Challenges in Engineering Conductive Protein Fibres: Disentangling the Knowledge

Sophia Roy[1,†], *Oliver Xie*[1,†], *Noémie-Manuelle Dorval Courchesne*[1,*]

[1]*Department of Chemical Engineering, McGill University, 3610 University Street, Wong Building, Montréal, Québec, Canada, H3A 0C5*

[†]*These authors contributed equally.*

**Corresponding author: Prof. Noémie-Manuelle Dorval Courchesne, noemie.dorvalcourchesne@mcgill.ca*

Abstract

Conductive protein materials are promising candidates for next-generation bioelectronics due to their genetically-customizable functionalities, biocompatibility, and bioactivity. We envision that they could be used in a variety of bio-friendly functional devices, including bio-electronic interfaces, bio-energy devices, and sensors. However, their practical uses are limited by gaps in our understanding of charge transport in proteins, and by challenges in establishing reliable data collection methods. Moreover, characterization protocols are not always designed with applications in mind, which hinders engineering developments. Here, we review the effects of sample preparation, environmental conditions (ie hydration level, pH, temperature), measurement scale (nano, micro, and macro), and geometrical considerations, on the measured electrical properties of proteins. We emphasize the need for standardized methods and collaborations across fields for the design of conductive protein materials, keeping in mind their end goal applications. Our objective for this review is to disentangle the knowledge on protein conductivity, and to clarify the current challenges, limitations, and future possibilities for these biological conductors.

1 INTRODUCTION

Bottom-up assembly, self-healing, flexibility, and biocompatibility – these are a few of the properties desired for next-generation electronics that can interact with the body and with the

environment.[1-3] With the growing focus on sustainability that is spurring the search for novel ecofriendly conductive materials,[4] the answer to our needs for modern electronics may very well be found in nature.

Long-range extracellular electron transfer has been found to occur in nature. Microbial species have been shown to wire their intracellular metabolic processes to external electron sinks such as metal ions or other microorganisms.[5-13] While some microorganisms, such as *Shewanella*, are believed to use mobile charge shuttles,[14-16] others produce intrinsically conductive extracellular protein structures that enable long-range conduction.[5,6,12,17] Of particular interest are the protein fibres of *Geobacter*, also referred to as pili, with conductivity comparable to that of organic conductive polymers.[18] Combining this discovery with advances in protein bioengineering allows for novel multifunctional materials to now be synthesized in microbial "factories." In fact, various types of synthetic conductive protein fibres have recently been engineered using recombinant DNA technologies and engineered microbes as chassis.[19-22] These novel conductive biomaterials hold promise in a variety of bioelectronic applications, ranging from electrode-biomolecule sensing units[23-25] to direct signal interfaces with living cells.[26-28]

In this review, we consider conductive protein materials in a general sense. These materials range from the self-assembled protein filaments—nanowires—secreted by microbial species to the more complex matrices—biofilms—which contain cells and other extracellular components (including protein nanowires, but also polysaccharides and other extracellular proteins). We chose this broad definition since researchers in the field compare results obtained from studies on both forms.[29-31] We believe that gaps in our understanding of these protein systems must be overcome before implementation in functional devices. Fundamental knowledge on the charge transport mechanisms that arise in conductive proteins is crucial to determine their most promising applications. This knowledge also goes hand-in-hand with an understanding of how these protein materials behave in different environments, interact at different scales, and respond to various stimuli. Due to the complexity of the task, few studies have fully elucidated these mechanisms. Moreover, while creative and diverse protocols have been used to characterize the

properties of conductive proteins, standard methodologies still need to be established to better compare innovative protein materials produced by different research groups. To this end, we aim to review the impacts of important variables in the design of electrical characterization experiments of both naturally occurring proteins and biomimetic synthetic analogs. We also aim to identify the material properties that will play a critical role in the way we can apply and utilize conductive proteins practically. Overall, we wish to bridge the gaps between fundamental investigations and applied research and development. We believe that this can be achieved by understanding what the challenges and limitations are when characterizing the electrical properties of proteins and introducing them into devices.

2 CHALLENGES IN CHARACTERIZING CONDUCTIVE PROTEIN MATERIALS

The characterization and engineering of conductive protein fibres involve multiple research groups approaching the challenge from various disciplinary perspectives. Most of the current research has focused on elucidating the charge transport mechanism of pili secreted naturally by the bacteria *Geobacter sulfurreducens*. Despite this effort, uncertainty over the conduction mechanism remains. One particularly controversial point is whether extracellular cytochromes are necessary to enable long-range extracellular electron transfer,[32-35] or whether, as evidence suggests, aromatic residue rich pili alone can facilitate electronic conduction.[18, 36-40] Dissimilar experimental conditions such as the state of the material and surrounding environment have been highlighted as possible culprits.[29, 30, 41, 42] The recent expansion of the field into the engineering of novel conductive protein materials has been based on design principles directly inspired by *G. sulfurreducens*,[19-22] making it all the more critical that *G. sulfurreducens*' electronic behaviour is well-understood. Without understanding how experimental conditions impact measurements, the rational design, accurate comparison, and evaluation of novel conductive protein materials become infinitely more difficult.

While the importance of environmental factors such as humidity,[43] pH,[6, 44] and temperature[6, 31, 35, 43, 45] on *G. sulfurreducens* pili nanowire conduction is well-accepted, their interpretation

has been framed by two main conduction theories—electron delocalization and electron hopping. Metallic-like conductivity, a concept first proposed to occur in protein nanowires by Malvankar et al, explains conductivity as the overlap of π-orbitals leading to electron delocalization between aromatic amino acid residues in the pili.[6] Organic metals—polymers in which metallic states originate from delocalized electrons—are an analogue of this mechanism.[46] Localized electron hopping between discrete sites is another possible conduction mechanism; modelling of *Geobacter* pili suggest that aromatic residues could function as sites of electron localization.[45, 47-49,50] Once again polymers serve as an analogue, where charge transfer can occur across various side chains or along the backbone in a series of redox reactions.[51, 52] However, the complex ways in which proteins can respond to these same environmental factors, not just electronically but also structurally, make simply fitting standard conduction models using this data unreliable. In this section, we aim to approach the problem from another angle. By analyzing the effects of certain experimental choices on conductive proteins as both an electronic material and a biological system, we hope to inspire researchers to reconsider the links between measurement conditions and results. Here, we address three main themes—the complex environment in which these proteins reside, the thermodynamic and kinetic effects of temperature, and how scale and geometry can lead to emergent properties. Unmasking why seemingly benign variations in testing setup can lead to vastly different electrical properties can help reinterpret previous data to form more accurate conclusions on transport mechanism and lead to more efficient designs of conductive protein materials for bioelectronic devices.

