The long-range effect induced by untying hydrogen bonds for single cell test using SECM

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ABSTRACT:

The enhanced positive feedback current of scanning electrochemical microscopy (SECM) is observed at a large separation between the tip and the substrate surface in the case of a 3-mercaptopropionic acid (3-MPA) self-assembled monolayer (SAM). The effect that induces the large separation (named the long-range effect) is confirmed by SECM and quantitatively studied by electrochemical quartz crystal microbalance (EQCM). A key finding is that the long-range effect results from untying hydrogen bonds beforehand formed between the hydroxyl group of tip redox species and the carboxyl group of 3-MPA SAM. It is found that the long-range effect is increased with the packing density of the 3-MPA SAM. On the basis of these findings,

the long-range effect is applied to the nondestructive test of living cells using SECM.

1. Introduction

Scanning electrochemical microscopy (SECM) has evolved as a successful test technique for characterization and electrochemical kinetic study of surfaces [1, 2]. Enhancing the feedback current of SECM, especially the positive feedback current, is one of the main directions of research over the past decade [3, 4]. The in-situ surface interrogation mode of scanning electrochemical microscopy (SI-SECM) operates a transient positive feedback current for the characterization of adsorbed species on the substrate electrodes, providing opportunities for fundamental breakthroughs in electrochemistry [5, 6]. This mode of SECM requires a current impulse to activate the adsorbed species on the substrate, and the tip-substrate separation (d) is less than one radius of the tip (a), $d/a < 1$ [7]. This approach is however not ideal for surface investigations. Hence a novel approach that allows a larger tip-substrate separation and provides a sustainable positive feedback current, would have a wider significance on SECM. In these respects, it is necessary to create a new way to extend the diffusion distance of redox species and increase the concentration of redox species in front of the tip.

The self-assembled monolayer (SAM) bonded on all kinds of material surfaces, has become increasingly popular and better known over the past twenty years [8-11]. The bio-applications of SAM have been recognized and studied extensively [12-15]. This suggests enormous potential of the functional SAMs for clinical applications. The physical adsorption, covalent and covalent coordinate bond methods have been

developed for molecules immobilization onto SAM [16, 17], such as covalent immobilization of proteins [18]. Among the intermolecular interactions developed for molecules immobilization on SAM, those based on weak interactions still need to explore [19].

Hence a new way is desirable to take advantage of the long-range interactions (e.g. hydrogen bonding) between the tip redox species and terminal groups of SAM on the substrate electrode. In consideration of the long-range interactions, ferrocenemethanol (described as FcCH_2OH) is selected as the tip redox species. This redox species benefits the formation of hydrogen bonding and electrostatic attraction because of the obvious distinction of electric charge between the reduced form (FcCH_2OH) and the oxidized form ($\text{Fc}^+\text{CH}_2\text{OH}$). Besides, the hydroxyl group in FcCH_2OH molecule provides the foundation for formation of hydrogen bonds [20]. It is common to graft molecules via intermolecular interactions with the aid of modified layers [21, 22]. The 3-mercaptopropionic acid (described as 3-MPA) is one of the popular functional molecules to graft protein molecules, and possesses a carboxyl group in the terminal end [23].

If the hydrogen bonding interaction between FcCH_2OH and 3-MPA is able to improve the concentration of redox species on the substrate surface and then release the redox species into the hemispherical diffusion profile of the tip by untying hydrogen bonds, the positive feedback current will be enhanced. That is to say, the tip could be positioned further to obtain a feedback current in the same intensity. This is beneficial for nondestructive test of living cells. Therefore, it

becomes a useful strategy that initially adsorbs the tip redox species by hydrogen bonds and then releases them in the SECM scanning process by disturbing the hydrogen bonds.

