High-resolution electrochemical STM of redox metalloproteins

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Abstract

Electrochemical studies of redox active metalloproteins have become an increasingly fruitful area of study in recent years, particularly with the single-molecule resolution capability of electrochemical scanning tunnelling microscopy (EC-STM) which provides both imaging and current-voltage spectroscopy under bipotentiostatic control. In this review, some of the most exciting advances in recent years are outlined, and directions for future research are considered.

Keywords: STM, Molecular electronics, Single-molecule break junction, Molecule-electrode interfaces, Electrochemical gating.

Introduction

This review centres on studies of redox active metalloproteins using the technique of electrochemical-scanning tunnelling microscopy (EC-STM). Its purpose is primarily to give a snapshot of recent research in the area, rather than an overarching description of the development of EC-STM, although a number of seminal works must be mentioned. For underpinning background material, both experimental and theoretical, the reader is therefore referred to the existing extensive literature. See, for example, the following (non exclusive) references [1–9] on EC-STM and related techniques. However, it is appropriate first to give a summary of essential concepts.

Experimental techniques

The study of single small organic molecules as well as proteins has seen enormous progress in recent years, following the invention [10] of scanning tunnelling microscopy (STM) and scanning probe techniques generally, to enable a host of physical properties (including electronic, electrical, mechanical and optical) to be examined on surfaces at the atomic scale. Electrochemical STM was developed soon after [11] as a powerful probe of single molecules electrochemistry, allowing not just simple molecular imaging, but current-voltage ($I - V$) characteristics of molecules to be examined when electrochemically 'gated' by a third electrode. The EC-STM technique is a combination of a simple STM with an electrochemical control circuit, outlined in the schematic of figure 1.

In the STM part, an electrical bias V_{tip} (typically 10-100 mV) is applied between the STM tip (which is insulated apart from the extreme end to minimise ionic currents) and the sample to be studied. A quantum mechanical tunnel current I flows across the gap between tip and sample. In this case the sample comprises a carefully prepared, suitably flat (ideally crystalline), conducting surface (often a metal such as gold) with attached individual protein molecules. Images are normally obtained by raster scanning the tip across the surface in constant-current mode, i.e. under computer feedback to control the height z of the tip above the sample, such that the tunnel current is a chosen fixed value 'set point' (typically a few nA or less). Since the current - distance relationship is exponential in nature $I(z) \sim \exp(-\beta z)$ where β is typically around 10nm^{-1}, a very precise and stable control of z is required.

To further examine the electrical properties of the molecules, rather than just their location and apparent height, a number of different approaches can be used. Conceptually, the simplest is to locate the tip above a molecule and measure the tunnel current as a function of applied tip bias (with feedback turned off) to produce an I versus V_{tip} curve. This can be done for different z. However, long term stability of the instrumentation might not always be sufficient to fix the tip position to do more than a few measurements; in this case a common approach is to perform multiple imaging for a range of set points and bias.

Sometimes, the nature of the surface and molecules (a rough surface, delicate molecules, weak attachment) is not conducive to good imaging, but valuable information can nevertheless be extracted. By measuring the current as the STM tip is repeatedly brought near to (or even in contact with – the 'break junction' or BJ method) the surface and withdrawn again – called the $I(z)$ or $I(s)$ method – it is possible to detect molecular attachment between tip and sample, and hence find the molecular conductivity [12, 13]. Repeating with a range of different tip bias enables full current-voltage characteristics to be determined [14]. These experiments are not simple; it is necessary to analyse many $I(z)$ curves and perform careful statistical analysis on them. Early experiments tended to select curves 'by eye', but more robust data selection techniques [15] are undoubtedly the way forward. There are also several variations of these STM type measurements in which the pulling rate between tip and sample is varied or the bias is changed during the $I(z)$ trace [16].

Another important variation, introduced several years ago [17, 18], is the $I(t)$ method in which an STM tip with fixed bias is located close above a molecule. Again, low drift is essential

for the reliable application of this method and interpretation of the results. Precise details of the measurement (in particular bias, and tip conditioning) also influence the results; it is necessary to have a stable STM tip, and not too high a bias, to get clear reproducible junction formation.

