KCTCCA, a peptide-based facilitator for bioelectrochemistry

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Electrochemical and scanning tunnelling microscopy (STM) studies have been carried out to investigate the suitability of the hexapeptide KCTCCA as a facilitator for bioelectrochemistry. The stable, quasi-reversible electrochemical response of cytochrome b_{562} on a KCTCCA modified gold electrode and the high degree of surface coverage of KCTCCA on gold (111), as observed by STM, indicate applicability of the molecule as an electrochemical facilitator.

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1. Introduction

Facilitators (earlier called promoters) (Eddowes and Hill 1977; Allen *et al* 1984) are small organic/biological molecules that are used to modify an electrode surface to generate a sterically and electrostatically compatible surface for bioelectrochemistry. Irreversible degradation due to direct adsorption of the protein molecules onto an electrode surface can be avoided, and the distance between electrode surface and the protein molecules can be reduced by means of electrostatic attraction leading to fast and detectable electron transfer between protein molecules and the facilitator modified electrode. The use of cysteine, its synthetic derivatives and small peptides as electrochemical facilitators (Di Gleria *et al* 1986; Barker *et al* 1990; Cooper *et al* 1993) adds a new dimension to the study of bioelectrochemistry. These molecules are biological in nature, can form a dynamic, conformationally flexible and versatile adlayer, and therefore, facilitate accommodation of many different protein orientations at the electrode surface. KCTCCA (Lys-Cys-Thr-Cys-Cys-Ala) is an important addition to this group of biological facilitators. The sequence of KCTCCA is so chosen that the electrode can be positively charged (through lysine), the molecules can be anchored on gold surface and maintain a horizontal orientation (through the three cysteine sites), and the electrode surface has hydrophilic (through threonine) as well as hydrophobic (through alanine) sites. It can promote electrochemical response of a number of primarily negatively charged proteins e.g. flavocytochrome *c* and methane monooxygenase apart from cytochrome b_{562}, a small heme protein consisting of 106 amino acid residues with a molecular weight of 12 kDa and a surface charge of – 2 at pH 7. In the present investigation, the KCTCCA/cytochrome b_{562} system has been studied by direct current (DC) cyclic voltammetry and the peptide adlayer has been studied by scanning tunnelling microscopy (STM). STM is known to provide direct 3-dimensional information on adsorbed atoms/molecules at ultra high resolution. Though atomic force microscopy (AFM) is generally preferred for imaging non-conductors, enhanced resolution of STM has made the technique more appropriate for imaging small biomolecules like amino acids (Feng *et al* 1988; Dakkouri *et al* 1996) and small peptides (Davies *et al* 1990). In this report, the observations of a quasi-reversible electrochemical response of cytochrome b_{562} on KCTCCA modified gold electrode and dense coverage of KCTCCA over large area on the gold (111) sur-

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Abbreviations used: DC, Direct current; STM, scanning tunnelling microscopy; TFE, 2,2,2-trifluoroethanol.

face are presented. These observations extend support towards applicability of the peptide as an electrochemical facilitator.

2. Materials and methods

2.1 *Electrochemistry experiment*

DC cyclic voltammetry experiments were carried out with a potentiostat (Autolab PGSTAT 10) from EcoChemie. Polycrystalline gold electrode was used as the working electrode, saturated calomel electrode (SCE) as the reference electrode and platinum gauge as counter electrode. The electrodes were set in a two-compartment electrochemical cell. The gold electrode was cleaned by polishing with alumina slurry followed by scanning in 1 M H_2SO_4 within scan range – 200 mV to 1700 mV at a scan rate 100 mVs^{-1} for 20 cycles. The electrode was finally sonicated for 5 min prior to modification. It was modified with 1 mM peptide solution in 2,2,2-trifluoroethanol (TFE) for 1 h by simple dipping method, then washed with AR ethanol and ultrapure milli-Q water (Millipore) of resistivity 18·2 MΩ·cm. The cyclic voltammetry experiments were carried out at room temperature with 70 μM cytochrome b_{562} (wild type) solution prepared in milli-Q water.

