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Review

Chitosan to Connect Biology to Electronics: Fabricating the Bio-Device Interface and Communicating Across This Interface

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Abstract: Individually, advances in microelectronics and biology transformed the way we live our lives. However, there remain few examples in which biology and electronics have been interfaced to create synergistic capabilities. We believe there are two major challenges to the integration of biological components into microelectronic systems: (i) assembly of the biological components at an electrode address, and (ii) communication between the assembled biological components and the underlying electrode. Chitosan

possesses a unique combination of properties to meet these challenges and serve as an effective bio-device interface material. For assembly, chitosan's pH-responsive film-forming properties allow it to "recognize" electrode-imposed signals and respond by self-assembling as a stable hydrogel film through a cathodic electrodeposition mechanism. A separate anodic electrodeposition mechanism was recently reported and this also allows chitosan hydrogel films to be assembled at an electrode address. Protein-based biofunctionality can be conferred to electrodeposited films through a variety of physical, chemical and biological methods. For communication, we are investigating redox-active catechol-modified chitosan films as an interface to bridge redox-based communication between biology and an electrode. Despite significant progress over the last decade, many questions still remain which warrants even deeper study of chitosan's structure, properties, and functions.

Keywords: bioelectronics; biofabrication; biosensing; catechol; chitosan; electrochemistry; electrodeposition; redox-activity; redox-capacitor; tyrosinase

1. Introduction: Integrating Biology into Electronics

1.1. The Opportunity. Why Interface Biology and Electronics?

During the 20th century, advances in biotechnology and microelectronics transformed the way we live our lives—whether by providing life-saving medications or by enabling the instantaneous communication across continents. Surprisingly, there have been relatively few examples in which the strengths of biology and electronics have been effectively leveraged to create synergistic capabilities. As suggested by the examples in Table 1, the interfacing of biology and electronics could be transformational by enabling enhanced abilities to; analyze (e.g., for health, safety or national security), acquire energy, or promote health. Yet the entries in Table 1 are only those opportunities that are obvious from today's perspective while facile technologies that integrate the powers of biology and electronics could provide unimagined capabilities. For instance, medicine has traditionally relied on drugs and surgery to treat pathologies yet the future of medicine may include implantable or ingestible devices capable of monitoring and maintaining physiological homeostasis.

Over a decade ago, our interdisciplinary team began investigating methods to interface biology and electronics. The aminopolysaccharide chitosan has proven to be an integral part of this quest. Here, we will review chitosan's unique properties and illustrate how these properties can be enlisted to perform important functions for the assembly of the biological components at device addresses and for establishing communication across this bio-device interface.

Table 1. Examples of opportunities enabled by bio-device integration.

Area	Possible Applications
Analysis	Biosensors—multiplexed analysis in hand-held devices Lab-on-a-Chip—high-throughput screening Smart fabrics—remote monitoring of first-responders
Energy	Biofuel cells—efficient conversion of chemical and solar energy Nanostructured batteries—compact storage of energy
Medicine	Devices to personalize medicine—theranostics Prosthetics—effective repair or restoration of function

1.2. Vision

Figure 1 illustrates our broad vision for integrating biology and electronics. At the left, we show a bio-device interface fabricated by assembly step(s) that physically localize the biological components (e.g., proteins, nucleic acids or cells) to an electrode address in a microfabricated system (e.g., an electrode array or microfluidic device). From an electronics standpoint, it would be ideal if assembly could be directed to occur in response to electrical signals because such signals can be imposed with exquisite control spatially, temporally and quantitatively [1–3]. From a biological standpoint, it would be ideal if assembly could be achieved rapidly, simply and under mild (e.g., aqueous) conditions, and without the need for reactive reagents. As will be discussed, chitosan allows these ideals to be realized—at least for the assembly of proteins [4].

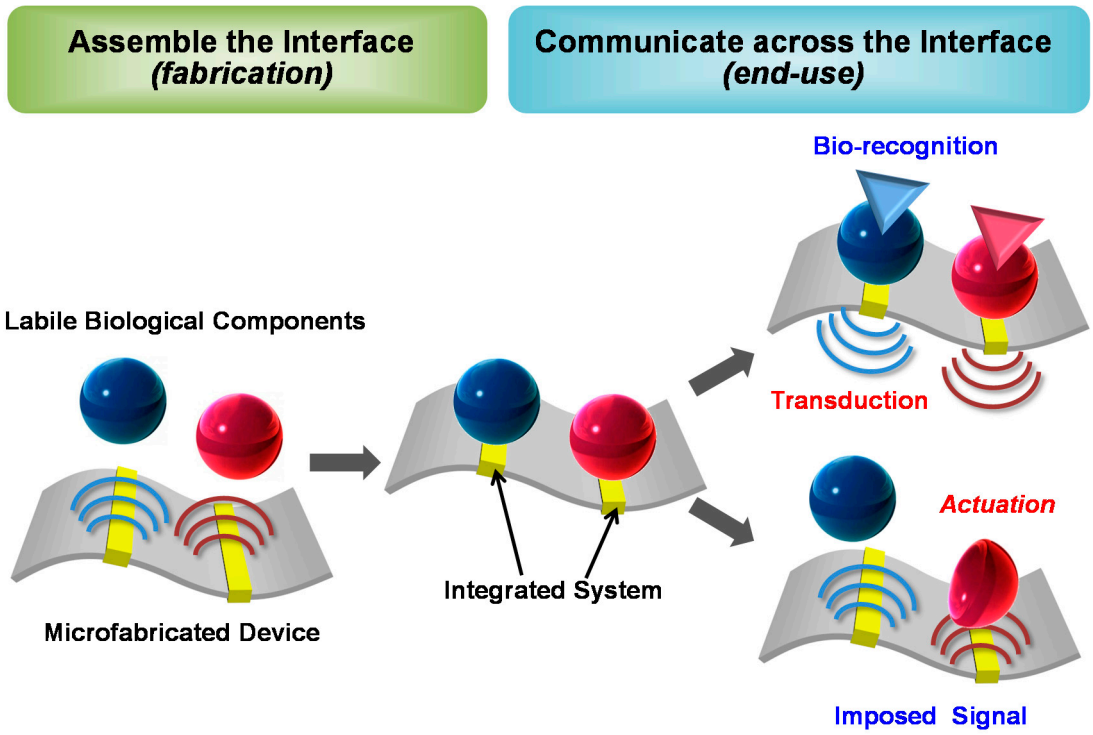


Figure 1. Broad vision for interfacing biology with electronics. We envision fabrication will enlist device imposed electrical signals to direct assembly of the bio-device interface, and communication will allow the interconversion of biological and electronic signals. Reproduced with permission from [5], published by Institute of Physics publishing, 2010.

The second part of the vision, illustrated at the right in Figure 1, is that an ideal bioelectronic device would allow ready communication between the biology and the electronics. Since many envisioned applications of bioelectronics involve sensing/analysis, then communication should allow biological recognition events to be transduced into device compatible (e.g., electrical) signals. Alternatively, communication in the opposite direction could allow device-imposed signals to actuate biology. For instance, if the assembled biological component is a protein, actuation could involve triggering its release from the interface (e.g., for controlled delivery) or actuation could involve inducing a conformational change to exert a force or to alter biological function (e.g., alter enzymatic activity) [6]. As will be discussed, we have recently begun using chitosan derivatives to promote such communication across the bio-device interface.

2. Why Chitosan?

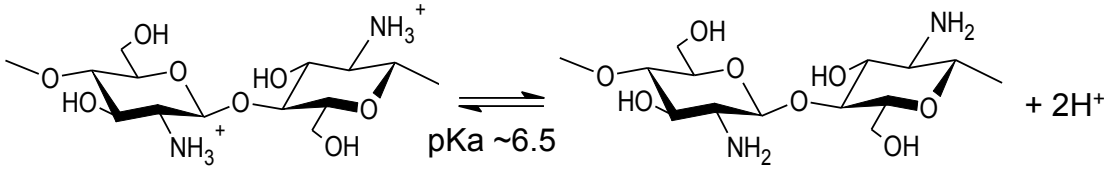
Chitosan is a linear aminopolysaccharide that possesses a unique combination of properties that have been found to be useful for a wide range of applications [4,7–13]. Because chitosan is generated by the partial de-acetylation of chitin, it is formally a co-polymer of glucosamine and *N*-acetylglucosamine residues. The distinguishing structural feature of chitosan is its primary amine at the C-2 position of the glucosamine residues. We should emphasize that chitosan's amine-rich chemical functionality is unique: we are unaware of other nature-derived polymers of such rich amine functionality and even synthetic amine-containing polymers are somewhat difficult to produce industrially. Not only is chitosan's amine functionality unique, it is important because many of chitosan's useful functional properties are conferred by these amine groups. Table 2 highlights chitosan's properties that are especially important for building and communicating across the bio-device interface.

