CHAPTER III

EXPERIMENTAL METHODS

3.1 Materials and Reagents

3.1.1 Chemicals

Mercaptoacetic acid (C1, ≥98%), 3-mercaptopropionic acid (C2, ≥99%), 11-mercaptoundecanoic acid (C10, 95%), 16-mercaptohexadecanoic acid (C15, 90%) were purchased from Sigma-Aldrich and 6-Mercaptohexanoic acid (C5, 99.3%) was purchased from Dojindo. They were used without purification. Di-potassium hydrogen phosphate (K_2HPO_4, ≥99%) and potassium di-hydrogen phosphate (KH_2PO_4, ≥99%) were purchased from Merck. Horse heart cytochrome c (Cyt-c) from Sigma-Aldrich was purified by HPLC. The water used in all experiments was purified by a Millipore system and its resistance was more than 18 $M\Omega$. Ethanol was a GC-quality (LiChrosolv, purity ≥99.9%) from Merck. All other chemicals were of highest purity grade available.

3.1.2 Buffer Solution

Potassium phosphate buffer solution was prepared by mixing 0.1 M K_2HPO_4 and 0.1 M KH_2PO_4 to obtain a neutral solution, pH 7.0. Then, the stock solution was diluted with Milli-Q water to achieve a final concentration of 10, 30, 50 and 60 mM corresponding to an ionic strengths of 22, 66, 110 and 132 mM, respectively.

3.2 Electrode Preparation

3.2.1 Preparation of the SEIRA Active Surface

Thin gold (Au) films were formed on the flat surface of a semi-cylindrical shaped silicone (Si) substrate (20 x 25 x 10 mm of W x L x H) by electroless (chemical) deposition technique as described by Miyake et al [23]. The flat surface of the Si-prism was polished with aluminum oxide powder (1-5 μm grain size), and rinsed with Milli-Q water prior to exposure with 40% wt NH_4F for 2 min in order to remove an oxide layer on the Si-surface and to terminate it with hydrogen. Alternatively, the reaction was stopped immediately when hydrogen gas bubbles appear on the Si surface. Subsequently, the deposition of a thin Au layer onto the surface of the semi-cylindrical Si-prism was performed in a water bath at a temperature controlled of 65 ^{0}C. A mixture of plating solution $(0.3\,M\,Na_2SO_3 + 0.1\,M\,Na_2SO_3 \cdot 5H_2O + 0.1\,M\,NH_4Cl) + 0.03M\,NaCl_4Au \cdot 2H_2O + 2\%\,HF$ (1:1:1 in volume) was dropped onto the hydrogen-terminated Si-surface and exposed for 60 s. In this step the thin Au-film was formed according to

$$Si^0(s) + 6F^-(aq) \rightarrow SiF_6^{2-}(aq) + 4e^- \quad (3.1)$$

$$AuCl_4^-(aq) + 3e^- \rightarrow Au^0(s) + 4Cl^-(aq) \quad (3.1)$$

The reaction was stopped by rinsing the surface with the Milli-Q water. Among utilisation, the Au-surface was covered with the Milli-Q water to prevent contaminations and changes in the morphology of the metal clusters.

NOTE: *All glass stuffs were cleaned with a mixture of concentrated HNO_3:H_2SO_4 (1:1) solution.*

3.2.2 Electrochemical Cleaning of the SEIRA Active Surface

Prior the measurement, the Au-surface was cleaned electrochemically with cyclic-voltammetric method in 0.1 M H_2SO_4 with a potential range between 0.1 and 1.4 V (5 cycles) until the course of the cyclic voltammograms was constant. The electro-chemical setup consisted of a Au working electrode, a Ag/AgCl reference

electrode and a platinum (Pt) counter electrode. If an oxidation process started around 1.1 V and a "smooth oxidation loop" was formed (Figure 3.1). This implies that the surface was unadulterated.

