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Debbie S. Campbell-Rance

Tran T. Doan

Michael C. Leopold
University of Richmond, mleopold@richmond.edu

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Sweep, Step, Pulse, and Frequency-Based Techniques Applied to Protein Monolayer Electrochemistry at Nanoparticle Interfaces

Debbie S. Campbell-Rance, Tran T. Doan, and Michael C. Leopold*

Department of Chemistry, Gottwald Center for the Sciences, University of Richmond Richmond, VA 23173

Abstract

Protein monolayer electrochemistry (PME), a strategy using synthetic platforms to study the electron transfer (ET) properties of adsorbed proteins, has been successfully applied to proteins adsorbed at monolayer-protected gold cluster (MPCs) assembled films, an adsorption interface shown to be an effective alternative, compared to traditional self-assembled monolayer (SAM) films, for the immobilization and study of ET proteins. Within PME studies, cyclic voltammetry (CV) remains the most commonly applied electrochemical technique in spite of several limitations that occur when the sweep technique is used at either platform. In particular, CV for PME at MPC films results in analysis complications stemming from the increased charging current inherent to electrochemical interfaces incorporating MPCs with capacitive properties. In this study, multiple electroanalytical techniques involving step (chronocoulometry, CC), pulse (square wave voltammetry, SWV), and frequency-based impedance (electrochemical impedance spectroscopy, EIS) measurements, are applied to monolayers of adsorbed *Pseudomonas aeruginosa* azurin and horse heart cytochrome c at both MPC film assemblies as well as traditional SAMs. Electrochemical parameters (formal potential, electroactive surface coverage, double-layer capacitance, and ET rate constant) measured from these various techniques are directly compared and offer insight into the performance and reliability of each technique's effectiveness in PME. While certain techniques result in measurements indistinguishable from CV, others offer distinct differences Moreover, the application of alternative techniques reveals systemic limitations and complications within the electrochemical analysis that we further explore, including strategies for applying fast scanning techniques like SWV as well as the construction of MPC platforms with controlled levels of charging current that enable successful impedance analysis. The application of more advanced electrochemical techniques to developing electrochemical interfaces such as MPC film assemblies allows for a greater understanding of not only PME but also the applicability and effectiveness of these techniques to optimize the measurement of specific electrochemical parameters.

Keywords: Protein monolayer electrochemistry, Monolayer-protected clusters, Self-assembled monolayers, Heterogeneous rate constant, Surface concentration, Cyclic voltammetry, Chronocoulometry, Square wave voltammetry and Electrochemical impedance spectroscopy

* To whom correspondence should be addressed. Email: mleopold@richmond.edu. Phone: (804) 287-6329. Fax: (804) 287-189

1. Introduction

Many biological processes of interest, including photosynthesis and respiration, are sustained by electron transfer (ET) reactions of proteins adsorbed to bio-membranes [1, 2]. These biological systems have inspired the design of electrochemically active protein film assemblies that allow for ET studies relating to biosensing, bioelectronics and bioreactors for catalytic chemical reactions [1-3]. The success of these devices depends to a large extent on how well the protein-substrate interfaces are designed and understood [4, 5].

One success in the construction of redox protein film assemblies with reproducible electrochemical responses has been attributed to a technique called protein monolayer electrochemistry (PME) [6, 7]. In PME, diffusion is eliminated by confining the protein to the electrode surface in order to facilitate unmediated ET reactions [8-10]. Kinetic and thermodynamic properties of the redox active protein are probed simultaneously using electrochemical techniques [10-12]. The most widely investigated proteins via the PME strategy have been equine cytochrome c (cyt *c*) and azurin (AZ) from *Pseudomonas aeruginosa*. PME of cyt *c* has been characterized by Bowden [6, 11-16], Niki [17, 18], Waldeck [19, 20], Gray [21] and Leopold [22] and their respective co-workers. AZ has attracted the attention of many researchers because of its similarity to cyt *c* in size, function, and structure [10, 23]. AZ allows for an alternate interaction with the PME platform as it is able to bind hydrophobically [24] rather than electrostatically [10, 22] like cyt *c*, greatly simplifying the interface by not requiring the presence of interfacial functionality (e.g., carboxylic acid terminal groups) for protein immobilization. Contributions to the study of the electrochemical properties of surface-confined AZ include the groups of Cavalleri [23], Ulstrup [24-26], Armstrong [27], Gray [28], Martin [29], and Leopold [30, 31].

The traditional platforms for PME include Langmuir-Blodgett (LB) films, self-assembled monolayers (SAMs) and monolayer-protected cluster (MPC) film assemblies [6, 13, 31, 32] Even though this PME strategy has been used successfully to investigate the ET of proteins and determine valuable thermodynamic and kinetic parameters, the technique is not without limitations. Broadened voltammetry is a common anomaly of this assembly, resulting in full width at half-maximum (FWHM) values that deviate significantly from the theoretical value of 90 mV for an optimally adsorbed system [33]. This non-ideality has been previously attributed by Bowden and coworkers to the presence of a heterogeneous population of adsorbed cyt *c* at the

SAM interface [14]. In another report they demonstrate that the topography of the SAM's underlying gold support is a contributing factor to interfacial heterogeneity [16]. Thus, uniform adsorption of the redox protein to a suitable substrate is central to the optimization of the PME platform. An additional limitation is the low signal-to-background current ratio, a consequence of monolayer or sub-monolayer protein coverage and/or adsorption of protein in non-optimal ET orientations [13].

In light of these limitations, research aimed at optimizing PME strategies is ongoing and includes the exploration of new materials able to interface electrode with protein. One approach is based on adsorbing proteins to citrate stabilized gold nanoparticles (NPs) to improve direct ET [34-36]. This idea is motivated by the fact that NPs have unique properties such as large surface-to-volume ratio (increase protein coverage), biocompatibility with protein (preserving its electroactivity), and the ability to act as a conduit for ET to occur between electrode and protein [35, 37-39]. This approach, however, has been shown to exhibit slow kinetics, increased background signals and non-ideal electrochemistry [40, 41]. An alternative PME strategy developed in our lab involves adsorbing proteins to covalently networked films of alkanethiolate-stabilized gold NPs known as monolayer-protected clusters (MPCs) [22, 30, 31, 42]. Prior studies using the MPC film assemblies focused on the voltammetry of cyt *c* and AZ. In the case of cyt *c*, MPC films featuring a variety of linking methods and different core sizes were studied. This work established a clear dependence of background charging current on the linking mechanisms used during the film assembly with covalent, dithiol interparticle linking resulting in low charging current [22]. Studies of AZ adsorbed at MPC film assemblies looked at the distance dependence of ET kinetics by varying the chain length of the alkanethiolate SAM and found a notable lack of distance dependence for heterogeneous ET reactions [30]. Another report used cyclic voltammetry (CV) and atomic force microscopy (AFM) to show that MPC film assemblies provided a more homogeneous adsorption interface that yielded cyclic voltammograms with FWHM values less than 110 mV compared to the traditional PME which has values in the range 120 - 130 mV [31].