In the neutral medium, like pH 7.0, the ionization of the weak acid 3-MPA is feeble. The oxidation product of tip redox species, $\text{Fc}^+\text{CH}_2\text{OH}$, facilitates the ionization of the carboxyl group by attracting the negative charged carboxylate ions. This leads to untying of hydrogen bonds between 3-MPA and FcCH_2OH . Consequently, the released FcCH_2OH molecules will diffuse to the tip and enhance the feedback current of the tip. For these reasons, a new mode of SECM based on SAM modified substrate electrode is applied to our work. In the SECM experiment (Scheme 1), the substrate gold electrode is modified with SAM of 3-MPA which possesses a carboxyl group in the terminal end. FcCH_2OH that equips with a hydroxyl group acts as the tip redox species. Before the test of the approach curve, a certain amount of tip redox species FcCH_2OH are bonded on the 3-MPA SAM by hydrogen bonds. Interestingly, we find that the positive feedback current is enhanced, resulting from untying hydrogen bonds beforehand formed between the tip redox species and the 3-MPA SAM. The ionization of the carboxyl group is driven by the electrostatic attraction between $\text{Fc}^+\text{CH}_2\text{OH}$ and the carboxylate ions. As a result, the released redox species diffuse into the range of the hemispherical profile in front of the tip. This brings to an interesting phenomenon that the tip feedback current of approach curve continuously increases in a very large tip-substrate separation. The effect that causes the feedback current increasing from 10 to 60 times radius of the micrometer tip is

defined as the long-range effect. To the best of our knowledge, this is the first work to enhance the feedback current of SECM by using a SAM. Furthermore, the resulting long-range effect is applied to the single cell test using SECM which is beneficial for cell tests without affecting the natural metabolic pathway and cell morphology.

2. Experimental

2.1 Chemicals and materials

3-mercaptopropanoic acid (3-MPA), ferrocenemethanol (FcCH_2OH), potassium ferricyanide, potassium ferrocyanide and potassium chloride were purchased from J&K Scientific Co., Ltd., (Beijing, China). All chemicals were used with no further purification. Fetal bovine serum, Dulbecco's modified eagle's medium (DMEM) were purchased from Gibco Life Technologies (USA). Water ($18.2 \text{ M}\Omega \text{ cm}^{-1}$) used in the experiments was generated by a Milli-Q filter (Research UV, Hetai Instrument Co. Ltd., Shanghai, China). The gold disk electrode, Ag/AgCl reference electrode and the platinum SECM tip with a radius of $5 \mu\text{m}$ (Part number: CHI116) were purchased from CH instruments, Inc.

2.2 Preparation of the substrate electrode with the 3-MPA SAM

The polycrystalline gold disk electrodes were polished with alumina slurry to achieve a mirror-like surface and then cleaned by sonication. They were then washed with water and etched in $0.05\text{M H}_2\text{SO}_4$ solution by cycling the potential from 0 to 1.5 V (vs. Ag/AgCl, sat. KCl) at a scanning rate of 50 mV s^{-1} and dried with pure nitrogen gas. 3-MPA SAM was prepared on clean surface of polycrystalline gold disk electrode. The procedure involved the immersion of the gold electrode into a 0.2 M solution of

3-MPA in ethanol at room temperature for 2 hours, followed by ultrasonic cleaning in water.

2.3 Electrochemical measurements

Electrochemical measurements were carried out with CHI920C scanning electrochemical microscopy. For cyclic voltammetry, a gold disk electrode was employed as the working electrode, a platinum wire as the counter electrode and an Ag/AgCl electrode with saturated KCl solution as the reference electrode. For the SECM approach curve test, a micrometer Pt disk electrode of 10 μm diameter was employed as the SECM tip and a 3-MPA SAM gold electrode as the substrate electrode. Electrochemical Impedance Spectroscopy measurements were performed in an electrochemical cell containing 5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ and 5 mM $\text{K}_4[\text{Fe}(\text{CN})_6]$ in the frequency ranging from 100 kHz to 1 Hz at 0.25 V.

