2.54 – 2.62 (1H, CH_2), 2.64 – 3.05 (6H, CH_2), 3.08 – 3.19 (1H, CH_2), 3.36 (4H, NH_2), for aromatic protons see Table 1; ^{13}C NMR (75 MHz, $CDCl_3$, TMS): $\delta = 29.42, 31.48, 32.60, 34.55, 120.29, 120.46, 122.06, 122.26, 123.69, 123.83, 128.44, 133.76, 140.32, 140.36, 144.91, 145.45$; IR (KBr): $\tilde{\nu} = 675, 726, 803, 1429, 1509, 1562, 1620, 2857, 2928, 3002, 3222, 3336, 3360, 3430\ cm^{-1}$; MS (70 eV): m/z: 238 [M^+], 119 [main, $C_8H_7NH_2^+$], 91 [$C_7H_7^+$], 77 [$C_6H_5^+$], 65 [$C_5H_5^+$].

4-Amino[2.2]paracyclophane (**3a**) was polymerized by using a self-designed CVD installation consisting of a sublimation zone, a pyrolysis zone, and a deposition chamber equipped with a sample holder.[7] Compound **3a** (30 mg) was placed in the sublimation zone and a copper substrate was fixed on the sample holder being cooled to 8 °C. The pressure was adjusted to 0.1 mbar and the pyrolysis zone was heated to 750 °C. Subsequently, **3a** was sublimed slowly resulting in a transparent film of polymer **4** on the copper substrate. XPS (referenced to hydrocarbon at 285.0 eV): C_{1s}: 93.9 % (calcd: 94.1 %), N_{1s}: 6.1 % (calcd: 5.9 %) atom %; IR (grazing angle of 85°): $\tilde{\nu} = 814, 865, 1155, 1286, 1424, 1516, 1583, 1618, 2863, 2946, 3012, 3047, 3359, 3431\ cm^{-1}$.

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Active-Site Structure and Dynamics of Cytochrome *c* Immobilized on Self-Assembled Monolayers—A Time-Resolved Surface Enhanced Resonance Raman Spectroscopic Study**

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Self-assembled monolayers (SAM) of alkanethiol derivatives on metal electrodes provide numerous possibilities for immobilizing redox proteins.[1] In the past few years, such devices have gained increasing interest in (nano)biotechnology. In particular, the current and potential importance of immobilized enzymes for the design and development of biosensors,[2] bioelectronic systems,[3] or biocatalytic cells[4] has stimulated a large number of experimental studies in this field. Customizing the catalytic functions and optimizing the efficiencies of these systems represent a challenge in current research as they require a detailed characterization of the structural and functional properties of the redox-active adlayers as well as of the dynamics of the processes involved. Such investigations impose high demands on the sensitivity and selectivity of the analytical tools. In most cases, microscopic and electrochemical methods such as atomic force microscopy and cyclic voltammetry (CV) are employed which, however, do not provide information about the molecular structure of the species involved in the redox process. In this respect, time-resolved surface-enhanced resonance Raman (TR-SERR) spectroscopy, which exclusively probes the vibrational spectra of the redox sites solely of the immobilized species, is a powerful alternative approach for analyzing molecular structure and dynamics of the adsorbed enzymes.[5]

So far, TR-SERR spectroscopy was restricted to redox proteins immobilized on bare Ag electrodes which, however, do not represent systems of general biotechnological applicability due to potential denaturation of proteins directly adsorbed on the Ag surface. In this work, we have employed TR-SERR spectroscopy for the first time to probe the heterogeneous electron transfer (ET) process of a heme protein immobilized on a SAM-coated Ag electrode. As a test redox protein we chose cytochrome *c* (Cyt-*c*) which has been studied by various electrochemical techniques including CV, electrochemical impedance, and electroreflectance.[6–8]

After electrochemcial roughening of the Ag electrode,[5] SAMs of 11-mercaptounadecanoic acid (11-MUDA; Aldrich) were prepared according to previously published procedures.[9] For the adsorption of Cyt-*c*, the electrode was dipped in a solution containing 2 μM of the purifed protein (horse heart, Sigma) as well as 12.5 mM KCl and 12.5 mM phosphate buffer (pH 7.0) for 30 min. Subsequently, this

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buffer was used for rinsing the electrode to remove unbound Cyt-*c* prior to inserting the electrode into the SERR cell containing a Cyt-*c*-free solution of the same electrolyte composition. SERR spectra were measured with 413-nm excitation using a home-built rotating Ag electrode to avoid laser-induced desorption and protein degradation.[5b] TR-SERR measurements were carried out by probing the SERR spectra during the time intervals Δt (typically 500 μs) after a delay time δ with respect to a potential jump from an initial potential E_i to a final potential E_f. The method and the experimental set-up are described in detail by Wackerbarth et al.[5b, 10]

