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Distance Dependence of Electron Transfer Kinetics for Azurin Protein Adsorbed to Monolayer Protected Nanoparticle Film Assemblies

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Abstract

The distance dependence and kinetics of the heterogeneous electron transfer (ET) reaction for the redox protein azurin adsorbed to an electrode modified with a gold nanoparticle film is investigated using cyclic voltammetry. The nanoparticle films are comprised of non-aqueous nanoparticles, known as monolayer-protected clusters (MPCs), which are covalently networked with dithiol linkers. The MPC film assembly serves as an alternative adsorption platform to the traditional alkanthiolate self-assembled monolayer (SAM) modified electrodes that are commonly employed to study the ET kinetics of immobilized redox proteins, a strategy known as protein monolayer electrochemistry. Voltammetric analysis of the ET kinetics for azurin adsorbed to SAMs of increasing chainlength results in quasi-reversible voltammetry with significant peak splitting. We observed rate constants ($k°_{ET}$) of 12-20 sec^{-1} for the protein at SAMs of shorter alkanethiolates that decays exponentially (β = 0.9/CH_2 or 0.8/Å) at SAMs of longer alkanethiolates (9-11 methylene units) or an estimated distance of 1.23 nm and is representative of classical electronic tunneling behavior over increasing distance. Azurin adsorbed to the MPC film platforms of increasing thickness results in reversible voltammetry with very little voltammetric peaks splitting and nearly negligible decay of the ET rate over significant distances up to 20 nm. The apparent lack of distance dependence for heterogeneous ET reactions at MPC film assemblies is attributed to a two-step mechanism involving extremely fast electronic hopping through the MPC film architecture. These results suggest that MPC platforms may be used in protein monolayer electrochemistry to create adsorption platforms of higher architecture that can accommodate greater than monolayer protein coverage and increase the Faradaic signal, a finding with significant implications for amperometric biosensor design and development.

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Introduction

Over the last two decades, the study of electron transfer (ET) properties of redox proteins has centered on a strategy known as protein monolayer electrochemistry (PME).[1,2] Used as a means to simplify the analysis of ET kinetics, the PME approach involves the confinement of redox proteins to a synthetic platform via adsorption or specific immobilization to eliminate diffusional aspects of protein interactions. The adsorption platform or working electrode subsequently serves as the redox partner for the electroactive protein layer that can be easily oxidized or reduced under potentiostatic control with simple voltammetry experiments. The direct, unmediated electrochemistry of redox proteins at electrodes is an important tool for the fundamental study of biological ET processes including but not limited to protein redox chemistry involved with both cellular respiration and photosynthesis.[1-4] Likewise, PME serves as a tool for studying redox protein behavior at man-made materials, a significant aspect of bioanalytical chemistry aimed at developing biocompatible materials as well as amperometric biosensors involving redox proteins.[5-8] Indeed, PME systems have been utilized as model systems in this area of study to further our fundamental knowledge of protein adsorption, interfacial chemistry and ET characteristics.[5-8]

Self-assembled monolayer (SAM) modified electrodes designed to mimic the redox partners of specific proteins effectively addressed several problematic aspects of the PME strategy.[2] Most notably, SAM modification of the electrode provided significant control over background charging current that, if unchecked, obscured the Faradaic responses and complicated voltammetric peak analysis. Second, the use of SAMs to immobilize proteins allowed for, at the time, an unprecedented degree of control of the binding chemistry at the protein/electrode interface. These attributes allowed the strategy of immobilizing redox proteins to SAMs to become the predominant approach to studying biological ET of a variety of important model proteins.[2,9] Notable within this body of work are studies of nonspecific adsorption of proteins to SAMs including reports on cytochrome c (cyt c) by the Bowden,[9,10] Waldeck,[11,12] Niki,[12] and Gray groups,[13] study of azurin (AZ) by Martin,[14] Ulstrup[15] and Niki,[16,17] and investigations of ferritin by Zapien's group.[18] These reports of PME spawned work that included the exploration of both AZ and cyt c at mixed SAMs[19,20] as well as covalent attachment of the target proteins to engineered SAMs.[11,21,22] Taken collectively, this research supports the

effectiveness of the PME strategy for studying the adsorption and electrochemical behavior of immobilized redox proteins where simple voltammetry experiments can be used to readily report essential thermodynamic and kinetic properties of the proteins such as formal potential, surface coverage, ET rate constant, and the very important distance dependence of ET.

In spite of its success, PME does suffer from several limitations that affect the quality of the results achieved with the technique.[2] First, the aforementioned examples of PME often display dispersed or non-ideal electrochemical properties and broadened voltammetric peaks, a consequence of a lack of molecular level control at the protein/SAM interface.[23,24] Second, the PME strategy is by definition limited to a monolayer or less of protein coverage which, in turn, gives the systems an inherent low signal-to-background current ratio.[2] This limitation is partially addressed with the use of SAMs which act as a low dielectric spacer between the protein and electrode and drastically reduce the double layer capacitance of the system and the associated background charging currents.[2] Additionally, efforts have been made to immobilize greater than a monolayer of protein and therefore improve the signal-to-background ratio but these attempts resulted in detrimental, in terms of signal collection, decay of the ET over the larger distances between the protein and the electrode. This ET decay is commonly seen with redox proteins adsorbed at SAMs of increasing alkanethiolate chain lengths where a dramatic drop off in ET rate constant is observed that is consistent with an electron tunneling mechanism.[9,11,15-17,25, 26] Thus, these limitations of PME persist and are a focus of research aimed at optimizing the PME strategy.[2]