While the primary electrochemical technique utilized in most PME reports is CV, our study seeks to expand the scope of this interrogation to include step, pulse and frequency based electrochemical techniques applied to both SAM and MPC adsorption platforms. Even though CV is capable of estimating both the kinetic and thermodynamic properties of the adsorbed protein, it is one of the least effective electrochemical techniques in terms of discriminating against

unwanted non-Faradaic (background) current [33]. Consequently, the accuracy with which surface coverage is evaluated is a concern since it is possible that portions of the protein signal are obscured by the background signal [30]. The discrimination of background current has been shown to be particularly important with electrode interfaces featuring additional capacitive components such as NPs [22]. In this report, double potential step chronocoulometry (CC), square wave voltammetry (SWV) and electrochemical impedance spectroscopy (EIS) are used for electrochemical analysis of adsorbed monolayers of cyt *c* and AZ at various SAM and MPC modified electrodes. The results from these techniques were compared to traditional CV analyses of the same systems. The selection of these particular techniques is strategic in that, compared to CV they are specifically geared to discriminate against charging current, a benefit that, in theory, should result in greater sensitivity (i.e. increased signal-to-noise ratios) and more accurate measurements of protein surface coverage [43-45]. Hence the focus of our work is to establish the usefulness and adaptability of these alternative electrochemical techniques in PME scenarios, including those incorporating NPs as a part of the protein adsorption platform.

2. Experimental Details

2.1. Reagents and Materials

Ultrapure water (UP H_2O, 18 MΩ) was used to prepare all solutions and rinse glassware and electrochemical cells. All thiols were purchased from Sigma-Aldrich and were used without further purification. Thiol solutions (5 mM) containing neat ethanol were used for SAM assembly as previously described [30, 46]. Gold electrodes (Evaporated Metal Films Inc.) were electrochemically cleaned prior to SAM assembly [12]. Similar protocol was followed in the preparation of *Pseudomonas aeruginosa* azurin and equine cytochrome *c* as reported in prior studies [22, 30, 31]. Alternate purification protocols were also explored on AZ solution prepared from the lyophilized protein in 4.4 mM potassium phosphate buffer (KPB, pH 7.0, μ = 10 mM) to determine if samples contained more than one population of AZ on the surface (Supplementary Data).

2.2. Equipment

Cyclic voltammograms (100 mV s^{-1}, unless otherwise noted), chronocoulograms and square wave voltammograms were recorded with a CH Instrument potentiostat (Model 400) while impedance spectra were recorded with the Gamry Reference 600 potentiostat, both of which incorporated a Faraday cage during measurements. SWV simulations were acquired with the DigiElch Professional (Version 6.F) for simulating electrochemical processes. In order to create the square wave voltammograms, the software required the input of the relevant experimental parameters from the SWV electrochemical waveform as well as the ET coefficient (α), the formal potential ($E^{\circ\prime}$) and the heterogeneous rate constant (k_{et}) .

2.3. MPC Synthesis

MPCs were prepared using established protocols (Brust reaction) [22, 30, 31]. In this preparation, chloroauric acid ($HAuCl_4$) and 1-hexanethiol (C6) or 1-dodecanethiol (C12) served as the precursor and capping agent, respectively. The MPCs produced possessed an average core composition of ~225 gold molecules (Au_{225}) with ~75 C6 or C12 thiolate ligands and average core diameter of 2.03 ± 0.95 nm as determined using nuclear magnetic resonance and transmission electron microscopy analyses, respectively and was in agreement with literature reports [30, 31].

2.4. Electrode Modification Procedures

Evaporated gold substrates were mounted in electrochemical sandwich cells [22] where a built-in viton ring further defined a 0.32 cm^2 working electrode area. The electrochemical circuit was completed by a coiled platinum wire counter electrode and a Ag/AgCl (sat'd KCl) reference electrode. The as-received gold was electrochemically cleaned by voltammetric cycling as previously described [12]. Immediately after cleaning, the gold substrate was rinsed repeatedly with UP water and ethanol, exposed to a 5 mM ethanolic thiol solution, allowed to sit 24 or 48 hr for shorter (C6 and C10) or longer (C14, C16 and C18) chain length thiols, respectively, and then rinsed with ethanol and water [46]. As described below in the electrochemistry section (Section 2.5), CV was used to confirm the presence of the SAM [46]. At this point the SAM was used for traditional PME or further modified with NPs (MPCs) as described below.

MPC films were assembled on SAM modified electrodes by exposing it to a 5 mM ethanolic solution of a dithiol (1, 9-nonanedithiol, NDT or 1, 16-hexadecanedithiol, HDT)

linking ligand for 1 hr. This was followed by successive rinsing with ethanol, water and dichloromethane (CH_2Cl_2) before exposure to the C6- or C12-protected MPCs solution in CH_2Cl_2 (1 mg/1 mL). The electrochemical cell with MPC treated electrode was stirred with a slow stream of nitrogen for 1 hr to affix the first dithiol-linked MPC layer to the substrate. The dithiol-linked MPC assembly was built up by repeatedly immersing the substrate in the dithiol linker for 20 min (stirred under N_2 gas) and then in the C6 or C12 MPC solution for 1 hr. The substrate was rinsed with CH_2Cl_2 in between each treatment of linker and MPC solutions [30, 31]. This general procedure was typically repeated three times to form a film of approximately three layers of MPCs (i.e., MPC_3). Prior work using TEM cross-sectional analysis suggests that these three layer MPC films have an estimated thickness of 7-8 nm [30]. The assembly of each layer of dithiol-linked MPC on the substrate was monitored via CV and/or EIS as described in the electrochemistry section below.

