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Review

Electro-Biofabrication. Coupling Electrochemical and Biomolecular Methods to Create Functional Bio-Based Hydrogels

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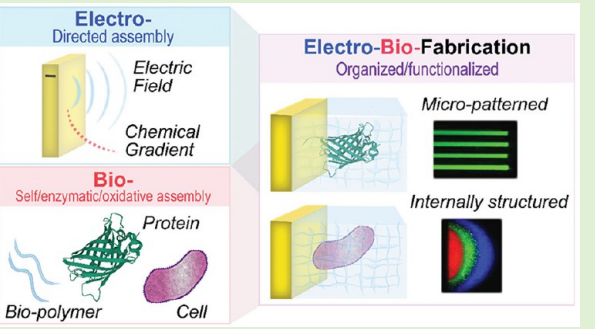
ABSTRACT: Twenty years ago, this journal published a review entitled "Biofabrication with Chitosan" based on the observations that (i) chitosan could be electrodeposited using low voltage electrical inputs (typically less than 5 V) and (ii) the enzyme tyrosinase could be used to graft proteins (via accessible tyrosine residues) to chitosan. Here, we provide a progress report on the coupling of electronic inputs with advanced biological methods for the fabrication of biopolymer-based hydrogel films. In many cases, the initial observations of chitosan's electrodeposition have been extended and generalized: mechanisms have been established for the electrodeposition of various other biological polymers (proteins and polysaccharides), and electrodeposition has been shown to allow the precise control of the hydrogel's emergent microstructure. In addition, the use of biotechnological methods to confer function has been extended from tyrosinase conjugation to the use of protein engineering to create genetically fused assembly tags (short sequences of accessible amino acid residues) that facilitate the attachment of function-conferring proteins to electrodeposited films using alternative enzymes (e.g., transglutaminase), metal chelation, and electrochemically induced oxidative mechanisms. Over these 20 years, the contributions from numerous groups have also identified exciting opportunities. First, electrochemistry provides unique capabilities to impose chemical and electrical cues that can induce assembly while controlling the emergent microstructure. Second, it is clear that the detailed mechanisms of biopolymer self-assembly (i.e., chitosan gel formation) are far more complex than anticipated, and this provides a rich opportunity both for fundamental inquiry and for the creation of high performance and sustainable material systems. Third, the mild conditions used for electrodeposition allow cells to be co-deposited for the fabrication of living materials. Finally, the applications have been expanded from biosensing and lab-on-a-chip systems to bioelectronic and medical materials. We suggest that electro-biofabrication is poised to emerge as an enabling additive manufacturing method especially suited for life science applications and to bridge communication between our biological and technological worlds.



1. INTRODUCTION

In 2005, the two senior authors copublished a review in which we suggested a vision for biofabrication which, after some modification, was refined to "building with biological or biomimetic materials and mechanisms".[1] Later, the terms "biofabrication", like the term "biomaterials", were refined (by others) with the focus of the "bio" prefix being on the application (e.g., regenerative medicine) not the method of fabrication.[2−5] While our use of the term "biofabrication" may not be consistent with common usage, we believe the underlying vision remains intact.

Our 2005 review was motivated by two discoveries. First, the pH-responsive aminopolysaccharide chitosan was observed to electrodeposit: localized electrical inputs could direct this polymer to self-assemble over a hierarchy of length scales onto an electrode surface.[6−9] Because this directed self-assembly is spatially, temporally, and quantitatively controllable, and because it can be performed from aqueous solution (e.g., within a covered microfluidic device), we envisioned this method could offer unique opportunities for interfacing biology with microfabricated electronic devices.[10−12] Second, biomolecular mechanisms were used to couple protein-based functionality to electrodeposited hydrogels.[13] Specifically, a protein was engineered to have a fusion tag that enabled this protein to be enzymatically assembled to chitosan.[14−19] In this Review, we illustrate how an extensive set of collaborators have

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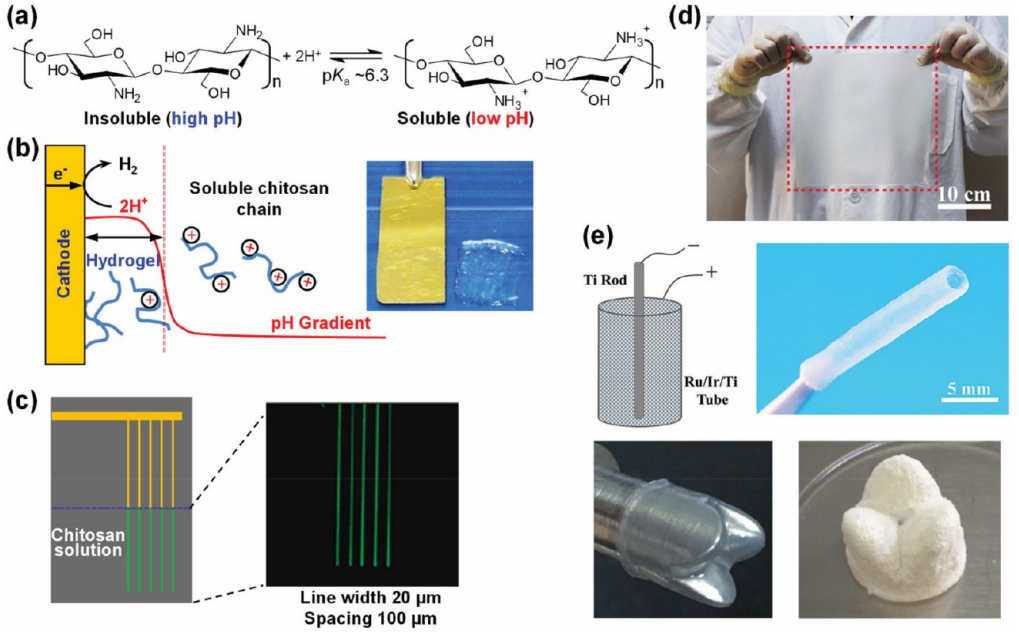

Figure 1. Chitosan's pH-responsive self-assembling properties enable its electrodeposition. (a) Chitosan's primary amines serve as reversible "switches" for chitosan's solubility. (b) Cathodic neutralization mechanism for chitosan's reversible electrodeposition. Chitosan's electrodeposition (c) onto a patterned electrode is spatially selective (Adapted with permission from ref 7. Copyright 2003 ACS), (d) is scalable based on the electrode's surface area, and (e) can conformally coat complex surfaces. Adapted with permission under a Creative Commons CC BY 4.0 License from ref 35. Copyright 2022 John Wiley and Sons.

broadened the original vision and expanded the range of applications.

Here, we not only summarize progress but also review how advances in electro-biofabrication methods have revealed gaps in our fundamental knowledge and, conversely, how a deeper fundamental understanding has enabled the fabrication of increasingly complex soft matter assemblies that can be tailored to specific needs. Further, we explain why we believe electro-biofabrication, and especially electrodeposition, is poised to emerge as an alternative and complementary additive manufacturing technology.

Much of our research has focused on the amino-polysaccharide chitosan, which is obtained from the partial deacetylation of chitin. Chitin is the second most abundant polysaccharide on earth as it is used as a structural polymer in various organisms such as fungi, insects, and crustaceans.[20−25] Chitosan is a linear copolymer of glucosamine and *N*-acetylglucosamine although the sequence of these residues cannot be easily controlled or measured (Figure 1a shows only glucosamine residues). Chitosan is the nature-derived polymer with the highest content of primary amines, and this is important because primary amines can confer important properties to polymers. First, primary amines can confer pH responsiveness. For instance, at low pH, chitosan's amines are protonated and the polymer is a water-soluble cationic polyelectrolyte; at high pH, chitosan's amines are deprotonated and the polymer is neutral and insoluble, often forming a hydrogel. Interestingly, chitosan's pK_a is low (about 6.3) compared to other primary amines, and thus, chitosan undergoes its sol−gel transition at near neutral pH conditions. Second, the neutral form of a primary amine has an unshared pair of electrons which make it nucleophilic, and this allows relatively simple coupling chemistries to be used to covalently graft functional groups onto the polymer. In a simplistic way, chitosan's amines can be thought of as switches (albeit not perfectly digital): at low pH, these amines are protonated which makes chitosan cationic, soluble, and unreactive, while, at high pH, the amines are deprotonated which makes chitosan neutral, insoluble, and reactive.

2. ELECTRO-BIOFABRICATION: FROM CUING SELF-ASSEMBLY TO CONTROLLING EMERGENT STRUCTURE AND PROPERTIES

2.1. Cathodic Neutralization Mechanism. In 2001, we were one of two groups who independently reported that chitosan could be electrodeposited[9,26] by a cathodic neutralization mechanism illustrated in Figure 1b.[8,27] Cathodic electrolysis reactions (e.g., with H_2O or H_2O_2) can generate a localized region of high pH adjacent to the cathode, and if this electrochemical reaction is performed in a slightly acidic solution of chitosan, then a gel is observed to deposit on the cathode surface (Figure 1b). Mechanistically, the chitosan chains in the high pH region undergo deprotonation which cues their self-assembly into a hydrogel network that is cross-linked through the reversible formation of crystalline network junctions.[28] The photographs in Figure 1b show that the electrodeposited film is typically transparent and remains attached to the electrode, although it can be peeled away to form a free-standing film.

From a processing standpoint, there are several important features of electrodeposition. First, it is controllable with the thickness of the deposited film being controlled by the electrical input (e.g., current or voltage) imposed at the electrode.[29−34] Second, it is spatially selective as illustrated by deposition of a fluorescently labeled chitosan onto the patterned electrodes in Figure 1c.[7] Third, it is scalable to larger surface area films as illustrated by the photograph in Figure 1d.[35] Fourth, it can coat complex surfaces conformally as illustrated in Figure 1e.[35] Finally, and maybe most importantly, electrodeposition is simple in that it uses

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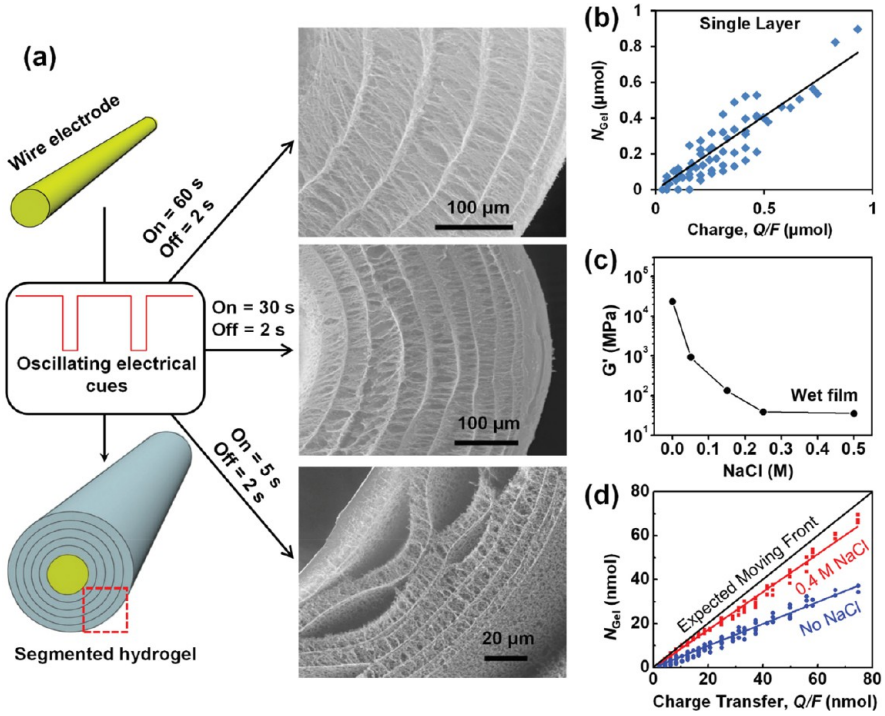

Figure 2. The emergent structure during electrodeposition is controlled by conditions in ways that are not yet understood. (a) Oscillating electrical inputs (ON-state 0.5 mA; OFF-state 0 mA) can generate segmented output structures as shown by the SEM images. (b) A simple moving front model predicted a linear relationship with a slope of 1. Adapted with permission from ref 38. Copyright 2014 Royal Society of Chemistry. (c) The presence of salt in the deposition solution profoundly affects the structure/properties of the deposited films. Reproduced with permission from ref 29. Copyright 2013 Royal Society of Chemistry. (d) The addition of salt into the deposition solution leads to better agreement with the moving front model, presumably by screening the electric field and suppressing chain migration. Adapted with permission from ref 39. Copyright 2018 ACS.

inexpensive electrochemical instrumentation under ambient conditions with low voltages (typically less than 5 V), rapid as it requires seconds to minutes (e.g., the film in Figure 1d was generated in 20 min), and reagentless. Because of the simplicity of chitosan's electrodeposition, it has been adapted by many groups around the world.