One variation of PME being explored is the incorporation of colloidal metallic nanoparticles (NPs) into the system.[27] NPs are targeted for interacting with biomolecules, particularly redox proteins, because of several advantageous characteristics that have been identified:[28-33] (1) large surface-to-volume ratios that allow for greater numbers of biological adsorbates; (2) biomolecules adsorbed to NP experience greater freedom of orientation and are more likely to maintain their native structures upon adsorption; (3) electroactivity of redox species is preserved upon adsorption to NPs;[34] (4) an ability to act as conduits for ET reactions and; (5) properties of NPs such as core size and interfacial chemistry, shown to have significant influence on protein adsorption and subsequent electrochemistry, including ET kinetic effects,[35] can be easily manipulated. Studies involving NP-modified electrodes as a platform for adsorbed or cross-linked redox proteins are dominated by reports focused on the direct, adsorbed

electrochemistry of the redox proteins such as cyt c at water soluble, citrate stabilized NPs (CS-NPs).[36-45] Careful examination of many of these reports shows that the use of CS-NPs in this capacity often results in protein electrochemistry that has erratic or high background charging currents that contribute to poorly defined peaks, inadequate protein stability (i.e., rapid denaturation), and/or slow, quasi-reversible ET kinetics.

Recent work in our group has explored an alternative approach using electrodes modified with networked films of non-aqueous, alkanethiolate protected nanoparticles, known as monolayer protected clusters (MPCs), as a platform for redox protein adsorption and electrochemistry.[27] Due to their unique properties, MPCs have been extensively researched in recent years, including excellent work by Rotello and coworkers focused on MPC interaction with biomolecules in solution.[30,46] Our initial study[27] focused on the electrochemistry of cyt c adsorbed to MPC film assemblies of various architectures, including different core sizes, MPC peripheral ligands varying in both chain length and terminal functional groups, and the linking mechanism used to assemble the film. Cyt c electrochemistry was evaluated almost exclusively on MPC films comprised of five layers of NPs, including four layers of unfunctionalized MPCs followed by a terminal, interfacial layer of carboxylic acid functionalized MPCs. The primary aim of this first study was to simply establish the feasibility of using MPC films within the PME strategy, with the goals of achieving stable, repeatable cyt c voltammetry that is specifically controlled by the molecular properties of the MPCs in the interfacial layer. While many of these goals were met in the study, it also was successful in establishing the dependence of background signal on the linking mechanism employed during film assembly. However, several major aspects of the MPC film used in this capacity were left undefined or unaddressed. Preliminary results from the study suggested that the MPC film may be masking the surface topography of the underlying gold substrate, a known source of creating heterogeneous adsorption sites and broadening of voltammetric peaks. Likewise, some results suggested that if the MPC films could be engineered for optimal adsorption there was a significant effect on ET rates, namely a seemingly inconsequential decay of ET rates of significant distances.[27] In both of these cases, however, because the five layer films were arbitrarily chosen for the study, the optimal number of the MPC layers and the exact role of the layers was not known, especially in terms of its effect on the ET kinetics.

In this report, we define the ET kinetic aspects of protein/MPC film assembly systems with a detailed presentation of the ET rate constant distance dependence compared to traditional SAM systems. A complicating factor for using cyt c as a model protein to be adsorbed to the MPC films is that a functionalized layer of MPCs is required at the interface. Attachment of carboxylic acid functionalized MPCs as the outermost layer of NPs in the films introduces a variable into the system that may have influenced the observed results (i.e., the surface charge density at the MPC film interface may vary from nanoparticle to nanoparticle as well as film to film and is difficult to control), especially the observed ET kinetics of the electrostatically adsorbed cyt c, a protein with ET kinetics known to be affected by a mixed SAM adsorption platform.[27] Thus, in order to isolate the protein ET kinetics as a function of the MPC films itself, the interfacial chemistry involved with immobilizing the protein (e.g., electrostatic adsorption between carboxylate groups on the MPC and cationic cyt c) required simplification. To accomplish this, our current study focuses on the electrochemistry of a blue copper protein, azurin (AZ), which binds to organic platforms via a well-known, simple hydrophobic interaction.[15] The use of AZ at MPC films allows for more effective control of the interfacial chemistry because it eliminates the need to functionalize the outermost layer of MPCs in order to immobilize protein. In addition to extensive analysis of AZ ET kinetics as a function of MPC film structure and assembly, a primary aspect of this work is the extensive physical characterization of the assembly of the MPCs into a film and the verification of the thickness of MPC films. The establishment of synthetic platforms that exhibit lower ET distance dependence may have important implications for the eventual construction of scaffolds with greater than a single protein monolayer capacity and, thus, more effective signal-to-background ratios.[27]

Experimental Section

Materials and Methods

Chemicals. All chemicals were of reagent grade quality and used as received unless otherwise noted. All aqueous solutions and buffers were generated with 18 MΩ ultra-purified (UP) water.