2.5. Film Characterization and Protein Monolayer Electrochemistry

As in prior reports where the PME platform contained MPC layers, CV was used to monitor film formation and growth by analyzing the double layer capacitance (C_{dl}) recorded when scanning between 100 and 400 mV at 100 mV s^{-1} in 4.4 mM KPB [22, 30, 31]. C_{dl} measurements were calculated from these cyclic voltammograms by inserting the average total current ($I_{tot}/2$) at 120 mV, a potential where there is minimal Faradaic current and accounting for voltammetric sweeps in both the cathodic and anodic direction, into Eq. (1), where ν is the sweep rate (V s^{-1}) and A is the area of the WE (cm^2).

$$C_{dl}(\mu F \cdot cm^{-2}) = \frac{I_{tot}}{2\nu A\ 10^{-6}} \quad (1)$$

The successive layering of the MPCs within the assembled films is confirmed in this manner with each layer of MPCs increasing the charging current as expected [22]. In the case of EIS, C_{dl} was monitored by taking impedance measurement at open circuit potential (OCP) within frequency ranges 0.01 Hz - 1000 Hz using the same electrolyte solution after rinsing with copious amounts of ethanol (SAM modified electrodes) or CH_2Cl_2 (MPC films) followed by UP water and 4.4 mM KPB.

PME was evaluated using CV, CC, SWV and EIS after 200 μL of 5 - 10 μM AZ or cyt *c* in 4.4 mM KPB was added to the electrochemical cell and refrigerated for 1 hr. before being rinsed with KPB and degassed (10 min.). For CV experiments, the surface concentration (Γ_{ads}) of AZ or cyt *c* at the film assemblies were determined by integrating the area under the cathodic curve. As in prior studies, to determine the apparent k_{et} from CV Laviron's simplest model for a surface-confined species was employed[47]. In CC experiments, the potentials were stepped from an initial value (E_i) of ~300 mV to potentials before and after the reducing wave of the CV. In the SWV experiments, the square wave amplitude (E_{sw}) was 25 mV, the potential increment (ΔE) was 4 mV, the pulse width (t_p) was 20 ms and the frequency (*f*) was 25 Hz, unless otherwise stated. Frequencies identical to those used in control EIS experiments (0.1 - 1000 Hz) were used for impedance measurements of the film systems (SAM and MPC) with adsorbed protein with the potential set to the protein's E°′ as measured by CV.

3. Results and Discussion

3.1. Cyclic Voltammetry Analysis of Azurin at C6 SAM vs. C6 MPC Film Assembly.

The cyclic voltammograms of AZ adsorbed to the SAM and MPC film assemblies are shown in Fig. 1. The voltammetric peaks achieved from both assemblies are well-defined but also show non-ideal voltammetry in that the FWHM of the cathodic wave is larger than that of the theoretical value (90 mV) for an ideally adsorbed species undergoing a ET reaction [14, 30, 31, 33]. While FWHM values for the cyclic voltammograms of AZ at these two interfaces showed peak broadening, the issue is more severe in the case of the SAM assembly (121 ± 4 mV) compared to the MPC assembly (106 ± 8 mV). This trend is consistent with prior reports [30, 31] and supports the theory of heterogeneous protein adsorption related to substrate (gold) topography put forth by Bowden and coworkers [14, 16]. Similar cyclic voltammograms are easily obtained for cyt *c* at the same assemblies (not shown) [22].

Thermodynamic and kinetic information such as E°′, Γ_{ads} and k_{et} are readily derived from CV and are presented in Table 1 for both types of assemblies. The apparent E°′ of AZ at the two different film assemblies are comparable to literature E°′ values (95 mV) at a SAM assembly [48, 49]. The E°′ of AZ at the MPC platform (100 mV) is slightly more positive than that at the SAM platform (82 mV). A comparison of the E°′ of the native protein in solution (102 mV) to that of

the two assemblies suggests that the MPC film assembly promotes the native structure of the protein to a greater degree than the SAM [48]. Table 1 shows Γ_{ads} data, determined by integrating the area under the cathodic peak of the cyclic voltammogram to find the charge passed of adsorbed species (Q_{ads}) and applying Eq. (2):

$$\Gamma_{ads} = \frac{Q_{ads}}{nFA} \quad (2)$$

where n is the number of electrons involved in ET (1), A is the area of electrode (0.32 cm^2) and F is the Faraday constant [33]. The average Γ_{ads} of AZ at the C6 SAM and C6 $(MPC)_5$ assembly are 11.66 ± 1.14 pmol cm^{-2} and 7.26 ± 1.18 pmol cm^{-2}, respectively. Even though Γ_{ads} of AZ at the MPC platform is lower than its coverage at the SAM modified substrate, it is still consistent with reports of near-monolayer protein adsorption [22, 31, 50, 51]. That being said, the discrepancy between these two measurements is directly addressed with other electrochemical techniques as described in subsequent sections and shows that the sensitivity of a particular technique or the presence of indiscriminate charging current may be critical factors that have to be considered for these measurements.

The apparent k_{et} for each assembly were determined by Laviron's method [47]. This analysis yields k_{et} values of 11.30 s^{-1} ± 1.87 and 11.25 ± 2.10 s^{-1} for the SAM and MPC films, respectively, that are indistinguishable (95% confidence). Similar k_{et} values are obtained even though the distance between the protein and the electrode surface is significantly greater in the MPC films compared to the SAM assembly. This lack of distance dependence in k_{et} values are supported by the findings of Vargo et al. in a prior report [30], which proposed a very fast electron hopping mechanism through the MPC films compared to the traditional electronic tunneling mechanism in the SAM assembly [30, 52-54]. While it is clear that CV remains a powerful tool for studying the thermodynamic and kinetic aspects of protein ET, it is much less clear if pulse, step, or frequency based methods can be applied without complications to the same protein monolayers and deliver additional or similar experimental results, the major focus of our current investigation.

3.2. Chronocoulometric Analysis of Azurin at C6 SAM vs. C6 MPC Film Assembly

CC has been used successfully to determine electroanalytical parameters such as the geometric surface area of an electrode, diffusion coefficients of redox species, the amount of adsorbed electroactive species, heterogeneous ET k_{et} and the mechanistic pathway of chemical reactions coupled to ET reactions [55-57]. In our experiments, double potential step CC is used to determine the Γ_{ads} of AZ adsorbed at SAM and MPC modified film assemblies. Here, we are specifically interested in the ability of CC to separate the charging current of the SAM or MPC based adsorption platforms/film assemblies from the Faradic current associated with the adsorbed protein. To the best of our knowledge CC has not been applied to PME at a NP modified electrode.

