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Santa Barbara

**Electrochemically Driven Structural Dynamics and Spectroscopy
of Biomolecular Systems**

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in Chemical Engineering

by

Yin-Chen Lin

Committee in charge:

Professor Michael Gordon, Chair

Professor Daniel E. Morse

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June 2026

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June 2026

Electrochemically Driven Structural Dynamics and Spectroscopy of Biomolecular Systems

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by

Yin-Chen Lin

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Curriculum Vitae

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Y.-C. Lin, C. Yang, S. Tochikura, D. E. Morse, L. Sepunaru, and M. J. Gordon, “Electrochemically Driven Optical Dynamics of Reflectin Protein” *Adv. Mater.*, 2411005 (2025).

Y.-C. Lin, E. Masquelier, Y. Al Sabeh, L. Sepunaru, M. J. Gordon, and D. E. Morse, “Voltage-Calibrated, Finely Tunable Protein Assembly” *J. R. Soc. Interface*, 20, 20230183 (2023).

Y.-C. Lin, S. Aulia, M.-H. Yeh, L.-Y. Hsiao, A. M. Tarigan, and K.-C. Ho, “Graphene Quantum Dots Induced Defect-Rich NiFe Prussian Blue Analogue as an Efficient Electrocatalyst for Oxygen Evolution Reaction” *J. Colloid Interface Sci.*, 648, 193-202 (2023).

Y.-C. Lin, C.-H. Chuang, L.-Y. Hsiao, M.-H. Yeh, and K.-C. Ho, “Oxygen Plasma Activation of Carbon Nanotubes-Interconnected Prussian Blue Analogue for Oxygen Evolution Reaction” *ACS Appl. Mater. & Interfaces*, 12, 42634-42643 (2020).

C.-H. Lin, **Y.-C. Lin**, C.-L. Yeh, L.-Y. Lin, K.-C. Ho, “Cobalt/Cobalt Phosphide/Nitrogen-doped Carbon Derived from Zeolitic Imidazolate Framework-11@Zeolitic Imidazolate Framework-12 Core-shell Structure as Efficient Electrocatalyst toward Water Oxidation” *Int. J. Hydrogen Energy*, 101, 1279-1287 (2025).

S. Aulia, **Y.-C. Lin**, L.-Y. Chang, Y.-X. Wang, M.-H. Lin, K.-C. Ho, and M.-H. Yeh, “Oxygen Plasma-Activated NiFe Prussian Blue Analogues Interconnected N-doped Carbon Nanotubes as a Bifunctional Electrocatalyst for a Rechargeable Zinc-Air Battery” *ACS Appl. Energy Mater.*, 5, 9801-9810 (2022).

C.-H. Lin, S. Perumal, **Y.-C. Lin**, L.-Y. Hsiao, L.-Y. Lin, K.-C. Ho, “Two-Dimensional Vanadium Carbide (V_2C) MXene Boosted Nickel Iron Phosphide Derived from Prussian Blue Analogue for Electrocatalytic Oxygen Evolution Reaction” *Int. J. Hydrogen Energy*, 111, 40-51 (2025).

Presentations

American Institute of Chemical Engineers Annual Meeting (poster) *November 2025*
Title: Electrochemically Driven Structural Dynamics and Spectroscopy of Biomolecular Systems

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Title: Electrochemically Driven Structural Dynamics and Spectroscopy of Biomolecular Systems

69th Biophysical Society Meeting (poster) *February 2025*
Title: Electrochemically Driven Optical Dynamics of Reflectin Protein Films

245th Electrochemistry Society Meeting (presentation) *May 2024*
Title: Electrochemically Driven Optical Dynamics of Reflectin Protein

UCSB ChE First Year Graduate Student Symposium (presentation) *September 2022*
Title: Electrochemically Driven Structural Dynamics and Spectroscopy of Reflectin and Other Proteins

Abstract

Electrochemically Driven Structural Dynamics and Spectroscopy of

Biomolecular Systems

by

Yin-Chen Lin

The diverse structure and function of biological systems often depend on the dynamic conformations and assemblies of biomolecules. In particular, electrostatic interactions arising from ionizable amino acid residue side chains play a central role in regulating biomolecular structure *in vivo* through changes in pH or post-translational modifications. Developing methods that can actively trigger and control assembly is therefore essential to understand protein function and to gain insight into the biophysical mechanisms that govern folding and assembly pathways.

This thesis presents an electrochemical approach for precisely modulating electrostatic interactions in biomolecules by neutralizing protonated amino acid residues, thereby enabling the study of protein structural evolution driven by changes in Coulombic interactions and intermolecular assembly. Electrochemistry was combined with *in situ* spectroscopies (e.g., dynamic light scattering, circular dichroism, ellipsometry, and surface plasmon resonance) to simultaneously trigger, control, and observe protein assembly. This electrochemically coupled spectroscopic approach was first demonstrated on freely diffusing reflectin proteins, the biomolecules responsible for

dynamic color change in squid, and revealed that the electrochemical regulation closely mirrors the physiological transitions of reflectin, enabling precise, real-time analysis of conformational and assembly dynamics. These methods were then applied to reflectin thin films, showing that applied voltage modulates both the refractive index and thickness of the films, mimicking osmotic dehydration and shedding light on the color-changing mechanisms in squid skin. Building on this, engineered RM1 (the most conserved motif in reflectin) peptides were investigated using electrochemical correlative spectroscopy, demonstrating that the essential features of reflectin tunability can be recapitulated by a minimal motif-based construct. At the same time, these results highlight the importance of native sequence architecture in promoting efficient and homogeneous assembly. More broadly, this work shows that reflectin behavior is governed by a delicate balance between electrostatic interactions and hierarchical assembly, providing a strong foundation for the future design and control of reflectin-inspired responsive biomaterials.

The approaches developed in this thesis enable electrical control and real-time observation of biomolecular systems, providing tools to study macromolecular dynamics, uncover biophysical mechanisms, design functional materials, and bridge the biotic–abiotic interface.

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Chapter 1

Introduction

1.1. Biomolecular systems of interest: Reflectin proteins

Reflectin proteins are a family of unique, cephalopod-specific proteins best known for their role in dynamic light reflection and iridescence. They were first identified in animals such as squid, where they are highly enriched in the stacks of plasma membrane-bounded lamellae (iridosomes) within the specialized iridocyte skin cells (Figure 1.1), which help these animals rapidly alter their appearance for camouflage, communication, and predator avoidance [1-4]. In addition to their biological significance, reflectins have attracted growing interest as inspiration for next-generation applications in optical materials and devices [5-8].

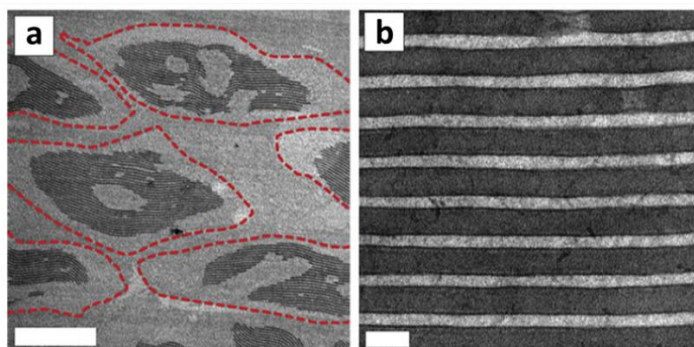


Figure 1.1. TEM images of ultrathin cross-sections of iridophore cells fixed with uranyl acetate. (a) Scale bar = 5 μ m. (b) Scale bar = 200 nm. Dark, electron-dense areas correspond to reflectin-filled lamellae. Cell boundaries are outlined in red for clarification. Adapted with permission from [1]. Copyright 2010 Elsevier.

Reflectin A1, the majority species, is a block copolymer-like peptide chain of *ca* 350 amino acid residues consisting of six repeating conserved domains alternating with cationic linkers (Figure 1.1), of which the positive charge is dominated by a total of 31 histidines [9, 10].

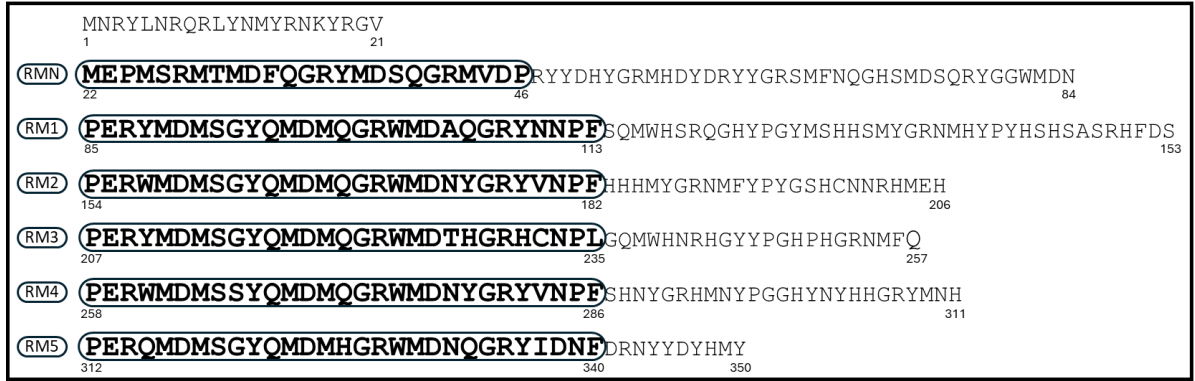


Figure 1.2. Sequences of *D. opalescens* reflectin A1. The conserved N-terminal domain is circled and labeled as RMN; other repeat domains are circled and labeled as RM1-RM5.

In vivo, progressive charge neutralization by acetylcholine (ACh)-activated phosphorylation overcomes the Coulombic repulsion of reflectin's cationic linkers to drive condensation, folding and assembly [11, 12]. Assembly then triggers osmotic and Gibbs–Donnan-mediated dehydration of the Bragg lamellae, reducing the platelet thickness and spacing, while increasing refractive index, thus dynamically tuning the color of the reflected light [13-15]. This regulatory mechanism underlying camouflage is illustrated in Figure 1.3. *In vitro*, pH-titration, charge screening, and genetic engineering function as surrogates for *in vivo* phosphorylation, progressively neutralizing the initially cationic reflectin to monotonically drive assembly calibrated with the extent of charge neutralization [16, 17]. More recently, reflectin assembly has also been triggered electrochemically through the electroreduction of histidine residues [10], suggesting that electroreduction can likewise serve as a surrogate for phosphorylation (discussed in more detail in Section 1.2). This finding provided the conceptual foundation for the research presented in this thesis.

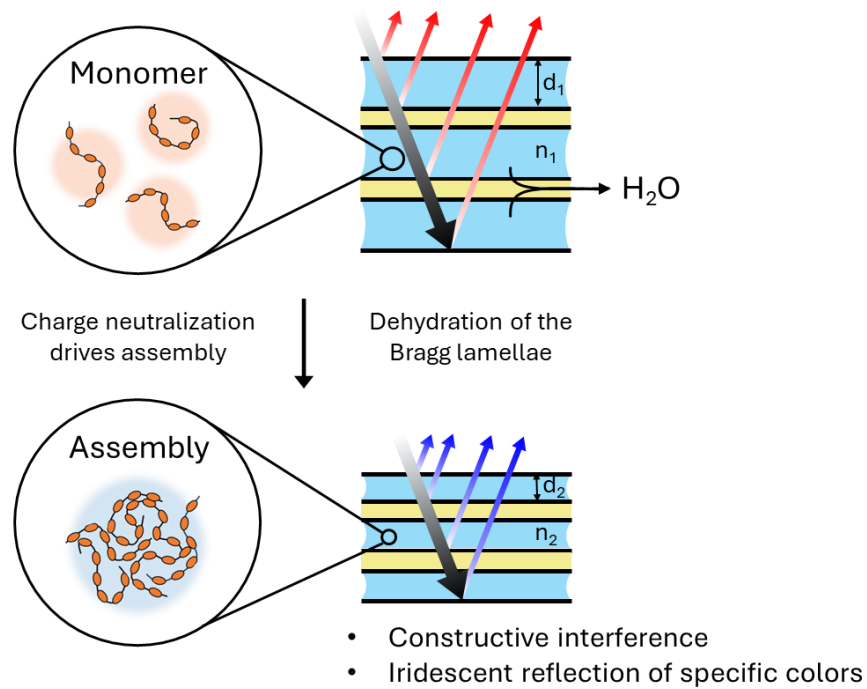


Figure 1.3. Schematic illustration of reflectin-mediated tunable coloration in squid iridophores. In the unassembled state (top), reflectin proteins remain monomeric, resulting in iridophore lamellae with a larger spacing (d_1) and lower refractive index (n_1), which preferentially reflect longer-wavelength light. Upon assembly (bottom), reflectin proteins condense and increase the local protein density, leading to reduced lamellar spacing (d_2) and an increased refractive index (n_2). These structural changes shift the reflected light toward shorter wavelengths, enabling dynamic color modulation.

1.2. Electrochemical control of biomolecules

Electrochemistry, the study of interactions between electrical inputs and chemical species, provides a powerful means of tuning protein electrostatic interactions. Its versatility lies in the ability to control electrochemical potential and current, as well as electrolyte composition, concentration, and electrochemical cell architecture, making it a highly adaptable platform for manipulating protein charge states. Because many biomolecules are highly sensitive to local changes in protonation, redox state, ionic strength, and interfacial environment, electrochemical methods offer a particularly effective route for regulating structure, assembly, and function in a reversible and controllable manner.

In proteins and peptides, electrochemical stimulation can alter charge distribution either directly, through electron transfer involving redox-active residues, or indirectly, by modifying the local chemical and electrical environment near an electrode surface through the formation of an electrical double layer. These changes can strengthen or weaken Coulombic interactions, shift conformational equilibria, and promote or suppress self-assembly. As a result, electrochemistry serves not only as a tool for probing biomolecular behavior but also as a mechanism for actively driving structural transitions.

This concept is supported by recent work showing that dissociable protons in free amino acids and peptide-incorporated residues can be directly electroreduced at a bare platinum electrode, with formal reduction potentials that correlate linearly with their corresponding pKa values. Using differential pulse voltammetry (DPV), Liang et al. demonstrated that proton dissociation equilibria of charged amino acids, including

histidine, lysine, aspartic acid, and glutamic acid, can be probed, and that distinct protonatable groups in short peptides can also be resolved [18]. As described previously, reflectin is a phosphorylation-regulated protein whose self-assembly is driven by charge neutralization within its cationic, histidine-rich linker regions. Liang et al. further showed that electroreduction of histidine residues can induce reflectin assembly, establishing electrochemical proton reduction as a label-free, selective, and highly sensitive surrogate for conventional methods [10].

1.3. Probing structural dynamics of biomolecules by spectroelectrochemistry

The integration of electrochemical control with spectroscopy provides a powerful approach for studying dynamic biomolecular systems by simultaneously manipulating and monitoring biomolecular behavior in real time. Electrochemistry enables precise control over protonation state and ionic strength, both of which strongly influence protein structure and assembly. Spectroscopic techniques, in turn, provide direct insight into molecular structure, conformation, and optical properties. When combined, these methods make it possible to directly correlate external electrochemical stimuli with structural and functional responses at the molecular level.

A major advantage of this approach is the ability to perform *in situ* and *operando* measurements, where structural changes are observed during the actual application of electrochemical bias rather than before or after the process. This is particularly important for responsive systems such as reflectin protein, whose assembly and optical properties are highly sensitive to transient changes in local charge environment. Traditional *ex situ* characterization often misses intermediate states or reversible transitions, whereas electrochemical spectroscopy can capture these processes as they occur, providing a more complete understanding of the underlying mechanisms.

In this thesis, several spectroscopic methods were employed to probe different aspects of reflectin protein dynamics. Dynamic light scattering (DLS) reports on the hydrodynamic size of protein species in solution, making it particularly useful for tracking electrochemically induced assembly and disassembly. Circular dichroism (CD)

provides information on secondary structure by measuring differential absorption of circularly polarized light, allowing conformational transitions such as random coil-to-helical changes to be followed in real time. For example, Masquelier et al. demonstrated that electrochemically driven CD measurements can be used to monitor charge-neutralization-induced folding and assembly of K18, a core peptide domain of tau, revealing progressive secondary structural transitions and assembly behavior under applied electrochemical bias [19]. For reflectin thin films, ellipsometry measures changes in film thickness and refractive index by analyzing the polarization state of reflected light, thereby revealing electrochemically driven swelling, condensation, and water redistribution within the film. Surface plasmon resonance (SPR) further complements these measurements by detecting changes in the optical response of the film–metal interface, offering an independent readout of refractive index changes associated with reflectin condensation.

Together, these techniques provide a multidimensional view of reflectin protein behavior across solutions and film architectures. By monitoring how electrochemically induced changes in charge state affect assembly, conformation, thickness, and refractive index, it becomes possible to directly connect nanoscale structural dynamics with macroscopic optical function. Such insight is essential not only for understanding biological photonic systems, but also for designing bio-inspired electrochromic devices with controllable and reversible optical performance.

1.4. Thesis scope

This thesis focuses on developing electrochemical correlative spectroscopies to simultaneously trigger, control, and observe protein conformational changes and assembly processes. These methods are demonstrated in the case of reflectin protein, where progressive charge neutralization controlled by phosphorylation drives condensation, folding and assembly. In Chapter 2, we first demonstrate that electrochemical reduction enables finely tunable and reversible control over the rate and extent of reflectin assembly via a voltage-calibrated mechanism. Electrochemical correlative dynamic light scattering, circular dichroism, and UV-Vis spectroscopy reveal that this regulation closely mirrors the physiological transitions of reflectin, allowing precise, real-time analysis of conformation and assembly dynamics. In Chapter 3, we fabricate thin films of reflectin via drop-casting onto conductive substrates to mimic the platelet structures of squid iridocytes. Using electrochemical correlative ellipsometry and surface plasmon resonance, we show that applied voltage modulates both the refractive index and thickness of reflectin films, mimicking osmotic dehydration and shedding light on the color-changing mechanisms in squid skin. These results support the hypothesis that charge neutralization governs water content within platelet-like structures, thereby tuning their optical properties. Chapter 4 is motivated by the need to confirm and extend the roles and mechanistic contributions of specific sequence elements in reflectin proteins. To simplify the system and isolate the determinants of reflectin's behavior, engineered RM1 peptides were designed to test whether a single conserved domain flanked by cationic linkers is sufficient to reproduce the charge-neutralization-dependent,

reversible, and cyclable folding and assembly behavior observed in native reflectin. In doing so, this chapter further examines how sequence reduction affects assembly, while demonstrating that the complexity of the cationic linker region can be reduced from 14 different amino acid types to a single residue without eliminating the essential responsive properties of the system. In Chapter 5, we discuss future work aimed at gaining a deeper understanding of reflectin film stability and the mechanisms that govern its electrochemical and optical behavior. These studies will help pave the way for bio-inspired materials and devices that seamlessly integrate biological principles with synthetic systems, thereby bridging the biotic–abiotic gap.

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Chapter 2

Electrochemically Calibrated Reflectin Protein Assembly

Adapted from the following publication:

[1] **Y.-C. Lin**, E. Masquelier, Y. Al Sabeh, L. Sepunaru, M. J. Gordon, and D. E. Morse, “Voltage-Calibrated, Finely Tunable Protein Assembly,” *J. R. Soc. Interface*, 20, 20230183 (2023).

Reflectin proteins from squid provide a remarkable model for understanding how charge regulation can control hierarchical protein assembly and, in turn, tunable optical behavior. In this chapter, we show that electrochemical reduction can serve as a surrogate for phosphorylation-driven charge neutralization, enabling the controlled and reversible assembly of reflectin A1 in real time. By combining electrochemistry with dynamic light scattering, circular dichroism, and UV absorbance spectroscopy, we demonstrate that applied voltage finely calibrates both the extent and rate of reflectin assembly, closely paralleling the physiological behavior of the native protein. These results establish electrochemical control as a powerful tool for probing the coupling between protein charge state, conformation, and assembly. The experimental protocols used for these measurements are described in Section 2.6.

2.1. Differential pulse voltammetry of reflectin protein

To identify the electrochemical reduction potential of histidines inside reflectin, differential pulse voltammetry (DPV) was performed on 25 μM reflectin in 40 mM NaCl (to ensure conductivity) at pH 3. This electrolyte condition was chosen to guarantee full protonation of all the protein's constituent amino acids, sufficient ionic conductivity and a low background of UV absorption in the wavelength region probed. The DPV of reflectin (Figure 2.1) exhibits a distinct electroreduction wave at -0.45 V with a shoulder around -0.57 V (black, solid curve). In comparison with the blank solution (black, dashed curve), the reduction peak at -0.45 V is the signature of hydronium reduction in the acidic, aqueous solvent [2, 3, 4]. The shoulder at -0.57 V, which becomes

increasingly evident after deconvolution (orange curve), corresponds to reduction of the imidazolium side-chain of histidine residues in the protein [2, 5].

