Electrochemical Scanning Tunneling Microscopy Analysis on Protein Based Electronic Devices

Ajay Kumar Yagati¹, Ji-Young Lee², Eun-Sook Nam³, Sungbo Cho¹,⁴ and Jeong Woo Choi¹,²,⁴,∗

¹Department of Biomedical Engineering, Gachon University, Yeonsu-gu, Incheon, 21936, Republic of Korea
²Department of Chemical and Biomolecular Engineering, Sogang University, Seoul, 04107, Republic of Korea
³Sogang-Binggrae Food Advanced Analysis Research Center, Sogang University, Seoul, 04107, Republic of Korea
⁴Interdisciplinary Program of Integrated Biotechnology, Sogang University, Seoul, 04107, Republic of Korea

ABSTRACT

Scanning probe microscopy (SPM) techniques demonstrate one of the most promising tools to investigate the physical and chemical properties of materials at nanoscale and become the most common and important characterization tools in the field of nanotechnology. Among many SPM methods electrochemical scanning tunneling microscopy (EC-STM) technique is one technique that directly provides three-dimensional real-space images with in-situ interfacial electrochemical studies and allows locally measured properties of nanostructured materials at atomic resolution. Furthermore, EC-STM based studies provides information on solution covered areas of electrode surfaces, metal deposition, charge transfer, potential-dependent surface morphology, corrosion, semiconductors, and various applications such as protein conductance measurements in nanobioelectronics. Therefore, in this review, we summarize the electrochemical scanning tunneling microscopic investigation on protein based electrode structures and their applications towards novel bioelectronic devices along with recent developments in ECSTM techniques with future prospects in the field of nanobiotechnology.

KEYWORDS: Review, Protein, Electrochemical, Tunneling, Azurin.

1. INTRODUCTION

There is a tremendous growth in nanotechnology in recent years with innovative tools and methods that enabled us to study and analysis of atoms, molecules and larger atomic structures.¹,² Further, with the development of new techniques and instrumentation, enabled the analysis and manipulation at atomic scale, which paved the path for the design and production of advanced nanostructured materials.³ The invention of scanning tunneling microscope (STM) by Binning and Rohrer in 1982 has provided a new high resolution tool⁴ to look at the surfaces and their achievement was recognized by the Nobel Prize. The other form of scanning tunneling microscopy, such as atomic force microscopy⁶ provides the information about the surface topography and surface forces.⁷ The advancement in techniques led to various detection methods and enabled to detect surface structures in liquid environment with the help of an electrochemical cell with in situ electrochemical properties.⁸,⁹ At present, STM is a powerful tool for analyzing metallic and semiconductor surface with real-space visualization of surface at atomic scale. By exploiting the SPM hardware in conjugation with optical detection methods enabled to study the biological systems such as live cells and applications in near-field optical systems.¹⁰,¹¹ SPM possess various operational modes which can be tuned according to
Ajay Kumar Yagati

Ji-Young Lee

Eun-Sook Nam

Sungbo Cho

Jeong Woo Choi
the imaging requirements such as probe-sample interactions, force or surface potential mapping and to analyze the conductance properties of redox molecules and tuning their tunneling properties. Thus, the scanning probe microscopy tools are most advanced and powerful tool in the field of nanotechnology and led the advancement of science and technology. Therefore, here we outline the various types of scanning probe techniques, such as Scanning Tunneling Microscopy (STM), scanning tunneling spectroscopy (STS) and electrochemical scanning tunneling microscopy (ECSTM) while focusing the ECSTM methods by describing with examples on protein based conductivity measurements.

1.1. Scanning Tunneling Microscopy

Scanning tunneling microscopy (STM) is based on the concept of quantum mechanical tunneling. When a voltage is applied between a sharp metal probe (tip) and the surface of an electrically conductive material, tunneling current will be produced if the tip is positioned a few nanometers from the surface. According to quantum mechanics, an electrical current will be produced under these circumstances without the need for the probe tip to physically touch the surface. The separation distance between the tip and the sample is roughly one-hundred thousandth of the thickness of the sheet of a paper. A typical tunneling current is on the order of one nA and the applied voltage is typically less than one volt.

The magnitude of the tunneling current is very sensitive to any change in the tip/sample separation distance and it is the sensitivity that makes it possible to monitor and detect changes in the separation distance. A piezoelectric tube is used to control the position of the tip in three-dimensions relative to the sample. The probe tip can be scanned parallel to the surface using computer control. The tip’s position perpendicular to the surface is determined from the output of a feedback circuit, which sends a voltage signal to the particular piezo element that moves the tip towards or away from the surface in order for a preset tunneling current to be maintained. As the probe tip is scanned over the surface, the topographic data were collected by the computer. An image of the surface is then displayed on the computer monitor from this data.

STM was originally designed for the investigation of conductive or semi-conductive material. It is very useful on high-resolution imaging or organic/biomaterials. The reason for this is the completely different nature of the characteristic binding forces in the surface region of such substances. STM possesses following important parameters which are the horizontal coordinates (x, y), the height (z), the bias voltage (V) and the tunneling current (I). Based on the utilization of these parameters, the modes of operation of STM was distinguished as:

(a) constant current mode, where I and V are kept constant, x and y are allowing to change with movement of the scanning tip, and z is measured;
(b) while in constant height mode where z and V are kept constant, x and y will change during the scan, and I is measured; and in
(c) scanning tunneling spectroscopy (STS), which is a whole set of modes with variation of V. The most commonly used method in STM measurements is the constant current mode, in which the STM tip is allowed to scan over the surface of the sample by keeping the bias voltage and tunneling current constant. To achieve the constant voltage and current the feedback system adjusts the vertical position of the STM tip by varying voltage Vz in the piezoelectric element. This mode of operation maintains a constant gap between the sample and the tip while scanning the surface topography of the sample as shown in Figure 1(a). The material surface structure height is determined directly from Vz that produce a surface topography as a function of the needle position z (x, y).

In constant height mode of operation, the surface is scanned with the STM tip kept at a constant voltage Vz in the z-piezoelectric element while measuring the tunneling current (I) as a function of the needle position (Fig. 1(b)). The voltage V between the tip and the sample is kept constant, and the servo system feedback is turned off. In this case, the surface bump will be reflected in higher tunneling current when passed by the needle. This mode enables to perform high scan speeds as the servo system was disabled. This mode of operation is suitable to study the dynamic processes in real time perhaps the recording the surface structures in a video format. This mode possess drawback as it is difficult to quantify the surface topography based on the changes in tunneling current. Scanning tunneling spectroscopy (STS) is a set of methods of scanning tunneling microscopy in which the voltage between the tip and the sample is varied in order to obtain the information on the local electronic structure of the surface.