The data presented below are analyzed according to equations given by Bard and Stankovich in their seminal work on the use of CC for estimating the total Faradaic charge for the reduction of a surface confined species [43]. The WE potential is stepped from an initial potential (E_i = 300 mV), where no Faradaic processes occur, to a value sufficiently negative of the protein's $E^{\circ\prime}$ that immediately reduces the AZ monolayer while the charge passed during the steps is measured. The strategy can also be reversed by applying oxidative steps before and after the $E_{p,a}$ of the adsorbed species and measuring the charge passed. A depiction of the CC waveform applied in these experiments, along with an illustrated example of reductive steps used in the analysis of adsorbed AZ, is provided as part of the Supplementary Data.

During a potential step, in the absence of diffusing electroactive species, the total charge (Q_{total}) is given by Eq. (3) below; where Q_{dl} is the double layer charging and Q_{ads} is the charge passed of the adsorbed protein.

$$Q_{total} = Q_{dl} + Q_{ads} \quad (3)$$

If C_{dl} is relatively constant under conditions of changing potential within the specified potential window of PME experiments, then Q_{dl} is given by Eq. (4); where E_i is the initial potential and E_j are the final step potentials (E_{j1}, E_{j2}, E_{j3}…).

$$Q_{dl} = C_{dl} (E_j - E_i) \quad (4)$$

If Eqs. (3) and (4) are combined, Q_{total} can be derived in terms of C_{dl} and Γ_{ads} according to Eq. (5) and a plot of Q_{total} vs. (E_j - E_i) yields a straight line where the slope and y intercept are defined as C_{dl}

and $nF\Gamma_{ads}$, respectively. Thus, from a set of CC experiments with different potential steps (E_i to E_{j1}, E_{j2}, E_{j3}…) one can readily calculate estimates of both C_{dl} and Γ_{ads}.

$$Q_{total} = C_{dl} (E_j - E_i) + nF\Gamma_{ads} \quad (5)$$

Here, CC experiments are used to investigate both the SAM and MPC protein adsorption platforms. Since a critical factor of the CC is the nature of the background charging current, it is useful to take a closer look at the CV response of AZ at these individual interfaces along with their corresponding backgrounds in the absence of the adsorbed protein (Fig. 2). Upon closer inspection of these results, several observations emerge that must be considered for CC analysis. First and foremost, there is a rather distinct difference between the charging current/background signals of the two interfaces. The SAM exhibits a relatively constant level of charging current across the potential widow whereas the MPC film shows a notable decrease in the background current toward negative potentials. As will be shown later, while this difference in background signal makes the use of CC more complex, it does not necessarily completely negate the application of the technique to MPC interfaces. Indeed, the background observed with MPC film assemblies is relatively constant compared to successful CC analysis of adsorbed species found in the literature[43]. The second observation evident from Fig. 2 is that the background signal decreases, rather significantly in the case of the MPC film, upon adsorption of AZ. This secondary observation will be discussed later in the report and is simply noted here.

The application of CC to SAM interfaces with adsorbed AZ follows the aforementioned analysis theory effectively. Figure 3 illustrates a set of typical chronocoulograms for AZ adsorbed to a C6 SAM interface. Each scan represents Q_{total} for the potential step either before or after the $E_{p,c}$ of AZ. As illustrated, the potential steps after $E_{p,c}$ (Fig. 3b) yield Q_{total} an order of magnitude higher than those steps prior to $E_{p,c}$ (Fig. 3a). The point charges measured from the chronocoulograms are used to prepare the plot of Q_{total} vs. (E_j - E_i) shown in Fig. 3c and corresponding inset. Figure 3c inset is a plot of Q_{total} vs. (E_j - E_i) for a C6 SAM modified electrode where the potential steps of interest for AZ have been applied in the absence of the protein (control system). We note, in particular, that this plot, comprised of potential steps both before and after $E_{p,c}$ is extremely linear (i.e., $R^2 = 0.9998$) and has an intercept of ca. zero, an indication that the background (C_{dl}) for the system is relatively constant across the potential window (i.e.,

similar slopes for each set of steps) and that, in the absence of AZ, there is only non-Faradaic charging current.

Figure 3c shows plots for CC experiments with AZ adsorbed to C6 SAM. In this case, the plot with an intercept of approximately zero is derived from the point charges acquired from applying potential steps before the cathodic wave and the plot with the non-zero intercept is derived from potential steps after AZ $E_{p,c}$. The non-zero intercept of Fig. 3c is a consequence of AZ Faradaic current and allows for the measurement of protein Γ_{ads} via Eq. (5). Both plots showed similar slopes (C_{dl}) regardless of the stepping potential or protein adsorption. C_{dl} and Γ_{ads}, as measured by CC are comparable to values determined using CV for protein adsorbed to SAMs (Table 2). We note that the small difference in C_{dl} before and after the $E_{p,c}$, both in the presence and absence of AZ, have little effect on the outcome of the analysis.

CC results before and after AZ adsorption at an MPC interface, comprised of 3 layers of dithiol (NDT) linked MPC layers anchored at a C6 SAM, are shown in Fig. 4. Application of the same set of potential steps and charge measurements reveal a plot with an increased slope a value of C_{dl} (7.5 μF cm^{-2}) for the MPC film compared to the SAM results (3.1 μF cm^{-1}) presented in Fig. 3, both without AZ. The more than two-fold increase in C_{dl} at the MPC interface is attributed to the known capacitive properties of the MPCs [22, 31]. Indeed, C_{dl} measurements made during the layer-by-layer assembly of the MPC films shows a progressively greater capacitance that is directly proportional to the number of MPC networked into the film [30, 31]. As seen in Fig. 4a, potential steps during CC analysis before and after $E_{p,c}$ reveal, as with the SAM system, a linear trend (i.e., R^2 = 0.9998) with a near zero intercept. This plot suggests that even though an inconsistent background is observable with CV (Fig. 2b), it may not have a substantial impact on the CC analysis at MPC films using these particular potential steps. Even when separate regression analysis is performed with each set of steps, the slopes of the two trendlines differ only slightly (Supplementary Data). We further note that this observation is reversible in that oxidative steps before and after $E_{p,a}$ reveal similar trends (Supplementary Data).

Potential stepping during CC provides estimations of the C_{dl} (i.e., slopes) for the MPC film that are potential dependent to a degree. The specific values of C_{dl} estimates during each set of steps (both reductive and oxidative) both before and after the peak potentials and in the presence and absence of AZ are included in a table found in the Supplementary Data. In general, the results are summarized by being consistent with the background signals from CV (Fig. 2) –

reductive steps before $E_{p,c}$ result in larger C_{dl} estimates than potential steps to more reducing potentials (negative). Conversely, oxidative steps before $E_{p,a}$ reveal smaller values of C_{dl} compared to steps after $E_{p,a}$(more positive potentials). In this respect, the dependence of C_{dl} on the direction of the potential steps, along with the choice of potentials, may both have an impact on the subsequent analysis described below.