Azurin Preparation and Purification. A plasmid containing the gene for *Pseudomonas aeruginosa* Azurin (AZ) was provided as a kind gift from Dr. Corey Wilson at Rice University. Purification of the wild type protein was performed using osmotic shock[47] and based on a

procedure previously described.[48] Briefly, a 5 mL culture of *E. coli* Top 10 with plasmid pAZU was grown overnight at 37°C with shaking (200 rpm) in 2xYT broth supplemented with ampicillin (100 μg/mL). This starter culture was used to inoculate fresh Terrific Broth supplemented with ampicillin (100 μg/mL). After 16 hours at 37°C, the cells were harvested by centrifugation at 3000g for 10 min and resuspended in 20% sucrose(w/v), 50 mM Tris (pH 8.0), and 1 mM EDTA (pH 8.3). The cells were incubated at room temperature for 15 min and were collected by centrifugation at 5000g for 15 min. The periplasmic proteins were released by resuspension in ice cold ultrapure water. Treated cells were shaken on ice for 15 min and centrifuged at 5000g for 15 min. The crude preparation containing AZ was treated with potassium ferrate and copper sulfate to a final concentration of 0.1 mM and 1.0 mM, respectively. The light blue supernatant was spun for 10 min at 5000g to clear the precipitated salts and applied to a CM-sepharose (Sigma-Aldrich) cation exchange column equilibrated with 50 mM NH_4OAc buffer, pH 3.9. An intense blue band containing AZ was eluted with 50 mM NH_4OAc buffer (pH 4.5). An absorption ratio at A_{625} and A_{280} was determined for protein purity, where a ratio of approximately 0.53 is considered to consist of pure AZ.[47] Purified protein was buffer exchanged into 4.4 mM potassium phosphate buffer (pH 7.0), lyophilized (Labconco Corporation), and rehydrated with UP water prior to use (Supporting Information).

MPC Synthesis. Hexanethiolate protected MPCs, average structure of $Au_{225}(C6)_{75}$, were synthesized via the well-known Brust reaction[49] from gold salt $HAuCl_4$ previously crystallized from aqua-regia reflux of 99.99% gold shot. Briefly, the $HAuCl_4$ is dissolved in water, mixed with toluene containing the phase transfer reagent tetraoctylammonium bromide which subsequently transfers the gold to the nonaqueous layer. Hexanethiol in a ratio of 2:1 with the gold salt is added to the separated organic phase and stirred for 30 minutes until the solution is a pale yellow. The reaction flask is then chilled in an ice bath for 30 minutes prior to the steady addition of chilled, aqueous sodium borohydride as a reductant. The reaction is stirred overnight and rotary evaporated to dryness prior to being precipitated with the addition of reagent grade acetonitrile. The specific thiol-to-gold ratio, temperature, and speed of reactant addition are contributing factors for producing MPCs with an *average* core composition and diameter of Au_{225} and 2.03(±0.95) nm, respectively. As described in previous work by our group,[27] the average diameter of the MPC cores was verified using TEM imaging (see Supporting Information).

MPC Film Assembly. MPC films were assembled on gold substrates using previously established procedures.[27] Briefly, gold substrates were mounted in electrochemical sandwich cells where they served as the working electrodes (described below). The gold was electrochemically cleaned in a solution of 0.1 M H_2SO_4 and 0.01 M KCl. Clean gold substrates were then exposed to a 5 mM hexanethiol solution of ethanol (EtOH) overnight to form an initial SAM. The SAM-modified gold was washed successively with EtOH and UP water prior to treatment with a 5 mM solution of nonanedithiol (NDT) or linker ligand in EtOH for one hour. After one hour, the gold electrodes were rinsed successively with EtOH, water, and methylene chloride (CH_2Cl_2) before being exposed to a solution of hexanethiolate-MPCs (~1 mg/mL C6 MPC in CH_2Cl_2) to anchor the first dithiol-linked MPC material to the substrate for approximately one hour, during which the solution in the cell was slowly agitated (stirred) with a slow N_2 bubble. The process of immersing the gold in NDT linking molecule solution and rinsing followed by exposure to the C6 MPC solution and rinsing (termed a "dip cycle") was repeated multiple times in order to form a dithiol-linked MPC film assembly on the gold substrate. Assembly of the film at each step was monitored with electrochemical measurements of double-layer capacitance and voltammetry of solution redox species at the film structure as described in the electrochemistry section below. For the gold substrates being modified only with SAMs, each was exposed to a 5 mM solution of thiol overnight before being rinsed and analyzed or used further.

Electrochemistry

Instrumentation and Equipment. Cyclic voltammetry was performed with CH Instruments potentiostats (Models 650A and 610B). The electrochemical sandwich cell, described and used in previous studies by our laboratory and others, featured a Ag/AgCl (sat. KCl) reference electrode (Microelectrodes, Inc.), a platinum wire (Sigma-Aldrich) counter electrode, and an evaporated gold substrate (EMF Corporation, Ithaca, NY) as a working electrode where a Viton o-ring defines the electrode area (0.32 cm^2). During all measurements described below, the cell was housed in a Faraday cage.

Film Characterization and Protein Monolayer Electrochemistry. Film growth could be successfully monitored by systematically measuring the double-layer capacitance (C_{dl}) of the film system at various steps[27] or by collecting and assessing the voltammetry of 5 mM potassium ferricyanide ($K_3Fe(CN)_6^{-4}$) in solution (0.5 M KCl, *aq*) at the film interface.[50] C_{dl} measurements were made by running cyclic voltammetry from 0.1 to 0.4 V (vs. Ag/AgCl, KCl) at 100 mV/sec in 4.4 mM potassium phosphate buffer (pH = 7.0, μ= 10 mM) and measuring the total current at 120 mV (vs. Ag/AgCl, KCl) as has been previously shown. Redox probing voltammetry of the ferricyanide redox couple ($Fe(CN)_6^{-3/-4}$) was accomplished by scanning the potential window of -0.2 V to +0.6 V at 50 mV/sec and noting qualitative changes in peak shape and quantitative changes in both peak current (i_p) and peak separation (ΔE_p).