In 2008, the Domard group was studying chitosan's pH-induced gelation and reported that, if the gelation process was systematically interrupted, then a complex layered structure was generated (they referred to this as an onion structure).[36,37] Inspired by this report, the Shi group investigated the effects of interrupting electrodeposition.[38] As illustrated in Figure 2a, they used a wire electrode and repeatedly switched the imposed input current between an ON-state and an OFF-state (the imposed current was set to zero) in a 1.0% chitosan solution. As illustrated by the SEM images in Figure 2a, the electrodeposited film possessed a segmented structure with segments grown during the ON-state and boundaries formed during the OFF-state. Importantly, this procedure is highly controllable; changes in the ON time led to changes in the thickness of the segments,[38] while changes in the OFF time led to changes in the thickness of the boundaries.[39]

While the ability to electrodeposit segmented structures of Figure 2a is reproducible, the underlying mechanisms responsible for the emergence of this complex morphology are not understood. In an initial effort to deepen our understanding of electrodeposition, we developed a moving front mathematical model.[38] This model assumes that each electron transferred at the electrode results in the removal of a proton from the chitosan chains (i.e., chitosan is the only "buffer" in the deposition solution) with the chains closest to the electrode being progressively deprotonated. The model also assumes that, once a chain is deprotonated, it immediately undergoes gelation. Finally, the model assumes the chains are stationary in that they are not moving during deposition (the chains remain in the same spatial location, although their protonation/gelation state can be switched). Importantly, this model links the observable output response (the growth of the hydrogel, which can be measured in real-time by microscope-based imaging) to the imposed electrical input (the cumulative number of electrons transferred at the electrode, which is sensitively measured by the electrochemical instrument).

Qualitatively, this moving front model is consistent with the experimental observation that the depositing chitosan hydrogel grows from the cathode as a front and this gelation front co-localizes with a pH front (i.e., the gelation front has a steep pH gradient separating the high pH gel region adjacent to the cathode from the low pH bulk solution).[31] Quantitatively, the model predicts that Figure 2b should show a linear relationship between the gel thickness measurements (y-axis) and the electrical input measurements (x-axis),[38] and the experimental results seemed to be reasonably consistent with this prediction. The model also predicted a slope of 1, and the deviation of this experimentally observed value from 1 was a harbinger of what we did not know.

2.2. Low Energy Interactions Control the Emergent Structure. Early in our studies, there were several lab members electrodepositing chitosan hydrogel films, each from a different chitosan solution. While chitosan films were always being deposited, there were remarkable differences in

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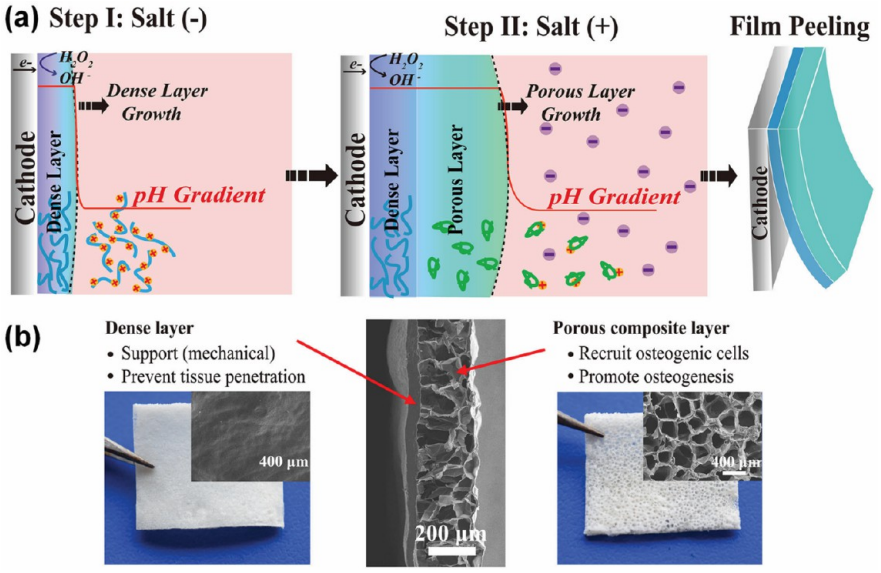

Figure 3. A two-step electrodeposition method to electrofabricate a Janus film. (a) A dense layer is formed by deposition from a low-salt solution, while a porous layer is formed by deposition from a high-salt solution. (b) Structure of the Janus film. Adapted with permission from ref 42. Copyright 2019 John Wiley and Sons.

the appearance and mechanical properties of these films. After considerable effort, we recognized the importance of the salt content of the deposition solution. Figure 2c shows the mechanical properties of chitosan hydrogels deposited from chitosan solutions containing different levels of salt (measurement by quartz crystal microbalance with dissipation; QCM-D).[29] The differences in this observed mechanical property were notable because they were very large (3 orders of magnitude for the wet films) and because these differences were not expected.

Salt is known to screen electrostatic interactions and also to screen an electrical field; thus, we next attempted to better understand the roles of salt and the electrical field on electrodeposition. Experiments showed that, in the absence of salt, chitosan chains were observed to migrate toward the cathode.[39] Qualitatively, it is not surprising that the cationic chitosan chains moved toward the negative electrode; however, it seemed surprising to us given the comparatively low voltages and shorter times compared to gel electrophoresis. In the presence of salt, experiments showed a significant suppression of this migration. Molecular modeling studies from the Shen group supported these experimentally observed effects of salt on the field-induced migration[39] and also suggested salt could partially collapse the individual chitosan chains.[40] In addition to driving chain migration, the imposed electric field also appears to align the chitosan chains during electrodeposition as measured by quantitative polarized light microscopy.[39] It has not yet been possible to establish agreement between experimental and molecular modeling results[41] to resolve how the electrical field promotes chain alignment in the depositing film.

While much remains to be learned about the electric field effects on chitosan's electrodeposition, our understanding of the salt effects has improved. Specifically, when chitosan is electrodeposited in the absence of salt, the electric field induces chain migration, thus recruiting chains into the growing hydrogel film: this slows the observed growth of the gel front since more chains/volume must be neutralized; however, the gels deposited in the absence of salt are more dense (i.e., higher polymer content and lower water content). In comparison, the addition of salt suppresses chain migration leading to a gel front that grows more rapidly and yielding a deposited gel that has a lower polymer density, higher porosity, and lower mechanical strength. Returning to the moving front model, Figure 2d shows that, in the presence of salt, the observed behavior approaches the behavior predicted (i.e., a linear relationship with a slope approaching 1).[39] We believe that this better agreement between experiment and predictions from the moving front model occurs because the suppression of chain migration by salt addition makes the experiment more consistent with the model assumption that the chains are stationary (i.e., the model ignores field-induced chain migration).

In the above discussion, we explained how the experimentally observed salt effects are driving fundamental studies to understand molecular-level details of the electrodeposition mechanism, but clarification of these salt effects is also enabling better control of the emergent hydrogel structure during chitosan's electrodeposition. For instance, the Qu group used a two-step electrodeposition step to create a Janus hydrogel film as illustrated in Figure 3a.[42] Importantly, the two faces of these Janus films are composed of the same material (i.e., chitosan) but are fabricated to have markedly different morphological structures. In the first step, electrodeposition was performed using a chitosan solution with no added salt in order to deposit a dense film. Next, the electrode coated with this dense film was transferred into a salt-containing chitosan solution to generate a porous film. The Janus film structure (Figure 3b) is useful for guided bone regeneration because the dense film provides mechanical support and serves as an external barrier to prevent fibroblast cells from infiltrating the wound site, while the porous layer serves as a scaffold for the ingrowth of bone-forming osteogenic cells.

The observation that salt has such a dramatic effect on the emergent structure of the depositing chitosan film emphasizes the importance of the low energy interaction mechanisms that are responsible for chitosan's self-assembly. Specifically, electrostatic repulsions between the protonated amines drives

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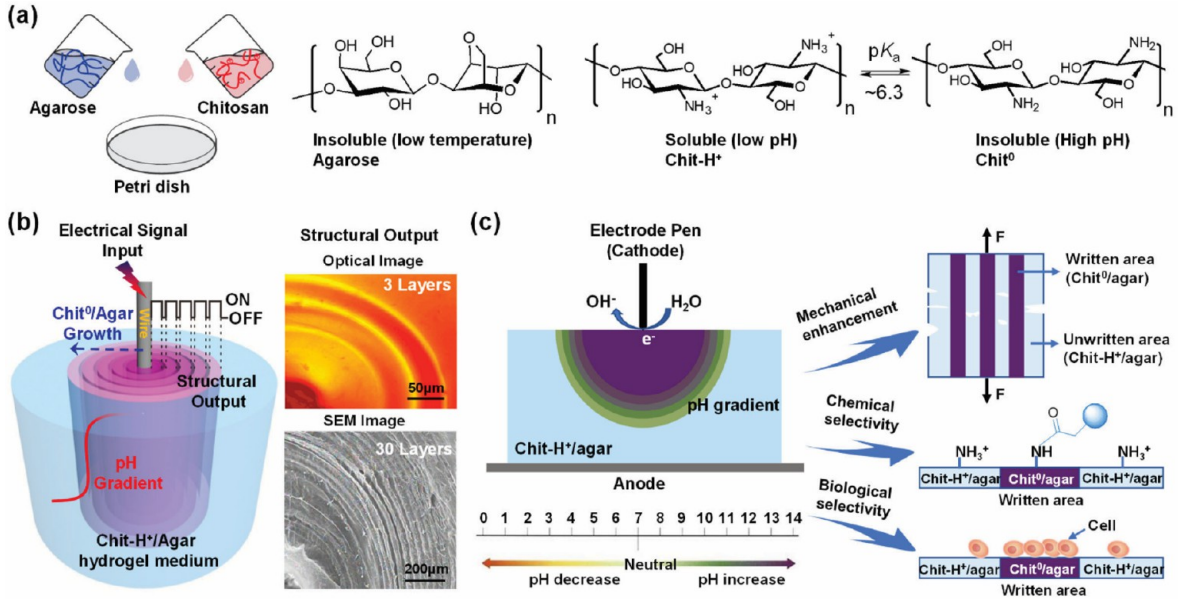

Figure 4. Transduction of electrical inputs into structural outputs by a dual-responsive medium. (a) A blend of chitosan (pH-responsive) and agarose (thermally responsive) can yield a dual responsive "medium" in which a single polymeric network or an interpenetrating network of two polymers can form. (b) Oscillating electrical inputs generated a complex segmented output structure. Reproduced with permission from ref 84. Copyright 2016 ACS. (c) Electrode writing imposes a transient electrical signal that patterns the medium with structurally and functionally different regions. Adapted with permission from ref 86. Copyright 2018 John Wiley and Sons.

cationic chitosan chains to be soluble, while hydrogen bonding and hydrophobic interactions provide the driving force for neutral residues from different chains to associate to form the crystalline network junctions that serve as the physical cross-links in chitosan's hydrogel.[28] Changes in the balance of these interactions during the deposition process can alter the hydrogel's emergent structure in ways that are not entirely predictable. From a practical perspective, salt is a simple and easily controllable process variable that can be used to manipulate the emergent structure as illustrated in Figure 3b.

Another easily controllable process variable is temperature, which is known to affect the balance between hydrogen bonding and hydrophobic interactions. Recent experimental studies demonstrate that chitosan could not be electrodeposited at higher temperatures (50 °C), while the dynamics of electrodeposition at subambient temperature (5 °C) were observed to be complex, even suggesting that the deposited chains may be able to reconfigure after gel formation.[35] These experimental studies are driving a new round of molecular modeling studies to discern the mechanistic basis for this temperature effect.

2.3. A Versatile Tool for Thin Film Coating. It is well-known that electrodeposition is an important tool for fabricating a variety of surface coatings on conducting materials, although in this Review, we only focused on electrodepositing biologically derived hydrogels. Readers who are interested in other aspects of the electrodeposition are directed to several reviews on various topics including conducting polymers,[43−45] polymer-based functional coatings (e.g., paints,[46,47] proteins/enzymes,[48−51] and polyelectrolyte coatings[52,53]), low-molecular-weight hydrogelators,[54−59] metal-phenolics,[49,51] and metal nanostructures.[60−63] In addition, using electrode reaction and surfactant (i.e., electrophoretic deposition) to fabricate surface coatings of inorganic and/or polymeric composite materials has found a variety biomedical applications.[64−68] Further, functional surface coatings can also be achieved by co-depositing a mixture of stimuli-responsive materials and additional functional components. For instance, chitosan[69−71] or alginate[72−74] were used to co-deposit inorganic nanoparticles onto metallic implant materials,[75] and pH responsive Fmoc-amino acids were used to co-deposit thermal responsive agarose[76] or gelatin[77] that served as a template for further biofunctionalization.[78] These capabilities, when appropriately enlisted, offer significant benefits for fabrication such as simplicity, rapidness, low cost, and exquisite temporal and quantitative control of the electrical cues to guide assembly.

