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REGULAR ARTICLE

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Transition metal complexes as promoters of direct electron transfer from gold electrodes to cytochrome c

SRIPARNA RAY[a,b], DINESHKUMAR YADAV[a,c], SHIVRAM S GARJE[c] and SHYAMALAVA MAZUMDAR[a,*]

[a]Department of Chemical Sciences, Tata Institute of Fundamental Research, Homi Bhabha Road, Colaba, Mumbai 400 005, India
[b]Department of Chemistry, Faculty of Science, School of Basic Sciences, Manipal University Jaipur, Dehmi Kalan, Jaipur, Rajasthan 303007, India
[c]Department of Chemistry, University of Mumbai, Kalina, Mumbai, Maharashtra 400098, India
E-mail: shyamal@tifr.res.in

Dedicated to Prof. (late). Bhaskar G Maiya.

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Abstract. Direct electrochemical responses of cytochrome c on a gold electrode modified by thiol-containing monolayer of Schiff base (SB) or its metal complexes (M-SB, M = Fe, Mn, Cr) have been investigated to determine the role of the monolayer in promoting heterogeneous electron transfer. The monolayer of the SB prepared *in situ* by the sequential association of L-cysteine followed by conjugation with salicylaldehyde on a freshly cleaned gold electrode formed a stable thin film of cytochrome c. The thin films of cytochrome c on SB or M-SB modified electrode showed quasi-reversible cyclic voltammetric signals, and the observed midpoint potential agreed with that reported earlier. The surface coverage (Γ) of the active cytochrome c in the thin film was found to vary with the nature of the metal ion in M-SB, and the value of Γ increased in the order: SB < Mn-SB < Cr-SB < Fe-SB in the monolayer, suggesting that the metal ion coordination may be important for the stability of the monolayer of M-SB and the formation of the thin film of the protein. The electron transfer rates (k_s) were found to be faster with the SB or M-SB monolayers compared to many other small promoters reported earlier. The k_s values were however almost independent of the metal binding to the SB, indicating that the electron transfer across the monolayer of the SB complex may not be the rate determining step for the heterogeneous electron transfer from the gold electrode to cytochrome c.

Keywords. Direct electron transport; Cytochrome c; Cyclic voltammetry; AFM; Resonance Raman.

1. Introduction

Among the various heme-containing metalloproteins, the cytochromes are the most versatile and ubiquitous in nature. Most prominent among them is cytochrome c that is located in the intermembrane space in the mitochondria and plays the key role in the final step of respiratory electron transfer.[1–4] Unlike most of the hemeproteins, cytochrome c consists of a covalently linked heme (heme c) bound to the amino acid chain through thioether linkage with two cysteine residues (Cys 14 and Cys 17) and the axially coordinated histidine (His 18) forming a very stable heme cavity.[5] The heme in cytochrome c accepts electrons from the complex III (cytochrome b-c1 complex) located at the inner mitochondrial membrane (cristae of mitochondria) and delivers the electron to the outer side of cytochrome c oxidase (complex IV) and transfers electrons to the cytochrome c oxidase complex in the inner mitochondrial membrane.[3,4,6] Studies of the electron transfer properties of cytochrome c has provided immense information on the mechanism of the

*For correspondence

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redox processes in the protein. Cytochrome c undergoes rapid redox state changes from Fe(III)-heme to Fe(II)-heme form on accepting the electron and reverses back to the Fe(III) form on donating the electron. The electron transfer properties of the protein, especially, the rate of electron transfer, the individual reactivity characteristics of the reactants and the distance between the redox centers are very important for efficient electron transfer function of the protein. In view of understanding the electron transfer properties of cytochrome c, extensive electrochemical studies were carried out.[7–9] Graphite and related electrodes, were shown to give quasi-reversible electrochemistry of cytochrome c and related systems.[10–15] The bare metal electrodes, such as gold, platinum, etc., on the other hand do not show any electrochemical response of cytochrome c primarily because of irreversible denaturation of the protein on the electrode surface leading to passivation of the electrode, while suitable modification of the electrode could promote electrochemistry of cytochrome c.[9–11,16,17]

The electron transfer between the electrode and the protein requires the association of the protein without affecting the structure of the protein. Extensive studies on electrode modification for efficient electron transfer between the electrode and the protein has been reported in literature.[7,9–11,14,16,18–21] Small bifunctional molecules such as bis(4-pyridyl) bisulphide, other small molecules as well as surfactants and lipids were shown to act as promoters of direct electrochemistry of the protein.[9,14,22] Thin films of immobilized proteins on the electrode surface have been used to achieve direct electrochemistry of cytochrome c and other proteins.[11,23–26] Earlier studies have also demonstrated that immobilization of certain proteins on the electrode surface could promote direct electrochemistry of another protein.[27–30]

Immobilization of the protein on a chemically modified electrode forming a self-assembled monolayer of the protein provides a simple method of designing active protein electrodes important for biosensors and other applications. Several electrode modifiers have earlier been investigated for direct electrochemistry of proteins immobilized on the electrode surface.[12,18–26,31,32] Thiol containing molecules have been shown to preferentially bind to the gold electrode and many such compounds were shown to promote electrochemistry of cytochrome c and related proteins.[9,18–20,22] Schiff bases constitute an interesting class of ligands that are formed at ambient conditions and they can bind a wide variety of metal ions in situ forming the corresponding metal complexes. Self-assembled monolayers of such simple ligand molecules and their metal complexes formed on the electrode surface could provide a stable surface to immobilize the protein. There have been no studies on the electron transfer properties of the Schiff bases and their metal complexes and it would be interesting to investigate the self-assembled layers of the Schiff base complexes as promoters of direct electrochemistry in thin films of a redox protein.