1.2. Scanning Tunneling Spectroscopy

The scanning tunneling spectroscopy of metals, semiconductors or biological materials primarily focus on elastic...
tunneling current changes associated with the local density of states (LDOS),\textsuperscript{16} \( \rho_s(r, E) \), shown in Eq. (1). To a good approximation, \( \rho_s(r, E) \) is proportional to \( dI/dV \) when the tip is far from the substrate and the density of states of the tip is reasonably smooth.

\[
I = \int_0^V \rho_s(r, E)\rho_t(r + eV - E)T(E, eV, r)\,dE \quad (1)
\]

In a real tunneling device; such as metal-insulator-metal (M-I-M) tunnel diode, or a substrate and STM tip,\textsuperscript{17} there are many electrons and the Pauli principle plays a key role. A simple model for the conduction electrons in a metal assumes that the one begins by removing the valence electrons, then spreads the remaining positive charge into a uniform distribution (jelly) producing a simple constant potential box. Into this box the valence electrons are returned, 2 at a time into each energy level, until the metal is just neutrally charged. The energy of the last electron to go in is the Fermi energy, \( E_F \), and the energy required to just remove it from the metal is the work function, \( \Phi \).

If there are no molecules in the barrier region (tunneling gap), the current is approximately proportional to the voltage difference between the two metals (called the bias) and \( \exp(-Ad\sqrt{\Phi}) \). This current is supposed to be due to elastic tunneling since the electron loses no energy to the barrier.

If the gap (barrier) between the electrodes is not a vacuum, Eq. (1) must be modified in several ways. The simplest effect is a reduction in the effective barrier height. For an insulator or semiconductor, it may only require a volt or two of energy above \( E_F \) for the electron to reach the conduction band in the barrier, while the work function may be 4 to 6 volts. In these cases, the work function in \( I = eV\exp(-d\sqrt{\Phi}) \) is replaced by barrier height, \( \Phi_B \), where \( \Phi_B \) is approximately the difference in energy between the bottom of the conduction band (in the insulator) and the Fermi energy in the electrodes at zero applied bias. If individual molecules are present in the barrier, several new interaction mechanisms can affect the tunneling current. The best known of these is inelastic electron tunneling and is the basis for inelastic electron tunneling spectroscopy (IETS). In IETS the moving electronic charge interacts with the time varying molecular dipoles (electronic or vibrational) to induce excitation of the molecule in the barrier with concomitant loss of energy by the electron. This process is similar to a Raman photon process by considering vibrational motion with frequency \( \nu \) and energy spacing \( h\nu \) as shown in Figure 2. An excitation from the ground vibrational state to the first excited vibrational state with a corresponding loss of energy by the tunneling electron. If the applied voltage is less than \( h\nu/e \), the inelastic channel is closed because the final states for the tunneling electron, the electronic levels in the metal electrode of the appropriate energy, are already filled.

To obtain an IETS spectrum we can plot \( d^2I/dV^2 \) versus \( V \) and expect to see peaks whenever the energy difference between the ground and excited state (electronic or vibrational) just matches the applied bias voltage. It is important to note that IETS bands appear at the same bias magnitude independent of sign, although the intensities may differ.

1.3. Electrochemical Scanning Tunneling Microscopy (ECSTM): A Promising Tool for Imaging with In-Situ Electrochemical Analysis

Electrochemistry has been used in a wide range of science and technology because of easy control of electrochemical potentials of interfaces and adsorbed molecules,\textsuperscript{18} which initiate electron transfer and redox reactions.\textsuperscript{19} This provides us a way to regulate the redox process for studying the basic phenomena how the local environment responds to the electron transfer process. Thus in-situ ECSTM technique (in-situ electro chemical scanning tunneling microscopy technique), which is based on the scanning tunneling microscopy technique used in conjunction with electrochemistry, is highly useful technique which is capable of measuring the small changes in the surface changes\textsuperscript{20} which occur as a result of the electrochemical process as shown in Figure 3. The method was used to study the electrode surface at atomic scale resolution...
and single molecule electron transfer in electrochemical environments.\textsuperscript{21,22} The electrochemical potentials of the sample and the tip are controlled independently against the reference electrode by a bipotentiostat. The substrate potential determines the redox state of molecules tethered on the substrate electrode, and the difference between the tip potential and the substrate potential sets the bias voltage for electron tunneling.

In the subsequent sections in this review, we focus on the developments on ECSTM experiments to study the applications of this tool in nanotechnological innovations.\textsuperscript{23,24} The control of voltage in order to achieve novel structures, and the application of oxidation and reduction potentials to study the single molecule conductivity were also discussed.

## 2. ELECTROCHEMISTRY WITH SCANNING TUNNELING MICROSCOPY

### 2.1. Structure of Single Crystal Surfaces, Adsorptions and Self-Assembly of Molecules

The formation of self-assembled monolayers (SAM)\textsuperscript{25} or self-organizing molecules are natural and spontaneous processes that play vital role in bottom-up strategies in the development of molecular electronic devices, biorecognition and biosensors. Mostly, thiols immobilized on gold (Au) electrodes are extensively studied and explored in various conditions such as ultrahigh vacuum,\textsuperscript{26} air\textsuperscript{27} and in solution state.\textsuperscript{28} Several studies were conducted to examine the SAM properties such as their adsorption, electrical and electrochemical properties, thermal and chemical stabilities, however to understand the morphology of the SAM molecules, SPM were considered to analyze the single molecular structures. These SPM based methods are utilized to unravel the SAM domain size and defect density which could affect the properties of the electronic devices based on the SAM materials. Several reports on the studies of single molecule characterization of SAM and therefore here we consider an example on the adsorption and assembly of 2-mercaptobenzimidazole (MBI) on Au electrode surface.\textsuperscript{29} The MBI molecules are examined with the help of ECSTM for understanding the adsorbed layer (adlayer) structures and potential dependent liquid/solid interface charge transfer process. \textit{In-situ} STM measurements shows that MBI molecules could form oriented molecular cluster lines along the reconstruction line direction at 0.55 V. However, when the electrode potential shift towards negative these molecules will undergo a disordered structural transition to form an ordered striped adlayers on the desorption region on Au. The \textit{in-situ} the cyclic voltammogram analysis on MBI modified Au electrode depicted two anodic peaks at 0.24 and 0.58 V which was attributed to the adsorption of MBI and the cathodic peak observed at 0.18 V was attributed to the desorption of MBI as shown in Figure 4(a).

Figure 4(b) represents a typical large-scale STM image of an MBI-modified Au surface in 0.1 M HClO\textsubscript{4} at 0.55 V. The MBI molecules form a multilayer structure with molecular clusters on the top layer. Interestingly, the clusters arrange along the direction of the reconstruction lines. The average distance between neighboring lines is 12 nm, which is about twice the distance separating the pairs of reconstruction lines. When the potential is shifted negatively to 0.15 V, the disordered multilayer disappears and an ordered monolayer appears on the Au surface. The disordered-ordered transition is reversible shown in Figure 4(c). The bright spots and the dark rods in Figure 4(c) are ascribed to the thiol sulfur atoms and the heteroaromatic rings of MBI, respectively. The sulfur group in thiol SAM always appears bright in the STM and the interdistance between the neighboring atoms was 0.4 nm. The heteroaromatic ring was resembled as dark rods in the STM image. Therefore, STM is a powerful tool for gathering information about the arrangement of molecules on metals. MB SAMs observed by STM will be helpful in understanding the mechanism of detecting metals using MBs molecules as chelating agents and the stability of MB-modified electrodes.