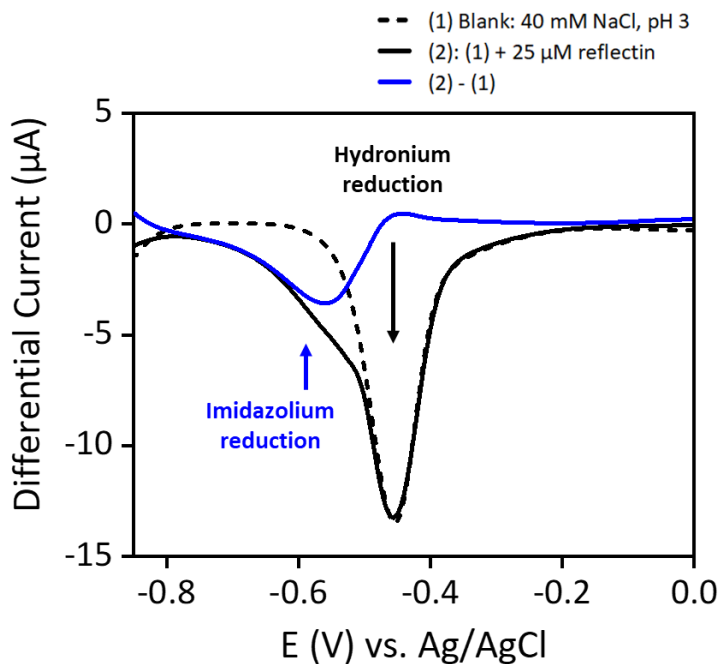


Figure 2.1. Differential pulse voltammogram of 25 μM reflectin in 40 mM NaCl, 1 mM perchloric acid, pH 3. Blue curve is the deconvoluted voltammogram of reflectin after subtraction of the differential pulse voltammogram without reflectin (dashed line) from the DPV with reflectin (black).

2.2. Voltage-calibrated reflectin assembly

Potentials below and above reflectin's identified reduction peak at -0.57 V versus Ag/AgCl were selected to analyze the time-dependent evolution of reflectin assembly size during electrochemical activation by electrochemical correlative dynamic light scattering (E-DLS), allowing us to simultaneously drive and quantitatively monitor these processes using the experimental set-up shown in Figure 2.2.

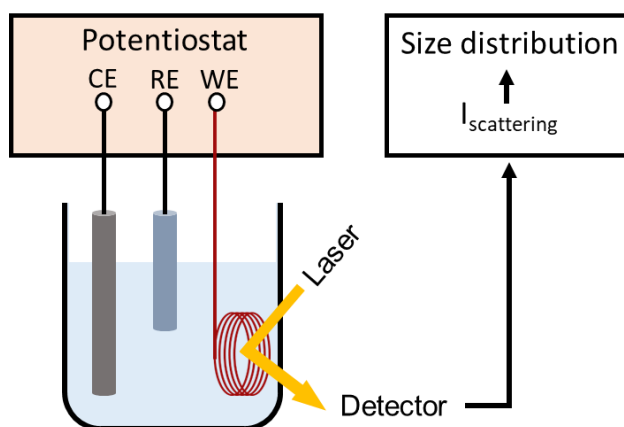


Figure 2.2. Cell configuration for electrochemical correlative dynamic light scattering (E-DLS). WE = working electrode; CE = counter electrode; RE = reference electrode.

The subthreshold potential of -0.475 V, sufficient to reduce hydronium ion, produces no change in reflectin size over that of the *ca* 10 nm monomer (Figure 2.3a). This result indicates that the electrochemically driven pH gradient resulting from hydronium reduction near the working electrode is insufficient to trigger reflectin assembly. By contrast, the slight increase in applied voltage to -0.57 V triggers metastable assembly over 25 min, with rapid return to the starting size of the monomer

when the potential is returned to open circuit potential (OCP) (i.e. equilibrium potential at which no current is flowing in the cell) (Figure 2.3c). The observed metastability of assembly can be attributed, in this kinetically controlled regime, to the rate of assembly being just barely able to compete with disassembly caused by the surrounding low-pH environment. At the higher potential of -0.6 V, reflectin assembly size is stable and homogeneous. Again, disassembly to monomers quickly occurs when the applied voltage is turned off, indicating rapid and complete reversibility (Figure 2.3d). Similarly, progressively larger assemblies are observed at -0.7 and -0.8 V, with rapid disassembly to the monomers driven by diffusion into the low pH environment and the return to local acidic environment at the electrode surface, once the voltage is turned off (Figure 2.3e,f). In addition, the pH gradient that arises from a small level of water electrolysis at a potential of -0.8 V may also contribute to reflectin assembly. As such, potentials below -0.8 V that can generate larger pH gradients have not been discussed here.

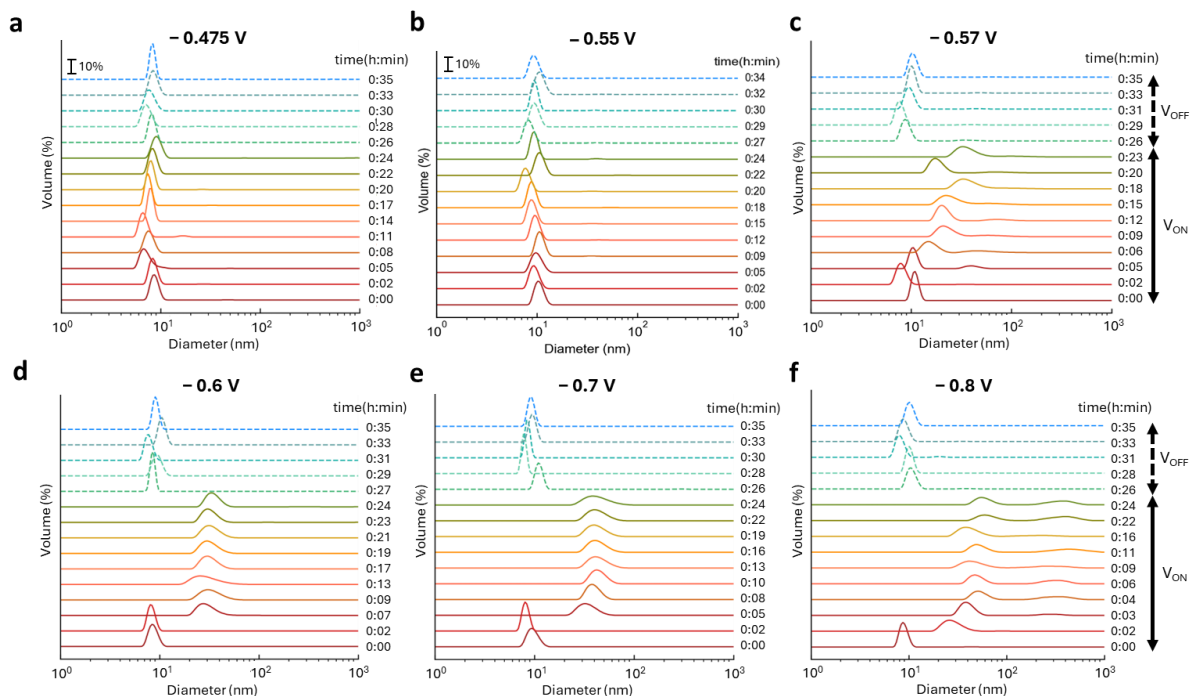


Figure 2.3. E-DLS of particle size distribution of 2 μM reflectin in 40 mM NaCl, 1 mM perchloric acid, pH 3 at applied voltages of (a) -0.475 V , (b) -0.55 V , (c) -0.57 V , (d) -0.6 V , (e) -0.7 V , and (f) -0.8 V . DLS was continuously measured during application (V_{ON}) and after the potential was returned to open circuit potential (OCP) (V_{OFF}) (solid and dashed lines, respectively).

Under apparent steady-state conditions (after 25 min), the final sizes of reflectin assemblies are seen to have increased monotonically as a function of negative potential applied to the working electrode (Figure 2.4a). After a threshold, the results are in close parallel to the protein's assembly in proportional response to the neuronal signal and its charge-neutralizing phosphorylation *in vivo*, and to charge neutralization by pH titration, charge screening and genetic engineering *in vitro* [6, 7]. At -0.8 V , reflectin exhibits the largest assembly size ($60 \pm 20\text{ nm}$), compared with the results at -0.7 V ($50 \pm 20\text{ nm}$),

−0.6 V (35 ± 15 nm), −0.57 V (27 ± 13 nm) and −0.475 V (10 nm = approximate diameter of the monomers). Although the size distribution by volume at −0.8 V displays a mixture of two reflectin assembly sizes, only the smaller of these is shown in Figure 2.4b, since it accounts for 99.6% of the particle number. Data are consistent with the hypothesis that at progressively higher voltages, histidine residues with slightly greater than their prototypical pKa, possibly due to differences in local sequence environment or steric hindrance, are electroreduced to yield progressively larger assemblies [5]. These histidine residues with various pKa may be observed and evidenced by the relatively broad reduction peak of reflectin (Figure 2.1).

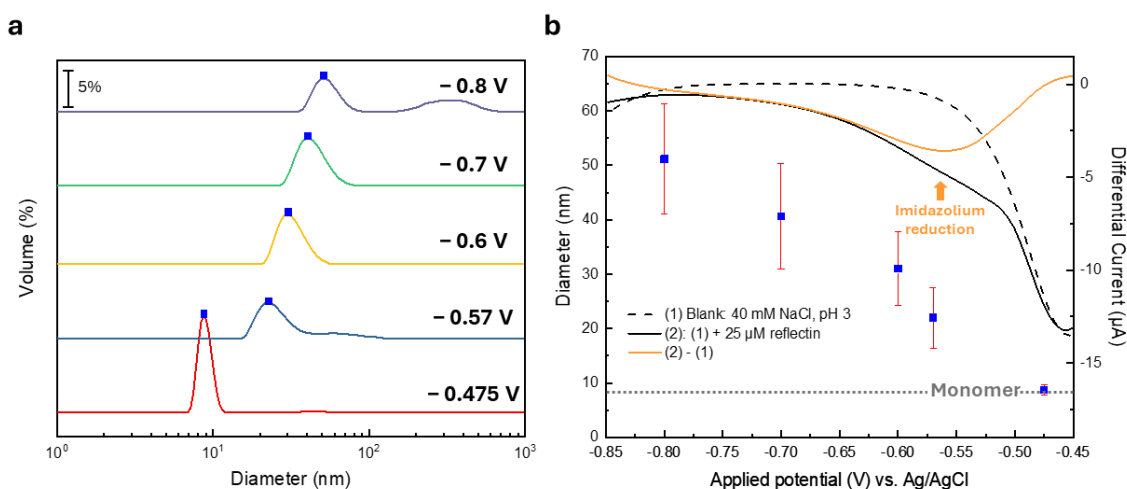


Figure 2.4. Reflectin assembly size as a function of voltage. (a) Final reflectin size distributions and (b) assembly sizes (left axis) measured by DLS with specified voltage applied for 25 min; error bars indicate full width at half maximum. DPV (right axis) was included for reference. All solutions were 2 μ M reflectin in 40 mM NaCl, 1 mM perchloric acid, pH 3.

Interestingly, the threshold for assembly seen in figure 2.4b indicates a small barrier, suggesting that electroreduction of a sufficient number of histidine residues is required for assembly. This suggestion is experimentally confirmed by the inability of -0.55 V, a voltage capable of reducing the imidazolium of free histidine [5], to trigger assembly of reflectin A1 (Figure 2.3b). This barrier was similarly observed in the protein's pH dependent assembly with values lower than the pKa of histidine being ineffective [7].

The relation between the rate of reflectin assembly and voltage was further investigated by analyzing assembly size over the first 10 min after application of -0.6 , -0.7 and -0.8 V (Figure 2.5a). The results unequivocally reveal that more negative potentials rapidly drive assemblies to the same sizes, owing to both the electroreduction of more histidines and the potentially faster electron transfer rate indicated by the broad and asymmetric reduction peak in reflectin's DPV (Figure 2.4b). For example, the formation of assemblies of ca 30 nm diameter requires approximately 2 and 6 min with applied potentials of -0.6 and -0.8 V, respectively. Thus, both the rate and size of reflectin assembly increase monotonically as a function of electrical signal strength. The observed lag time before the onset of any detectable increase in diameter (Figure 2.5a) is progressively decreased with more negative potentials (Figure 2.5b), consistent with our hypothesis that more rapid assembly occurs under more negative potentials. (Although the lag times observed for the two lowest voltages are the same, this is a consequence of the relatively low temporal resolution of analysis, as indicated by the larger assembly size at -0.6 V relative to that at -0.57 V.)

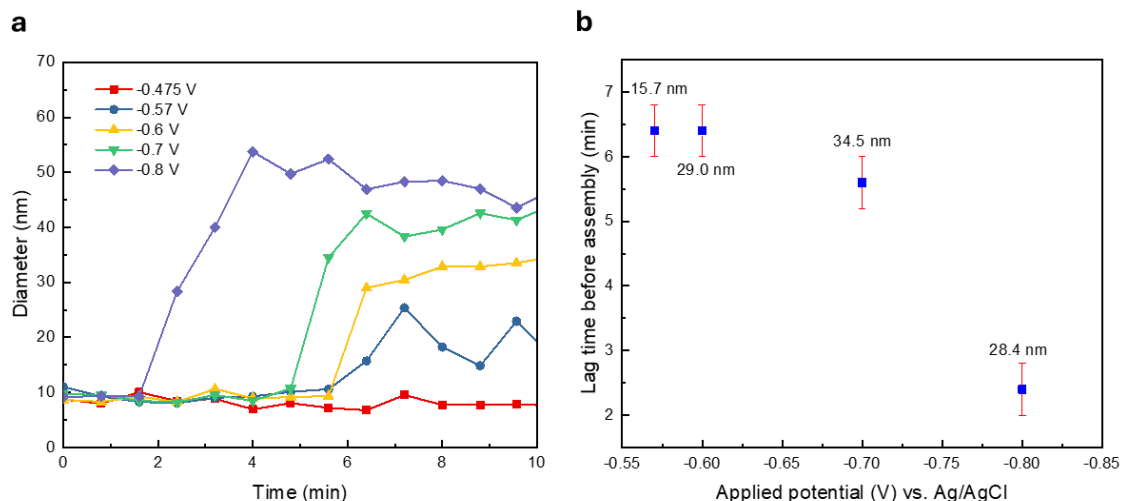


Figure 2.5. Reflectin assembly rate as a function of voltage. (a) Evolution of assembly size as a function of applied voltage over the first 10 min. (b) Lag time between application of the specified potential and first observed increase in assembly size (i.e. larger than monomer, approx. 10 nm) in a. Vertical error bars correspond to the time between sampling points. All solutions were 2 μ M reflectin in 40 mM NaCl, 1 mM perchloric acid, pH 3.

To gain further insight into the assembly process triggered by electrochemistry, the count rates of back-scattered photons from the DLS photodetector at different potentials were measured simultaneously with the determination of size, since the intensity of Rayleigh scattering depends on both the sixth power of the diameter and the absolute number of light-scattering particles [2]. Initially, similar count rates were observed at each voltage analyzed, since each sample contained the same concentration and number of reflectin monomers (Figure 2.6a). The insufficient driving force of -0.475 V did not increase count rate as a function of time (Figure 2.6a), consistent with the lack of assembly already noted (Figures 2.3-2.5). By contrast, count rates began to climb at

increasingly faster rates and reached correspondingly higher plateaus, monotonically as a function of progressively greater negative potentials (Figure 2.6a), consistent with the assembly sizes described above (Figures 2.3-2.5). The stable plateau values are parallel to the results previously observed using pH titration as a surrogate for charge neutralization resulting from phosphorylation *in vivo*, in which the assemblies remained stable with both time and dilution. Significantly, the count rate in all samples falls precipitously as soon as the applied voltage is turned off, consistent with the disassembly to monomers as described above.

These results are consistent with the evolution of particle size distributions in Figure 2.3. Figure 2.6b reveals the lag time at different potentials before any onset of increase in the count rate as seen in Figure 2.6a. The more negative voltages exhibit progressively shorter delay, indicating that less time is required for sufficient charge neutralization to trigger assembly. This ‘lag’ in count rate increase (Figure 2.6b) falls more rapidly as a function of voltage than the corresponding lag in growth of assembly size (Figure 2.5b), simply because the count rate of photon scattering is a function of both particle number and particle diameter. The latter power law dependency greatly magnifies the contribution of relatively small increases in particle diameter (shown above each voltage in Figure 2.5b). The slope of count rate also increases monotonically with increasingly negative applied voltage (Figure 2.6c), consistent with voltage-dependent increases in both assembly particle number and size (cf. Figure 2.5b).

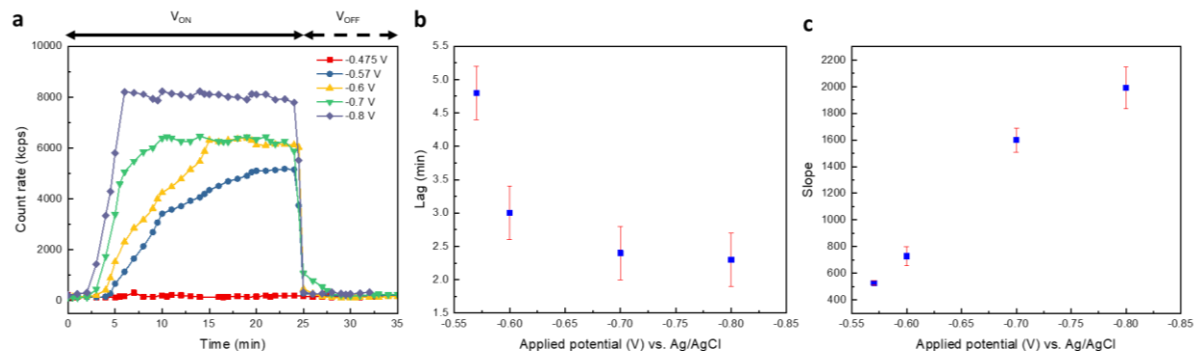


Figure 2.6. Plateaus of count rate show dynamic arrest of reflectin assembly. (a) Count rates from the DLS photodetector, continuously measured during and after application of the specified voltage. (b) Lag time between application of specified potential and increase of the count rate. Error bars correspond to the measurement time. (c) Slope of the count rate versus time from panel a in units of (kcps min⁻¹) for different applied potentials, calculated from a linear fit of the initial rise in count rate. Error bars show $\pm$ s.d. All solutions were 2 μ M reflectin in 40 mM NaCl, 1 mM perchloric acid, pH 3.

To further investigate the phenomenon of dynamic arrest, E-DLS was performed on reflectin A1 at three different concentrations (1, 2 and 3 μ M) with an applied potential of -0.7 V (electronic supplementary material, Figure 2.7). All measurements were conducted in 40 mM NaCl to ensure consistency. Interestingly, we observe that the final assembly size does not depend on the initial monomer concentration. The presence of similar sizes confirms the precise and finely tunable relationship between the extent of charge neutralization and the resulting size of reflectin assemblies that *in vivo* ensures the accurate calibration between the triggering neurochemical signal and the resulting changes in skin color of squid [7].

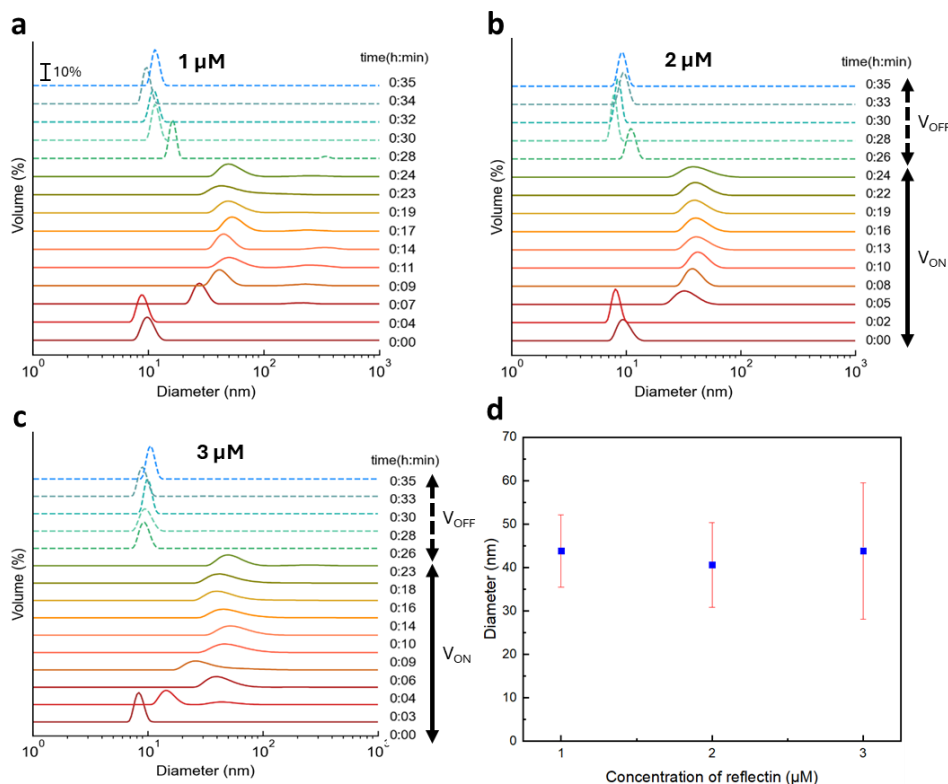


Figure 2.7. Reflectin assembly size is independent of concentration. E-DLS of reflectin particle size distribution at -0.7 V with (a) 1, (b) 2, and (c) 3 μM reflectin in 40 mM NaCl, 1 mM perchloric acid, pH 3. DLS was continuously measured during and after the potential is switched off (solid and dashed lines, respectively). (d) Final assembly sizes of 1, 2, and 3 μM reflectin in (a-c). Error bars show the full width at half maximum of reflectin particle size distribution.

Since the assembly sizes are comparable at these three concentrations, the increases in the plateaus of count rate (Figure 2.8a, b) primarily depend on the number of assembled particles in the system. In contrast, little if any difference in the count rate slope is observed as a function of reflectin concentration, consistent with the unchanged rate of electron transfer and thus unchanged kinetics of assembly at the electrode (Figure 2.8c).