Protein electrochemistry was performed in the following manner. After completing assembly (described above) of either a SAM modified gold substrate or a MPC film assembly, the cell was rinsed with fresh CH_2Cl_2 several times (pure ethanol for SAM-modified gold substrates), followed by copious rinsing with 4.4 mM potassium phosphate buffer (KPB). The cells were injected with 200 μL of ~5-10 μM AZ in KPB (pH = 7.0, μ= 10 mM) and allowed to sit for one hour in the refrigerator (6-7°C). Cells were allowed to come to near room temperature, rinsed well with KPB (pH = 7.0, μ= 10 mM), refilled with KPB, and degassed with N_2 for 10 minutes. Unless otherwise stated, protein electrochemistry experiments were run in the potential window of -0.25 V to +0.25 V at 100 mV/sec with KPB (pH = 7.0, μ= 10 mM) as the supporting electrolyte. The average surface concentration of AZ at the MPC film assemblies, as determined by integrating the voltammetric peaks, was 6.4 (±2.9) pmol/cm^2, a value consistent with existing reports for near monolayer coverage of AZ at SAMs.[14-16] As previously shown, apparent electron transfer rate constants (k°_{ET}) were determined by applying Laviron's simplest model for an adsorbed species and involving collection of a series of voltammograms at increasingly faster sweep rates to achieve quasi-reversible peak splitting (≤ 200 mV).[9,51,52]

Microscopy

Transmission Electron Microscopy. Transmission Electron Microscopy (TEM) imaging of MPC materials was accomplished using a JEOL 1010 Microscope operating at 80-100kV. Samples of MPC were drop-cast from toluene onto 400 mesh copper grids coated with Formvar (Electron

Microscopy Sciences). Image analysis to determine average core size and polydispersity of the samples was performed using Image J software.

Cross-sectional TEM imaging of the MPC films was achieved by re-embedding en face embedded films.[53] Briefly, MPC films grown on cut glass slides were attached to clean, standard microscope slides using Embed 812 epoxy resin (EMS). A '00' BEEM capsule was filled with resin and inverted over the film and allowed to polymerize (18 hrs at 60°C). After cooling to room temperature, the mounted slides were heated for 20 seconds on a cast aluminum hot plate at 200° C to facilitate removal of blocks with attached en face films. A portion of the block face was cut away just below the film surface using a jeweler's saw. The sliver of material was then re-embedded using a flat mold (source) with the film side facing the interior of the well. After the second resin polymerization, thin sections were prepared on a Leica UCT ultramicrotome using a diamond knife (Diatome). Great care was taken to assure the film was cut perpendicular to the knife's edge. Sections were collected on carbon coated Formvar support films and imaged using the TEM described above.

Atomic Force Microscopy. Substrates of evaporated gold on mica (Agilent-Molecular Imaging) were immersed in piranha solution (a 2:1 mixture of concentrated H_2SO_4 and 30% H_2O_2) for 10 minutes to remove all organic material. ***Warning****: Piranha solution reacts violently with organic material and should be handled with extreme caution*. The gold/mica substrates were then rinsed with UP water and dried under a stream of N_2 before imaging the clean, bare gold surface with an AFM (MFP-3D from Asylum Research). After imaging, the Au/mica sheets were immersed in a solution of C6 thiol in ethanol for 3 hours to prepare a SAM. The slides were then treated with a solution of nonanedithiol (NDT) in ethanol for 20 minutes, followed by a N_2-bubbled solution of C6 MPCs in CH_2Cl_2 for 1 hour. These steps (known as "dip cycles" were repeated for multiple layer deposition of MPC films, usually 3-4 cycles.[54] After the final exposure to the MPC solution, the sheets were rinsed with CH_2Cl_2 and mounted on glass microscope slides for imaging. AFM imaging was performed on an MFP-3D microscope from Asylum Research in non-contact (AC) mode, using SSS-NCRH SuperSharpSilicon AFM tips (nominal frequency f_o = 330 kHz, typical tip radius of curvature 2 nm) from Nanosensors. Typical 1 μm^2 images were scanned at 0.5 Hz with free-air amplitude A_o = 0.3 V and setpoint amplitude A = 0.23V.

Results and Discussion

Protein monolayer electrochemistry (PME) is a strategy for studying the ET properties of simple metalloproteins adsorbed to synthetic platforms. The following PME results focus on studying the ET kinetics of *P.aeruginosa* azurin (AZ), a single copper, redox protein with the structure shown in **Figure 1** at two different types of interfaces, namely self-assembled monolayers (SAMs) and nanoparticle film assemblies. AZ (14.6 kDa, ~3.5 nm diameter) is a highly stable, structurally well-characterized, globular protein involved in ET processes related to bacterial respiration and oxidative stress. Yielding a strong absorption band (ε = 5000-6000 $M^{-1}cm^{-1}$) that makes it intensely blue in color (Supporting Information) and highly reproducible voltammetric behavior, AZ is a well-known model protein for studying biological ET.[14-17, 55-59] Among its attributes in this respect, AZ possesses a predominance of hydrophobic amino acid subunits on one side of its structure (Figure 1) which comprise a hydrophobic binding pocket that has been used to easily immobilize the protein to hydrophobic surfaces. Our previous studies[27] in this area have focused on cytochrome c, a protein of similar size that binds via electrostatic interactions where the immobilization is highly dependent on the surface charge density engineered into the interface. Thus, the hydrophobic binding of AZ to synthetic platforms is significant in that it represents a simplified and more consistent interface where any observed kinetic effects can be directly attributable to the MPC film rather than interfacial chemistry such as the number of carboxylic acid groups (charge) per MPC at the interface.

PME of Azurin at Self-Assembled Monolayers.

