and structures of biomolecules [1]. For example, the cysteine residues present in p53, a tumor-suppression transcription factor protein, can modulate the protein binding to its consensus DNA duplexes [2–5]. Since cysteine, the most easily oxidizable amino acid [6], is known to be susceptible to form dimers, determination of cysteine residues present in peptides/proteins is of great biological relevance. Thus far, a great number of methods have been developed to determine sulfhydryl groups. The use of the Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid)) for the spectrometric identification [7], fluorescence measurements based on the reaction of proteins with a thiol-specific reagent [6], and the thiol detection using Western analysis [8–10] are just a few of the many methods reported. However, these methods are mainly limited to peptides or proteins in solution and not applicable to sulfhydryl groups present in surface-bound molecules. Quantification of sulfhydryl-containing groups in various biomolecules affixed onto surfaces is becoming increasingly important in view of the development and characterization of microarrays and

heterogeneous biosensors (particularly the electrochemically-based ones [11]).

Sulfhydryl groups present in biomolecules can also be tagged with various chromophores and electroactive moieties through the use of heterobifunctional reagents. For example, many *N*-hydrosuccinimide [12] and maleimide (Mi) [13] containing heterobifunctional reagents are commercially available for labeling proteins with fluorescence tags. Labeling biomolecules with electroactive label is also an attractive alternative in bioanalysis, because voltammetric techniques are simple and sensitive (particularly to surface-confined species), do not require sophisticated instrumentation, and can circumvent certain limitations in other methods (e.g., photobleaching in fluorescence detection) [14]. Conceivably, the introduction of an electroactive label that reacts specifically with the sulfhydryl groups should allow biomolecules that are either electro-inactive or do not exhibit reversible voltammetric responses to be detected. Hill and coworkers [15, 16] have successfully synthesized a sulfhydryl-specific reagent, *N*-(2-ferrocenyl-ethyl)maleimide (Fc-Mi), to label the cysteine groups in select proteins and peptides, thus yielding detectable voltammetric signals. In their work, excess amounts of Fc-Mi are mixed with a

protein or peptide in solution. The Fc-labeled biomolecules were then separated from the reaction mixture via column chromatography and subsequently analyzed using cyclic voltammetry (CV). While well-resolved voltammetric waves were observed, the method involves sample pretreatment (chromatographic separation) and was only applied to solution species. Furthermore, the relationship between the labeling efficiency and the observed voltammetric signals was not elucidated. Recently, we developed an amplified voltammetric scheme to detect surface-confined peptides and proteins by labeling peptidic species and proteins with Fc-capped gold nanoparticle-streptavidin conjugates [17, 18]. The presence of a large number of Fc moieties enhances the detection of a small number of cysteine-containing peptides or proteins at the electrode surface. However, the large size of the Fc-capped nanoparticle-streptavidin conjugate was found to be quite inefficient in binding to the biotinylated peptides or proteins [18]. As a result, only about 1% of the total sulfhydryl groups at the surface were linked to the redox labels. It appears that a small electroactive molecule that can react with thiol groups is more desirable when a high labeling efficiency is sought.

In this paper, we report the use of Fc-Mi to tag surface-confined peptides containing sulfhydryl groups. Peptides are first attached onto *N*-hydroxysuccinimide (NHS)-terminated alkanethiol self-assembled monolayers (SAMs) [19]. Fc-Mi molecules are then linked onto the sulfhydryl groups on the cysteine residues [20] and detected voltammetrically. Alternatively, biomolecules containing cysteine residues can be pre-labeled with Fc-Mi and directly adsorbed onto electrodes modified with NHS-terminated SAMs. A quartz crystal microbalance and mass spectrometry were used in conjunction with CV to gauge the total number of cysteine groups per biomolecule labeled and to probe the relationship between the voltammetric response and the labeling efficiency. We also applied this methodology to a sandwich DNA assay in which Fc-Mi is attached to the thiol linker on a detection oligonucleotide (ODN) probe. The analytical performance of this method was evaluated through the analyses of a complementary ODN target and ODN targets with varying numbers of mismatching bases.