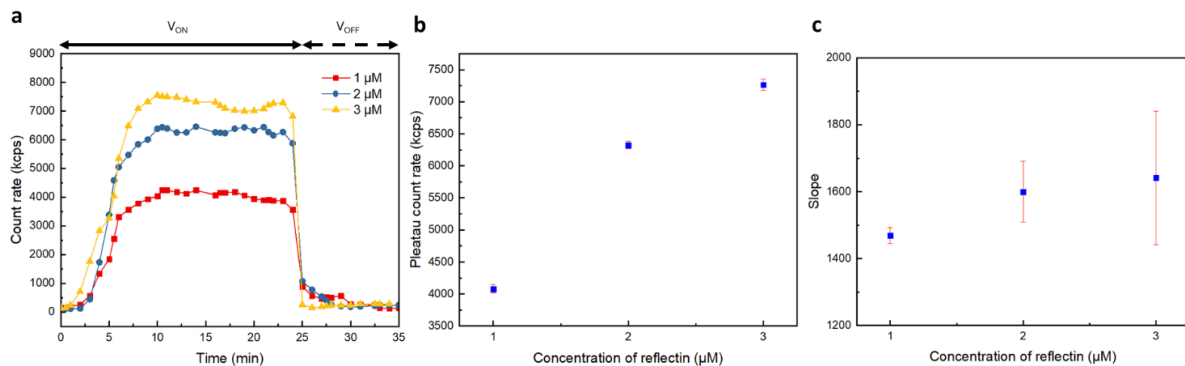


Figure 2.8. Reflectin assembly rate is independent of concentration. (a) Count rates from the DLS photodetector, continuously measured during and after the potential (-0.7 V) is switched off, (b) plateau count rate, and (c) slope of the count rate calculated from onset to plateau with 1, 2, and 3 μM reflectin in 40 mM NaCl, 1 mM perchloric acid, pH 3. Error bars show mean $\pm$ standard deviation.

2.3. Voltage-controlled conformational change of reflectin

To evaluate the conformational response of reflectin to heterogeneous charge neutralization resulting from the electroreduction of histidine residues, electrochemical correlative circular dichroism (E-CD) and UV absorbance were measured during electrochemistry (Figure 2.9). The ellipticity of reflectin shows a strong minimum near 200 nm at 0 min, corresponding to a random coil conformation of the protein [8]. Control CD analyses in the absence of added protein or salt are shown in Figure 2.10 for comparison. At a low potential of -0.475 V, the ellipticity and absorbance remain stable over time (Figure 2.9a,c), demonstrating no structural change at a potential that produced no assembly. In marked contrast at -0.7 V, ellipticity gradually evolves near 200 nm (Figure 2.9b), suggesting significant structural changes during assembly. Previous analyses showed that the CD spectrum of reflectin assemblies is consistent with the presence of both α -helix and β -sheet structures [7, 8]. The unresolved spectra of these structures at 208–220 nm may partially be due to the inhomogeneous nature of the sample, consisting of assemblies near the electrode and monomers in the surrounding acidic medium. Voltage- and time-dependent changes in UV absorbance (Figure 2.9d) parallel those in CD and assembly, with significant hypochromicity at *ca* 197 nm, corresponding to the induced conformational transition from a random coil to α -helix [9,10]. This transition is attributed to the strong dipole–dipole interactions between the chromophoric peptide bonds as they take up the α -helical conformation [11,12]. Notably, as soon as the potential is switched off, the protein reverts back to the random coil conformation in response to the low pH environment (Figure 2.9b,d). This result is in

good agreement with the aforementioned disassembly as seen by E-DLS size distribution and count rate.

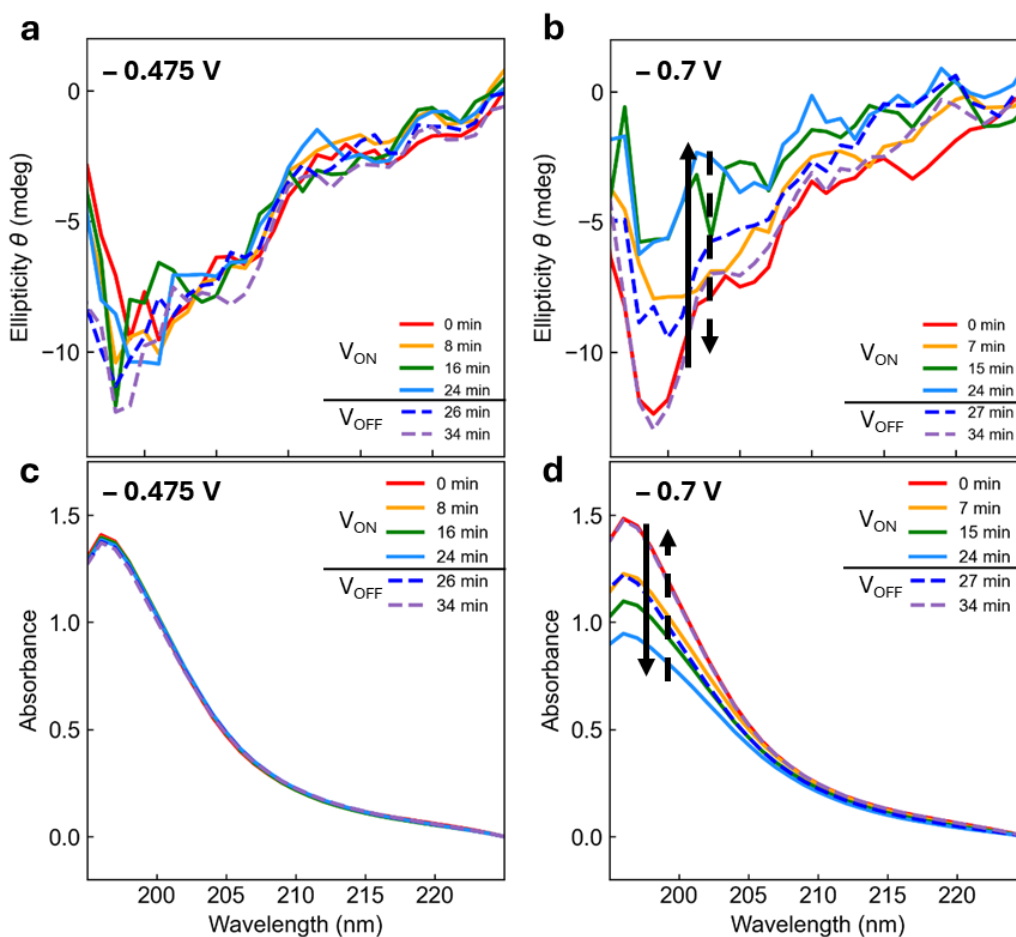


Figure 2.9. Electroreduction of reflectin drives secondary folding. Dynamic evolution of ellipticity (a,b) and absorbance (c,d) of 2 μ M reflectin in 40 mM NaCl, 1 mM perchloric acid, pH 3 exposed to an applied potential of (a,c) -0.475 V and (b,d) -0.7 V. CD was continuously monitored during this exposure (V_{ON}) and after the potential was returned to OCP (V_{OFF}) (solid and dashed curves, respectively). Solid and dashed arrows indicate the temporal evolution of CD and absorbance signals during and following exposure to potential, respectively.

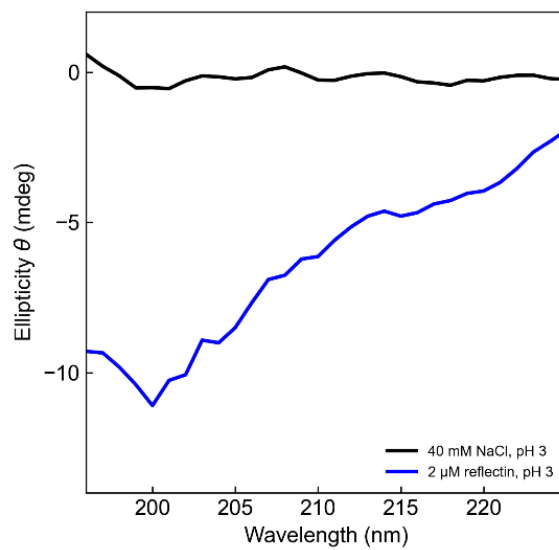


Figure 2.10. Control CD analyses in 1 mM perchloric acid, pH 3, as in Figure 2.9, but containing either 40 mM NaCl but no reflectin (black) or 2 μ M reflectin but no NaCl (blue).

2.4. Reversibility and cyclability of reflectin assembly

In efforts to mimic the reversibility and cyclability of reflectin folding and assembly occurring in the tunable squid iridocytes, E-DLS was conducted with potential switched on and off for several cycles (Figure 2.11). Figure 2.11a shows the similar rise and fall of count rate in each of four cycles, and the corresponding sizes at indicated times are displayed in Figure 2.11b. These results indicate that reflectin A1 reversibly switched through multiple cycles of assembly and complete disassembly. This is the first time that such electrically driven, reversible assembly has been demonstrated for any protein. While this switching behavior of reflectin also can be driven by cyclical changes in pH [13], the need for dialysis (to prevent increases in salt concentration) make that process slow, while the electrochemical process shown here is quicker and potentially more practical for future applications such as photochemical switching.

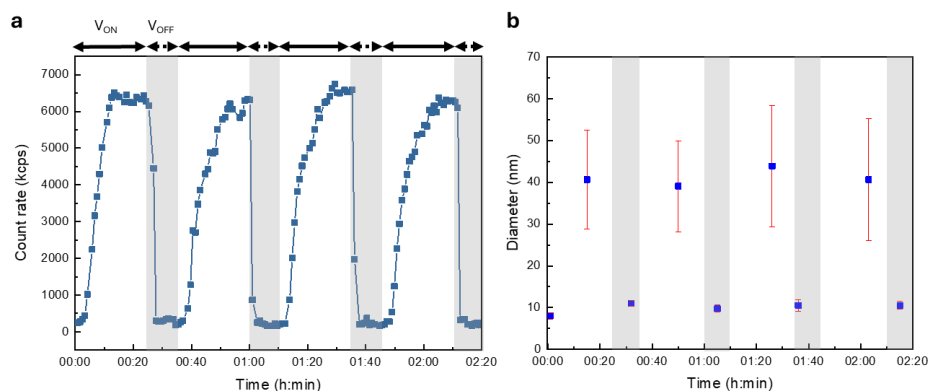


Figure 5. Reversibility and cyclability of reflectin's electro-assembly. (a) Count rate of reflectin assembly and disassembly measured by E-DLS as a function of time during four cycles of 25 min exposure to -0.7 V (V_{ON}) followed by 10 min at OCP (V_{OFF}). (b) Corresponding sizes at stages indicated in (a). Error bars show the full width at half maximum of reflectin particle size distribution. Reflectin ($2 \mu\text{M}$) in 40 mM NaCl, pH 3.

2.5. Discussion and summary

The results reported here build on the prior demonstration that charge neutralization by low-voltage electroreduction can serve as a surrogate for signal-dependent phosphorylation to trigger protein folding and assembly [2, 14]. What is new here is the demonstration that protein assembly, which can be proportionally driven by phosphorylation *in vivo*, can be size-proportionally driven by small increments of DC voltage *in vitro*. The rate of assembly (as inferred from the DLS count rate, which convolves both number density and size) also appears to increase with voltage. The physical mechanism and utility of this process differ completely from those of AC field-mediated concentration, liquid–liquid phase segregation and crystallization of macromolecules [15–18].

The data presented demonstrate for the first time that low-voltage electrochemical reduction monotonically, reversibly and cyclically fine-tunes the rate and size of reflectin A1 assembly as a function of voltage in acidic media. Assembly is triggered, at least in part, by what appears to be direct deprotonation of the imidazolium side-chains of histidine residues in reflectin at a Pt surface at a potential above reflectin's identified reduction peak of -0.57 V. Inspection of the DPV data (figure 1a), however, reveals that a pH gradient will exist at these potentials, extending from alkalinity in the vicinity of the working electrode to the acidity of the bulk solution, thus raising the possibility that some charge neutralization may result from electrochemically driven pH titration. Related spectro-electrochemical analyses of charge neutralization-driven conformational transition of polylysine confirm that direct electroreduction of protein does occur at the

electrode surface under these conditions [3]. Our current investigations are seeking to define the relative contributions of these two processes and the factors that control them.

E-DLS was used to simultaneously trigger and dynamically monitor the evolution of reflectin assembly size from the *ca* 10 nm diameter monomers (at zero applied voltage) to 15–40 nm diameter assemblies at -0.57 V; 20–50 nm at -0.6 V; 30–70 nm at -0.7 V and 40–80 nm at -0.8 V. The precise relationship between voltage and the resulting size is governed by the protein's rapid dynamic equilibration of assembly size as a function of its net charge [7]. This behavior, responsible for reflectin's precise calibration between neuronally driven, phosphorylation-mediated charge neutralization and color in the biological (squid skin cell) system, is likely to result from the balance between the protein's weak, short-range attractive forces and its stronger, long-range repulsive (primarily Coulombic) forces, as characteristic of many colloidal systems [19, 20] and consistent with the observed tuning with charge [7] and voltage. The rate of assembly also increases monotonically with applied voltage, permitting very fine tuning.

The mechanism by which charge neutralization reversibly and cyclically controls reflectin's structure and assembly is fairly well understood [7, 13, 21]. Reflectin A1 is a cationic, alternating block copolymer-like protein, with six histidine rich cationic linkers separating six unique conserved domains, and functions like a concatenate of alternating opposing mechanical springs [13]: analyses showed that Coulombic repulsion of the cationic domains opposes the thermodynamic drive of the conserved domains to condense and fold [7, 13, 21]. Progressive charge neutralization, by phosphorylation *in vivo*, or by genetic engineering, pH-titration (or electroreduction) *in vitro*, progressively

overcomes that Coulombic repulsion, allowing condensations and folding of the conserved domains to alpha and beta structures creating newly emergent hydrophobic surfaces suggested to facilitate hierarchical assembly [2, 7, 13, 21]. Sites of phosphorylation are located in several of the cationic linkers [22], consistent with the results of deletion analyses and titration analyses [13] confirming that the ‘Coulombic switch’ governing assembly is spatially disseminated along the protein chain. Previous genetic deletion experiments showed that the reduction of any specific histidine is not required to trigger folding and assembly; instead, it is simply the net charge balance of the protein that matters [13].

The systematic variation in lag time with voltage before the onset of increase in count rate (measured by E-DLS) indicates that the applied potential is critical to determining the time required to achieve sufficient charge neutralization for assembly. E-CD and UV absorbance were analyzed to evaluate the conformational response of reflectin to charge neutralization resulting from electroreduction. The results confirmed the electrochemically driven evolution from the initial random coil conformation of the fully protonated reflectin monomer (with Coulombic repulsion of the cationic linkers preventing both folding and assembly [7, 23]) to α -helix and β -structures concomitant with assembly.

Previous analysis of the interacting effects of salt concentration and pH on reflectin’s structure and assembly showed that the principal effect of salt is through charge screening [20]. This contributes an independent axis of charge neutralization that can be manipulated to increase or decrease a protein’s sensitivity to electrochemically

driven folding and assembly, thus potentially increasing the power of the methods described here.

In summary, our findings demonstrate that electrochemistry can be used as a surrogate for stimulus-regulated phosphorylation, precisely regulating protein charge state to enable the simultaneous fine tuning and analysis of protein folding and assembly. The significance, wide generality and potential utility of this finding extends far beyond the squid's signal transducing reflectin, as signal-controlled phosphorylation is one of the most evolutionarily ancient and widely distributed mechanisms regulating protein structure and function throughout the kingdom of life. As one further example, we note that low-voltage electroreduction drives assembly of K18, the core peptide of tau—the brain protein that is pathologically driven by excess phosphorylation to assemble as amyloid in Alzheimer's and related neurodegenerative diseases [14]. The structural features shared by reflectin and K18 that enable their responses to be tuned by electroreduction include their thermodynamic drive to fold and assemble [21]; the restriction of that drive by the Coulombic repulsion of their cationic residues (primarily histidine in the case of reflectin, and lysine in the case of K18) initially maintaining both as intrinsically disordered proteins, and their physiological regulation by signal-activated phosphorylation, progressively neutralizing Coulombic repulsion to drive proportional folding and assembly. For both reflectin and K18, low-voltage electroreduction (of His⁺ or Lys⁺, respectively) acts as a surrogate for charge-neutralizing phosphorylation to permit the proteins' entropically driven [21] folding and assembly. Our results with reflectin (from squid) and K18 (from human brain) suggest that this regulatory

mechanism, in which cationic, Coulombic repulsion inhibits the drive for protein folding and assembly, and signal-dependent phosphorylation progressively overcomes that inhibition in a quantized manner, may in fact be widely distributed in biological systems. In the case of reflectin A1, the rates and sizes of assembly are closely and monotonically calibrated with voltage, and reversible and repeatedly cyclable, all in close parallel with these behaviors finely tuned by charge neutralization physiologically by phosphorylation, and in vitro by pH titration, charge screening and genetic engineering [7]. These results open new avenues for manipulating other macromolecular systems, designing advanced functional materials and devices, and bridging the biotic–abiotic interface.

2.6. Experimental protocols

2.6.1. Reflectin source, expression and purification

Recombinant reflectin A1 from the squid, *Doryteuthis opalescens*, was produced from a codon-optimized DNA construct, expressed in Rosetta 2 (DE3) *E. coli*. It was then purified by centrifugal collection of the protein expressed as inclusion bodies, followed by chaotropic solubilization, cation exchange and reverse-phase chromatographies, all as previously described [13]. The purified protein was lyophilized and stored at -80°C until solubilized for use [7]. Purity and functional integrity were confirmed by SDS-PAGE and assembly as a function of pH as measured by DLS, also as previously [7].

2.6.2. Reflectin solubilization and sample preparation

Lyophilized reflectin was dissolved in 0.22 μm -filtered sodium acetate buffer (25 mM, pH 4.5), and concentration determined by spectroscopic measurement of A280 [7]. For electrochemical measurements, solubilized reflectin was then diluted into 40 mM NaCl, 1 mM HClO_4 , pH 3 to final protein concentrations of 1, 2, 3 and 25 μM . Final pH values were readjusted to 3.0 by the addition of HClO_4 as needed.

2.6.3. Differential pulse voltammetry (DPV) measurements

Reflectin DPV was conducted in 20 μl droplets of analyte (25 μM reflectin in 40 mM NaCl, 1 mM HClO_4 , pH 3) using an Autolab M204 electrochemical workstation with a standard three-electrode configuration consisting of a 2 mm diameter Pt disc as working electrode, a Pt wire as counter electrode, and a fritted Ag/AgCl in 1 M KCl aqueous solution as reference electrode. The analyte solution was centered on the working electrode faced upward with the reference electrode on top and the counter electrode

inserted between the working and reference electrode. Before each measurement, the Pt disc working electrode was polished successively with 3, 1, 0.25 and 0.05 μm MetaDiTM polycrystalline diamond suspensions (Buehler, Lake Bluff, IL, USA) on a micro-fibre cloth polishing pad, after which the polished working electrode was sonicated in water for 2 min. DPV measurements were performed with a potential step size of 5 mV, pulse height of 10 mV, pulse duration of 50 ms and interval time of 0.5 s [24].

2.6.4. Electrochemical correlative dynamic light scattering (E-DLS)

E-DLS measurements of reflectin (2 μM in 40 mM NaCl, 1 mM HClO_4 , pH 3) were conducted using Autolab M204 electrochemical workstation used in chronoamperometry mode with a three-electrode configuration consisting of a Pt coil as working electrode, a Pt wire as counter electrode and a fritted Ag/AgCl in 1 M KCl aqueous solution as reference electrode assembled in a DLS compatible optical cuvette and sealed with a plug to avoid evaporation and dust. DLS was carried out with a Malvern Zetasizer Nano ZS, always at the same scanning frequency, as previously described and illustrated [2]. All samples were measured with a 632.8 nm HeNe gas laser with a beam diameter of 0.63 mm ($1/e^2$) and detected by an avalanche photodiode (quantum efficiency (QE) greater than 50% at 633 nm) in a back-scattering configuration at 7° from normal. Measurements were performed with 1.2 ml sample volumes at 25°C at the potentials indicated for 25 min and then followed with bias off for 10 min. Cyclability of reflectin was evaluated at -0.7 V for 25 min and followed with bias off for 10 min for four cycles.

2.6.5. Electrochemical correlative circular dichroism (E-CD)

In situ CD measurements of reflectin (2 μ M in 40 mM NaCl, 1 mM HClO₄, pH 3) were performed on a Jasco J-1500 spectropolarimeter with constant N₂ flushing, temperature controlled at 25°C and a Gamry electrochemical workstation used in chronoamperometry mode with a three-electrode configuration consisting of a Pt mesh (52 mesh Pt wire of 100 μ m diameter folded in two, geometric area = 8 $\times$ 16 mm²) as working electrode, a Pt mesh (52 mesh Pt wire of a 100 μ m diameter folded in two, geometric area = 4 $\times$ 8 mm²) as counter electrode, and a fritted Ag/AgCl in 1 M KCl aqueous solution as reference electrode fitted in a 0.2 cm pathlength quartz cuvette, as previously described and illustrated [3]. All potentials are expressed as versus Ag/AgCl. Both ellipticity and absorbance were monitored simultaneously and recorded between 195 and 225 nm with a spectral width of 1 nm. Measurements were performed with 0.8 ml sample volumes at the potentials indicated for 25 min and then followed with bias off for 10 min.