Figure 2 depicts a schematic of the traditional approach to PME using self-assembled monolayers (SAMs) for the immobilization of AZ as well as a typical cyclic voltammogram for the system. Analysis of the cyclic voltammetry of AZ at SAMs results in protein surface coverage, formal potential, and peak shape similar to that reported in the literature.[14-17, 55-59] By performing cyclic voltammetry (CV) of azurin adsorbed to methyl-terminated alkanethiolate ($H_3C(CH_2)_nS$-) SAMs of increasing chainlength (i.e., number of methylene units, n), the observed voltammetric peak shapes clearly transitions from nearly reversible kinetics at shorter chainlengths to quasi-reversible kinetics at longer chainlengths (**Figure 3A, inset**). The effect of slower ET kinetics for the AZ voltammetry at longer chainlengths is easily observed if the voltammetric peak separation (ΔE_p) is tracked over SAMs of increasing number of methylene units as seen in **Figure 3A**. While the SAMs of shorter thickness (n<9) exhibit $\Delta E_p \leq 50$ mV, an

abrupt and steady increase in the peak splitting is seen in the voltammetry of AZ at SAMs comprised of alkanethiolates with 9-11 methylene units and continuing with a sharp increase at values of n greater than 11, eventually sloping sharply (i.e., ~100 mV/CH_2) with SAMs of the longest chainlengths having ΔE_p of approximately 100 and 300 mV, respectively.

The kinetic effect observed from the peak separation is also reflected in the corresponding ET rate constants ($k°_{ET}$) of AZ determined for each SAM system. To assess the distance dependence of the ET reaction, **Figure 3B** shows the rate constant as a function of protein separation from the gold electrode (i.e., as a function of both methylene units and estimated distance).[60, 61-63] For SAMs of shorter chainlengths, the apparent ET rate constant (k_{app}) is seemingly independent of distance (methylene units, n) before an exponential decay of the rate is observed at 11 methylene units or an estimated distance of 1.23 nm.[60-63] As has been shown elsewhere for protein systems involving both cytochrome c and AZ at SAMs,[9,11,12,15,17] the exponential dependence can be modeled with the following simple equation (1):

$$k_{app} = k_{n=0} \bullet \exp(-\beta \Delta n) \qquad (1)$$

where $k_{n=0}$ is the extrapolated rate at a distance of zero (n=0), Δn is the distance in terms of methylene units, and β is the decay factor. Equation 1 can be viewed as a simplified approximation of the Marcus equation for non-adiabatic ET through an electronic tunneling mechanism:[9]

$$k_{ET}° = \upsilon \cdot \exp[-\beta \cdot d] \exp(-\Delta G^*/RT) \qquad (2)$$

where $k_{ET}°$ is the standard ET rate constant at zero free energy of reaction, υ is the frequency factor, d is the ET distance, ΔG^* is the activation energy, and β is the electronic tunneling factor. In either case, a plot of $\ln(k_{ET}°)$ versus ET distance, in this case the methylene unit separation, will yield an estimate of β, shown to be between 1.0-1.1 CH_2^{-1} (0.78-0.85 $Å^{-1}$) for electron tunneling ET of ferrocene at SAMs.[64-68] Analysis of our data in Figure 3B results in a β determination of 0.9 CH_2^{-1} or 0.8 $Å^{-1}$. This result is in excellent agreement with established findings for studies involving simple redox proteins undergoing an electron tunneling mechanism at a SAM adlayer, including both AZ and cytochrome c where a β range of 0.9-1.2 CH_2^{-1} is typically found.[9,11,16,14-17] Of

significance to our current study and one of the established shortcomings of SAM-based PME strategies is the decay of the current signal, a factor that limits protein coverage in such systems to a monolayer or less adsorbed only a very short distance from the electrode surface.[2]

Assembly of Nanoparticle Platform for Protein Adsorption

As an alternative platform to SAMs, nanoparticle film assemblies comprised of dithiol-linked monolayer protected clusters (MPCs) of similar diameter to AZ were constructed. Previous research has shown that initial exposure of a glass substrate silanized with 3-mercaptopropyltrimethoxy silane to a MPC solution results in anchoring nanoparticles being adhered to the surface.[27, 69, 70] Subsequent exposures of that glass slide to a solution of nonanedithiol, the dithiol linker, followed by immediate immersion in a solution of MPC results in the build-up of a covalently linked MPC film assembly that is easily verified with UV-Vis spectroscopy (Supporting Information). Our prior work[27] has also shown that similar MPC films can be assembled within electrochemical cells on SAM-modified gold substrates. More specifically, gold electrodes modified by hexanethiolate SAMs can be exposed to dithiol solutions of nonanedithiol to create mixed SAMs of hexanthiolates and dithiol linker ligands. Upon exposure of the mixed SAM to a solution of MPCs, the thiols protruding from the SAM allow for the formation of an anchoring layer of MPCs. As before, subsequent alternating exposures to nonanedithiol and MPC results in the near layer-by-layer assembly of a MPC network on the electrode surface.[27]

The assembly of MPC films at gold substrates can be monitored via spectroscopy (see Supporting Information), electrochemistry, and microscopy. As shown by our prior studies,[27] cyclic voltammetry performed in a narrow potential window that is void of Faradaic responses yields a measure of the charging current of the film. This background signal, quantified as the double-layer capacitance (C_{dl}) of the system, can be used to track MPC film growth. That is, with each exposure of the gold substrates to a combination of nonanedithiol and MPC solution (i.e., a "dip cycle" as described in the Experimental Details section) there is a corresponding increase in the C_{dl} of the system, an indication that MPCs, which behave as small capacitors,[71] are being added to the substrate with each exposure to the MPC solution or dip cycle. **Figure 4A** shows example voltammograms that systematically increase in current magnitude with each dip

cycle. Likewise, with current directly proportional to and translated directly to C_{dl},[27] the quantitative trend for C_{dl} over several dip cycles is shown in **Figure 4A, inset**.