2.2 *STM experiment*

2.2a *Set up, probes and image acquisition*: The STM studies were carried out in ambient conditions at room temperature using a TopoMetrix Discoverer SPM (TMX 2000) with 1 μm scanner. The tips were mechanically cut from a 0·25 mm diameter Pt-Ir (90 : 10) wire. The tips used were tested for atomic level imaging on highly oriented pyrolytic graphite (HOPG) before each experiment. The gold substrates were annealed in butane flame for few seconds till red hot to produce high quality surfaces of (111) orientation. Imaging was carried out in the constant current mode with tunnel currents (I_t) 0·3–0·7 nA and a bias voltage (V_b) ± 200 to ± 800 mV. The scan rates were 7–20 times the magnitude of the scan size. The images were subjected to a first order 2D leveling during image acquisition.

2.2b *Sample preparation with KCTCCA*: The gold substrate was annealed and cooled for a short period (1–2 s) under argon jet, the substrate was then immersed in 1 mM peptide (from Peptide Synthesis Facility, Oxford Centre for Molecular Sciences, Oxford University, UK) solution prepared in TFE (99%, Aldrich). It was incubated for 30 min to 1 h and then gently rinsed with AR ethanol to remove the weakly bound material followed by drying in a covered box for 1 h and immediately imaged.

3. Results and discussion

In figure 1, the electrochemical response of cytochrome b_{562} on polycrystalline gold electrode, modified with KCTCCA, is shown. A quasi-reversible response with a peak separation of 90 mV [E_c = – 0·01 V and E_A = 0·08 V] is observed. No voltammetric response was observed in any of the control experiments carried out with peptide modified electrode in the absence of protein molecules and the unmodified electrode in the presence of protein solution at similar scan parameters. The modified electrode could be used for a maximum period of 5–6 days. After this time period, a weak voltammetric response was observed indicating electrode fouling. Though drastic passivation of the electrode by adsorption of protein molecules was not observed at KCTCCA modified surface, a quasi-reversible electrochemical response may indicate electrode passivation to some extent. This is not unexpected, given the electrode coverage by facilitator molecules can remain incomplete according to the Langmuir adsorption theory and protein molecules can undergo irreversible adsorption on the unoccupied areas.

From the quasi-reversible response on KCTCCA modified electrode, it is evident that the e' transfer rate between cytochrome b_{562} and gold electrode is not as fast as in some other cases e.g. response of the heme protein cytochrome *c* on hydroxyalkane thiol [$HO(CH_2)_nSH$, where n = 3,4] modified gold electrode. However, reversible behaviour of cytochrome *c* on hydroxyalkane thiol modified gold surface shifted towards irreversibility with increase in alkane chain length from 3 to 11 carbon atoms (Terrettaz *et al* 1996). The number of intermediate atoms between thiol group and the solvent exposed functional groups – NH_3^+, – OH and – CH_3 in KCTCCA varies from 3 (for the peptide bond region) to 8 atoms (for the lysine side chain region). This creates a situation similar to hydroxyalkane thiols of intermediate chain length (n = 5–8). This factor of molecular thickness along with electrode passivation may explain the observation of relatively slow e' transfer on peptide modified electrode compared to the reversible behaviour. Methods of electrode modification other than simple immersion in the molecular

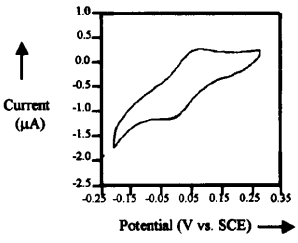

Figure 1. Electrochemical response of wild type cytochrome b_{562} on KCTCCA modified gold electrode (scan rate = 20 mVs^{-1}).

solution may generate better coverage and therefore, enhance the rate of e' transfer between protein molecules and the modified electrode.