2.6.6. Use of perchloric acid

Perchloric acid was used to maintain reflectin in the monomeric state at the start of each experiment. Acidic pH previously had been shown necessary to maintain the protein's excess of cationic amino acid residues (His, Lys and Arg) in their fully charged state, thus ensuring the high Coulombic repulsion needed initially to prevent condensation, folding and assembly [7, 21]. The choice of perchloric acid was dictated by its transparency in the far UV, in contrast with the high absorption of many other common acids, thus permitting sensitive, interference-free analyses of voltage-induced folding by CD.

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Chapter 3

Electrochemically Driven Optical Properties of Reflectin Protein Film

Adapted from the following publication:

[1] **Y.-C. Lin**, C. Yang, S. Tochikura, D. E. Morse, L. Sepunaru, and M. J. Gordon, “Electrochemically Driven Optical Dynamics of Reflectin Protein” *Adv. Mater.*, 2411005 (2025).

In this chapter, we extend our electrochemical approach from freely diffusing reflectin to thin-film architecture. By combining electrochemistry with ellipsometry and surface plasmon resonance, we directly monitor how applied voltage modulates the optical properties of reflectin films in real time, revealing a reversible, voltage-calibrated change in both thickness and refractive index. These measurements provide insight into the coupling between charge neutralization, film dehydration, and optical modulation, and help explain how reflectin-based structures generate tunable color in squid. The experimental protocols for these measurements are described in Section 3.6.

3.1. Differential pulse voltammetry of reflectin protein film

Differential pulse voltammetry (DPV) was performed on histidine and reflectin films in 40 mM NaCl at pH 3 to identify redox features associated with the electroreduction of histidine inside reflectin (see experimental protocols). This electrolyte condition was selected to ensure complete protonation of every amino acid in the reflectin and adequate ionic conductivity. Figure 3.1 shows the DPV of histidine using a Pt disk and the Pt/Si substrate as working electrodes. DPV with the Pt disk electrode reveals three distinct electroreduction waves with peaks at -0.41 , -0.59 , and -0.80 V that correspond to hydronium reduction (black arrow), deprotonation of imidazolium (red arrow), and deprotonation of NH^{3+} (yellow arrow), respectively [2, 3].

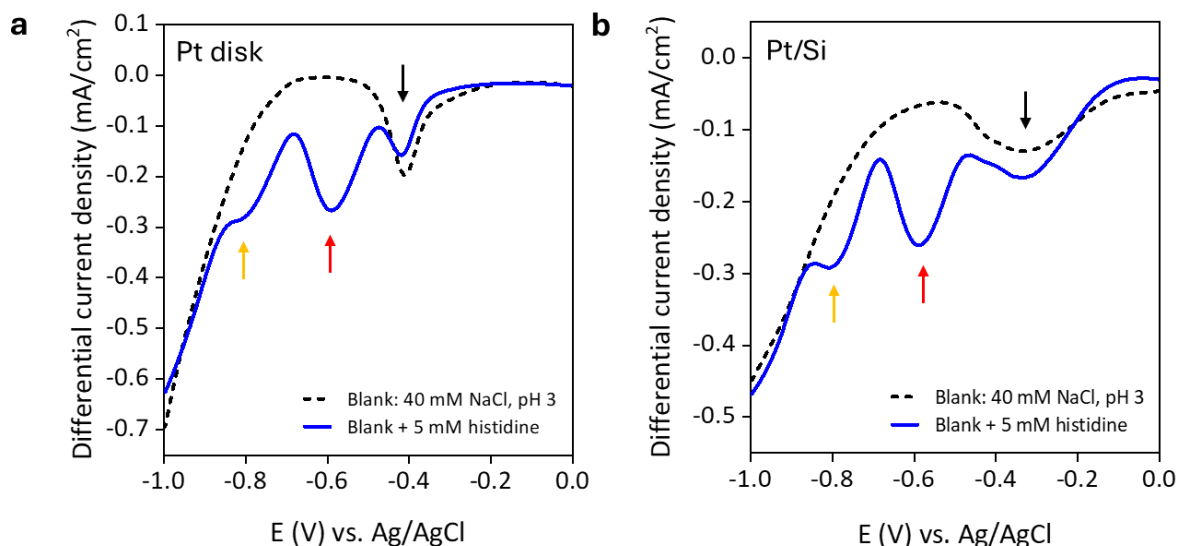


Figure 3.1. Differential pulse voltammetry (DPV) of 5 mM histidine in 40 mM NaCl aqueous solution at pH 3 using (a) commercial Pt disk and (b) Pt/Si as working electrodes. Arrows indicate the redox features of hydronium reduction (black arrow), deprotonation of imidazolium (red arrow), and deprotonation of NH_3^+ (yellow arrow).

Similarly, the DPV scan with a Pt/Si electrode, used as the working electrode for electrochemical correlative ellipsometry (E-Ellipsometry), exhibited the same three reduction waves, albeit with slightly broader peaks than observed with the Pt disk. This phenomenon is attributed to the amorphous structure of Pt resulting from electron beam deposition and shows that the homemade Pt/Si substrate exhibits comparable catalytic efficiency for proton electroreduction, thereby allowing low overpotentials similar to those of a commercial Pt disk.

Figure 3.2 shows the DPV of reflectin films with different thicknesses employing a Pt/Si substrate as a working electrode. The results exhibit a clear electroreduction wave with a peak at approximately -0.4 V and a shoulder at -0.55 V, corresponding to

hydronium reduction (black arrow) and reduction of the imidazolium side-chain of histidine residues in the protein (red arrow), respectively. The onset of water electrolysis occurs at ca. -0.8 V, which is readily apparent in the blank [4]. These findings align with prior results obtained with freely diffusing reflectin in solution using a Pt disk as the working electrode [1]. In addition, the current of the imidazolium reduction peak gradually increases with an increasing number of deposited reflectin layers, indicating that a greater amount of reflectin immobilized on the Pt/Si surface undergoes electroreduction. The estimated thickness of reflectin for 1, 2, and 3 layers of deposition is 45, 60, and 74 nm, respectively, from ellipsometry as described in the experimental protocols. The impact of varying amounts of immobilized reflectin, resulting in different film thicknesses, on changes in optical properties during electrochemical activation are discussed later.

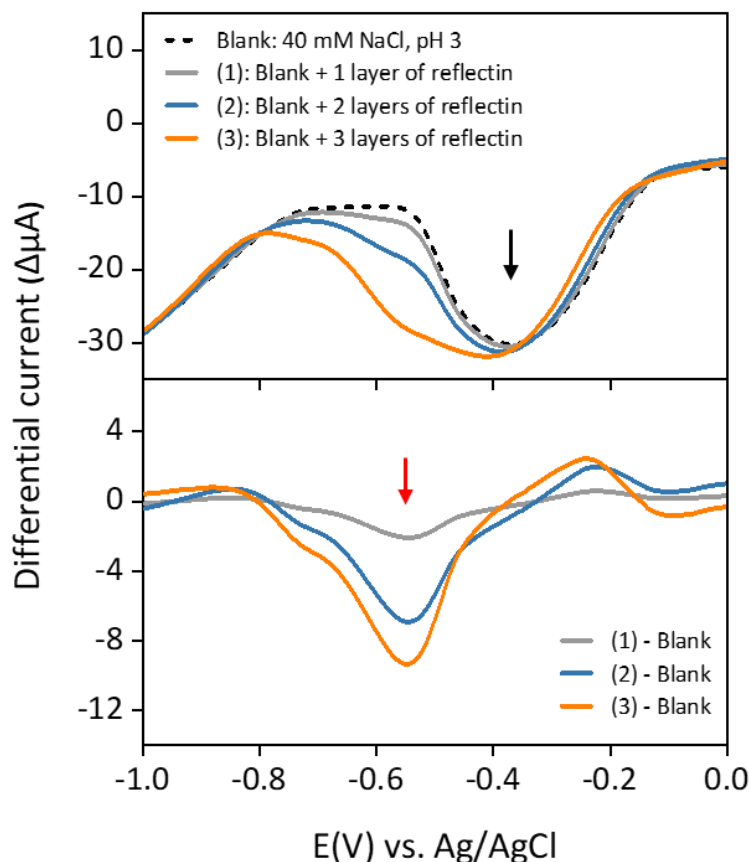
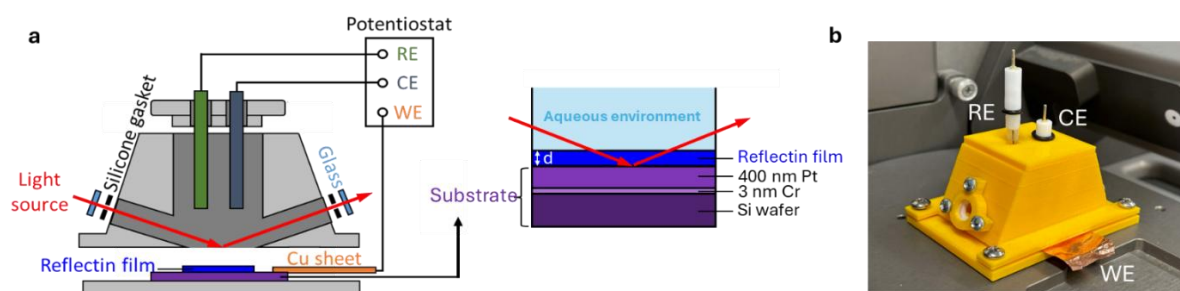


Figure 3.2. Differential pulse voltammograms (DPV) of reflectin films show direct electroreduction of histidine residues. The top panel shows the DPV for different reflectin film thicknesses in 40 mM NaCl aqueous solution at pH 3. The bottom panel shows the deconvoluted voltammograms of reflectin films after subtraction of the DPV without analyte (blank, dashed line) from that with analyte. Arrows indicate the redox features of hydronium reduction (black arrow) and reduction of the imidazolium side-chain of histidine residues in the protein (red arrow). The estimated thickness of reflectin for 1, 2, and 3 layers of deposition is 45, 60, and 74 nm, respectively, measured by ellipsometry.

3.2. Electrochemical correlative ellipsometry of reflectin films

Electrochemical correlative ellipsometry (E-Ellipsometry) was used to investigate the change in optical properties and thickness of reflectin films under different bias conditions using the 3D-printed cell configuration depicted in Figure 3.3. In this setup, the Pt/Si substrate (WE) with reflectin sample on the surface was edge contacted with a Cu ribbon to apply bias. The cell consisted of two windows for the probe beam entrance and exit, and a hole in the bottom to expose the sample. The top had two holes machined to fit the counter (CE) and reference electrodes (RE). In addition, a silicone gasket was placed accordingly to prevent solution leakage. The thickness (d) and refractive index (n) of reflectin films formed by drop-casting on the Pt/Si substrate to mimic the platelet structures in cephalopod iridophores were measured in situ during electrochemical activation to elucidate the color-changing mechanisms in squid skin. The ellipsometry modeling, as described in Figure 3.3, also takes into account the liquid solution (electrolyte) that interacts with light.



Furthermore, to assess the stability of reflectin films in 40 mM NaCl at pH 3 solution, ellipsometry measurements were performed over long times to observe if any changes in film thickness occurred; reflectin films showed excellent stability for at least 15 h, as shown in Figure 3.4.

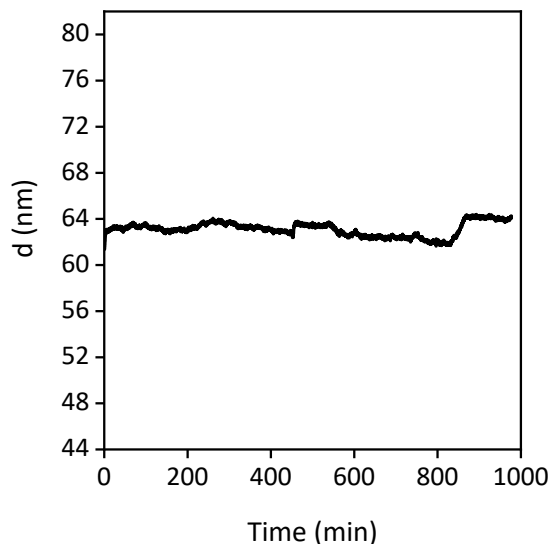


Figure 3.4. Stability of reflectin film in 40 mM NaCl at pH 3 measured by ellipsometry.

Figure 3.5 illustrates the variation in d and n of reflectin films when held at potentials below and above the identified reduction peak of reflectin (histidine) at -0.55 V versus Ag/AgCl for 15 min, followed by open circuit potential (OCP), i.e., equilibrium potential at which no current flows in the cell. The sub subthreshold potential of -0.4 V is only sufficient to electroreduce free hydronium ions, thus demonstrating no change in d or n over time. In contrast, a slight increase in applied voltage to -0.55 V, corresponding to reflectin's identified reduction peak (i.e., electroreduction of imidazolium in histidine),

results in a thickness decrease of 8 nm and an increase in refractive index of 0.022. These changes rapidly return to the initial state when the potential is returned to OCP, due to re-exposure of the film to a low pH environment when the voltage is turned off. At a higher applied voltage of -0.6 V, a more significant decrease in d (12 nm) and a larger increase in n (0.035) are observed. Once more, the observed changes rapidly revert to their initial state when the applied voltage is returned to OCP, indicating rapid and complete reversibility of the reflectin film. Similarly, increasingly pronounced changes in both d and n are observed at -0.7 V (13 nm; 0.041) and -0.8 V (15 nm; 0.045), with rapid reversal when the voltage is returned to OCP. In addition, it is important to acknowledge that a minor pH gradient is expected to arise as a consequence of water electrolysis at -0.8 V. This pH gradient may also contribute to indirect (non-faradaic) charge neutralization of reflectin, consequently leading to larger changes in film thickness and optical properties. As such, potentials more negative than -0.8 V, which could potentially generate larger pH gradients, were not studied. Control experiments of a bare Pt/Si substrate without reflectin measured at OCP, -0.4 , -0.7 , and -0.8 V for comparison (Figure 3.6) show negligible change (<0.004) in refractive index.

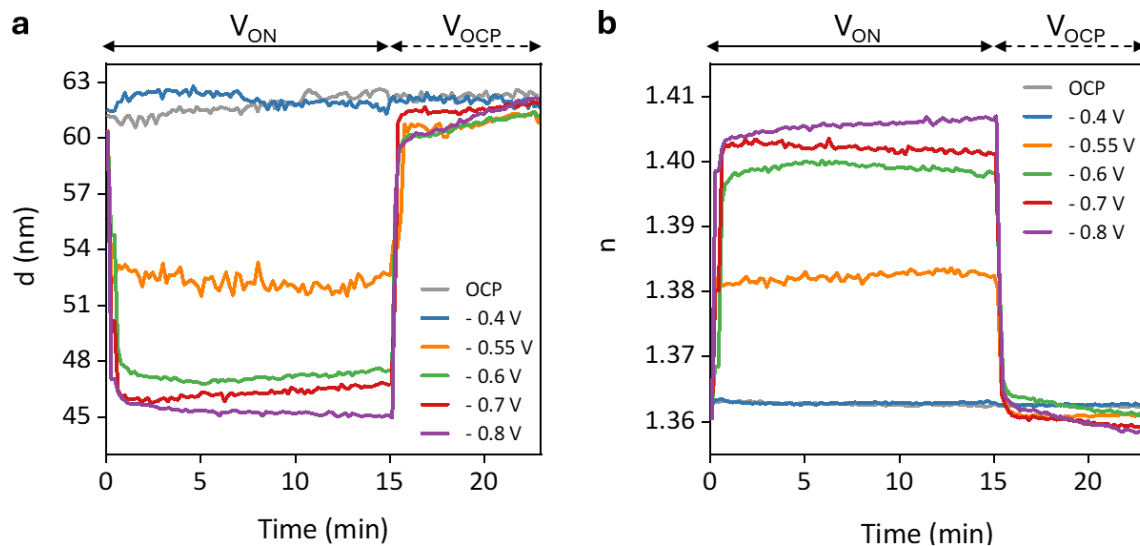


Figure 3.5. (a) Film thickness (d) and (b) refractive index (n) at a wavelength of 632.8 nm of a reflectin film on Pt/Si in 40mM NaCl at pH 3 from E-Ellipsometry, continuously measured during application (V_{ON}) and after the potential was returned to open circuit potential (OCP) (V_{OCP}) (solid and dashed lines, respectively).

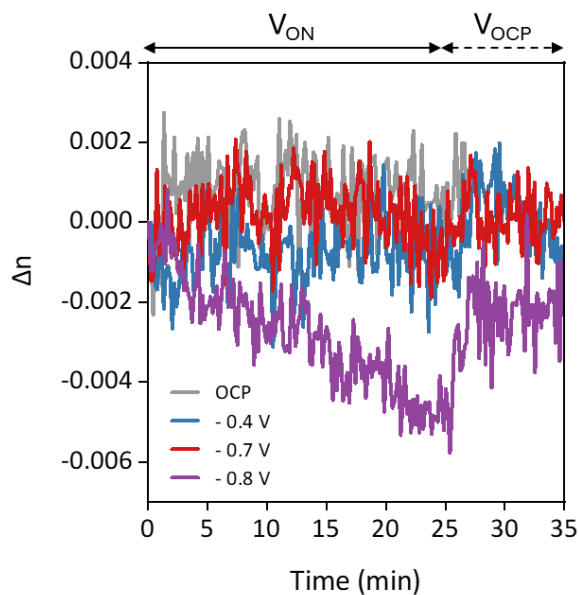


Figure 3.6. Change in refractive index (n) at a wavelength of 632.8 nm of bare Pt/Si substrate without reflectin film in 40 mM NaCl at pH 3 from E-Ellipsometry.

In a state of apparent steady equilibrium (after 15 min), it was observed that the final thickness of the reflectin film decreases, while the refractive index increases reciprocally, as a function of the negative potential applied to the working electrode (Figure 3.7).

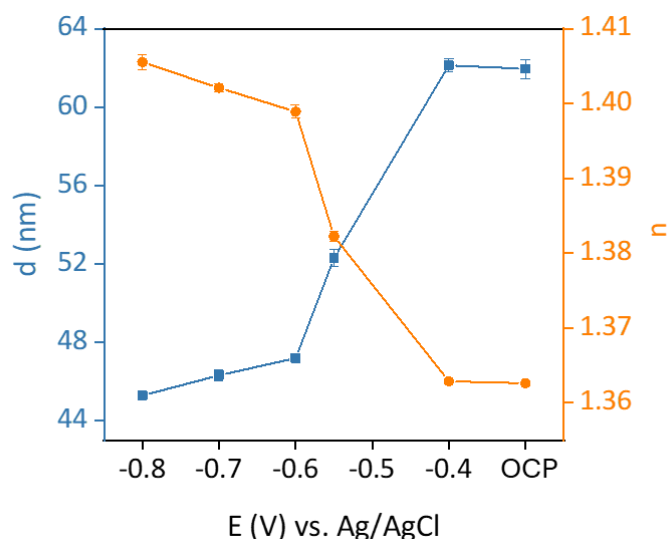


Figure 3.7. d and n at the plateau (cf. Figure 3.5) as a function of applied potential. Error bars indicate mean $\pm$ standard deviation obtained for a minimum of three measurements.

To account for the observed behavior of voltage-induced changes in thickness and refractive index, the structure of the reflectin film is hypothesized to dynamically change, as illustrated in Figure 3.8. The reflectin monomer is first immobilized on the electrode surface to form a homogeneous film, within which a certain volume fraction of water is contained, mimicking one of the platelet structures in cephalopod iridophores. When the applied potential is sufficient to reduce the histidine residues in reflectin, progressive charge neutralization overcomes the Coulombic repulsion of reflectin's cationic linkers,

thereby driving condensation of the film. This condensation would reduce the exposure of ionic groups on the reflectin surface, thus driving an efflux of water. Subsequently, the resulting partial dehydration leads to a further augmentation in the local protein concentration while concurrently decreasing film thickness, thus yielding the observed optical properties. As the applied voltage is returned to OCP, the reflectin film is then re-exposed to a low pH environment, resulting in the reversal of charge neutralization, rehydration, and swelling. Moreover, it is noteworthy that the extent of charge neutralization, controlled by the applied voltage, plays a crucial role in proportionally regulating the water volume fraction within the films, and subsequently influencing the resulting optical features as seen in Figure 3.7 and mimicking the process observed *in vivo*.

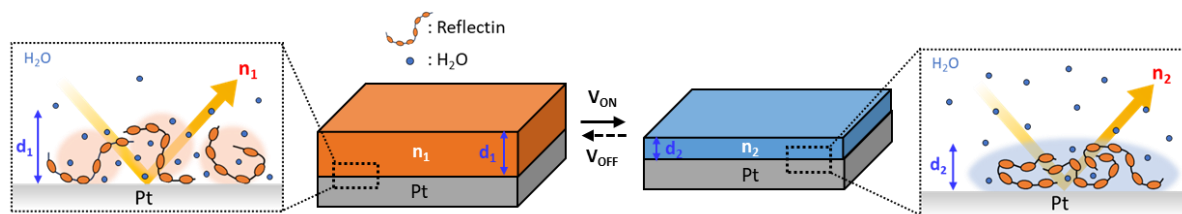


Figure 3.8. Schematic illustration of the hypothesized voltage-induced, reversible swelling and shrinkage of a reflectin film on a Pt working electrode. A swelled film of reflectin monomers (random coils) with thickness d_1 and refractive index n_1 condenses upon application of voltage (V_{ON}), resulting in water expulsion and densification of the protein film with a concomitant decrease in effective film thickness ($d_1 \rightarrow d_2$) and an increase in refractive index ($n_1 \rightarrow n_2$). When the voltage is turned off (V_{OCP}), the film goes back to its initial state. Orange arrows indicate the incident and reflected light used to probe film optical properties via ellipsometry.