Each exposure of the gold electrode to a surface modifier associated with the assembly scheme, either alkanethiols for a SAM or dip cycles for MPC attachment, can also be assessed by observing the voltammetry of a solution redox couple at the film interface.[50] Here, **Figure 4B** shows illustrative examples of cyclic voltammetry for the redox probe potassium ferricyanide ($K_4Fe(CN)_6$) at various stages of the film assembly process. As shown in the figure, the voltammetry of $Fe(CN)_6^{3/4}$ at bare gold reveals a reversible, diffusional response that indicates the probe molecule easily gains access to the gold electrode. Upon formation of a hexanethiolate SAM (C6 SAM) to the gold substrate (dip cycle #0), the voltammetry of $Fe(CN)_6^{3/4}$ is altered with significant decreases in the peak currents and an increase in peak splitting – both indicators of $Fe(CN)_6^{3/4}$ access/approach to the gold electrode being challenged by the added material. With each subsequent dip cycle involving MPCs (dip cycle > 0), we observe a systematic shift of decreasing peak current and increasing peak splitting from slower kinetics (**Figure 4B, inset**).[72] Taken collectively, the electrochemical results support the view that each exposure to MPC solution is adding a significant, layer-by-layer or less, amount of nanoparticles to form a networked film assembly.

Two forms of microscopy, atomic force microscopy (AFM) and transmission electron microscopy (TEM) were used to further characterize the assembled films. MPC films were grown on gold evaporated on mica substrates and imaged with the AFM. **Figure 5** shows representative AFM images of the same area of a gold on mica substrate before and after 4 dipping cycles (i.e., exposures to nonanedithiol/MPC solutions). Noted for being comprised of atomically flat plateaus, the gold on mica substrate's topography is clearly altered with the addition of the MPC film, taking on a "corrugated" or "bumpy" appearance in the image after the MPC film is assembled. Likewise, cross-sectional analysis from approximately the same area shows a significant change in topography, transitioning from a relatively flat trace to a trace revealing significant, and repetitious surface structure.[73] From AFM imaging, it is evident that before and after the assembly of a MPC film on the substrate there is a visible change in the topography of the substrate with the results suggesting that material is both building up on the surface and assembling with a somewhat regular pattern.

The electrochemical and AFM results suggest the deposition of significant amounts of material being deposited on the gold substrate, but offer little information on the thickness of the films being assembled. Perhaps the most unambiguous measure of film thickness is achieved with cross-sectional TEM analysis of the films,[53] an example of which is shown in **Figure 6**.[74] From the cross section analysis of these films, we estimate a thickness of approximately 10-12 nm for a film formed from five exposures to nonanedithiol. This measurement is in excellent agreement with ellipsometry measurements performed in a prior study on the same films and used to determine that the films were growing in a nearly layer by layer mechanism and packing almost completely interdigitated.[27] In this study, the TEM-cross sectional analysis provides a more direct measurement of the film thickness and an estimate of the ET distance from an adsorbate (protein) to the working electrode.

PME of Azurin at MPC Film Assemblies

MPC films can be used to act as protein monolayer electrochemistry platforms to obtain repeatable and stable cyclic voltammetry of the adsorbed protein as has been shown in prior work for cytochrome c. **Figure 7** represents a schematic of AZ adsorbed to an MPC film as well as a typical cyclic voltammogram obtained for such a system. Unlike previous studies with cytochrome c,[27] however, these systems represent a much simpler interface for protein adsorption since the binding interaction between AZ and the surface is strictly hydrophobic. This simplification allows for the isolation and study of the ET kinetics of the system. While not the focus of this particular study, it is worth noting that adsorption and redox thermodynamic properties such as near monolayer protein surface coverage and stable formal potential, respectively, were successfully measured for AZ at MPC films of varying thickness with results similar to AZ at SAMs.[57]

As shown with the SAM-based PME systems, kinetic parameters of ET for the AZ electrochemistry can be easily tracked over different MPC film thicknesses to determine the distance dependence of the redox reaction. **Figure 8** shows the calculated kinetic parameters of AZ at MPC films of varying thickness measured in both terms of methylene units as well as actual distance. Unlike the AZ/SAM system which showed a steady increase in peak splitting (ΔE_p) after ET through nine methylene units (see Figure 3A), a significant corresponding increase in ΔE_p is not observed for the AZ/MPC systems even though the distance of ET is much

greater (**Figure 8A**). While a change of >250 mV in ΔE_p was recorded for AZ/SAM systems of increasing ET distance, a total ΔE_p of only 10-15 mV was consistently observed for AZ at MPC films of increasing thicknesses (i.e., 1-8 MPC exposures or dip cycles) yielding a slope of only 0.012 V/dip in Figure 8A. This result is obtained in spite of the fact that the actual ET distance, from electrode to protein, is probably much greater with the MPC films compared to the SAMs (see below).