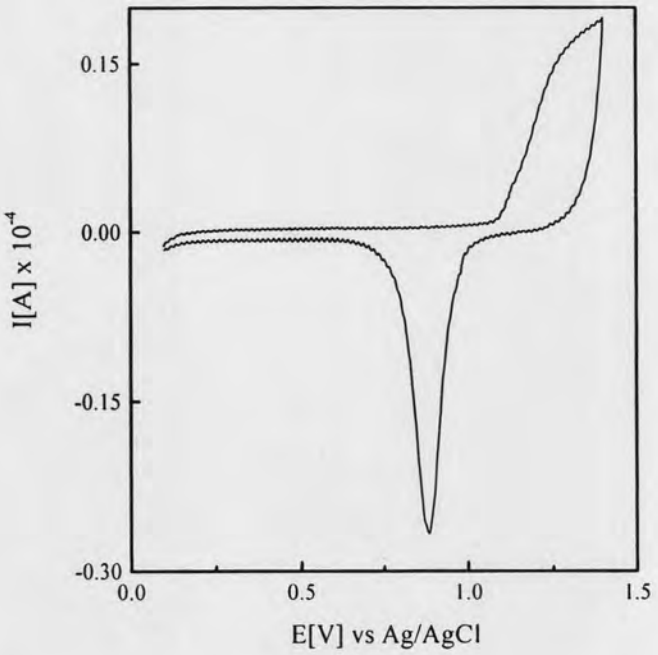

Figure 3.1 The cyclic voltammogram of an electrochemically cleaned Au-surface. The Au-film was formed at a temperature controlled of 65 ^{0}C and a deposition time of 60 s.

3.2.3 Optimization of the Gold Surface Preparation

To achieve a good surface enhancement of IR absorption spectra, a condition for the gold film preparation was optimized by varying the temperature and deposition time during the forming of the Au-films onto the Si-surface. The enhancement of the Au surface was investigated by measuring time-dependent peak intensity of a C=O stretching vibration of 1711-cm^{-1} band in the absorption spectra of C2-SAM, Figure 3.2. In this manner, an excellent enhancement of IR absorption was achieved by forming the Au-films with a water bath controlled temperature of 65 ^{0}C, and deposition times between 60 and 90 s. However, the gold film was too thick and easy to peel off at the edge when a deposition time of 90 s was used.

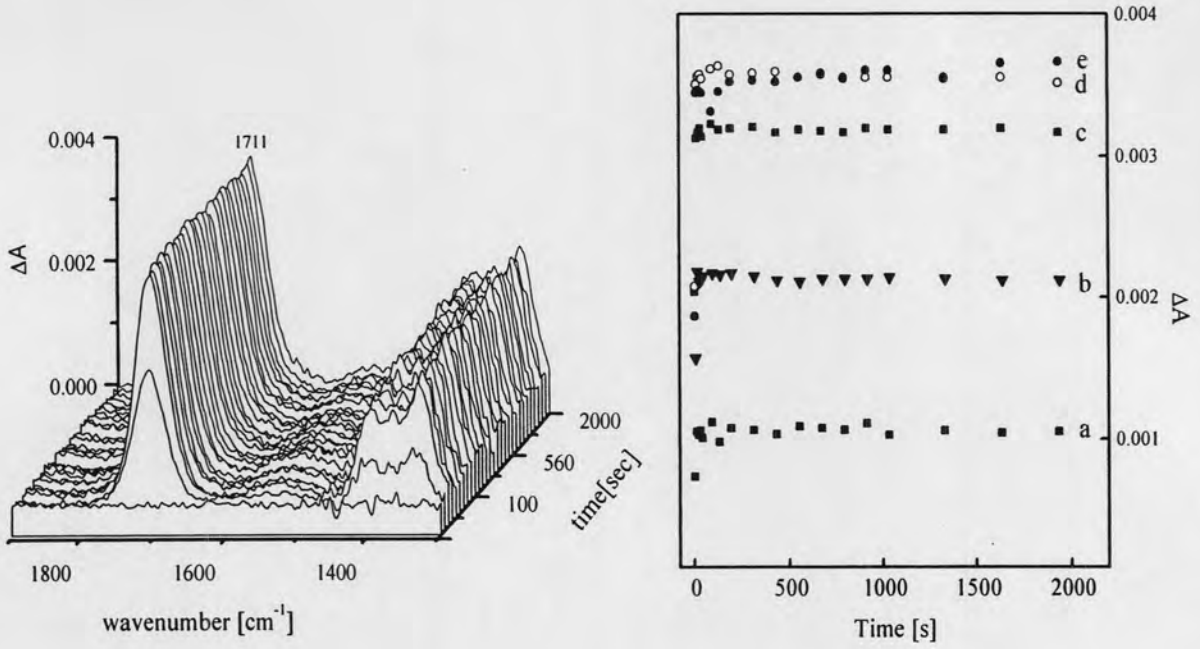

Figure 3.2 The time-dependent SEIRA spectra of a C2-SAM (Left) and the plot of the peak intensities of C=O stretching vibration of the 1711-cm^{-1} band as function of absorption time (Right) for different Au-film preparation; (a) 55 °C 60 s, (b) 55 °C 120 s, (c) 75 °C 60 s, (d) 65 °C 90 s and (e) 65 °C 60 s.