Our hypothesis is that at progressively higher voltages, histidine residues with slightly elevated pKa values, influenced by variations in the local sequence environment, undergo electroreduction, leading to a greater extent of charge neutralization [5]. These histidine residues, characterized by their varying pKa values, may be identified and evidenced by the relatively broad reduction peak of reflectin film, as shown in Figure 3.2.

E-Ellipsometry measurements were conducted at -0.7 V versus Ag/AgCl on reflectin films of varying thicknesses to investigate the changes in d and n under electrochemical activation. The hypothesis is that the change in optical properties of the reflectin film depends on the level of reflectin condensation and should be independent of the initial thickness. Different films were fabricated by drop-casting 1 to 5 layers of reflectin, corresponding to initial thicknesses (d_0) of 45, 60, 74, 83, and 91 nm, respectively, as measured by ellipsometry. Figure 3.9 depicts the evolution of d and n of reflectin films when held at -0.7 V for 15 min, followed by OCP. The change in d is 10, 14, 17, 19, and 21 nm for reflectin films with initial thicknesses of 45, 60, 74, 83, and 91 nm, respectively. This result suggests that the change in d is more pronounced in thicker films. However, the change in n remains nearly consistent across all five thicknesses.

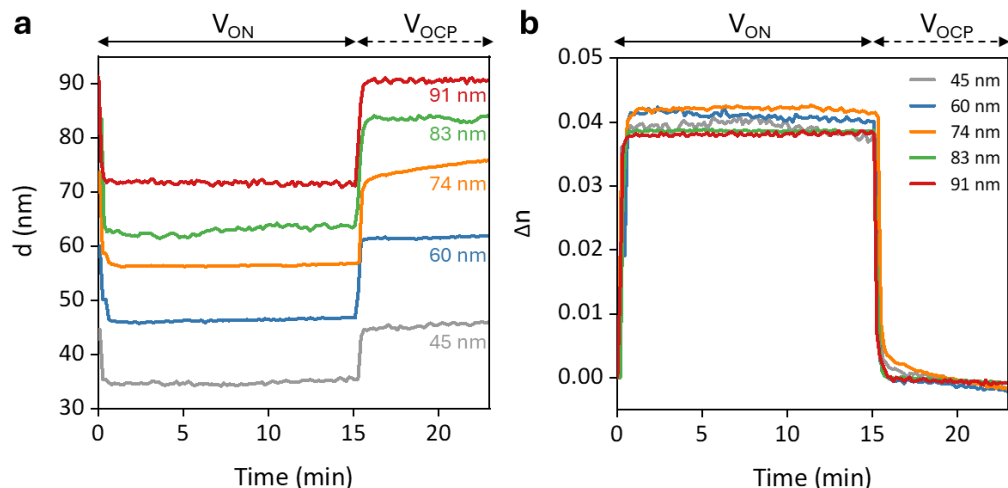


Figure 3.9. (a) Film thickness (d) and (b) change in refractive index (n) at a wavelength of 632.8 nm of reflectin films with different initial thickness (d_0) in 40 mM NaCl at pH 3 from E-Ellipsometry, continuously measured during an applied voltage of -0.7 V versus Ag/AgCl (V_{ON}) and after the potential was returned to OCP (V_{OCP}) (solid and dashed lines, respectively).

To elucidate this observation, Figure 3.10 summarizes the change in d and the final thickness as a percentage of the initial thickness (d/d_0). While a thicker film displays a greater variation in d , the d/d_0 remains relatively consistent across all three conditions ($\approx 76\text{--}78\%$), leading to a comparable change in n . This similar variation arises due to the same extent of charge neutralization at -0.7 V. Most significantly, the observation of similar changes validates the precise relationship between the extent of charge neutralization and the resulting level of reflectin condensation, similar to the calibration previously observed between charge neutralization and the extent of structural changes for reflectin in solution [1, 7]. This calibration guarantees the precise correspondence between the initiating neurochemical signal and the resulting alterations in the skin

coloration of squid *in vivo* [7]. Again, these changes quickly revert back to their initial states when the system is returned to OCP, owing to exposure to a low pH environment, as detailed previously.

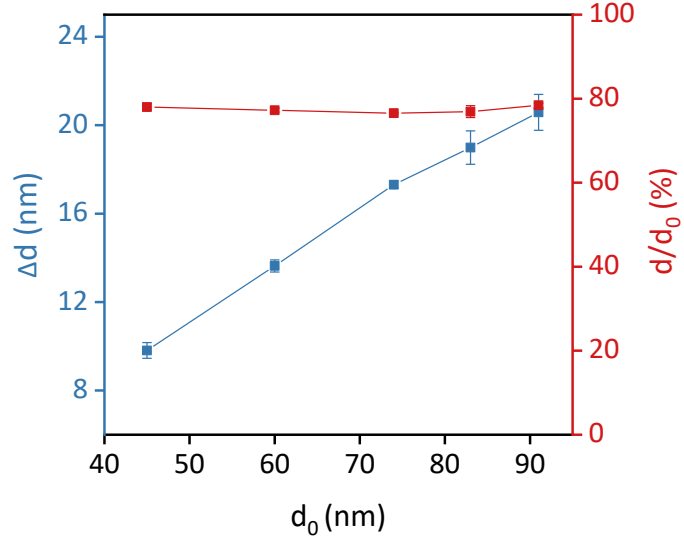


Figure 3.10. Change in d and final thickness as a percentage of the initial thickness (d/d_0) of reflectin films with different initial thicknesses. Error bars indicate mean $\pm$ standard deviation obtained for a minimum of three measurements.

3.3. Reversibility and cyclability of reflectin film

Cephalopods demonstrate an extraordinary ability for dynamic and reversible color alteration, where optical nanostructures formed with reflectin play a crucial role. To emulate the reversibility and cyclability of this biologically tunable and adaptive reflector, E-Ellipsometry measurements were conducted with reflectin films and applied voltage switched on for 3 min, followed by OCP for 3 min, and then repeated for a total of 30 cycles. Figure 3.11 illustrates the rise and fall of d and n across each cycle. As the voltage is turned on, d decreases and n increases; when the voltage returns to OCP, the d and n of the film revert back to nearly their initial values. These findings suggest a reversible and cyclable flux of water across the reflectin film, leading to shrinkage and swelling of the film, along with the corresponding change in the local concentration of reflectin, which directly influences its optical properties. This process is highly similar to the reversible flux of water across the invaginated and lamellar iridocyte membrane, a mechanism regulated by neurotransmitter- and signal transduction cascade-mediated phosphorylation and consequent condensation, folding, and assembly of the reflectin *in vivo* [8]. Notably, d remained relatively consistent over cycles when the voltage was turned on; however, a slight reduction in d over cycles is observed as applied potential is returned to OCP, indicating that the influx of water gradually diminishes each time the voltage is returned to OCP. This observation could be attributed to the loss of reflectin from the film or decay in flexibility of reflectin chains, possibly due to the random arrangement of reflectin on the electrode surface. This arrangement may hinder the influx of water over successive cycles. In addition, our previous research has demonstrated that

reflectin undergoes reversible transitions through multiple cycles of assembly and complete disassembly when the potential is alternated on and off [1]. That result is consistent with our current findings. Applications of these findings may contribute to the development of bio-inspired optical materials based on reflectin protein.

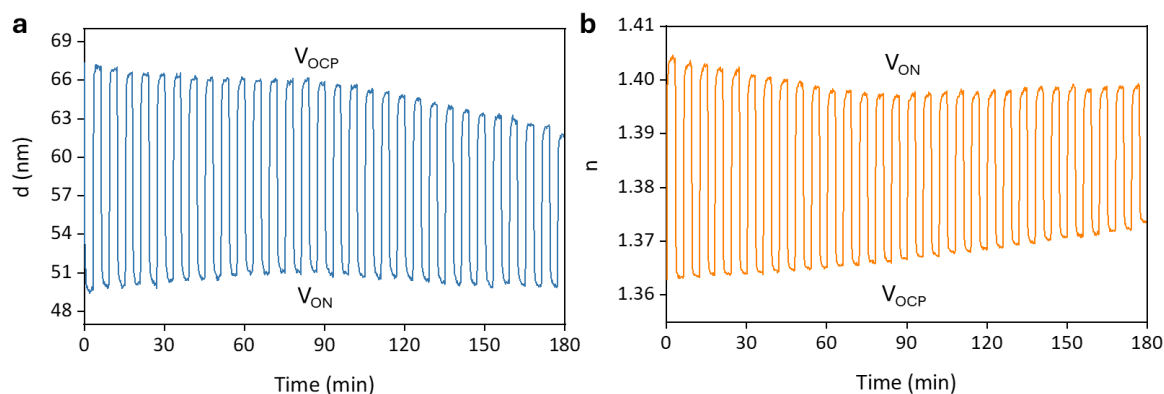


Figure 3.11. Electrochemical reduction drives reversible and cyclable shrinkage and swelling of a reflectin film. (a) Changes in film thickness (d) and (b) refractive index (n) of a reflectin film in 40 mM NaCl at pH 3 measured by E-Ellipsometry as a function of time during cycles of 3 min exposure to -0.7 V versus Ag/AgCl followed by 3 min at OCP.

3.4. Electrochemical correlative surface plasmon resonance of reflectin films

To independently verify the variation in n of the reflectin film during electrochemical activation, electrochemical correlative surface plasmon resonance (E-SPR) measurements also were conducted. The experimental setup for E-SPR is illustrated in Figure 3.12. In this setup, the Au thin film substrate functions as both the SPR sensing surface and the electrochemical working electrode (WE). The DPVs of histidine using a commercial Au disk and the Au substrate as working electrodes are compared and shown in Figure 3.13. Again, three similar distinct electroreduction waves were observed in both cases, indicating the comparable catalytic efficiency of the homemade Au substrate to that of a commercial Au disk for proton electroreduction. Notably, and as expected, the potentials required for hydronium reduction and histidine reduction using Au as WE are more negative compared to those using Pt. This difference is attributed to the better catalytic efficiency of Pt for proton electroreduction, leading to lower overpotentials. Figure 3.14 shows the DPV of reflectin film using the Au substrate as WE. The results consistently demonstrate a more negative potential required for electroreduction of the reflectin film on gold rather than platinum. As a result, the applied voltages required for E-SPR differ from those used in E-Ellipsometry.

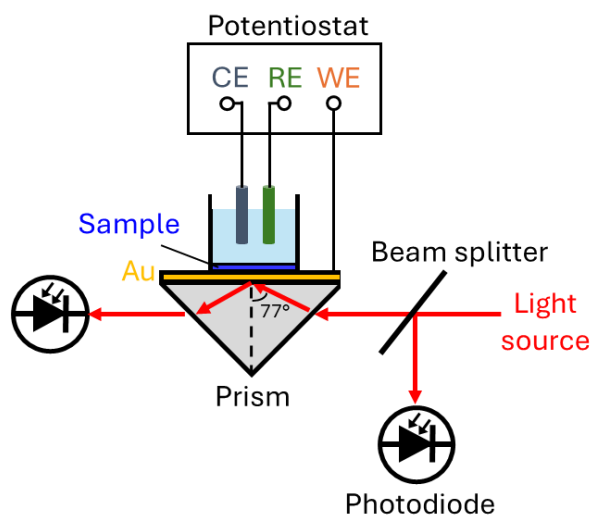


Figure 3.12. Schematic diagram of the designed electrochemical correlative surface plasmon resonance (E-SPR) setup.

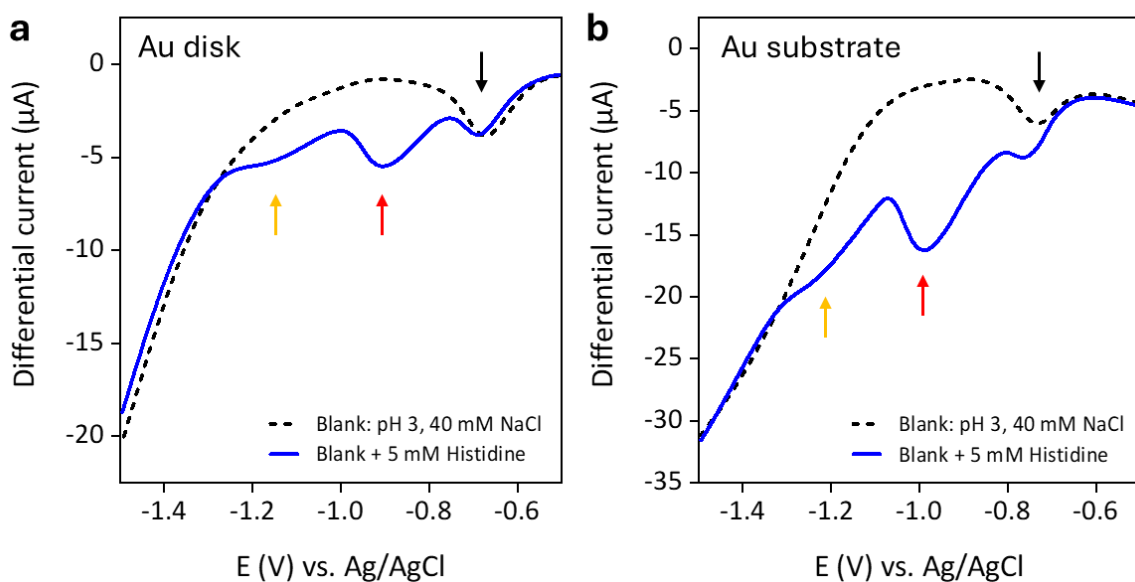


Figure 3.13. DPV of 5 mM histidine in 40 mM NaCl aqueous solution at pH 3 using (a) commercial Au disk and (b) Au substrate as working electrodes. Arrows indicate the redox features of hydronium reduction (black arrow), deprotonation of imidazolium (red arrow), and deprotonation of NH_3^+ (yellow arrow).

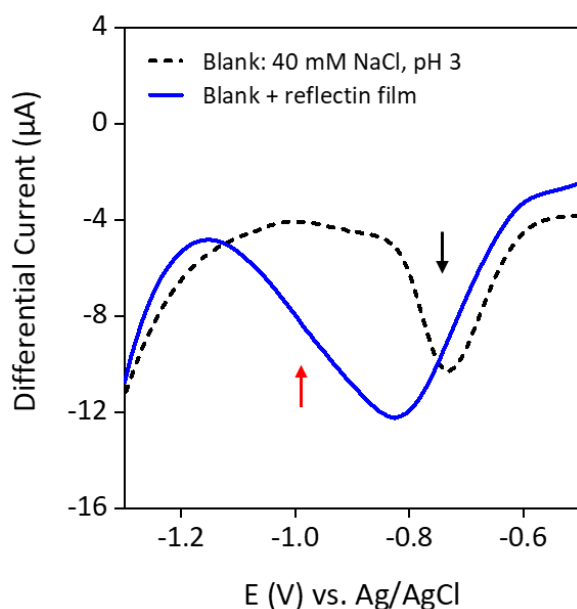


Figure 3.14. DPV of reflectin film in 40 mM NaCl aqueous solution at pH 3 using Au substrate as working electrode. Arrows indicate the redox feature of hydronium reduction (black arrow) and reduction of the imidazolium side-chain of histidine residues in the protein (red arrow).

To better understand the results from E-SPR, we first simulated the relationship between n of the sample on the Au substrate and the reflection signal obtained from this setup using the Fresnel equations, as depicted in Figure 3.15, and explained in more detail in the experimental protocols. Our simulation indicates that as n of the sample on the Au substrate increases from 1.36 to 1.40, corresponding to the application of a negative voltage to trigger reflectin film condensation, there is a concomitant increase in the reflection signal. Moreover, the reflection signal is expected to return to its initial state when the voltage is turned off, according to the results from E-Ellipsometry.

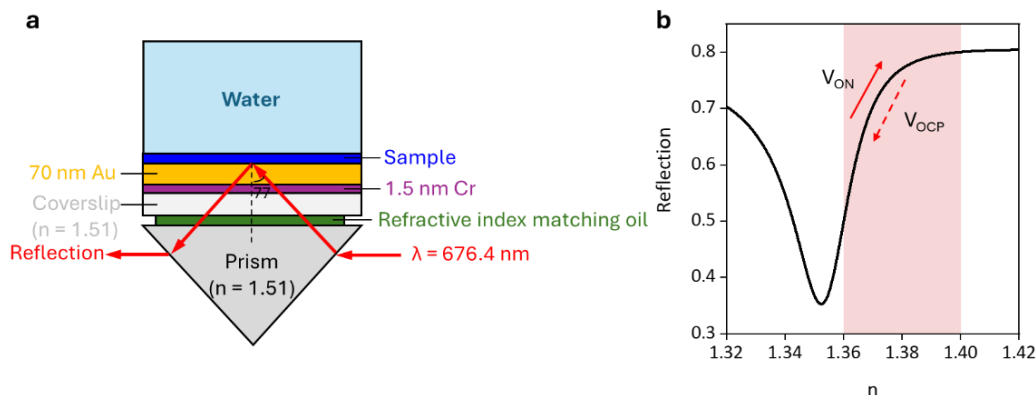


Figure 3.15. (a) Illustration depicting the stack structure used for E-SPR modeling. (b) Simulated refractive index (n) of the sample on the Au substrate and the corresponding reflection signal.

Figure 3.16 shows the E-SPR results at different applied potentials. At the applied voltage of -0.7 V , which corresponds to hydronium reduction (with the Au electrode), no change in SPR intensity is observed. At the more negative applied voltage of -0.9 V , the SPR intensity increases, indicating a corresponding increase in the reflection signal and n , as SPR intensity is positively correlated with the reflection signal. Increasingly pronounced changes in SPR intensity at -1.0 and -1.1 V are also observed. These changes quickly revert back to their initial states upon returning the potential to OCP, suggesting the reversibility of the reflectin film. Control experiments using a bare Au substrate without the reflectin film were conducted at -1.0 V for comparison, as shown in Figure 3.17. The results show a slight increase in SPR intensity, which is only one-tenth of that observed with the reflectin film. This slight increase can be attributed to the formation of the electric double layer as the voltage is applied [9]. The E-SPR results are thus in good agreement with those from E-Ellipsometry. Both techniques confirm that the

optical properties of the reflectin film can be interrogated, controlled, and calibrated by electrochemical reduction.

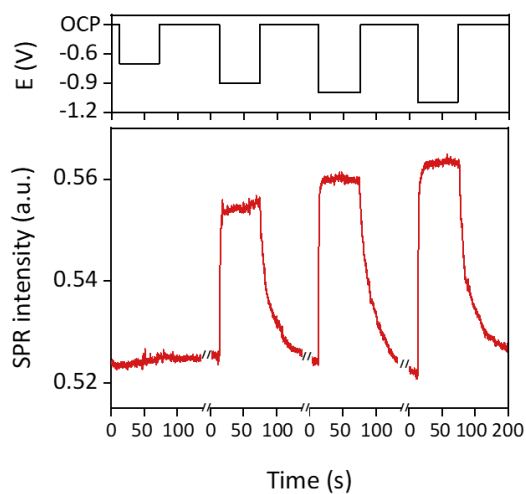


Figure 3.16. Top panel: applied voltage waveform (E (V) versus Ag/AgCl); bottom panel: SPR intensity of reflectin film in 40 mM NaCl at pH 3 from E-SPR, continuously measured during application of potentials and after the potentials were returned to OCP.

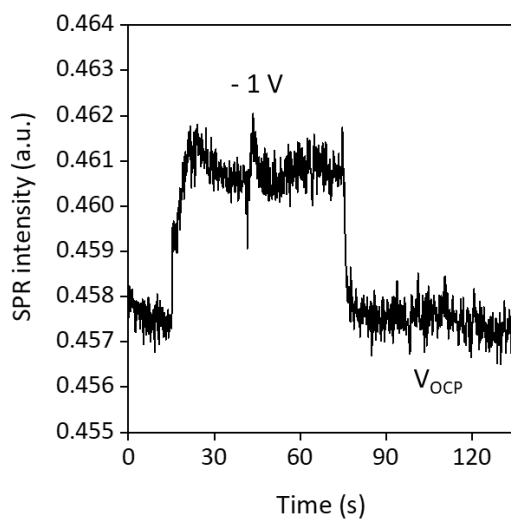


Figure 3.17. Change in SPR intensity of bare Au substrate without reflectin film in 40 mM NaCl at pH 3 from E-SPR, continuously measured during application of potentials and after the potentials were returned to OCP.

3.5. Summary

In this work, we replicate the platelet structures observed in cephalopod iridophores by fabricating reflectin thin films, obtained through drop-casting aqueous reflectin monomers onto Pt or Au working electrodes. The optical properties of these reflectin films were modulated via low-voltage electrochemical reduction of histidine residues, serving as an *in vitro* surrogate for charge neutralization by phosphorylation *in vivo*. Electrochemical correlative ellipsometry and surface plasmon resonance spectroscopy were used to dynamically follow and confirm changes in both thickness and refractive index of reflectin films under bias, as well as the reversal of charge neutralization, restoring the reflectin film to its initial state upon the removal of bias. Our results suggest that the extent of charge neutralization regulated by applied voltage enables tunable and reversible control of the water volume fraction within the reflectin film, thereby influencing the effective optical properties; specifically, the measurements showed that electrochemical reduction enables precise modulation of the refractive index from 1.36 to 1.40 at a wavelength of 632.8 nm, similar to measurements *in vivo* [6]. Moreover, electrochemical correlative surface plasmon resonance experiments unequivocally confirm the behavior of reflectin condensation, calibrated in accordance with the extent of charge neutralization, leading to alterations in optical features closely related to the calibration between the extent of charge neutralization and reflectin's structural changes in solution [7]. These findings bear resemblance to the discovery that the neuronally regulated flux of water modifies the geometry and optical characteristics of reflectin-based lamellae within the squid's biological Bragg reflectors *in vivo* [8].