Upon adsorption of AZ to the MPC film assembly, CC experiments yield a plot (Fig. 4b) with a nonzero intercept as a consequence of the charge passed from protein ET. In theory, the slope and intercept of this plot with AZ should translate directly into direct estimations of system C_{dl} and Γ_{ads}, respectively. Unfortunately, two issues preclude this straightforward analysis. First, as previously discussed, the directional dependence of the C_{dl} values suggest that reductive steps used in CC may result in a decreased slope and subsequently cause the intercept to *overestimate* Γ_{ads}. Likewise, oxidative steps would likely cause an *underestimation* of Γ_{ads}. A second issue in this analysis is the unexplained overall decrease in C_{dl} across the potential window upon adsorption and reduction of AZ. It is this phenomenon that is both markedly more pronounced at the MPC system compared to the SAM system and is identified as the primary cause of the altered slope in Fig. 4b. That is, even though a substantial change in slope is observed, it cannot be completely attributed to the inconsistent background current as it did not have nearly the same effect when the same potential steps were applied in the absence of AZ (Fig. 4a). Moreover, we note that the C_{dl} estimation (slope) for the potential steps after $E_{p,c}$ and in the presence of the protein are in complete alignment, i.e., no statistically significant difference, with the CV analysis (see Table 2).

While the reasons for the abrupt change in the slope upon AZ adsorption and reduction are not completely understood, we can speculate that it may be related to the protein representing an adsorbate with a redox center encased in a protein shell. In every case examined, the adsorption and subsequent stepped reduction of AZ at the MPC film interface was coupled with a corresponding and rather significant decrease in C_{dl}. The same effect, to a lesser extent, was observed with AZ at SAM interfaces. Control experiments (not shown) with adsorbed ruthenium hexamine, a simple redox molecule, at MPC films yielded an abrupt *increase* in C_{dl} upon reductive stepping. The different behavior between the two adsorbates suggests that the insulating protein structure and/or its specific interaction at the two interface is a critical factor that impacts the CC results. Nevertheless, the CC analysis described, with careful assessment of

the background current and choice of step potentials, remains a legitimate pulse technique for estimating C_{dl} and Γ_{ads} in protein monolayer electrochemistry.

3.3. Square Wave Voltammetry Study of Adsorbed Azurin

Square wave voltammetry (SWV) is an important electrochemical technique used to study the mechanisms, kinetics and thermodynamics of electrochemical reactions. It offers several advantages compared to other techniques including high sensitivity with extremely fast scanning capability, differentiation between processes with fast and slow kinetics and effective separation of non-Faradaic and Faradaic current signals [58]. The applied waveform in a SWV experiment (Fig. 5 inset) consists of a sequence of square wave potentials of fixed heights superimposed on a voltage staircase. In this study, SWV was used to determine the ET kinetics of AZ on alkanethiol SAM and MPC platforms by adopting the approach used by Reeves et al., who determined the ET kinetics of cyt *c* at carboxylic acid SAMs [44]. Like Reeves and coworkers, our study employs the peak separation (ΔE_p) in square wave voltammograms as a diagnostic tool to determine apparent k_{et}, which were then compared to values derived from CV using Laviron's method.

3.3.1. Square Wave Voltammetry Study of Azurin at C14 and C16 SAM and at C12 $(MPC)_3$ Assemblies Anchored by C14 SAM

Reeves and co-workers showed that monitoring ΔE_p in SWV experiments provides an alternative approach for estimating k_{et} [44]. This model assumes that the experimental system contains a uniform substrate with similarly oriented adsorbates at the interface. To accommodate this requirement in our experiments, we used SAM film assemblies incorporating long chain alkanethiols (C14 and C16) as they are usually more well-behaved, stable and have a lower defect density compared to shorter chain SAMs [46]. Figure 5 represents typical square wave voltammograms of AZ adsorbed on C14 and C16 SAMs. The peak potentials of the voltammograms were averaged to determine estimates for E°′ which, for AZ at C14 and C16 SAMs, were 77 ± 5 mV and 74 ± 3 mV, respectively. These values are in relative agreement with E°′ values determined for the same systems using CV (75 ± 3 mV for AZ at C14 SAM, and 77 ± 1 mV for AZ at C16 SAM) as shown in Table 3.

The ΔE_p of a square wave voltammogram is dependent on t_p which is directly linked to k_{et} via Eq. (6), where Λ is the dimensionless ET rate constant [44].

$$\Lambda = k_{et} t_p \quad (6)$$

A working curve ($\alpha = 0.5$ and $n = 1$) was created through a series of SWV simulations using the DigiElch-Professional (Version 6.F) software for modeling electrochemical processes. A waveform identical to that used in the SWV experiments was employed for the simulations, where t_p = 20 ms, ΔE = 4 mV, E_{sw} = 25 mV, and f = 25 Hz. Simulated square wave voltammograms with different ΔE_p values were obtained by varying the value of k_{et} (0.5-15 s^{-1}) provided to the software. Calculated values of Λ were obtained by applying these k_{et} values used in the simulation and t_p of 20 ms to Eq. (6). A working curve that plots Λ vs. ΔE_p was established to facilitate the determination of k_{et} from experimental square wave voltammograms with ΔE_p of 10 mV or more. Figure 6 is the generated working curve that is used to determine k_{et} for AZ at SAM film assemblies. The data was fitted using two quadratic equations, one for ΔE_p between 10 and 40 mV (Eq. (7)) and the other for $\Delta E_p \geq 40$ mV (Eq. (8)).

$$\Lambda = 5.0 \times 10^{-5} \Delta E_p^2 - 0.004\, \Delta E_p + 0.1064 \qquad (\Delta E_p = 10 - 40 \text{ mV}) \quad (7)$$

$$\Lambda = 6.0 \times 10^{-6}\, \Delta E_p^2 - 0.001\, \Delta E_p + 0.0566 \qquad (\Delta E_p = 10 - 40 \text{ mV}) \quad (8)$$

SWV experiments performed on AZ at SAMs of C14 and C16 thiols produced experimental ΔE_p values of 10 - 15 mV and 80 mV, respectively. Application of Eqs. (7) and (8) to these peak splitting yielded values of Λ which, using eqn. (6) translated into apparent k_{et} values of 3.28 ± 0.14 s^{-1} and 0.75 ± 0.01 s^{-1}. These results are in agreement with k_{et} values (4.77 ± 0.95 s^{-1} and 0.97 ± 0.09 s^{-1} for C14 and C16 SAMs, respectively) obtained from CV experiments (Table 3). This model failed for AZ at short chain SAMs because the SWV response on these thinner films yields ΔEp values of < 10 mV and, in some cases, no separation at all, meaning the

ΔE_p values fall out of the range of our working curve. Reeves et al. attributed this failure to the apparent instability of the protein at these thinner films [44].

