CHEMCOMM
Communication
www.rsc.org/chemcomm

Immobilization of cytochrome c at Au electrodes by association of a pyridine terminated SAM and the heme of cytochrome

Hiromichi Yamamoto, Haiying Liu and David H. Waldeck*

Department of Chemistry, University of Pittsburgh, Pittsburgh, Pennsylvania 15260, USA. E-mail: dave@pitt.edu

Received (in Cambridge, UK) 23rd February 2001, Accepted 23rd April 2001
First published as an Advance Article on the web 9th May 2001

Electrochemical methods are used to demonstrate that cytochrome c can be immobilized on electrodes that are coated with self-assembled monolayers of 4-pyridinyl–CO_2–$(CH_2)_n$–S ($n > 6$) through interaction between the pyridine terminal unit and the heme of the cytochrome.

One avenue toward better sensors and enzyme electrodes is the use of a long tether, whose terminal functionality can selectively bind a biomolecule. Here this strategy is explored with the redox protein cytochrome c. Electron transfer reactions with cytochrome c have been extensively studied, both to clarify its function in biological systems and to provide a canonical example of a redox active protein.[1] Cytochrome c has been immobilized on CO_2H terminated self-assembled monolayers (SAMs) through electrostatic interactions between the carboxylate and the exterior of the protein[2] and by covalent linkage.[3] This work investigates the ligation between the terminal unit of a 4-pyridinyl–CO_2–$(CH_2)_n$–S ($PyCO_2$–C_n, n = 2, 6, 11, 16) SAM and the heme of horse-heart cytochrome c. The data show that immobilization of cytochrome is achieved by the pyridine functionality when the alkane tethers are long enough, n = 6, 11, 16. In addition, the immobilization causes a large negative shift of the formal potential $E^{\circ\prime}$ that does not occur for other immobilization strategies. The data also show that the electron transfer rate for the oxidation is enhanced over that for the reduction; *i.e.* an asymmetry in the rates becomes evident. These data suggest that the pyridine interacts with the iron in the cytrochrome's heme.

The experiments used a three-electrode (Pt auxiliary, Ag/AgCl reference) electrochemical cell with a working electrode that was a Au ball (*ca.* 1 mm diameter) to which the disulfide was self-assembled (see ESI† for details on the construction of the electrode and its characterization). Capacitance measurements were used to assess the quality of the SAM. For the immobilization studies the SAM coated electrodes were incubated in a cytochrome c solution and measurements were made in 25 mM phosphate buffer solution at pH 7. Details on the preparation of the SAM materials are given in the ESI†. For the studies with OH terminated layers and $PyCO_2$–C_2, in which the cyt c is not immobilized, the purified cytochrome c was 30–50 μM in the buffer solution (see ESI† for further details).

Fig. 1 shows cyclic voltammograms for some of the systems that were studied. Fig. 1(a) and (b) show voltammograms for SAMs that have pyridinal functionalities. The solid line curves show the electrode's response after it has been removed from the incubation solution, rinsed, and placed in contact with a buffer/electrolyte solution that does not contain cytochrome c. The data in Fig. 1(a) shows no faradaic response for the buffer solution because cytochrome c does not adsorb onto the surface of $PyCO_2$–C_2, but does show a response when the electrode contacts a solution containing cytochrome c (dashed line). Fig. 1(b) shows that some cytochrome adsorbs on the $PyCO_2$–C_{11} SAM and displays a negative shift in the apparent formal potential. A corresponding experiment using a SAM in which the pyridine nitrogen is blocked with a methyl group shows no faradaic current. Hence the adsorption of cytochrome c requires that the nitrogen atom of the pyridine be accessible to the protein and the alkane chain be long enough. Fig. 1(c) shows voltammograms for HO-terminated films,[4] in contact with cytochrome c solution (dashed line). The solid line voltammogram is the same system with 0.8% pyridine (by volume) added to the solution. Two redox waves (apparent $E^{\circ\prime}$ of −17 and −325 mV) are found upon pyridine addition. The redox wave at −17 mV is assigned to the redox reaction of unmodified cytochrome c and the wave at −325 mV is assigned to the redox reaction of cytochrome c in which pyridine is coordinated to the heme. In this system, a positive potential sweep oxidizes the iron, and complexation with the pyridine occurs (or is strengthened). As the voltage is swept back negative the pyridine is not removed until the iron is reduced at the more negative reduction potential of the pyridine-coordinated moiety. To summarize, these data show that $PyCO_2$–C_{11} SAMs immobilize cytochrome c and cause an $E^{\circ\prime}$ shift that is consistent with an interaction between pyridine and the heme.