Taken together, this work introduces novel avenues for investigating the biophysical mechanisms underlying color change via reflectin condensation, as well as for developing advanced bio-inspired functional materials and devices that bridge the biotic-abiotic gap.

3.6. Experimental protocols

3.6.1. Reflectin expression, purification, and solubilization

Recombinant *Doryteuthis opalescens* reflectin A1 was synthesized using a codon-optimized DNA construct, expressed, and chromatographically purified as previously described [10]. Briefly, Rosetta 2 (DE3) *E. coli* cells were grown in liquid cultures from freshly plated transformants containing 50 mg mL⁻¹ kanamycin and 37 mg mL⁻¹ chloramphenicol overnight for selection of the recombinant DNA plasmid. Expression was then induced at A₆₀₀ ≈ 0.6 and allowed to proceed for ≈ 6 h at 37°C. Afterward, cells were centrifuged and stored in a -80°C freezer until purification. Purification of reflectin inclusion bodies was performed on BugBuster medium (Novagen, Inc., Madison, WI). Purified inclusions bodies were solubilized in 5% acetic acid, 8 M urea, 6 M guanidinium HCl. To remove guanidinium, inclusion bodies were further dialyzed in 5% acetic acid, 8 M urea. Purification of reflectin was performed by ion exchange over a HiTrap XL (GE Healthcare) cation-exchange column eluted with a gradient of 5% acetic acid, 6 M guanidinium. Fractions consisting of reflectin were collected and subsequently diluted in 5% acetic acid, 8 M urea. The eluted reflectin was concentrated and loaded onto a reverse-phase HPLC column equilibrated with 0.1% trifluoroacetic acid (TFA) in an aqueous solution and then eluted with a gradient of acetonitrile in 0.1% TFA. The resulting purified protein was lyophilized and frozen in a -80°C freezer until solubilization for use. Purity and functional integrity were assessed by SDS-PAGE using 10% trisacetate gels (Life Sciences, Carlsbad, CA, USA) and assembly as a function of pH as measured by dynamic light scattering (Zetasizer Nano ZS, Malvern). Lyophilized reflectin was

dissolved in 0.22 μm -filtered sodium acetate buffer (25 mM, pH 4.5), and its concentration was determined using the Beer–Lambert law by measuring the absorbance of solubilized protein at 280 nm on a NanoDrop UV/Vis spectrophotometer. The molar extinction coefficient used for the calculation was $120685 \text{ M}^{-1} \text{ cm}^{-1}$.

3.6.2. Sample preparation

Pt/Si substrates were made by electron beam deposition of Pt onto an undoped Si (100) wafer (University Wafer, MA, USA). Preceding the deposition process, the Si wafer was cleaned by sonicating sequentially in acetone and isopropyl alcohol (IPA) for 3 min each, and then rinsing with deionized (DI) water (18.2 M Ω -cm resistivity). For electron beam deposition, an Inficon IC/5 controller equipped with a quartz crystal deposition monitor was used for *in situ* film thickness measurement. The deposition process was conducted at a vacuum pressure of $\approx 3 \times 10^{-6}$ Torr, with substrate rotation employed to ensure uniform film thickness across the substrate. A 3 nm layer of Cr was deposited first at a rate of $1 \text{ \AA s}^{-1}$ as an adhesion layer, followed by 400 nm of Pt at a rate of $0.5 \text{ \AA s}^{-1}$ for the initial 10 nm, $1 \text{ \AA s}^{-1}$ for the next 20 nm, and $1.5 \text{ \AA s}^{-1}$ beyond 20 nm. Similarly, Au substrates were prepared using an analogous methodology, with 1.5 nm of Cr deposition onto glass coverslips, followed by 70 nm of Au deposition. For reflectin film formation, solubilized reflectin was diluted into 1 mM HClO₄, pH 3 to a final protein concentration of 2 and 10 μM . Final pH values were readjusted to 3.0 by the addition of HClO₄ as necessary. The diluted reflectin solution was drop-casted (as 25 μL aliquots) onto the substrate and dried using a vacuum desiccator connected to the building vacuum system (≈ 520 torr). The drying process was carried out for 30 min. This

process was repeated to obtain different reflectin film thicknesses. The dried samples were immersed in a beaker of DI water for 10 s before measurement to remove any excess reflectin.

3.6.3. Differential pulse voltammetry (DPV)

All DPV measurements were conducted in 40 mM NaCl, 1 mM HClO₄, pH 3 using an Autolab M204 electrochemical workstation with a standard three-electrode configuration (working electrode (WE): 2 mm diameter Pt disk, Pt/Si substrate, 2 mm diameter Au disk, or Au substrate; counter electrode (CE): Pt wire; reference electrode (RE): fritted Ag/AgCl in 1 M KCl aqueous solution). Before each measurement, the Pt disk was polished sequentially using 3, 1, 0.25, and 0.05 μ M MetaDiTM polycrystalline diamond suspensions. Similarly, the Au disk was polished in 1, 0.3, and 0.05 μ m alumina slurry for 2 min at each size. The polished working electrode was then sonicated in water for 2 min, rinsed with DI water, and used for the experiment. DPV measurements were performed with a potential step size of 5 mV, pulse height of 10 mV, pulse duration of 50 ms, and interval time of 0.5 s.

3.6.4. Electrochemical correlative ellipsometry (E-Ellipsometry)

E-Ellipsometry measurements were performed on an alpha-SE (spectroscopic) ellipsometer (J. A. Woollam, Lincoln, NE, USA) at a 70° angle of incidence from vertical at a wavelength of 632.8 nm using a 3D printed, custom electrochemical cell made of polylactic acid (PLA) fabricated with an Original Prusa i3 MK3S+ 3D printer (Prusa Research, Prague, Czech Republic) with a nozzle diameter of 0.4 mm and a layer

thickness of 0.2 mm; see Figure 3.3 for design details. Optical constants and thicknesses of reflectin films were described using a Cauchy dispersive model, with fits to the ellipsometry parameters Ψ and Δ carried out over the entire visible measurement range (380–900 nm). A Princeton Applied Research 263A potentiostat was used in chronoamperometry mode with a three-electrode configuration consisting of a Pt/Si substrate as WE, a Pt wire as CE, and a fritted Ag/AgCl in 1 M KCl aqueous solution as RE. Measurements were performed in 40 mM NaCl, 1 mM HClO₄, pH3 at the potentials indicated for 15 min and then followed with open circuit potential (OCP) for 10 min. All potentials are referenced to Ag/AgCl. Cyclability of reflectin films was evaluated at -0.7 V for 3 min and followed with OCP for 3 min for several cycles.

3.6.5. Electrochemical correlative surface plasmon resonance spectroscopy (E-SPR)

The design of the E-SPR setup is based on the Kretschmann SPR configuration and consists of a Kr-ion laser ($\lambda = 676.4$ nm) at 77° incidence, prism, Au thin film substrate, and photodiodes (Thor Labs PDA-36A) as detectors. The Au substrate, serving both as the SPR sensing surface and WE simultaneously, was placed on top of the prism using refractive index matching immersion oil. For E-SPR, a 3D printed, custom electrochemical cell made of PLA was placed on top of the Au substrate. Electrochemical measurements were conducted using a Princeton Applied Research 263A potentiostat in chronoamperometry mode, employing a three-electrode configuration comprising the Au substrate as WE, a Pt wire as CE, and a fritted Ag/AgCl electrode in a 1 M KCl aqueous solution as RE. Measurements were carried out in 40 mM NaCl, 1 mM HClO₄, pH 3 at

the potentials indicated for 60 s and then followed with OCP. All potentials are referenced to Ag/AgCl. Simulation of the reflected/transmitted light signals for the aforementioned SPR sensing thin film stack and geometry was carried out with MatLab using the generalized N-layer Fresnel solution approach of Abelès[11] and Hansen [12]. The corresponding code is provided below.

SPR simulation of reflection as a function of refractive index

```
%% Optical constants and layer thicknesses
n_prism = 1.51; % Refractive index of prism
n_Cr = complex(3.07125, 3.36); % Complex refractive index of Cr
n_Au = complex(0.1726, 3.4218); % Complex refractive index of Au
t_Cr = 1.5e-9; % Cr thickness, m
t_Au = 66e-9; % Au thickness, m
lambda = 676.4e-9; % Wavelength, m
%% Refractive index sweep
n_medium = 1.32:0.001:1.42; % Refractive index of dielectric medium
reflection = zeros(size(n_medium));
for i = 1:length(n_medium)
    [theta, R, ~] = SPRcurve(n_prism, n_Cr, n_Au, n_medium(i), ...
        t_Cr, t_Au, lambda, 30, 85, 500);
    reflection(i) = R(428); % Reflection at ~77 degrees
end
%% Plot refractive index versus reflection
figure;
plot(n_medium, reflection, 'LineWidth', 1.5);
xlabel('Refractive index');
ylabel('Reflection');
title('Reflection as a Function of Refractive Index');
%% SPR transfer-matrix function
function [theta, R, T] = SPRcurve(n1, n2, n3, n4, t2, t3, lambda, amin, amax, pts)
% n1: prism
% n2: Cr adhesion layer
% n3: Au layer
% n4: semi-infinite dielectric medium
epsilon(1) = complex(real(n1)^2 - imag(n1)^2, 2*real(n1)*imag(n1));
epsilon(2) = complex(real(n2)^2 - imag(n2)^2, 2*real(n2)*imag(n2));
epsilon(3) = complex(real(n3)^2 - imag(n3)^2, 2*real(n3)*imag(n3));
epsilon(4) = complex(real(n4)^2 - imag(n4)^2, 2*real(n4)*imag(n4));
n_real = real([n1, n2, n3, n4]);
d(2) = t2;
d(3) = t3;
f = complex(0, 1);
```

```

nlayer = 4;
for i = 1:pts
    theta(i) = (amin + (i - 1)/pts*(amax - amin))*pi/180;
    q1 = sqrt(epsilon(1) - n_real(1)^2*sin(theta(i))^2)/epsilon(1);
    qn = sqrt(epsilon(nlayer) - n_real(1)^2*sin(theta(i))^2)/epsilon(nlayer);
    for j = 2:nlayer-1
        beta = d(j)*2*pi/lambda*sqrt(epsilon(j) - n_real(1)^2*sin(theta(i))^2);
        q = sqrt(epsilon(j) - n_real(1)^2*sin(theta(i))^2)/epsilon(j);
        em(j,1,1) = cos(beta);
        em(j,1,2) = -f*sin(beta)/q;
        em(j,2,1) = -f*sin(beta)*q;
        em(j,2,2) = cos(beta);
    end
    emtot = eye(2);
    for k = 2:nlayer-1
        emtot1 = [em(k,1,1), em(k,1,2); ...
                  em(k,2,1), em(k,2,2)];
        emtot = emtot*emtot1;
    end
    rp = ((emtot(1,1) + emtot(1,2)*qn)*q1 - ...
          (emtot(2,1) + emtot(2,2)*qn)) / ...
          ((emtot(1,1) + emtot(1,2)*qn)*q1 + ...
          (emtot(2,1) + emtot(2,2)*qn));
    tp = 2*q1 / ((emtot(1,1) + emtot(1,2)*qn)*q1 + ...
                 (emtot(2,1) + emtot(2,2)*qn));
    R(i) = rp*conj(rp);
    T(i) = tp*conj(tp);
end
end

```

3.7. References

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Chapter 4

The Beating Heart of Reflectin A1: A Charge-Regulated, Cyclable Machine

Manuscript in preparation by:

Y.-C. Lin, A. Bowser, L. Sepunaru, M. J. Gordon, D. E. Morse, “The Beating Heart of Reflectin A1: A Charge-Regulated, Cyclable Machine”

Previous chapters have shown that full-length reflectin A1 exhibits reversible and electrochemically tunable assembly behavior controlled by charge neutralization. However, the extent to which these properties depend on the native multiblock architecture of reflectin remains unclear. In particular, it is important to determine whether a minimal conserved reflectin motif can recapitulate the dynamic assembly behavior of the full-length protein. In this chapter, engineered peptides are investigated to evaluate how sequence composition, linker charge, and local structural constraints influence pH-dependent and electrochemically driven assembly. The behavior of these simplified constructs is compared directly with full-length reflectin A1 using dynamic light scattering (DLS), circular dichroism (CD), and electrochemical correlative dynamic light scattering (E-DLS). Together, these studies provide insight into the minimal sequence features required for reversible reflectin assembly and help establish design principles for tunable reflectin-inspired biomaterials.

4.1. Sequence of full-length reflectin A1 and engineered RM1 peptides

The tunable reflectin A1 from the loliginid squid *D. opalescens* is essentially a block-copolymeric protein, consisting of six highly conserved domains alternating with cationic linker regions. As shown in Figure 4.1, the reflectin A1 sequence contains an N-terminal region followed by five highly similar reflectin motifs (RMN, RM1–RM5), illustrating its characteristic repetitive architecture. Although each motif differs slightly in sequence composition, RM1 was selected as a minimal reflectin-derived module because it represents one of the conserved repeat motifs within reflectin A1. Using RM1 allows

comparison between the full-length protein and a simplified sequence that retains the essential motif architecture, thereby helping to determine whether the behavior of reflectin A1 can be recapitulated by a single repeat unit.

Three peptides are investigated here (Figure 4.1). These peptides differ in two ways: the RM1 core sequence and the flanking linkers. RM1-His contains the RM1 core sequence with an octa-histidine linker, whereas RM1*-His and RM1*-Gly are based on the shortened RM1* core sequence, which lacks the proline residues on both sides of the RM1 motif, and differ only in their flanking regions, which consist of either an octa-histidine or an octa-glycine linker, respectively. In other words, the “His” and “Gly” versions were designed to examine the role of linker composition and positive charge, whereas the RM1 and RM1* variants were used to assess the effect of a slightly altered core motif sequence. The proline residues were retained in RM1 because proline imposes conformational constraints that may influence the structural and assembly properties of the motif and commonly flank beta structures in numerous proteins [1]. RM1* lacks these prolines, allowing us to evaluate how removing this sequence context affects peptide behavior.



Figure 4.1. Sequences of *D. opalescens* reflectin A1 and three RM1 peptides. Conserved N-terminal domains are circled and labeled as RMN; other repeat domains are circled and labeled as RM1-RM5. RM1 is highlighted in pale orange, whereas RM1*, which lacks the proline residues on both sides of the RM1 motif, is highlighted in pale yellow.

4.2. pH-dependent charge distribution

The tunability of *D. opalescens* reflectins is controlled by a neurotransmitter-initiated signaling cascade that culminates in phosphorylation. This process progressively neutralizes the proteins' positive charge, thereby driving reversible, cyclic condensation and hierarchical assembly. Because reflectin behavior is strongly influenced by electrostatic interactions, charge modulation is a key determinant of reflectin function.

To better understand how sequence design influences peptide charge distribution, Figure 4.2 summarizes the expected side-chain charge patterns of reflectin A1 and two of the above-described peptides at pH 4, pH 6, and pH 7. RM1*-His is not shown here because its charge distribution is identical to that of RM1-His, as proline residues do not contribute charge. The pKa values used to calculate amino acid residue' side-chain charges were 3.9, 4.2, 6.2, 8.3, 10.1, 10.7, and 12.1 for aspartic acid, glutamic acid, histidine, cysteine, tyrosine, lysine, and arginine, respectively. This calculation assumes that each residue of the same type has the same pKa value. At pH 4, most titratable residues in the linker regions of reflectin A1 are protonated, so these regions carry an overall positive charge. In contrast, the conserved domains contain both positively and negatively charged residues, resulting in a slightly net positive charge. RM1-His shows a charge distribution very similar to that of reflectin A1 in both the linker regions and the conserved domain, as the octa-histidine linkers contribute a total of 16 positive charges at low pH. In contrast, the octa-glycine linker is neutral, so the overall charge is dominated by the conserved domain. This difference influences both intermolecular and intramolecular interactions, leading to distinct assembly behaviors. At pH 6,

approximately 39%, 99%, and 98% of histidine, aspartic acid, and glutamic acid residues, respectively, are deprotonated, resulting in a decrease in the net charge of both the linker regions and conserved domains of reflectin A1. Similar trends can be observed in RM1-His. In RM1*-Gly, however, the decrease in charge is limited to the conserved domain, as glycine is neutral. As the pH increases to 7, approximately 86% of histidine residues are deprotonated, leading to a decrease in the net charge of the linker regions in both reflectin A1 and RM1-His. The charge distribution of RM1*-Gly remains similar to that at pH 6. The pH dependence of these peptides is particularly important because pH acts as a direct regulator of the residues' charge states. As protonation and deprotonation shift with pH, the balance of attractive and repulsive interactions along the chain changes as well. This makes charge distribution a useful framework for interpreting the different behaviors of these peptides in the following section.

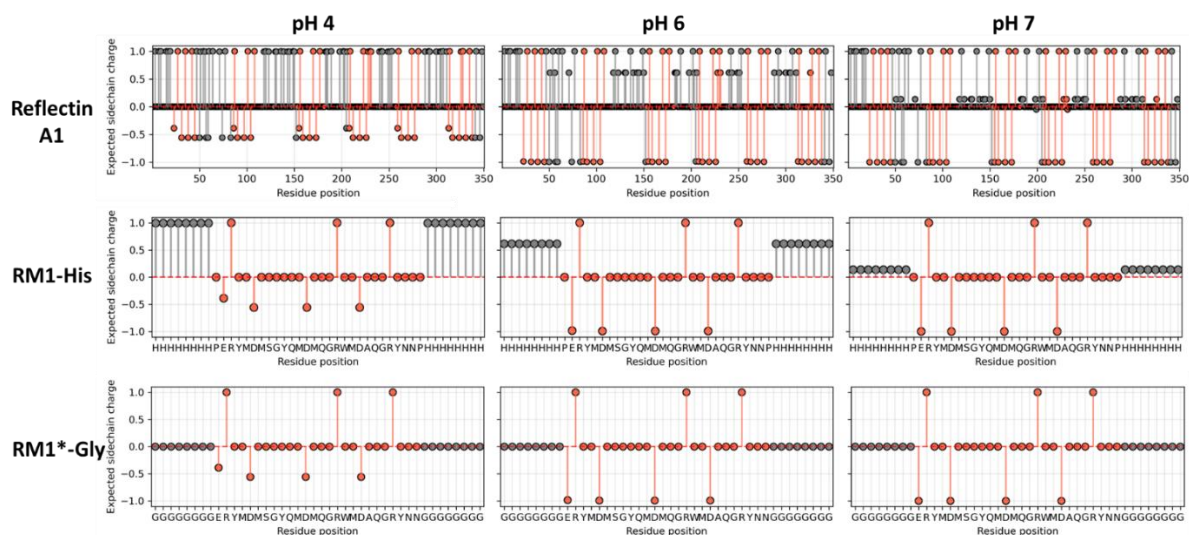


Figure 4.2. Expected side-chain charge patterns of reflectin A1, RM1-His, and RM1*-Gly at pH 4, 6, and 7. Side-chain charges were calculated using pKa values of 3.9, 4.2, 6.2, 8.3, 10.1, 10.7, and 12.1 for aspartic acid, glutamic acid, histidine, cysteine, tyrosine, lysine, and arginine, respectively, assuming uniform pKa values for each residue type. The corresponding code is provided in experimental protocols.

4.3. DLS and CD analysis of pH-dependent assembly of reflectin A1 and peptides

Dynamic light scattering (DLS) is used to characterize the size distribution of particles formed by reflectin A1 and peptides as a function of charge neutralization (Figure 4.3a). The DLS data show that reflectin A1 exhibits distinct size populations at each pH, with the particle size of 8 ± 1 nm (approximate diameter of the monomers) at pH 4, 30 ± 3 nm at pH 6, and 121 ± 14 nm at pH 7. This confirms that the full-length protein can adopt different assembly sizes depending on the extent of charge neutralization, as noted above. For RM1-His, the monomer size is 4 ± 1 nm at pH 4, slightly smaller than that of reflectin A1. Only a slight increase in size is observed as pH increases to 6, likely because the overall net charge of RM1-His remains highly positive and the electrostatic repulsion is too strong to allow significant assembly (Figure 4.4a). As pH increases to 7, the overall net charge of RM1-His decreases, leading to assembly with a size of 231 ± 17 nm. RM1*-His follows a similar general pattern to RM1-His. RM1*-Gly displays a different profile, with large assemblies formed at pH 4 and dissociation to monomers as the pH increases to 6 and 7. This behavior is primarily driven by changes in the net charge of the conserved domain (Figure 4.2).