2.4. Electroassembly in a Dual-Responsive Medium: Electromolecular Information Transduction. While the above studies considered the electrodeposition of chitosan, a different set of experiments were performed by the Shi group to examine how electrical inputs could be imposed on a hydrogel composed of two stimuli-responsive self-assembling polysaccharides. In initial studies, Figure 4a shows they blended an acidic solution of chitosan with a warm solution of the thermally responsive agarose polysaccharide.[79] This blend was cooled to allow the agarose (Agar) network to form, while the chitosan chains remained protonated and disassembled (designated as Chit−H^+). To demonstrate that the chitosan chains in this Chit−H^+/Agar network could still undergo pH-responsive self-assembly, they immersed the gel in base and observed the appearance of an X-ray diffraction peak, consistent with the formation of chitosan's crystalline network junctions. Also, they observed that the base-treated hydrogel was mechanically stronger consistent with the formation of a chitosan network that was interpenetrating within the agarose network. This process was reversible as reimmersion of the gel into an acid solution disassembled the chitosan network.

The chitosan−agarose hydrogel is an interesting system as it forms an interpenetrating network of two reversibly responsive polymers that respond to independent and orthogonal stimuli. Specifically, the chitosan network can be cued to assemble or

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disassemble in response to pH changes, while the agarose network can be cued to assemble or disassemble in response to temperature changes. Thus, the chitosan−agarose system offers exciting shape-shifting capabilities.[80−85]

In essence, the chitosan−agarose system can be thought of as a dynamically responsive medium for information storage. In initial studies, Figure 4b shows that a wire electrode was inserted into this medium and then electrical input was imposed to provide the pH cues to induce chitosan chains to self-assemble.[84] When an oscillating electrical input was imposed, a segmented output structure was generated, and this illustrates the broader point that complex electrical inputs can be used to generate information-containing patterns in supramolecular structure. Importantly, the pattern can be erased and the medium dissolved by treating with acid and heat, while cooling regenerates a medium that can be reprogrammed with another pattern.[80,82,87]

In a later study, Figure 4c shows the electrode was used as a "pen" to write onto the surface of the Chit−H^+/Agar. As in the previous example, this cathode pen provided the stimulus for a transient local increase in pH that induced chitosan to undergo deprotonation and gel formation. As in the previous studies, the patterns could be erased (by acid treatment) and the pen could be used to write a new pattern.[79,86,88] There are two fundamentally interesting features of this work. First, it illustrates the switching capabilities of chitosan: writing switches the amines from a protonated to a deprotonated state, and this also switches important functional properties (i.e., Figure 4c illustrates that the patterned region is functionally different from the unpatterned region).[86] Mechanically, the electrode-based deprotonation induces the assembly of the chitosan network that interpenetrates the agarose network and locally strengthens the medium in the patterned region. Depending on the written pattern, films can be generated with anisotropic mechanical properties. Chemically, the amines in the patterned region are neutral with reactivities that are different from those in the unpatterned region. Because of these chemical differences, the patterned region can selectively undergo reactions with various electrophilic agents and can also chelate silver. Biologically, fibroblast cells were observed to selectively adhere to the patterned region of the film, although the underlying reason for this difference is not known.[86]

The second important feature of this work was more puzzling: why is the written pattern stable and able to persist for days? If cathodic writing creates a transient local change in pH and this induces chitosan's hierarchical (i.e., supramolecular) assembly, then why does this assembled structure not dissociate after the pH gradient dissipates? This question was answered from molecular modeling studies. Specifically, Figure 5 shows that the self-assembled crystalline regions tend to be hydrophobic with little water and significant interchain hydrogen bonding.[86,89] The crystallite's local microenvironment stabilizes the neutral state of chitosan, essentially lowering its pK_a. This structure-induced pK_a shift enables the patterned region with neutral chitosan chains to stably persist adjacent to a region in which the chitosan chains are protonated.

2.5. Summary. Overall, these studies demonstrate that chitosan's electrodeposition by cathodic electrodeposition is a deceptively complex process. The imposed electrical input has two components that cannot be easily dissociated from each other. The current is associated with the electron transfer

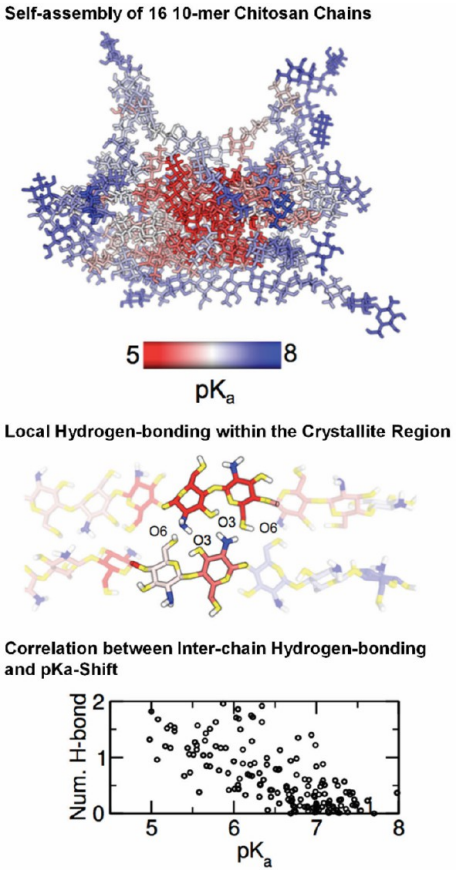

Figure 5. Self-assembly of chitosan chains stabilizes the neutral state (i.e., lowers the pK_a). Self-assembly of chitosan chains leads to localized hydrophobic regions in which little water is present and interchain hydrogen bonds are formed (e.g., these crystalline network junctions serve as the physical cross-links in chitosan's hydrogel). Reproduced with permission from ref 86. Copyright 2018 John Wiley and Sons.

electrochemical reaction that yields the pH change that induces chitosan's self-assembly. The potential (i.e., voltage) provides an electric field that can drive the protonated chains to migrate toward the electrode, and this field may also alter the conformation and alignment of the chains as they are depositing. It also seems possible that the electric field may alter the strength of the interactions (e.g., hydrogen bonds) responsible for self-assembly. Because chitosan's self-assembly relies on a large number of weak physical interactions, it is difficult to fully understand how process conditions (e.g., imposed electrical input sequence, salt levels, and temperature) can be tailored to control the emergent structure. Nevertheless, these process variables provide a rich design space, and each incremental increase in our fundamental understanding has made it possible to generate films of increasing complexity (e.g., hydrogels with complex internal structure).

3. COUPLING ELECTRODEPOSITION WITH OTHER METHODS

Chitosan's electrodeposition uses top-down electrical cues to induce bottom-up self-assembly, and as a result, electrodeposition can generate hydrogels with complex internal structure and is spatially selective (if a patterned electrode is used to provide the cues). Further, electrodeposition can be performed from solution without the need for direct contact

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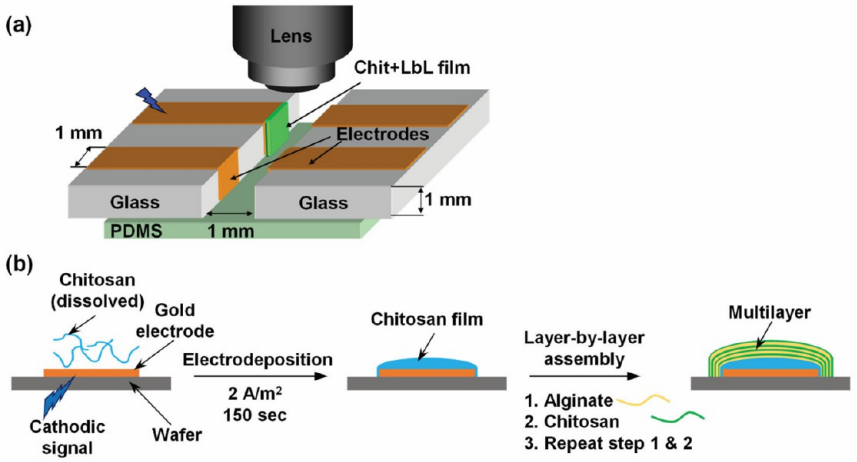

Figure 6. Chitosan's electrodeposition confers spatial selectivity to layer-by-layer (LbL) self-assembly. (a) Chitosan hydrogel films can be electrodeposited onto a side-wall electrode within a microfluidic device. (Note that the transparent cover of the device is not shown for clarity.) (b) Sequential contacting with alginate and chitosan allows a polyelectrolyte multilayer to be grown onto the electrodeposited chitosan "template".

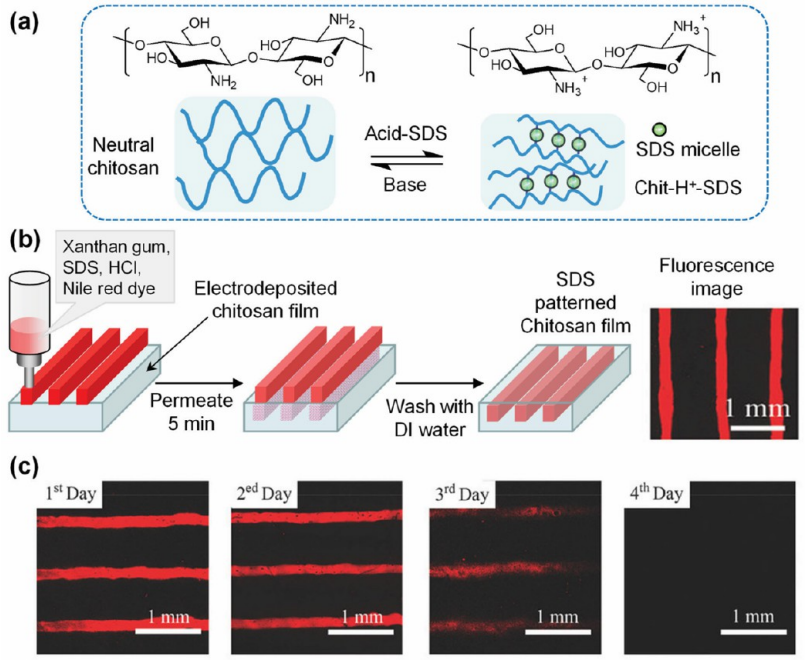

Figure 7. Chitosan's cross-linking can be switched between two reversible physical mechanisms. (a) Neutral chains can form crystalline network junctions, while protonated chains can be electrostatically cross-linked by multivalent anions (e.g., SDS micelles). (b) 3D printing with acidic SDS allows hydrogels to be patterned to have spatially varying properties (e.g., anisotropic mechanical properties). (c) The 3D printed patterns are stable because the electrostatic interactions between chitosan and SDS stabilize chitosan's protonated state (i.e., increase the pK_a). (Note: the red color in the printed areas is from the hydrophobic Nile red fluorescence dye.) Adapted with permission from ref 91. Copyright 2017 John Wiley and Sons.

(e.g., required by alternative printing methods) or line-of-sight (e.g., required by alternative photolithographic methods). In addition, the deposited film can be retained on the electrode surface (e.g., for electrochemical biosensing applications)[22] or peeled from the electrode (e.g., for food, pharmaceutical, or medical applications). It is also possible to couple the electrodeposition of chitosan with other fabrication methods.