2.1 Proteins, water, and ions: a complex environment

The presence of an aqueous environment—a necessity for protein structural stability—poses a unique challenge for the electrical characterization of conductive protein materials. Direct impacts on measurements can be caused by water hydration or ionic species in solution. The correlation between structure and conductivity can cause further complications; specific folding and spatial orientations may be required for proper charge conduction through assembled proteins. Modelling shows that hydration can affect protein structure.[53] Furthermore, proteins

are charged species, and their charge varies with their amino acid composition. Changes in pH can affect their overall charge, and consequently their secondary, tertiary, and quaternary structures. In addition, the protonation or deprotonation of amino acids may affect the electronic environment of the protein and, thus, their tendency to trap electrons or ions. Disentangling this web of causes and effects for protein conduction in aqueous conditions remains a challenging task.

2.1.1 Effect of hydration level on electron transfer rate

The importance of water hydration on electron transfer in small protein assemblies has been well-documented. The significant body of work conducted on electrically active single proteins such as azurin,[54, 55] bacteriorhodopsin,[54, 56, 57] and cytochromes[58-60] for use in solid-state monolayer junctions has shown that while proteins retain electronic functionality in a dry state, fundamental differences in charge transport behaviour remain when compared to fully aqueous conditions.[61] Charge transport in aqueous solutions has been described in terms of the hopping model.[62, 63] Solid-state conduction meanwhile has been thought to happen via tunnelling mechanisms, where proteins can behave as conjugated molecules.[59, 64] The parallel between these two mechanisms and what has been described for *G. sulfurreducens* conduction raises the question: how does hydration influence charge transport in long-range protein network conduction?

Electrical characterization for protein materials has been conducted mostly in aqueous environments. Protein networks, either purified or as part of a biofilm, form the bulk of aqueous environment response studies.[31, 43] A positive correlation has been reported between the level of hydration and conductivity for *G. sulfurreducens* biofilms (Figure 1A); this was explained as the result of increased ionic mobility, the proposed rate-limiting step in the redox model.[43] While water hydration's effects on DNA conductivity have been explained using ionic mobility,[65, 66] water molecules can affect electron transfer in proteins via more complex mechanisms. Proton-coupled electron transfer is one such example. This charge transport mechanism often occurs in biological systems and involves the transfer of both an electron and a proton between redox

active sites.[67] Depending on their access to redox active amino acids, water molecules can act as a proton acceptor in short-range proton transfers when coupled to more distant electron transfers.[68] Electron transfer between amino acids has also been reported to be directly affected by the presence of water molecules, which lower the activation energy requirements for electron transfer or even the formation of new charge transfer pathways due to strong hydrogen bonding effects.[69, 70] In such cases, aromatic amino acids such as tyrosine, tryptophan, and phenylalanine can serve as sites for electron localization and act as relay stations for electrons moving between donor and acceptor species.[71-74] Based on this knowledge, an alternative conduction pathway has been proposed in *G. sulfurreducens* pili, where aromatic amino acids contribute to conduction not via electron delocalization but by acting as charge localization centres themselves.[47, 48, 75, 76] Recently published work reveals more complex interactions could be at play; thin films of *G. sulfurreducens* protein fibres have been shown to generate current when a water concentration gradient is present in the film.[77] In this example, the passage of water molecules in nanopores formed by the protein network is thought to allow for the formation of an ionization gradient or a concentration gradient in mobile protons that could contribute to conduction in the material.

Electron transfer measurements are commonly achieved by electrochemical or spectroscopic methods. In such techniques, protein samples are often found in a solvated state. Conductance measurements for proteins in the dried state can also be achieved with methods such as laser-flash quench.[78] For instance, measured electron transfer rates for the cytochrome C protein differ considerably between the two techniques requiring different states.[61] Protein immobilization into a constrained morphology arises under solid state conditions, leading to fewer available electron transfer pathways than in solvated conditions.[79] This effect is especially pronounced for electrode-material interfaces where the orientation of the protein relative to the electrode can strongly affect the interface electron transfer rate thereby altering measures of conductivity.[80] Proteins can be immobilized into the optimal configuration for

higher transfer rate to the electrode; for instance, one newly developed solution for a synthetic glucose dehydrogenase, an orientation-dependent enzyme, is to attach the enzyme to a gold binding peptide. Depending on the fusion site, the peptide-enzyme complex stabilizes into specific configurations; with some configurations resulting in higher efficiency for the electron transfer between the electrode and the enzyme.[81]

2.1.2 The essential role of solvation on protein structure

The structure of a protein is a crucial determinant of its conductive properties. For example, in both localized hopping and metallic-like conduction, the distances and orientation between aromatic residues or cytochrome group have been indicated as critical (Figure 1B).[37, 47, 76, 82, 83] Tightly packed amino acids residues are believed to be necessary for electron conduction.[37] This rationale has been proven effective in engineering curli fibres where proteins with denser aromatic amino acid residues profiles were found to be more conductive.[19] Protein folding also decreases the reorganization energy needed in redox reactions in cytochrome electron transfer.[84] Just like the proximity of aromatic rings increases conductivity, cytochrome groups can be similarly affected. The close packing and alignment of heme groups (4-6 Å) are thought to be a requirement for electrical conduction.[34] The aqueous environment directly affects protein folding and assembly, which can, in turn, affect these critical distances between electrically-active groups.

One major driving force for folding is the hydrophobic effect. Water molecules at the interface of proteins have been demonstrated not to lose hydrogen bonding in comparison to bulk water molecules, but only rotational entropy. The hydrophobic effect suggests that a certain level of hydration is necessary for the stability of proteins. For instance, modelling of β-helix proteins show hydrogen bonds perpendicular to its strands. These protein-water interactions are found to be essential to the structure's stability (Figure 1C). Hydration can also be directly affected by other environmental variables such as temperature. As temperature increases, the number of hydrogen bonds available for the protein to form with surrounding water molecules decreases, therefore the protein has to rely on its own hydrogen bonding interactions, which favours more

compact secondary and tertiary structures.[85] The consequences of temperature on protein structure will be further discussed in section 2.2.

The level of hydration becomes most relevant when designing conductive proteins for use in devices under a hydrogel form. Conductance changes due to the effect on protein structure are to be expected. The hydrogel state can be defined as a polymeric three-dimensional network that has the ability to absorb water and that matches the flexibility of biological tissues.[86, 87] These properties become even more useful as the hydrogel's response to stimuli can be transmitted electronically and mechanically. For instance, applications in biomedical engineering require current transfer to excitable biological cells or recording signals sent by electroactive cells.[86, 88] To furthermore increase conductivity, commonly used protein hydrogel combine nanoparticles of graphene or CNT (carbon nanotubes) to the matrix.[89, 90]

2.1.3 Impact of ions on protein material electronic properties

The ionic environment and pH at which an experiment is performed can affect conductivity measurements. Varying pH can induce structural changes in the protein, which can change the distance between electron conducting centres. In addition, the ionic environment can impact the electrical performance of the material depending on the conduction pathway. Unravelling how an ion-rich environment can impact measurements is key to understanding how conductive protein materials might behave in real-world applications.