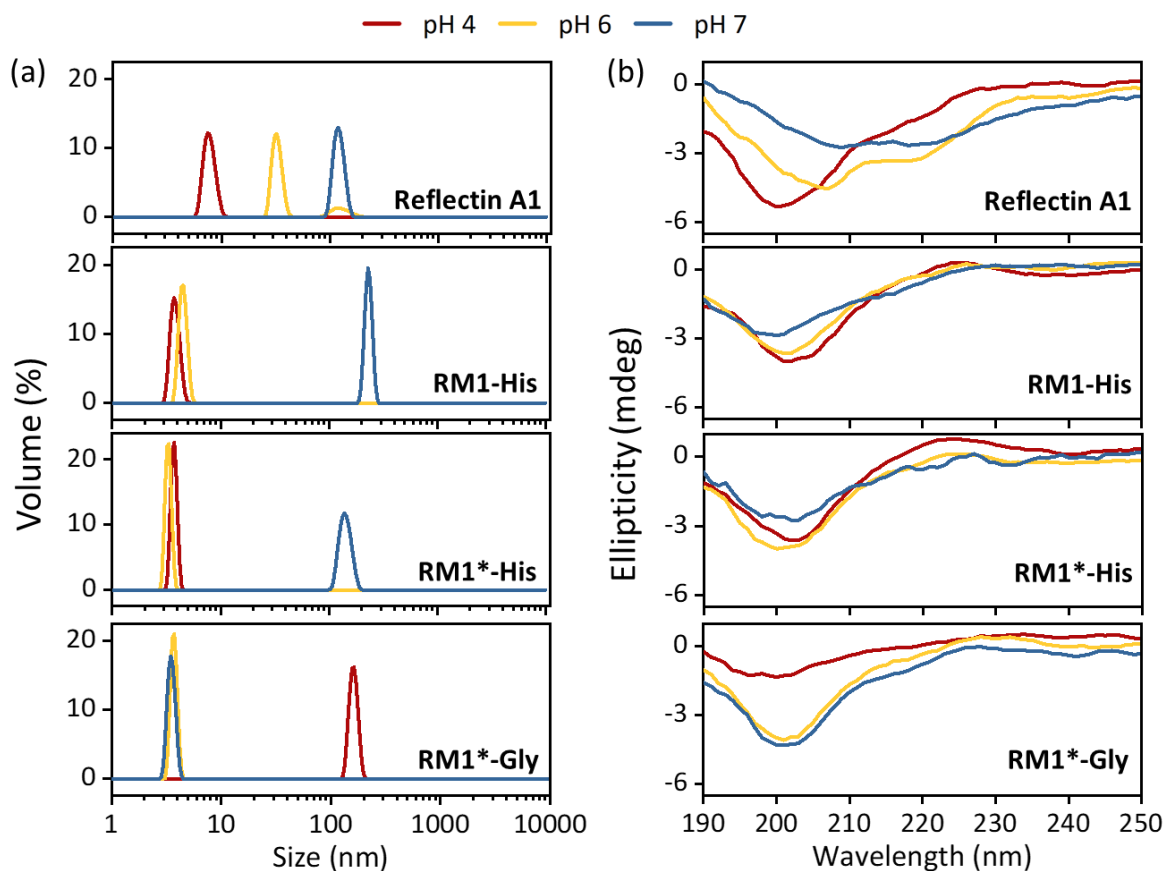


Figure 4.3. (a) DLS and (b) CD spectra of 25 μM reflectin A1, 150 μM RM1-His, 150 μM RM1*-His, and 150 μM RM1*-Gly in pH 4 (15 mM sodium acetate), 6 (15 mM MES), and 7 (15 mM MOPS).

Figure 4.4 summarizes the calculated overall net charge and particle sizes of reflectin A1 and peptides across different pH conditions. For reflectin A1, the net charge is 1.43, 0.78, and 0.34 per 10 amino acids (AAs) at pH 4, 6, and 7, respectively. This gradual decrease in net charge leads to a corresponding increase in assembly size (Figure 4.4b). For RM1-His, the net charge is 3.84, 2.54, and 0.64 per 10 AAs at pH 4, 6, and 7, respectively. The relatively high net charge at pH 6, with its still significant electrostatic repulsion between monomers inhibiting large assembly. Interestingly, the decrease in net

charge of RM1-His as pH increases from 6 to 7 is more pronounced than that of reflectin A1, apparently making its assembly size more sensitive to small pH changes in this range. For RM1*-Gly, the net charge shifts from 0.21 to -0.22 and -0.23 as the pH increases from 4 to 6 and 7, respectively, conversely permitting assembly only at the lowest pH, and inhibiting assembly at pH 6 and 7. With the flanking octa-glycines contributing no significant net charge (their amino and carboxyl termini essentially cancelling one another, the slight negativity of the conserved domain accounts for this peptide's observed “reverse” assembly behavior.

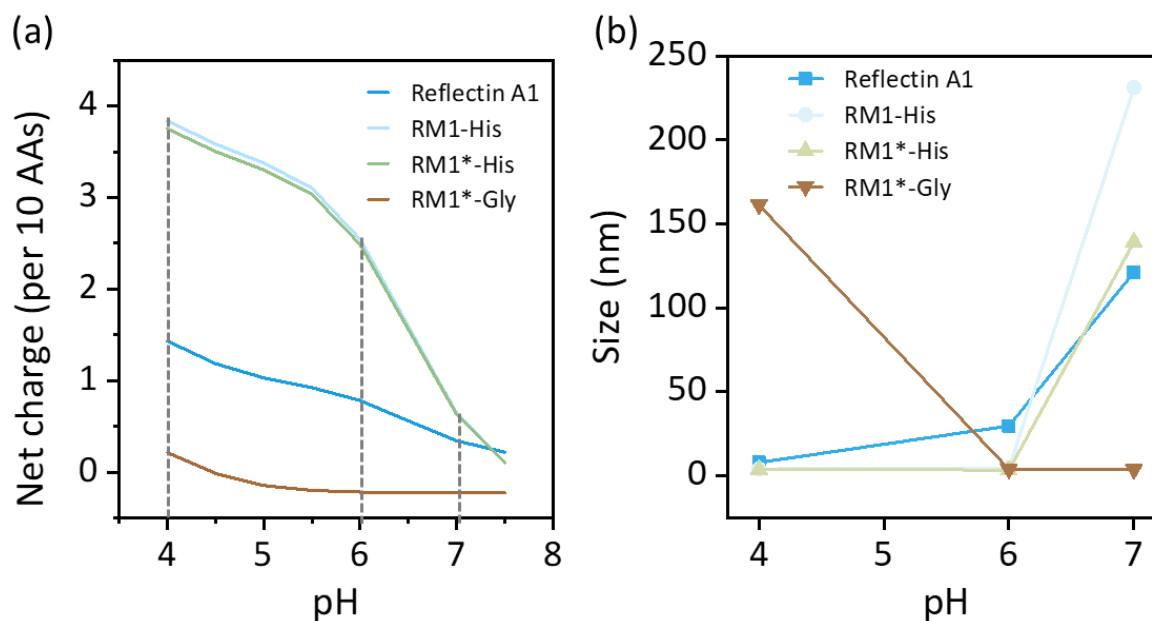


Figure 4.4. (a) The calculated overall net charge and (b) particle sizes measured from DLS of reflectin A1 and three peptides across different pH conditions.

Circular dichroism (CD) was used to characterize the conformational response of reflectin A1 and the 3 peptides as function of pH (Figure 4.3b). For reflectin A1, the

ellipticity shows clearly negative minimum near 200 nm at pH 4, corresponding to an extended-random coil conformation [2]. As pH increases to 6 and 7, the ellipticity becomes less negative. This shift in the strong minimum and shoulder suggests significant structural changes during assembly. Although these data do not allow precise quantification of individual structural populations, previous analyses showed that the CD spectrum of reflectin assemblies is consistent with the emergence of both α -helical and β -sheet structures as charge neutralization and consequent folding and assembly proceed [3]. In contrast, RM1-His shows a smaller conformational change as pH increases to 7 despite its equivalent molar concentration and significantly larger assembly size (Figure 4.3a), likely because the full-length protein contains multiple conserved motifs and cationic linkers that enable cooperative folding and inter-domain assembly. By comparison, RM1-His contains only one conserved domain, giving it far fewer cooperative contacts and much less capacity to reorganize into a new structure. RM1*-His exhibits behavior similar to that of RM1-His. Consistent with its opposite assembly behavior, RM1*-Gly shows the opposite trend, with a pronounced negative minimum at high pH, corresponding to a random coil conformation, and a loss of random coil character at pH 4. These results are in good agreement with the DLS data, which show assemblies RM1*-Gly at pH 4 and monomeric species at pH 6 and 7.

4.4. Reversibility and cyclability

Electrochemical correlative dynamic light scattering (E-DLS) was performed to analyze time-dependent evolution of reflectin A1 and RM1-His assembly size during electrochemical activation. RM1-His is compared with reflectin A1 because its assembly behavior is closely similar to reflectin A1 (Figure 4.3). Figure 4.5 shows the E-DLS behavior of reflectin A1 and RM1-His under an applied potential of -0.7 V versus Ag/AgCl for 25 min, followed by 15 min with the voltage turned off. This potential is selected to ensure sufficient driving force for deprotonation of histidine residues [4].

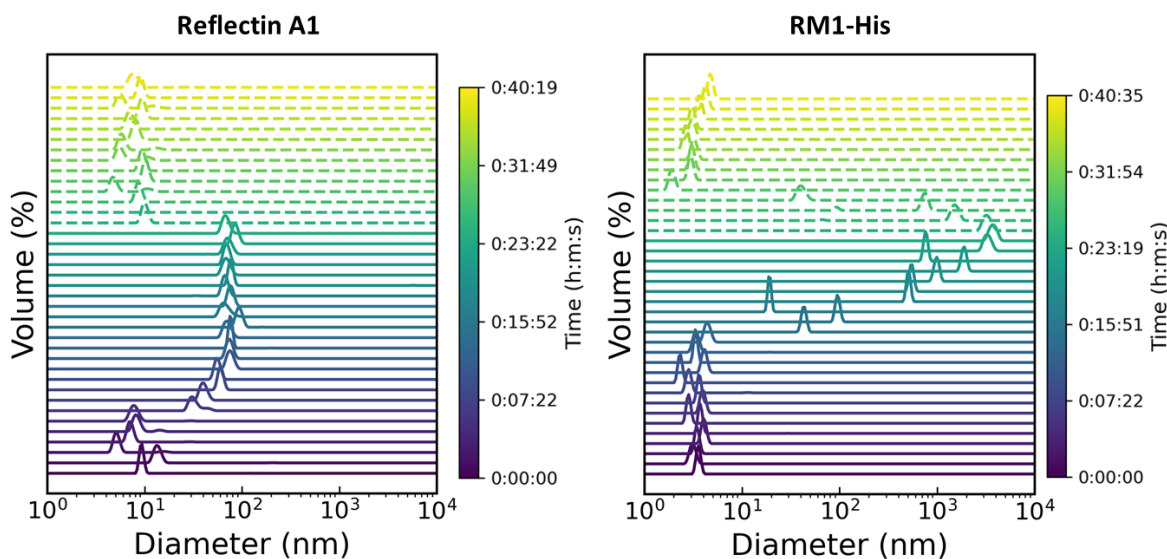


Figure 4.5. Reversibility of reflectin A1 and RM1-His' eletro-assembly measured by E-DLS under an applied potential of -0.7 V versus Ag/AgCl for 25 min (solid lines), followed by 15 min with the voltage turned off (dashed lines). Reflectin A1 (2 μ M), RM1-His (12 μ M) in 40 mM NaCl, 1 mM HClO₄, pH 3.

For reflectin A1, the particle size increases from approximately 10 nm monomers to approximately 70 nm assemblies after 5 min of applying the potential, and then rapidly returns to the monomeric size when the potential is turned off, driven by diffusion into the low pH environment and the return to local acidic environment at the electrode surface, consistent with previous reports [5]. The assembly remains stable and relatively homogeneous. Most importantly, the assembly process is fully reversible. RM1-His shows slightly different behavior from reflectin A1. The sample also begins as monomers, but after a longer applied potential, at least 10 min, the distribution then shifts abruptly, populating multiple size regimes. It then slowly returns to the monomeric size when the potential is turned off, indicating that the assembly process is also reversible. The longer time required for assembly of RM1-His than for reflectin A1 is likely because RM1-His remains highly positively charged at pH 6, which increases electrostatic repulsion at the initial stage (Figure 4.4a). In addition, its smaller initial assemblies may diffuse away from the working electrode more readily because of their higher diffusivity. It is worth noting that RM1-His assemblies appear to be more heterogeneous than the stable and homogeneous reflectin A1 assemblies. This may be due to the more pronounced change in net-charge change of RM1-His, making its assembly size more sensitive to small pH changes in this range (Figure 4.4a).

To determine whether the heterogeneity of RM1-His is due to pH variation in the electrochemical cell rather than intrinsic properties of the peptide, incremental additions of RM1-His monomer are made successively to a buffered solution at pH 7 to assess whether the assemblies have reached equilibrium, maintaining a constant size

distribution, or whether each successive addition of monomers adds to the size of the previous assemblies (Figure 4.6). The results show that additional RM1-His added to the solution do not progressively increase the size of the initially formed assemblies; instead, each addition formed new assemblies of approximately constant size as measured by DLS. This behavior is consistent with the “dynamic arrest” of the size of reflectin A1 assemblies proportional to the extent of charge neutralization, suggested to arise from a balance between short-range attraction and long-range repulsion [3, 6, 7]. Additionally, a pH jump experiment performed under the same solution conditions as the E-DLS measurements to test the sensitivity of assembly size to pH variation (Figure 4.7) shows that RM1-His remains monomeric at pH 6 (5 ± 1 nm) but formed progressively larger assemblies at pH 6.3 (102 ± 6 nm), pH 6.5 (164 ± 4 nm), pH 7 (286 ± 31 nm), and pH 7.5 (605 ± 65 nm). The sharp rise in size suggests a highly pH-sensitive assembly process rather than the more gradual response observed for reflectin A1.

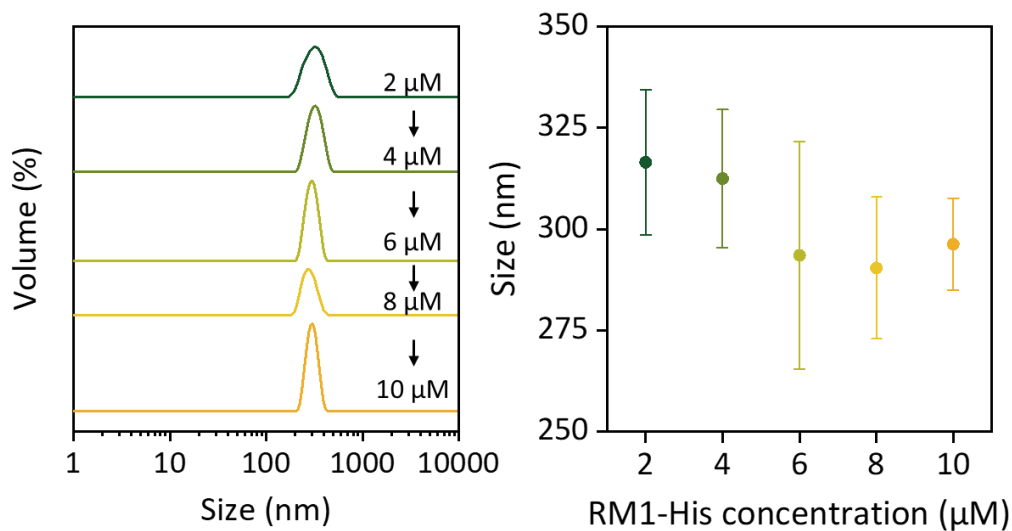


Figure 4.6. Assemblies of RM1-His are stable, as seen in these results of incremental additions of RM1-His monomers (pH 4) to the same 15 mM MOPS, pH 7 solution. RM1-His assembly sizes are measured by DLS.

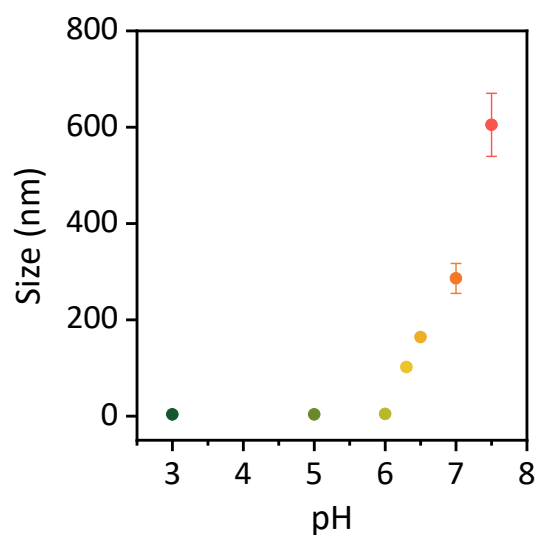


Figure 4.7. Assembly size of RM1-His as function of pH. RM1-His (12 μM) is prepared in 40 mM NaCl and 1 mM HClO₄ at pH 3, and the pH is subsequently increased to 7. pH is adjusted by incremental additions of 1 mM NaOH.

To mimic the repeatable cycling of reflectin folding and assembly in tunable squid iridocytes, E-DLS is performed with the potential switched on and off over several cycles (Figure 4.8). These results show that both reflectin A1 and RM1-His reversibly cycled through repeated rounds of assembly and complete disassembly. Notably, this is the first demonstration that a single conserved domain flanked by cationic linkers can exhibit reversible, cyclable assembly behavior similar to that of reflectin A1, albeit it does so more slowly, and to a larger size than the whole protein, as seen in the prior reversibility study above.

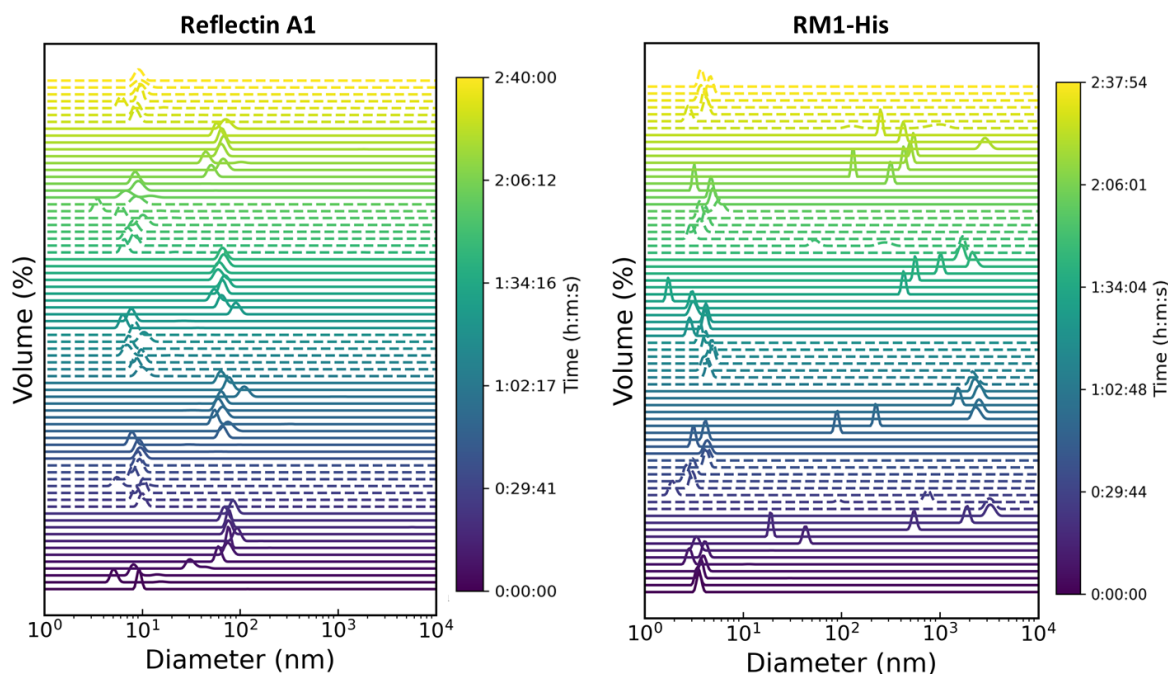


Figure 4.8. Cyclability of reflectin A1 and RM1-His' eletro-assembly measured by E-DLS during four cycles of 25 min exposure to -0.7 V versus Ag/AgCl (solid lines) followed by 15 min with the voltage turned off (dashed lines). Reflectin A1 (2 μ M), RM1-His (12 μ M) in 40 mM NaCl, 1 mM HClO₄, pH 3.

4.5. Summary

In summary, this work demonstrates that both the full-length reflectin A1 and engineered RM1-based peptides exhibit strong pH-dependent and electrochemically driven assembly behavior, but with some sequence-dependent differences. Reflectin A1 shows robust, reversible, and cyclable transitions in size, apparently reflecting the cooperative contributions of multiple conserved motifs and cationic linkers. By comparison, RM1-His retains the ability to respond to changes in charge state, but its assembly is significantly more sensitive to pH due to the sharp net charge transition during histidine deprotonation, which results in greater heterogeneity in assembly size during electrochemical cycling. On the other hand, RM1*-Gly shows an opposite trend, with assembly favored at low pH and suppressed at higher pH values; this behavior is driven by the intrinsic charge distribution of the conserved domain, as the neutral glycine linkers lack titratable side chains. Together, these results show that the essential features of reflectin tunability can be recapitulated by a minimal motif-based construct, while also highlighting the importance of native sequence architecture in promoting efficient and homogeneous assembly. More broadly, these findings confirm that reflectin behavior is governed by a delicate balance between electrostatic interactions and hierarchical assembly, providing a robust foundation for the future design and control of reflectin-inspired responsive biomaterials.

4.6. Experimental protocols

4.6.1. Reflectin A1 and peptides

Recombinant reflectin A1 from the squid, *Doryteuthis opalescens*, was produced from a codon-optimized DNA construct and expressed in *E. coli* Rosetta 2 (DE3). It was purified by collecting the expressed inclusion bodies by centrifugation, followed by chaotropic solubilization, cation-exchange chromatography, and reverse-phase chromatography, as previously described [7]. The purified protein was lyophilized and stored at -80°C . Purity and functional integrity were assessed by SDS-PAGE and assembly as a function of pH as measured by dynamic light scattering (Malvern Zetasizer Nano ZS, Malvern). Lyophilized reflectin A1 was solubilized in 0.22- μm -filtered sodium acetate buffer (25 mM, pH 4.5). Protein concentration was determined by measuring absorbance at 280 nm. RM1-His, RM1*-His, RM1*-Gly were synthesized by Biomatik. For DLS and CD measurements, reflectin A1 was diluted to 25 μM in 15 mM sodium acetate buffer at pH 4, 15 mM MES buffer at pH 6, and 15 mM MOPS buffer at pH 7. The peptides were dissolved to 150 μM in the same buffers. For electrochemical measurement, reflectin A1 was diluted to 2 μM in 1 mM HClO_4 , pH 3, 40 mM NaCl; RM1-His was dissolved to 12 μM in the same solution.