2. Experimental

2.1. Reagents

Oxidized and reduced forms of glutathione (GSSG and GSH, respectively), (dimethylaminomethyl)-ferrocene, 3,3'-dithiobis(succinimidylpropionate) (C_3 -NHS), and ethanolamine were acquired from Sigma-Aldrich. 4-(dimethylamino)-pyridine was obtained from Schuchardt (Hohenbrunn, Germany). The pentapeptide (A5, Ala-Ala-Ala-Ala-Ala) was synthesized by Sangon Co., LTD (Shanghai, China). The hexapeptide, Lys-Cys-Thr-Cys-Cys-Ala (denoted as FT), was purchased from Bachem California Inc. (Torrance, CA). ODN capture probes with their 5' ends modified with amino groups and detection probes with their

3' ends attached to a thiol linker were both obtained from Sangon Co., LTD (Shanghai, China). The capture and detection probes have sequences of 5'-NH₂-(CH₂)₆-TTT TTG GAG CAC CCA CGT GTC CTG GCC-3' and 5'-AGG AGA GCT CAG TTT ACT AGT GCC TTT-3' (CH₂)₆-SH, respectively. Three different target sequences (mismatching sequences underlined) were chosen to evaluate the selectivity of the method: 3'-GGG TGC ACA GGA CCG GTC ATA TCC TCT CGA GTC AAA TGA -5' (complementary to the capture probe), 3'-GGG TGT ACA GGA CCG GTC ATA TCC TCT CGA GTC AAA TGA -5' (one base mismatching to the capture probe), and 3'-GCG TGT ACA GGC CTG GTC ATA TCC TCT CGA GTC AAA TGA -5' (four-bases mismatching to the capture probe). Fc-Mi was synthesized according to literature procedures [15, 16]. All solutions were prepared with deionized water treated using a water purification system (Simplicity 185, Millipore Corp.).

2.2. Electrodes

The gold working electrodes have a diameter of 2 mm. A platinum wire and a 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 supporting electrolyte was a 0.1 M KClO₄ solution. All electrochemical experiments were conducted at the ambient temperature.

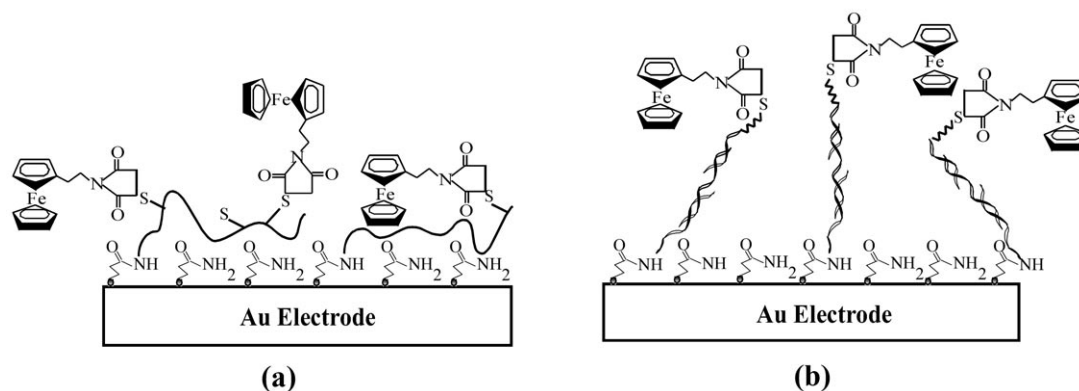
2.3. Procedures

2.3.1. Peptide Immobilization and Tagging with *N*-(2-Ethyl-Ferrocene)maleimide

Alkanethiol SAMs terminated with C_3 -NHS were first attached onto Au electrodes by immersing the electrodes into a 2.0 mM C_3 -NHS in ethyl acetate for 24 h. The electrodes were then washed sequentially with ethyl acetate and deionized water and soaked into a phosphate buffer containing peptides for 18–24 h. The peptide-covered electrodes were immersed into a 4.0 mM Fc-Mi solution dissolved in ethanol or DMSO for about 20 h. This step was followed by an extensive wash with water to rid any possible nonspecifically adsorbed Fc-Mi of the electrodes.