2.4 EQCM analysis

The electrochemical quartz crystal microbalance (EQCM) measurement of the self-assembling process of 3-MPA on gold substrate was carried out in QCM 922 (Princeton Applied Research) controlled by PARSTAT 4000 electrochemical work station. Prior to the experiment, the surface of QCM Au electrode was cleaned according to the manufacturer's protocol. After establishing a baseline for 40 minutes, 3-MPA (0.2 M solution in ethanol) was introduced into the QCM chamber. All the measurements were performed at room temperature. The increase in the mass of SAM was measured by Sauerbrey's equation [24]:

$$\Delta m = \frac{-A\sqrt{\rho\mu}}{2F^2} \times \Delta F$$

Where A is the surface area of the gold electrode ($A=0.2 \text{ cm}^2$), ρ is the density of quartz, μ is the shear modulus of quartz, F is the inherent frequency (9 MHz) of the quartz crystal and ΔF is the frequency decrease of the quartz crystal.

2.5 Cell culture and single cell test using SECM

Cells were cultured in Dulbecco's modified-eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (5% CO_2 , 37 °C). After 48 hours, cells were trypsinized and collected. Prior to the SECM test, the cells were cultured in DMEM⁻ (lacking serum) containing 0.5 mM FcCH_2OH for one hour as previously reported [25]. Biased at 0.4 V in 0.5 mM FcCH_2OH , the micrometer tip was positioned $\sim 30 \mu\text{m}$ over the substrate electrode. The reported result for one-line cell scanning in this experiment was the mean value of measurements for ten individual cells, and the error bars represent the standard deviation ($n=10$).

3. Results and discussion

Cyclic voltammetry (CV) and electrochemical impedance spectra (EIS) were carried out to prove the formation of SAM and describe the surface coverage of the SAM [26, 27]. Figure 1 shows the CV curves (a) and EIS (b) of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ at the bare Au substrate electrode, 3-MPA SAM modified substrate electrode, respectively. The relevant electrochemical data are listed in Table 1. As can be seen, the redox species $[\text{Fe}(\text{CN})_6]^{3-/4-}$ at 3-MPA SAM substrate electrode presents one couple of reversible redox peaks which is similar with the redox species $[\text{Fe}(\text{CN})_6]^{3-/4-}$ at the bare Au substrate electrode. For 3-MPA SAM substrate electrode, both current of anodic peak

(I_{pa}) and cathodic peak (I_{pc}) have a little decrease and the separation potential between anodic peak and cathodic peak (ΔE_p) becomes bigger than the bare Au substrate electrode. In the experiment of EIS, the Randles equivalent circuit [28] was used to fit EIS data and determine the charge transfer resistance (R_{ct}). The EIS curves show that the R_{ct} value of 3-MPA SAM electrode (282.1 ohm) is slightly higher than the bare electrode (254.6 ohm). The results indicate the formation of the 3-MPA SAM. Further, the surface coverage (θ) of 3-MPA SAM was determined by comparing the area of the reduction peak of surface gold oxide [29, 30] in 0.1 M H_2SO_4 before and after modification which was calculated according to the equation (1).

$$\theta = \frac{Q^0 - Q}{Q^0} \times 100\% \quad (1)$$

Where Q and Q^0 are the amount of electric charge exchanged during the electroadsorption of oxygen ($C\ cm^{-2}$) of Au electrode with and without 3-MPA SAM respectively. The θ of the 3-MPA SAM modified Au is calculated as 21.3% which indicates a low surface coverage of the SAM (Fig. 1c). This is reasonable because 3-MPA is short thiol molecule. More important, the lower surface coverage of the 3-MPA SAM makes the electrode suitable to serve as a substrate for SECM, because the surface defects of the 3-MPA SAM modified electrode allow electron transfer between the tip redox species and the substrate gold surface.

The micrometer tip electrode of 5 μm radius was characterized by steady-state voltammetry. Figure 2(a) shows a normal steady-state voltammogram. The S shaped curve starts with a current close to zero and increases until it reaches a steady state that resulting from the limited diffusion of $FcCH_2OH$ to the tip. It proves that the tip