3.2.4 Self-Assemble Monolayers

1 mM of C1, C2, C5, C10 and C15 were always freshly prepared in order to prevent the decomposition of the thiol (SH) headgroup. The formation of SAM was carried out followed the protocol described by Murgida et. al [26,60]. C1 and C2 were dissolved in Milli-Q water and exposed to the surface of Au-electrode for 12 hs; C5, C10 and C15 were dissolved in ethanol and exposed for 24 hs. Afterwards, the unbound monolayer can be easily removed by rinsing the Au-electrode with the solvent.

3.3 SEIRA Spectro-Electrochemical Measurements

3.3.1 SEIRA Spectro-Electrochemical Set-Up

The SEIRA spectro-electrochemical cell consists of a one-compartment cylindrical body. Measurements were done in a Kretschmann-ATR configuration using a Si crystal coated with an Au film on the top of the surface. The Si crystal was incorporated into a home-built three-electrode cell that was constructed on the basis of a previously published design [6]. The Au-film on the Si crystal, a Pt wire, and a Ag/AgCl (3 M KCl) electrode served as working, counter, and reference electrode, respectively, Figure 3.3. A Au-plate was used to establish electrical contact with the gold film that was sealed with a teflon coated o-ring (inner diameter of 8 mm). The total filling volume of the cell was 6 mL. Electrode potentials were controlled by an Autolab PGSTAT 12 potentiostat with the related GPES software. All potentials cited in this work refer to the Ag/AgCl electrode. The IR beam was coupled into the single reflection Si-crystal with an angle of incident of 60°. The source of mid-IR radiation was a globar. The SEIRA spectra were recorded from 4000 to 1000 cm^{-1} with a spectral resolution of 4 cm^{-1} on a Bruker IFS66v/s spectrometer equipped with a photoconductive MCT detector. 400 scans were co-added for a spectrum. Neither baseline corrections nor any smoothing procedures were applied to the data.

3.3.2 Immobilisation of Cytochrome c

Concentrated Cyt-c was injected into the buffer solution containing SEIRA spectro-electrochemical cell to achieve a final concentration of 2 μM. Absorption spectra of Cyt-c immobilised on a bare Au-electrode or on SAMs coated Au-electrode were measured. A reference spectrum of the buffer solution was measured in the absence of Cyt-c. The absorption spectra of Cyt-c were collected at open-circuit potential (OCP > 0 V). The buffer solution was constantly stirred by bubbling Argon (Ar) gas during the measurement to prevent a precipitation of the protein. The temperature was set to 23 °C by a thermostat water bath (HAAKE Fisons F3 and K). For each spectrum, 390 scans were used, requiring approximately 1 min for the

acquisition. The equilibrium state of Cyt-c immobilised on a bare Au-electrode or on a SAM coated electrode was reached after about 1 hr.