Table 2. Important properties of chitosan for building the bio-device interface and establishing communication across this bio-device interface.

Property	Details
Stimuli-responsive (pH-responsive)	Chitosan undergoes a soluble to insoluble transition with a change in pH
Self-assembly (self-association)	Chitosan's interchain associations can yield a 3-dimensional hydrogel network
Polycationic	Positive charges on chitosan's amines can undergo electrostatic interactions with (poly)anions
Nucleophilic	Unshared electrons on chitosan's amines enable chemical modification under facile conditions
Metal-binding	Chitosan chelates metals through interactions with its amino and hydroxyl groups
Oxidizable	Chitosan (like many polysaccharides) can be partially oxidized to generate reactive moieties (e.g., aldehydes)

Because of the amines, chitosan is a weak polyelectrolyte that possesses pH-responsive properties (Scheme 1). At low pH, chitosan's primary amines are protonated making chitosan a cationic polyelectrolyte that is soluble in aqueous solution. At high pH, these amines are deprotonated making chitosan neutral and insoluble. Importantly, chitosan undergoes it's soluble to insoluble transition near

its p*K*a (≈ 6.2–6.6) [14–19], which is within a convenient range for biological applications. Interestingly, chitosan's pKa is considerably less than the value of 7.7 reported for the glucosamine monomer [18,20].



Scheme 1. Chitosan's amines confer pH-responsiveness.

Chitosan chains can undergo self-associations to form a three-dimensional hydrogel network. At low pH when the chitosan chains are polycationic, these self-associations are suppressed by the electrostatic repulsions and chitosan is soluble in aqueous solution. However, as the pH is increased and chitosan loses its charge the balance between the attractive and repulsive interactions is altered [21–23] such that the chains can undergo self-associations to form the hydrogel [24–27]. The process of chitosan's hydrogel formation can be viewed as either (i) a reversible sol-gel transition that results from the formation of physical (*i.e.*, non-covalent) crosslinks, or (ii) a triggered self-assembly of a supramolecular network. Irrelevant to the words used to describe this process, hydrogel formation serves to organize structure over a hierarchy of length scales from individual macromolecular chains to the macroscale [28].

There are two important points concerning chitosan's self-assembly. First, despite decades of study, the molecular-level details of chitosan's structure and properties are not well understood [26,29]. Unresolved questions include; how do the solution conditions (e.g., salt concentration) modulate the chitosan chain conformation, the sol-gel transition pH, and the hydrogel network [30]? How does a competition between intramolecular and intermolecular associations affect network structure (aggregates *vs.* gels)? and how does the network structure affect the mechanical properties of the electro-deposited film? Second, the interactions that hold the chains together are strong (yet non-covalent) and once the structure is formed it tends to be stable. As will be discussed, self-assembly through strong non-covalent interactions provides interesting opportunities to build structure [31,32]. However, the sol–gel transition is reversible and the hydrogel can be dis-assembled by re-dissolving the chitosan chains in mild acid.

Table 2 illustrates further properties conferred by chitosan's amines. At low pH, the protonated amines make chitosan a polycation which promotes electrostatic interactions with (poly)anions (chitosan is often used for polyelectrolyte complexation) [33–35]. At high pH, the deprotonated amines have an unshared pair of electrons that are nucleophilic and can be readily modified by electrophiles (standard grafting/conjugation chemistries can be used to generate chitosan derivatives with diverse functional properties). Further, this chitosan's amines can undergo chelation interactions with metals [36–39]. A final property of chitosan (not necessarily related to the primary amine) is that it can be partially oxidized [40–45]. Partial oxidation is often used to generate reactive aldehydes moieties that facilitate covalent grafting to the polysaccharide [46–49].

In summary, chitosan possesses a unique combination of properties that can be enlisted to realize the vision illustrated in Figure 1.

3. Fabrication

3.1. The Fabrication Challenge

Many people have recognized the potential of bioelectronics [50–54] and various approaches have been developed to assemble biological components at electrode addresses (e.g., electrode arrays or fluidic devices). While there has been considerable progress, many challenges remain and generic methods for fabricating the bio-device interface don't yet exist. In our opinion, the fabrication challenges arise because the materials and methods used to build electronic systems are profoundly different than those used for the creation of biological systems as illustrated in Table 3 [55–57].

Table 3. Divergent fabrication paradigms for electronics and biology.

Aspect	Biological Fabrication	Microfabrication
Fabrication paradigm	Bottom-up & hierarchical	Top-down & monolithic
Common materials	Soft (e.g., proteins & polysaccharides)	Hard (e.g., silicon & metals)
Approach to control chemistry	Enlist molecular recognition	Exclude contaminants
Approach to control defects	Correct & heal	Strive for defect-free fabrication
Final structure	Dynamic & adaptable	Static & permanent

Device microfabrication typically employs top-down, monolithic methods to generate patterned surfaces from hard materials (e.g., silicon wafers). In contrast, biology self-assembles from the bottom-up often using soft matter. For instance, biology uses genetic information to code for a protein's amino acid sequence while this sequence contains the information needed for the protein to fold (≈1 nm) and also to undergo the interactions required for hierarchical assembly (e.g., for the protein to associate with other subunits or to insert into a membrane).

A requirement common for both microfabrication and biological synthesis is that chemistry must be controlled to create structure with high precision. However, the strategies used to control chemistry are very different. During microfabrication, chemistry is controlled largely by excluding contaminants using clean rooms, ultra-pure reagents, and high vacuum processing. In contrast, biology controls chemistry using molecular recognition during binding, catalysis and assembly steps.

Finally, microfabricated and biological systems can have vastly different lifetimes. For instance, microfabrication generates "permanent" systems: electronic devices often become obsolete before their structures and functions are degraded. In contrast, biology is built from labile components that are often assembled using non-covalent mechanisms: this approach allows biological systems to respond, adapt and heal. Biology even has mechanisms to guide its own destruction: enzymes allow the selective degradation of protein and polysaccharide structures, while programmed cell death dissipates structure and function at the cellular level. The difference in lifetimes between a microfabricated system and the biological components embedded within this system can impose severe limitations as discussed in the next section.

In summary, the materials, mechanisms and construction "paradigms" are markedly different between device microfabrication and biological synthesis. Efforts to assemble biological components at a bio-device interface must bridge these differences and must especially accommodate the labile nature of biology.

3.2. Generic Fabrication: Constraints

Our fabrication goal is to create methods that are broadly applicable for the assembly of biological components at electrode addresses. To realize this goal, we believe that assembly approaches will need to satisfy the four major constraints listed as follows [5,58]:

- Post-fabrication biofunctionalization. Biological components (e.g., proteins and cells) are inherently unstable compared to traditional electronic devices, and traditional microfabrication methods are "bio-incompatible". Thus, we believe the best strategy is to complete microfabrication of the electronics before adding biological functionality. In fact, we envision the biofabrication steps could be done on-site by end-users just prior to use;
- Erasable biofunctional films. Biofunctional films that can be washed away after use will permit re-use of the electronic device (e.g., a microfluidic device) and thus relax cost constraints imposed by single use systems;
- Fabrication from water. Water is the medium of biology and thus fabrication methods must embrace and guide interactions in water to generate hydrogel-based interfaces between the biology and the electronics;
- Fabrication must be simple, rapid and programmable. We use electrical signals for spatiotemporal programmability and molecular recognition for chemoselectivity.

We envision satisfying these constraints as illustrated in Figure 2. First, we believe the best approach for accommodating the differences between biology and fabrication is to develop methods that allow the biological components to be incorporated into the device after the device has been fully microfabricated [59,60]. As indicated in Figure 2, we believe post-fabrication biofunctionalization will allow the most advanced methods to be used to generate the electronics without regard for the lability of the biological components: these components will be assembled after the device has been fully fabricated. By separating device fabrication from bio-functionalization, it is also possible to envision that biofuntionalization could be performed by end-users closer to the point of application. For instance, we envision the possibility that post-fabrication biofunctionalization could be performed at a small support lab associated with a physician's office, a mobile lab for environmental or security monitoring, or even a kitchen of a restaurant or grocery store.

