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Electron Transfer Reactions of Cytochrome *c* Using the Mixed Monolayer Au Electrode Composed of Co^{III} Complex and Hydroxyl-Terminated Alkanethiols

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We prepared the mixed monolayer-modified Au electrode composed of negatively charged Co^{III} complex (**1**) and hydroxyl-terminated alkanethiols (C_n; n = 2, 6), **1/C_n–Au**, using the "step-by-step" immobilization method; the *low-density* monolayer of **1** (**1–Au**) was firstly constructed with a spacing and then C_n molecules were filled in the gap of **1**. In the cyclic voltammetric measurement of horse heart cytochrome *c* (cyt *c*) using **1/C_2–Au**, only a pair of oxidation-reduction wave was observed with almost the same peak separation as the case of **1–Au**. Since the electron transfer rate by using the single monolayer of C_2 was much faster than that of **1–Au**, it clearly indicates that the modification of C_2 does not cause the phase-separation and C_2 molecules on Au do not influence to the electron transfer reaction with cyt *c*. In contrast, **1/C_6–Au** showed faster electron transfer rate than the cases of **1–Au** and **1/C_2–Au**, but exhibited slower rate than that of single monolayer of C_6. These findings indicate that cyt *c* firstly interacts with negatively charged **1**, and then electron transfer reaction proceeds *via* C_6 molecules on Au electrode.

Key words: mixed monolayer, electron transfer reaction, cytochrome *c*, modified electrode, negatively charged Co^{III} complex

1. INTRODUCTION

In electrochemical studies for metalloproteins, a number of promoters have widely been applied. The promoters are defined as the modified molecule which can promote electron transfer reaction between proteins and electrode surface, and are often represented by X–R–Y, where X is a binding group to the electrode surface, R is a bridging unit, and Y is an interacting group with protein surface [1-7].

In the case of horse heart cytochrome *c* (cyt *c*), a mitochondrial electron transfer protein, the modified Au electrodes with 4-mercaptopyridine and 2-mercapto-hexanol can promote the heterogeneous electron transfer reaction, but those with benzenethiol and alkanethiol cannot promote this reaction [2,3,6,7]. Various researchers have pointed out the importance of hydrogen bonding between electrode surface and positive residues (lysine-NH_3^+) around heme crevice of cyt *c* [1,2,4]. However, it cannot unambiguously define that a protein surface has a positively charged, negatively charged, hydrophilic, or hydrophobic character. Mixed monolayer with two components will be needed to construct the more appropriate bio-interface. However, it is difficult to prepare the homogeneously mixed monolayer because two different molecules often cause the phase-separated structure on Au surface [7,8].

We have previously reported the mixed monolayer composed of negatively charged Co^{III} complex (**1**) and hexanethiol using the "step-by-step" immobilization method; the *low-density* monolayer of **1** (**1–Au**) was firstly constructed with a spacing and then hexanethiol was filled in the space of **1** [9,10]. This electrode can promote the electron transfer reactions with cyt *c*. In the case of *densely packed* monolayer of **1**, the redox

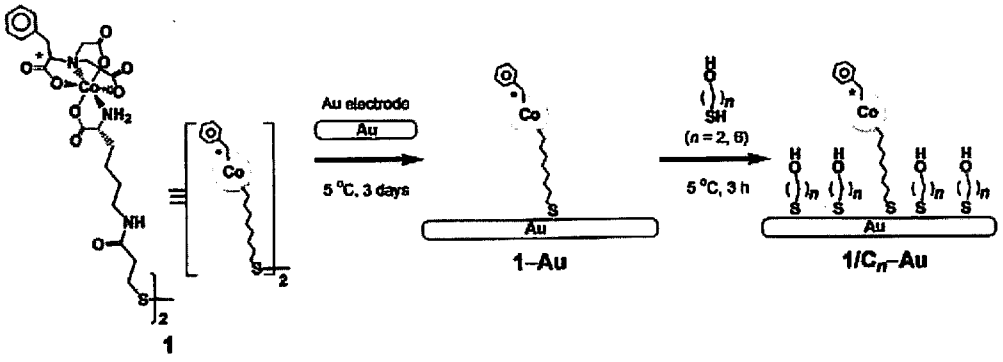

Fig. 1 Schematic view of complex **1** and modified Au electrodes with **1** (**1–Au**, **1/C_n–Au**)