Rheology and stability of protein compounds depend on whether pH conditions are below or above the protein's isoelectric point (pH at which the surface charge of the protein is null).[91] For polymer-based hydrogels, the solvent concentration gradient around the hydrogel impacts hydrogen bonding.[92] Similarly for proteins, the ionic strength of the buffer will change the charge repartition on the protein's amino acids side chains. Charges are involved in salt bridges interactions which stabilize the tertiary and quaternary structures of the protein.[91] As a consequence, protein conductivity is affected by pH, as seen for *G. sulfurreducens* pili, in Figure 2A.[18] Changes in the overall charge of an individual protein subunit in these pili is a function of

pH, as reported in Figure 2B, further supporting the hypothesis that side chain charges have a direct effect on protein conductivity.[37]

The effect of pH on conductivity can be quantified using fluorescence spectroscopy. Fluorescence peaks allow the investigation of the electronic state of amino acids incorporated as mutations which are presumed to enhance conductivity. This technique is restricted to intrinsically fluorescent amino acids (ie, phenylalanine, tryptophan, and tyrosine), and the presence of peaks attributed to $\pi - \pi$ stacking interactions between the rings of these aromatic residues can inform on protein folding.[93] For instance, this technique was applied to a synthetic helical biomimetic peptide designed to be conductive through the incorporation of phenylalanine residues. With varied pH or buffer concentrations, a shift in the location of the fluorescence peaks and a change in their intensity were observed for this peptide.[20] The peptide was titrated with sodium bicarbonate, causing an increase in pH with time, and resulting in a decrease in peak intensity. This evidence suggests that the conformation of the peptide was dependent on pH, which had an effect on the formation of peptide fibrils and on their conductivity. Therefore, measuring conductivity of proteins and peptides at different pH values can lead to varying results depending on the effects of ions and pH on conduction pathways.[20]

Proteins are used in biological sensors that convert a change in a physiological input into an electric signal. pH can interfere in the sensor's readings, as it affects protein conduction pathways. In certain cases, pH interference can be removed by incorporating point mutations to the engineered peptide. This solution applies to the yellow fluorescence protein, which is used for monitoring fluctuations in the chloride pumping achieved by transmembrane proteins. By changing the mutant's isoelectronic point, the pH sensitivity of the peptide can be reduced. The chloride sensor is most useful for cell wall monitoring when changes in anionic concentration are efficiently monitored by the protein sensor even if different gradients cause pH to fluctuate.[94]

When characterizing a protein material, the pH to which it will be exposed is to be determined prior testing. On the same note, the current sensing response will not be consistent if the

environmental pH value diverges from test to test.

Ion-rich environments must also be considered both in experimental setups and applications especially when using biofilms. Conductivity measurements on live *G. sulfurreducens* biofilms have been performed with acetate present as metabolite for the cells.[6, 29] Redox conduction driven either by concentration gradients or by an electric field has been proposed as one possible conduction mechanism.[50] Concentration driven conduction is caused by the formation of reduced and oxidized species near each electrode, with electrons flowing to maintain the steady state condition near the electrodes. Fickian diffusion can be used to describe how electrons move under this gradient; its current-voltage response can be described as mass transfer limited, resulting in a plateau of current at high potentials as seen in Figure 2C.[50, 95] This process requires electrolytes in solution to provide counterion diffusion thus ensuring neutral overall charge[96]; the process will thus directly depend on the ionic environment. Meanwhile, electric field driven transport occurs when ions are not as permeable in the material and instead the difference in potential energies of electrons induced by the external field causes electron flow to occur.[50, 95] This process is not mass transfer limited, and the current-voltage response is exponential at higher potentials (Figure 2D) while linear around zero potential.[50]

Control over these two redox mechanisms is required to obtain relevant results, and the electrochemical characterization of conductive polymers has given us many tools to help with this control.[95, 96] In the characterization of conductive *G. sulfurreducens* biofilms, both concentration-driven[97] and electric-field driven[43] redox conduction have been reported. Differences in the ionic environment, specifically the presence or absence of acetate, might account for these different mechanisms. Moreover, another study reported that the mechanism of charge transport in live biofilms of *G. sulfurreducens* was entirely mediated by redox reactions between the cells and the electrode, with charge neutrality provided by the movement of ions in solution.[98] The complex chemical composition and, therefore, electronic properties of biofilms make the comparison of experiments on biofilm to those on purified proteins—as some researchers have done—potentially misleading.[31, 34, 99] Moreover, individual protein nanowires

are already challenging for electronic characterization. Figure 2E shows the modelled structure of *G. sulfurreducens* with aromatic amino acids highlighted in yellow and basic or acidic residues in blue and red, respectively.[37] This complex structure gives rise to many physical-chemical phenomena. Charge trapping caused by changes in the electronic environment due to ions or pH effect on neighbouring amino acids can further complicate electrical characterization. Physical (conformational) and chemical (impurity) disorder in polymers have been cited as key reasons for the formation of these trapping states.[100] The myriad of results obtained via electrochemical investigation of conductive protein materials suggests that a deeper understanding of the impact of an ionic environment on electronic properties is required.

2.2 The link between conduction mechanism and temperature

Temperature is an important variable when dealing with conductive protein materials. Electrical characterization has traditionally been performed as a function of temperature since each charge transport mechanism shows a unique temperature-conductivity correlation. Charge transport via electron delocalization sees decreasing conductivity with increasing temperature due to thermal scattering events, a behaviour observed in metals.[101] Electron hopping processes show the inverse trend, due to the presence of an activation energy barrier. Increased thermal energy allows electrons to cross this barrier and move sites.[102] The observation of these temperature-conduction behaviours form part of the evidence in support of the metallic-like conduction and electron hopping mechanisms in *G. sulfurreducens* pili.[6, 35] In proteins, however, temperature's effect on structural stability and the possible existence of multiple conduction mechanisms makes drawing conclusions from this method more complex.

2.2.1 Temperature affects protein structure

Electronic states are related to thermal energy states. Electrons require a certain potential to hop from one step to another. As a result, change in surrounding temperature impacts electron transfer rates.[45] We previously mentioned that higher temperatures cause a decrease in tertiary structures from the loss of favourable hydrogen bonds with water molecules on the surface of proteins.[85] Loss of protein folding directly impacts stability and electron transport by the same

effect. As a consequence, the required alignment and distance in between amino acids are lost. To quantify the effect of temperature only, the conductivity of proteins dried on gold electrodes was measured. A decrease in temperature has effectively been demonstrated to augment electron transfer rate, suggesting metallic-like conduction at the core of protein.[45]