4.6.2. Circular dichroism (CD)

CD measurements of reflectin A1 (25 μM) and RM1 peptides (150 μM) were performed on a Jasco J-1500 spectropolarimeter with constant N_2 flushing, temperature controlled at 25°C using a 0.1-mm-pathlength demountable cuvette (Starna Cells, Inc., Atascadero, CA). Ellipticity was recorded between 190 and 250 nm with a spectral width

of 1 nm.

4.6.3. Dynamic light scattering (DLS)

DLS measurement of reflectin A1 (25 μ M) and RM1 peptides (150 μ M) were conducted with a Malvern Zetasizer Nano ZS (Worcestershire, UK). The sample was measured with a 632.8 nm HeNe gas laser with a beam diameter of 0.63 mm ($1/e^2$) and detected with an avalanche photodiode (quantum efficiency greater than 50% at 633 nm) in a back-scattering configuration at 7° from normal. Measurement was performed at 25° C. At least three independent measurements were performed for each experimental condition, with each measurement consisting of three technical replicates. All measurements and volume fittings were carried out using Zetasizer software (version 7.13).

4.6.4. Electrochemical correlative dynamic light scattering (E-DLS)

E-DLS measurements of reflectin A1 (2 μ M in 40 mM NaCl, 1 mM HClO₄, pH 3) and RM1-His (12 μ M in 40 mM NaCl, 1 mM HClO₄, pH 3) were conducted using BioLogic SP-300 potentiostat used in chronoamperometry mode with a three-electrode configuration consisting of a Pt coil as working electrode, a Pt wire as counter electrode and a fritted Ag/AgCl in 1 M KCl aqueous solution as reference electrode assembled in a DLS compatible optical cuvette and sealed with a plug to avoid evaporation and dust. DLS measurements were performed using a Malvern Zetasizer Nano ZS, as previously described. Measurements were performed with 1.2 ml sample volumes at 25° C under an applied potential of -0.7 V for 25 min, followed by 15 min with the bias turned off.

4.6.5. Calculation of expected side-chain charge patterns of reflectin and peptides

```
import numpy as np
import pandas as pd
import matplotlib.pyplot as plt
from typing import Optional, Dict
def sidechain_partial_charge(
    sequence: str,
    pH: float,
    pKaOverrides: Optional[Dict] = None,
    domains: Optional[Dict[str, tuple]] = None,
    doPlot: bool = True
):
    """
    Calculate expected sidechain charge and optionally color by domain.
    Parameters
    -----
    sequence : str
        1-letter amino acid sequence
    pH : float
        pH value
    pKaOverrides : dict, optional
        pKa overrides
    domains : dict, optional
        e.g. {"N_term": (1, 10), "RM1": (11, 25)}
    doPlot : bool
        Whether to plot
    """
    sequence = sequence.upper()
    n = len(sequence)
    if pKaOverrides is None:
        pKaOverrides = {}
    # default pKa
    defaultpKa = {
        'D': 3.9,
        'E': 4.2,
        'H': 6.2,
        'C': 8.3,
        'Y': 10.1,
        'K': 10.7,
        'R': 12.1
```

```

}
acidSet = {'D', 'E', 'C', 'Y'}
baseSet = {'H', 'K', 'R'}
perCharge = np.zeros(n)
pKaUsed = np.full(n, np.nan)
typeUsed = ['none'] * n

# =====
# charge calculation
# =====
for i in range(n):
    aa = sequence[i]
    if aa not in acidSet and aa not in baseSet:
        continue
    pKa = defaultpKa.get(aa)
    # residue override
    if 'res' in pKaOverrides and aa in pKaOverrides['res']:
        pKa = pKaOverrides['res'][aa]
    # position override
    if 'pos' in pKaOverrides:
        key = str(i + 1)
        if key in pKaOverrides['pos']:
            pKa = pKaOverrides['pos'][key]
        elif f'p{key}' in pKaOverrides['pos']:
            pKa = pKaOverrides['pos'][f'p{key}']
    f_prot = 1.0 / (1.0 + 10.0 ** (pH - pKa))
    if aa in baseSet:
        perCharge[i] = f_prot
        typeUsed[i] = "base"
    else:
        perCharge[i] = -(1.0 - f_prot)
        typeUsed[i] = "acid"
    pKaUsed[i] = pKa
# =====
# domain assignment
# =====
domain_label = ['none'] * n
if domains is not None:
    for dom, (start, end) in domains.items():
        for i in range(start - 1, end):
            if 0 <= i < n:
                domain_label[i] = dom
color_map = {

```

```

    "Linker1": "gray",
    "RM1": "tomato",
    "Linker2": "gray",
}
colors = [color_map.get(d, "black") for d in domain_label]
# =====
# output table
# =====
result = {
    'sequence': sequence,
    'pH': pH,
    'perResidueCharge': perCharge,
    'totalSidechainCharge': np.sum(perCharge),
    'detail': pd.DataFrame({
        'Position': np.arange(1, n + 1),
        'Residue': list(sequence),
        'pKaUsed': pKaUsed,
        'Type': typeUsed,
        'Domain': domain_label,
        'ExpectedCharge': perCharge
    })
}
# =====
# plotting
# =====
if doPlot:
    plt.figure(figsize=(5.6, 2.4))
    positions = np.arange(1, n + 1)

    plt.vlines(positions, 0, perCharge,
               colors=colors, alpha=0.7, linewidth=1.5)

    plt.scatter(positions, perCharge,
               c=colors, s=50, edgecolor='k')

    plt.axhline(y=0, color='red', linestyle='--', alpha=0.7)

    plt.xlabel('Residue position')
    plt.ylabel('Expected sidechain charge')
    #plt.title(f'Sidechain partial charge vs position (pH = {pH:.2f})')

    plt.xlim(0.5, n + 0.5)
    plt.ylim(-1.1, 1.1)

```

```

plt.grid(True, alpha=0.3)
# legend
import matplotlib.patches as mpatches
unique_domains = sorted(set(domain_label))
handles = [
    mpatches.Patch(color=color_map.get(d, "black"), label=d)
    for d in unique_domains
]
#plt.legend(handles=handles)
if n <= 60:
    plt.xticks(positions, list(sequence))
plt.tight_layout()
plt.savefig("Test.png", dpi=300)
plt.show()
return result
seq = "GGGGGGGGERYMDMSGYQMDMQGRWMDAQGRYNNGGGGGGGG"
domains = {
    "Linker1": (1, 8),
    "RM1": (9, 34),
    "Linker2": (35, 42),
}
out = sidechain_partial_charge(seq, pH=7, domains=domains)
print(out["totalSidechainCharge"])

```

4.7. References

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Chapter 5

Future Work

Building on the findings presented in this thesis, future work should focus on extending the electrochemical understanding of reflectin films from static characterization to dynamic, time-resolved behavior. While the present studies have demonstrated that reflectin's charge regulated folding, assembly and optical properties can be modulated electrochemically, several important questions remain regarding the decay of the optical response in reflectin films. In particular, combining electrochemical impedance spectroscopy (EIS) with ellipsometry offers a promising route to resolve how electrical processes at the electrode interface are linked to changes in refractive index and thickness. These studies will help establish a more complete picture of how reflectin films function as adaptive, electrochemically tunable biomaterials.

5.1. Dynamic electrochemical impedance spectroscopy of reflectin films

Electrochemical processes at an interface often involve several coupled phenomena, including electron transfer, double-layer capacitance, and mass transport, making it difficult to assign the measured response to a single mechanism [1, 2]. EIS offers a powerful framework because it separates these contributions through their distinct time scales and frequency dependence, allowing interfacial kinetics and transport processes to be analyzed within the same experiment. When integrated with ellipsometry, this approach enables the simultaneous characterization of charge transport and optical response, providing a direct link between electrical dynamics, refractive index changes, and film thickness variations.

To gain deeper insight into the decay in film thickness and refractive index

observed over cycling in Chapter 3, EIS was therefore coupled with ellipsometry, establishing a foundation for this strategy and demonstrating the feasibility of coupling these techniques to monitor reflectin film behavior in real time.

Figure 5.1 presents the equivalent circuit model and the measured impedance spectra, shown as Nyquist plots, under applied (ON) and non-applied (OCP) voltage conditions. The Nyquist plots indicate that the electrochemical response of the reflectin film is strongly dependent on the applied potential, reflecting changes in interfacial charge transfer, capacitance, and ion transport as the film undergoes swelling and shrinkage. The five-element equivalent circuit includes the bulk solution resistance (R1), double-layer capacitance (C1), charge-transfer resistance associated with hydronium reduction (R2), reflectin film capacitance (C3), and charge-transfer resistance within the reflectin film (R3). Both the double-layer capacitance and the reflectin film capacitance were modeled as constant phase elements (CPEs) because they represent parallel, indistinguishable current pathways through the film and the electrode support. The proposed equivalent circuit (Figure 5.1a) successfully modeled each impedance spectrum obtained under applied and non-applied voltage conditions (Figure 5.1b). The overall impedance equation of the equivalent circuit used for fitting is shown in Equation 5.1:

$$Z(\omega) = R1 + \left[Q1(j\omega)^{\alpha1} + \frac{1}{R2 + \left(\frac{1}{\frac{1}{R3} + Q3(j\omega)^{\alpha3}} \right)} \right]^{-1} \quad (5.1)$$

The exponent α , which dictates the non-ideal capacitive behavior of the system, was typically ~ 0.8 ; this deviation from the ideal capacitive value of $\alpha = 1$ arises from the contribution of these bifurcated pathways and the inherent heterogeneity of the reflectin film interface. The fitted CPE parameters (Q) were converted to effective capacitance (C) values using the Brug's formula:

$$C1 = \frac{(R1Q1)^{\frac{1}{\alpha1}}}{R1} \quad (5.2)$$

$$C3 = \frac{(R3Q3)^{\frac{1}{\alpha3}}}{R3} \quad (5.3)$$

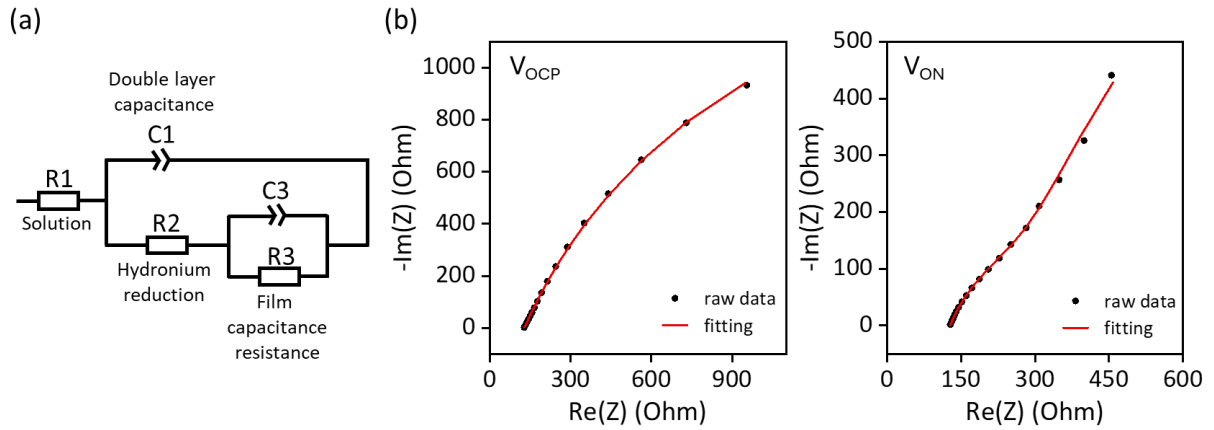


Figure 5.1. (a) Equivalent electronic circuit used for fitting impedance spectra includes bulk solution resistance ($R1$), double-layer capacitance ($C1$), charge-transfer resistance associated with hydronium reduction ($R2$), reflectin film capacitance ($C3$), and charge-transfer resistance within the reflectin film ($R3$). (b) EIS data and equivalent circuit fits obtained under applied (V_{ON}) and non-applied voltage (V_{OCP}) conditions.

To examine the evolution of the reflectin film over repeated cycles, the applied potential was periodically alternated between V_{ON} and V_{OCP} . Figure 5.2 shows the refractive index, film thickness, and five electrical parameters extracted from the equivalent circuit. Specifically, when the voltage is turned on, the film thickness decreases while the refractive index increases; conversely, upon returning the potential to OCP, both parameters revert to nearly their initial values. These dynamics suggest a reversible and cyclable flux of water across the reflectin film, driving periodic shrinkage and swelling. This process leads to an adjustable alteration in the local concentration of reflectin, which directly modulates its optical properties, consistent with the findings presented in Chapter 3. The electrical parameters derived from the equivalent circuit provide further insight into the underlying processes. The stable bulk solution resistance ($R1$) confirms that the measured changes are localized to the interface rather than fluctuations in the bulk medium. $C1$ exhibits a periodic modulation synchronized with the applied potential, indicating the distribution of ions and the effective thickness of the double layer change. When a negative potential (V_{ON}) is applied, the electrode surface becomes increasingly charged, attracting counter-ions toward the interface. This results in a higher local ion concentration and compression of the diffuse layer, effectively reducing the double-layer thickness and increasing the capacitance. In contrast, at open circuit potential, the driving force for ion accumulation diminishes, leading to a more diffuse ion distribution and expansion of the double layer. The increased effective separation distance consequently results in a decrease in capacitance. The charge-transfer resistance of hydronium ($R2$) also shows pronounced dependence on the applied potential. The

increase in resistance under V_{ON} indicates that hydronium reduction competes with electroreduction of the reflectin film. The periodic modulation of the film capacitance (C_3) and resistance (R_3) demonstrates that the reflectin film undergoes significant change in dielectric properties, likely involving ion uptake and expulsion, which in turn dictates its optical properties. Under V_{ON} , C_3 increases while R_3 decreases, suggesting that shrinkage of the reflectin film enhances conductivity and increases capacitance due to a more compact structure, consistent with the expected biophysical response of the film to charge neutralization. Notably, although the response remains largely reversible over cycles, a gradual change in magnitude is observed, indicating cumulative structural changes within the film, potentially arising from irreversible rearrangement, or loss of reflectin material to the solution.

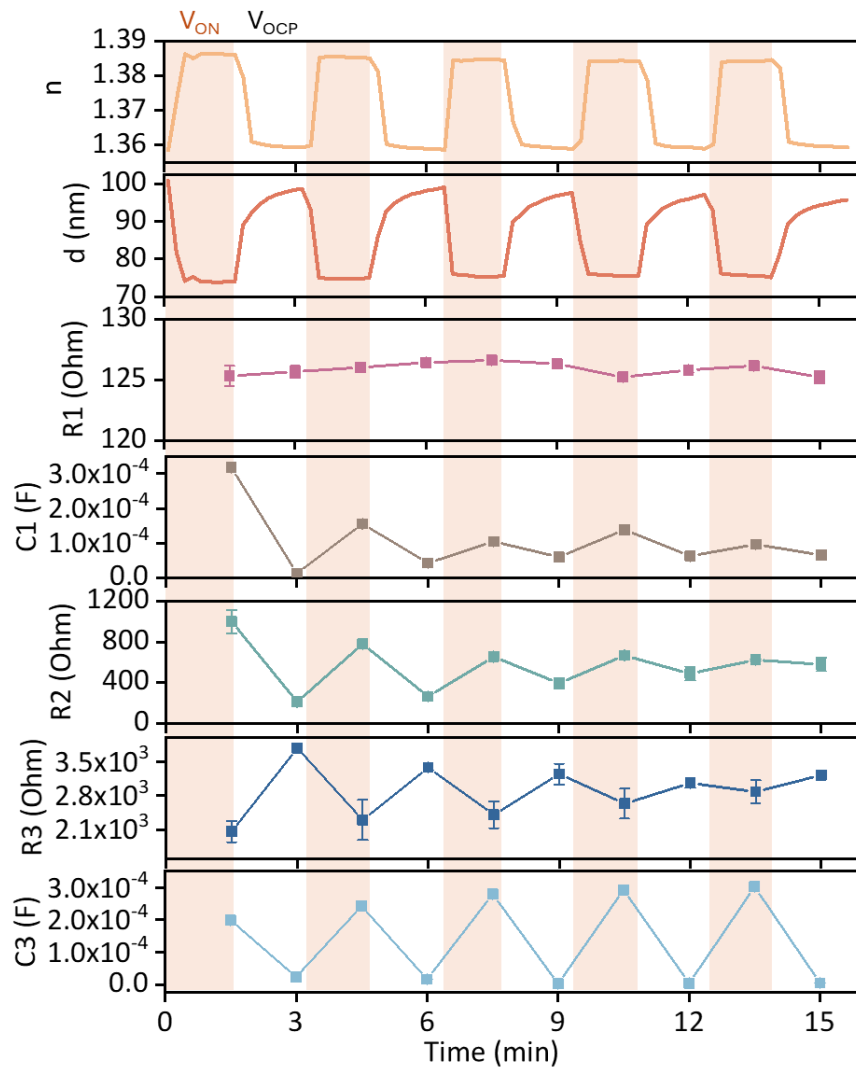


Figure 5.2. Refractive index at wavelength of 632.8 nm, film thickness, bulk solution resistance (R1), double-layer capacitance (C1), charge-transfer resistance associated with hydronium reduction (R2), reflectin film capacitance (C3), and charge-transfer resistance within the reflectin film (R3) measured by EIS coupled with ellipsometry as a function of time during cycles of exposure to -0.7 V versus Ag/AgCl followed by OCP. Reflectin film on Pt/Si in 40 mM NaCl at pH 3.

The results demonstrate that the optical responses, specifically changes in refractive index and film thickness, are closely correlated with electrochemical processes occurring at both the interface and within the film. However, the current measurements primarily probe macroscale properties and do not directly resolve molecular-level structural changes within the reflectin film. To address this limitation, future work will explore spectroscopic approaches that are sensitive to nanoscale organization. One promising strategy is the implementation of Förster resonance energy transfer (FRET) within the reflectin film. By incorporating suitable donor-acceptor fluorophore pairs, FRET measurements could provide nanoscale insight into changes in intermolecular interactions and protein organization during electrochemical cycling. Such measurements would enable direct correlation between structural rearrangements at the molecular level and the observed macroscopic optical and electrical responses. Ultimately, integrating FRET with EIS and ellipsometry could provide a comprehensive, multiscale understanding of reflectin film behavior and clarify whether the observed performance degradation arises from irreversible conformational changes, protein aggregation, or material loss. These insights could, in turn, inform strategies to improve film stability and guide the design of more robust and responsive bio-inspired functional materials.

5.2. Experimental protocols

5.2.1. Reflectin

Reflectin was synthesized using a codon-optimized DNA construct, expressed, and chromatographically purified as previously described [3]. The purified protein was lyophilized and stored at -80°C until solubilized for use. Lyophilized reflectin was dissolved in 0.22 μm -filtered sodium acetate buffer (25 mM, pH 4.5), and concentration determined by spectroscopic measurement of A_{280} [4].

5.2.2. Sample preparation

Pt/Si substrates were made by electron beam deposition of Pt onto an undoped Si (100) wafer (University Wafer, MA, USA) as previously described [5]. For reflectin film formation, solubilized reflectin was diluted into 1 mM HClO_4 , pH 3 to a final protein concentration of 2 μM . Final pH values were readjusted to 3.0 by the addition of HClO_4 as necessary. The diluted reflectin solution was drop-casted (as 100 μL aliquots) onto the substrate and dried using a vacuum desiccator connected to the building vacuum system (≈ 520 torr). The drying process was carried out for 1 hr. This process was repeated to obtain desired film thickness. The dried samples were immersed in a beaker of DI water for 10 s before measurement to remove any excess reflectin.

5.2.3. Electrochemical impedance spectroscopy (EIS) coupled with ellipsometry

EIS was performed over a frequency range of 1 Hz to 1 MHz using an AC perturbation amplitude of 10 mV. The AC signal was superimposed on DC bias. Measurements were conducted using a BioLogic SP-300 potentiostat with a three-electrode configuration consisting of a Pt/Si as working electrode, a Pt wire as counter

electrode and a fritted Ag/AgCl in 1 M KCl aqueous solution as reference electrode assembled in a 3D printed, custom electrochemical cell made of polylactic acid (PLA) fabricated with an Original Prusa i3 MK3S+ 3D printer (Prusa Research, Prague, Czech Republic) with a nozzle diameter of 0.4 mm and a layer thickness of 0.2 mm. Ellipsometry measurements were performed on an alpha-SE (spectroscopic) ellipsometer (J. A. Woollam, Lincoln, NE, USA) at a 70° angle of incidence from vertical at a wavelength of 632.8 nm. Optical constants and thicknesses of reflectin films were described using a Cauchy dispersive model, with fits to the ellipsometry parameters Ψ and Δ carried out over the entire visible measurement range (380–900 nm). Measurements were performed in 40 mM NaCl, 1 mM HClO₄, pH 3. All potentials are referenced to Ag/AgCl. Cyclability of reflectin films was evaluated at −0.7 V (V_{ON}) for three consecutive EIS measurements, followed by three EIS measurements at open circuit potential (V_{OCP}) for several cycles.

5.3. References

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