2.3.2. Attachment of *N*-(2-Ethyl-Ferrocene)maleimide onto DNA-Modified Electrodes

C_3 -NHS SAM-covered electrodes were exposed to a mixed solution of 10.0 mM (pH 7.0) phosphate buffer saline (PBS) solution comprising 0.3 M NaCl and 0.4 μM capture probe. These electrodes were then washed with water and soaked in a 0.1 M ethanolamine solution for 1 h to eliminate the unreacted NHS ester groups at the electrode surface. For



Scheme 1. Schematic representation of the cross-linking reactions investigated: the nucleophilic substitution of the NHS-terminated alkanethiol SAM by the amino groups on the peptide molecules (a), and the capture probes whose 5' ends modified with amino groups (b) followed by the attachment of the Fc-Mi via an electrophilic addition with the sulfydryl groups on the pet. For clarity, the peptide, ODN, and alkanethiol molecules are not drawn to scale.

DNA hybridization, 10 μL aliquots of TNE solutions (10.0 mM Tris-HCl + 1.0 mM EDTA + 0.1 M NaCl) containing target ODNs were cast onto the electrodes and the hybridization reactions were allowed to proceed for 3 h in a humidified Styrofoam chamber. After the surface was thoroughly rinsed with a washing buffer, the electrodes were immersed in a TNE solution containing 1 μM detection probe for another 3 h. Upon completion of the two consecutive hybridization reactions, the electrodes were rinsed with a washing buffer and water. Fc-Mi was attached to the surface by soaking the electrodes in a 2.0 mM Fc-Mi solution for about 20 h.

2.4. Instruments

Electrochemical experiments and frequency measurements were conducted with a CHI 440 electrochemical workstation (CH Instruments, Austin, TX) in a conventional three-electrode cell. For the QCM measurements, 9.995-MHz AT-cut crystals coated with gold films on both sides (QCM active area = 0.212 cm^2 , ICM Technologies, Oklahoma City, OK) were used. The gold-coated crystals were cleaned with a piranha solution and rinsed with deionized water. To verify the completeness of labeling cysteine residues in the peptide sample, electrospray mass spectrometry (ES-MS, Apex II, Bruker) or matrix-assisted laser desorption-time of flight mass spectrometry (MALDI-TOF Biflex II, Bruker) was used to analyze the reaction products.

3. Results and Discussion

Scheme 1 illustrates the principle behind the voltammetric detection of surface-confined biomolecules containing sulfydryl groups. Peptides (Scheme 1a) and ODN capture probes with the amino tether groups (Scheme 1b) were anchored onto NHS-terminated SAMs through the nucleophilic substitution reaction between the amino groups and

the NHS ester groups at the electrode [19]. In Scheme 1a, the peptide immobilization step was followed by a Michael-type electrophilic addition reaction of the Fc-Mi molecules with cysteine groups [20], whereas in Scheme 1b, the Fc-Mi attachment was carried out after the sandwich hybridization reaction involving the target ODNs and the detection probe ODN that contains a thiol linker. The number of Fc-Mi groups attached might be dependent on the structure, molecular orientation, and surface coverage of the immobilized biomolecules. Consequently, only the Fc-Mi groups that are positioned in close vicinity of the electrode are accessible for facile electron transfer (ET) reactions.

3.1. Voltammetric Behavior of Fc-Mi Linked to Surface-Confined Peptides

Curve a in Figure 1 is a cyclic voltammogram (CV) of Fc-Mi anchored onto an electrode covered with GSH (the reduced form of glutathione). The anodic peak appears at 0.265 V (E_{pa}), with the full width at half-height of the cathodic peak of 0.173 V and the peak potential separation (ΔE_{p}) of 0.112 V. The plot of anodic peak currents (i_{pa}) against the scan rates between 0.01 and 0.5 V s^{-1} was linear with a correlation coefficient of 0.9979, suggesting that Fc-Mi was surface-confined [14]. To demonstrate specificity of the method to the surface-confined sulfhydryl groups, we conducted an experiment in which GSH was replaced by GSSG (the oxidized form of glutathione). No voltammetric peak was observed (curve b). This is understandable, because free thiol groups are absent in GSSG for the Fc-Mi attachment. We also performed a control experiment in which a C_3 -NHS modified electrode was directly exposed to a Fc-Mi solution (curve c). While a tiny peak appeared, we should note that nonspecific binding is much smaller than the signals associated with the specific tagging by the Fc-Mi molecules.