3.1. Coupling with Other Additive Manufacturing Methods. Figure 6a shows chitosan's electrodeposition onto a side-wall electrode of a fluidic device (note that the transparent cover of the device is not shown for clarity). After electrodeposition, the fluidic channel can be rinsed, and the electrodeposited chitosan can serve as a "template" for the layer-by-layer (LbL) self-assembly of a polyelectrolyte complex.[90] Specifically, the sequential filling of the channel with solutions of chitosan (pH 5.6) and alginate (pH 6.2) with intermediate rinsing steps allowed a chitosan−alginate multilayer to be grown on the chitosan template, as illustrated in Figure 6b. When the enzyme glucose oxidase was included in the alginate solution, this enzyme could be incorporated within the assembled film and could then be used for the electrochemical detection of glucose. Thus, electrodeposition provides the template that confers spatial selectivity to LbL self-assembly, while LbL provides a generic means to incorporate functionality (e.g., enzymatic activity) into the assembled films.[90]

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The Dong group used 3D printing to pattern previously electrodeposited chitosan films. Specifically, they electrodeposited a chitosan film onto a titanium foil to generate a neutralized hydrogel film that was physically cross-linked by crystalline network junctions. If they treat these films with an acidic solution containing sodium dodecyl sulfate (SDS) micelles, Figure 7a shows the neutral chitosan chains are protonated, the crystalline network junctions disassemble, and strong electrostatic cross-links form between the cationic chitosan chains and SDS micelles (this network is designed Chit−H^+−SDS).[91] If this Chit−H^+−SDS film is further treated with base to deprotonate the chitosan, the electrostatic interactions are lost, and the neutral chitosan chains can reassemble to form crystalline network junctions. Thus, this film can be cross-linked by two different physical mechanisms and these mechanisms can be repeatedly switched (i.e., the cross-linking can be reconfigured). Interestingly, these two cross-linking mechanisms confer different mechanical properties: films cross-linked by crystalline network junctions are elastic, while the SDS-cross-linked films offer greater mechanical strength but are viscoelastic.[91−93]

As illustrated in Figure 7b, 3D printing was used to pattern Chit−H^+−SDS regions into the neutral chitosan film. Because the different cross-linking mechanisms conferred different mechanical properties, patterning enabled the creation of films with anisotropic properties.[91] There are two important extensions of this work. One extension focused on the stability of the printed pattern. As illustrated in Figure 7c, the printed patterns were stable for days in water, and the loss of the pattern seemed to be due to the SDS micelles being diluted into the aqueous phase rather than diffusion across the film. Analogous to Figure 4c, the stability of the pattern was puzzling since the pattern contained regions with protonated chitosan chains (engaged in electrostatic cross-links with SDS) adjacent to regions with neutral chitosan chains (assembled into crystalline network junctions) under conditions in which no pH gradient is expected to persist. Again, molecular modeling provided a mechanistic explanation for this stability: the electrostatic interaction with SDS stabilized the protonated state of chitosan's amines (i.e., shifted the pK_a upward).[94]

A second important extension involves the blending of chitosan and gelatin to create a multiresponsive medium. Specifically, the reversible and orthogonal physical cross-linking mechanisms of chitosan were integrated with the reversible thermally induced sol−gel transitions of the protein gelatin to generate a medium that could respond in predictable ways to various stimuli (pH, temperature, and multivalent anionic cross-linkers). This chitosan−gelatin−anion system was used with 3D printing to enable the fabrication of complex hydrogel structures.[95,96]

3.2. Coupling with Biomolecular Methods. In addition to the two reversible and orthogonal cross-linking mechanisms discussed above (Figure 7a), chitosan can undergo interactions through additional mechanisms, and such mechanisms have often been used to attach proteins to chitosan to confer biological functionality. As noted, chitosan's neutral amines are nucleophilic and a variety of coupling reagents have been used to covalently conjugate functional groups (i.e., proteins) to chitosan. In addition, chitosan can chelate with metals, and this has also been used for cross-linking and even as an electrodeposition mechanism (e.g., a copper electrode can be purposefully oxidized to convert the metallic atoms into soluble ions that can serve to cross-link chitosan).[97,98]

Two biomolecular-based methods have been used to confer protein-based functionality to chitosan. One method is protein engineering in which a short sequence of amino acid residues is "genetically fused" to the C- or N-termini of a protein (i.e., a fusion tag) to facilitate its assembly with chitosan. For instance, proteins with histidine tags could undergo nickel-mediated assembly to chitosan.[99] The second method is the use of enzymes to catalyze the conjugation of proteins to chitosan.[100,101]

These two biomolecular methods were combined for the tyrosinase-mediated oxidative assembly of tyrosine-tagged proteins to chitosan, as illustrated in Figure 8a. Tyrosinase

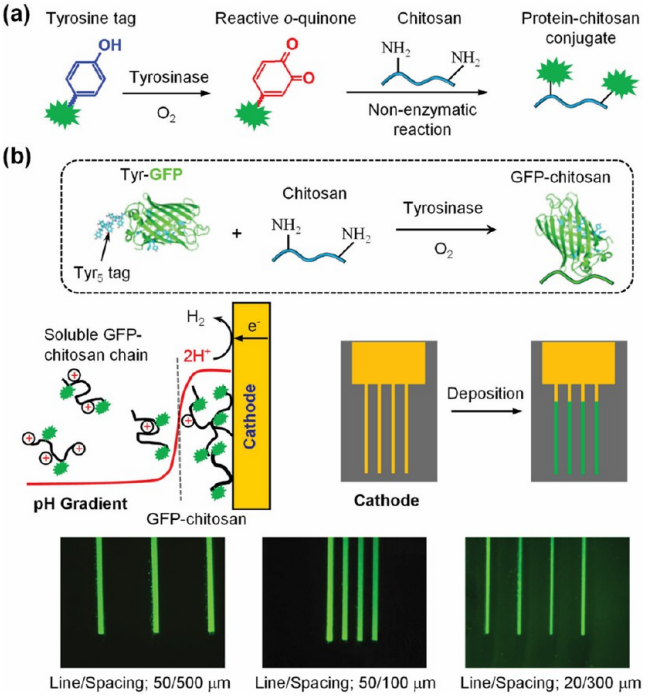

Figure 8. Biomolecular mechanisms can be used to functionalize chitosan. (a) Proteins engineered to have a short sequence of accessible tyrosine residues can be enzymatically oxidized by tyrosinase to generate a reactive quinone that can non-enzymatically graft to chitosan. (b) Enzymatic conjugation of a tyrosine-tagged green fluorescent protein (GFP) to chitosan generated a pH-responsive self-assembling GFP−chitosan conjugate that can be electrodeposited. Adapted with permission from ref 13. Copyright 2003 ACS.

uses molecular oxygen to oxidize the accessible tyrosine residues of the fusion tag to generate reactive *o*-quinones. These *o*-quinones undergo a non-enzymatic reaction with chitosan's amine groups,[19,100] although the chemistry is complex and not entirely resolved.[102,103] The use of tyrosinase to generate reactive *o*-quinone moieties that undergo subsequent covalent-bond-forming reactions mimics the tyrosinase-catalyzed cross-linking of the mussel glue protein.[104]

Figure 8b illustrates that the tyrosinase-mediated conjugation of a fusion-tagged protein (i.e., Tyr-GFP) to chitosan can generate a pH-responsive self-assembling protein−chitosan conjugate. This conjugate could then be electrodeposited onto a patterned electrode.[13] These steps could be interchanged as chitosan could be first electrodeposited and then enzymatically conjugated with a fusion-tagged protein.[14,16,17,104−106] In essence, conjugation to chitosan confers pH-responsive self-

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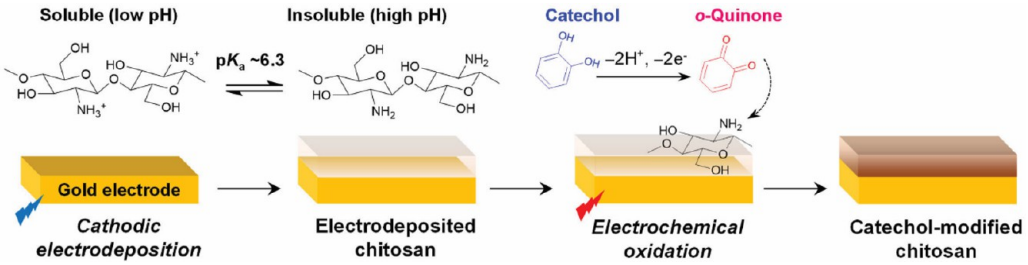

Figure 9. Two-step electrofabrication of a catechol−chitosan hydrogel film. A chitosan film is cathodically electrodeposited, and then, the chitosan-coated electrode is immersed in a catechol solution for the anodically induced oxidative grafting of catechol to chitosan. Adapted with permission from ref 112. Copyright 2017 John Wiley and Sons.

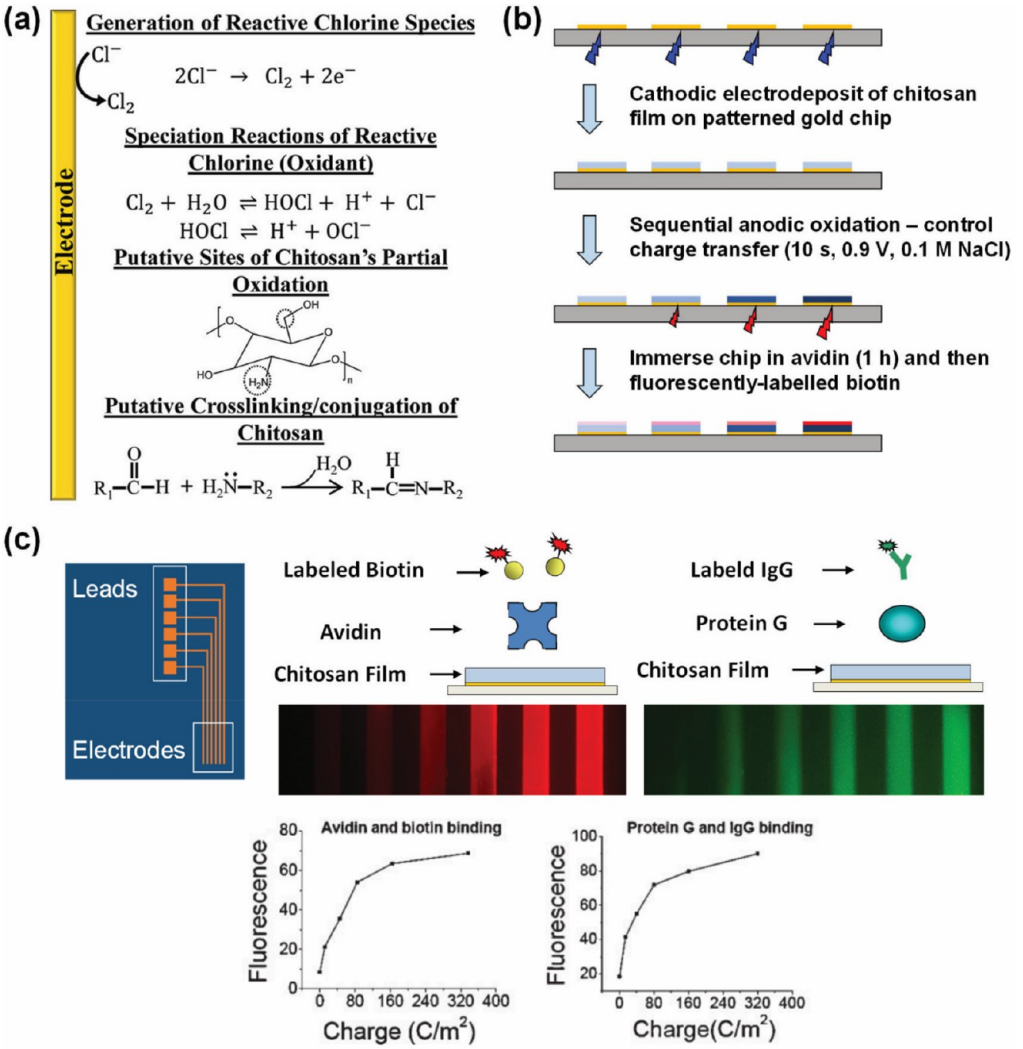

Figure 10. Anodically generated HOCl can oxidatively activate chitosan. (a) Mechanism proposed for the anodic activation of chitosan and its subsequent coupling reactions. Reproduced with permission from ref 113. Copyright 2012 ACS. (b) A three-step method to deposit, activate, and conjugate proteins to chitosan: Avidin and Protein G were used as simple models to demonstrate quantitative assembly. (c) Spatially selective and quantitatively controlled protein assembly onto an electrode address. Adapted with permission from ref 114. Copyright 2009 John Wiley and Sons.

assembling capabilities to the protein, enabling it to be assembled into higher ordered structures (e.g., an electrodeposited hydrogel). Finally, tyrosinase could be used to add a peptide linker to chitosan films and this linker could be used for subsequent protein conjugation through an orthogonal enzymatic mechanism.[107]

3.3. Coupling with Electrochemical Oxidative Methods. Biology uses a range of oxidants and oxidative mechanisms to perform various functions. As mentioned, tyrosinase is used to oxidize dopamine residues to induce cross-linking of the mussel glue protein.[78,104,108,109] Insects use tyrosinase to oxidize lower molecular weight phenols and catechols for wound sealing and hardening (i.e., sclerotization) of its cuticle (i.e., shell).[110,111] Catechols can also be oxidized electrochemically, and this was used to induce cross-linking of a previously electrodeposited chitosan film. As illustrated in

