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# Adsorption of Microorganisms using the $Fe^{III}$-Artificial Siderophore-Modified Au Electrode Surface.

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The hydroxamate-typed artificial siderophore, tris[2-{3-(*N*-acetyl-*N*-hydroxamino)propylamido}-propyl]aminomethane (**TAPPA**) was prepared and its $Fe^{III}$ complex, **$Fe^{III}$-TAPPA** was modified on Au electrode surface. **$Fe^{III}$-TAPPA** indicated biological activity for *Microbacterium flavescens*, which is hydroxamate-typed siderophore auxotrophic gram-positive microorganism, suggesting that **$Fe^{III}$-TAPPA** was able to permeate the cell membrane of microorganism. The modification of $Fe^{III}$-siderophore complex was carried out by stepwise self-assembling method. The cyclic voltammetry of the resultant Au electrode, **$Fe^{III}$-TAPPA/Au** confirmed the surface modification of **$Fe^{III}$-TAPPA**. The adsorption experiments of *M. flavescens* with **$Fe^{III}$-TAPPA/Au** were clearly showed that **$Fe^{III}$-TAPPA/Au** could immobilize microorganisms. The images of the adsorption of *M. flavescens* were obtained by various microscopic methods. Quart crystal microbalance (QCM) measurements also suggested that **$Fe^{III}$-TAPPA/Au** was able to adsorb *M. flavescens*. The adsorption ability is due to the interaction between **$Fe^{III}$-TAPPA** on a Au electrode and receptor/binding protein in the cell membrane.

Key words: artificial siderophore, microorganism, quartz crystal microbalance, and self-assembled monolayer

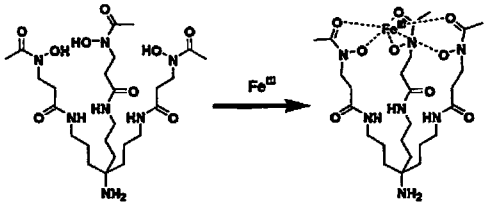
## 1. INTRODUCTION

The detection of microorganisms is one of the important problems in food and health industry [1-3]. Currently, the quick and high sensitive detection method is required. Various microorganisms-detection methods have been developed and used in the field of environment sanitation. The detection and identification methods for numerous microorganisms, bacteria, and fungi include gene analysis, interaction to specific peptides, sugar chains, and proteins on the cell surface. The interaction between siderophore and its receptor protein in the cell surface is one of those interactions.

Siderophores, which are secreted by microorganisms for iron uptake, are one of the most important bio-related materials [4]. Siderophore binds $Fe^{III}$ ion and forms very stable $Fe^{III}$-siderophore complex in aqueous media. The receptor and/or binding protein in the cell membrane recognizes this $Fe^{III}$-siderophore complex. Then the complex permeates in the cell through these proteins. The complex is reductively decomposed and obtained $Fe^{II}$ ion is used for various reactions in the cell.

Siderophores have also been applied for other purpose, such as therapeutic agents, drug conjugates as drug delivery system (DSS), and so on [5-7]. Powers et al. reported the detection probe of microorganisms by using native siderophores-immobilized substrates [8,9]. They have focused on the recognition and permeation behavior between $Fe^{III}$-siderophore complex and receptor/binding protein in the cell membrane.

In this study, we tried to apply for an artificial siderophore, which is model compound extracted the function of native one [10-12], as an adsorbing and detecting probe of microorganisms. We choose the hydroxamate-type artificial siderophore as the modified siderophore on the surface. Our group have been studied this typed artificial siderophores as functional models of native siderophores for several years [13-15]. Our artificial siderophores are able to change their structure systematically, so that we modulate their properties at our requests. To modify this artificial siderophores on the surface of substrate, new typed-siderophore involving terminal binding site, **TAPPA** and its $Fe^{III}$ complex, **$Fe^{III}$-TAPPA** was synthesized (Fig. 1).




**TAPPA** **$Fe^{III}$-TAPPA**

Fig. 1. Schematic views of $-NH_2$ group terminated artificial siderophore **TAPPA** and its $Fe^{III}$ complex **$Fe^{III}$-TAPPA**.

Herein, we report the stepwise modification of hydroxamate-based artificial siderophore on a Au electrode and its absorption behavior of microorganisms.

## 2. EXPERIMENTAL

All regents were commercial available and used without further purification. Milli-Q water was obtained by Milli-Q biocel A (Millipore).