### 2.2. Current Distance Spectroscopy and Break Junction Techniques

The bias voltage dependent STM imaging offers a possibility for spectroscopic studies on the sample properties. Scanning tunneling spectroscopy (STS) measurements enable to record the electronic properties of the sample on different surface sites. On the other hand, the current–distance (\textit{I–s}) measurements\textsuperscript{30} where the bias was kept constant and the tip-sample distance was varied and the current were measured. Therefore, in this analysis the current decay factor \(\beta\) of the surface was analyzed. The surface state wave-functions of the sample materials decay into the vacuum with an exponential dependence on
the distance. The proportional conductance \( G = G_0 e^{-\beta s} \) depends on the constant \( (G_0) \) quantum conductance and the decay factor \( (\beta) \). In case of molecules adsorbed on metal surfaces that forms a tunneling junction then the total conductance will be \( G_{\text{total}} = G_{\text{tip}} \cdot G_{\text{film}} \).

The electrochemical STM results have generally been interpreted using a one-dimensional tunneling model. According to this model, the relationship between tunneling current \( (I_t) \), tunneling voltage \( (V_t) \), and gap distance \( (s) \) is expressed by Eq. (2), if the density of states of the metal surfaces is assumed to be structure less and the energy barrier is rectangular.\(^{31}\)

\[
I_t \propto V_t \exp(-1.025 \sqrt{\varphi s}) \tag{2}
\]

Here \( \varphi \) is the tunneling barrier height given in eV, and \( s \) is in Å. Experiments revealed that the dependence of tunneling current on distance follows the above equation. As model example,\(^{31}\) the result of the tip-approach experiment obtained in a NaClO\(_4\) solution of 1 mM concentration was presented here. The bias voltage \( (V_b) \) was fixed at \(-100\) mV, which is applied at a substrate against the ground potential of a tip. The \( I_t - s \) curve shows that the tunneling current increases exponentially with decrease in the gap distance, only when the tip and the substrate are far apart. At small distances the \( I_t - s \) curve does not follow an exponential behavior.

Tunneling conductance, \( G_t = I_t / V_t \), can be calculated from the result of simultaneous measurements of \( I_t \) and \( V_t \). As can be seen in Figure 5, the \( \ln G_t \) versus \( s \) plots for the results were obtained in pure water and NaClO\(_4\) solutions of 1 mM and 100 mM concentration. The plots show good linearity in the investigated ranges of tunneling distance. This observation is in conformity with the \( G_t - s \) relationship in the one dimensional tunneling model given in Eq. (3).

\[
G_t = \frac{I_t}{V_t} = G_0 \exp(-1.025 \sqrt{\varphi s}) \tag{3}
\]

where \( G_0 \) is the conductance at \( s = 0 \) Å. The results obtained in pure water will fall slightly below the line at long distances (>15 Å), but the degree of deviation from linearity is within the experimental uncertainty and does not represent a general feature. Furthermore, the \( \ln G_t - s \) plot is modulated with weak periodic oscillations coinciding with the structure of interfacial water layer and those features can be disappeared after averaging the results of repeated tip-approach experiments.\(^{32,33}\) Whereas, the \( \ln G_t - s \) plots are linear, the corresponding \( \ln I_t - s \) plots are obviously curved due to the drop in \( V_t \) at decreased tunneling distances. This indicates importance to measure \( G_t = I_t / V_t \), not \( I_t \) alone, in the electrochemical STM experiment, because the \( V_t \) drop could be a significant factor at a relatively long distance. Also, the electronic properties of single molecules were studied for the development of novel molecular electronic devices. Molecular and nanoscale structures have been utilized for the demonstration of the developing functions such as rectification, negative differential resistance and single molecule transistor behavior. Break junction
techniques has been utilized to study the conductivity of single molecules as a function of redox state. So, therefore in this context, the molecule, 6-[1’-(6-mercaptohexyl)][4,4’]bipyridinium]-hexane-1-thiol iodide (6V6; see Fig. 6(a)), was chosen for analysis. The thiol groups at both ends of the molecule enabled to bind with the gold substrate and the STM tip. This molecule possesses a redox group which has readily accessible energy levels and is symmetrically placed between defined molecular tunneling bridges (the two alkyl chains) at either end. Furthermore, these molecules are highly stable in their redox states. When the STM tip was brought close enough to the Au surface, by increasing the tunneling current set point ($I_0$), there is a spontaneous formation of stable molecular wires between the tip and the sample was observed. Subsequently the tip was then lifted while keeping a constant $x-y$ position, and the current–distance ($I(s)$; $s =$ relative tip-sample distance) relation was measured.

As can be seen in Figure 6(b): two unique current–distance ($I-s$) curves were observed: The first one being a fast exponential decay typical of tunneling between a tip and a bare metal (curve 1 in Fig. 6(b)) and the other one is less abrupt decay followed by a characteristic current plateau ($I_w$) (curve 2). From these results in can be understood that the plateau is related to conduction through molecular wires which are chemically bonded to the tip and to the substrate. Furthermore, direct tunneling current does not have significant amount of contribution in the observed phenomena of separations. Also, the plateau is followed by another current decay at longer distances. A plot of $I_w$ versus $s_{1/2}$ ($s_{1/2} =$ distance for $I = I_{w/2}$) for more than hundred $I(s)$ scans were taken at different locations was presented in Figure 6(c). It was found that the average value of $s_{1/2}$ is about 2.4 ± 0.6 nm. Similarly, the end of the plateau is observed at approximately 2 nm from the initial set point distance ($s_0$). Therefore, an estimate $s_0$ places the tip-to-substrate distance at approximately 2.5 nm at the end of the plateau. This distance is close to the length of 6V6 molecule, which was confirmed by molecular modeling of the free molecule, produces a distance between the two sulfur atoms of 2.4 nm for trans oriented alkyl chains. Therefore, it clearly indicated the molecule was in fully extended conformation before disengagement from the tip as shown in Figure 6(a).

It is also observed that the decrease in current for longer tip-sample distance following a plateau (Fig. 6(b)) occurs over a distance range not just abruptly by the extension of molecular assembly before the breakage at either the tip or surface end. Moreover, the molecular assembly constitutes the molecular wire as well as group of surface and tip atoms. At sufficiently larger tip-sample separations, the chemical contact of the molecular wire to the tip or to the surface is broken and, thus, the current drops to zero. These events were recorded and three sets of data were present as shown in (Fig. 6(c)) as group 1 (squares), group 2 (circles), and group 3 (triangles). From the analysis, the measured $I_w$ cluster values are around integer multiples of a basic current value of (98 ± 16) pA. Therefore, it can be understood that the steps in conductivity was attributed due to the presence of a discrete number of molecules between the tip and the sample. Thus, it can be concluded that the lowest conductivity unit (group 1 events) and the respective subsequent steps (groups 2 and 3 events) correspond to conduction through a single molecule and the others to two and three molecules, respectively.