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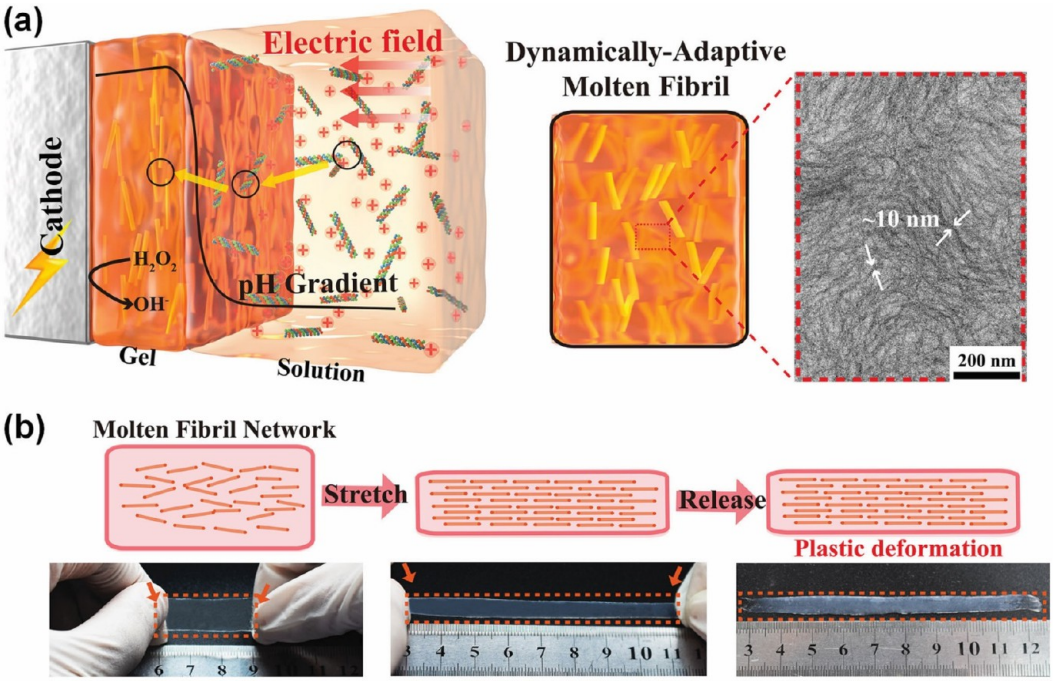

Figure 11. Electrodeposition of an intermediate molten fibril state of collagen. (a) Collagen's cathodic electrodeposition from an acidic solution (acid-solubilized collagen I; pH 3.5; pI 4.5) yielded a partially aligned and reversibly organized molten fibril state. (b) Stretching the molten fibril network aligns and densifies the network. Adapted with permission under a Creative Commons CC BY 4.0 License from ref 130. Copyright 2022 American Association for the Advancement of Science.

Figure 9, if a chitosan-coated electrode is immersed into a catechol solution and the underlying electrode is biased to an oxidative potential, then the catechol that diffuses through the film can be oxidized at the electrode and the quinone product that diffuses into the film can undergo grafting/cross-linking reactions with the chitosan film's amines.[102,103] As will be discussed later, this catechol grafting confers interesting properties to the chitosan film.

A common immune response in plants and animals is an oxidative burst, in which various reactive oxidants are generated to defend against a pathogen attack. Some of these same reactive oxidant species can be generated electrochemically, and some of these oxidants can react with chitosan. Specifically, when an anodic potential is imposed in the presence of NaCl, the reactive HOCl can be generated as illustrated in Figure 10a. Reactions between HOCl and chitosan are believed to "activate" chitosan by generating reactive aldehydes. Since such aldehydes can undergo covalent grafting to amines, this anodic oxidation enables a covalently cross-linked chitosan hydrogel to be electrodeposited at an anode.[113]

The anodically generated HOCl can also be used for protein assembly, as illustrated by a three-step procedure in Figure 10b. First, a chitosan film was electrodeposited through a cathodic neutralization mechanism, then this film was activated by anodic oxidation, and finally this activated chitosan film was contacted with a protein solution to allow the protein to graft to the activated chitosan film (possibly through lysine residues). Importantly, this anodic reaction can be quantitatively controlled by the extent of the electrical input (i.e., by the amount of charge transferred during oxidation, $Q = \int i\,dt$). Figure 10c illustrates that, if a patterned "chip" was fabricated with six independently addressable electrodes, it was possible to controllably activate each of the initially deposited films for subsequent protein assembly. Quantitatively controlled protein conjugation was demonstrated for two different protein assemblies: avidin which undergoes high-affinity binding with biotin and protein G which binds the constant region of IgG antibodies.[114]

It is interesting to note one additional study in which a detailed chemical analysis of anodically oxidized chitosan showed the generation of chloramine moieties. Chloramine functionality is useful as it can confer broad spectrum disinfectant activity against bacteria, fungi, and viruses. After peeling this chloramine-functionalized chitosan film from the electrode, it was tested as an antimicrobial wound dressing.[115]

3.4. Summary. There are many reasons why biopolymer-derived hydrogels are attractive materials systems: typically they are intrinsically safe (often edible) and sustainable; their aqueous nature makes them suitable for life science applications; and often their supramolecular organization endows them with capabilities to dynamically respond, heal, and reconfigure. In many applications, it would be desirable if additional chemical or biological properties could be added to such hydrogels, and biology provides useful lessons on how to covalently connect function-conferring molecules (e.g., proteins). By enlisting advances in biotechnology, it is possible to create proteins with specific information (e.g., fusion tags) to facilitate their integration into a hydrogel system through either enzymatic mechanisms or electrochemically induced oxidative mechanisms.

4. INTEGRATING ELECTROCHEMISTRY INTO MATERIALS SCIENCE AND BIOLOGY

In the previous sections, we focused on electrobiofabrication using chitosan as the example. In the remainder of this Review, we broaden the discussion to other biopolymer systems and emphasize a few unique opportunities for enlisting electro-

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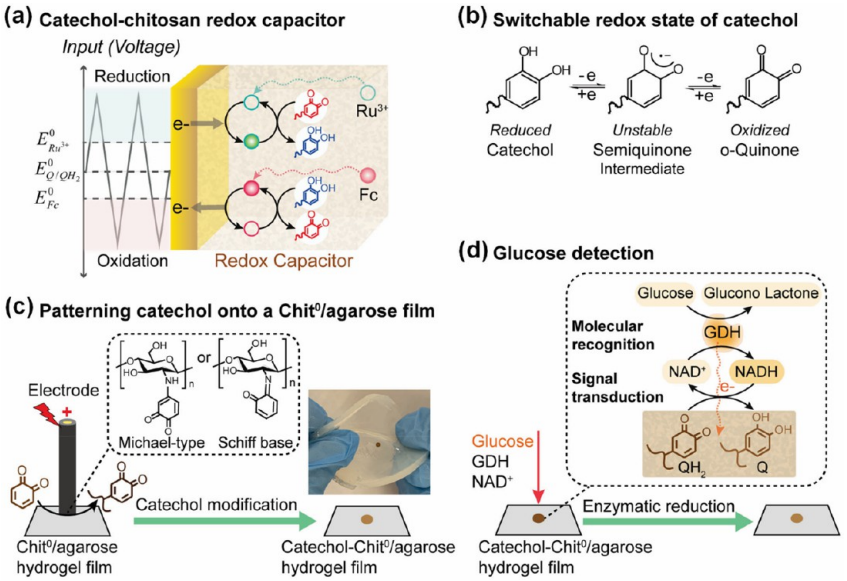

Figure 12. The catechol−chitosan film is redox-active but non-conducting. (a) Mediated electrochemical probing (MEP) was used to discern redox activities. Reproduced with permission from ref 142. Copyright 2019 ACS. (b) The redox state of the grafted catechol moieties can be repeatedly switched and thus can serve as a redox-based molecular memory. (c) Anodically writing of a catechol "dot" on a chitosan−agarose film generates a local region with redox activity. (d) When the redox-active "dot" accepts electrons, its optical properties change, and this provides a simple approach for cell-phone-based biosensing. Abbreviations: glucose dehydrogenase (GDH). Reproduced with permission from ref 79. Copyright 2021 ACS.

chemistry as a tool for fabrication, characterization, and applications.

4.1. Electroassembly of Collagen "Molten Fibrils". Collagen is the most abundant protein on earth where it can be organized into various hierarchical (i.e., supramolecular) structures that confer important structure-related functions: cross-linked microfibrils permit transparency (e.g., in the cornea); fibrils are present in load-bearing hard tissue composites (e.g., bone); and fibers/fiber bundles are present in connective tissue (e.g., tendons). Because collagen is intrinsically biocompatible, is resorbable, and offers important bioactivities (e.g., cell adhesion), it is a desirable biomaterial in regenerative medicine. Unfortunately, it has generally been impossible to fabricate collagen-based materials that recapitulate the diverse hierarchical structures observed in biology.[116−120]

Over 50 years ago, it was reported that dilute acetic acid solutions of collagen could "precipitate" in response to cathodic inputs that resulted in localized high pH.[121] To our knowledge, the next reports appeared nearly 40 years later[122,123] and emphasized that the emergent structure relied on both electrochemical electrolysis reactions (that induced a pH gradient) and the electric field. [It is important to note that unlike chitosan, which is a weak polyelectrolyte, proteins are ampholytes with complex pH-dependent solubilities such that protein electrodeposition resembles isoelectric focusing.] The focus of much of collagen's electrofabrication research has been driven by translational needs, and several reports describe the creation of dense and aligned collagen fibers using descriptive terms such as electrochemical compaction[124,125] and electrochemically aligned collagen.[126−129]

Recently, the Qu group reported an electroassembly method in which collagen could be partially assembled from a slightly acidic solution of individual molecules into an intermediate "molten fibril" state.[130] As illustrated in Figure 11a, the pH change is integral to the assembly process, while the electric field is important for inducing the collagen molecules to migrate and partially align within this molten fibril state. Importantly, these molten fibrils are reversibly organized (i.e., the molten fibrils can be redissolved in acid) and can respond to external stimuli. For instance, the as-deposited molten fibrils have structural features and optical properties consistent with the collagen found in the cornea,[131] while mechanical stretching aligned and densified the fibrils (Figure 11b) allowing subsequent treatments to generate higher-ordered structural features characteristic of the collagen found in tendons.[130]

4.2. Characterizing the Flow of Electrons through Hydrogels. After electrofabricating the catechol−chitosan (Cat−Chit) film in Figure 9, an obvious question was whether this film was conducting. Chemically, this film has similarities to the natural catechol-based pigment melanin which was suggested in the 1970s to offer semiconducting properties.[132−134] Initial studies showed that the Cat−Chit film was non-conducting: electrons did not flow through the film in response to an applied potential, and electrons could not be directly exchanged with the electrode. Presumably, the grafted catechols do not form an extended conjugated ring system, and most of the grafted catechols in the hundred-micrometer thick gel are too far from the electrode surface for direct electron exchange. We next asked whether the Cat−Chit film was redox-active: could the grafted moieties donate or accept electrons? To answer this question, we used diffusible mediators to shuttle electrons between the film and the electrode using a method of mediated electrochemical probing (MEP) illustrated in Figure 12a. From MEP we were able to show that the Cat−Chit films are redox-active and can be repeatedly and reversibly switched between an oxidized state (presumptive quinone moieties) and a reduced state (presumptive catechol moieties).[135] The ability to fabricate and characterize redox-active films has revealed four interesting opportunities for fundamental and applied research.

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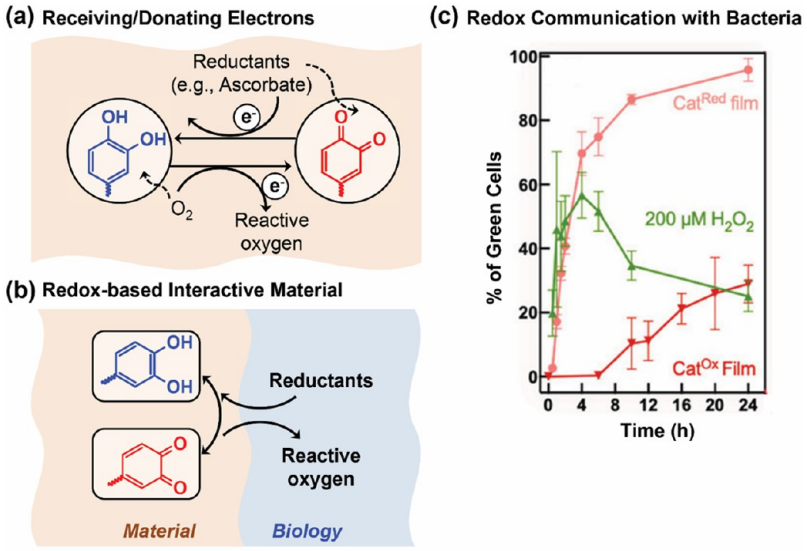

Figure 13. Catechol-containing films enable redox-based communication with biology. (a) A catechol−chitosan film that is capable of sustained ROS generation was shown to confer antimicrobial and wound healing properties. (b) A catechol-containing film was shown to be capable of interactive redox-based communication leading. (c) Redox communication with bacterial cells. Adapted with permission from ref 148. Copyright 2021 John Wiley and Sons.