The second constraint is that the biofunctional films that are interfaced to an electrode should be removable. As illustrated in Figure 2, erasable biofunctional films will allow devices to be reused and thus accommodate the divergent lifetimes between the device and the biological components.

Water is the medium of biology and as illustrated above assembly of the bio-device interface should be performed from aqueous solution. Finally, fabrication methods to build the bio-device interface should be simple. As will be discussed, we use electrical signals to trigger self-assembly of the bio-device interface because they are convenient and controllable. These electrical inputs are used to confer spatial-temporal specificity to assembly. To confer chemical selectivity to fabrication, we rely of biological molecular recognition mechanisms.

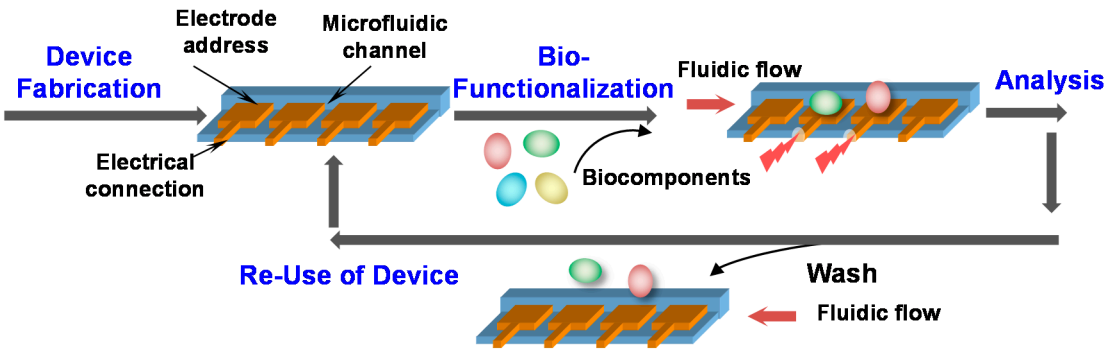


Figure 2. Generic fabrication approach for assembling the bio-device interface. We envision biological components (e.g., proteins) can be assembled at electrode addresses of a re-useable device through methods that allow post-fabrication biofunctionalization. Reproduced with permission from [5,58], published by Institute of Physics publishing, 2010 and American Chemical Society, 2005.

3.3. Biofabrication to Build the Interface

The overarching theme of our efforts to build the bio-device interface is to enlist materials, mechanism and lessons from biology to create generic biologically-based fabrication (*i.e.*, biofabrication) methods [61,62]. Biology is expert in creating structure at the nanoscale [63] and we enlist biotechnological methods to engineer proteins with added functionality that "code" for assembly. Further, biology is expert at assembling structure over a hierarchy of lengths scales and we enlist biological self-assembly and enzymatic assembly methods. As will be discussed, chitosan provides important stimuli-responsive self-assembly capabilities as well as the ability to be functionalized through enzymatic mechanisms.

3.4. Chitosan's Cathodic Electrodeposition

3.4.1. Mechanism

A decade ago, initial reports appeared demonstrating that chitosan's pH-responsive hydrogel-forming properties enabled it's triggered self-assembly at a cathode surface [64–69]. Figure 3 illustrates the neutralization mechanism responsible for chitosan's cathodic electrodeposition [69,70]. Chitosan is first dissolved into an acidic deposition solution (pH < 6) and then electrodes are immersed in this solution. Typically, either a controlled potential (voltage) or controlled current is applied to the electrodes to initiate the electrochemical reactions (e.g., water electrolysis reactions). The net effect of these cathodic reactions is to generate OH^- (or as illustrated in Figure 3 to consume H^+) to establish a pH-gradient with a high localized pH adjacent to the cathode surface. Chitosan chains near this cathodic region experience a high pH and their primary amines are deprotonated which induces the localized sol–gel reaction. The photographs in Figure 3 illustrate the formation of the electrodeposited hydrogel at a cathode surface. If deposition is performed for a sufficiently long time, the film can be peeled from the electrode to illustrate that the electrodeposited hydrogel film is stable. Typically, we do not remove the electrodeposited film from the electrode but rather this film serves as the bio-device interface.

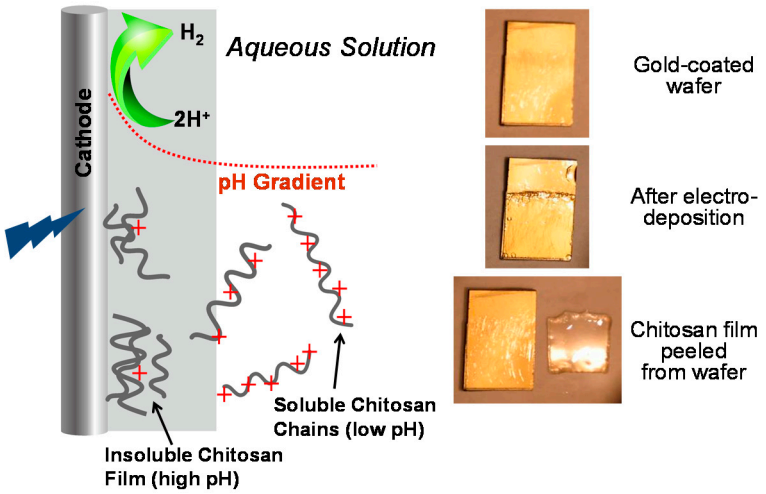


Figure 3. Chitosan's cathodic electrodeposition. Cathodic reactions generate the localized high pH adjacent to the electrode that neutralize chitosan and trigger its sol–gel transition.

From a chemistry perspective, the neutralization mechanism for cathodic electrodeposition relies on (i) chitosan's pH-dependent hydrogel-forming properties and (ii) cathodic reactions that yield the high-localized pH that induces chitosan to undergo its sol–gel transition. Several features of this mechanism are worth noting. First, because chitosan's pH-dependent sol–gel transition is reversible, the film generated by cathodic electrodeposition can be re-dissolved by adding acid. Second, if the electrode with the deposited film is removed from the deposition solution and added to neutral or basic solutions, the film remains adhered to the electrode surface in the absence of an applied potential/current. Third, sometimes readily reducible compounds (e.g., H_2O_2 or *p*-quinones) have been added to enable cathodic reactions to occur at lower applied voltages (*i.e.*, these compounds enable the pH gradient to be generated under milder conditions) [71,72]. For instance, cathodic reactions with H_2O, H_2O_2, and *p*-quinone occur at −1.4 V, −0.4 V and −0.1 V *vs.* Ag/AgCl, respectively [71]. Fourth, while the neutralization mechanism for chitosan's cathodic electrodeposition appears to be generally accepted, the relative contribution of electrophoresis (movement of charged chitosan chains in response to an applied potential) may not be fully resolved. Possibly, the importance of electrophoresis in chitosan's cathodic deposition may vary among different laboratories because different deposition conditions are often used; different acids to dissolve chitosan (e.g., HCl *vs.* acetic acid) [73], different solutions (alcohols are sometimes added) [74], and different electrical inputs (controlled currents or voltages).

From a functional perspective, the neutralization-gelation reaction in Figure 3 provides the mechanism that allows chitosan to "recognize" imposed electrical inputs and "respond" by assembling into a hydrogel film at the electrode surface. Importantly, electrodeposition couples the capabilities of imposing electrical inputs with high spatial and temporal control with the capabilities of chitosan's pH-responsive smart properties to form a hydrogel film rapidly and reversibly under mild aqueous conditions (without the need for reagents).

3.4.2. Spatiotemporal Controllability

Initial studies demonstrated that chitosan's cathodic electrodeposition can be controlled in the lateral dimension. To test lateral resolution, a silicon wafer was patterned with gold lines (*i.e.*, gold electrodes) of varying widths and spacing as illustrated in Figure 4. These patterned electrodes were immersed in chitosan solutions, deposition was performed for 2 min and then the wafer was removed from the deposition solution, disconnected from the power supply, and rinsed. The deposited chitosan films were then visualized by reacting with amine-reactive dye NHS (*N*-hydroxysuccinimide)-fluorescein and imaging using fluorescence microscopy. The fluorescence micrographs in Figure 4 show that the lines were well-resolved even for electrodes as thin as 20 μm [75]. Subsequent studies with more precisely patterned electrodes demonstrate deposition can be controlled at the micron scale [76].