wave of cyt *c* was not observed [10]. Moreover, single monolayer of hexanethiol cannot promote the electron transfer reaction with cyt *c*. Therefore, the modification of hexanethiol did not cause serious phase-separation on the modified Au surface.

Here in this manuscript, we report the preparation of the mixed monolayer composed of **1** and hydroxyl-terminated alkanethiols (C_n; n = 2, 6), **1/C_n–Au** (Fig. 1). C_n molecules can promote the electron transfer reaction with cyt *c* much faster than the case of **1** [3,5,6]. From the comparison with the electron transfer rate with cyt *c* using a series of modified Au electrodes, we discussed the surface condition of mixed monolayers and their electron transfer mechanisms with cyt *c*.

2. EXPERIMENTAL

2.1 Materials

All the chemicals and solvents were purchased from Wako Pure Chemical Industries, Tokyo Chemical Industry, Nacalai Tesque, and Peptide Institute. All reagents were used without further purification. Milli-Q water was prepared by using a Milli-Q biocel A (Millipore). Negatively charged Co^{III} complex (**1**) was synthesized from (*R*)-phenylalanine derivative according to the previously published method [9,10]. *Low-density* monolayer of 1 (**1–Au**) was prepared according to the previous methods and its surface coverage was *ca.* 4×10^{-11} mol cm^{-2} (identical calculated one: 5.2×10^{-10} mol cm^{-2}) [10].

2.2 Electrode preparation

1–Au was prepared by dipping a polycrystalline Au flag electrode (0.796 cm^2) in a 1 mM aqueous solution of **1** for 3 days at 5 °C. **1/C_2–Au** and **1/C_6–Au** were obtained by dipping **1–Au** in 2-mercaptoethanol (C_2) and 6-mercaptohexanol (C_6) solutions of MeOH (*ca.* 0.1 g/50 mL) at 5 °C for 3 h, respectively. Single monolayers of C_2 and C_6 (**C_2–Au**, **C_6–Au**) were also prepared by dipping bare Au electrode in the same C_2 and C_6 solutions at 5 °C for 3 h, respectively. These modified electrodes were rinsed with MeOH and water before electrochemical measurements.

2.3 Electrochemical measurements

Electrochemical measurements were performed by using a HZ-5000 automatic polarization system (HOKUTO DENKO). The cyclic voltammetry was recorded with each SAM as a working electrode, Pt wire as a counter electrode and Ag/AgCl (3.0 M NaCl) as a reference electrode. Ar gas was purged through the electrolyte solution for at least 15 minutes before each measurement.

The voltammetry measurements were performed in a 0.1 M phosphate buffer solution (pH 7.0, I = 0.1 M $NaClO_4$). $K_3[Fe(CN)_6]$ and $[Ru(NH_3)_6]Cl_3$ solutions (1 mM) were preprared by the same buffer solution. A 100 μM horse heart cyt *c* (purchased from Nacalai Tesque) solution was prepared by dialyzing in the same buffer solution a few times before use.