The SWV waveform applied to AZ at MPC assemblies was identical to the one applied to AZ at SAM assemblies except that the potential increment (ΔE) of the waveform was changed from 4 to 16 mV. An increase in ΔE affects the rate at which the voltammograms are scanned and is necessary to generate ΔE_p values consistent with the working curve. SWV experiments of AZ at 3 layered HDT-linked C12 MPC film anchored by C14 SAM yielded average ΔE_p value of 45 ± 2 mV for voltammograms recorded with scan rate of 400 mV s^{-1} compared to ΔE_p < 10 mV for the same system at 100 mV s^{-1}. Due to this change in ΔE, a new working curve was established to determine k_{et} values for AZ at the C12 $(MPC)_3$. This working curve, generated in a similar fashion to the one for AZ at SAM assemblies, and the quadratic equations used to fit the curve are given in the Supplementary Data. Based on the average ΔE_p (45 ± 2 mV) value obtained for AZ at C12 $(MPC)_3$ film assemblies, Λ was determined from the appropriate quadratic equation and this value applied to Eq. (6) along with t_p = 20 ms. The average k_{et} for AZ at C12 $(MPC)_3$ film assemblies from SWV and CV are compiled in Table 3. Similar k_{et} values were determined via SWV (4.48 ± 0.18 s^{-1}) and CV (4.24 ± 0.33 s^{-1}) and are comparable to k_{et} values at C14 SAM assemblies determined from SWV (3.28 ± 0.14 s^{-1}) and CV (4.77 ± 0.95 s^{-1}).

This comparison is noteworthy as it again suggests the addition of layers of MPC to a SAM adlayer has little effect on the ET rate through the film. This observation is consistent with the established thinking that ET through MPC films, even with MPCs having larger peripheral ligands, is fast relative to the electronic tunneling through a SAM which remains the rate determining mechanism in the assembly [30].

3.4. Electrochemical Impedance Spectroscopy Study of Adsorbed Azurin

Electrochemical impedance spectroscopy, an extremely sensitive technique to use in studying surface-confined proteins, measures the impedance of an electrochemical system by applying a small oscillating signal over a range of frequencies at a specified potential [10, 59]. The results of a typical EIS experiment are presented in the form of a Nyquist or Bode plot [33, 45], the former being a plot of the real vs. imaginary components of the impedance in accordance with the overall impedance expression shown as Eq. (9):

$$Z(\omega) = R_s + \frac{R_{CT}}{1 + j\omega R_{CT} C_{dl}} = R_s + \frac{R_{CT}}{1 + \omega^2 R_{CT}^2 C_{dl}^2} - \frac{j\omega R_{CT}^2 C_{dl}}{1 + \omega^2 R_{CT}^2 C_{dl}^2} = Z' + jZ \quad (9)$$

where ω is the angular frequency of the AC signal, C_{dl} is the double layer capacitance and R_s and R_{CT} are the solution and charge transfer resistance, respectively. The selection of the potential for EIS allows both Faradaic and non-Faradaic currents to be assessed independently depending if that potential is near the E°′ of the adsorbed species or not. Only the non-Faradaic components, R_s and C_{dl}, of the system are considered when the EIS experiment is performed at a potential away from the E°′ of the adsorbed species. In that case, C_{dl} can be obtained from the point where the maximum imaginary impedance (Z'') is found according to the relationship shown in Eq. (10) [45].

$$R_{CT} C_{dl} = \frac{1}{\omega_{max}} = \frac{1}{2\pi f_{max}} \quad (10)$$

When the Faradaic components of the system are considered, the experiment is performed at E°′ of the redox species attached to the monolayer; R_s and C_{dl} can be found directly from the plot but the fitting program, described below, is used to determine both non-Faradaic (R_s and C_{dl}) and Faradaic (charge transfer resistance, R_{CT}, and psuedocapacitance, C_{AD}) contributions [10, 45].

3.4.1. Equivalent Circuit Analysis

The equivalent circuits used to model the systems studied in this report are shown in Fig. 7. The impedance of the surface confined species was analyzed using a modified Randles circuit [10, 11, 60, 61]. This circuit has four different components: R_s, C_{dl}, R_{CT}, and C_{AD}, corresponding to the electrochemical charging/discharging process of the surface confined electroactive species [11, 61, 62]. The redox species Γ_{ads} and heterogeneous k_{et} are retrieved from the equivalent circuit (Fig. 7b) after modeling with a complex non-linear least square (CNLS) algorithm [11]. Once C_{AD} and R_{CT} are determined, Γ_{ads} and k_{et} are calculated according to their respective equations below where other variables have their usual meaning.

$$\Gamma = \frac{4RTC_{AD}}{F^2 A} \quad (11)$$

$$k_{et} = \frac{1}{2R_{CT}C_{AD}} \quad (12)$$

In this report, the experimental results are displayed on Cole-Cole plots (1/jωZ plane) instead of the typical Nyquist plots (Z plane) as this type of representation allowed us to more effectively track the capacitive properties of our electrochemical systems [6, 11, 17, 61]. The Cole-Cole plots are manipulations of the Nyquist and Bode plots (see Supplementary Data).
The shape of a typical Cole-Cole plot is a semicircle which is affixed to the origin on real axis, Re[1/jωZ], and its diameter is C_{dl} or (C_{dl} + C_{AD}) in the absence or presence of the redox species, respectively [11, 61, 62].

3.4.2. Electrochemical Impedance Spectroscopy of AZ at SAM Assemblies.

The Cole-Cole representation of AZ confined to SAM assemblies showed two different regimes depending on the methylene chain length of the thiol used [11, 62]. Two semicircles were observed in Cole-Cole plots when the assemblies contained long chain thiols as seen in Fig. 8. The greater the number of methylene units in the chain (C18 > C16 > C14), the more distinct the small high frequency semicircle (see Supplementary Data). The small high frequency semicircle represents the non-Faradaic charging of the double layer while the large low frequency semicircle represents the Faradaic process [11, 62]. In contrast, only one semicircle was observed in the Cole-Cole plot of AZ confined to SAM assemblies with short chain thiols. The non-Faradaic and the Faradaic contributions were not resolved into two time constants as illustrated in Fig. 8 insets, where the low frequency semicircle overlaps with the high frequency semicircle. The difference between the two regimes stems from the value of the time constant, $\tau = RC$, for the Faradaic and non-Faradaic components of the system [11, 62]. The regime representing the longer chain SAMs has time constants that are significantly different for the non-Faradaic and Faradaic contributions, while that representing the short chain SAMs has similar time constants. Since the capacitive components are usually of the same order of magnitude It is the resistive components of the cell (R_s and R_{CT}) that are responsible for differences in the time constants [11].