We also studied the dependence of the Fc-Mi oxidation peak current on the concentration of GSH used for its

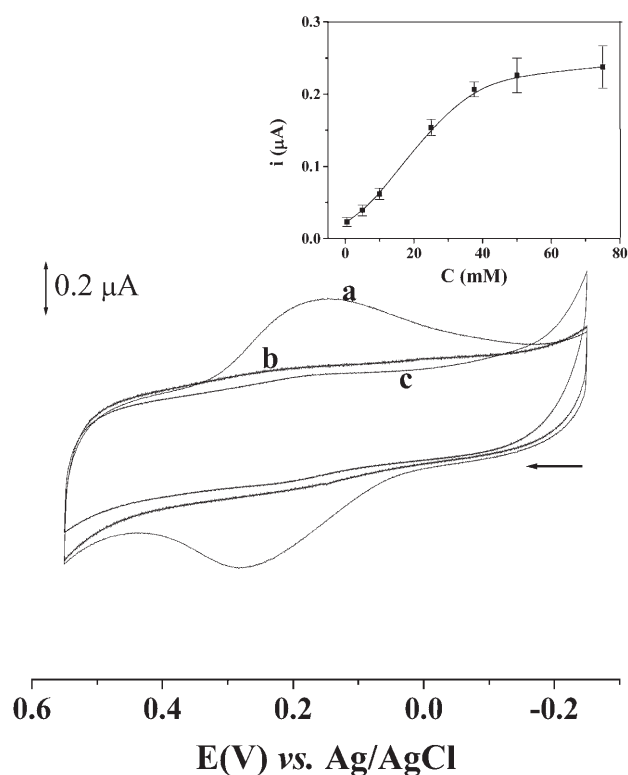


Fig. 1. CVs of a GSH-covered C_3 -NHS SAM (a), a GSSG-covered C_3 -NHS SAM (b) and a C_3 -NHS SAM (c) after exposure to a 4.0 mM Fc-Mi ethanol solution. All three curves were acquired upon transferring the various electrodes into a 0.1 M $KClO_4$ solution. The arrow indicates the scan direction and the scan rate was 0.1 V s^{-1} . The inset shows the dependence of anodic peak of Fc-Mi (attached to GSH anchored onto the C_3 -NHS SAMs) on GSH concentrations used to form the GSH-covered C_3 -NHS SAMs. The GSH concentrations studied are 0.5, 5.0, 10.0, 25.0, 37.5, 50.0, and 75.0 mM, respectively.

immobilization. As shown in the inset of Figure 1, i_{pa} increases with the GSH concentration between 0.5 and 40.0 mM. Beyond 40.0 mM, the curve begins to level off, suggesting a monolayer of GSH has been formed and for a given labeling efficiency, the maximum number of cysteine sites that can be tagged has been reached. In fact, a separate QCM measurement (Table 1) indicates that the surface coverage of a GSH film (Γ) formed in a 25.0 mM GSH solution is close to that of a full monolayer [21]. The amount of GSH adsorbate (Δm) can be deduced with the Sauerbrey equation, $\Delta m = -(f_0^2 \Delta f)/(N \rho_q)$, where Δf is the difference