Voltammograms were collected as a function of the voltage scan rate for each of the electrodes. For the HO–C_6 and $PyCO_2$–C_2 SAMs with cytochrome in solution, the peak current depended on the square root of the scan rate, indicating that the cytochrome is not immobilized on the electrode surface. For the HO_2C–C_{10} and the $PyCO_2$–C_n (n = 6, 11, 16) SAMs, the peak current was linear with the voltage scan rate, indicating that the

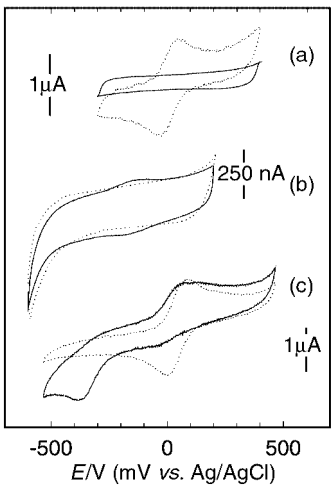

Fig. 1 Cyclic voltammograms are shown for gold electrodes that are coated with (a) 4-pyridinyl–CO_2–$(CH_2)_2$–S, (b) 4-pyridinyl–CO_2–$(CH_2)_{11}$–S, and (c) HO–$(CH_2)_6$–S SAMs. In (a), the solid line shows a voltammogram for an electrode that was incubated in 50 μM cytochrome solution for 30 min and placed in contact with a 25 mM buffer solution, and the dashed line shows the same electrode in contact with a 50 μM cytochrome c buffer solution. In (b) the solid line corresponds to the Py terminated layer after it was incubated in the cytochrome solution and the dashed line corresponds to the background curve. (c) Shows a voltammogram for cytochrome in a buffer solution (dashed line) and a voltammogram for a cytochrome solution containing 0.8% pyridine (solid line).

† Electronic supplementary information (ESI) available: full experimental details. See http://www.rsc.org/suppdata/cc/b1/b101767p/

 DOI: 10.1039/b101767p

Table 1 The apparent formal potential $E^{\circ\prime}$ and the change in peak potential between the high (20 V s^{-1}) and low (0.1 V s^{-1}) scan rates

	$E^{\circ\prime}$/mV[a]	$\Delta E_{p,ox}$/mV	$\Delta E_{p,red}$/mV
$PyCO_2(CH_2)_2S$–	5	—	—
$PyCO_2(CH_2)_6S$–	−159[a] ± 7	0	−11
$PyCO_2(CH_2)_{11}S$–	−152[a] ± 5	0	−21
$PyCO_2(CH_2)_{16}S$–	−147[a]	0	−44
$HO(CH_2)_6S$–	44 ± 2	—	—
$HO_2C(CH_2)_{10}S$–	13 ± 3	32[b]	−24[b]

[a] $E^{\circ\prime} = (E_{p,ox} + E_{p,red})/2$ at 100 mV s^{-1} scan rate and *vs.* the Ag/AgCl reference. [b] Shift in the peak potential upon change in scan rate from 0.6 to 10 V s^{-1}.

cytochrome is immobilized on the surface, as demonstrated by the data in Fig 1. The apparent formal potential, $E^{\circ\prime}$, for the cytochrome immobilized on pyridine are significantly shifted negative of the other systems (see Table 1). For the HO_2C–C_n SAMs, the adsorption is attributed to electrostatic and hydrogen bonding interactions between the lysine groups ($-NH_3^+$) on the cytochrome c periphery and the carboxylic acid group of the SAM.[2] The negative shift of $E^{\circ\prime}$ suggests a different adsorption state for cytochrome on the pyridinyl layers than on the carboxylic acid layers and a significant interaction of the pyridine with the heme. The nitrogen in the pyridinyl moiety of the SAM and the long alkyl chain are both needed for significant adsorption to occur.

Available literature results support the interpretation that the pyridine terminated SAMs coordinate to the heme. Recent Raman and NMR studies show that Met80, which is axially bound to the iron in the heme of cytochrome c, can be displaced by an imidazole or pyridine.[5] The apparent $E^{\circ\prime}$ values in the pyridine containing solution were found to be −17 and −325 mV, which is consistent with the reported result for yeast iso-1-cytochrome c that complexes with imidazole.[6] Thus, the large negative shift of the $E^{\circ\prime}$ (342 mV) by pyridine in solution is caused by the axial ligand exchange from Met80 to pyridine on the heme's iron. The 10–20 mV shift in the $E^{\circ\prime}$ of the unsubstituted cytochrome is consistent with the change in solution dielectric constant caused by 1% pyridine.[7] These observations strongly suggest that the large shift of 150 to 160 mV in the $PyCO_2$–C_n coated electrodes results from association of the pyridine with the heme. Although the potential shift observed on the SAM is about half of the shift found for the free pyridine, such a result is consistent with a reduced ability for optimal coordination when the pyridine is tethered to the SAM. Although a change in the local dielectric constant[7] or local field effects[8] may contribute to the potential shift, they are expected to be smaller than the shift observed here and show a chain length dependence.