Control experiments were conducted in 4.4 mM KPB on SAM assemblies prior to AZ attachment. Figure 9 is a representative comparison of the Cole-Cole plots for Au electrodes modified with alkanethiolate SAMs of different chain lengths. As expected, C_{dl} decreases as methylene units in SAM increase. That is, there is a progressive increase in the x-intercept at the low frequency end of the semicircle of the Cole-Cole plots as the SAM gets shorter and charging current or C_{dl} increases [6, 11, 62]. EIS spectra from control experiments and ones with AZ adsorbed on SAM assemblies of different chain lengths are computer fitted (CNLS) using the equivalent circuits (Fig. 7) and yielded the results shown in Table 4 which clearly illustrate excellent agreement between C_{dl} values obtained from CV and EIS experiments at the various SAMs [6, 62].

Values of C_{AD} are derived from Cole-Cole plots by subtracting the diameter of the semicircle of the Cole-Cole plots obtained in the presence and absence of the redox species. In addition to deriving C_{AD} directly from Cole-Cole plots, values are also obtained from computer fitting as well. The values from these methods are comparable (Table 4) and can each be applied to Eq. (12) to calculate estimates of Γ_{ads} [6, 11, 62]. Computer fitting and Cole-Cole plots yielded sub-monolayer Γ_{ads}, 6.57 ± 0.68 and 6.31 ± 1.2 pmol cm^{-2}, for AZ on C14 and C16 SAMs, respectively. These values are similar to those obtained from CV (7.56 ± 0.69 and 6.75 ± 0.81 pmol cm^{-2} respectively) [6].

Values of k_{et} are also determined from either computer modeling and/or Cole-Cole plot analysis. In the case of computer modeling, the value of C_{AD} is applied to Eq. (13) while k_{et} is evaluated directly from the large low frequency semicircle of the Cole-Cole plot by inputting the frequency (f°) that corresponds to the maximum value of the imaginary capacitance, -Im [1/jωZ], into Eq. (17) given below:

$$k_{et} = \pi f^{\circ} \qquad (16)$$

Computer fitting determined k_{et} values to be 8.07 ± 0.43 and 1.14 ± 0.07 s^{-1} for C14 and C16 assemblies, respectively. Cole-Cole analysis yielded similar k_{et}, 6.78 ± 0.75 and 1.05 ± 0.11 s^{-1} for AZ at the same film assemblies. Both methods agreed with values obtained from CV experiments using Laviron's method. Similar to the CV results, EIS analysis confirms that ET kinetics through the SAMs is chain length dependent [6, 30, 62]. Excellent agreement between

CV and EIS results reaffirms the view that AZ / C14 SAM / Au and AZ / C16 SAM / Au assemblies are well-behaved from an electrochemical perspective, reproducible, and highly stable [46].

In assessing EIS as a tool for PME, we note that the Cole-Cole plots of AZ adsorbed on C6 SAM assemblies showed a slight distortion at the low frequency end of the semicircle (Fig. 8 inset). As EIS is an inherently more sensitive technique, it is not surprising that corresponding CVs of the same systems were normal and showed no signs of abnormalities. Unfortunately, as a consequence of this distortion in the EIS spectra of short chain protein-SAM systems, we were unable to use the computer fitting model or Cole-Cole plot analysis to obtain values of C_{dl} comparable to those found by CV (Table 4). For example, the C_{dl} value from CV of AZ / C6 SAM / Au is ca. 3.00 μF cm^{-2} while the C_{dl} value from EIS experiments and fitting is ca. 7.00 μF cm^{-2}. An explanation for this observation has not been directly addressed in the literature but reports on the EIS behavior of AZ or cyt *c* at short chain SAMs by Bowden and Guo [11, 62] suggest a number of causes for this observation. It is well known that short chain SAMs lack the rigidity and organized structure of long chain SAMs [46, 63]. The greater defect density of the short chain SAMs leads to an interface with more inherent fluidity as well as an increase in the hydrophobicity of the adsorbed protein's microenvironment perhaps resulting in AZ having a more direct interaction with the gold surface, a factor that would directly affect the nature of the observed PME [24, 29, 30]. AZ, for example, is known to bind to alkanethiol SAMs via a hydrophobic pocket near the copper redox center or directly to the gold substrate through the disulfide bonds from the cysteine residue on the opposite side of the protein (see Supplementary Data). Thus, a hexanethiol SAM may have enough defect density to allow varying orientations of adsorbed protein – a situation that would impact the measured k_{et} [25].

Another cause of the distortion observed in EIS spectra of AZ film assemblies containing C6 SAMs may be related to the presence of multiple protein populations on the surface. To test this hypothesis, we conducted studies on purified and unpurified cyt *c* or AZ adsorbed onto a gold electrode that has been modified with short or long chain SAMs. Unpurified proteins were adsorbed on PME assemblies comprised of C10 SAMs because these SAMs contain enough methylene units to possess a more rigid and organized structure relative to C6 SAMs. In the case of cyt *c*, EIS results indicate that the unpurified protein likely contains more than a singular population of protein, resulting in a Cole-Cole plot with significant distortion. The purified cyt *c*

sample, however, results in a plot with a single undistorted semicircle, indicative of a more uniform population of protein (see Supplementary Data). Similar experiments were attempted with the AZ system since EIS spectra showed that unpurified AZ at a C6 SAM results in the same type of low frequency distortion (Fig. 8 inset). Unfortunately, attempts to chromatographically resolve the AZ into separate populations were unsuccessful (see Supplementary Data). The EIS spectra of AZ or cyt *c* on long chain SAMs showed no distortion, which suggests that the multiple protein population theory may not be as significant as the SAM structure argument.