The STM images of KCTCCA were taken on atomically flat triangular terraces of gold (111) substrate (figure 2). In figures 3 and 4, two characteristic images showing dense coverage of self-assembled structural array of KCTCCA over a large area are presented. From the cross-sectional diagrams, the distance between two consecutive rows is found to be 29 ± 1 Å, the width of the rows 38·5 ± 4 Å and the average height of the peaks 1·6 ± 0·3 Å. The height value appears unusually low compared to the expected real height (> 5 Å). A lower apparent height is, however, a usual observation in the STM images of biomolecules, since these molecules are not sufficiently conductive (resistivity: typically 10^{15}–10^{18} Ω m at room temperature) (Pethig 1979) and the tunnel probe either compresses the molecular layer or travels inside the film to detect the assigned tunnel current resulting in a smaller observed height. Control experiments were carried out by imaging bare gold (111) and TFE-treated gold (111) at the same scan parameters as used for imaging the peptide film. No major contamination, surface roughening and features like the peptide array were observed.

Dense arrays of KCTCCA have been observed over large areas of the gold (111) surface (figures 3 and 4). Many thiols and disulfides have been reported to form ordered and oriented self-assembled arrays on gold surfaces (Hähner *et al* 1993; Kooyman *et al* 1994; Nuzzo *et al* 1990), where the packing and ordering are determined by contributions of both chemisorption and intra-/interchain nonbonded interactions. For KCTCCA, a van der Waals type interaction appears to be responsible for array formation. An added factor can be the repulsive interaction between coadsorbed molecules. This is a possibility when the bulk concentration is quite high e.g. 1 mM as it is in the present case. Such repulsive interactions can lower the average heat of adsorption and reduce strength of the chemical bond. Consequently, the strength of adsorbate-adsorbate interaction relative to the adsorbate-substrate interaction should increase compared to a situation where the bond strength is unchanged. This creates a more favourable situation for generating a self-assembled array of chemisorbed molecules on the gold surface. The rows observed in figures 3 and 4 are not entirely parallel though over the whole scan area as a consequence of lack of registry of the molecular packing with the underlying gold lattice. This is not unexpected, given the thiols are not regularly spaced in the sequence KCTCCA, and the molecule may adsorb through all the three thiols for the obvious benefit of exothermic gold-thiol bond formation (Nuzzo and Allara 1983).

High resolution images of the peptide molecules were not obtained plausibly due to electron delocalization over the peptide backbone and/or tip-sample interactions that are unavoidable in STM imaging of biomolecules in ambient conditions. But importantly, self-assembled arrays of KCTCCA were observed to generate a dense coverage of peptide molecules over a large area on the gold electrode surfaces. Such an adlayer should prevent adsorption induced degradation of the protein structure and assist in electrochemical promotion. Furthermore, presence of sulphur atoms in the system and their involvement in direct interaction with the gold surface makes the molecule electronically addressable. The role of secondary structure of the peptide in electron mediation can be insignificant, since from circular dichroism studies it was found that the peptide remains in random coil state (a negative band between 190 and 200 nm, which is typical of a random coil structure, was observed; data not shown) even in a coil inducing solvent like TFE. It is evident from the present results that neither secondary structure of the peptide nor ordering/disordering of the molecules in the adlayer are crucial factors in electrochemical promotion. The more important factors controlling the facilitator action of KCTCCA appear to be the dense coverage over a large area of the electrode, electrostatic compatibility due to

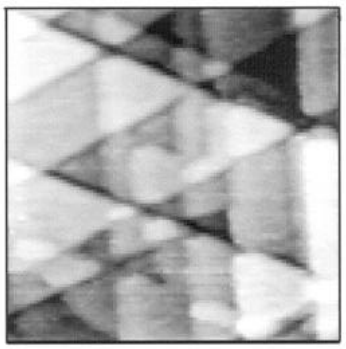

Figure 2. Gold (111) substrate; scan range = 300 × 300 nm^2.