The present report describes detailed studies of the formation of thiol-containing Schiff base and its metal complexes immobilized on a gold electrode. Direct electrochemistry of protein films of cytochrome c immobilized on the monolayers of the Schiff base has been investigated to understand the heterogeneous electron transfer through such a monolayer. The effect of transition metal ion binding to such ligands in promoting electron transfer from the electrode to the protein has also be investigated to unravel the possible role of metal ion binding to the Schiff base on the property of the monolayer. Result showed that the self-assembled layers of the Schiff base (2-((2-hydroxybenzylidene)amino)-3-mercaptopropionic acid) formed *in situ* on condensation of L-cysteine and salicylaldehyde at freshly cleaned gold electrode surface. Horse cytochrome c could be successfully immobilized on the modified gold electrode containing the monolayer of the Schiff base and its metal complexes, which acted as promoters of quasi-reversible electrochemistry of cytochrome c.

2. Experimental

2.1 *Chemicals*

Horse heart cytochrome c was obtained from Sigma, USA. It was further purified by passing through a Sephadex G-25 (PD-10) column equilibrated with 40 mM potassium phosphate buffer, pH 7.4. L-cysteine, Salicylaldehyde, ferrous chloride hexahydrate, chromium(III) chloride hexahydrate and manganese(II) chloride tetrahydrate were obtained from SD-Fine Chemicals, India and were used without further purification. All chemicals were of analytical grade.

2.2 *UV–vis spectroscopy*

UV–vis spectra were taken using PerkinElmer Lambda-750 spectrophotometer. The concentration of cytochrome c (in the reduced form) was determined

from the absorption spectrum of the protein, using an extinction coefficient of 29 mM^{-1} cm^{-1} at 550 nm.[33] The concentration of the protein used for the incubation of the electrode for the preparation of thin film of the protein was ~ 50 μM.

2.3 *Electrochemical studies*

Cyclic voltammetry experiments were carried out at room temperature using VersaSTAT 3F potentiostat in a three-electrode assembly with Ag/AgCl (3.5 M KCl) as the reference electrode, a platinum wire mesh as the counter electrode and a gold electrode as the working electrode. Platinum wire was obtained from Sigma–Aldrich. The gold and Ag/AgCl electrodes were obtained from CH-Instruments, USA. The gold electrodes were first polished on micro-cloth with alumina suspension (0.3 micron and 0.05 micron) for 5 min. Subsequently, they were cleaned electrochemically in 0.5 M sulfuric acid by potential cycling in the range of − 0.3 V to 1.5 V at the potential scan rate of 100 mV s^{-1} until the cyclic voltammogram, characteristic for a clean gold electrode, was obtained. The electrode potential values were reported with respect to the Ag/AgCl reference electrode.

2.4 *Preparation of monolayer of Schiff base on the gold electrode*

The pretreated freshly cleaned gold electrodes were immediately immersed in a 20 mM aqueous solution of L-cysteine in a nitrogen atmosphere and incubated for 2-4 h at room temperature. The free thiol groups of cysteine would form covalent bond with gold. The electrode surface containing the L-cysteine monolayer was thoroughly washed with water, dried under N_2 gas and subsequently incubated in a dry ethanolic solution of salicylaldehyde (20 mM) for overnight to form the Schiff base (SB) ligands on the electrode surface. The monolayer of the SB covalently attached to the gold electrode was washed with ethanol and dried under N_2 gas. Incubation the SB containing electrode in an ethanolic solution of metal salt (20 mM) of iron ($FeCl_2$), Mn ($MnCl_2$) or Cr ($CrCl_3$) at room temperature separately for overnight to form the corresponding M-SB in the self-assembled monolayer on the gold electrode. The electrodes containing self-assembled monolayer of the Schiff base (SB) or of the metal complexes of the Schiff base [M-SB, M = Fe(III), Mn(III), Cr(III)] were separately incubated into cytochrome c solution (50 μM) in 40mM phosphate buffer solution (pH 7.4) for overnight at 4 °C to form thin films of cytochrome c immobilized on the modified electrode. The electrode was then removed, rinsed with Milli-Q water and allowed to dry under nitrogen flow at 4 °C before further studies.

2.5 *Atomic force microscopy (AFM) measurements*

The experiments were performed with a commercially available Parks Systems XE-70. The cantilevers used in this experiment were n-type Si cantilevers. The cantilever was oscillated in the constant excitation mode. The typical spring constant was 30 N/m. No special tip treatment was carried out. Non-contact-AFM images were acquired in the constant frequency shift mode using a frequency modulation (FM) detection method. The bias voltage of both the cantilever and the sample were kept at zero during the NC-AFM imaging. Data analysis of the surfaces was done with the XEI software.

2.6 *Resonance Raman (rR) Study*

Resonance Raman spectra of the samples were recorded using a Horiba Yvon Raman spectrometer (T64000), with an inverted optical microscope adapted to a triple grating equipped with (1024 × 256) liquid N_2-cooled CCD. A Spectra-Physics model with an argon ion laser provided the excitation at 514 nm. The laser power at the sample was 5-6 mW and was focused using a 50X objective lens. Wavenumber calibration was conducted using the 521 cm^{-1} line of the silicon wafer. Spectra were recorded by scanning the 1200-1800 cm^{-1} region with a total acquisition time of 200 s, and data were averaged over five scans.