From the above analysis, it is concluded that the conductivity of a single molecule ($\sigma_m$) at $U_i = 0.2$ V is (0.49 ± 0.08) nS (where $U_i$ is the tip-to-substrate potential difference). The inset in Figure 6(c) shows a histogram of the current values observed in Figure 6(c). The obtained results are also compared with the conductivity results

![Fig. 6.](image)
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In which the wiring is realized by chemical attachment of nanoparticles to 6V6 molecules incorporated in a hexanethiol monolayer on gold which gave a value of \(0.56 \pm 0.03\) nS. Therefore, the results indicated that measurement of single molecule conductance was reliable and can be extended to examine the conductive of various other single molecules towards novel electronic devices.

2.3. ECSTM Based Nanostructure Formations

Metal atomic-size nanowires and single molecular junctions are the main focus in nano and molecular electronics for their quantum transport properties. Therefore, there is always a quest for innovative methods or procedures for the formation of atomic scale structures. Furthermore, electrochemical deposition of metals to form various nanostructures is a valuable route to modify the structures for nanoelectronic applications. ECSTM based technique with variety of tip-surface interactions nanostructured surfaces under high-vacuum conditions were prepared. A jump-to-contact based approach with ECSTM-break junction techniques enables to construct different nanostructures such as nanodots and nanowires. In this approach, the STM tip was coated with material of interest and transferred to the surface through jump-to-contact method. This process enables the formation of atomic sized nanowire when the tip retracts from the surface. This procedure was utilized to study the conductance behavior of many metals such as Cu, Ag, Pd and Fe, however room temperature measurement of metal conductance with complex electronic structure was found difficult. So, the utilization of this technique led to tip induced formation of nanoclusters at metal/solution interfaces, and also enabled the formation of large-scale arrays nanopatterns with high precision. Due to low decomposition potential of water which is 1.23 V, there is a fundamental limit for any deposition process in liquid environment. This limitation prevents the deposition of non-noble metals such as Fe or Al from aqueous solutions. However, these limitations can overcome by using ionic liquids, that is, room-temperature salt melts, which enable a potential window of 4–5 V for deposition. For example, Fe clusters in a 1-butyl-3-methyl-imidazolium tetrafluoroborate (BMI-BF\(_4\)) ionic liquid, was fabricated on electrode surface with a microprocessor-controlled pulsed tip approach. The tip potential was chosen to be between \(-0.75\) and \(-0.95\) V so that Fe bulk deposition onto the tip proceeded at a fairly high rate. For the 5 × 5 array of Fe clusters generated at intervals of 40 nm the clusters have an average height of 0.5 nm (Fig. 7(a)).

A large-scale array of 50 × 50 Fe nanoclusters with 20-nm intervals were fabricated by using 5-ms pulses at a rate of 1 cluster every 300 ms (Fig. 7(b)). In this process, the total time to perform the experiment to construct the array was about 14 min without any sign of depletion of Fe supply at the tip. In order to demonstrate the stability and durability of the device a ring with a diameter of 120 nm, composed of 48 Fe clusters was also created (Fig. 7(c)), with a cluster height of approximately 0.6 nm. The Fe nanoclusters possessed uniformed size however less perfect than the Cu clusters fabricated in aqueous solution and Zn clusters in ionic liquids. It is understandable that the potential-dependent morphology adds more complexity to the deposition procedures onto the tip for supply of Fe during the jump-to-contact processes, which in turn responsible for less uniform Fe cluster formations.

Fig. 7. STM images of Au (111) surfaces decorated with Fe clusters. (a) 5 × 5 Array, scan area 200 × 200 nm\(^2\); (b) 50 × 50 Large-scale array, scan area 1 × 1 mm\(^2\); (c) ring of 48 clusters with a ring diameter of 120 nm; z Pulse: 0.42–0.45 V. Reproduced with permission from [35], Y. M. Wei, et al., Small 4, 1355 (2008). © 2008, Wiley.
This approach can apply to organic molecules for controlled pattern of molecules with ECSTM. In an approach proposed by Song et al., by controlling the substrate potential a well ordered structure of methylene blue (MB) monolayers on Au (111) surface was obtained. Methylene blue (MB) is an electron mediator in many biological processes of living organisms and exhibits interesting redox behaviors. Electrochemical scanning tunneling microscopy (ECSTM) is an important technique to unravel the surface-potential-induced ordering with submolecular resolution. Electrochemical scanning tunneling microscopy (ECSTM) examined the monolayers of MB on Au (111) in 0.1 M HClO₄ and showed long-range ordered, interweaved arrays of MB with quadratic symmetry on the substrate in the potential range of double-layer charging. CV of the MB-adsorbed Au (111) electrode was performed in 0.1 M HClO₄. Figure 8 shows the CVs for a well-prepared Au (111) electrode in 0.1 M HClO₄ at a scan rate of 50 mVs⁻¹ in the absence and presence of MB molecules. Figure 8(b) presents the CV of Au (111) electrode in the presence of 10 μM MB in 0.1 M HClO₄ solution. The presence of the organic molecules in the solution resulted in a dramatic change in the CV shape, compared with that obtained in the pure HClO₄ solution. Two oxidative peaks appeared at 0.39 and 0.54 V and the corresponding reductive peaks appeared at 0.39 and 0.52 V, respectively.

A highly ordered, large area MB adsorbed layer of high quality molecular resolution ECSTM images were presented in Figure 9. The images clearly demonstrated that the MB molecules are closely packed with internal structure of MBs in a scan size of 7 × 7 nm². Each MB molecule clearly showed three bright spots denoted as indicated by the three solid ellipses (a)–(c) on the obtained ECSTM image. From the image, it is clearly noticeable that the structure looks elliptical rather than circular, especially the spot (b). This indicated the possible drift in the ECSTM measurements and abundant images were scanned and analyzed from different scan directions. However, the images obtained all clearly showed that similar elliptical spots which confirmed that the elliptical spots did result from the MB. The bright spots resulted from ECSTM imaging was due to the strong electronic coupling between the nitrogen and sulfur atoms of MB with Au having high electron densities of phenothiazine, nitrogen of dimethylamide functional groups and benzene rings in the MB molecule. The bright spots (a) and (c) are assumed to be mainly the benzene and nitrogen of dimethylamide functional groups in MB.