Table I. Peak separations calculated from the voltammograms of $[Fe(CN)_6]^{3-}$ and $[Ru(NH_3)_6]^{3+}$ using a series of electrodes at 50 mV s^{-1}

	$[Fe(CN)_6]^{3-}$	$[Ru(NH_3)_6]^{3+}$
	ΔE_p / mV	ΔE_p / mV
bare Au	67	62
1–Au	303	58
1/C_2–Au	174	59
1/C_6–Au	219	61

3. RESULTS AND DISCUSSION

3.1 Electron transfer behaviors of a redox marker

Cyclic voltammetry measurements of aqueous solutions of $[Fe(CN)_6]^{3-}$ and $[Ru(NH_3)_6]^{3+}$, as the qualitative redox marker against negatively/positively charged surface [11,12], were recorded using a series of modified Au electrodes and these peak separations (ΔE_p) at 50 mV s^{-1} were summarized in Table I. In the case of bare Au electrode, the ΔE_p values of voltammograms of $[Fe(CN)_6]^{3-}$ and $[Ru(NH_3)_6]^{3+}$ were near an ideal one for the case of a fully reversible system (56.5 mV [13]), respectively. The ΔE_p values of $[Ru(NH_3)_6]^{3+}$ indicated a reversible character in each case. However, voltammograms of $[Fe(CN)_6]^{3-}$ using a series of modified electrodes with **1** showed irreversible redox waves, which were explained to be due to the electrostatic repulsion between $[Fe(CN)_6]^{3-}$ and negatively charged surfaces of **1–Au** and **1/C_n–Au** [11].

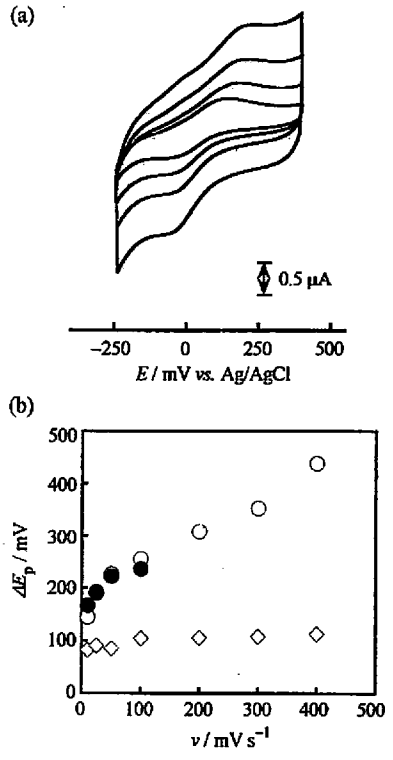

Fig. 2 (a) Cyclic voltammograms of cyt *c* using **1/C_2–Au** at the scan rates of 10, 25, 50, and 100 mV s^{-1}. (b) Relationship between scan rates and ΔE_p values of cyt *c* as measured with **1–Au** (open circle), **1/C_2–Au** (filled circle), and **C_2–Au** (open diamond).

These data clearly suggest that negatively charged **1** is associated with the surfaces of **1–Au** and **1/C_n–Au**, and is likely to influence the heterogeneous electron transfer reaction.

3.2 Electron transfer behaviors of cyt *c* using **1/C_2–Au**

Cyclic voltammograms of cyt *c* as measured with **1/C_2–Au** are shown in Fig. 2a. In the absence of cyt *c*; no redox wave was shown in this region. Only a pair of oxidation-reduction wave of cyt *c* was observed in each scan rate. The redox wave became broader with increasing scan rates, and its peak top was not detected over 100 mV s^{-1}. These behaviors are quite similar to the case of **1–Au** [9,10].

The ΔE_p values of cyt *c* were plotted against scan rates using **1/C_2–Au**, **1–Au**, and **C_2–Au** (Fig. 2b). **1/C_2–Au** gave irreversible redox wave and the peak separations were much larger than the case of **C_2–Au**. If two molecules, **1** and C_2, formed phase-separated structure, a reversible redox wave will be observed as well as the case of **C_2–Au**, because the *densely packed* monolayer of **1** does not give any redox wave of cyt *c* [10]. However, irreversible redox waves were observed in the case of **1/C_2–Au**, implying that the modification of C_2 does not cause the phase-separation. Moreover, the ΔE_p values were almost the same as those of **1–Au**. It clearly indicates that the overall electron transfer rates