This kinetic insensitivity to distance is reinforced in **Figure 8B** as the ET rate constant determined for AZ at MPC films of increasing thickness, starting with a hexanethiolate SAM as an anchoring layer with six methylene units, is plotted versus the number of dip cycles or MPC exposures, (#). Figure 8B also displays the rate as a function of distance. Rather than translating the dipping cycles to distances using strict geometry (e.g., hexanthiolate chainlength and average core diameter – see Supporting Information), we have chosen a more conservative estimation of the distance based on the experimental results and pre-existing findings for films of this nature by our lab and others. Specifically, because of the excellent agreement of our TEM cross-sectional analysis of the MPC films with the ellipsometry measurements we estimate a thickness of ~2.5 nm/dip cycle. Thus, a five dip cycle film would expectedly be ~12.5 nm in thickness. As seen in Figure 6, this appears to be a very reasonable estimation. In addition to being supported by our own measurements, these estimations are consistent with findings that dithiol-linked MPC films are almost completely interdigitated (Figure 8B, inset) rather than being "spaced" by the linker ligand (nonanedithiol) or arranged edge-to-edge.[75,76] Remarkably, we observe only a very small degradation of rate (slope = -0.095/dip) of $k_{ET}°$ even up to 8 dip cycles or a conservatively estimated distance of 20 nm, an order of magnitude larger distance than the exponential decay observed with AZ/SAM systems at 11 methylene units (~1.23 nm). In fact, films assembled from 8 dip cycles were visible to the naked eye and still yielded extremely fast (not degraded) ET rates for AZ. These results suggest that there is little or no ET distance dependence with the MPC film system.

Considering the aforementioned findings, if one were to even crudely apply the electronic tunneling mechanism/analysis described earlier for traditional PME involving SAMs to the MPC system, one would estimate a drastically different β decay factor. For example, a 5 dip cycle MPC film assembly offers an estimated distance of 12.5 nm (Figure 8B). If we assume the film to be nearly 100% interdigitated (i.e., spaced only by the length of a single hexanethiolate ligand

- Figure 8B, Inset),[75, 76] we can estimate a minimum number of 36 methylene units in the proposed electronic tunneling pathway. With the rate measured at such a film, an approximate β value of 0.01 CH_2^{-1} (vs. 0.9-1.2 CH_2^{-1} for SAM systems) can be determined. While this impractical β value is useful for illustrating the unique differences in ET dynamics and electronic coupling of the two film systems, it also suggests that the MPC film must feature a completely different ET mechanism.

The observation of long-range ET via nanoparticle films is not unprecedented. Fermin and coworkers[77] recently observed electronic communication between nanoparticles and the electrode over distances as large as 13 Å. Likewise, Ulstrup and coworkers[78] report a significant enhancement of the ET rate for the redox protein cytochrome c at a gold surface modified with a single monolayer of thioctic acid protected nanoparticles, an ET distance estimated at over 50 Å. Ulstrup suggests that this ET enhancement is the result of stronger electronic coupling between the gold nanoparticle and the protein. While we do not see an enhancement of the ET rate, we do observe an apparent indifference of AZ's ET to distance at the MPC film assembly. We believe the difference in the ET rate of AZ at MPC films versus SAMs can be attributed to the two film's inherently different ET mechanisms, namely electron hopping through the MPCs and classical electronic tunneling through the SAMs. Extensive study of electron self-exchange dynamics through MPC films by Murray has shown that electron hopping through MPC films of this nature occurs via a diffusion-like electron hopping process at an extremely high rate with an average first order rate constant (k_{HOP}) of approximately 2 x 10^6 s^{-1} and an electron self-exchange rate constant (k_{EX}) of nearly 2 x 10^8 $M^{-1}s^{-1}$.[79] Moreover, Murray has also investigated the dynamics of ET of MPC monolayers at gold electrodes and through cyclic voltammetry analysis similar to this current study, found a heterogeneous rate constant of approximately 100 s^{-1}.[52] Given these findings and our results, we propose that the ET reaction proceeds via a very fast electron-hopping mechanism through the film. The y axis linear extrapolation intercepts from the rate constant graphs for AZ at SAMs (Figure 3B) and AZ at MPC films (Figure 8B) are similar with values significantly smaller (12-20 sec^{-1}) than the aforementioned rates found by Murray et al. but representative of the AZ ET reaction. With the SAM systems, electronic tunneling through longer chainlengths eventually dominates, degrading the overall rate, an effect not observed at even greater distances with MPC films. It is interesting to note that if the MPC films are assembled with an initial anchoring SAM of significant chainlength, decanethiolate or

hexadecanethiolate SAMs for example, the observed Faradaic signal from AZ is severely diminished, manifested as low surface coverage (Supporting Information). This result suggests that to facilitate long range ET via an electron hopping mechanism, the initial layer of MPCs must be electronically coupled through a short distance to the electrode surface. In other words, the insertion of a highly ordered, low dielectric SAM of significant chainlength effectively decouples the electronic connection with the MPC-network part of the film and the ET reaction begins to exhibit more traditional, apparent distance dependence. This interpretation of the system is consistent with other results we have observed with polyelectrolyte-linked aqueous nanoparticles (not shown) as well as literature reports on NP assisted/mediated ET reactions which also identify electronic coupling and chemical contact within the conductive pathway as important factors.[77,78] Considering these results as a whole, it is suggested that the ET reaction of AZ at MPC films may be viewed as a two step process where because of the extremely fast rate of electronic hopping within the MPC film, the rate-limiting step of the reaction remains the ET from the copper protein core to the surface of the MPC film.[78]

Conclusion

Protein monolayer electrochemistry as a fundamental strategy to study biological ET reactions and biosensor development is limited by definition to a single monolayer of protein coverage. The distance decay of the ET reaction with traditional SAM platforms excludes the possibility of exceeding a monolayer of protein as the Faradaic current signal would likely be lost at larger ET pathways.[2] The most significant finding of this work is the lack of distance dependence of the ET rate for AZ adsorbed to MPC films. As a consequence and the basis of future work in this field, one can envision strategies to incorporate nanoparticles, specifically MPCs, into developmental biosensor schemes where structures of higher order architecture are engineered to allow for greater amounts of protein to be immobilized, thereby increasing the signal to noise ratios of such devices and expanding the usefulness of the strategy in this regard.