2.2.2 Multiple conduction pathways mask temperature behaviour

Crystalline inorganic materials such as metals or semiconductors exhibit clear temperature-conductivity correlations across large temperature ranges. As structural disorder increases, this relationship becomes less applicable across all temperature ranges. Conducting polymers are a good analog for protein nanowires; both possess a complex morphology of long junction-forming fibrils.[103] The temperature-conductivity relation in these polymers is similarly complex. Insulating behaviour is observed in low temperature regions while metallic behaviour is seen at high temperature.[103, 104] This insulator-metal transition has been shown to be a function of various external factors, including the morphology of the material.[105] In addition to these morphology challenges, multiple conduction pathways may further complicate the issue with some conducting polymers exhibiting mixed electronic-ionic transport.[106-108] This complexity is mimicked in proteins. In *G. sulfurreducens*, tunnelling has been proposed to occur within the C-terminal head of each alpha-helical protein subunit while hopping is the dominant mechanism through the N-terminal tail.[109] Temperature-conductivity behaviour might, therefore, vary depending on experimental protocol, specifically cross-sectional measurements of conductivity might reveal a different conduction pathway than fibre-length measurements[5, 12, 18, 34, 110] and which mechanism forms the rate-limiting step in electron transfer, a property that has previously been discussed to be influenced by other factors such as hydration and the ionic environment. The importance of consistent experimental conditions when conducting a temperature-conductivity study on these protein materials is demonstrated with *G. sulfurreducens* pili networks (Figure 3A). Vastly different temperature-conductivity relationships have been reported, which may be due to differing experimental conditions—specifically, how the pili networks were processed. Increasing conductivity with increasing temperature—consistent with

hopping transport—has been reported on living *G. sulfurreducens* biofilms, as seen in Figure 3B,[35] while decreasing conductivity with increasing temperature—band transport behaviour—was observed on free-standing biofilms (Figure 3C) or purified protein films (Figure 3C,D).[6, 31] In addition to these opposing trends, temperature-conductivity behaviour within a temperature range is not consistent. The behaviour reported by Malvankar et al shows a transition temperature where conductivity inverses its relationship with temperature (Figure 3C).[6] Ing et al meanwhile report two different slopes in the conductance-temperature graph marked by a transition temperature (Figure 3D).[31] The work done on *G. sulfurreducens* pili shows that unlike in crystalline materials, temperature may not be a straight-forward indicator of charge transport mechanism for protein materials.

2.3 Scale and geometry: an array of possibilities

Dimensional analysis of a given system can inform on the relationship of various properties with scale, which is of direct interest to chemical engineers. Diverse applications require knowledge of protein conductance from the macroscale down to the nanoscale. We will discuss scale of measurements by introducing differences in conductance for all three dimensions. Protein nanofibrils can be considered a one-dimensional system. Monolayers of protein fibres are two-dimensional while thin films with a significant thickness have an added third dimension. For each scale, the definition of conductivity (σ) is used to quantify protein conduction (refer to Equation (1)).

2.3.1 From nano to macro: emergent properties at different length scales

Conductance can be experimentally measured and related to a material's conductivity from the geometry of the measurement set-up:

$$\sigma = G \cdot \frac{l}{A} \qquad (1)$$

Measured parameters, namely conductance (G), length (l), and area (A), will change depending on the scale of the protein material and the testing set-up. The slope of current-voltage (IV)

sweeps performed using various instruments yields a conductance value, as shown in Equation (2):

$$G = \frac{1}{R} = \frac{I}{V} \qquad (2)$$

When isolated, engineered peptides can be used as nanowires.[111] The conductance of a single fibre is often measured by conductive atomic force microscopy (c-AFM). Two possible set-ups for this technique are illustrated in Figure 4A,B, where the circuit is closed by a gold electrode and the c-AFM tip. A voltage potential is applied to the two ends of the circuit and the current is measured.[18] Single fibre electrical characterization is also performed by carefully bridging small electrode gaps with an isolated protein filament.[5, 12, 18, 110]

Current can be passed longitudinally through a protein fibre to characterize its conductivity[18] In that case, the length, *l*, corresponds to the gap in between the two electrodes and the area is approximated as a disk with a radius, *r*, of the pilus.[45] A single protein fibre typically measures about a few μm in length and a few nm in diameter, allowing it to bridge two electrodes to complete a circuit (Figure 4B).[31, 112] Alternatively, current can be passed transversely; through the cross-section of the fibre (Figure 4A). In this case, *l* represents the cross-sectional height of the fibre and the area, *A*, can be taken as the probe tip contact area. Both methods yield considerably different values and cannot be compared directly.[113]

Theoretically, a monolayer of protein fibres does not exceed a few nanometers in thickness. Conduction mechanism theories developed for protein fibres may be thought to directly apply to these systems since they are formed from a single layer. However, the addition of protein fibres, as well as the formation of additional layers increasing the film thickness, raise new effects. Nanowires previously designed to facilitate extracellular long-range electron conduction are now being used as a bulk conductive biomaterial. As the protein network grows, proteins aggregate into small bundles, leading to a random arrangement of fibres that gives rise to unexpected conductive behaviours. For conductivity measurements, thin films are usually deposited on

interdigitated gold electrodes or in between two parallel electrodes for conductivity measurements (Figure 4C,D). IV sweeps are performed and the geometry of the sample is measured.[22] Conductivity is calculated using the length in between the electrodes and the width and thickness of the thin film.

Thicker films (starting at a few microns) are considered as three dimensional or bulk protein materials (Figure 4E). When the hydration content is high, protein films often take the form of hydrogels. Hydrogels exhibit percolation effects, which can be directly linked to conductivity. In fact, protein gelation can be described by a percolation model. From this model, variations in gel elasticity are typically proportionally related to conductivity by experimentally determined coefficients.[114] Elastic stretching of gels can lead to varying signal responses (Figure 4F). In this case, the percolation threshold corresponds to the lowest allowable concentration of fibres in the network, below which the hydrogel conductivity drops significantly.[115] Percolation representation is independent of the predominant conduction mechanism, and rather indicates film conduction as a function of fibre length and density of filament junctions.[115] For conductivity measurements on films of purified pili from *G. sulfurreducens*, deviations from the percolation model can be attributed to fibre junction resistance.[31] Higher occurrence of capacitance effect is to be expected for a random network of filaments. For living protein films, diffusion limitations of cytochromes occur at a thickness of 10 microns, inhibiting the redox reaction in the electron transfer process.[111] Bulk measurements are more likely to experience more charge trapping. Controlled dispersion of fibres into a specific alignment for thin film deposition could potentially reduce this effect.[116]

2.3.2 The importance of measurement setup

In addition to the geometry of the measured sample, the conduction path length may also affect the mechanism of charge transport. The length of electron conduction in living *G. sulfurreducens* biofilm was shown to be different depending on whether conductive pili was co-expressed with cytochromes.[99] Since a thicker biofilm results in longer electron conduction path lengths from the outermost layers to the anode, the authors propose a coordination effect between pili and

cytochrome is necessary to enable this longer-range electron transport.[99] Path length-dependent switching of conduction mechanisms has important consequences for measurement setup. In previously mentioned articles by Yates et al[29] and Malvankar et al,[6] the respective distances between electrodes were 5 μm, and 50 μm. Different conduction mechanisms were proposed for each electrode gap distance, moving with increasing path length from electron hopping via discrete centres to metallic-like delocalization.[6, 29] This switchover of mechanism has been observed on other organic molecules such as donor-bridge-acceptor systems or DNA, where electron tunnelling gives way to multi-step hopping as the distance the electron must travel increases.[117-122] In addition, spacing differences in interdigitated electrode arrays have been shown to change how quickly steady-state current is reached in electrochemical experiments.[123] Even electrode area has been reported to change current-voltage responses in *G. sulfurreducens* biofilms.[98] For all these reasons, electrode design requires careful consideration; differing geometries may lead to different observed conduction mechanisms and complicate comparison between various conductive protein materials.