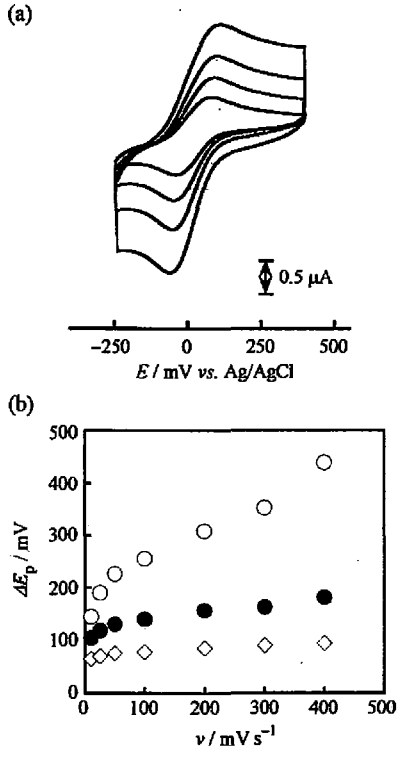

Fig. 3 (a) Cyclic voltammograms of cyt *c* using **1/C_6–Au** at the scan rates of 10, 25, 50, and 100 mV s^{-1}. (b) Relationship between scan rates and ΔE_p values of cyt *c* as measured with **1–Au** (open circle), **1/C_6–Au** (filled circle), and **C_6–Au** (open diamond).

(*k*) with cyt *c* are almost the same between **1/C_2–Au** and **1–Au** [13]. From these peak behaviors, C_2 molecules on Au surface do not influence to the electron transfer reaction with cyt *c*. To put it another way, **1** inhibits the electron transfer reaction between cyt *c* and C_2 molecule. Therefore, the electron transfer process between cyt *c* and **1/C_2–Au** is similar to the case of **1–Au**: Electron transfer reaction proceeds *via* the associated complex between cyt *c* and Co^{III} complex on **1/C_2–Au** [10].

3.3 Electron transfer behaviors of cyt *c* using **1/C_6–Au**

Cyclic voltammograms of cyt *c* as measured with **1/C_6–Au** exhibited only a pair of redox wave in each scan rate (Fig. 3a). The ΔE_p values of cyt *c* were plotted against scan rates using **1/C_6–Au**, **1–Au**, and **C_6–Au** (Fig. 3b). In the case of **1/C_6–Au**, the ΔE_p values were smaller than that of **1–Au**, but were larger than that of **C_6–Au**, suggesting that the modification of C_6 does not cause the phase separation as described above. This peak behavior is obviously different from the case of **1/C_2–Au**. The overall electron transfer rate (*k*) between cyt *c* and **1/C_6–Au** was faster than that of **1–Au**, indicating that C_6 molecules on Au surface are concerned with the electron transfer reaction of cyt *c*. However, negatively charged **1** also contributes to the reaction process with cyt *c*, because **C_6–Au** indicated much faster electron transfer reaction compared with **1/C_6–Au**. That is to say, positively charged cyt *c* firstly interacts with negatively charged **1**, and then electron transfer reaction proceeds *via* C_6 molecules on Au electrode.

3.4 Comparison between 1/C_n–Au

Proposed electron transfer mechanisms between cyt *c* and **1/C_n–Au** are summarized in Fig. 4. In both cases, positively charged cyt *c* is firstly attracted toward negatively charged **1** on Au surface. Then, electron transfer reaction with **1/C_2–Au** proceeds *via* the associated complex between cyt *c* and **1** on Au surface as well as the case of **1–Au** [9,10]. In the case of **1/C_6–Au**, C_6 molecules seem to promote the electron transfer reaction with cyt *c*, indicating that the association rate with C_6 molecules is much faster than that with **1**. Therefore, the rate determining steps are the association processes with **1** (**1/C_2–Au**) and C_6 (**1/C_6–Au**), respectively. It has been suggested that hydrogen bonding interaction between positive residues of cyt *c* and hydration layer on hydroxyl-terminated alkanethiol plays an important role in the promoting electron transfer reaction with **C_n–Au** [5]. Therefore, the different reaction behaviors using **1/C_n–Au** are dependent on the chain-length of C_n, that is, cyt *c* easily interacts (associates) with the longer alkanethiol, C_6.