3. Results and Discussion

Crystalline gold surfaces are known to facilitate the formation of thiol-containing self-organized assemblies.[34] The precise nature of the gold sulfur interactions have provided an excellent procedure for the formation of chemisorbed molecular films. Various long chain alkane thiols have been adsorbed onto gold surfaces, thereby forming self-assembled monolayers.[11] The cysteine molecules immobilized on the gold electrode through strong Au-S bonds in the array would form the monolayer of (2-((2-hydroxybenzylidene) amino)-3-mercaptopropionic acid (Schiff base, SB) by condensation with salicylaldehyde (Figure 1).

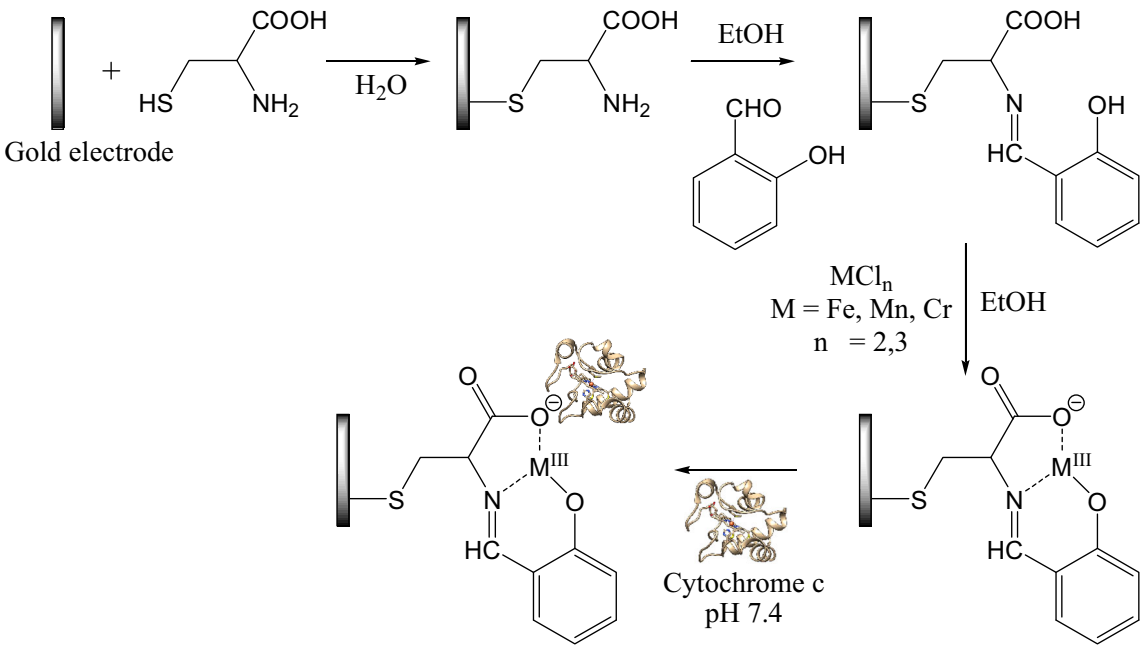

Figure 1. Schematic representation of electrode modification with M-Schiff base complex and cytochrome c, where M = Fe(III), Mn(III), Cr(III).

The SB would have three ligand centers viz., -C=N- (Azomethine), -COO (Carboxylic) and C-O (phenolic) groups, which may bind to metal ions to form the corresponding metal-Schiff base complex (M-SB) as shown schematically in Figure 1. Earlier studies have shown that transition metal ions can form strong complexes of the Schiff base[35] and formation of such complex on the electrode surface would form an array of M-SB on the electrode surface. The carboxylic acid (-COO) as well as the metal center may stabilize cytochrome c molecules on the SB or M-SB monolayer as depicted in Figure 1 forming a stable thin film of the active protein on the modified electrode surface. Incubation of unmodified electrode with cytochrome c is known to cause passivation of the electrode with adsorption of denatured/partially unfolded protein on the hydrophobic metal surface.[9] The electrode was thoroughly washed with cold Milli Q to remove any non-specifically bound protein before further experiment so that the electrochemical and other results solely correspond to the thin film of cytochrome c immobilized on the electrode.

3.1 *Characterization of modified electrodes: atomic force microscopy (AFM) studies*

The formation of the self-assembled monolayers of the Schiff base and their metal complexes showed distinct signatures in the morphology of the electrode surface. Atomic force microscopy (AFM) is most widely used as an easy and efficient method to map the surface topography of self-assembled layers. Freshly cleaned gold electrode showed very smooth surface with almost uniform height distribution detected in the AFM showed in Figure 2A exhibiting nonspecific step edges, which are in good agreement with typical AFM images of Au substrates. Presence of arrays of the Schiff base complex (eg., Fe-SB) on the electrode surface was detected in AFM (Figure 2B) showing periodic structure on the electrode surface. The distribution of the height profile also showed decrease in surface uniformity though the magnitude of the peak height detected by AFM seems too high for only the monolayer of the Schiff base. The height distribution detected in AFM studies depends on various factors including the nature of the AFM tip. Nevertheless, it provides qualitative information of formation of the array on the electrode surface. Immobilization of cytochrome c on the electrode modified with the Schiff base complex showed well-defined periodic distribution of the protein on the electrode surface suggesting formation of protein film on the self-assembled layer on the electrode. It was observed that the surface height analysis for the entire 5 μm × 5 μm scanning range in Figure 2C gave almost a monomodal height distribution, suggesting that the surface-attached globules were the cytochrome c molecules. It is noteworthy that the AFM images of the surface-