electrode is satisfactory to SECM experiments. In order to validate the concentration increment of FcCH_2OH molecules in front of the tip, as shown in Scheme 1, we designed a set of experiments considering whether there's hydrogen-bonding or not. The SECM approach curves approaching the bare gold electrode and 3-MPA SAM modified electrodes in 0.2 M KCl containing 0.5 mM FcCH_2OH were performed respectively. As a control experiment, the conventional positive feedback approach curve (black line) and negative feedback approach curve (red line) of SECM were observed for the tips approaching a bare gold and an insulator surface respectively, seen in Figure 2(b). The positive feedback current starts with a steady current and sharply increases until the tip-substrate separation reaches 5 times radius of the tip. However, a positive feedback curve with a flat slope was observed for the 3-MPA SAM modified substrate, the tip-substrate separation was close to 60 times radius of the tip (blue line in Figure 2(b)) on the initial point of the flat slope. This experiment phenomenon is very different with the SECM approach curves obtained by the bare gold substrate and the alkyl (as terminal group) SAM modified substrates [31]. The positive feedback approach curve with a flat slope suggests that the additional tip redox mediator (FcCH_2OH) has returned to the tip from outside the range of the hemispherical diffusion profile in front of the tip. The effect that causes the feedback current increasing from 10 to 60 times radius of the tip is defined as long-range effect. The result hints that the long-range effect is induced by untying hydrogen bonds beforehand formed between FcCH_2OH and 3-MPA SAM. The experimental approach curve for the tip approaching an insulator surface is well fitted to the Cornut theory

[32] (data not shown). However, the experimental approach curves for the tip approaching a 3-MPA SAM modified gold surface are difficult to fit because of the presence of the hydrogen bonding between 3-MPA SAM and tip redox species. So we take the positive feedback curve on gold as the "theoretical fitting" for positive feedback curve on 3-MPA SAM modified gold surface. The increment of feedback current for 3-MPA SAM modified gold surface is attributed to the long-range effect. In this way, the increment of feedback current induced by long-range effect can be quantified by the "theoretical fitting". Further, the approach curve towards 3-MPA SAM modified electrodes in 0.2 M KCl containing 2 mM $K_4(FeCN)_6$ (as tip redox species) were performed (green line). The positive feedback current starts with a steady current until the tip-substrate separation is less than 5 times radius of the tip. This can be understood in consideration of tip redox species $K_4Fe(CN)_6$ which cannot form hydrogen bonds with 3-MPA. Therefore, there is no redox species $K_4Fe(CN)_6$ bonded on the 3-MPA SAM. As a result, the long-range effect was not observed in 0.2 M KCl containing 2 mM $K_4Fe(CN)_6$.

The hydrogen bond, it is well known, could be hindered by the strong base or strong acid. So it is important to verify the proposed long-range effect under the strong basic or strong acidic condition. The SECM approach curves approaching 3-MPA SAM modified substrate under strong base or strong acid in 0.2 M KCl containing 0.5 mM $FcCH_2OH$ were measured respectively. Shown in Figure 3(a), under strong basic condition (pH 10.0), the positive feedback current starts with a steady current and increases until the tip-substrate separation reaches ~ 5 times of

the tip radius. It suggests that the hydrogen bonds are disrupted and the ionization of the carboxyl group which is disadvantageous for hydrogen bonds formation, are also promoted. Consequently the SECM approach curve has returned to conventional shape and the long-range effect has disappeared. Under strong acidic condition (pH 2.0), the positive feedback current starts with a steady current and increases until the tip-substrate separation reaches ~ 60 times of the tip radius, and the current increment is less than that under nearly neutral condition (pH 6.4). In this case, hydrogen bonds are also disrupted. However, the ionization of the carboxyl group is blocked by the hydrogen ions, which provides additional neutral 3-MPA molecules for hydrogen bonds formation. As a result, the long-range effect still exists but performs weaker than that under pH 6.4. These results further illustrate the importance of hydrogen bonds formation for the long-range effect.

Apparently, the feedback current is related to the diffusion behavior of tip redox species (FcCH_2OH). The SECM approach curves recorded at different approach rates are shown in Figure 3(b), the approach curves obtained at the approach rates in the range of $0.5 - 50 \mu\text{m s}^{-1}$ are almost overlapped, and obviously show the long-range effect. This indicates that the diffusion behavior of tip redox species would not be affected by the motion of the tip electrode. So the approach rate of approach curve experiments in this work is set at $10 \mu\text{m s}^{-1}$ which is commonly used in SECM approaching test for a $10 \mu\text{m}$ diameter tip [33].