First, MEP provides an especially versatile means to study the redox-based "flow" of electrons in aqueous systems. As mentioned, melanin was reported to have semiconducting properties, yet a half century later, researchers are still debating the relative importance of electronic vs ionic conductivity.[136] Similar questions emerge for conducting polymers: in aqueous systems electrons may be able to flow through the material, while the cationic counterions (i.e., dopants) can flow through an aqueous solution. The fact that the counterions are soluble in the medium while the electrons are not soluble creates a complex linkage between electron and ion flow, and this linkage has been difficult to understand. Using MEP, we observed that melanin-containing films (like Cat−Chit films[135,137,138]) are redox-active and can repeatedly exchange electrons with diffusible mediators.[139−141] It remains to be seen if/how measured redox activities are related to ionic and electronic conductivities.

Second, because catechol-containing films are redox-active but non-conducting, they offer unique molecular electronic properties.[138,142−144] For instance, Figure 12b shows the grafted moieties can exist in two stable redox states, oxidized (e.g., quinones) or reduced (e.g., catechols), and thus can serve as a two-state molecular memory.[79,142] A simple demonstration of this molecular memory was shown using a neutralized chitosan−agarose film with an anodically patterned catechol dot (Figure 12c). The unpatterned transparent regions are not redox-active, while the patterned dot with the grafted catechol is redox-active. If the patterned dot is initially oxidized, then it can accept electrons from the appropriate reducing agents. In this demonstration study, Figure 12d shows the reducing agent, NADH, was generated enzymatically by the enzyme glucose dehydrogenase (GDH), while the transfer of electrons to the patterned dot switches the grafted moieties from their oxidized to their reduced states. Importantly, the redox state can be observed optically (e.g., by cell phone imaging) because the oxidized state is darker in color and has a higher UV−vis absorbance compared to the reduced state.[145] From an application standpoint, this example illustrates a simple and sustainable platform for biosensing. From a fundamental standpoint, it is interesting to note that this molecular memory can be repeatedly switched, but these memory states are only stable for hours to days: the molecular-level reason for this instability is unclear. Possibly, a two-state model in which a two-electron transfer separates the oxidized and reduced states is too simple, as it ignores the unstable intermediate semiquinone state.

Third, catechol-containing films can interactively communicate with biology. While Figure 12d illustrates that catechol-containing films can accept electrons from biological reductants (i.e., NADH), Figure 13 shows that these films can also donate electrons to biologically relevant oxidants. Specifically, Figure 13a shows that the donation of electrons to the atoms of O_2 can generate reactive oxygen species (ROS). ROS are common antimicrobial, effector molecules of the immune system of plants and animals, and an electrofabricated, ROS-generating catechol−chitosan wound dressing was shown to confer antimicrobial activities and promote healing of an infected wound.[146,147] It was suggested in that study that ascorbate at the wound site might be a source of electrons used by the catechol film for the continued generation of ROS: according to this hypothesis, the catechol film catalyzes the transfer electrons from a relatively stable physiological reductant, ascorbate, to O_2 for ROS generation. Later studies illustrated in Figure 13b show that an electrofabricated synthetic catechol-based film could engage a bacterial population in redox communication by both accepting electrons from reductants generated by the bacterial cells and generating the products (presumably ROS) that upregulate stress-response genes.[148] Specifically, an *E. coli* regulon that responds to H_2O_2 was "re-wired" to express a green fluorescent protein. Figure 13c shows that the response of these cultures to H_2O_2 (positive control) is similar to the response when these cultures were incubated with a previously reduced catechol-based film (designated "Cat^{Red} film"). Incubation of these cultures with a previously oxidized film ("Cat^{Ox} film") resulted in a delayed response: presumably, the culture provided the reducing equivalents to convert the oxidized film to its reduced state, thus allowing the film to generate H_2O_2 in the presence

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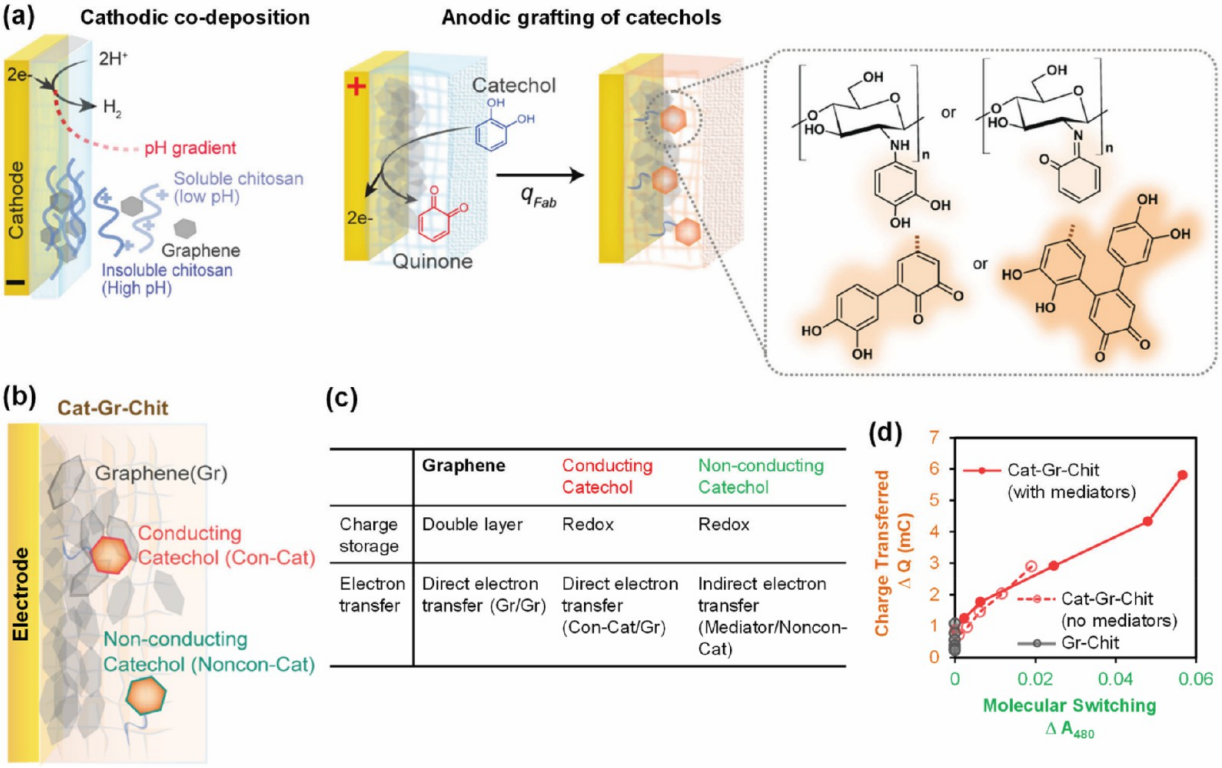

Figure 14. Electrofabricated catechol−graphene−chitosan composite films. (a) The composite is fabricated by cathodic co-deposition of graphene and chitosan, followed by anodic grafting of catechol moieties. (b) The composite contains two functional populations of catechol: one is conducting (can directly exchange electrons), and the other is non-conducting (requires mediators for electron exchange). (c) Properties conferred by graphene and the two catechol populations. (d) Summary plot showing how the redox-state switching of the conducting and non-conducting catechols (*x*-axis) enhance the ability to transfer electrons into and out of the film (*y*-axis). Adapted with permission from ref 153. Copyright 2022 John Wiley and Sons.

of O_2. More broadly, we believe MEP could become a new tool for redox biology[149−151] both to understand the redox activities of native materials (e.g., melanins) and for the design of redox-based interactive materials.[151,152]

Fourth, an electrofabricated composite catechol−graphene−chitosan (Cat−Gr−Chit) film was shown to offer synergistic conducting and redox properties.[153] Figure 14a shows the two-step electrofabrication of this composite: a graphene−chitosan film was first assembled by cathodic co-deposition from a solution of chitosan with dispersed graphene, and then, catechol was anodically grafted to the graphene−chitosan film. Figure 14b illustrates that this film contains graphene and two functional populations of catechols. One population is redox-active and conducting: presumably these conducting catechols are in intimate contact with the graphene. The second population is redox-active and non-conducting, and these non-conducting catechols cannot exchange electrons with graphene but can exchange electrons with diffusible mediators. Using a variety of spectroelectrochemical methods, it was possible to resolve the functional properties of these individual components (Figure 14c). Graphene conferred metal-like conductivity to the film and enabled charge storage through an electrical double layer mechanism. The conducting catechols could undergo direct redox-state switching (via graphene) and could store charge through a redox capacitance mechanism. The non-conducting catechols required diffusible mediators for redox-state switching and conferred an additional redox capacitance. Using dynamic measurements, Figure 14d shows that the ability to transfer charge into and out of the film (the *y*-axis) was comparatively small for a film containing only graphene, while the redox-state molecular switching of the catechol-containing films (*x*-axis) allowed more charge to be transferred into and out of the film. Using this knowledge, it was possible to fabricate a catechol−graphene−chitosan composite and use redox mediators to achieve capacities within 20% of the highest-reported values for aqueous-based energy materials.

4.3. Electroassembling Living Bioelectronics. Our original motivation for building with biological materials/mechanisms was to create a mutually compatible bioelectronic interface that accesses the power of electronics to assemble, characterize, and communicate with biological systems. Our primary experimental focus with living systems has been with microbial systems that are integral to biotechnology (e.g., for protein, genetic, and metabolic engineering) and also form the complex ecosystems of the gut microbiome and plant rhizosphere.

Our initial goal was to develop the capabilities to electroassemble living microbial populations at an electrode "address". An obvious candidate material for electroaddressing is the polysaccharide alginate which is a component in bacterial biofilms,[154,155] and is commonly used as a matrix to immobilize microbes in biotechnology and a scaffold for tissue engineering studies.[156−158] A few years after the electrodeposition of chitosan was reported, the acidic polysaccharide alginate was reported to be electrodeposited: first through an anodic neutralization mechanism[74] and then by the anodic mechanism illustrated in Figure 15a.[159] Specifically, a

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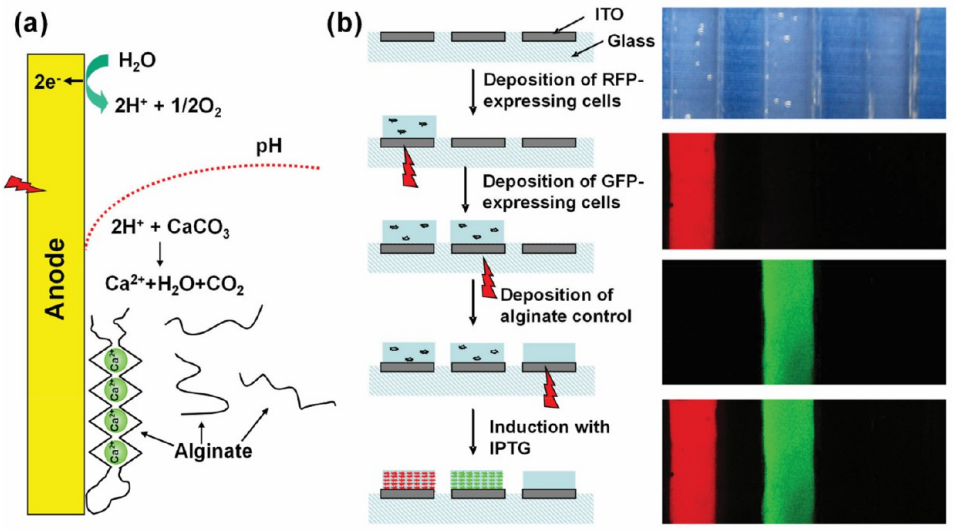

Figure 15. Anodic mechanism for the electrodeposition of Ca^{2+}–alginate hydrogels. (a) Mechanisms for Ca^{2+}–alginate electrodeposition. (b) Sequential co-deposition to assemble different bacterial populations at different electrode addresses of a patterned glass slide. Abbreviations: indium tin oxide (ITO); red fluorescent protein (RFP); green fluorescent protein (GFP); and isopropyl β-D-1-thiogalactopyranoside (IPTG). Adapted with permission from ref 159. Copyright 2009 John Wiley and Sons.