The detailed preparation of **TAPPA** and **$Fe^{III}$-TAPPA** was as follows:

**TAPPA.** ABP (8.43 mmol) [16-18] and 1,7-diamino-4-(3-aminopropyl)-4-nitroheptane (2.53 mmol) [15,19] were dissolved in MeOH (20 mL). The MeOH solution (15 mL) of EDC (8.43 mmol) was added to this solution and the resultant solution was stirred for several hours at room temperature. After complete evaporation, the resulting residue was dissolved to AcOEt (100 mL) and washed with 10 % citric acid, $H_2O$, and brine. A Au film on a mica substrate was prepared by a vapor deposition method. After drying with $MgSO_4$, the solution was evaporated completely. The pale yellow oil product was obtained. To the MeOH (30 mL) solution of the resulting oil was added 10 % Pd-C (catalytic amount). The resultant mixture was stirred for several days under 3 atm $H_2$ gas pressure condition. After removal of Pd-C with celite, the solution was evaporated completely. The pale reddish oil was obtained. Yield 66.4 %. $^1H$ NMR (300 MHz, $D_2O$, 25 °C, vs. DSS): d = 3.89 (t, 6H; $CH_2$), 3.20 (t, 6H; $CH_2$), 2.54 (t, 6H; $CH_2$), 2.19 (s, 9H; $CH_3$), 1.62 (m, 6H; $CH_2$), 1.53 ppm (t, 6H; $CH_2$).

**$Fe^{III}$-TAPPA.** The $H_2O$ solution of **TAPPA** and the AcOEt solution of $Fe^{III}(acac)_3$ (equimolar amount of **TAPPA**) was combined and vigorously stirred for 3 h at room temperature. The $H_2O$ layer was collected and evaporated completely. The deep red oily solid was obtained quantitatively. ESI-TOF MS m/z = 643.4 [M + $H^+$].

A Au film with 1,000 Å thickness was deposited on a cleaved mica substrate (14 x 14 mm) at 1.0 Å $s^{-1}$. The resultant Au film was annealed with hydrogen gas flame before dipping into each sample solution.

UV-vis absorption spectra were measured by a Jasco V-570 spectrophotometer. $^1H$ NMR spectra were measured with a Gemini 300 MHz NMR spectrometer. Cyclic voltammograms were recorded by Hokuto Denko HAG-1512/BP electrochemical analyzer. ESI-MS was measured by a Micromass LCT API-TOF MS with a nanoelectrospray source.

*M. flavescens* (ATCC No. 25091) was obtained from American Type Culture Collection (ATCC) and cultured by liquid Arthrobacter medium 424 at 30 °C with shaking 100 rpm. Biological activity was recorded by ADVANTEC TN-1506 bio-photorecorder.

The optical microscopic observation was measured by Nikon ECLIPSE LV-100D microscope with a CCD camera and a differential interferometer module. Scanning electron microscopy (SEM) images were recorded by HITACHI S-5000 scanning electron microscope. All samples were pre-treated by spatter depositing with Pt. Atomic force microscopy (AFM) images were recorded by a SHIMADZU SPM-9600 scanning probe microscope.

10 MHz AT-cut quart crystal (Meidensha) was used for QCM measurement. **$Fe^{III}$-TAPPA** was modified on the crystal surface by same method in the case of deposited Au film. All QCM measurements were recorded by Hokuto Denko HQ-101C QCM controller connected to Hokuto Denko HAG-1512/BP electrochemical analyzer.

## 3. RESULTS and DISCUSSION

### 3.1. Preparation and characterization of TAPPA and $Fe^{III}$-TAPPA

The hydroxamate-typed artificial siderophore, **TAPPA** was synthesized on the basis of previous method [15-19]. Terminal -$NH_2$ group of tripodal ligand was introduced for modifying it on a Au electrode. **$Fe^{III}$-TAPPA** was easily obtained by ligand exchange reaction between **TAPPA** and $Fe^{III}(acac)_3$ complex [19].

The LMCT band ($O_{hydroxamate}$ to $Fe^{III}$) was observed at $\lambda_{max}$ = 420 nm at neutral pH range, suggesting that the structure of **$Fe^{III}$-TAPPA** was stable in solution. Fig. 2 shows the spectral change of **$Fe^{III}$-TAPPA** due to the pH. In the acidic media, one of three hydroxamate-arms of **$Fe^{III}$-TAPPA** suffered the protonation and released $Fe^{III}$ ion, resulting in the intensity decreasing of its LMCT band and shifting to low energy field. This is typical behavior for hydroxamate-typed siderophores [20].