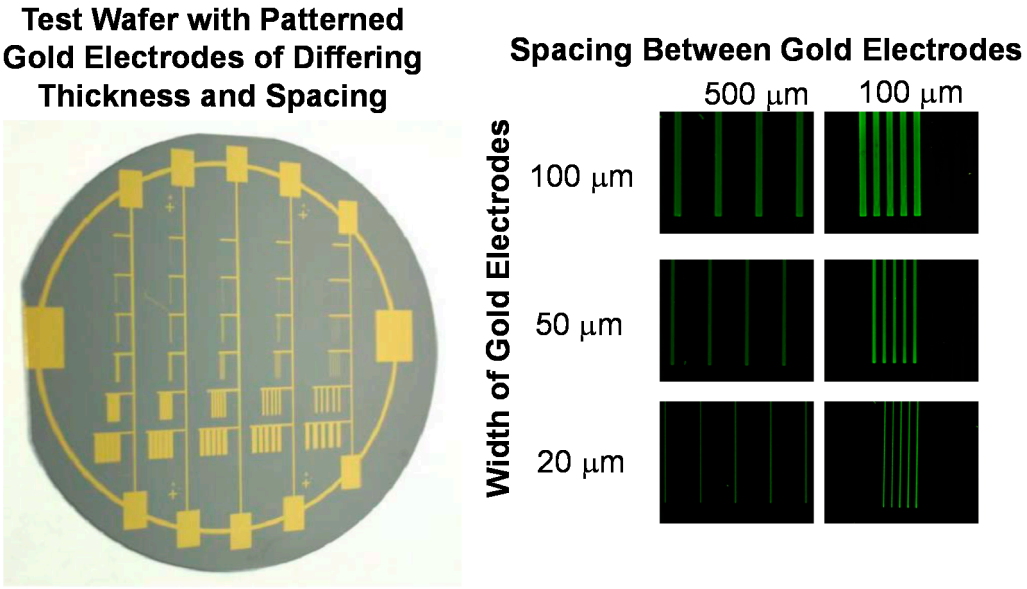


Figure 4. Spatial control of chitosan's electrodeposition. Chitosan was deposited onto gold electrodes that had been patterned onto a silicon wafer. The test wafer was designed to have electrodes of varying width and spacing. After deposition, chitosan was fluorescently labeled to facilitate visualization. Reproduced with permission from [75], published by American Chemical Society, 2003.

Cathodic electrodeposition also allows assembly to be controlled in the normal direction to generate hydrogel films of specific thicknesses. To study this capability, the simple fluidic test device of Figure 5a was fabricated. This device has (i) a 1 mm × 1 mm channel cross-section, (ii) multiple 1 mm × 1 mm gold electrodes fabricated onto the glass sidewalls, and (iii) a transparent polydimethylsiloxane (PDMS) cover to enable real time optical and spectroscopic observation of the deposition process [77]. Figure 5b shows an image of a thick deposited chitosan gel. As illustrated by this photograph, chitosan's electrodeposition generates a three-dimensional hydrogel that can occupy considerable volume.

Images like those in Figure 5b can be collected over time during the course of the deposition process and image analysis allows the geometric features (*i.e.*, thickness) of the deposits to be measured. Figure 5c shows results for deposition experiments performed over time at varying current densities. The important results from Figure 5c are that deposition is rapid occurring over the course of

seconds to minutes, and is controllable with thicknesses varying as a function of time and the intensity of the electrical input signal (*i.e.*, the current density) [77].

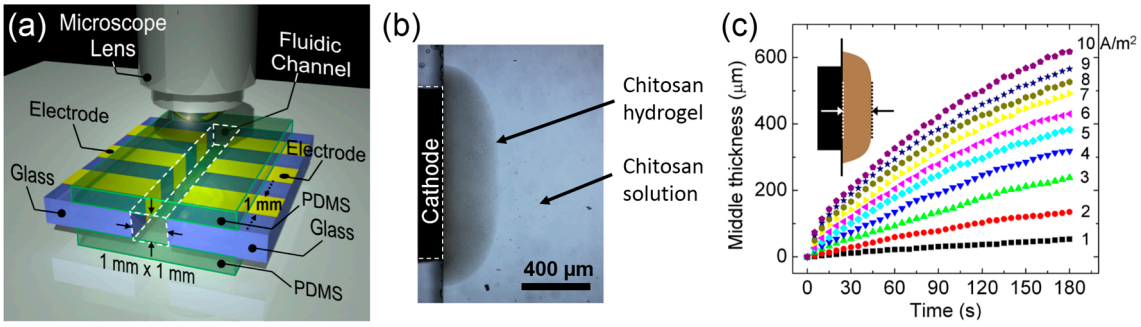


Figure 5. Cathodic electrodeposition allows control of chitosan film thickness. (**a**) Fabricated fluidic device used for *in situ* observation of chitosan's electrodeposition. (**b**) Photograph of a chitosan film electrodeposited onto the sidewall electrode address. (**c**) *In situ* measurements of chitosan's film thickness as a function of time and current density. Reproduced with permission from [77,78], published by Royal Society of Chemistry, 2010 and 2011.

3.4.3. Electrodeposition as a Moving Front

The test device of Figure 5a was also used to examine additional features of chitosan's electrodeposition. In particular, pH indicator dyes could be used to monitor the pH profile generated within the fluidic device. In a control study, the pH indicator dye was added to a solution (without chitosan), this solution was pumped into the fluidic channel and a constant current density (4 A/m^2) was applied to two opposing electrodes (there was no fluid flow during the time the electrodes were biased). After 85 s, the image in Figure 6a shows a pH gradient progressing from acidic conditions near the anode (on the right) to basic conditions near the cathode (on the left) [77]. Next, the pH indicator dye was added to the chitosan deposition solution that was then added to the channel and images were collected after deposition had been initiated. The images in Figure 6b show two regions are obvious, the basic region adjacent to the cathode where the hydrogel had deposited and the acidic region outside the gel. The insert in Figure 6b indicates that these two regions are separated by narrow interface region where the pH is observed to transition from the gel phase (pH ≈ 10) to the bulk deposition solution (pH ≈ 5).

The results in Figure 6 indicate that chitosan's gelation proceeds as a moving front with the gelation front co-localizing with the pH front [77]. In an independent study with a different experimental system, Dobashi and co-workers [79] demonstrated that the pH and gelation fronts are co-localized with a third front that separates an anisotropic chitosan gel from an isotropic chitosan solution. Initial mathematical modeling efforts have employed a moving front model to characterize the dynamics of chitosan's cathodic electrodeposition [80].

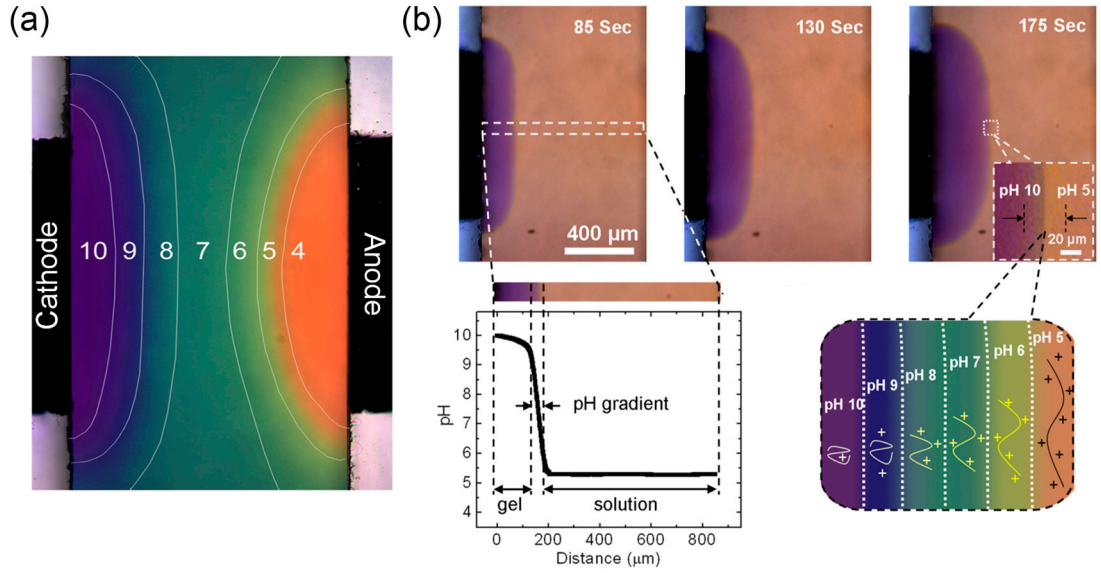


Figure 6. Measurements of the electrode induced pH gradient indicate that chitosan electrodeposition occurs as a moving front of both pH and gelation. Electrochemical pH gradient (**a**) in the absence of chitosan and (**b**) in the presence of chitosan. Reproduced with permission from [77], published by Royal Society of Chemistry, 2010.