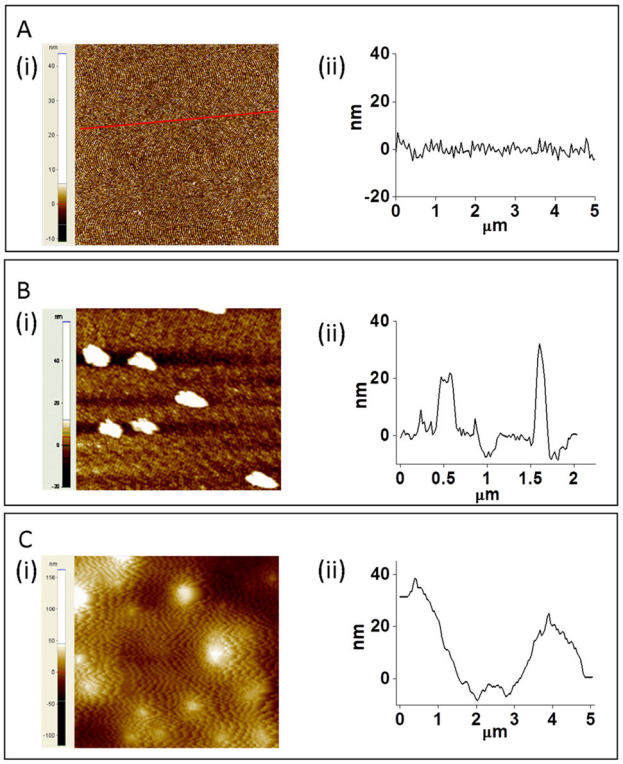

Figure 2. A (i) AFM images of bare Au substrate containing polycrystalline Au(111) plane. (ii) Distribution of height of the polycrystalline Au(111) plane along the line profile. B (i) AFM images of Au substrate modified with Fe(III) Schiff base complex. (ii) Distribution of height of the Fe(III) Schiff base complex on Au substrate along the line profile. C (i) AFM images of immobilized cytochrome c on Au substrate modified with Fe(III) Schiff base complex. (ii) Distribution of height of the cytochrome c on Au substrate modified with Fe(III) Schiff base complex along the line profile.

attached cytochrome c are stable, and reproducible even after repetitive scans.

3.2 *Raman spectral studies*

Resonance Raman spectroscopy can be used as a sensitive structural probe to determine any conformational changes in the protein. The resonance Raman spectra of the cytochrome c immobilized onto the modified gold electrode containing the monolayer of Schiff base complexes was studied in order to determine whether there was any alteration in the conformation of the heme center in the protein. Figure 3 shows the resonance Raman spectra of cytochrome c immobilized on the electrodes modified with Schiff base and metal-Schiff base complex, obtained by excitation of a heme electronic transition (at 514 nm). Figure 3 also shows the rR spectrum of cytochrome c

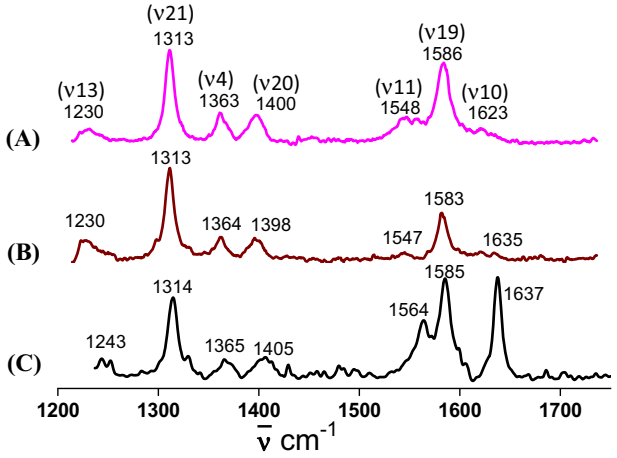

Figure 3. (A) Resonance Raman spectra of thin film of Cytochrome c immobilized on gold electrode modified with monolayer of Mn(III)-Schiff base complex; (B) Resonance Raman spectra of thin film of Cytochrome c immobilized on gold electrode modified with monolayer of Fe(III)-Schiff base complex; (C) Resonance Raman Spectrum of the protein in phosphate buffer solution. All measurements were taken using excitation at the Q band (514 nm) and incident powers of 5-6 mW at room temperature. The spectra were recorded with data accumulated over 200 secs averaged over five scans.

in solution in presence of separately synthesized Schiff base complex. The resonance Raman (rR) spectroscopy of cytochrome c has been extensively studied earlier.[36–38] The rR spectrum of the cytochrome c obtained on excitation of the heme transition (at 514 nm) corresponds to transitions due to various vibrational modes of the heme (porphyrin ring). As the heme moiety in cytochrome c is rigidly anchored to the protein matrix,[5] the heme resonance Raman signals show certain signatures of distortion of the heme ring. The resonance Raman transitions were assigned based on the earlier report of complete assignment of resonance Raman spectrum of the protein.[37] The spectrum observed in case of the cytochrome c immobilized on the metal-Schiff base (M-SB) showed two strong bands at 1313 and 1586 cm^{-1} assigned to ν21 and ν19, respectively, and the other bands at 1233 (ν13), 1363 (ν4), 1400 (ν20), 1548 (ν11) and 1623 (ν10) cm^{-1}, respectively, were of medium intensity and the relative intensities of the bands agreed with earlier reports.[37,38] The observation of the characteristic heme in-plane skeletal vibrational frequencies in the resonance Raman spectrum of cytochrome c immobilized onto the chemically modified electrode support that the heme center of the protein was not perturbed and thus the folded (tertiary) structure of the protein remained intact when associated with the SB. The SB and M-SB consists of free carboxylate group