The electrochemical part is based on a potentiostat, which is used to control a potential V of the electrolyte solution with respect to the working electrode (WE) or substrate and measure the resulting current I_m flowing to a counter elecrode (CE). Similar to a four-terminal measurement used to eliminate contact effects in determining a resistance, the potentiostat uses a separate current path to the path where the potential is measured. This both prevents instabilities in the current and allows measurement with respect to a reference electrode (RE) maintained at equilibrium. Importantly, in combination with the STM, the potentiostat thus allows *electrochemical gating* of redox active protein molecules which are deposited on the substrate. This is analogous to electrostatic gating of the channel in a field effect transistor (FET). The electrolyte provides electrostatic coupling to the applied external potential although, as it depends on the exchange of charge between ions in solution and the molecule of interest, electrochemical gating is inherently slower than electrostatic gating as it is limited by diffusion of ions. The electrochemical environment of a single protein molecule may also be influenced by the physical presence of the STM tip. One feature actually in favour of electrochemical gating [19] is that, with a short Debye screening length, any charge on the molecule in the EC-STM set up is screened by ions of the solution; the effective gate potential environment of the molecule is thus arguably better controlled than for an electrostatic gate, despite the remoteness of counter and reference electrodes. On the other hand, the polarisation of the effective molecule-ion may influence electronic properties.

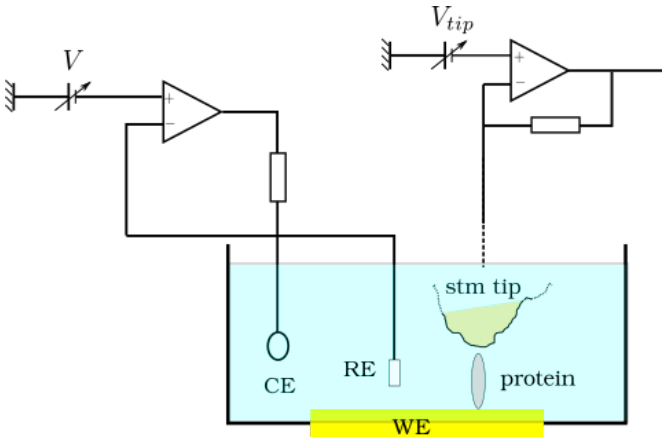

Figure 1: Schematic of EC-STM. In this implementation a (bi)potentiostat controls the potentials on both the STM tip and the surrounding solution with respect to the grounded working electrode.

Outline theoretical description

Electron motion across any molecule which bridges two electrodes (i.e. STM tip and sample) can often be divided into two, non exclusive, limits [20, 21] where the electron can (i) tunnel directly between the contacts but mediated by the molecule or (ii) tunnel onto the molecule and off again, but with an energy relaxation (due to inelastic phonon scattering) as it resides on the molecule. The first limit describes coherent tunnelling (maintaining phase information) while the second is incoherent. To ascertain which is the dominant mechanism in any particular molecular system requires more information than simple $I - V$ characteristics. This may be provided for example by examining the length dependence of conductance of a homologous series of molecules such as alkanedithiols [22, 14], by measuring temperature dependence, or by examining gate bias dependence. (Hopping transport, which can dominate in longer molecules, is not considered here.)

Reference [23] presents a treatment of coherent electron tunnelling between metallic contacts mediated by a single molecular level, which is commonly the case for small organic molecules. The electrochemical potentials (Fermi levels) of the contacts can be on resonance (energetically aligned with) or off resonance with the molecular level. (The latter picture is in fact not essentially distinct from simple tunnelling, if a non-rectangular barrier is admitted. A Simmons-type tunnelling, assuming a rectangular barrier, is usually sufficient to describe off-resonant tunnelling.) Figure 2 illustrates this model when the relevant energy level is a LUMO of an isolated molecule, which is unoccupied when close to the electrodes. In the lower figure, with a shift of levels due to gating, an electron can tunnel onto the LUMO from a filled state of the same energy in contact 1, and tunnel off again to an empty state in contact 2. This should be viewed (and usually is, for small organic molecules) as a single quantum step. The LUMO level will in general be partially occupied (reduced) since it actually has an intrinsic energy width Γ governed by the coupling strength between the molecule and contacts - the DOS is no longer a delta function. (The relevant wave functions are delocalised over the molecule and overlap the contacts. Γ can be related to the lifetime τ of the state through the uncertainty principle $\tau = \hbar/\Gamma$.) A two-step tunnelling will occur if vibrations in the molecule interact with the electron (destroying phase information, and possibly lowering or raising its energy) during the tunnelling time; if τ is long (weak coupling) then a two-step process is more likely.