3.4.4. Structure and Properties of Cathodically-deposited Chitosan

The above discussion suggests that chitosan's cathodic electrodeposition is well-understood and highly-reproducible. In a sense, it is true that cathodic electrodeposition is well-behaved: the phenomena is simple to observe and has been reproduced in many labs around the world [8,81]. However, chitosan's electrodeposition can be considered to be a self-assembly process involving strong non-covalent interchain associations [82], and as a result, the structure and properties of the deposited films are highly sensitive to the deposition conditions. Once the films are formed at the cathode, they do not readily "anneal" to an equilibrium structure (although they can be re-dissolved in acid). Two recent studies demonstrate the potential for controlling structure and properties by controlling the conditions used for electrodeposition.

In the first study, chitosan was electrodeposited from solutions containing different levels of salt (*i.e.*, NaCl). Intuitively, the added salt would be expected to screen electrostatic repulsions of the protonated chitosan chains leading to a tendency toward more collapsed chain conformations and also a tendency for aggregates to form upon neutralization. Deposition under such high salt conditions might be expected to yield a looser network of aggregates with fewer elastically-active inter-polymer network junctions. Experimentally, chitosan films were deposited from solutions containing varying levels of salt and after deposition the films were rinsed and then probed using a quartz crystal microbalance with dissipation (QCM-D). Figure 7 shows that the strength (*i.e.*, the elastic modulus) of the resulting wet films varied by several orders of magnitude depending on the salt concentration in the initial deposition solution [83]. This result demonstrates that film properties are highly dependent on deposition conditions: the good news is that there is a rich design space available to manipulate conditions to tailor film properties, but the bad news is that the sensitivity to small variations in conditions can lead to considerable difficulty in precise reproducibility. In our opinion, the critical

barrier to controlling film properties is our limited understanding of the hierarchical interactions responsible for gelation. For instance, the expectation suggested above that salt additions would tend to favor more collapsed chitosan chain conformations is simplistic as several studies have shown that chitosan's rigidity resists such chain collapse [84].

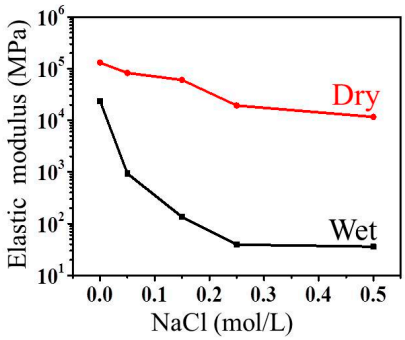


Figure 7. The properties of electrodeposited chitosan films are sensitive to the conditions used during the deposition process (*i.e.*, the addition of salt to the deposition solution). The elastic modulus was measured by quartz crystal microbalance with dissipation. Reproduced with permission from [83], published by Royal Society of Chemistry, 2013.

In a second study, chitosan was deposited onto a stainless steel wire using on-off pulses of input current. As illustrated by the schematic in Figure 8 chitosan layers are "grown" during the on-pulses, while interfaces were generated during the off-pulses. The scanning electron microscope (SEM) images in Figure 8 show the resulting "output" multilayer structures generated using different input sequences. Importantly, a moving front model could reasonably describe the multilayer growth dynamics [80]. More broadly, the observations in Figure 8 are consistent with several recent studies that demonstrate multilayer hydrogel structures can be generated by imposing conditions that result in interrupted gelation [85–89]. Remarkably, there is a limited fundamental understanding of how interruptions in gelation cause changes in structure, and how such phenomena can be controlled to create complex and arbitrarily-defined structures in soft matter.

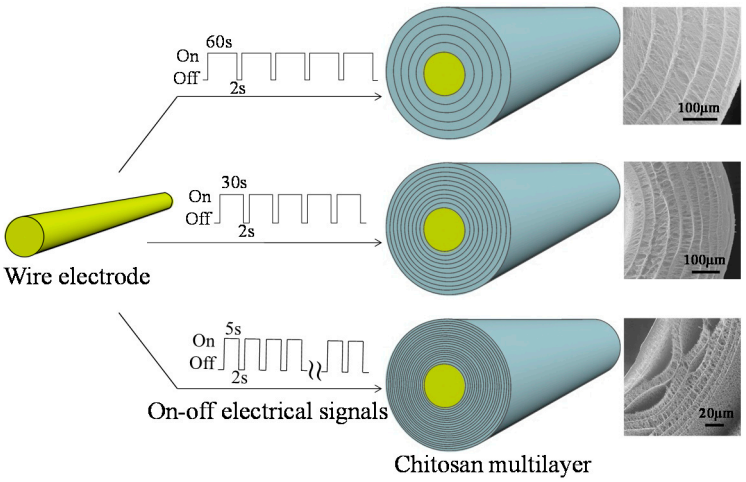


Figure 8. The structure of the electrodeposited chitosan film can be "coded" by the sequence of electrical stimuli used to induce and interrupt chitosan's cathodic electrodeposition. Adapted from Yan *et al.* [80].

3.4.5. Co-Deposition

One of the most useful features of chitosan's electrodeposition is the possibility to co-deposit and entrap various other materials in the chitosan film as illustrated in Figure 9. Shortly after chitosan's cathodic electrodeposition mechanism was reported, several studies demonstrated that co-deposition provides a simple means to localize a protein at an electrode interface and thus confer biological functionality (*i.e.*, enzymatic activity) to the deposited film. Such biofunctionalization is particularly convenient for creating enzyme-based electrochemical biosensors [66,67,90–92]. These studies demonstrated that deposition conditions could be developed to be sufficiently mild to preserve the protein's catalytic function. Subsequently, a large number of studies have extended this work for the co-deposition of various proteins [93–106] and many of these studies have been described in a recent review on the use of chitosan for electrochemical biosensing [8].

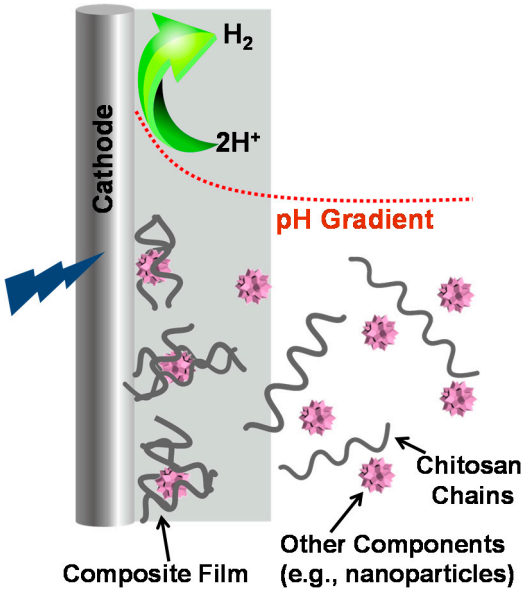


Figure 9. Co-deposition allows various biological and non-biological components to be assembled with and entrapped in the electrodeposited chitosan film.

In addition to proteins, it is also possible to co-deposit various other materials for incorporation into the electrodeposited chitosan film [68,107–111]. In many cases, co-deposition aims to generate composite coatings (e.g., biocompatible coatings) [112–115]. With respect to interfacing biological components to electronics (the topic of this review) many materials have been co-deposited with chitosan (e.g., carbon nanotubes [71,116,117], quantum dots [118] or other nanoparticles [119–121]) with the goal of facilitating electron transfer and signal transduction (e.g., to facilitate electron transfer from an entrapped enzyme to the electrode).