Since the results show the diffusion behavior of tip redox species would not be affected by the motion of the tip electrode, the concentration gradients of FcCH_2OH

molecules should be linearly related to tip-substrate separation according to the Fick's law of diffusion. The closer to the substrate of the tip electrode is, the higher concentration of FcCH_2OH molecules becomes. Precisely because of the concentration gradients, the positive feedback current of the approach curves have continuously increased. In order to prove the process of diffusion, the steady-state voltammograms under different tip-substrate separation were performed. The steady-state voltammograms of 0.5 mM FcCH_2OH at different tip-substrate separation from the 3-MPA SAM modified substrate in 0.2 M KCl are shown in Figure 4 (a), the oxidization limiting diffusion current (I_∞) is gradually increased with the lessening of the tip-substrate separation. The limiting diffusion current is almost linearly growing in the range from 60 to 10 times radius of the tip, seen in Figure 4(b). The increment amount of released FcCH_2OH molecule is growing with the decrease of the tip-substrate separation distance. This indicates the diffusion of FcCH_2OH is related to the distance and ensures the appearance of the long-range effect. The steady-state voltammograms of 0.5 mM FcCH_2OH at different distances from the bare gold substrate in 0.2 M KCl are shown in Figure 4(c). I_∞ is essentially a constant in the range from 60 to 10 times radius of the tip (Fig. 4d). Also, the steady-state voltammograms of 2 mM $\text{K}_4(\text{FeCN})_6$ at different distances from the 3-MPA SAM modified substrate in 0.2 M KCl are shown in Figure 4(e). I_∞ remains a certain value until the tip-substrate separation is less than 5 times radius of the tip (Fig. 4f). The results shed light on the explanation for the long-range effect (Scheme 2). It was hard to take the FcCH_2OH molecules off from the 3-MPA SAM as if they were "glued" to

the surface by hydrogen bonds. However, the $\text{Fc}^+\text{CH}_2\text{OH}$ ions generated in front of the tip, would diffuse to the substrate surface thus attracting the carboxylate anions by electrostatic interaction. This leads to the ionization of the carboxyl group, so as to the disruption of the hydrogen bonds between FcCH_2OH molecules and the 3-MPA SAM. As a result, the additional molecules of FcCH_2OH would return to the tip which finally causes an enhanced feedback.

We next consider the influence of packing density of the SAM on the long-range effect. The 3-MPA SAMs with different packing densities were formed by controlling the assembly time. And the assembly process was quantitatively investigated by electrochemical quartz crystal microbalance (EQCM). Figure 5(a) shows an exponential decrease of frequency in the first 10 minutes and a nearly linearly decrease of frequency after that. This indicates the formation of 3-MPA SAM on the gold surface, which results in a rapid assembly process in the first 10 minutes and a slow assembly process in the following. The assembly reaction has reached the equilibrium point in 60 minutes. At this equilibrium point, the mass gain of 3-MPA SAM is calculated as ~ 15 ng based on the changes in frequency. The packing density of 3-MPA SAM (assembling for 60 minutes) on the gold surface calculated from the increase in the mass is $\sim 4 \times 10^{14}$ molecules cm^{-2} , almost equal to the packing density of alkanethiols $\text{CH}_3(\text{CH}_2)_5\text{SH}$ SAM on gold surface (4.6×10^{14} molecules cm^{-2}) [34]. The ionization of the weak acid 3-MPA is feeble in the neutral medium, so the density of the undissociated 3-MPA molecules is approximately equal to the packing density of 3-MPA SAM on the gold surface. And the density of the undissociated 3-MPA