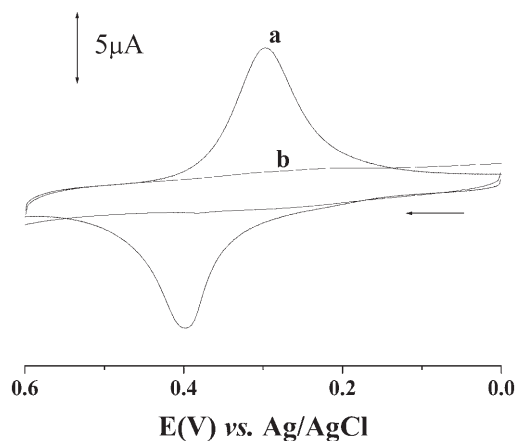


Fig. 2. CVs of FT-covered (curve a) and A5-covered (curve b) C_3 -NHS SAMs after immersing the electrodes in an ethanol solution containing 4.0 mM Fc-Mi and subsequently transferring into a 0.1 M $KClO_4$ solution for voltammetric characterization. The scan rate was 0.1 V s^{-1} and the arrow indicates the scan direction.

in resonance frequencies between the quartz crystals with and without GSH, f_0 is the resonance frequency of fundamental mode of the crystal, N ($= 1670 \text{ kHz mm}$) is the shear modulus, and ρ_q ($= 2.648 \text{ g cm}^{-3}$) is the density of quartz. It is also possible that the steric hindrance inherent in a denser surface coverage might have impeded the reaction of Fc-Mi with the sulfhydryl groups. This trend is similar to what we observed previously with the Fc-capped nanoparticle/streptavidin conjugates [17]. The method is sensitive, as the submonolayer surface coverage of GSH adsorbate film measured from QCM ($216 \pm 9 \text{ pmol/cm}^2$ for 0.5 mM GSH used for adsorption, the lowest point in the inset of Figure 1), can be easily determined.

We then applied this method to the study of a larger oligopeptide. FT, a hexapeptide with a sequence of Lys-Cys-Thr-Cys-Cys-Ala, is the thionein fragment in the α -domain of metallothionein (MT) [22]. The successful immobilization and tagging of FT are evidenced by the well-resolved peaks (Fig. 2a). In the case of the pentapeptide A5 (Ala-Ala-Ala-Ala-Ala), which contains no sulfhydryl groups, no Fc redox wave was observed (Fig. 2b). Note that the average peak current in Figure 2a ($0.612 \mu\text{A}$) is much greater than that associated with GSH ($0.062 \mu\text{A}$ in Figure 1a). As shown in Table 1, the Γ value of FT is about 2.4 times as great as the

Table 1. Voltammetric parameters and surface coverage values of the two types of peptides studied and the corresponding labeling and detecting efficiencies.

| | E_{pa} (V) | ΔE_p (V) | i_{pa} (μA) | $\Gamma_{(\text{Fc})}$ (pmol/cm^2) [a] | $\Gamma_{(\text{Biomolecules})}$ (pmol/cm^2) [b] | Labeling efficiency | Detecting efficiency (%) [c] |
|-----|--------------|------------------|----------------------------|---|---|---------------------|------------------------------|
| GSH | 0.265 | 0.112 | 0.062 | 46 ± 1 | 632 ± 4 | 7.2 ± 0.2 | 86.8 |
| FT | 0.396 | 0.101 | 0.612 | 109 ± 1 | 549 ± 2 | 6.6 ± 0.1 | 66.2 |

[a] Measured by integration of the Fc-Mi anodic peak;

[b] Measured using QCM;

[c] Determined by completely labeling the cysteine groups followed by immobilization;

Γ value of GSH. Such a value is in reasonable agreement with the ratio of the cysteine contents between FT and GSH (3:1; Recall that, in the aforementioned FT sequence, there are three cysteine residues).