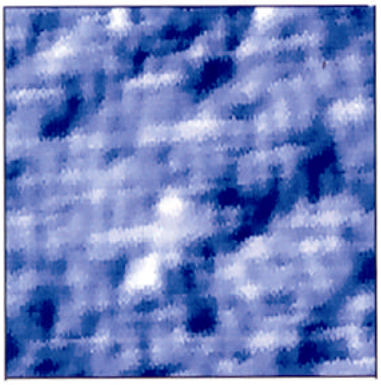

Figure 3. Self-assembled peptide array on gold (111) (I_t = 0·5 nA; V_b = – 800 mV; scan range = 80 × 80 nm^2; z range = 0–0.49 nm).

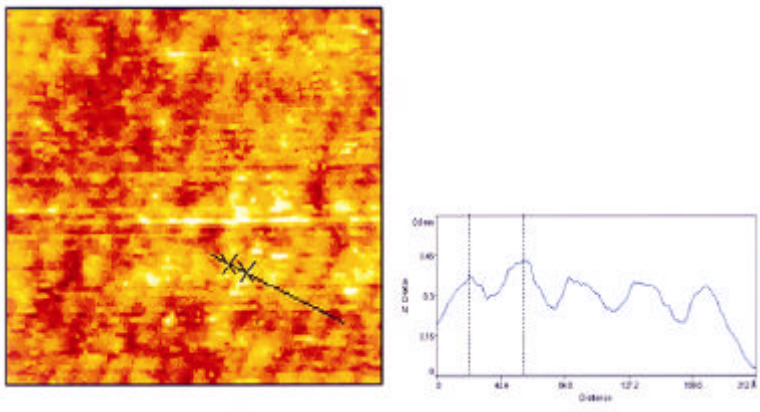

Figure 4. Peptide array with a cross-sectional diagram (I_t = 0·7 nA; V_b = – 800 mV; scan range = 66 × 66 nm^2; z range = 0–0·59 nm).

presence of lysine, and the gold-sulphur tunnel routes generated due to strong gold-thiol interactions.

The presence of – OH containing threonine and – CH_3 containing alanine in KCTCCA are supposed to impart hydrophilic and hydrophobic nature respectively to the peptide film. The versatility of this peptide can be enhanced further due to presence of the charge from – NH_3^+ of lysine side chain. Earlier also, a facilitator like hydroxyalkane thiol exhibited versatility with respect to promoting electrochemical response of both +vely and –vely charged proteins. It appears the versatile behaviour of hydroxyalkane thiol can be attributed to the hydrophilic character of the facilitator film due to presence of the hydroxyl groups. Proteins with predominantly hydrophilic surface patches, whatever the surface charge may be, +ve or –ve, therefore enjoy compatibility with the hydroxyalkane thiol modified surfaces. The purpose behind the present work was, however, not to create a surface of very specific characteristic determined by any one of the factors charge/hydrophilicity/hydrophobicity/thickness of the film but to mimic the biological surface – a system influenced by all these factors as close as possible. Earlier, the concept of a functionalized electrode surface originated from the dream to mimic the protein surfaces that are responsible for protein–protein interactions relevant in biological *e′* transfer. Though over the last two decades, surface functionalization with small organic molecules has led to a variety of surfaces of technological importance, especially in sensor technologies, yet the attempts to create a truly biological and therefore biocompatible surface are being continued. The present endeavour is an important addition to that attempt.

In the present manuscript, it has been shown that a biological molecule of size bigger than an amino acid but smaller than a protein can be used as a facilitator. This success may lead to the use of protein molecules as facilitators fulfilling the dream of construction of a bio electrode/protein chip. Important progress has already been made towards this end. Oriented immobilization of the protein molecules on gold electrode surface via gold-thiol bond formation is now possible (Mukhopadhyay *et al* 2004). This strategy of oriented immobilization has also aided solvent exposure of the interaction sites on protein surface and subsequent complex formation with the partner enzyme molecules (Mukhopadhyay *et al* 2002).

4. Conclusion

In conclusion, the possibilities of the use of a medium size peptide as an electrochemical facilitator has been discussed. This has ramifications towards application of bigger peptides and small proteins in constructing bioelectrodes important in sensor technology.

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