The similar appearance of the spots indicated as (a) and (c) strongly suggests that the two functional groups of each MB molecule are located at same adsorption sites. The bright spot (b) was assumed to be mainly the phenothiazine in MB. Therefore, the bright spots exhibited elliptical forms and were positioned with a grooved shape rather than keeping along the same line. The distance between the outside of the spots (a) and (c) along the long axis is 1.4 ± 0.1 nm, which is in accordance with the length of each individual molecule. Also, the spots (a), (b), and (c) were found to have a corrugation height of about 0.1 nm, which is comparable with that observed with benzene, naphthalene, anthracene, and coronene. Therefore, from these analysis forms the ECSTM measurements the molecular plane of each MB molecule is proposed to be parallel to the Au (111) surface. Hence, a set of three bright spots were attributed from one MB molecule with a flat-lying orientation on the Au (111) surface as shown in Figure 9. This approach of understanding the conductance of metal with the influence of molecule-electrode interactions is an important issue which enables to develop single molecular junctions.

Fig. 8. Typical cyclic voltammograms of Au (111) electrode in 0.1 M HClO₄ (a) and 0.1 M HClO₄ + 10 μM MB (b). The potential scan rate was 50 mVs⁻¹. Reproduced with permission from [39], Y. H. Song and L. Wang, Microsc. Res. Tech. 72, 79 (2009). © 2009, Wiley.

Fig. 9. High-resolution ECSTM image (about 7 × 7 nm²) of MB adlayer formed on the Au (111) surface in 0.1 M HClO₄ at 0.25 V versus RHE. The triple set of arrows indicates the directions of atomic rows of Au (111) surface. Tip potential and tunneling current were 0.18 versus RHE and 2.83 nA, respectively. Reproduced with permission from: [39], Y. H. Song and L. Wang, Microsc. Res. Tech. 72, 79 (2009). © 2009, Wiley.
3. APPLICATION OF ECSTM IN PROTEIN ELECTROCHEMISTRY FOR NANOBIOELECTRONIC DEVICES

Towards the ultimate miniaturization of the electronic devices, biomolecules have been considered as the building blocks for future development of electronic devices. Thus, single biomolecules especially the redox proteins also emerged as building blocks and has shown great opportunity in the development of molecular bioelectronic devices like diodes, transistor and robust memory switching elements. On the other hand, ECSTM with a possibility to observe nanoscale resolution with combined electrochemistry enabled to develop electrochemical protein based devices.

3.1. ECSTM Studies Towards Protein Based Memory Devices, Transistors

The electrochemical properties of self-assembled protein molecules immobilized on conductive substrates which are well swollen in electrolytic solutions has achieved much attention in recent days in order to study the fundamental properties, kinetics of charge transport and also for the potential applications towards innovation bioelectronics devices based on the redox properties of the biomolecules. Much attention has paid for possible implementation of hybrid electronic devices by the self-assembly of bio molecules conjugated with other inorganic or organic molecules. Toward the development of hybrid architectures, biomolecules such protein, RNA and DNA molecules were suited due to their size and intrinsic properties similar to inorganic nanomaterials. Furthermore, to analyze the redox properties of protein architectures scanning probe microscopy operated under electrochemical control was utilized. Recently, many works were reported based on SPM to analyze the conductivity of the proteins and various redox molecules adsorbed on flat metallic surfaces. Several theoretical assumptions and explanations were proposed for understanding the STM signal involving resonant tunneling due to nuclear relaxation or due to redox reactions.

The in-situ ECSTM technique (in situ electro chemical scanning tunneling microscopy technique), which is based on the scanning tunneling microscopy technique used in conjunction with electrochemistry, is highly useful technique which is capable of measuring the small changes in the surface changes which occur as a result of the electrochemical process. For some electro-active bio or organic thin films, mass changes, which occur upon electrochemical oxidation and reduction due to insertion or expulsion, respectively, of counter ions, coinions and/or solvent into and out of the thin film have been elucidated quantitatively using in situ ECSTM technique.

A recombinant Azurin (Az) redox protein, in which the cysteine residues were incorporated to bind with the noble materials without using any chemical linkers. The modified proteins were allowed to adsorb on Au-nanodots pattern fabricated on indium tin oxide (ITO) surface. Scanning tunneling microscopy and in situ cyclic voltammetry of protein monolayers adsorbed on the Au-dots were performed with a bipotentiostat. The electrochemical cell was housed with Pt and Ag wires as counter and reference electrodes and filled with 50 μl of 10 mM HEPES buffer pH 7.0. The schematic diagram of the ECSTM experimental set-up and immobilization of cysteine modified Az onto Au-nanodot is shown in (Figs. 10(a, b)). Scanning tunneling spectroscopy (STS) measurements were performed at a set point of 500 pA and 100 mV bias. Current–voltage (I-V) spectra were recorded by positioning the tip on the top of the redox protein, and the feedback loop has been disengaged, the tunneling current being monitored by ramping the bias in the range of ±300 mV.

In-situ cyclic volammetry measurements were performed to investigate the electron transfer function of Az monolayer on Au-dots. Representative data shown in Figure 11, reveals a robust electrochemical response with a midpoint potential of 200 mV versus Ag/Ag⁺. From the cyclic voltammograms, redox peaks due to Az were observed and with no peaks at the bare Au-dot electrode, which means that the Az monolayer is well adsorbed on
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Fig. 11. (a) Representative cyclic voltammogram for a bare dot and (b) monolayer of the Az adsorbed on the Au-dot electrode in 10 mM HEPES electrolyte solution. Scan rate is 50 mV/s (inset shows the bare and Az immobilized Au-dots in each case respectively). (c) $I$–$V$ curves of the Az/Au-dot system (yellow, continuous line) and of bare Au-dots (red, continuous line) at an engaged tunneling current of 500 pA and at an engaged bias of 0.1 V. Each curve has been averaged over 10 different sites; for each site an average over 10 curves has been performed. Reproduced with permission from [61], A. K. Yagati et al., Thin Solid Films 518, 634 (2009). © 2009, Elsevier.

the dots. The formal redox potential ($E_{1/2}$), calculated as $E_{1/2} = (E_{pa} + E_{pc})/2$, is 195 ± 10 mV, which appears to be shifted to more negative values than that reported for Az directly anchored on gold (270–280 mV). The separation between the anodic and cathodic peaks $\Delta E_{pc}$ is 150 mV (at a ramp rate of 50 mV/s) and it is dependent on ramp rate showing a quasi-reversible kinetics.

STS measurements in air were conducted to examine the conductive properties of Az adsorbed on Au-nanodots. Measurements were performed through ramping the voltage in range of ±300 mV by disengaging the feedback loop temporarily by positioning the STM tip on the protein. It was observed that the shape of tunnel $I$–$V$ curve depends substantially on the STM tip position point over the protein globule at which the electron tunneling was measured. The $I$–$V$ curve shown in Figure 11(c) over the center of the protein molecules was asymmetric with respect to that obtained for bare Au-dot with features recorded in a double-tunnel junction configuration, an STM tip-protein molecule-conducting substrate. This behavior in the current profile for Az adsorbed nanodots were expected from various factors such as if the molecule is a donor–acceptor pair or may be conformational changes driven by the electric field or even to schottky barrier effects, but it is mainly dominated by the air gap between the tip and protein.