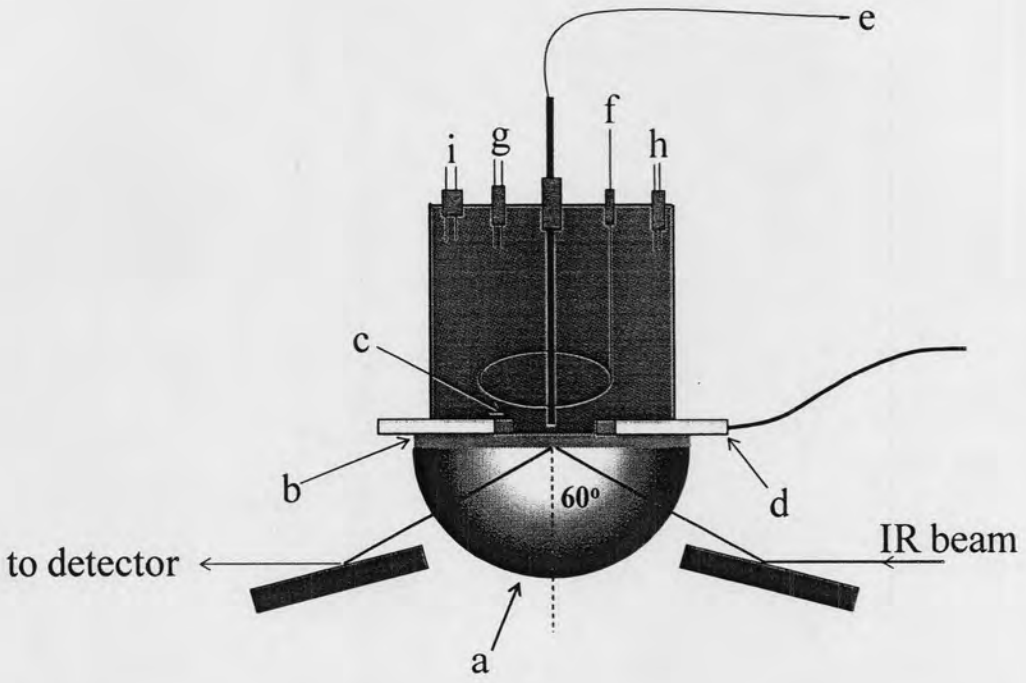

Figure 3.3 The SEIRA spectro-electrochemical cell consists of (a) a Si ATR-crystal, (b) a thin Au-film working electrode, (c) a teflon coated o-ring, (d) a Au plate for the contact with the working electrode, (e) a Ag/AgCl reference electrode, (f) a Pt counter electrode, (g) a gas inlet, (h) a gas outlet and (i) a sample inlet.

3.3.3 Stationary SEIRA Investigations

3.3.3.1 Self-Assembled Monolayers

The potential-dependent SEIRA difference spectra of SAMs were measured after the monolayer formed an ordered structure on the Au-electrode. A single-beam spectrum recorded at -0.1 V was used as the reference. The potential-dependent spectra were measured at various electrode potentials, comparable to those applied within the measurements of immobilised Cyt-c (-0.2, -0.15, -0.10, -0.05, 0.00, 0.05, 0.10, 0.15 and 0.20 V, respectively) using newly measured background

spectrum in each case. All spectra were measured after equilibration of the system at the respective electrode potential for 3 min to achieve a good signal-to-noise. During the measurements, which were carried out at ambient temperature (23 °C), the solution was purged with Ar. All experiments were carried out in a 10 mM potassium phosphate buffer corresponding to an ionic strength (I) of 22 mM. The reference potential and the applied potentials used for recording the difference spectra could be varied for specific purposes.

3.3.3.2 Native B1 State of Cytochrome c

Redox-induced SEIRA difference spectra of Cyt-*c* were measured after Cyt-c was immobilised on the Au-electrode. A single-beam spectrum recorded at -0.1 V, where the adsorbed Cyt-*c* is fully reduced, was used as the reference. Sample spectra were measured at different electrode potentials of -0.08, -0.06, -0.04, -0.02, 0.00, 0.02, 0.04, 0.06, 0.08, 0.10, 0.125 and 0.15 V, respectively, using a newly measured background spectrum in each case. All spectra were measured after equilibration of the system at the respective electrode potential for 60 s. During the measurements which were carried out at ambient temperature (23 °C), the solution was purged with Ar. All experiments were carried out at pH 7.0 with 10 or 30 mM phosphate buffer corresponding $(\mathrm{I} = 22$ and 66 mM, respectively$)$.

3.3.3.3 Non-Natve B2 State of Cytochrome c

After Cyt-c absorption on the bare Au-electrode, the potential-dependent SEIRA difference spectra of Cyt-*c* were measured in the potential range from -0.5 V to +0.15 V. Series of single-beam spectra at each potentials were recorded, until the spectra revealed that an equilibrium was reached. This was done by checking the absorbance change, ΔA, as function of time, until no change could be observed anymore. The absorbance change is calculated using the subsequence single-beam spectrum relative to the previous one according to $\Delta A = -\log\left[I_{t+1} / I_t\right]$, where I_t and I_{t+1} are the single-beam spectrum at specific time and the corresponding subsequent time, respectively. The reference single-beam spectrum was recorded at