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Supporting Information Available:

TEM characterization of MPC materials; UV-Vis spectra tracking growth of dithiol-linked MPC films assembled on glass and azurin characterization; electrochemical characterization of lyophilized verses as-prepared azurin; additional examples of AFM and cross-sectional TEM analysis of MPC films; AZ electrochemistry at MPC films with anchoring SAMs of varying chainlength; and MPC film estimates based on geometry. These materials are available free of charge via the Internet at http://pubs.acs.org.

Captions

Figure 1. Ribbon diagram of the globular protein azurin (14.6 kDa; ~3.5 nm average diameter)[56,59] showing copper redox core in orange (*left*). Structure of azurin rotated 90° about the y axis and space filled to show the hydrophobic binding pocket (*right*). The types and number of hydrophobic or non-polar amino acids found within the binding pocket include Ala(2), Gly(2), Leu(3), Met(4), Val(1), Pro(2), Thr(1), Tyr(1), are shown in yellow with the orange histidine used to mark the location (not at the surface) of the single copper redox core. Comparatively, the rest of the protein surface of azurin is comprised of polar or charged amino acids (e.g., Lys, Asp, Asn).[55]

Figure 2. **(A)** Schematic depicting traditional strategy of protein monolayer electrochemistry where azurin is adsorbed to an alkanthiolate self-assembled monolayer;[14-17, 55-59] **(B)** Typical cyclic voltammetry of azurin adsorbed to a hexanethiolate self-assembled monolayer collected at 100 mV/sec in 4.4 mM potassium phosphate buffer at pH = 7.

Figure 3. **(A)** Peak splitting (ΔE_p) of cyclic voltammograms for AZ at SAMs of varying number of methylene units (n) in the alkanthiolates [$H_3C(CH_2)_nS$-] comprising the films; **Inset**: Typical AZ voltammograms collected at 100 mV/sec in 4.4 mM potassium phosphate buffer at pH = 7 for representative SAMs of different chainlengths: hexanethiolate (C6), dodecanthiolate (C12), hexadecanethiolate (C16), and octadecanethiolate (C18) SAMs. **(B)** ET rate constant as a function of SAM thickness in terms of both methylene unit separation, n (*upper x axis*) and estimated distance in nm (*lower x axis*) between the AZ and the electrode surface.

Figure 4. **(A)** Cyclic voltammetry of MPC films for one to five exposures to MPC and nonanedithiol (dipping cycles). Voltammograms were collected at 100 mV/sec in 4.4 mM potassium phosphate buffer at pH = 7. **Inset**: Plot of double layer capacitance (C_{dl}) of MPC films versus the number of dipping cycles. **(B)** Cyclic voltammetry of 5 mM potassium ferricyanide [$K_4Fe(CN)_6^{-4}$] probe molecule at bare gold, hexanethiolate SAM, MPC film after one dip cycle, MPC film after two dip cycles, and MPC film after 3 dip cycles (see legend). Voltammograms were collected at 50 mV/sec in 0.5 M KCl solution. **Inset**: Potential different between oxidation and reduction peaks of $K_4Fe(CN)_6^{-4}$ voltammetry as a function of the number of MPC dipping cycles used to assemble the films (Note: Dip cycle "0" is the hexanthiolate SAM modified electrode prior to any MPC exposure.)

Figure 5. Atomic force microscopy (AFM) results of gold-coated mica substrates before (*left*) and after (*right*) assembly of 4 layers (4 dip cycles) of a MPC film. **Top**: AFM images of the same area of the substrate; **Bottom**: Cross-sectional analysis of roughly the same area of the substrates (*designated with white arrows in images above*).

Figure 6. Transmission electron microscopy (TEM) cross-sectional analysis imaging of a 5 dip cycle (5 layer) dithiol-linked MPC film assembly. **Inset**: Typical TEM image of as-prepared MPCs used to create the assembled film (Note: Inset figure has a different length marker). TEM analysis (Inset) indicates an *average* diameter of ~ 2 nm for the MPC cores and an estimated overall nanoparticle diameter (i.e., including peripheral ligands) of 3.9-4.1 nm, only slightly larger than the AZ protein (Supporting Information).

Figure 7. (A) Schematic representation of azurin adsorbed to a dithiol-linked MPC film assembly. **(B)** Typical cyclic voltammogram for AZ adsorbed to MPC film assembly collected at 100 mV/sec in 4.4 mM potassium phosphate buffer at pH = 7.

Figure 8. (A) Peak splitting (ΔE_p) of cyclic voltammograms for AZ at MPC films of increasing thickness (i.e., increasing MPC layers from dip cycles). **Inset**: Example voltammograms of AZ at MPC films created with two and three dip cycles which show almost negligible ΔE_p. **(B)** ET rate constant as a function of MPC film thickness in terms of the number of dip cycles used to create the film (*upper x axis*), the estimated distance in nm (*middle x axis*) between the AZ and the electrode surface, and minimum number of methylene unit separation, n (*lower x axis*). **Inset**: Schematic illustration of fully interdigitated MPC film assembly used to determine minimum number of methylene units. (Note: Dip cycle "0", indicated by × is the hexanthiolate SAM modified electrode prior to any MPC exposure.)

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(73) These images are representative of consistently achieved results from cross-sectional analysis of multiple locations on the same substrate as well as sampling of different gold on

mica substrates, all of which displayed the trends shown in Figure 5 (Supporting Information).

(74) These images are representative of consistently achieved results from cross-sectional analysis of multiple locations on the same substrate as well as sampling of different gold on mica substrates, all of which displayed the trends shown in Figure 6 (Supporting Information).

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