Furthermore, the redox properties of Az proteins such as oxidation and reduction potentials can be utilized for charge storage and erase and these two states can be read by the application of open-circuit or equilibrium potential towards in order to demonstrate the system as a nanoscale biomemory device. Towards this, ECSTM technique was well suited to examine the oxidation and reduction states with cyclic voltammetry and simultaneous it is possible to visualize the topography of the molecules in these three states. Thus measurements were performed with Az on bulk Au substrate with an electrochemical cell that was housed with Pt and Ag wires as counter and reference electrodes and filled with 10 mm HEPES buffer at 7.0. The redox states of the protein molecule and open circuit potential was used for charge write, read and erase function of the proposed device. The redox states of the Az can be controlled by the applied potential. Application of oxidation potential causes the transfer of electrons from the Az molecules to the Au surface results in storage of positive charge (write) and application of reduction potential causes the electron back to the protein molecule thereby ‘erasing’ the stored charge. These two states can be ‘read’ by the application of open circuit potential or cell equilibrium potential. Application this open circuit potential generally doesn’t affect the charged states hence no electron transfer will occur at these states.

The topography for the immobilized Az molecules on Au surface was obtained with ECSTM under these three distinct conducting states as shown in Figure 12. Tip induced potential changes can be observed one specific...
region on Az molecule. Applying an oxidation voltage (486 mV) causes transfer of electrons from the immobilized Az molecules into the Au substrate, and positive charges stored in Az molecules. On contrary, reduction voltage (278 mV) was applied to pass electron transfer reverse into Az molecules, to erase the stored charge. For reading these charged states, open circuit potential (OCP) was utilized. Az molecules attained a stable equilibrium state between assembled Az and electrolytes when OCP was applied. ECSTM images were obtained at three distinct conducting states as mentioned above (at 486 mV, at 278 mV, at 80 mV). The typical bright spots which are seen when tuning the substrate potential in a region, appear to be strongly potential-dependent of redox potentials.65 Such kind of behavior is consistent with a resonant nature of the current measured in STM experiments in the Au adsorbed Az molecules.

According to the Marcus–Gerischer model,66 the maximum of the density of unoccupied states (oxidized state) of a metalloprotein is at energy equal to its formal potential plus the reorganization energy. To interpret an ECSTM experiment one has to relate these quantities; (a) time-scale of the electron transition, (b) spanning from a genuine resonance process where a molecule is not reduced, to the involved potentials. In case of single-electron transfer, Schmickler derived the following relationship between the working electrode potential \( E_{\text{max}} \), corresponding to the maximum in the tunneling current, the bias voltage \( E_{\text{bias}} \), applied between the tip and the substrate, the midpoint potential \( E_m \) of the electro-active species, and the reorganization energy \( \lambda \).67

Towards the development of a nanoscale memory device it is expected that these results might be interrelated with current flowing and charge storing defined as writing, reading and erasing. The results are described in Figure 12 with 50 nm scale three surface images (set point current = 4.33 nA, scan rate = 3.01 Hz). Figure 12(a) indicated assembled Az at 80 mV. There are three or four lump little aggregated on Au surface. Figure 12(b) pointed at assembled Az at 486 mV (in write step) and Figure 12(c) showed assembled Az at 278 mV (in erase step). The shape and height have been changed more and more as specific potential applied. Figures 12(d–f) shows the three dimensional profiles of the Figures 12(a–c) respectively.

These studies were further extended in order to achieve a complete nanoscale biomemory device based on ECSTM. To address, this Az protein was immobilized on Au nanopattern and with ECSTM the charge states were exploited in order perform memory functions and also direct visualization of Az/Au was achieved. To achieve Au nanopattern, nanosphere lithography method68 was adopted to achieve controlled 2D ordered gold nanoarray on ITO substrate by spin coating polystyrene spherical particles followed by thermal evaporation. As a result, nanooarray of ordered hexagonal Au pattern was evolved from the colloidal monolayer. With the effective immobilization of cysteine modified Az on the patterned Au arrays, a technique was discussed to develop a nanoscale memory based on the ECSTM experiments on the redox reaction of Az film in aqueous solution. Well-adsorbed Az molecules on Au hexagonal pattern on ITO surface were observed form electrochemical scanning probe microscopy experiments. The application of proper bias potentials will ensure the mechanism of the memory device functioning. Clear memory switching for charge storage and erase functions is observed, which will lead to the implementation of nanoscale bio devices and open important new perspectives for developing a nanoscale memory device.

The schematics for the experimental set-up and the nanobiomemory principle are shown in Figure 13. Electrochemical experiments on Az/Au-ITO were conducted by ECSTM with in-situ cyclic voltammetry. Experiments were carried out in 10 mM Hepes buffer solution, where the Az/Au-ITO substrate acts as working electrode (vs. Ag/Ag+) as shown in Fig. 13(a). The biomemory mechanism is shown in Figure 13(b).

EC-STM imaging was performed in constant-current mode on the Az adsorbed on the Au nanotriangles. Initially cyclic voltammetry was performed to figure out redox properties of Az protein. It is found that, reduction at −0.07 V and oxidation at 0.36 V for the Cu²⁺/Cu⁺ redox process. Then, a set of images of the same sample area was obtained at constant bias while varying the potential. It was observed from the imaging that there was a variation in the conductance of the redox molecules upon varying the potential which was reflected in a difference in apparent height with respect to the background. Figures 13(c and d) shows the images for the applied potential of 0.36 and −0.07 V respectively for a bias voltage of 100 mV for oxidation and reduction states of Az molecules. It was assumed that at \( E_{\text{bias}} = 0 \) V, the redox state of Az is vacant with energy higher than the Fermi energy of the substrate.
and tip. When a negative bias was applied to the substrate and the tip was kept at the positive potential, the vacant state energy and fermi energy of the substrate were matched, allowing electrons to be transferred from the substrate to the biomolecule, which lowers the thermal activation allowing the biomolecule to transfer the electron to the tip.

It is understood that from the images that there were two conductive states for charge storage. A brighter spot emerged, which was interpreted as a small island of oxidized Az molecules containing a Cu center. When a voltage of $0.36\, E_{\text{ox}}$ was applied, an electron tunnel formed adsorbed Az molecules on the Au substrate, and lead to storage of positive charges in the azurin molecules. In contrast, when a voltage of $-0.07\, E_{\text{red}}$ was applied, the electrons were transferred back to Az molecules and the stored charge was erased, which was performed to erase the stored charge. In this case, the brighter spot on the Au triangle disappeared, which confirmed that the oxidized Az molecules were now reduced. The bright spots on the Au nanotriangles, which contained Az, were attributed to electron tunneling enhancement due to the presence of the copper active site. These morphological changes appeared to be strongly dependent on the redox potentials. This behavior is consistent with the resonant nature of the current measured in the STM experiments of the Au adsorbed Az molecules.