In the case of a redox active protein molecule however, as is established by classical electrochemistry studies of electron transfer in proteins, it is further essential to consider both thermally induced fluctuations of the molecular energy levels (internal vibrations and surrounding solvent fluctuations) and the energy of the structural change or 'reorganisation' of the molecule between oxidised and reduced states. EC-STM data has thus been widely and successfully described through the Kuznetsov-Ulstrup (KU) model [24, 7, 25] of *electron transfer*. In this model, near the equilibrium potential a two-step process occurs where both the oxidized and reduced forms of a molecule contribute to the current flow between tip and substrate. Thermal fluctuations effectively result in *probability distributions* for transfer between oxidised and reduced states (arising from the Boltzmann factor and an assumed quadratic dependence of these states' energy levels on configuration coordinate of

the molecule) written as the oxidised (or reduced) distributions $D_{ox}(E)$ (or $D_{red}(E)$). The nature of the model yields normalised

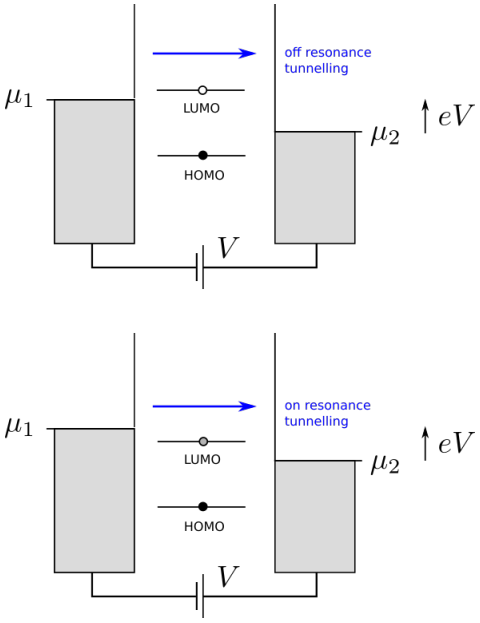

Figure 2: Schematic of electron tunnelling between an STM tip (positive bias V, right) and substrate (left) as mediated by a molecular energy level. The vertical scale is energy. The horizontal scale suggests the contact positions as well as the density of states (DOS). The DOS is shown as constant for the metals and a delta function for the molecular levels. (The molecular levels actually have an intrinsic energy width Γ due to coupling with the metallic states.) Electrochemical potentials μ_1 and μ_2 are separated by eV due to the bias. At zero bias, the metals' Fermi levels are aligned within the HOMO-LUMO gap, while with bias the molecular levels are lowered by $eV/2$ if symmetrically coupled to the contacts. Open (closed) circles indicate unoccupied (occupied) molecular states. Top: The closest molecular level (LUMO in this example) lies above μ_1, but a current can flow through the broadened tail state of the LUMO. Bottom: The LUMO lies between μ_1 and μ_2, leading to enhanced current.

Gaussian distributions of width $\sqrt{2kT\lambda}$ where λ is the reorganisation energy. The process of electron transfer might thus be viewed [26–29] as proceeding (for example) through an empty molecular level which momentarily enters the energy window between μ_1 and μ_2. While the level is in this energy window, a degree of (coherent or incoherent) tunnelling can proceed, but when reorganisation of the protein occurs from the effect of the now (at least partially) reduced level, the level will relax (by an amount 2λ) to take it below the energy window. Fluctuations can take the now fully reduced level back into the energy window at some stage to allow further tunnelling. This picture has been developed into a quantitative analytic description of the $I-V$ characteristics of tunnelling through a redox protein.