3.4.3. Electrochemical Impedance Spectroscopy Study of Azurin at C12 $(MPC)_3$ Film Assemblies

Previous research from our laboratory involving the use of MPCs as an alternative platform for PME studies focused mainly on CV experiments [22, 30, 31]. In this report, we have used EIS to study protein ET at MPC platforms as well. In a similar fashion to CV, EIS can be used to measure the C_{dl} of the system and monitor the assembly of MPC layers, with each layer of MPCs adding to the overall film capacitance [30, 31]. As previously stated, we were unable to use the computer fitting model or Cole-Cole plot to acquire data for AZ adsorbed at C6 SAM film assemblies either because the ET kinetics of AZ on these assemblies is too fast to measure using the current EIS fitting softwares and/or adsorption of the protein on the short chain SAM is unstable due to the lack of rigidity and organization in these SAMs [62]. As a result, MPC film assemblies were purposely designed with low capacitance. More specifically, MPC assemblies consisting of HDT-linked C12 $(MPC)_3$ films at C14 SAM were constructed. This strategy doubles the peripheral thickness of the NP's insulation in an effort to minimize the overall additive capacitance of incorporated MPCs [22]. Three layered HDT-linked, C12 MPC films at C14 SAM, monitored via Cole-Cole plots, are illustrated in Fig. 10a. Unlike the C6 SAM / $(C6\ MPC)_3$ / Au system, which showed an increase in C_{dl} with each exposure to MPC (see Supplementary Data), the measured C_{dl} of the long chain system (C14 SAM / $(C12\ MPC)_3$ / Au) showed an initial decrease in C_{dl} with the addition of the first layer of MPC before a steady increase with subsequent MPC attachments as seen in Fig. 10. This initial decrease is thought to

be a result of the first layer of MPCs interdigitating with the longer chain, dithiol amended SAM to create a very low dielectric barrier that lowers the overall C_{dl} [22].

Given the lower overall C_{dl} of the system, AZ was subsequently adsorbed to the HDT-linked C12 $(MPC)_3$ / C14 SAM /Au assembly and analyzed via EIS. An example of a typical Cole-Cole plot from these experiments is displayed as the inset in Fig. 10a. The resulting electrochemical parameters derived from EIS (Table 5) are in excellent agreement with CV determinations and suggest that the computer fitting program is only able to accurately model data from MPC systems where the capacitance is kept low. Similar Γ_{ads} (sub-monolayer) values were obtained for the AZ / $(C12\ MPC)_3$ / C14 SAM / Au (6.03 ± 0.35 pmol cm^{-2}) system (Table 5) and the traditional AZ / C14 SAM / Au (6.57 ± 0.68 pmol cm^{-2}) platform (Table 4). This is not surprising since the protein binds to the assembly via hydrophobic interactions and the C14 SAM and C12 MPCs should offer essentially the same interface. Comparable k_{et} values were obtained at the AZ / $(C12\ MPC)_3$ / C14 SAM / Au (7.34 ± 1.0 s^{-1}) and AZ / C14 SAM / Au (8.07 ± 0.43) film assemblies. This result again reinforces the observation that k_{et} values are not enhanced due to the presence of the MPCs but rather that the mechanisms of ET through the AZ / C12 $(MPC)_3$ / C14 SAM / Au and AZ / C14 SAM / Au film assemblies differ on a fundamental level [30, 52]. Murray et al. has shown that electrons travel extremely fast (electron hopping) through the MPC films [53] while ET occurs through the SAM via electron tunneling [6, 20, 24, 30, 51]. In the case of these film assemblies, if the ET mechanism were the same, one would expect ET kinetics for the MPC film assembly to be slower than that of the SAM since the electron must travel over greater distance to reach the electrode within the MPC-SAM hybrid films.

4. Conclusions

Strategies for effective PME analysis over the last decade have often focused on the adsorption interfaces including both SAM and MPC films, the latter of which offers ET over greater distances and a more homogeneous adsorption environment for immobilized proteins. With the advent of new materials being incorporated in PME schemes, the complexity of the analysis has also increased with the suggestion that the application of simple CV to certain nanoparticle interfaces may not be as effective as it had been with the traditional SAM platforms due to the inherent increase in capacitive charging current observed with the nanomaterials and the multi-step nature of constructing the more complex films. The present study delves into

protein electrochemistry comparing, not only these two different interfaces (SAMs vs. MPC films), but also the application of more advanced electroanalytical techniques, including pulse/step potential and frequency-based methods, to the analysis of protein ET. In the latter respect, this report represents some of the first systematic investigations of electroactive proteins adsorbed to nanoparticle modified electrodes and comparing the results to that of traditional SAM platforms.

The cumulative results of this report have suggested that while CV remains an effective tool for estimating most electrochemical parameters for these systems, it does, as shown by our comparisons have the potential to underestimate the determination of Γ_{ads} in cases where charging current is considerably higher (i.e., the use of shorter chain SAMs or the incorporation of MPCs at the electrode interface). Based on the work presented, if given these conditions, experimenters should independently measure Γ_{ads} using the presented CC analysis which also provides excellent estimations of C_{dl} or consider the use of EIS (*vide infra*). In terms of applying electrochemical pulse techniques such as SWV to these PME systems, it remains a viable technique for both E°´ and ET kinetic determinations but, under our current understanding, is relatively ineffective for studying other electrochemical parameters, including Γ_{ads}. Its use in estimating k_{et} requires significant pre-measurement development in terms of simulating square wave voltammograms to create a suitable working curve. That being said, SWV of PME allows for much faster and more sensitive electrochemical analysis of the protein ET, properties advantageous for the development of real-time biosensors relying on amperometric signaling. Aside from CV, our work establishes that for PME at either type of adsorption platform, EIS is the most comprehensive method in determining most of the electrochemical parameters of interest. Indeed, the results show that C_{dl}, Γ_{ads}, k_{et} can be accurately determined at both interfaces using EIS and, while we cannot directly assess the uniformity of adsorbates in terms of FWHM like CV, distortion in low frequency end of the EIS scan can yield a qualitative indication of the same phenomenon. Unfortunately, of these techniques EIS is also one of the more complex theories of analysis within the spectrum of electrochemical methods.

Ultimately this work outlines the effectiveness and applicability of an array of electrochemical methods for PME at both SAMs, the traditional adsorption platform of this strategy over the last 20 years, as well as at MPC film assemblies, a more recent innovation to adsorbed ET protein studies. It is our hope that this work will serve as a tool for others exploring

the ET properties of adsorbed proteins to properly select appropriate electrochemical methods for the most effective analysis, particularly with electrodes modified with capacitive elements like these nanoparticles. We envision such research would eventually aid in the development of amperometric biosensors and biocompatible interfaces.

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