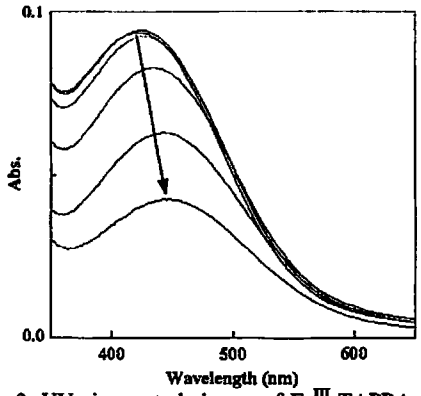



Fig. 2. UV-vis spectral change of **$Fe^{III}$-TAPPA** due to the pH. The pH was changed from 6.6 to 2.4.

The cyclic voltammogram of **$Fe^{III}$-TAPPA** indicated irreversible redox wave at $E_{pc}$ = –0.74 V *vs.* Ag/AgCl, assignable to $Fe^{II/III}$ of **$Fe^{III}$-TAPPA** by the comparison with other similar structural artificial siderophores [15].

### 3.2. Biological activity of $Fe^{III}$-TAPPA

To examine the cell-permeation ability of **$Fe^{III}$-TAPPA**, the growth-promoting activity was investigated by using gram-positive bacterium *Microbacterium*

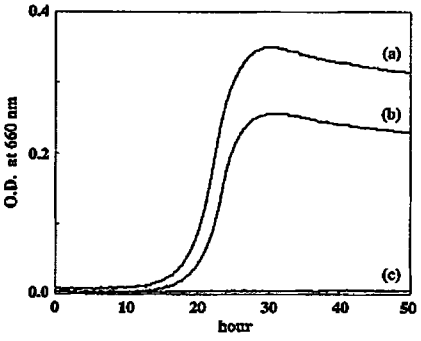



Fig. 3. Growth curves of *M. flavescens* in liquid media, as followed by monitoring the increase in optical density at 660 nm with (a) native siderophore, (b) **$Fe^{III}$-TAPPA**, and (c) no siderophore. Each siderophore concentration is 1 mM.

*flavescens* (ATCC No. 25091). This bacterium is known not to able to synthesize any siderophore by itself and can use only hydroxamate-typed siderophores for the growth. Fig. 3 shows that the biological activity of $Fe^{III}$-TAPPA is comparable to that of native siderophore (desferrioxamine mesylate). In the absence of any siderophores, *M. flavescens* could not growth, suggesting that $Fe^{III}$-TAPPA was able to permeate the cell membrane. Thus, $Fe^{III}$-TAPPA is strongly interacted to its receptors and/or binding proteins.

### 3.3. Preparation of $Fe^{III}$-TAPPA/Au

$Fe^{III}$ complex-modified Au substrate, $Fe^{III}$-TAPPA /Au was constructed by the stepwise modification of $Fe^{III}$-TAPPA on a Au electrode. At first, an annealed Au electrode, which was prepared by vapor deposition of Au onto a clean mica surface, was immersed in DTSP solution of DMSO for several minutes. The resultant active ester-modified electrode was dipped into $Fe^{III}$-TAPPA solution of $H_2O$ for one day (Fig. 4). The terminal -$NH_2$ group of $Fe^{III}$-TAPPA and activated ester on Au electrode reacted and formed amide bond, resulting in immobilizing $Fe^{III}$-TAPPA on a Au electrode.

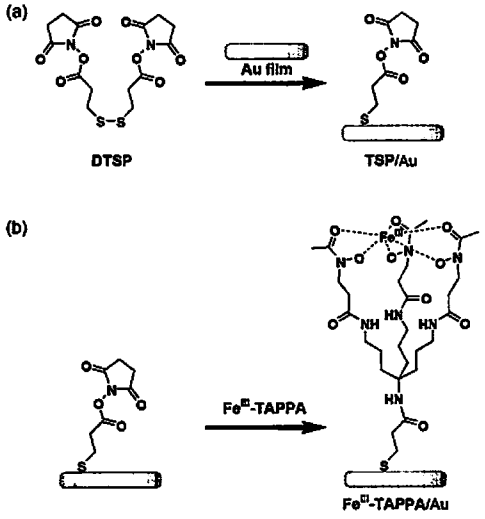



Fig. 4. Schematic views of the surface modification process of $Fe^{III}$-TAPPA. (a) Self-assembly of TSP monolayer on the deposited Au film by spontaneous splitting of DTSP. (b) Cross-linking of $Fe^{III}$-TAPPA with the TSP monolayer.

The surface modification of $Fe^{III}$-TAPPA was checked by the cyclic voltammetry of $Fe^{III}$-TAPPA/Au in a phosphate buffer solution. The irreversible redox wave was observed at $E_{pc}$ = –0.76 V *vs.* Ag/AgCl (Fig. 5). In the comparison with $Fe^{III}$-TAPPA in aqueous solution, this was assigned to $Fe^{II/III}$ of $Fe^{III}$-TAPPA modified on the Au electrode surface. Moreover, the peak current of this wave was proportional to the scan rate (Fig. 5 inset), meaning that this redox wave was originated from the surface immobilized species [21]. Thus, $Fe^{III}$-TAPPA was introduced on the Au electrode surface.