(Figure 1) which may form electrostatic bonds with surface exposed lysine residues of cytochrome c. Furthermore, the metal center coordinated to the SB would also be able to bind to ligand amino acids on the protein surface. Thus, the protein may be immobilized on the monolayer of the SB or M-SB through polyvalent interactions retaining the overall structure. It is however not possible to determine whether cytochrome c would have any preferred surface patch to associate to the SB or M-SB, and the possibility of the presence of multiple orientations of the protein immobilized on the electrode cannot be ruled out in the present case.

3.3 *Electrochemistry of cytochrome c on modified gold electrodes*

The bare gold electrode does not show any electrochemical response of Cytochrome c and the electrode surface was found to become passivated on incubation of freshly cleaned electrode in cytochrome c solution (Figure S1, SI). Earlier studies have shown that the proteins tend to irreversibly adsorb at the metal electrode surface causing denaturation of the protein and passivation of the electrode.[9,10,39,40]

Voltammetric studies of cytochrome c involve redox reaction of the heme center that is embedded in the protein cavity. The structure of the porphyrin ring around the metal ion (Fe) properties of the axial ligands and the nature of the protein environment play a key role in tuning the redox potential as well as the redox potential of the protein. The structure analyses of cytochrome c in oxidized (PDB code 1HRC.PDB) and reduced (PDB code: 1GIW.PDB) suggested that the conformation of the heme center, as well as the coordination geometry of the metal ion, remains almost the same in both oxidized and reduced forms. The redox potential of the heme in cytochrome c thus can be easily determined by the conventional concept of mid-point potential from the Faradaic signals in the cyclic voltammetric studies of the protein. The thin film of cytochrome c is expected to be formed by immobilization on the Schiff base or its metal complex bound to the gold electrode. The monolayer of Schiff base or its metal complex would serve as promoters and also immobilize the protein forming a thin film of the protein on the electrode. Figure 4 schematically shows the redox processes during the voltammetric experiments of cytochrome c immobilized on the gold electrode through the promoter layer of SB or M-SB. The electrochemical cell contained an aqueous buffer

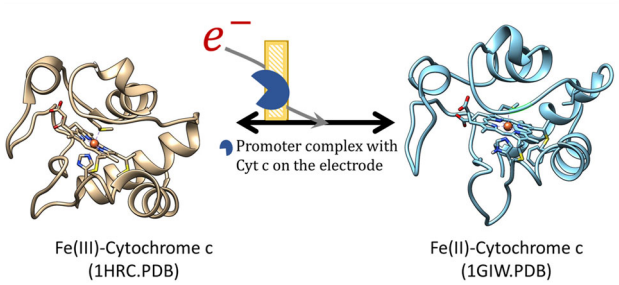

Figure 4. Schematic representation of electrochemical reduction of Fe(III)-cytochrome c (Crystal structure 1HRC.PDB) forming Fe(II)-cytochrome c (Crystal structure 1GIW.PDB) immobilized on the gold electrode containing monolayer of the Schiff base complex as promoter for electrochemistry.

with supporting electrolyte in which the working gold electrode containing the thin film of the protein is dipped along with the counter and reference electrode. So, the electrochemical response (Faradaic) arises solely from the redox changes in cytochrome c in the potential range of the experiment. The promoter monolayer formed by the SB or M-SB [M=Fe(III), Mn(III), Cr(III)] did not show any redox signal in the experimental potential range. We selected three first row transition metal ions viz., iron, manganese and chromium which showed redox potential much below -200 mV (vs Ag/AgCl) when complexes with the Schiff base (Figure S2, SI). Cyclic voltammetric studies showed well-defined quasi-reversible signals (Faradaic signals) from the thin film of cytochrome c at the scan rates from ~ 5 mV s^{-1} and above < 100 mV s^{-1} with the free Schiff base as well as different metal-Schiff base complex modified protein electrodes (Figure 5 (A-D)). The value of the midpoint potential ($E_{1/2}$) for cytochrome c in the thin film was found to be 0.05 ± 0.02 V (vs Ag/AgCl), which agrees with earlier reported value of redox potential of the heme in cytochrome c.[10] This supports that the coordination geometry as well as the tertiary structure around the heme remained unaffected on immobilization of the protein on the promoter monolayer at the gold surface. The anodic and cathodic peak currents were observed to be almost equal in all cases. The peak currents deviated significantly from linearity when plotted against square root of scan rate supporting that there was no Faradaic response from any diffusible cytochrome c that may have been released from the thin film on the electrode. It is thus important to note that the electrochemistry of cytochrome c referred only to the thin film of the protein. The peak currents were found to be directly