3.4.6. Integration with Other Assembly Methods

Chitosan's electrodeposition is simple and versatile and can be coupled with other aqueous based thin-film assembly methods. In particular, the layer-by-layer (LbL) assembly of polyelectrolyte multilayers has become a simple and effective means to create thin films with diverse functional

properties [122–124]. Figure 10a shows that chitosan's electrodeposition can be coupled with the LbL assembly—in this case for the assembly of alginate-chitosan multilayers. In this study, chitosan was first electrodeposited onto either a gold electrode patterned onto a two-dimensional silicon surface, or onto a sidewall electrode of the fluidic device of Figure 5a. This deposited chitosan film serves as the "template" onto which the first alginate layer can be assembled by electrostatic interactions. Sequential contacting between solutions of alginate (0.2% alginate, pH = 6.2), and chitosan (0.2% chitosan, pH = 5.6), enabled a multilayer to be generated as illustrated in Figure 10a [125]. As indicated by the quartz crystal microbalance (QCM) measurements of Figure 10b, the LbL assembly steps add small, reproducible amounts of biopolymer to the film. Interestingly, recent studies indicate that chitosan and alginate may form particularly strong electrostatic complexes because of geometric matching between the polycationic sites of chitosan and the polyanionic sites of alginate [126]. This observation illustrates a broader point that polysaccharides possess unique structures and undergo specific interactions that can confer important functional properties.

In subsequent studies, the model biosensing enzyme glucose oxidase (GOx) was incorporated into the multilayers by dissolving this enzyme into the alginate solution used for multilayer assembly. As illustrated in Figure 10c, GOx catalyzes the selective oxidation of glucose to generate H_2O_2 which can be detected electrochemically. We assembled a GOx-containing multilayer on a side-wall electrode of the fluidic device in Figure 5a, and as shown in Figure 10c this enzyme-functionalized electrode could repeatedly detect the presence of glucose in the solution introduced into the fluidic channel [125]. The important points of this proof-of-concept study are: (i) chitosan's electrodeposition confers spatio-temporal selectivity for programmable assembly of the film; (ii) LbL provides the benefits of precise control of film thickness and protein incorporation; (iii) both assembly methods can be performed from water in a covered fluidic channel satisfying the constraints imposed in Figure 2; and (iv) the biosensing film is assembled on an electrode surface which is convenient for electrochemical biosensing applications [125]. Also important to note is that the polysaccharide hydrogel film is expected to provide a biologically compatible microenvironment that preserves protein structure and function (*i.e.*, to retain GOx's enzymatic activity) [127,128].

It should be noted that in the study of Figure 10, the electrode was only used for the initial chitosan electrodeposition step and it was not biased for any subsequent LbL assembly steps. Recent studies suggested that electrical inputs could be employed for such multilayer assembly steps. For instance, cathodic inputs could be imposed during each chitosan assembly step and anodic inputs could be imposed during each alginate deposition step [129] (Note: the weakly acidic polysaccharide alginate can be electrodeposited by an analogous neutralization mechanism in which anodic reactions are utilized to generate the low pH conditions necessary to protonate alginate's carboxylate groups [130]). When electrical inputs were used during the chitosan and alginate assembly steps relatively thick films (10–20 μm) could be generated.

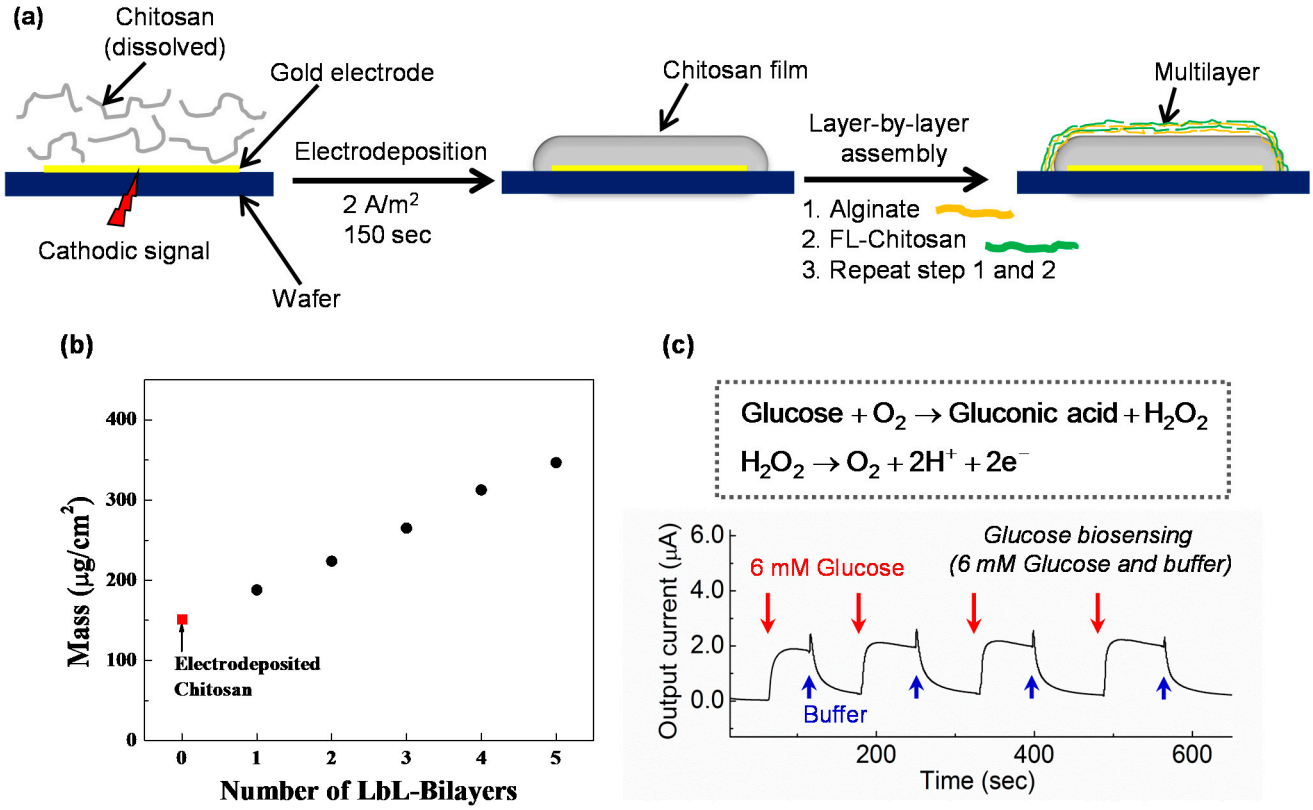


Figure 10. Coupling chitosan's electrodeposition with layer-by-layer assembly of chitosan-alginate multilayers. (**a**) Schematic of assembly steps; (**b**) Quartz crystal microbalance with dissipation (QCM-D) measurments showing controlled assembly of multilayer film; (**c**) Assembly of model enzyme glucose oxidase (GOx) confers biosensing function to the assembled multilayer. Reproduced with permission from [125], published by John Wiley and Sons, 2011.

In summary, chitosan's cathodic electrodeposition results from a neutralization mechanism and couples the strengths of electronics for imposing electrical signals with exquisite spatial, temporal and quantitative control, with the intrinsic capabilities of chitosan to self-assemble in response to these imposed electrical inputs. Operationally, chitosan's cathodic electrodeposition is simple, rapid, spatially-selective and occurs under mild conditions. Because this method enlists chitosan's intrinsic ability to self-assemble, the films are generated without the need for reagents, and thus cathodic electrodeposition is potentially compatible with labile biological structures (e.g., proteins and liposomes can be co-deposited). Further, assembly methods that do not require reactive reagents are intrinsically safe and environmentally friendly. Finally, chitosan's self-assembly is reversible and films generated by cathodic electrodeposition can be dis-assembled by adding acid to re-dissolve chitosan. While much progress has been achieved over the last 10–15 years, much remains to be learned about the molecular level and hierarchical interactions that are responsible for the structures and properties of the electrodeposited films.

3.5. Chitosan's Anodic Electrodeposition

3.5.1. Mechanism

Recently, an anodic mechanism was reported to electrodeposit a covalently crosslinked chitosan film [131]. In contrast to cathodic electrodeposition, anodic deposition (i) results from chemical (*vs*. physical) interactions; (ii) is irreversible; and (iii) relies on chitosan's ability to be partially oxidized [132]. The proposed mechanism is illustrated in Figure 11a and results from four chemical steps.