Measurement voltage range is another important consideration. If charge conduction in conductive protein material is indeed redox driven, electrochemistry gives an appropriate model of how voltage affects charge conduction behaviour. [97] The Butler-Volmer equation describes current density behaviour at various potentials.[124] A transition from a kinetic-controlled regime to a mass-limited regime occurs at a certain potential; the current density goes from linearly increasing to a plateau during this transition. For non-electrochemical systems, a transition voltage can exist where the potential applied matches the tunneling barrier of the system.[97, 125] More complicated multiple-transport regime behaviour has been observed in other conductive organic molecules,[126, 127] and though the exact mechanism of transition is not well-understood, the existence of different current responses at various potential windows makes this a crucial factor when characterizing conductive proteins.

The current-voltage responses of various engineered proteins have been just as diverse. Synthetic alpha helical peptides displayed both power law behaviour (Figure 5A),[20] and linear current-

voltage responses (Figures 5B).[22] Modified curli fibre proteins from *E. coli* with an attached aromatic rich peptide sequence displayed linear responses (Figure 5C),[21] while aromatic substitutions on the protein itself displayed a transition from linear to power law depending on the voltage applied (Figure 5D).[19] Small differences in measurement setup may explain some of the differences in result. The voltage range used varied from ±0.8 V to ±5 V. The dependence of how a specific conduction mechanism responds to an applied potential as previously described may account for these differences in result. Choosing small voltage ranges may also mask exponential behaviour; as seen in Figure 5D, the current response is near linear, below around 1 V while increasing exponentially afterwards. Furthermore, other factors talked about in this review such as water content or electrode geometry were not consistent across these studies. Though all measured samples were dried at ambient or humid conditions, as reported by Phan et al, water content is a function of both room humidity and temperature, and thus could have varied greatly, leading to uncontrolled effects on conductivity.[43] The lengths between electrode contacts used for measurements were as small as 5 μm[20, 22] to as large as 175 μm[21].

Combined with varying aspect ratios and electrode design (interdigitated versus lined), differing conduction mechanisms may have occurred depending on which was most favourable. Therefore, the intrinsic electrical properties of these materials may not be fully understood, since variations in electrical behaviour could be artifacts of measurement conditions. While all the engineered proteins whose current-voltage responses were shown in Figure 5 took design inspiration from *G. sulfurreducens*, the variety of results observed only highlights the need for standardization in the engineering and characterization of conductive protein material.

3 NEXT STEPS IN ENGINEERING CONDUCTIVE PROTEIN MATERIALS

The past decade has seen significant strides made in our knowledge of how conductive protein materials work. Their unique ability among biological molecules to conduct electrons over micrometer-length range has made the design and engineering of novel conductive protein materials a key research interest. From increasing the utility of *Geobacter* pili through surface

functionalization and scalable production,[128, 129] to the synthesis of new self-assembled conductive protein fibres,[19-22] recent advances point to the fabrication of functional devices integrating these materials as the logical next step. Nevertheless, challenges such as those highlighted in the previous section hinder the shift from fundamental investigation of material properties to integration in relevant applications. Here, we propose that the use of a standard engineering workflow can help research groups bridge this gap. Given the multitude of factors influencing protein fibre conductivity, we believe that such a workflow should be application-oriented, testing materials under working conditions. The complexity of the molecules and mechanisms involved would make characterizing protein long-range conduction under every possible condition a resource intensive task. Cutting through the clutter by identifying and testing a few key parameters can allow faster device development. The five key steps we believe to be essential in the workflow for novel conductive protein engineering are illustrated in Figure 6. In the previous section, we presented an overview of the major conditions impacting protein conduction. Here, we discuss improvements to the development cycle, highlighting one step in particular—computer-aided rational design (Figure 6B).

Most engineered conductive proteins utilize closely spaced aromatic residues as the principal motifs for conveying electronic properties and have been engineered accordingly.[19-22, 110] Proper folding and spatial orientation of these aromatic residues form a crucial role in governing the electrical behaviour of protein fibres. Despite the accepted importance of structure, the engineering process of novel fibres still uses computational modelling and simulation *ex post facto*—after design and synthesis have been completed—rather than as a tool to guide rational design. Alpha-helical peptides designed with abundant aromatic residues in stacking formations were simulated via molecular dynamics by both Ing et al[22] and Creasey et al,[20] but used only to compare with experimentally derived structural results rather than drive the design process. For engineered conductive curli fibre, molecular dynamics simulations were equally used to verify the structural stability of mutant fibres. While a model of the native curli protein was consulted

beforehand to identify favourable mutation sites, computational modelling was not used to simulate the structure or charge transfer behaviour of mutants.[19]

With the ever-increasing power of computational modelling tools, we propose to use them to guide the design of novel conductive proteins. By identifying promising mutants and discarding others through computer-aided design, time and resources can be saved during the characterization steps. A variety of computational tools have already been developed and used. First principles quantum-mechanical calculations coupled with molecular dynamics was used to explore the charge transport mechanisms of *Geobacter* pilin in solvated conditions.[47, 48] New tools such as eMap,[130] which allows the identification of hole or electron hopping pathways, as well as the variety of molecular dynamics software available,[131] could be integrated into the rational engineering workflow of conductive proteins, not just as verification tools but as the first step in design. Though challenges remain, increased collaboration between research groups focused on modelling and groups doing fundamental research on conductive proteins can lead to the development of computational models that more accurately predict the structural and electrical behaviour of protein fibres.

4 CONCLUSION

In this review, we highlighted the need for a set standard of testing protocols to reduce variability between results presented by research groups. The effects of many factors on protein conductivity make extracting relevant data and comparing protein materials difficult. The new engineering workflow that we propose is built from an application-focused approach to reduce time spent in the development cycle of novel conductive protein materials.

Obstacles remain on the road to fabrication of devices integrated with conductive protein materials. Gaps in our understanding on how both naturally occurring and engineered fibres conduct charges and respond to environmental stimuli hinder the transition from fundamental research to application. Further investigation into the electrical properties of conductive protein biomaterials and how these properties can be controlled is necessary before integrating conductive protein materials into functional devices. Promising applications for material such as

biohybrid photo electrochemical cells,[132] transistors,[133, 134] microbial fuel cells,[135-137] and flexible circuits in the nano and microscale.[138]

Multifunctional peptides exhibiting biocompatibility,[139] biodegradability,[90] or used as living materials[140] represent a gold mine for novel device designs, stemming from the many unique properties that can be introduced from this class of material. A coordinated, holistic approach to conductive protein biomaterial development involving researchers across many disciplines is necessary in order to fully realize the promise of this novel class of materials for next-generation, sustainable, electronic devices.