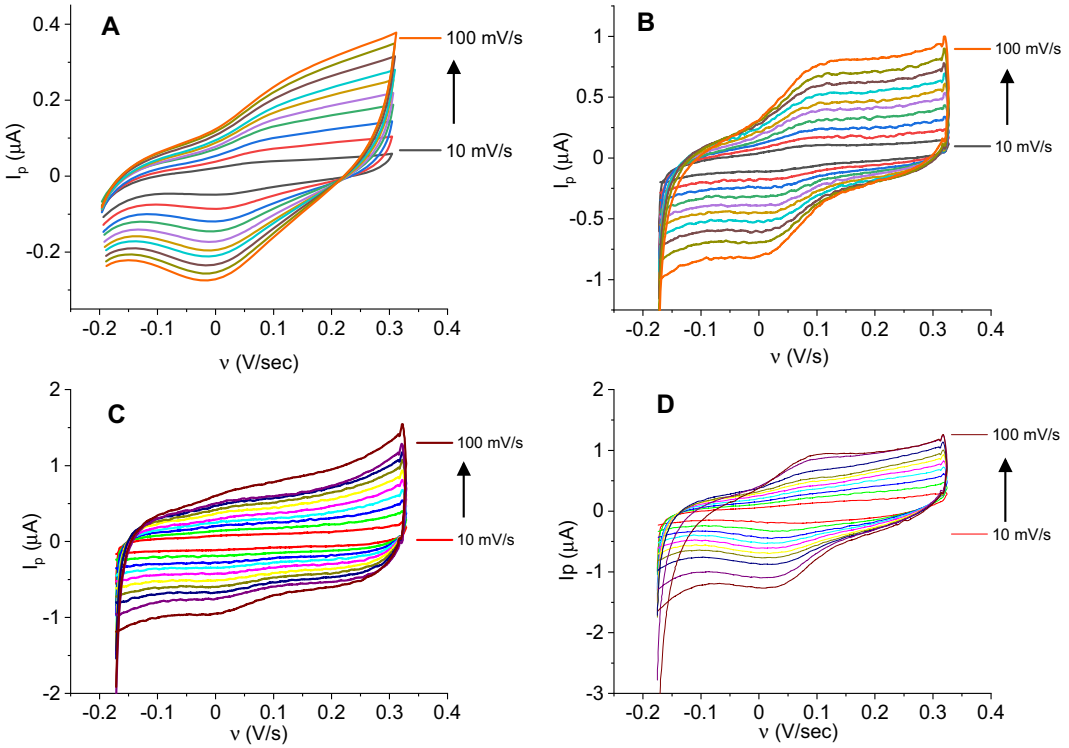

Figure 5. Cyclic voltammetric response of cytochrome c immobilized as thin film on the Gold electrode modified with monolayer of (A) Schiff base ligand (SB); (B) Chromium complex of the Schiff base (Cr-SB); (C) Manganese complex of the Schiff base (Mn-SB); (D) Iron complex of the Schiff base covalently bound to the gold surface through thiol bond of cysteine. Electrochemical cell contained 40 mM phosphate buffer, pH 7.4, 40 mM KCl at room temperature in nitrogen saturated solution at different scan rates (10-100 mV/sec).

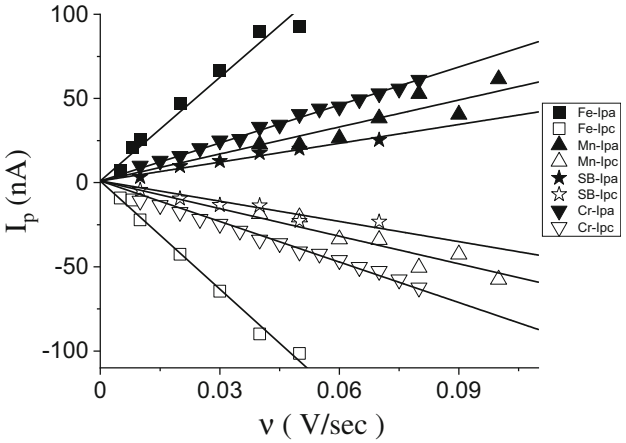

Figure 6. Plot of peak current vs. scan rate of Schiff base conjugated Cyt c on gold electrode in 40 mM phosphate buffer, pH 7.4, 40 mM KCl at room temperature in nitrogen saturated solution at different scan rates. The electrode area is 0.0314 cm^2.

proportional to the scan rate (Figure 6) in the cyclic voltammetric experiments. This indicates that the electrochemical response indeed corresponds to the protein immobilized as thin film on the on the electrode surface. The peak current of the Faradaic peaks for a surface confined reactant is given by[40]

$$I_p = \frac{n^2F^2}{4RT}A\Gamma v \quad (1)$$

where n = number of electron transferred, F = Faraday's constant, R = universal gas constant, T = absolute temperature in Kelvin ($T = 298$ K), A = surface area of the electrode, Γ = surface coverage or the concentration of the redox active molecules adsorbed in mol/cm^2, v = scan rate.

Using the known value of the electrode area ($A = 0.0314$ cm^2) and the slope of the plot of I_p *vs* for the cyclic voltammetric responses of cytochrome c immobilized on the electrode through different metal Schiff base complexes, the surface coverage Γ was estimated. The surface coverage Γ was found to depend on the nature of the promoter molecule bound onto the electrode. The free Schiff base (SB) bound onto the gold electrode $(1.3 \pm 0.1) \times 10^{-11}$ mol/cm^2, and binding of the metal ion to the Schiff base showed an increase in the surface coverage of the electroactive centers on the electrode surface for cytochrome c electrochemistry. The value of Γ increased with the nature of the metal ion in the following order: Mn(III), $(1.8 \pm 0.1) \times 10^{-11}$ mol/cm^2 < Cr(III), $(2.6 \pm 0.1)\times$

10^{-11} mol/cm^2 < Fe(III), $(7.1 \pm 0.2) \times 10^{-11}$ mol/cm^2. This result suggested that cytochrome c binds to the electrode surface through the metal Schiff base complexes and binding of the protein to the Schiff base complex depends on the nature of the metal ion present in the promoter. The observation of different surface coverages of monolayers of the SB and the metal complexes for cytochrome c electrochemistry possibly indicates the effect of binding of the metal ion on the monolayer of the Schiff base complex formed on the electrode surface.