The first step, that initiates electrodeposition, is the anodic oxidation of NaCl to generate Cl_2. This electrochemical reaction is well known and forms the basis for industrial chlor-alkali process. In the second step, Cl_2 reacts with water to form reactive HOCl species [133,134]. This reaction is also well known as well as the resulting pH-dependent speciation reactions shown in Figure 11. The third step suggested in Figure 11 is the reaction of HOCl to partially oxidize chitosan to generate reactive aldehyde species. Several studies have reported the partial oxidation of chitosan to generate aldehydes, although the reaction details may not be completely resolved [40–42,44,47]. The generation of aldehyde moieties is convenient because they can readily undergo reaction with primary amines to form Schiff base linkages in a fourth chemical reaction step (not shown in Figure 11) [48,135]. If a Schiff-base is formed between a partially-oxidized residue of one chitosan chain and the primary amine of a second chitosan chain, then this Schiff base can serve as a crosslink to generate a covalently-crosslinked film.

Physical and chemical evidence support the anodic deposition mechanism of Figure 11a [131]. For instance, anodically deposited films were observed to swell (but not dissolve) under acidic conditions—consistent with expectations of a covalently-crosslinked hydrogel network.

While there has been much less study of chitosan's anodic deposition mechanism (compared to cathodic electrodeposition), we believe anodic electrodeposition has an important potential advantage. Specifically, anodic deposition may provide a simple, one step method to deposit a chitosan hydrogel while simultaneously conjugating a protein to this hydrogel through the same Schiff-base mechanism that can crosslink chitosan chains.

To illustrate the potential for simultaneous chitosan deposition-protein conjugation, we examined the assembly of the GOx biosensing enzyme. As mentioned in Figure 10b, GOx-catalyzed oxidation of glucose generates H_2O_2 which is redox-active and can be detected electrochemically. In the experiment, GOx (680 U/mL) was added to the deposition solution (1% chitosan dissolved in acetic acid with 0.15 M NaCl), this mixture was added to the fluidic channel of the device in Figure 5a, and electrodeposition was initiated by applying an anodic potential to a sidewall electrode (constant current of 4 A/m^2 for 90 s). After rinsing the channel, the film-coated electrode was then used for glucose detection. For glucose detection the underlying electrode was poised to +0.6 V (*vs*. Ag/AgCl) and the current associated with H_2O_2 oxidation was measured. The results in Figure 11b show that when the channel was filled with solutions containing glucose, a current was observed and the steady state current increased with glucose concentration. These results demonstrate that the GOx-enzyme in the anodically-deposited chitosan retains its biocatalytic function. Potentially, anodic chitosan deposition provides a rapid, generic, single step method to assemble protein-containing hydrogels to an electrode address.

We should emphasize that anodic deposition is more sensitive to conditions than cathodic deposition and inadequate attention to details can lead to irreproducibility. Since Cl^- is integral to the deposition mechanism this ion must be included in the deposition solution. Also, the HOCl species appears to be the most reactive oxidant for partial oxidation [136–139] and thus controlling the local pH appears critical to anodic deposition. For our anodic deposition studies, we prepared solutions by dissolving chitosan in acetic acid (and not HCl). We believe the choice of acid is important because acetic acid serves as a buffer to maintain a pH ≈ 5 adjacent to the anode and this is near the optimum pH for the HOCl species [131].

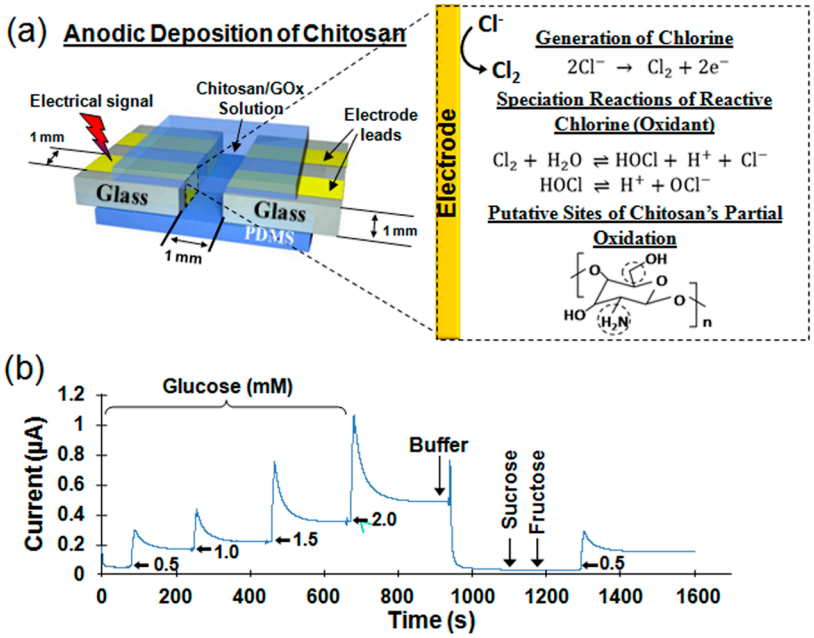


Figure 11. Anodic deposition of chitosan results from a sequence of chemical reactions that results in a covalently crosslinked network and possibly with covalently conjugated proteins. (**a**) Proposed deposition mechanism; (**b**) Results with the model biosensing enzyme glucose oxidase (GOx). Reproduced with permission from [131], published by American Chemical Society, 2012.

3.6. Biofunctionalizing Electrodeposited Chitosan Films

When we consider biofunctionalization of an electrode address, we are generally concerned with the assembly of either biomacromolecules (proteins or nucleic acids), or the assembly of viable cells (bacteria or eukaryotes). We use chitosan primarily for the assembly of proteins to impart either selective binding properties [140] or enzymatic catalytic activities [141]. In contrast, when our goal is to assemble cells at electrode addresses, we generally favor alternative stimuli-responsive biopolymers such as alginate [78,142–146], agarose [147] and gelatin [148]. Chitosan possesses several properties that facilitate protein assembly in/on an electrodeposited film through entrapment within the hydrogel network, covalent grafting to the polysaccharide backbone, or attachment through non-covalent associations. As previously mentioned, proteins could be assembled by co-deposition (Figure 9), layer-by-layer assembly (Figure 10), or anodic deposition (Figure 11). Additional approaches to assemble proteins to chitosan are listed in Table 4.

Table 4. Methods to couple proteins to chitosan to confer biological function.

Method	Details
Chemical conjugation	Glutaraldehyde Carbodiimide Epoxy Partial oxidation
Non-covalent binding	Electrostatic (Strept)avidin-biotin Metal chelation His-tagged protein
Electrochemical	Electro-click
Enzymatic	Tyrosinase

Since protein conjugation to chitosan is important for many technological applications (e.g., controlled delivery and regenerative medicine) there are several reports that illustrate the broad range of covalent conjugation methods available to create protein- conjugates [149–151]. Thus, we will only briefly mention these methods but refer the interested reader to the extensive body of literature available. Common methods to covalently conjugate proteins to chitosan include methods based on glutaraldehyde [152], carbodiimide [153,154], glyoxal [155] and epoxy [156] chemistries.

Non-covalent methods or a combination of covalent and non-covalent methods have also been used to assemble proteins to chitosan films. For instance, proteins can be bound to chitosan films through non-specific physical (e.g., electrostatic) interactions. In addition, chemical methods have been utilized to alkynylate an electrodeposited chitosan film to enable a subsequent electrically induced click ("electro-click") reaction to covalently conjugate proteins to the film [157].

In addition to the chemical methods described above, proteins have been assembled to electrodeposited chitosan films through mechanisms common to biotechnology. For instance, biotin moieties have been covalently attached to chitosan and then proteins have been assembled to the biotinylated chitosan through biospecific (strept)avidin-biotin binding [158,159]. In addition, chitosan's ability to chelate metals (e.g., nickel) enables a nickel-mediated assembly of proteins if the proteins have been engineered with hexa-histidine fusion tags that are commonly used to facilitate protein purification [160].