Current developments

The power of STM and related techniques is in obtaining single molecule data, which is lost in bulk ensemble studies, and in opening the way to observing dynamical behaviour, revealing a more fundamental understanding of general molecular interactions. Although proteins which are not redox active can be examined with STM [30], there has been extensive use of EC-STM for gating studies of metalloproteins. Early seminal experiments on the blue single-copper redox active protein azurin [31–33] demonstrated electrochemical gating at the single molecule level. Much work has since centred on this protein, which is robust and can attach to Au surfaces through cysteine residues. For instance, the BJ method in EC-STM was used [34] and confirms the gating observations of the earlier imaging studies. In a recent development, single molecule redox events switching events [35] have been reported for azurin, over an accessible time scale. These are challenging experiments which depend on variation of experimental parameters (sample potential with respect to redox potential in this case) and careful interpretation. Evidence for control of single azurin molecule conductance has even been obtained [36] through the application of voltage pulses. This raises the question of the degree of molecule-substrate coupling and provides further support to the two-step model of electron transfer.

Apart from the possibilities opened up by *time*-resolved measurements, the question naturally arises as to the maximum *spatial* resolution attainable on protein molecules. EC-STM imaging experiments on proteins generally reveal rather featureless structures, although evidence of sub-molecular features were in fact observed [37] in azurin several years ago. Recently, rather detailed sub-molecular features of single streptavidin proteins in solution have been resolved with STM [38], where advantage was taken of carefully optimised conditions. It would be extremely interesting to get improved sub-molecular observation of redox site(s) under electrochemical control.

The nature of the contact between any molecule and surface is well known to be crucial to the conductance measured in STM. Much work has depended on naturally occurring cysteine residues which link to a metal such as gold through a thiol bond. An interesting method to control protein-substrate linking is offered through protein engineering and has been used [39] to attach *cyt-*b_{562} to gold surfaces in specifically chosen orientations. Such methods should be extended to a broader range of proteins, linkers, and substrates in future. It is also worth noting that, if a protein molecule is intimately (covalently) linked to both the STM tip and substrate, strong electronic coupling of molecular levels to the continuum of metallic levels may also be important, as suggested by a number of authors [37, 39, 29, 40].

Theoretical development of EC-STM is also not static. The consensus description is the KU two-step model of charge transfer to describe EC-STM for redox-active molecules. However, Bâldea [41] has considered an alternative approach, perhaps valid for strong tip and substrate coupling, applying instead a Newns-Anderson framework to detailed $I-V$ measurements on azurin which were originally fitted by the authors using the two-step formula. Although both models have a number of adjustable parameters, Bâldea's claim was the use of consistent parameters for different gate biases (reduced and oxidised molecules) to describe the results quantitatively. Interestingly, a suppression of the solvent reorganisation in the nanogap compared to bulk solution was deduced. Further theoretical work should be of interest here.

In any case, the detailed role of the complex surrounding medium is also increasingly being focussed upon in STM-based

experiments [42–45] as well as EC-STM [46]. In terms of general effect on molecular conductivity, local water molecules or ions can both influence the average local electrostatics (shifting molecular levels) and have important dynamical effects. The local structure of water molecules at interfaces is, for example, well known to be partly ordered. Recently, Matyushov *et al* [47–50] have explored how experimentally rather smaller reorganisation energies than expected from atomistic simulations might be explained, examining the case [47] of cytochrome *c*. They extend the Marcus/Gerischer model to distinguish between two types of reorganisation energy representing medium polarisation and (a typically large) thermal fluctuation contribution, the latter being linked to a heterogeneous region surrounding the protein cofactor. The arguments are interesting and would perhaps apply *a fortiori* to the local geometry of the EC-STM set up. Recently, although not directly applicable to proteins, the application of non-aqueous electrochemical gating using ionic liquids and small redox-active molecules has been particularly revealing [51, 19] in understanding the effect of the surrounding medium.

The general environment of a protein is intimately linked to its overall physical properties. References [1] and in particular [52, 53] include good discussions of how the various processes of electron flow between a protein and its environment (electrode or electrolyte) might be categorised, and point to the distinction between mechanisms involved of 'electron transport' (essentially, protein-mediated electron current between two adjacent metallic contacts) and 'electron transfer' (electrons moving between donor and acceptor sites within a protein, or between a surrounding electrolyte and redox centre in the protein). In all cases however, the driving mechanism is a difference in the electronic electrochemical potential μ, which in EC-STM is tuned by the applied bias between tip and substrate.