FIGURE CAPTIONS

FIGURE 1: Hydration level affects electron transfer in proteins. A, increasing conductivity of *G. sulfurreducens* is observed as the hydration of the biofilm increases. The water content of conductive protein films may affect the kinetics of electron transfer or the structural stability of the protein.[43] B, Changes in folding may change the distance between aromatic amino acids that contribute to charge transport, this distance is a key parameter in charge transfer kinetics.[47] C, Water solvation is necessary to promote proper protein folding via the hydrophobic effect and hydrogen bonding which stabilize protein structure.[53] A, Reproduced from Phan et al with permission from the PCCP Owner Societies. B, Reproduced from Feliciano et al with permission from the PCCP Owner Societies. C, Reprinted from *Computer Methods in Applied Mechanics and Engineering*, Vol. 197, S. Keten, M. J. Buehler, pp. 3203-3214, Copyright 2008, with permission from Elsevier.

FIGURE 2: The ionic environment can strongly affect how conductive proteins behave electronically. A, Lower pH values result in increased conductivity in wild-type *G. sulfurreducens*. This may be due to several reasons, including structural changes that result in changes of aromatic amino acid conformation.[18] B, *G. sulfurreducens* undergoes changes in overall charge as a result of pH variations. Changing charge may induce structural or aggregation differences compared to neutral pH conditions.[37] C, Theoretical current-voltage responses under various assumed conditions are shown, such as predicted results for a sample under concentration-gradient -driven charge transfer. The plateau occurs as mass transfer limits the availability of charge carriers. D, A current-voltage sweep of an electric-field-driven charge transfer is shown, where only the applied potential limits the current. Environmental effects or measurement setups might induce switching between both exponential and linear behaviours in a single material.[50] E, The complex structure of *G. sufurreducens* is shown as predicted via homology modelling. Aromatic amino acids (phenylalanine and tyrosine) are shown in yellow, while basic and acidic amino acid residues are shown in blue and red, respectively. The electronic environment of proteins can be strongly affected by changes in pH or ions due to

amino acid residues.[37] A, Reproduced from Adhikari et al with permission of by The Royal Society of Chemistry. B, Reproduced from Malvankar et al. C and D, Reproduced from Strycharz-Glaven et al, with permission from The Royal Society of Chemistry. E, Reproduced from Malvankar et al.

FIGURE 3: Temperature is often used as an indicator to determine the charge transport mechanism. Different temperature-conductivity behaviours are reported in: A, *G. sulfurreducens* by different research groups.[47] The current response of *G. sulfurreducens* was investigated at a fixed voltage and changing temperature to produce the temperature dependence of conductivity. All experiments were conducted in buffered conditions, with: B, performed on live *G. sulfurreducens* biofilms.[35] C, Investigation of both free-standing biofilm and purified pili was conducted.[6] D, Temperature-conductivity measurements were performed on purified films containing protein only.[31] While B, Yates et al report hopping-like dependence where higher temperatures result in higher conductivity, C, Malvankar et al and D, Ing et al report metallic-like temperature dependence; increasing conductivity with decreasing temperature. A, Reproduced from Feliciano et al with permission from the PCCP Owner Societies. B, Reproduced from Yates et al with permission from the PCCP Owner Societies. C Reprinted by permission from *Nature Nanotechnology*, N.S. Malvankar et al, Copyright 2011. D Reproduced from Ing et al with permission from the PCCP Owner Societies.

FIGURE 4: Varying measurements set-ups are used for measuring the conductivity of protein materials at different length scales. Single fibres correspond to protein materials in the nanoscale. Since the radius of the fibre is negligible compared to its length, the system can be considered as one dimensional. A, Voltage is usually applied across the length and current passing through the fibre is measured via c-AFM. B, Current can also be measured across the diameter of the fibre via c-AFM where the potential is applied below and above the fibre. When thickness reaches the microscale, protein films are considered as two dimensional. C, Fibres are deposited onto interdigitated gold electrodes; or D, other metallic designs of electrodes with contact-pads where probes measure the current passing through the thin film. While more layers of protein fibres are

added to reach a considerable thickness, other properties of the material arise. E, Bulk conduction can occur in thicker protein films, which can be integrated into circuits to measure its conductivity. F, Elasticity and gelation properties can be used for signal response and transmission measurements. The protein film is placed between metallic electrodes that measure change in electrical resistance while mechanical deformations are induced to the material using clamps.

FIGURE 5: The potential window chosen for characterizing engineered conductive protein materials varies among research groups. Some have characterized up to 5 V, while others remain below 1 V with differing current responses observed. While A[20] displays very little linearity in the current-voltage response, both B[22] and C[21] display relatively linear trends. Finally, D[19] displays a transition from linear at low potentials to non-linear at high potentials. In A, synthetic alpha helical peptides that were aromatic rich (six aligned phenylalanines) were measured. B, is also a synthetic alpha helical peptide (ACC-hex fibres) with three phenylalanines buried in the hydrophobic core of the assembled structure. Aβ are amyloid-β fibres used as a comparison. C and D, are mutations of naturally occurring *E. coli* curli fibre protein subunits (WT), with C attaching an aromatic residue rich sequence (aromatic residue tripeptide of either all histidine, phenylalanine, tyrosine, or tryptophan) to the C-terminal of the curli fibre subunit, and D mutating one row of aligned residues on the exterior of the β-helical structure to aromatic residues (phenylalanine, tryptophan, tyrosine, or histidine). The corresponding protein structures are shown by each current-voltage plot. All proteins shown self-assemble into larger fibres which aggregate into films. A, Reprinted with permission from R. C. G. Creasey, A. B. Mostert, A. Solemanifar, T. A. H. Nguyen, B. Virdis, S. Freguia, B. Laycock, *ACS Omega* **2019**, 4, https://pubs.acs.org/doi/10.1021/acsomega.8b02231. Copyright 2019 ACS Nano. Further permissions should be directed to the ACS. B, Reprinted (adapted) with permission from N. L. Ing, R. K. Spencer, S. H. Luong, H. D. Nguyen, A. I. Hochbaum, *ACS Nano* **2018**. Copyright 2018 American Chemical Society. C, Reproduced from Kalyoncu et al – Published by The Royal Society of Chemistry. D, Reproduced from Dorval Courchesne et al with permission from IOP

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FIGURE 6: The ideal workflow for engineering novel conductive protein materials. A, The first step when engineering conductive protein materials is to determine its conduction mechanism. B, This can be facilitated by protein modelling, which indicates the most probable pathways for electron transfer. C, When the design for conduction is optimized, the synthesis of the protein of interest needs to be confirmed. D, Next, it is crucial to establish electrical characterization procedures that reflect the end-use of the proteins and their role in a desired device. At this stage of collecting experimental data on proteins, hydration level, pH, temperature, as well as spacing and geometric considerations of the electrical set-up all need to be carefully taken into account. E, When conductivity results meet requirements for the potential device's functional use, the focus shifts to the integration of the protein material within a prototype. Added desired properties of the conductive material such as biocompatibility, interfacial response and signaling or biodegradability can also be tested for.