The efficiency of labeling the number of sulfhydryl groups per surface-confined peptide molecule was estimated by comparing the voltammetric results to QCM measurements. By integrating the Fc-Mi anodic peak, the total number of detectable sulfhydryl tags can be determined. The ratio of this value over the total number of peptide molecules measured by QCM yielded the labeling efficiency (Table 1). It is interesting to note from Table 1 that the percentage of the sulfhydryl groups tagged by Fc-Mi molecules does not appear to be significantly different between the two small peptides studied. While the greater cysteine content of FT should favor the attachment of a higher number of Fc-Mi molecules, its larger size may have imposed more steric hindrance around the cysteine residues for their reaction with Fc-Mi. Furthermore, even if most of the cysteine residues were tagged, the rates at which Fc-Mi molecules undergo ET reactions with the underlying electrode would depend on the distance between Fc-Mi and the electrode. To probe this possibility, excess Fc-Mi was added into a GSH solution (molar ratio of 15:1) or a FT solution (molar ratio of 25:1). Based on the absence of molecular ions corresponding to GSH and FT and the predominance of the adduct peaks of GSH/Fc-Mi or FT/Fc-Mi in the MS spectra, we ascertained that all of the cysteine residues in both peptides can indeed be tagged in homogeneous solutions. The as-prepared Fc-Mi-tagged peptides were then immobilized onto the NHS-terminated SAMs. The peptide Γ values estimated from the voltammetric responses (62.3 ± 4.7 pmol/cm² for GSH and 34.4 ± 2.1 pmol/cm² for FT) were both found to be smaller than those determined from QCM measurements (71.8 ± 5.9 pmol/cm² for GSH and 52.0 ± 4.4 pmol/cm² for FT). These discrepancies substantiated our initial speculation that not all of the Fc-Mi molecules bound to these peptides have participated in the interfacial ET reactions. Note that, in the case of GSH, the extent of discrepancy is less. This is conceivable, since the Fc-Mi tags present in the smaller GSH molecule should be positioned closer to the electrode. Interestingly, the detecting efficiencies in this case are 86.8% for GSH and 66.2% for FT, both of which are much greater than the labeling efficiencies given in Table 1. It appears that allowing the peptide sample to react with the Fc-Mi tag results in a greater number of Fc-Mi molecules attached from solution to the peptidic species pre-immobilized onto surface. Therefore, the labeling/detecting efficiency of a sulfhydryl-containing biomolecule is dependent on the structure of the biomolecules of interest, the steric hindrance (how deep the sulfhydryl groups are buried, the presence of a substrate surface, and the crowdedness of the adjacent molecules), and the size of the redox tag. As far as the size of the redox tag is concerned, we should note that all the efficiencies measured herein are much greater than those in our previous method using bulky Fc-capped nanoparticle/streptavidin conjugates [18].

3.2. DNA Analysis via Voltammetric Detection of the Fc-Mi Tag Molecules

Having explored the viability of the method for voltammetric detection of surface-confined peptides, we extended our approach to gene analysis in the manner illustrated in scheme 1b. Curve a in Figure 3 depicts the CV response at an electrode modified with a 27-mer capture probe after consecutive hybridization reactions with a 39-mer complement (curve a) and an ODN detection probe, and the final Fc-Mi attachment. ΔE_p of curve a is 84 mV, and the anodic peak currents (i_{pa}) were also found to be proportional to the scan rates within the range examined (0.01 – 0.2 V s⁻¹). These characteristics again suggest that the Fc moieties are surface-bound and can undergo facile electron-transfer reactions with the underlying Au electrodes [14]. For comparison, the voltammogram is overlaid with signals observed at electrodes modified with the same ODN capture probe upon hybridization reactions with the single-base (curve b) and four-base (curve c) 39-mer mutants. The anodic peak intensities in curves b and c decreased by approximately 49% and 88% with respect to the i_{pa} value of curve a, suggesting high level of sequence specificity. In a separate experiment, we examined the relationship between the i_{pa} values and the quantities of ODN samples (Fig. 4). As can be seen, the signal intensities increase with the sample quantity over a range of 10 fmol to 10 pmol and begin to level off at a higher sample quantity (e.g., 10 pmol). The plateau can be explained on the basis of the complete utilization of most, if not all, of the capture probes immobilized onto the electrode surface.