4. CONCLUSION

We tried to prepare the mixed monolayer composed of negatively charged Co^{III} complex (**1**) and hydroxyl-terminated alkanethiol (C_n; *n* = 2, 6), **1/C_n–Au**, by applying the "step-by-step" immobilization method. As measured with **1/C_n–Au**, cyclic voltammograms of $[Ru(NH_3)_6]^{3+}$ exhibited reversible redox waves and those of $[Fe(CN)_6]^{3-}$ showed irreversible redox waves, re-

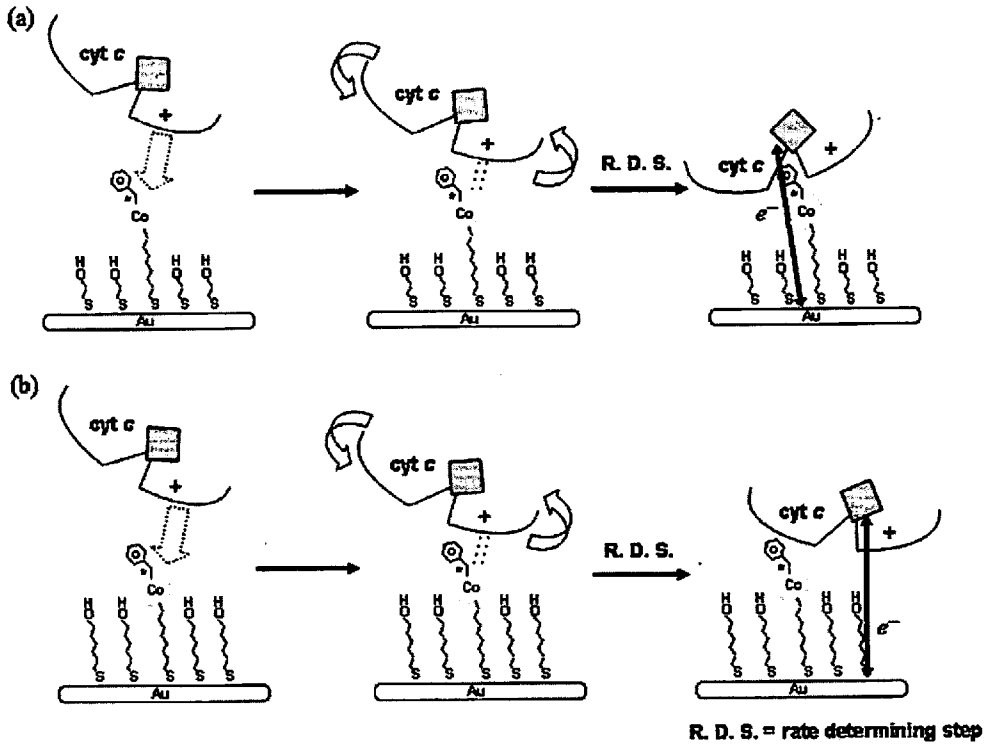

Fig. 4 Proposed mechanisms of the electron transfer reaction of cyt *c* using **1/C_2–Au** (a) and **1/C_6–Au** (b).

spectively. Voltammograms of cyt *c* using **1/C_n–Au** gave only a pair of redox wave in each scan rate. Electron transfer rate between cyt *c* and **1/C_2–Au** was almost the same as the case of **1–Au**. On the contrary, in the case of **1/C_6–Au**, electron transfer rate was faster than that of **1–Au** and was slower than **C_6–Au**. From these behaviors, positively charged cyt *c* firstly interacts with negatively charged **1** in both cases, and then electron transfer reaction proceeds *via* **1** (**1/C_2–Au**) and C_6 molecules (**1/C_6–Au**), respectively.

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