Current–time measurements were obtained on Az/Au and on a bare Au pattern as working electrode. Currents were recorded by applying oxidation ($0.36\, V$ vs. Ag/Ag$^+$) and reduction ($-0.07\, V$ vs. Ag/Ag$^+$) potentials in 10 mM HEPES buffer solution as shown in Figures 14(a, c). The cyclic voltammetry measurement recorded on both bare Au and Az/Au with Ag/AgCl electrode are also shown, where bare Au does not reveal any peaks however Az/Au shows broad peaks corresponds to oxidation and reduction of Az and with its currents increased linearly with increase in scan rate, which is characteristic of surface-confined electroactive species (Figs. 14(b, d). Sharp current transitions were observed for both the oxidation and reduction potentials on the Az/Au pattern indicating that the device can be switched ON and OFF for charge storage. However, the bare pattern does not show any faradaic currents rather it shows a pulsed waveform similar to the applied voltages which behaves as a pure conductor and cannot store any charge. Additionally, we observed that the reducing current produced the same magnitude of oxidation current in which we assumed that the oxidized Az molecules were completely reduced. The switch response to a sequence for write and erase pulses were examined to further demonstrate the potential of the system to develop a nonvolatile...
memory device. Short 2 ms write (0.36 V) and erase (−0.07 V) pulses were applied, and the readings were observed for 15 cycles. As shown in Figure 14(e) both switch states were completely stable and reversible. The redox potentials were continuously applied to switch ON and OFF to estimate device stability, which we observed based on the stable redox peaks by CV. The redox properties were maintained for up to $10^5$ cycles with little change in peak currents. In addition, the switching robustness at fast-voltage pulses for write and erase sequences in the range of $10^{-6}$ s did not show degraded current values. Based on this, the present device showed good endurance for charge storage without loss of any magnitude in the faradaic current.

### 3.2. Single Molecule Charge Transport and Switching

Single molecule conductance between two metal junctions has attracted much attention in the field of molecular electronics. The molecular switching between the on and off states has been achieved where the conductance of the molecular junction can be controlled electrostatically with a third (gate) terminal. The role of the source and drain can be represented by the tip of the STM and the substrate (working electrode 1 and 2). Conductance measurements in an electrochemical environment where the solid–liquid interface forms have advantageous as it enables to control the potential drop between each working electrode and the reference electrode. Therefore, here the electrochemical gate will modulate or controls the charge transport between the two working electrodes. The effective gate-molecule distance is estimated by the double layer thickness at the electrode-electrolyte interface, which is of size of few solvated ions. The formation of electrochemical double layers which has thickness of few nanometers acts as a separation layer for ion movements even at high concentration levels. This double can act as reproducible gating element for studying the conductivity of single molecules. This gate voltage can be controlled through variations in electrochemical potentials and can be applied to various biomolecules, organic molecules to understand the conductivity properties and hence to develop a single molecular transistor for future bioelectronic devices.
For example, in the case of redox based molecules or nanowires, this methodology can be utilized to switch the molecule between their redox states. A schematic illustration of electrolyte gating of a single-molecule bridge is shown in Figure 15. A single viologen molecule is coupled between the STM tip and the substrates in which the tip and substrates will act as a source and drain electrodes resembling a conventional transistor. Furthermore, the counter and reference electrode combination acts as a gate electrode coupled to a bipotentiostat with independent electrochemical potential control of the substrate (working electrode 1, or “source”) and the STM tip (working electrode 2, or “drain”). Thus the potential difference between these two electrodes was applied to the molecular junction; Figure 15(b) shows the redox group is gated by the close proximity of electrolyte ions.

This mechanism of electrochemical gating of single molecules to utilize the single molecule electrochemical transistor like configuration where the molecule will bridge between two electrodes typically made of noble materials. Electrolyte gating where the redox active molecule is attached to the substrate but not the STM tip, or occasionally vice versa. To understand this strategy, as a model example, viologen molecular wires were the first electrochemical redox system studied in this single-molecule junction configuration, with the molecule anchored to the source and drain electrodes through chemisorbed thiol contacting groups. The viologen molecules are of particular interest due to the electrochemical reduction process ($V^{2+}/V^{+}$ redox system).

Figure 16 shows a typical voltammetric curve resulted from viologen molecules having thiol groups at each end enabled to couple Au electrode (HS-6V6-SH) for a low and a densely packed high coverage molecular adlayer.

A highly ordered viologen molecules were identified in at atomic resolution which are arranged in a zig-zag manner composed of sulfur atoms, the alkyl chains and the viologen moiety. However, at high coverage the molecules were disordered. Hence, low coverage phase was used to study the single molecule conductance in the present study as a function of redox state.

The break junction method was performed under electrochemical potential control in which the current-distance traces were recorded by retracting the tip. The results indicated some characteristics plateaus of 0.2 to 0.3 nm separated by abrupt steps. The obtained curves were statistically analyzed which lead to single molecular conductance data of the oxidized and reduced form of the viologen bridge by appropriate substrate and tip polarization (Fig. 17).

The single molecule conductance value of viologen molecule coupled to Au electrodes was constant and possessed a good stability in the oxidized viologen dication $V^{2+}$. The conductance was found increasing about 50% for more negative potentials such as at $E = -0.7$ V. This tendency in single-molecule redox switching is credited to the higher electron density and the higher degree of conjugation of the radical cation as compared to the dication.

Recently, a large number of metalloproteins has evolved as possible candidate for next generation...
Review of biomemory, electronics to carry out many important functions towards the development of biomemory and bioprocessing devices. Single-protein conductance was measured with an ECSTM using the STM-BJ approach on the blue copper protein Az in buffer solution and under bipotentiostatic control shown in Figure 18. This redox center makes the protein capable of accepting and transporting electrons by switching its redox state (Cu$^{1+/2+}$).

Similarly, using ECSTM in a STM-BJ approach, Az immobilized on Au was studied under electrochemical control in buffer solution. Current–distance was presented in Figure 18(b) present current steps that resemble those reported for small organic compounds using the same setup. These steps were absent in control experiments performed on a clean gold surface (compare red traces of Figure 18(b) with black traces in the inset of Figure 18(b), and thus they were interpreted as transient formations and ruptures of molecular junctions with Az bridging the two electrodes (the Au substrate and ECSTM probe, see Figure 18(a)). Collecting hundreds of current steps during one experiment allowed building conductance histograms and then single-Az conductance was calculated. By performing histograms at different sample potentials, conductance modulation with overpotential (“redox gating”) was demonstrated. In Figure 18(c), the conductance of Az (red circles) as function of electrochemical gate potential. It can be noticed that Az conductance depends on potentials applied, while the conductance obtained in control experiments on non-redox Zn-Az junctions (black squares) remains potential independent. This demonstrates again that the Cu center is fundamental for the electron transfer process and that the conductance can be modulated with electrochemical potentials. Such conductance modulation is analogous to the modulation of current in a field effect transistor and possesses yields of on/off ratio of 20 in conductance modulation (Fig. 18(b)). The in-situ behavior of single Az, was estimated by having the ECSTM tip at a controlled distance from the surface, the current was recorded in order to detect spontaneous formation of single-Az junctions. Upon the detection of such single-Az junctions, the reference electrode potential (acting as gate electrode) was swept. Conductance of a single Az determined in situ was found to be modulated with the electrochemical gate. These results constituted a proof of concept of a single-wired protein transistor.