The heterogeneous electron transfer from the gold electrode to cytochrome c through the self-assembled monolayer of SB or M-SB as the promoters. The rate of heterogeneous electron transfer between the electrode and the immobilized cytochrome c (k_s) was estimated by Laviron's method. The value of the transfer coefficient (α) was determined from the variations in the E_{pa}, E_{pc}, with the scan rate (ν), given by the equations 2a and 2b:

$$E_{pa} = E^0 + \frac{2.303RT}{(1-\alpha)nF} log\left(\frac{(1-\alpha)nF}{RTk_s}\right) = \left[E^0 + \frac{2.303RT}{(1-\alpha)nF} log\left(\frac{(1-\alpha)nF}{RTk_s}\right)\right] + \frac{2.303RT}{(1-\alpha)nF} log(v) \quad (2a)$$

$$E_{pc} = E^0 - \frac{2.303RT}{\alpha nF} log\left(\frac{\alpha nF}{RTk_s}\right) = \left[E^0 - \frac{2.303RT}{\alpha nF} log\left(\frac{\alpha nF}{RTk_s}\right)\right] - \frac{2.303RT}{\alpha nF} log(v) \quad (2b)$$

where E_{pa}, E_{pc}, are respectively the anodic and cathodic peak potentials at a given scan rate, v. E^0 is the apparent midpoint potential, $n = 1$ is the number of electrons transferred and α is the transfer coefficient. The ratio of the slopes of the plots of E_{pa} and E_{pc} against log(v), were used to estimate the value of the transfer coefficient (α) for each promoter complex associated to the gold electrode (Figure S3 in Supplementary information). The value of α was found to slightly vary with the nature of the metal ion bound to the Schiff base (M-SB) associated to the gold electrode as the promoters for electrochemistry of cytochrome c and the average value of α for the metal-free Schiff base and metal complexes of the Schiff base monolayers was found to be 0.5 ± 0.07. We assumed a value of 0.5 for α in subsequent analyses of the results.

The rate of heterogeneous electron transfer (k_s) between the electrode and cytochrome c was determined by Laviron's method from equation 3 at different scan rates:

$$\Delta E_p = \frac{2.303RT}{nF\alpha(1-\alpha)}(\alpha \log(1-\alpha) + (1-\alpha)log(\alpha) - log\left(\frac{RT}{nF}\right) - log(k_s)) + \frac{2.303RT}{nF\alpha(1-\alpha)} log(v) \quad (3)$$

where ΔE_p is the peak potential separation. Using the average value of α (0.5) and from the plot of ΔE_p *vs* log(v) (Figure 7), the heterogeneous electron transfer rate constants for the immobilized cytochrome c on the chemically modified gold electrodes were calculated for different Schiff base promoter complexes. The values of k_s were found to be as follows: Cr(III)-SB (3.6 ± 0.1 s^{-1}), SB (3.2 ± 0.2 s^{-1}), Fe(III)-SB (5.3 ± 0.1 s^{-1}), and Mn(III)-SB (5.3 ± 0.3 s^{-1}). This result indicates that the self-assembled Schiff base and its metal complexes immobilized on the gold electrode promoted electron transfer between the electrode and cytochrome c bound to the promoter. Diffusion of the protein to the electrode surface is known to affect the heterogeneous electron transfer from the electrode to the protein in solution. The rate of electron transfer (k_s) to cytochrome c in solution to an unmodified gold electrode was earlier[21] estimated to be ~10^{-6} ms^{-1} which was shown to enhance to 1.4–1.9 ms^{-1} in presence of 4,4'-

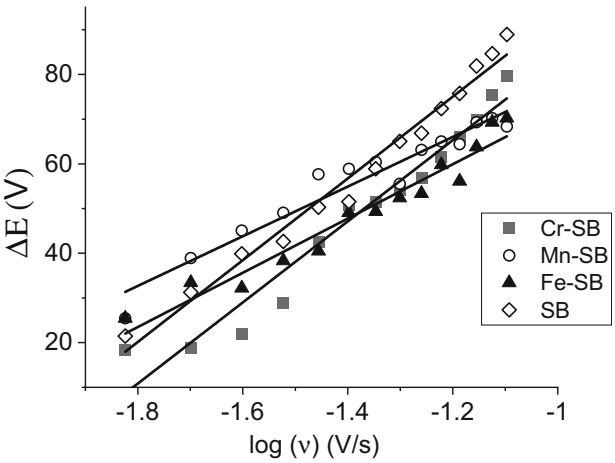

Figure 7. Plot of ΔEp vs. log ν for Cytochrome c on modified Au electrode containing monolayer of Schiff base (SB) and its metal complexes (M-SB) in 40 mM phosphate buffer, pH 7.4, 40 mM KCl at room temperature in nitrogen saturated solution at different scan rates [M=Fe(III), Mn(III), Cr(III)].