The intensity of the stationary SERR spectra of Cyt-*c* bound to the SAM-coated Ag electrode was found to be lower by not more than a factor of 2 compared to Cyt-*c* adsorbed on a bare Ag electrode. These findings, which are in line with previous observations,[11, 12] imply that the distance-dependent attenuation of the surface enhancement still allows the measurement of high-quality SERR even at a separation of about 19 Å between Cyt-*c* and the electrode. During the experiments, the SERR intensity decreased by less than 10%, indicating an efficient electrostatic binding of Cyt-*c* to the carboxylate groups of the SAM through the lysine-rich domain around the exposed heme edge of the protein.[13] The analysis of the SERR spectra focused on the region between 1300 and 1700 cm^{-1} that includes the vibrational bands of the heme that are characteristic of the various oxidation, spin, and ligation states of Cyt-*c*.[13] Hence, this spectral region is of particular importance for identifying Cyt-*c* species that exhibit different active site structures.[5] Except for a slight broadening of the bands, the SERR spectra, measured at $+0.05$ and -0.4 V,[14] are essentially identical to the respective resonance Raman (RR) spectra of oxidized and reduced Cyt-*c* in solution, implying that the structure of the heme pocket remains unchanged upon binding to SAMs of 11-MUDA. These findings are in contrast to those for Cyt-*c* adsorbed on a bare Ag electrode where electrostatic interactions induce a potential-dependent conformational equilibrium between the native form of Cyt-*c* (state B1) and a new state (state B2) that lacks the axial Met-80 ligand and exhibits a strong negative shift of the redox potential.[5b, 13, 15] Thus, the analysis of the redox process of Cyt-*c* at the 11-MUDA-coated Ag electrode is substantially simplified such that only two spectrally different species have to be considered, that is, the reduced and the oxidized forms of Cyt-*c* in the (native) B1 state. Consequently, superpositions of the component spectra[5] of these two species yield very good and unambiguous fits to all measured SERR spectra between $+0.05$ and -0.4 V so that the potential-dependence of the redox equilibrium could be determined with high accuracy. The Nernst plot yields an excellent linear correlation with a slope corresponding to $n = 0.96$. The redox potential was determined to be -0.031 V which is about 40 mV more negative than the redox potential of Cyt-*c* in solution.[16] This potential shift can be rationalized in terms of a potential drop at the redox site due to its separation from the electrode by the SAM.[5c, 17]

TR-SERR experiments were carried out by applying potential jumps to the final potential of -0.031 V, that is, the redox potential, by using delay times between 500 μs and 40 ms. As an example, the TR-SERR spectrum measured at $\delta = 8.5$ ms after a potential jump from $E_i = -0.074$ V is displayed in Figure 1 along with the stationary SERR spectra measured at E_i and E_f. Also in the TR experiments, the SERR spectra reveal no contributions of Cyt-*c* species other than the native reduced and oxidized state B1 so that the component spectra of these two species allow a consistent fit to the experimental spectra on the entire time scale. Thus, the time-dependent concentration changes can be analyzed in terms of a one-step relaxation process that refers to the heterogeneous ET between the adsorbed Cyt-*c* and the Ag electrode. Indeed, a semilogarithmical plot of the deviations from the equilibrium concentrations as a function of δ yields a straight line with a very good correlation and an intercept close to the theoretical value of zero (Figure 2a). From the slope, the formal heterogeneous rate constant (at $E_f = E^0$) is determined to be 43 s^{-1}, which is somewhat smaller than the values derived from electrochemical experiments at SAM-coated Au electrodes (60–72 s^{-1}) by electrochemical methods.[6d, 8]

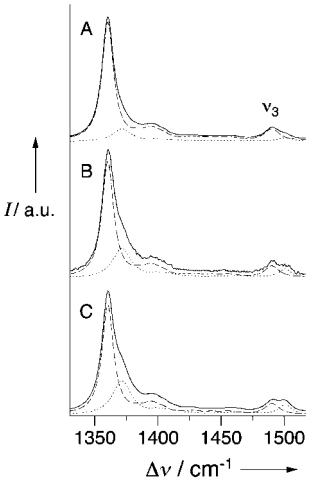

Figure 1. SERR spectra of Cyt-*c* adsorbed on an 11-MUDA-modified Ag electrode for a potential jump from $E_i = -0.074$ V to $E_f = -0.031$ V. Traces A) and C) are the stationary SERR spectra measured at E_i and E_f, respectively. Trace B) shows the TRSERR spectrum measured at 8.5 ms after the potential jump. The dashed and the dotted lines indicate the component spectra of the reduced and oxidized Cyt-*c*, respectively.