The dependence of the voltammogram's peak position on the voltage scan rate can be used to quantify the electron transfer rate constant. For the $PyCO_2$–C_6, $PyCO_2$–C_{11}, and $PyCO_2$–C_{16} SAMs the oxidation waves do not shift with increasing scan rate; *i.e.* the rate remains too fast for the instrument to resolve. In contrast, the reduction peaks shift (see Table 1) and show a dependence on the alkane chain length of the SAM, reflecting the impact of the tunneling barrier thickness[9] on the electron transfer rate. This asymmetry in the transfer rate is consistent with stabilization of the heme by the pyridine. Such an interaction would enhance the oxidation rate and inhibit the reduction rate for which a dependence of the peak position on the voltage scan rate is evident. The increase in the reduction peak's potential shift with scan rate as the alkane chain length increases reflects a slowing of the electron transfer rate because of an increased tunneling barrier width. A more quantitative analysis of the electron transfer kinetics is underway and will be reported elsewhere.

This study shows that cytochrome c can be immobilized on $PyCO_2$–C_nS SAMs with alkane chains of $n > 6$, and the adsorption occurs through association of the pyridine with the cytochrome's heme. This latter conclusion is supported by the large potential shifts, an induced asymmetry in the reduction and oxidation rates, and other literature results. Fig. 2 depicts the mode of binding that is envisioned for this system. In contrast to electrostatic binding with surface functionalities, the pyridine associates directly with the redox center, which should create a better defined geometry between the protein and the electrode. This approach will allow the electron transfer kinetics to be explored without the limitations imposed by an inhomogeneous distribution of protein orientations with respect to the electrode. On a practical level, this strategy of ligating to metal redox centers or binding to a biomolecule's active site promises improved selectivity for the electrochemical detection of biomolecules.

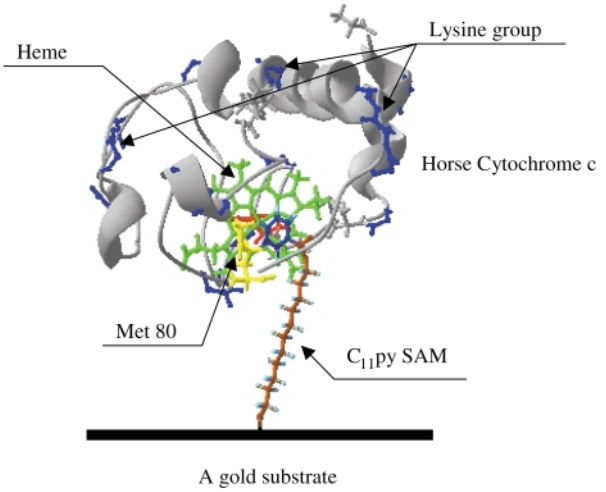

Fig. 2 Illustration of the association of a pyridine tether to the heme of cytochrome c. Also shown are the lysine groups that are present on the protein surface.

We are grateful to Professor J. Shin, A. R. Lajmi, and A. M. Napper for helpful advice. We acknowledge the Department of Energy for partial support of this work. We thank Hach Corporation for loan of the VoltaLab PGZ407 during part of this study.

Notes and references

1 M. Fedurco, *Coord. Chem. Rev.*, 2000, **209**, 263.
2 M. J. Tarlov and E. F. Bowden, *J. Am. Chem. Soc.*, 1991, **113**, 1847; M. Collinson, E. F. Bowden and M. J. Tarlov, *Langmuir*, 1992, **8**, 1247; S. Song, R. A. Clark, E. F. Bowden and M. J. Tarlov, *J. Phys. Chem.*, 1993, **97**, 6564.
3 C. J. McNeil, D. Athey and H. Wo, *Biosens. Bioelectron.*, 1995, **10**, 75.
4 The cytochrome does not adsorb onto these films. See S. Terrettaz, J. Cheng, C. J. Miller and R. D. Guiles, *J. Am. Chem. Soc.*, 1996, **118**, 7857.
5 T. G. Spiro and X. Y. Li, *Resonance Raman Spectra of Heme and Metalloproteins in Biological Applications of Raman spectroscopy*, Wiley, NY, 1988, vol. 3, p. 1; S. Hirota, T. Ogura, I. K. Shingawa, S. Yoshikawa and T. Kitagawa, *J. Phys. Chem.*, 1996, **100**, 15 274; S. R. Yeh and D. L. Rousseau, *J. Biol. Chem.*, 1999, **274**, 17 853; M. Smith and G. Mclendon, *J. Am. Chem. Soc.*, 1981, **103**, 4912; J. C. Ferrer, J. G. Gullemette, R. Bogumil, S. C. Inglis, M. Smith and A. G. Mauk, *J. Am. Chem. Soc.*, 1993, **115**, 7507; G. Liu, Y. Chen and W. Tang, *J. Chem. Soc., Dalton Trans.*, 1997, 795.
6 B. A. Feinberg, X. Liu, M. D. Ryan, A. Schejter, C. Zhang and E. Margoliash, *Biochemistry*, 1998, **37**, 13 091.
7 S. G. Sivakolundu and P. A. Mabrouk, *J. Am. Chem. Soc.*, 2000, **122**, 1513.
8 D. H. Murgida and P. Hildebrandt, *J. Am. Chem. Soc.*, 2001, **123**, ASAP.
9 H. Finklea, *Electroanal. Chem.*, 1996, **19**, 109.