Table 1. Surface coverage, electron transfer rate and transfer coefficient from electrochemical studies of cytochrome c on different modified electrodes.

System	Γ (pmol/cm$^{2)}$	k_s(/sec)	α	Ref
Immobilized Cytc on ITO electrode	14 ± 2	18	0.5	[15]
Immobilized Cytc on $HS(CH_2)_{10}COOH$/gold electrode	11.6	72	0.5	[18]
Immobilized Cytc on [$HS(CH_2)_{15}COOH$]/gold electrode	–	0.1	0.5	[20]
Immobilized Cytc on $HS(CH_2)_{15}COOH$/gold electrode	17 ± 2	0.4 ± 0.1	0.5	[19]
Immobilized Cytc on N-acetylcys/gold	–	3.4 ± 1.2	0.5	[17]
Cytc on 4,4'-bipyridine (4-bipy) and bis(4-pyridyl) disulphide ($(4\text{-}PyS)_2$)/gold electrode	∼ 5.2	30 ± 5	0.5	[21,22]
Immobilized Cytc on Cys/GNPs/Chits/MWNTs/GC electrode	∼ 12	0.97	0.5	[16]
Cytc in buffer on GC modified graphene electrode	-	1.65	0.5	[13]
Immobilized Cytc on Mn(III)-SB/gold electrode	18 ± 1	5.3 ± 0.3	0.5	This work
Immobilized Cytc on Cr(III)-SB/gold electrode	26 ± 1	3.6 ± 0.1	0.5	This work
Immobilized Cytc on Fe(III)-SB/Gold electrode	71 ± 2	5.3 ± 0.1	0.5	This work
Immobilized Cytc on SB/gold electrode	13 ± 1	3.2 ± 0.2	0.5	This work

bipyridyl and analogous modifiers of the gold electrode. Chemical modification of GC electrode with graphene nanosheets was reported[13] to promote electron transfer to cytochrome c in a buffered solution of the protein with an enhanced rate constant of 1.65 s^{-1}. Earlier study[16] of direct electrochemistry of cytochrome c immobilized on nano-hybrid film containing gold nanoparticles embedded in self-assembled layer of chitosan with multiwalled carbon nanotubes on glassy carbon electrode showed the heterogeneous electron transfer rate of 0.97 s^{-1}. Table 1 shows that the heterogeneous electron transfer rates (k_s) determined in the present case using the self-assembled mono-layer of the thiol containing Schiff base and its metal complexes were thus faster than many of those reported earlier using different electrode modifiers.

The thiol linked Schiff base complexes thus seem to help the protein to form a stable thin film supported by the monolayer on the gold electrode, and also efficiently promote electron transfer from the electrode to the protein, possibly through the aromatic ring of the ligand. It is important to note that the overall rate of electron transfer (k_s) through the monolayer of the promoter to cytochrome c would also depend on the interface between the electrode and the monolayer, as well as on the interface between the monolayer and the protein along with the efficiency of the monolayer in promoting electron transfer. The association of the protein onto the monolayer needs to be strong enough to form a stable structure while maintaining the integrity of the protein. Moreover, since the heme center in cytochrome c is closer to one surface on the protein, the rate of heterogeneous electron transfer to the protein would depend on the specific orientation of the protein-bound onto the monolayer of the promoter. The present results indicate that the observed rate of heterogeneous electron transfer (k_s) does not seem to depend on the nature of the metal ion [Fe(III), Mn(III), Cr(III)] bound to the Schiff base, though the metal complexation seems to enhance the active surface coverage on the electrode. However, the presence of transition metal bound to the Schiff base is expected to facilitate electron transfer across the monolayer of the promoter. This result thus suggests that electron transfer across the Schiff base complexes is not the rate-determining step in the heterogeneous electron transfer from the gold electrode to cytochrome c through the monolayer of the promoter.

4. Conclusions

Efficient electron transfer from gold electrode to cytochrome c immobilized on the electrode was achieved through monolayers of thiol-containing Schiff base (SB) and its metal complexes (M-SB). The Schiff base ligands were formed *in situ* on condensation of L-cysteine and salicylaldehyde on gold electrode surface. The monolayer of the Schiff base and its metal complexes showed distinct morphological changes on the gold electrode detected by AFM studies. Thin film of cytochrome c was formed on the electrode containing the monolayer of the Schiff base as well as of the metal complex of the Schiff base. AFM studies showed distinct effects of formation of the thin film of the protein on the electrode. The surface coverage (Γ) of the redox active molecules in the thin film was found to vary with the nature of the

metal ion bound to the Schiff base, and the value of Γ increased in the order: SB < Mn(III)-SB < Cr(III)-SB < Fe(III)-SB in the monolayer formed on the electrode. This indicates that the cytochrome c molecules possibly bind to the Schiff base complexes through electrostatic interaction between the surface lysine residues of the protein and the carboxylate and metal ion coordination of the monolayer of the SB complex. Heterogeneous electron transfer between the gold electrode and cytochrome c in the thin film mediated by the layer of the SB or M-SB was found to be faster than several earlier reports on electrode modifiers as promoters. The overall electron transfer rates were however found to be almost independent of the metal binding to the SB, indicating that the electron transfer through the monolayer of the Schiff base complex may not be the rate determining step in the heterogeneous electron transfer from the electrode to the protein.

Supplementary Information (SI)

Figures S1-S3 are available at www.ias.ac.in/chemsci.

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