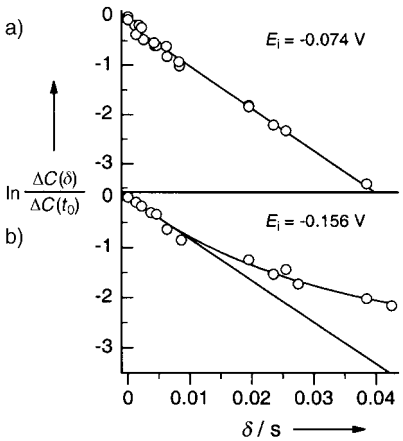

Figure 2. Semilogarithmic plot of the concentration changes of the reduced Cyt-*c* as a function of the delay time δ as determined by TR-SERR spectroscopy. $\Delta C(\delta)$ and $\Delta C(t_0)$ denote the deviations from equilibrium concentrations for $E_f = -0.031$ V at $t = \delta$ and $t = 0$, respectively. The upper diagram (a) refers to a potential jump from $E_i = -0.074$ V to $E_f = E^0$, the lower one (b) to a potential jump from $E_i = -0.156$ V to $E_f = E^0$.

By applying larger potentials jumps from more negative initial potentials to $E_f = E^0$, the dynamics of the ET becomes more complex as shown in Figure 2b for $E_i = -0.156$ V. Whereas at delay times shorter than about 7 ms, the semi-logarithmical plot of the concentration changes reveals a linear behavior with the same slope as determined for $E_i = -0.074$ V (Figure 2a), at longer delay times substantial deviations from linearity are found.[18] These deviations indicate that at large potential jumps a second and relatively slow non-Faradaic process interferes with the ET of the adsorbed Cyt-*c*. Since the analysis of SERR spectra and the ideal Nernst behavior rule out the involvement of conformational transitions that include changes of the heme pocket structure and, thus, alterations of the redox potential, it is concluded that there is a potential-dependent equilibrium between adsorbed Cyt-*c* species that solely differ by the ET kinetics. Such differences may result from different orientations of the heme with respect to the SAM which in turn may be brought about by electrostatic binding through different lysine residues in the binding domain of Cyt-*c*. Since this part of the protein surface includes about 10 lysine residues,[19] variations of the heme orientations may easily be achieved without changing the distance of the redox center from the Ag electrode.[20] Following this interpretation, it may be that at potentials close to E^0, Cyt-*c* is predominantly bound through an orientation that enables a fast ET. Decreasing the potential may thermodynamically favor an orientation of the bound Cyt-*c* that is associated with a slower ET rate. Consequently, very negative values for E_i should lead to a substantial population of this latter species. Thus, the additional slow phase in the relaxation kinetics (Figure 2b) may either reflect directly the ET of this species or its reorientation followed by a fast ET. At small potential jumps, the equilibrium distributions among the different populations are likely to be similar at E_f and E_i so that the relaxation process can adequately be approximated by a single exponential (Figure 2a).[21, 22]

According to this interpretation, at potentials above E^0 essentially all the adsorbed Cyt-*c* molecules should be adsorbed in the orientation that is favorable for the ET. This conclusion is in fact confirmed by measurements with large potential jumps from $E_i = +0.1$ V to E^0. In this case, the ET kinetics is monophasic with a rate constant of 39 s^{-1} that is nearly identical to that obtained for small potential jumps from negative potentials.

In contrast to TR-SERR spectroscopy, electroactive and -inactive species cannot be identified and distinguished by CV and its applicability for analyzing the ET kinetics of adsorbates is questionable or, at least, limited.[23] However, a coupling of Faradaic and non-Faradaic processes may be indirectly reflected by electrochemical experiments. Thus, the peak broadening observed in cyclic voltammograms of Cyt-*c* on SAM-modified Au electrodes was attributed to a dispersion of kinetic or thermodynamic parameters of the redox process.[6a–c] On the basis of the present TR-SERR results, the electronic coupling constant is suggested to be the crucial parameter as it should sensitively depend on the orientation of the adsorbed Cyt-*c* even though the possible orientations are not likely to be associated with different ET distances.[20] On the other hand, distributions of reorganization energies or redox potentials, as suggested previously by Bowden et al.,[6b] can be ruled out since differences in these parameters should be sensitively reflected by differences in the redox site structures. However, the SERR spectra do not reveal any indication for such a structural heterogeneity of the bound Cyt-*c* (see above).

These results illustrate the principle advantages of TR-SERR spectroscopy, which is suitable for monitoring time-dependent processes of adsorbed redox proteins directly and selectively and simultaneously providing information about the active site structures of the various species involved. Thus, TR-SERR spectroscopy represents a powerful approach to analyze the dynamics and mechanism of complex interfacial processes of immobilized enzymes and promises to become an important analytical tool for investigating molecular structures and testing the performance of nanoscale devices in biotechnology.

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[21] A coupling between ET and protein reorientation has also been proposed recently for Cyt-*c* on SAM-coated Au electrodes;[8b] however, in that study protein reorientation was suggested to be much faster than found in the present work.
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