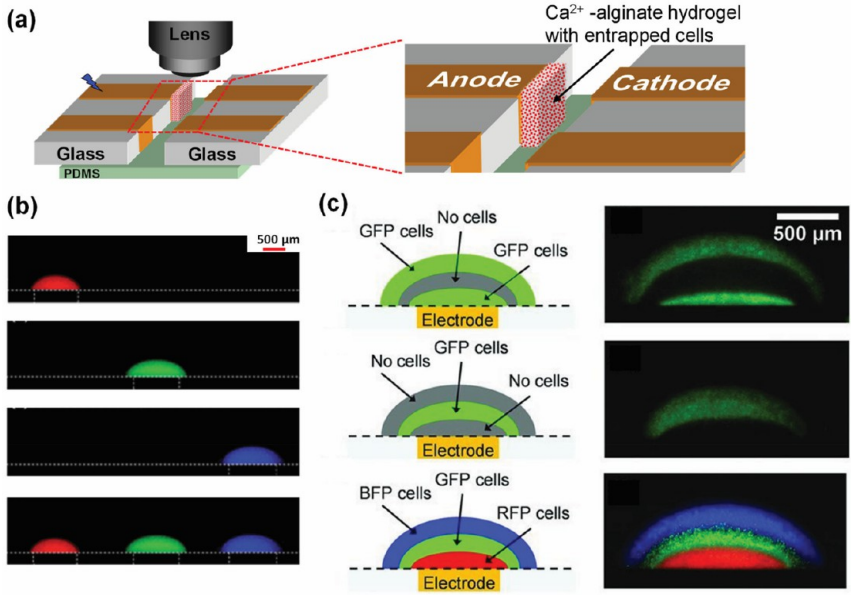

Figure 16. Sequential Ca^{2+}–alginate co-deposition to "electroaddress" bacterial populations within a microfluidic device. (a) Co-deposition allows a bacterial population to be assembled at a specific side-wall electrode address. (b) Sequential co-deposition of bacterial populations at different side-wall electrode addresses. Adapted with permission from ref 161. Copyright 2011 RSC. (c) Sequential co-deposition of bacterial populations to the same side-wall electrode address. Abbreviations: red fluorescent protein (RFP); green fluorescent protein (GFP); and blue fluorescent protein (BFP). Adapted with permission from ref 162. Copyright 2012 John Wiley and Sons.

deposition solution was prepared with sodium alginate and insoluble $CaCO_3$ particles, and an anodic electrolytic reaction was used to generate H^+ that solubilized $CaCO_3$ to locally generate the Ca^{2+} ions that could induce the formation of a Ca^{2+}-cross-linked alginate hydrogel.[160]

Importantly, the Ca^{2+}–alginate electrodeposition mechanism is biocompatible in that it uses mild conditions (3 A/m^2 for 2 min), while the $CaCO_3$ solubilization mechanism is also a buffering mechanism that prevents large pH excursions. In initial studies shown in Figure 15b, a glass slide was patterned with transparent indium tin oxide (ITO) and then sequential co-deposition steps were performed to electroaddress *E. coli* populations that express either red fluorescent protein (RFP) or GFP and also to electroaddress a control Ca^{2+}–alginate hydrogel. Experimentally, RFP-expressing *E. coli* cells (OD_{600} of 1) were diluted 10-fold with an alginate–$CaCO_3$ suspension (0.9% alginate, 0.23% $CaCO_3$) and the left-most electrode was biased anodically at 3 A/m^2 for 2 min. After washing with 1% NaCl, a GFP-expressing *E. coli* cells–alginate–$CaCO_3$ suspension (0.1 OD_{600}, 0.9% alginate, 0.23% $CaCO_3$) was co-deposited (3 A/m^2 for 2 min) on the middle electrode. After rinsing with NaCl, calcium alginate (without cells) was electrodeposited at the right-most electrode (the control electrode). After adding the chemical inducer isopropyl β-D-1-

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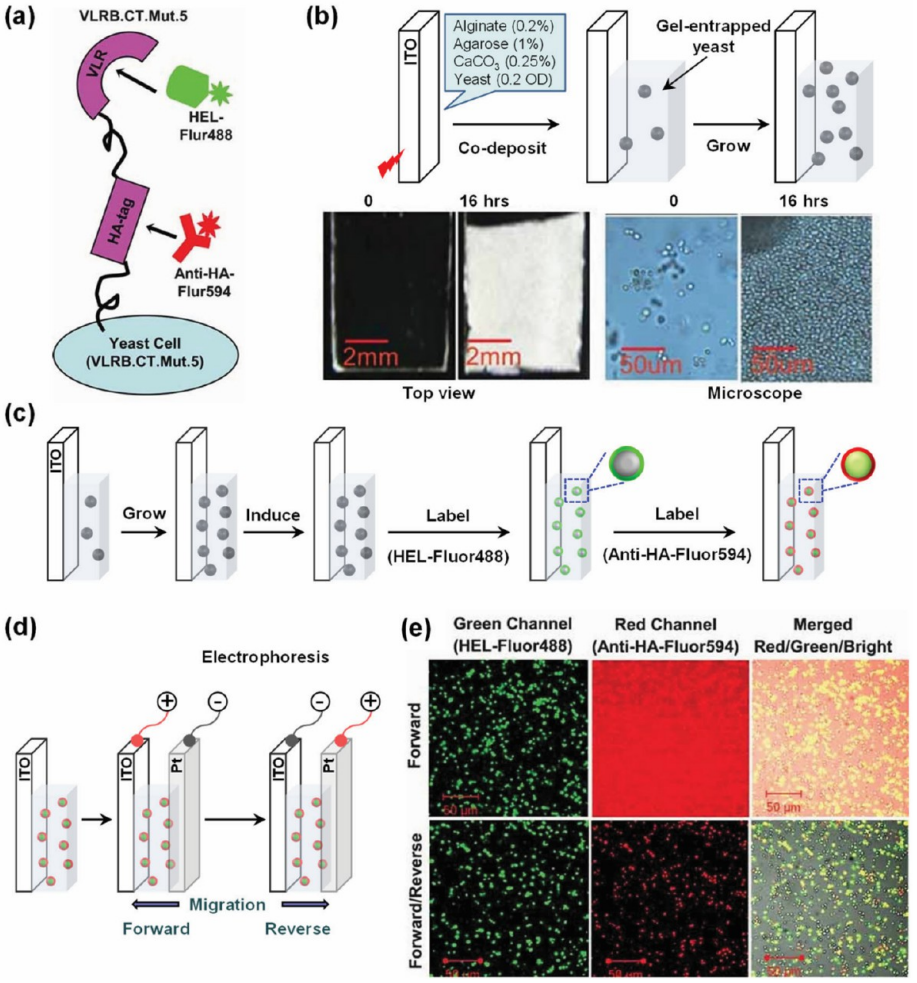

Figure 17. An electrodeposited yeast population (designated VLRB.CT.Mut5) can be cultivated and characterized for the expression of surface-bound proteins. (a) These yeast cells were engineered to express a hemagglutinin (HA) tag and a receptor (designated VLR) on their cell surface. (b) After co-deposition within an agarose−alginate hydrogel, the gel-entrapped yeast could be grown. (c) Process for growing and inducing these yeast clones and then analyzing their surface-expressed proteins: VLR binds with the small protein hen egg lysozyme (HEL), and HA binds with an antibody reagent (anti-HA). (d) Electrophoresis was used to accelerate contacting with the large anti-HA reagent. (e) Fluorescent images show co-localization of the two fluorescently labeled probes (HEL and anti-HA) at the cell surface. Adapted with permission from ref 163. Copyright 2010 John Wiley and Sons.

thiogalactopyranoside (IPTG) and incubating for 16 h, the fluorescence images show no fluorescence in the control gel, while the electrodes with the RFP- and GFP-expressing cells show appropriate fluorescence at their individual addresses.[159]

Figure 16a shows that sequential Ca^{2+}−alginate co-deposition can be extended to electroaddress different fluorescent-protein-expressing *E. coli* populations at side-wall electrodes within a microfluidic device.[161] Figure 16b shows individual populations assembled on separate electrode addresses, while Figure 16c shows a model biofilm with segregated populations of bacteria assembled at a single electrode address.[162]

In the next stage of the research, we aimed to adapt biological methods to characterize an electroaddressed population. In many cases, biological characterization methods involve the detection of molecular-based structures and functions. For instance, antibodies are commonly used as reagents to detect specific molecules (e.g., by immunoanalysis), and receptor activity can be detected by observing binding with its ligand. Further, such analyses often use molecular reagents that are labeled with fluorescent moieties to enable visualization through fluorescence microscopy. For our studies, we collaborated with the Pancer group, which was using yeast cells to produce specialized types of receptors (designated VLR). Specifically, Figure 17a shows one yeast clone (designated VLRB.CT.Mut5) that was engineered to have an inducible surface-expressed protein with both a hemagglutinin (HA) tag and a VLR capable of binding the model protein hen egg white lysozyme (HEL).[163] Thus, the goal for our demonstration study was to electroaddress a hydrogel containing these yeast clones, cultivate these cells within the hydrogel to expand their population and induce expression, and then characterize these cells by probing the hydrogel to detect HA and VLR on the cell surfaces. One challenge is that hydrogels composed of the anionic alginate do not allow the penetration of small anions or large proteins (e.g., the immunoreagent anti-HA-Flur594) into the hydrogel, and this limited our ability to detect the cell-surface-expressed HA protein.

To overcome this limitation, we used the neutral polysaccharide agarose, which is commonly used in biotechnology for gel electrophoresis. Because agarose lacks acidic

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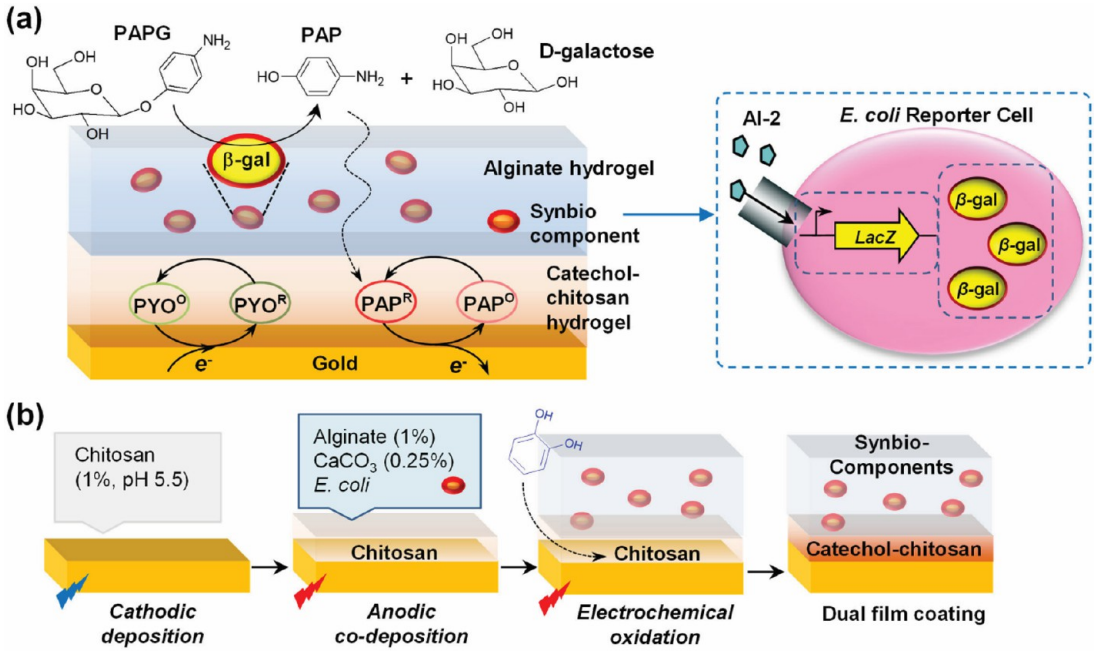

Figure 18. A dual film system to enable redox-based bioelectronic communication. (a) The alginate-based hydrogel contained *E. coli* reporter cells that were programmed to recognize the quorum sensing autoinducer-2 (AI-2) and respond by expressing an enzyme capable of generating a redox-active molecular signal (PAP). The cataechol−chitosan film amplified the redox-based PAP currents. (b) Three-step electrofabrication of this dual film system. Adapted with permission from ref 112. Copyright 2017 John Wiley and Sons.