Finally, although not the focuss of the present review, it is worth noting that electrochemical atomic force microscopy (EC-AFM) measurements can yield interesting information complementary to that of EC-STM since they allow knowledge and control of the force acting between tip and sample, as well as providing reliable height measurement of a protein. For example single molecule redox active Cu-azurin shows evidence [54] of electrochemical modulation of its height, attributed to conformational changes. This is perhaps not surprising in view of the importance of the metal site to protein stability [55].

Progress towards functional devices

A major driving force behind research into functional biomolecules is for active sensing components in device applications for bioelectronics [1, 56, 53]. Due to their inherent molecular function and exquisite recognition properties, protein molecules have become a particular focus [57, 52, 58, 59] in recent years. Proteins which are electrically active are of obvious potential for electronic sensing – if they can be linked to external circuitry. Furthermore, creation of single (or few) molecule devices can provide temporally-resolved information which is lost in bulk ensemble studies, allowing a more fundamental understanding of molecular interactions and behaviour such as transient motions and intermediate states.

To build a protein-based bioelectronic device there are two important prerequisites:

(i) Robust, reproducible and directed physical linking of protein molecules to an electronically active surface (typically a semiconductor). This means that the device can be reliable, long-lived and give a consistent transduction signal. Consistency means that it is possible to distinguish between the subtle changes of signal that would otherwise be masked by random variation.

Although thiol end groups, widely applied in single molecule studies, may occur naturally or be introduced into proteins as a direct means of attachment to metal contacts [39, 60], it is less straightforward to contact the technologically important materials silicon, GaAs or – our focus here – carbon allotropes. Functionalisation of nanoscale devices based on graphene and carbon nanotubes (CNTs) provides an important step in the development of single/few molecule electronic components and miniaturised bio/chemical sensors [61, 58, 62].

(ii) A change in the electronic or morphological properties of the protein, arising from a biorecognition event, should yield an adequate transduction signal. The great sensitivity of the electron network in CNTs and graphene to surface electrostatics makes them especially favourable, and single-walled CNT field-effect transistors are an attractive platform for protein-based biosensors [63, 64].

Much has been learnt about creating biologically active hybrid materials from proteins and CNTs or graphene. Passive adsorption (mostly through hydrophobic interactions) is commonly used to create these interfaces, but while this interaction is intimate, it is also weak and ill defined. An obstacle to achieving a strong and stable linkage with good electrostatic communication between a protein and the electronically active sp^2 system of CNTs is their inherent chemistry. Covalent attachment is the preferred route but there is no inherent compatible chemistry in either the protein or CNT. Thus CNTs are usually first chemically oxidised to introduce the required reactive groups (e.g. ketone, carboxyl or alcohol). Oxidation is difficult to control, leading to wide-scale perturbation of bond π and σ networks that give CNTs their conductance characteristics. Additionally, to functionalise oxidised CNTs with protein, further chemical modification is required to add appropriate linking groups. Such linking groups increase spacing between the CNT and protein and generally rely on reactions with primary amine groups such as lysine amino acids. Lysine residues are common in proteins and are commonly found distributed across the protein surface. Thus, as well as losing intimacy, a single defined and optimal protein interface with CNTs or graphene is generally difficult to achieve. Future advances are thus likely to depend on novel approaches like protein engineering to achieve directed covalent linking. There is also potential to modify protein properties to allow detection, via binding, of a chosen molecular type.

Concluding remarks

High resolution (single or a few molecules) studies of redox active metalloproteins have advanced rapidly in recent

years, driven by interest in understanding basic mechanisms, huge potential for biosensors, and technological advances in measurement. Time-resolved behaviour of single protein molecules under electrochemical control are becoming technically feasible, potentially providing statistics of switching, and may lead to much deeper insight of the nanoscale electron transfer mechanism. Theoretical approaches continue to develop in the areas of (i) electrostatic details around the STM tip and (ii) tunnelling/transfer to include all relevant time scales correctly (iii) the degree of electronic coupling between tip/molecule/substrate. Biosensor applications still require much development. One major obstacle is in going beyond research experiments to make scalable devices. Graphene is an obvious platform here, but there have been promising advances [65] in creating arrays of selected CNTs for FET devices. There is no doubt that nanoscale studies of redox active metalloproteins promise fascinating results for years to come.

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