The surface coverage of $Fe^{III}$-TAPPA on $Fe^{III}$-TAPPA/Au, *ca.* 9.0 x $10^{-11}$ mol $cm^{-2}$, was estimated from its redox wave. The total Au-S bonds estimated from its reductive desorption wave was *ca.* $10^{-9}$ mol $cm^{-2}$, suggesting that $Fe^{III}$-TAPPA was not modified densely. This results indicates that the coupling reaction of the terminal -$NH_2$ group of $Fe^{III}$-TAPPA and activated ester on the Au electrode is not easy because of the steric hindrance among DTSP molecules. When the dipping time of DTSP solution was long, the coverage of $Fe^{III}$-TAPPA tended to decrease. This result is derived from the densely packed DTSP monolayer.

### 3.4. Microscopic observation for the adsorption of *M. flavescens* on $Fe^{III}$-TAPPA/Au

Fig. 6a shows optical microscopic image of $Fe^{III}$-TAPPA/Au after incubating *M. flavescens* on its surface. Many black spots were observed on $Fe^{III}$-TAPPA/Au. Although similar experiments were carried out on the DTSP-modified Au electrode as control, no spot was observed on it. To clarify what these black spots were, scanning electron microscopy (SEM) and atomic force microscopy (AFM) were performed. The SEM image (Fig. 6b) clearly showed the adsorption of *M. flavescens* on $Fe^{III}$-TAPPA/Au. Same result was also obtained by AFM measurement. These results suggest that $Fe^{III}$-TAPPA/Au can adsorb *M. flavescens*.

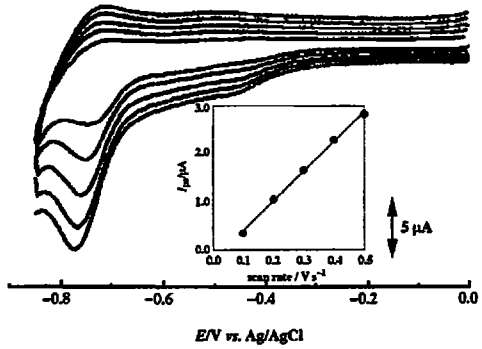



Fig. 5. Cyclic voltammogram of $Fe^{III}$-TAPPA in 0.1 M $NaClO_4$ (pH 7.0). Scan rate is 0.1 V $s^{-1}$. The inset shows the relationship between scan rate and peak current of $E_{pc}$.

### 3.5. QCM measurements for the adsorption of *M. flavescens* on substrate $Fe^{III}$-TAPPA/Au

The quartz crystal microbalance (QCM) was measured to investigate more detail of the adsorption behavior of *M. flavescens* on $Fe^{III}$-TAPPA/Au. The QCM result indicated the significant frequency decrease by the injection of *M. flavescens* broth. When the broth without *M. flavescens* was injected, the decreasing of frequency was hardly observed. The frequency decrease indicates increasing of the surface gravity on $Fe^{III}$-TAPPA/Au, suggesting the adsorption of *M. flavescens* on its surface. On the contrary, no crucial frequency decrease was observed in the case of the DTSP-modified Au electrode as control. This result well corresponded with the observation of various microscopy as discussed above. The application of this

system for other microorganisms, bacteria, and fungi is now proceeding.

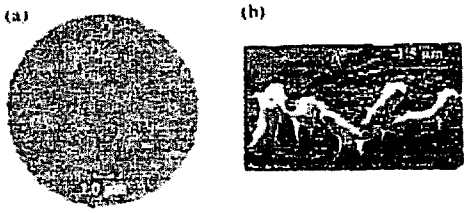



Fig. 6. Microscopic image of $Fe^{III}$-TAPPA/Au after adsorption of *M. flavescens*. (a) optical microscope image and (b) ist SEM image.

## 4. CONCLUSION

We demonstrated the adsorption of microorganisms by using the artificial siderophore-modified $Fe^{III}$-TAPPA/Au. The microscopic and QCM measurements clearly indicated the adsorption of *M. flavescens* on $Fe^{III}$-TAPPA/Au. Current detection limit of *M. flavescens* with $Fe^{III}$-TAPPA/Au was *ca.* $10^5$-$10^6$ CFU $mL^{-1}$. However, we expect that this artificial siderophore-modified $Fe^{III}$-TAPPA/Au has a potential for the detection and/or probe device of microorganisms.

Acknowledgments

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