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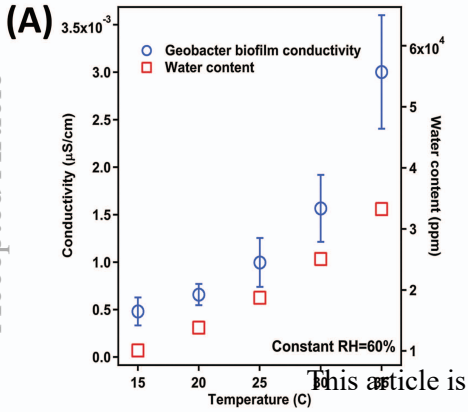
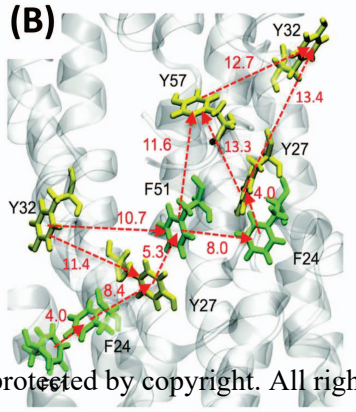
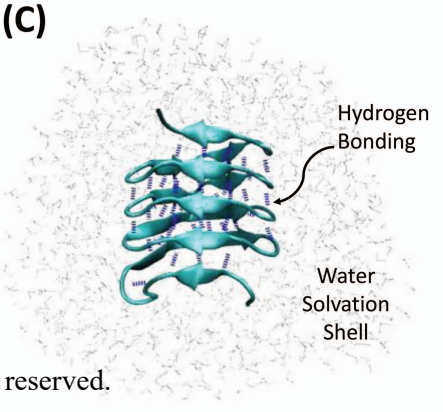
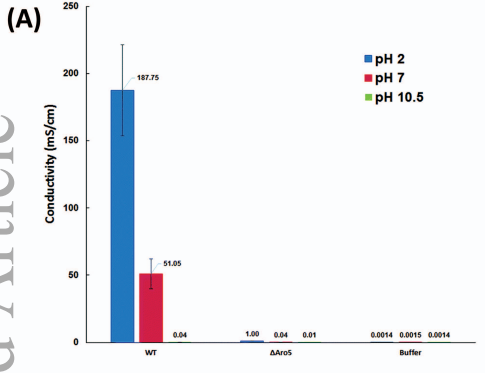
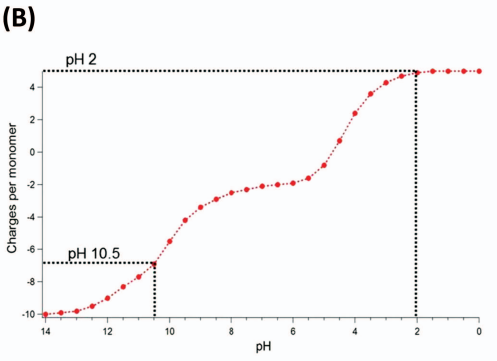
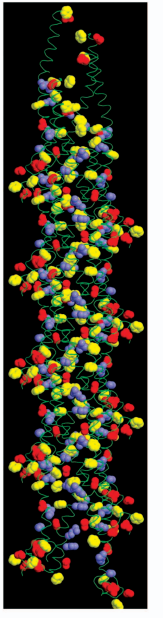


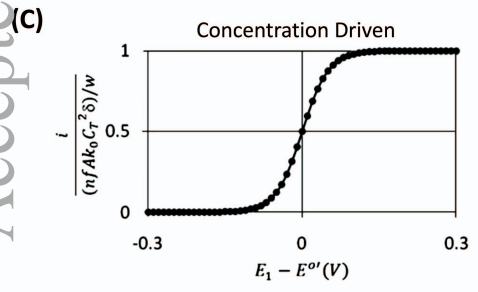


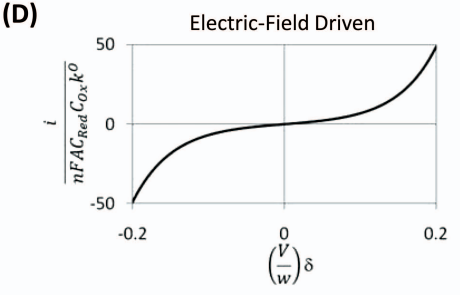






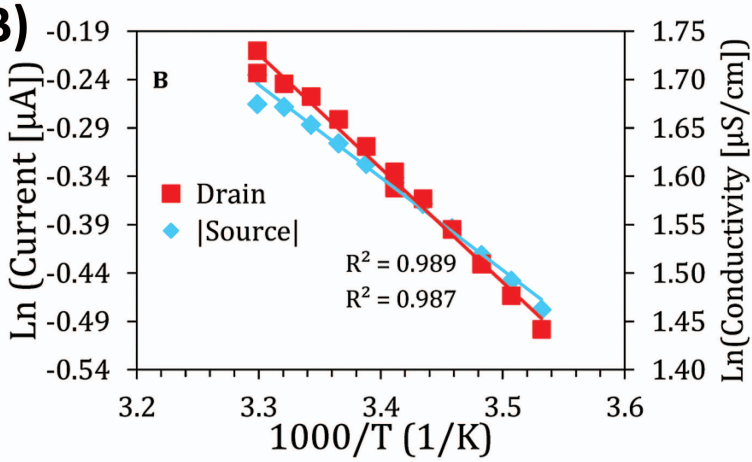






(A)

(B)



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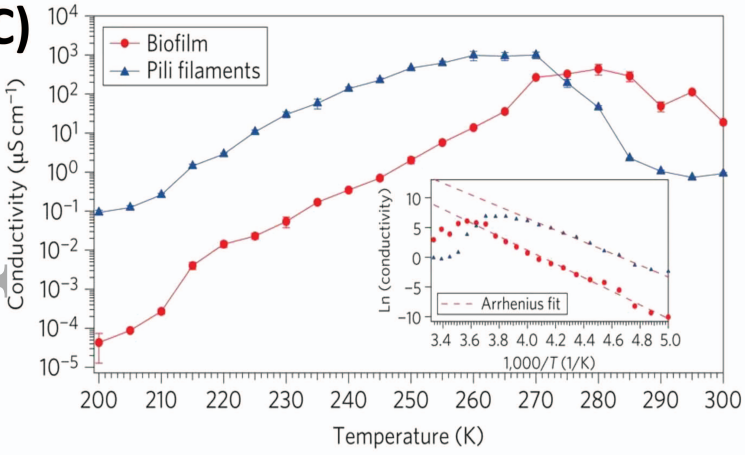