(carboxy) or basic (amine) functional groups, it is not pH-responsive, and thus, there are no known mechanisms for agarose's direct electrodeposition. However, agarose can be co-deposited from a warm deposition solution containing alginate and $CaCO_3$. After cooling, the co-deposited agarose can undergo its thermally responsive gel formation. Like the chitosan−agarose hydrogel of Figure 4, the alginate−agarose hydrogel can form an interpenetrating network. After the agarose network forms, the Ca^{2+}−alginate network can be dissociated by treatment with citrate, which binds Ca^{2+}. We observed that co-deposited alginate−agarose hydrogels with low levels of alginate allow penetration of the anti-HA immunoreagent.[163]

Experimentally, these yeast cells were blended into a deposition solution containing alginate (0.2%), agarose (1.0%), and $CaCO_3$ (0.25%) and electrodeposited onto an ITO-coated glass slide (2 A/m^2 for 1 min). After overnight incubation, the images in Figure 17b show a considerable increase in the yeast cell population within this electro-deposited hydrogel (additional measurements suggest a doubling time of 3 h).[163]

Figure 17c illustrates a procedure in which the electro-deposited yeasts were grown (3 h) and induced (4 h) and then contacted for 1 h with the relatively small HEL protein (15 kDa; labeled with a green fluorophore) that easily penetrated into the hydrogel to bind with the surface-expressed VLR receptor. Because the larger anti-HA antibody (150 kDa; labeled with a red fluorophore) could not easily penetrate into the hydrogel, a second electrode was added, as illustrated in Figure 17d, and the anti-HA antibody was driven into the film by electrophoresis (1 V mm^{-1} for 20 min) to allow binding with the surface-expressed protein. After binding, the unbound anti-HA was removed from the hydrogel by reversing the direction of the electrophoresis (1 V mm^{-1} for 30 min). The confocal images in Figure 17e show the expected co-localization of the two fluorescent probes. This result illustrates the coupling of electro-biofabrication with in-film bioprocessing (for cell growth and induction) and characterization by electrophoretic-based immunoanalysis.

Currently, the focus of the research is bioelectronic communication, and for this, we are using redox as a modality that enables bidirectional communication. Redox is a native modality for biological communication (i.e., redox signaling)[164−166] while this modality is also accessible to electronics via electrochemistry. Thus, there are growing efforts to develop an electron-based redox bioelectronics that is fundamentally different from conventional bioelectronics that interfaces through biology's ionic electrical modality. For instance, synthetic biology has been used to enable sensing (to convert a molecular signal into a redox response that can be detected at an electrode)[105,167,168] and actuation (to convert electrode-imposed redox signals into altered gene expression and motility).[169,170] In the example of Figure 18a, a dual film system was electrofabricated in which a synthetic biology (synbio) reporter cell was entrapped within a Ca^{2+}−alginate hydrogel that was assembled above a catechol−chitosan hydrogel. The genetic circuitry for this synbio construct was reprogrammed to recognize the quorum-sensing signaling molecule Autoinducer-2 (AI-2) and respond by expressing the "reporter" enzyme β-galactosidase (β-gal) that converts a redox-inactive substrate (4-aminophenyl β-D-galactopyranoside; PAPG) into a redox-active product (4-aminophenol; PAP) that can be electrochemically detected.[112]

Electrobiofabrication of this dual film involved three steps, as illustrated in Figure 18b. First, chitosan was cathodically electrodeposited onto a gold electrode (4 A/m^2, 1 min). Second, a bio-hydrogel was assembled directly on top of the chitosan film by blending these *E. coli* reporter cells (optical density = 3) with sodium alginate and $CaCO_3$ and anodically co-depositing the hydrogel (4 A/m^2, 1 min). Finally, the

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electrode with this dual hydrogel coating was immersed in a catechol solution (5 mM) and an anodic potential was imposed (0.5 V, 1 min) to oxidatively graft catechol moieties onto the chitosan. Often Ca^{2+}−alginate films are soft, and thus, we performed a brief stabilization by incubation in a $CaCl_2$ solution (e.g., 1% for 15 min). Functionally, the alginate biohydrogel transduces the quorum sensing AI-2 signal into a redox signal (PAP), while the catechol−chitosan film serves to amplify the PAP-based redox signal.[112,171]

4.4. Summary. Studies with chitosan have revealed many interesting features of electro-biofabrication, which are being generalized to other systems and applications. Observations that the emergent structure of the electrodepositing chitosan is sensitive to subtle changes in conditions (e.g., salt and temperature) illustrate the rich design space available for tailoring hydrogel structure and function. Although these effects are not entirely understood, they have enabled unprecedented control of the hierarchical structures of collagen-based biomaterials. The observation that electrode-imposed cues can be benign is enabling the fabrication of living material systems, while the observation that redox is a modality that can span bioelectronic communication is enabling bidirectional communication that promises to connect our biological and technological worlds. And the observation that films can be fabricated with synergistic conducting and redox properties may enable a new type of molecular electronics for energy and information processing applications.

5. CONCLUSIONS AND PERSPECTIVES

It is useful to consider what we learned in the nearly 20 years since the initial review that proposed building hydrogels with biological materials/mechanisms. It is especially interesting to consider what was not anticipated. As detailed below, we believe the biggest surprise is the extent to which electrochemistry can be applied as a tool for materials science and the biological sciences.

5.1. Electro-. The initial observation that chitosan could be electrodeposited showed that electrical inputs yielded pH cues that directed chitosan chains to assemble into an organized supramolecular structure. Since then, it has become clear that the electrode can also impose oxidative cues that induced covalent cross-linking/conjugation. Controlling the imposed electrical inputs to control these pH and oxidative cues is reasonably well understood. However, we did not anticipate the difficulties in understanding the relationship between the electrical inputs, the electric field, and how this field serves as a cue that controls the emergent structure.

Twenty years ago, we viewed a hydrogel as a volume-spanning polymer network containing mostly water and were concerned with whether we needed to distinguish a difference between a gel and a film (we decided this distinction is not important). We did not anticipate that the self-assembling biological polymers could be cued to generate hydrogel films with such complex internal structures (e.g., Figures 2a and 3b). We also had not anticipated electrode-induced covalent cross-linking mechanisms (e.g., electrode generation of HOCl or oxidation of mediators).

While chitosan's electrodeposition showed that electrically imposed cues could induce hydrogel formation, the focus then and now is for the hydrogels to perform functions. Our focus was biological functions, and we initially focused on molecular-based biological function (e.g., conferred by nucleic acids[172,173] and proteins[13,101]), while cell-based function awaited the discovery of alginate's electrodeposition. Other groups focused on non-biological functions and used co-deposition mechanisms to create composite films and especially composites with ceramics.[33,70,71,73,174−182] A major recent focus of many groups (including ours) are functions conferred by integrating catecholic moieties (including polydopamine) with electrodeposited films.[146,147,174,183,184]

Chitosan's electrodeposition showed that electrochemistry offered interesting opportunities for materials fabrication, but probably the biggest unanticipated spin-off from a materials science perspective was the development of electrochemical methods for materials characterization. Specifically, mediated electrochemical probing (MEP) was integral to demonstrating the catechol−chitosan films are redox-active but non-conducting. Initially, it was not apparent if/why a redox-active but non-conducting material would be useful until we looked to biology where electron "flow" generally does not involve conducting mechanisms but rather redox reaction networks (e.g., the respiratory electron transfer chain is a spatially organized redox reaction pathway). The ability to fabricate and characterize redox-active materials is opening new opportunities to enable communication with biology through a native biological redox modality.[115,185] Thus, we envision that MEP may emerge as an important tool to understand the networked flow of electrons in redox biology[149] and also potentially to change the way we think about building electronics to function in aqueous systems.[186]

In terms of future opportunities, we believe electrochemistry is an un(der)tapped resource for materials science and biology. Electrochemistry converts low voltage electrical inputs into pH and redox cues (i.e., diffusible H^+/OH^- and oxidants/reductants) that can induce a switching of protonation and redox states. Biology routinely equips its biochemicals (e.g., proteins) with moieties that possess thermodynamic properties (pK_a and $E°$) that allow their state-switching under near-physiological conditions, and biology often uses such state-switching to alter a biochemical's structure as a mechanism to process information (e.g., for signal transduction). Electrodeposition demonstrates that imposed electronic inputs can induce state-switching and changes in hierarchical structure. Thus, electrochemistry provides an interesting information processing bridge between the electronic logic of modern technology and the molecular logic of biology. Potentially, electrochemistry can be used to fabricate hydrogels/films with complex information encoded in their structure, and potentially, electrochemistry may allow the information in a complex chemical/materials system to be decoded electronically.

5.2. -Bio-. In our original definition of biofabrication, one focus of the "bio" prefix was stimuli-responsive self-assembling of polymers from biology. Chitosan turned out to be a very nice starting point, as it illustrated several distinct features of biological polymers. Cathodic electrodeposition created chitosan hydrogels that were physically cross-linked through crystalline network junctions. However, it has become clear that alternative mechanisms could be used to alter structure or confer function (e.g., electrostatic, metal chelation, and oxidation), and these options provide a rich design-space for fabrication and also, in some cases, for the creation of dynamically responsive hydrogel systems. At the level of the individual sugar residue, the protonation state of the glucosamine residues serves as a type of switch controlling these interactions. Importantly, the pK_a of this switch is

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sensitive to local interactions: the residue's pK_a shifts upward when it is engaged in electrostatic interactions (e.g., with SDS micelles) and downward when it is engaged in interchain hydrogen bonding and hydrophobic interactions in the crystalline region. In hindsight, this pK_a shift is not surprising, but the fact that it was not anticipated emphasizes how little is really understood about the self-assembly of biopolymers (e.g., the role of cooperativity in self-assembly).

The second initial focus of the "bio" prefix was the use of biomolecular methods to confer function. Specifically, protein engineering enables the genetic fusion of amino acid residues to proteins that facilitate their coupling to materials. For chitosan, histidine tagged proteins can be coupled through a nickel-mediated mechanism,[99] and tyrosine tagged proteins can be conjugated through a tyrosinase-mediated mechanism. In other cases, lysine or glutamine tags enable transglutaminase-mediated conjugation and cysteine tags enable oxidative conjugation through disulfide bond formation. In some of these examples, enzymes are used to induce covalent bond formation. In other examples, oxidative mechanisms are used for conjugation and the use of mediators to electrochemically induce covalent coupling offers exciting opportunities to precisely attach function-conferring components.[49,50,53,55,187,188]

Probably the biggest unanticipated spin-off from a biological perspective was the development of electrochemical methods for sensing and actuation. When we began this work, we were concerned that electrical inputs would have detrimental effects on living systems. In our initial efforts to co-deposit bacteria within Ca^{2+}−alginate hydrogels, we emphasized the importance of $CaCO_3$ as a buffering agent to prevent large pH excursions from neutrality. The observations that biological oxidants and reductants could serve as mediators and electrochemical probing could be performed with living systems dramatically changed our perspective. [We should note that we were not the first to use mediated electrochemistry to probe living systems,[189] and there is considerable active research in this area.[190−193]] Now, rather than fearing that electrical inputs will be harmful, biological systems are being designed to communicate with electrodes both for sensing (e.g., Figure 18) and actuation (e.g., electrogentics).[105,150,151,167−170,194−197]

In terms of future opportunities, we believe that redox biology provides many lessons to apply or mimic for the creation of functional materials. Biology often uses diffusible oxidants that engage somewhat specific chemistries to generate cross-linked materials: oxidation of catechol moieties is integral to the setting of the mussel glue and hardening of the insect cuticle; the oxidative generation of phenolic free radicals is integral to the formation of dityrosine cross-links of proteins and synthesis of lignin; the oxidative deamination of lysine is used to cross-link collagen; and the oxidation of cysteine residues to disulfide cross-links is a common mechanism in redox-responsive transcription factors for the upregulation of stress-response genes. Electrochemistry allows some of these oxidants to be generated directly (e.g., H_2O_2 and HOCl) or can enlist mediators to transmit oxidative redox signals, and advanced methods in biology (protein engineering and synthetic biology) allow biological components to be engineered to receive/respond to these redox transmissions. While many of the above examples involve irreversible mechanisms, some reversibly redox-active materials (e.g., melanin and dietary antioxidants) appear to offer important antimicrobial, antioxidant, radical scavenging, and redox signaling capabilities. Thus, we believe that redox serves as a modality that enables electrochemical inputs to engage different biologically relevant reaction chemistries to build structure and confer function.

5.3. -Fabrication. Twenty years ago, the authors of the original review had a nagging concern of over-reach: conflating two observations (chitosan's electrodeposition and the enzymatic conjugation of a fusion-tagged protein) into a broader vision for integrating biological components into electronic devices. Was chitosan the only biopolymer that could be electrodeposited, or was it just the first to be observed? As discussed throughout this Review, it is now clear that many polymer hydrogel films can be electroassembled, various methods (including electrochemical methods) are available to confer function to these hydrogels, and applications extend beyond bioelectronics.

Since the original review, there have been remarkable advances in additive manufacturing, with 3D printing emerging as a platform technology that is appropriate for industrial manufacturing and also for small-scale maker spaces. We envision that electro-biofabrication could emerge as an alternative and complementary additive manufacturing platform technology. Typically, electro-biofabrication is simple, rapid, cheap, and reagentless. It can be performed serially (e.g., to functionalize addresses of an electrode array; Figure 10b) or in parallel (Figure 8b), and it is also scalable (Figure 1d) and conformal (Figure 1e). Further, since electro-biofabrication often uses biological materials and mechanisms, the processes and products tend to be intrinsically safe and sustainable.

Thus, the initial review "Biofabrication with Chitosan"[1] launched an exciting journey and we look forward to seeing where the next 20 years take us and who will take us there!

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Notes

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