molecules is equal to the density of hydrogen bonds beforehand formed in the 3-MPA SAM. While the density of untied hydrogen bonds observed in the approach curve calculated by electron charge is 3.4×10^{11} molecules cm^{-2} (integral area above I_{∞} in Fig. 2(b)). It can be seen that the density of hydrogen bonds beforehand formed in the 3-MPA SAM is obviously higher than the density of untied hydrogen bonds in the enhanced positive feedback, which verifies the mechanism we proposed. Further, the approach curves approaching substrate electrodes with different packing densities of 3-MPA SAM are presented in Figure 5(b). The tip-substrate separation, when the current starts to increase, is increasing with self-assembly time of 3-MPA SAM. This demonstrates the positive correlation between the separation distance and the packing density of the 3-MPA SAM. Now it is clear that why the 3-MPA SAM affects the approach curve. The redox species are beforehand bonded on the SAM by hydrogen bonds. However, the hydrogen bonds will be untied by the ionization of the carboxyl group because the $\text{Fc}^+\text{CH}_2\text{OH}$ ions generated by the tip attract the negative charged carboxylate ions, leading to the escape of FcCH_2OH from 3-MPA SAM. And the diffusion of escaped FcCH_2OH is found to follow the Fick's law of diffusion. As a result, the amount of the released molecules are linear increasing when the tip approaches to the 3-MPA SAM modified substrate, which triggers the long-range effect.

In order to achieve the nondestructive test of living cells using SECM, one-line scanning model of SECM test for HeLa cell on 3-MPA SAM modified substrate was carried out as an example. It has been reported that the redox species function on

the HeLa cell leads to a positive feedback [25]. In short, the redox species FcCH_2OH facilitate the intracellular generation of glutathione. The additional glutathione reacts with $\text{Fc}^+\text{CH}_2\text{OH}$ to generate FcCH_2OH on the cell membrane, leading to a positive feedback. Figure 6 shows the one-line scanning model over the cell in 0.5 mM FcCH_2OH . The tip was set at $\sim 30\text{ }\mu\text{m}$ (6 times radius of the tip) above the substrate electrode. The current responses (ΔI_a) for single HeLa cell on the 3-MPA SAM modified substrate were $\sim 68.6\text{ pA}$ while the responses (ΔI_b) for the HeLa cell on the bare substrate were $\sim 17.4\text{ pA}$ (inset of Fig. 6c). The current signal for single cell test was obviously enhanced, and the background current on the 3-MPA SAM was also higher than that on the bare substrate. Though the increasing of the background current decreases the resolution, the resolution of single cell with the 3-MPA SAM has been increased. This suggests that the increase of resolution is over compensating the decrease of resolution induced by background current. The current enhancement may be attributed to the additional FcCH_2OH , which is induced by the long-range effect. This observation uncovers the previously unknown property of FcCH_2OH outside of cells, and demonstrates that the test for the cells with 3-MPA SAM modified substrate was more sensitive than that with bare gold substrate. In addition, the current curve (in black) shows two valleys ($x=26\text{ }\mu\text{m}$ and $x=46\text{ }\mu\text{m}$) which is consistent with the results reported by Janine Mauzeroll et al [35]. This is a result from the small perturbations of diffusion triggered by the edge of the cell membrane. It can be seen that the long-range effect induced by 3-MPA SAM provides a reliable approach which allows long range nondestructive detection of single cell.

Without tip disturbances within the surroundings, the natural behavior of the cells could be precisely sensed by SECM, making the SECM technique practical for clinical use in the future. In addition, it has been confirmed that the cell lines derived from cancers have distinctive morphological property [36], indicating that there are potentials for the development of SECM-based sensors for differentiating cancer cells with normal cells.

4. Conclusions

To summarize, we used SECM to quantitatively demonstrate the long-range effect induced by untying hydrogen bonds on the 3-MPA SAM surface. It is found that the hydrogen bonds exist between the carbonyl group of 3-MPA SAM and the hydroxyl group of ferrocenemethanol. The redox species FcCH_2OH are bonded on the SAM by hydrogen-bonding. However, the hydrogen bonds will be untied by the ionization of the carboxyl group because the $\text{Fc}^+\text{CH}_2\text{OH}$ ions generated by the tip attract the negative charged carboxylate ions, leading to desorption of FcCH_2OH . On the basis of the discoveries, an approach of SAM modification on the substrate electrode of SECM is proposed, which is applied to the nondestructive test of single living cell.

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Scheme 1

Figure 1

Figure 2

Figure 3

Figure 4

Scheme 2

Figure 5

Figure 6