More recently, the current–voltage characteristics of single Az were recorded both in tunneling and wired configurations, which yield the lowest transition voltage reported to date (0.4 V).

The $I–V$ measurements in single biomolecules such as protein or DNA is crucial for the development of novel bioelectronic devices in which ECSTM plays an important role to analyze the properties in the native environment. ECSTM analysis along with other spectroscopic molecules enabled to understand the properties of the biomolecule. Recently, transition voltage spectroscopy (TVS), through which molecular level positions can be determined in molecular devices without applying extreme voltages, has been applied for organic monolayer and also for non-redox proteins. TVS spectroscopy for redox proteins which is useful for understanding voltage dependence of molecular conductance is especially relevant for the mechanism of ET in redox-active molecules.

Here, azurin was covalently immobilized on Au substrate and ECSTM was employed under bipotentiostatic control using Ag/AgCl as a reference electrode to examine the redox properties of the protein. Measurements were performed in both tunneling configuration (no physical contact between the STM probe and the protein) and the wired configuration (probe is in contact with the protein). In tunneling configuration of ECSTM, like normal STS spectroscopy, the $I–V$ curves were obtained by positioning the probe over a region with a high protein surface concentration once the imaging is finished. The $I–V$ curves for the reduced azurin depicts two distinct behaviors one relatively linear and the other more rectifying behavior which is not observed on bare Au electrode.
This rectifying behavior is due to the applied bias voltage such as the reduced and oxidized states of azurin. From the I–V characteristics, the conductance (G) of the tunneling gap in the presence of azurin was calculated from the relation $G = I/V$ to be between $10^{-4}$ $G_0$ and $10^{-5} G_0$. To get better understanding of the obtained I–V curves, TV value that can be used to describe the electrochemical potential dependence of the azurin conductance in the context of TVS. Individual I–V curves and plotted $\ln(I/V^2)$ versus $1/V$ displayed minima in the curve, called transition voltage (TV) value, Figure 19, which is not visible in bare Au samples. Several ET studies of redox molecules have shown that a transition in conductance occurs when the application of an external potential results in the alignment of the molecular energy levels and the Fermi level of the electrodes. Here, the low TV value obtained for azurin suggests that the effective barrier for tunneling through a solution is lower than the barrier observed in pure tunneling processes (i.e., tunneling through vacuum), in agreement with experimental and theoretical works on tunneling through an electrochemical environment. In particular, in the context of two-step ET in a redox molecule, a transition was predicted by theory to occur in the range where the effective voltage in the redox center is higher than the reorganization energy of the molecule.

For the determination of TV in the conductance of the redox protein, the tunneling current for the two-step electron transfer mechanism, $I_T$, is given by:

$$I_T = e k p (e U_{\text{bias}}) \frac{\omega}{2 \pi} \left[ \exp \left( \frac{e^2}{4 \hbar k T} (\lambda + \xi \eta + \gamma U_{\text{bias}})^2 \right) \right]^{-1}$$

in which is the electronic transmission coefficient; $\rho$ is the density of states in the metal near the Fermi level; $\omega$ is the nuclear vibration frequency; $k$ is Boltzmann’s constant; $T$ is the temperature; $U_{\text{bias}} = U_F - U_S$ is the potential difference between probe and sample electrodes; $\lambda$ is the reorganization energy; $\eta$ is the overpotential, given by $\eta = U_r - U_A$, where $U_A$ is the redox potential of an azurin molecule; and $\gamma$ and $\xi$ are two model parameters describing the shifts in $U_{\text{bias}}$ and $\eta$ at the redox center, respectively. The parameters $\gamma$ and $\xi$ are related to the electronic
coupling of the molecule with the probe and substrate, respectively.

To obtain values of the parameters for azurin, we fit the experimental $I-V$ curves to Pobelov and Wandlowski's numerical equation:

$$I_T = 1820kU_{bias} \left\{ \exp \left[ \frac{9.73}{\lambda}(\lambda + \xi \eta + \gamma U_{bias})^2 \right] 
+ \exp \left[ \frac{9.73}{\lambda}(\lambda + U_{bias} - \xi \eta - \gamma U_{bias})^2 \right] \right\}^{-1} (5)$$

in which $I_T$ is expressed in nA, potentials are in V, and $\lambda$ is in eV. In this expression, typical values for $\omega$ in a liquid and $\rho$ in a metal were used, and $\xi$, $\gamma$, $\lambda$, and $\eta$ were left as free model parameters. In the wired junctions, a TVS spectrum is also determined but a negative low value was determined. This minimum is due to the stronger coupling with the probe electrode, which lowers the energy barrier between the levels of the STM probe electrode and the molecule. Thus, in wired junctions, the TV is related to the contact resistance, as commonly found for single-molecule junctions. Hence, these measurements help in characterizing redox proteins and understanding their performance in biological ET chains and molecular electronic devices.

4. SUMMARY AND OUTLOOK

In this review, we began focusing on the advancement of scanning probe microscopic techniques such as STM, STS and ECSTM and their capabilities in atomically resolving the surface structures and electrical/electrochemical properties. In recent years, ECSTM technique has become an increasingly versatile tool in the advancement of molecular electronics. With the capability to study these structures and functions with tunneling, liquid format with nanoscale resolution, there are many possibilities for the ECSTM technique to utilize in various bioelectronics applications. We discussed some of the application of ECSTM and its capabilities with selected articles of our own work on charge transport in biomolecular structures on Au surface in resolving the conductance of proteins and also applied to various organic and inorganic molecules in understanding the properties and for structural analysis. This review also presented that many biomolecules can be imaged at single-molecule level using ECSTM, once a suitable strategy for its immobilization on an atomically flat electrode is found. However, certain factors influencing the tunneling gap and role of ionic strength needs to be considered. Further, electrochemical atomic force microscopy (ECAFM) can be complimented in analyzing the mechanical information.
of single molecular structures and electron transport phenomena. Furthermore, the electron transport phenomena in complex biomolecules, cellular biology and molecules with multiple redox systems requires deeper understanding however with computer simulations and advancement in nanotechnology will provide novel concepts and applications in molecular bioelectronics.

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References and Notes

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86. Z. Li, B. Han, G. Meszaros, I. Pobelov, T. Wandlowski, A. Blaszczyk, and M. Mayor, Faraday Discuss. 131, 121 (2006).