The electrochemical DNA analysis via detection of the Fc-Mi tag is simple, selective, and reproducible. Moreover,

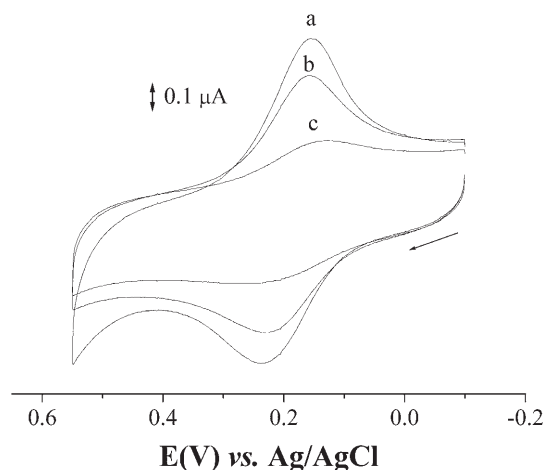


Fig. 3. CVs acquired at electrodes covered with a 27-mer capture probe after room-temperature hybridization in a TNE buffer containing (a) 1.0 μ M complementary 39-mer target, (b) 1.0 μ M 39-mer mutant containing one base mismatching the sequence of the capture probe, and (c) 1.0 μ M 39-mer target with a four-base mismatch. Hybridization with these targets was followed by another hybridization with an ODN detection probe and the Fc-Mi attachment. The scan rate was 0.05 V s⁻¹, and 0.1 M KClO₄ was used as the electrolyte solution.

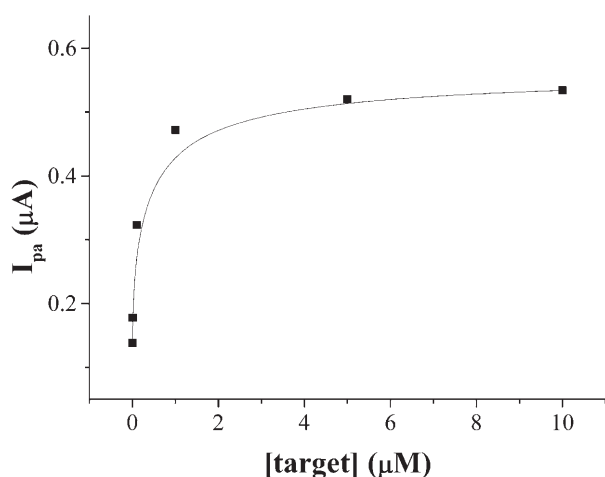


Fig. 4. A plot of i_{pa} values of Fc-Mi resulted from a sandwich DNA assay against the quantity of the ODN target in the sample solution.

the ODN target (sample) does not need to be labeled, simplifying the sample pretreatment. Note that the lowest point shown in Figure 4 corresponds to 10 fmol of ODN target in the sample solution. Such a low detection level is remarkable considering that no signal amplification scheme is involved in the current method. Our approach also compares well with certain simple and label-free methods, such as flow injection QCM [23–25], surface plasmon resonance [26], and conventional molecular biology techniques [27].

4. Conclusions

We demonstrated in this work that *N*-(2-ethyl-ferrocene)-maleimide can be used to effectively tag surface-confined, sulfhydryl-containing peptides and oligodeoxynucleotides (ODNs) adsorbed onto the electrode surface. The reversibility of the voltammetric peaks given rise by the Fc-Mi-tagged peptides/ODNs is comparable to that of their counterparts pretagged with Fc-Mi in the solution phase, but the procedure does not require chromatographic separation of the Fc-Mi-tagged biomolecules from the unreacted Fc-Mi [15, 16]. The signal intensities, however, are higher for the latter scenario, indicating that the efficiency in labeling the biomolecules in the solution phase is greater. By comparing the quartz crystal microbalance, voltammetric, and mass spectrometric studies, we found that not all of the Fc-Mi linked to the sulfhydryl groups can participate in the ET reactions and, interestingly, the Fc-Mi labeling/detecting efficiency is not strongly dependent on the cysteine contents of the immobilized biomolecules. Rather, the efficiency is governed by the structure of the biomolecule, the surface parameter, and the size of the redox tag. The small size of the Fc-Mi molecule resulted in a greater cysteine-labeling efficiency than nanoparticle covered with electroactive molecules. The analytical performance of electrochemical gene analysis via detection of the

Fc-Mi tag was evaluated through the analyses of a complementary ODN target and ODN targets with varying numbers of mismatching bases. The method described herein is straightforward and can provide insight about relationship between the labeling efficiency and the observed analytical response, aiding effective constructions of electrochemically-based biosensors.

5. Acknowledgements

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