the potential of -0.50 V, while the sample spectra were measured at the potential of -0.475 V, -0.40 V, -0.30 V, -0.20 V, -0.10 V, 0.00 V, +0.10 V and +0.15 V, respectively. After the potential jumps were carried out, a new single-beam spectrum was collected in the equilibrium state at the reference potential of -0.50 V. The SEIRA difference spectra were recalculated using the last single-beam spectrum at equilibrium state according to $\Delta A = -\log\left[I_{ox} / I_{red}\right]$, where I_{ox} and I_{red} are the single-beam spectrum at an oxidised state at various potentials and at the fully reduced state with a potential of -0.50 V, respectively. The 10 mM potassium phosphate buffer (I = 22 mM) was bubbled with Ar gas during the measurement. The temperature was set to 26 °C.

3.3.4 Time-Resolved SEIRA Measurements

Time-resolved SEIRA experiments were carried out in the same SEIRA electro-chemical setup described in Section 3.3.1 using the potential jump technique. Spectra acquisition was synchronized with the potential jumps controlled by a home-made pulse delay generator. Series of time dependent single-channel spectra of Cyt-c were collected when a potential jump was carried out from the reference potential (Cyt-c largely reduced) to the redox potential, and for the reverse jump. Depending on the time scale under examination, either step-scan or rapid-scan TR-SEIRA measurements were carried out. The photoconductive MCT detector equipped with a fast amplifier was used in the DC coupled mode for the step-scan measurement. In the case of the rapid-scan measurements, the AC-coupled mode was utilised.

Step-scan measurements were carried out for the faster ET processes when Cyt-c was electrostatically adsorbed on a C5- or C10-SAM. The time resolution was set to 100 μs covering the whole time-range for the redox reaction from 0 to 26 (250) ms for a single experiment in the case of C5-SAM (C10-SAM). The single-channel spectra were first measured at an applied electrode potential of -0.1 V for 5 (30) ms as a reference, then a potential jump to the redox potential at ca. +0.04 V for 7 (70) ms was carried out. Subsequently, the potential was set back to the initial value of -0.1 V for a relaxation time of 14 (150) ms. 1558 (200) coadditions were done to improve the

signal-to-noise (S/N) ratio. Nine subsequently recorded sample single-channel spectra were averaged for the investigations with C10 to increase the S/N ratio further. An optical filter (< 1828 cm^{-1}) was used to reduce the number of the data points. Measurements in the rapid scan mode were done when Cyt-c was adsorbed on C15-SAM. The time resolution was set to 500 ms and the experiment was repeated 256 times to increase the S/N ratio. In the same way as described before, single-channel spectra were measured with an applied electrode potential of -0.10 V (10 s) as reference, then the potential was set to the redox potential at ca. +0.04 V (20 s) and set back to the initial potential of -0.10 V (40 s).

Afterward, a three-dimensional (3D) data set of the single-channel spectra was produced, and further manipulated by a software macro. The single-channel spectra at negative potentials were averaged as the reference (I_{ref}). At the redox potential, nine subsequently recorded single-channel sample redox spectra (I_f) were averaged to increase the spectral S/N for the spectrum measurements. They were converted into the time-dependent difference spectra by using the relationship $\Delta A = -\log\left[I_{ref} / I_f\right]$, where I_f and I_{ref} are the corresponding "averaged" single beam spectra at the redox potential and at the initially reduced state, respectively.

3.4 Cyclic Voltammetry Measurements

Cyclic voltammetric (CV) measurements using a staircase method were performed with the same Autolab PGSTAT 12 potentiostat together with the SEIRA electrochemical cell described above. After Cyt-c immobilised on C5-, C10- and C15-SAM, the CV measurements were carried out in Cyt-c-free 30 mM potassium phosphate buffer $(I = 66\ \text{mM})$ at pH 7.0. Potentials were swept with variation of a scan rate from the initial potential $(E_{initial})$ to the final potential (E_{final}) and swept back to $(E_{initial})$ for three cycles. A step potential was 5 mV and an alpha value of 0.25 was used. The last cyclo-voltammogram was used for the evaluation.

3.5 Data Evaluation

The second derivative spectra were obtained using the "Savitzky-Golay" algorithm, which is implemented in the OPUS software (Bruker) with a smoothing of 9 points. The anodic and cathodic peak potentials were obtained with the help of pick peaks tool by OriginPro 7.0 software.