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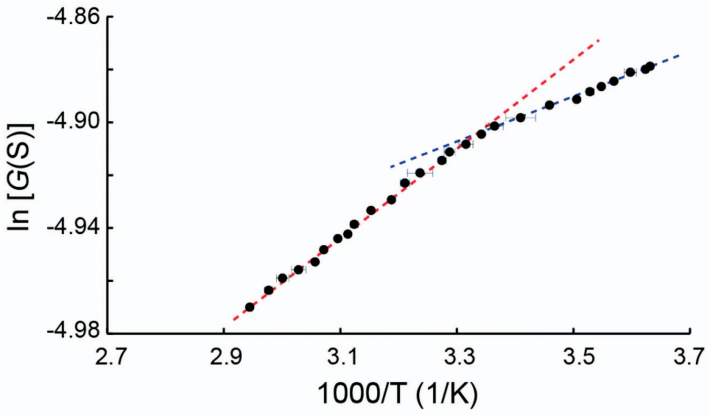
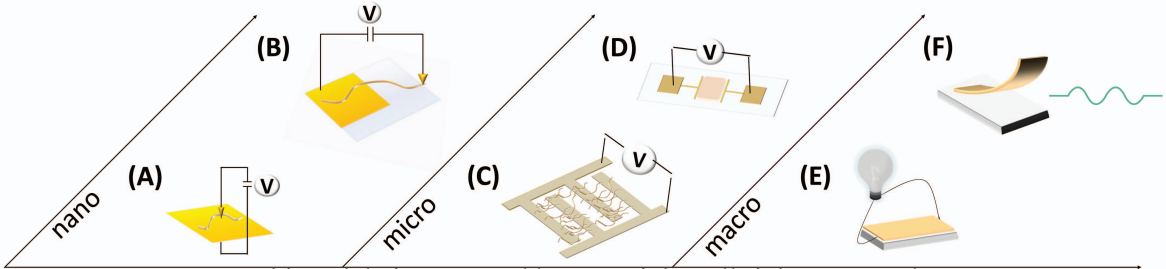
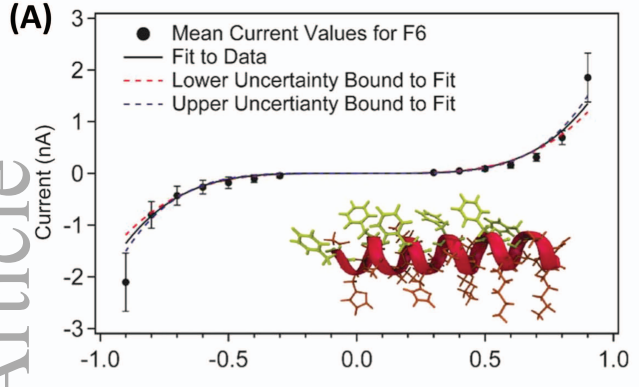
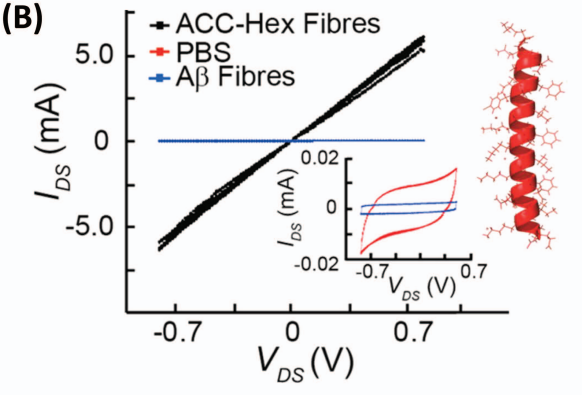
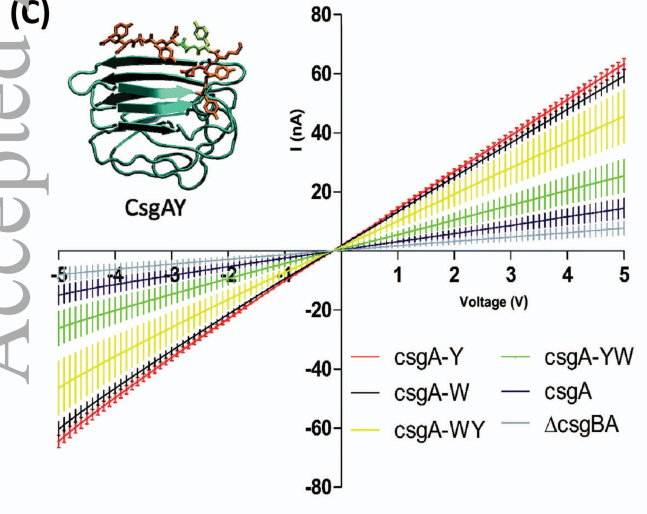
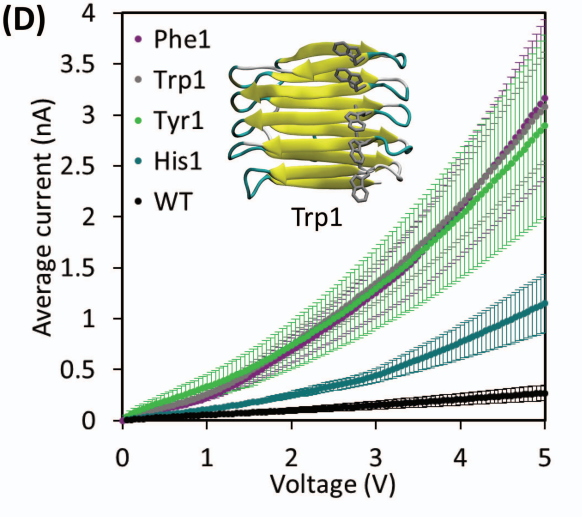


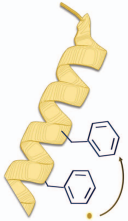




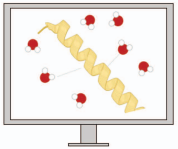




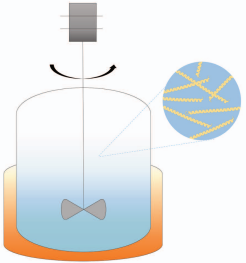




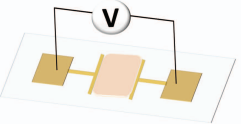
(A) Design of Conduction Mechanism



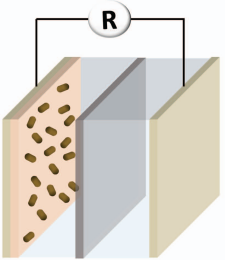
(B) Protein Modelling



(C) Biosynthesis Process



(D) Material Characterization



(E) Device Integration & Testing