Recently, enzymes are being more fully enlisted for the fabrication and modification of materials [161–177]. Oxidative enzymes especially, tyrosinases, laccases, and peroxidases have been used to generate derivatives of chitosan [178–180] and have been extended to generating protein-chitosan conjugates [181]. Figure 12a shows that tyrosinase converts the phenolic tyrosine residues of proteins to *o*-quinone moieties that can undergo subsequent uncatalyzed reactions to covalently conjugate the protein to chitosan (see below for details of quinone-chitosan reactions). Tyrosinase has been used to mediate the grafting of peptides [182], open-chain proteins (e.g., gelatin [183,184] and silk proteins [185–190]) and proteins with globular structures (e.g., fluorescent proteins [191], binding proteins [140] and enzymes [141,192–194]) to chitosan. For the case of globular proteins, the tyrosine residues may be inaccessible (*i.e.*, tyrosinase cannot access these residues and convert them into *o*-quinones). In these cases it is possible to genetically engineer the protein with a short sequence of

tyrosine residues (*i.e.*, a tyrosine tag) [165,195] to provide accessible residues for tyrosinase oxidation and subsequent chitosan conjugation [191,196,197].

Figure 12b illustrates one example of tyrosinase-mediated protein conjugation of an IgG (Immunoglobulin G)-binding protein. In this study, the IgG-binding protein (Streptococcal protein G) was engineered to have a penta-tyrosine fusion tag which allows it to be conjugated to chitosan using tyrosinase. Protein G binds to the constant (Fc) region of IgG antibodies and thus orients the antibodies for antigen recognition [140]. In a separate study, tyrosinase was used to graft a peptide or protein tether to electrodeposited chitosan. Tethers that contain either lysine or glutamine residues can then be used to graft proteins through a separate enzymatic conjugation method involving a microbial transglutaminase as illustrated in Figure 12c. For instance, Figure 12d shows that tyrosinase was used to graft a gelatin tether to an electrodeposited chitosan film after which transglutaminase was used to graft proteins to the tether [198]. Thus, tyrosinase and translutaminase provide orthogonal methods to assemble proteins onto chitosan.

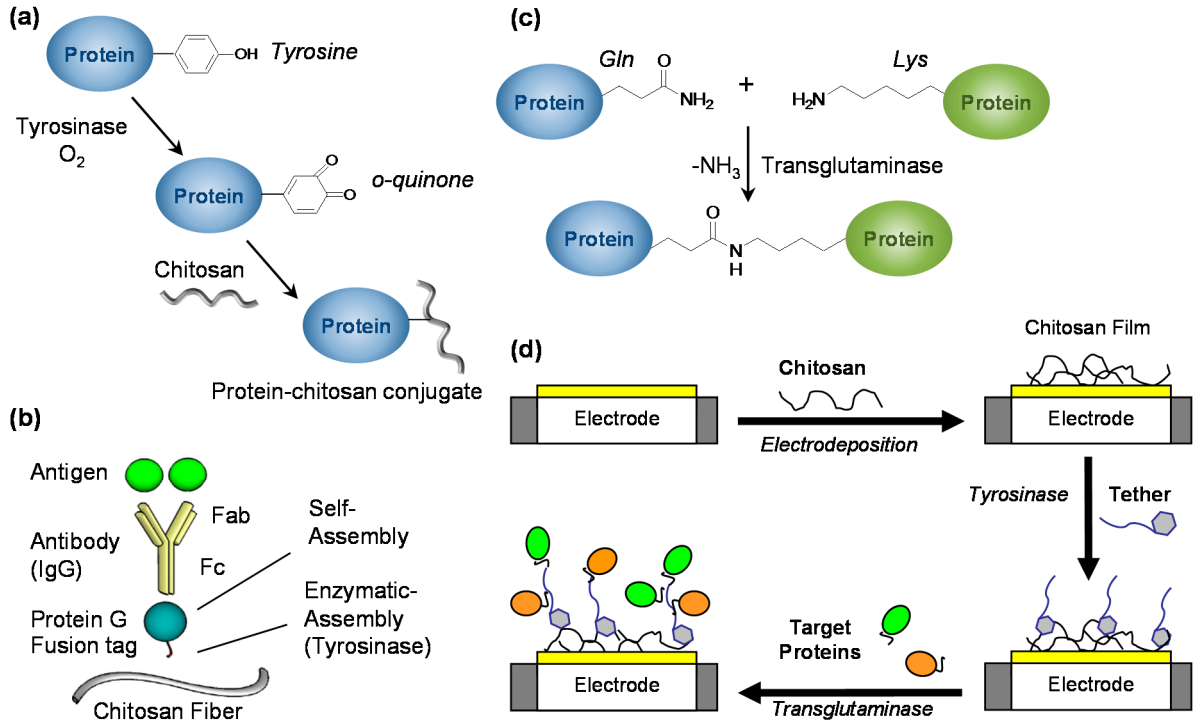


Figure 12. Enzymatic methods for assembling proteins to chitosan. (**a**) Tyrosinase oxidizes tyrosine to *o*-quinone residues that can react with chitosan; (**b**) Example of enzymatic assembly of a functional protein (IgG-binding protein) to chitosan. Reproduced with permission from [140], published by John Wiley and Sons, 2009; (**c**) Microbial transglutaminase provides an alternative enzymatic assembly method; (**d**) Orthogonal enzymatic methods to assemble proteins to chitosan. Reproduced with permission from [198], published by American Chemical Society, 2009.

In summary, chitosan offers unique properties both for the assembly of a hydrogel film at an electrode address and for its biofunctionalization by the incorporation of biological components (especially proteins). Protein incorporation can involve interactions that are physical, chemical or biological as illustrated in Table 4.

4. Communication

4.1. The Communication Challenge

Individually, electronic and biological systems are expert at acquiring, analyzing, storing and transmitting information. However electronic and biological systems use entirely different information processing approaches. As implied by the name, electronic systems employ electrons (or photons) to process information. In contrast, biology rarely uses electrons or photons for information processing: rather biology processes information using ions (e.g., Ca^{2+}) and molecules (e.g., hormones and neurotransmitters). Thus, establishing effective communication between electronics and biology must somehow bridge these different signal processing modalities.

4.2. Redox to Connect Bio-device Communication

It is not obvious how to bridge signaling modalities, and our efforts are focused on "connecting" biology with devices through electron-based communication. We believe that if we can succeed in establishing bio-device connectivity through electron-based communication, then it should be possible to access the immense capabilities of electronics for speed, computational power and wireless communication. While there have been efforts to create direct electrical connections to biology (*i.e.*, to enzymes [199]), we envision an indirect but potentially more generic approach. The obvious challenge to using electrons to communicate between biology and electronics is that free electrons do not exist in the aqueous solutions that are characteristic of biological systems. Rather, as indicated in Figure 13, the bridging of bio-device communication using electrons essentially means linking electrochemistry at the device-water interface with redox-biology.

Electrochemistry focuses on the electrical potentials and currents at an electrode-solution interface and links the transfer of electrons across this interface to the oxidation-reduction (redox) reactions occurring on the solution side of the interface. Because electrical potentials and currents can be readily measured and controlled, electrochemical methods are highly sensitive and rapid, while the instrumentation is relatively inexpensive. Electrochemistry also acquires information in a convenient format for information processing as will be discussed later.

From a biological perspective, redox reactions may provide an important "interface" into biology. As noted, free electrons do not generally exist in solution and thus biological electron transfer reactions involve redox reactions in which an electron(s) is transferred from one chemical species to another. Electron transfer pathways (e.g., respiration and photosynthesis) are commonly used by biology for energy harvesting. In addition, biology uses NADPH (Nicotinamide adenine dinucleotide phosphate) as a diffusible electron carrier (mediator or shuttle) to provide reducing equivalents for biosynthesis. Also, redox-active species are known to be important in health and disease. For instance, reactive oxygen species (ROS) are generated by the transfer of electrons to O_2 and these chemicals are integral components to a healthy immune response (*i.e.*, an oxidative burst) but are also prominent components in free radical theories of aging. Further, ascorbate (vitamin C) and thiol species (e.g., cysteine and glutathione) are important antioxidants for redox homeostasis and oxidative stress. More recently protein cysteine residues have been observed to serve as redox-switches that can be important in cellular signal transduction pathways. In summary, redox may provide a unique means to

access biological information related to energetics, biosynthesis, health and disease, as well as providing an interface into biological signal transduction pathways that impact gene expression and phenotypic behavior.

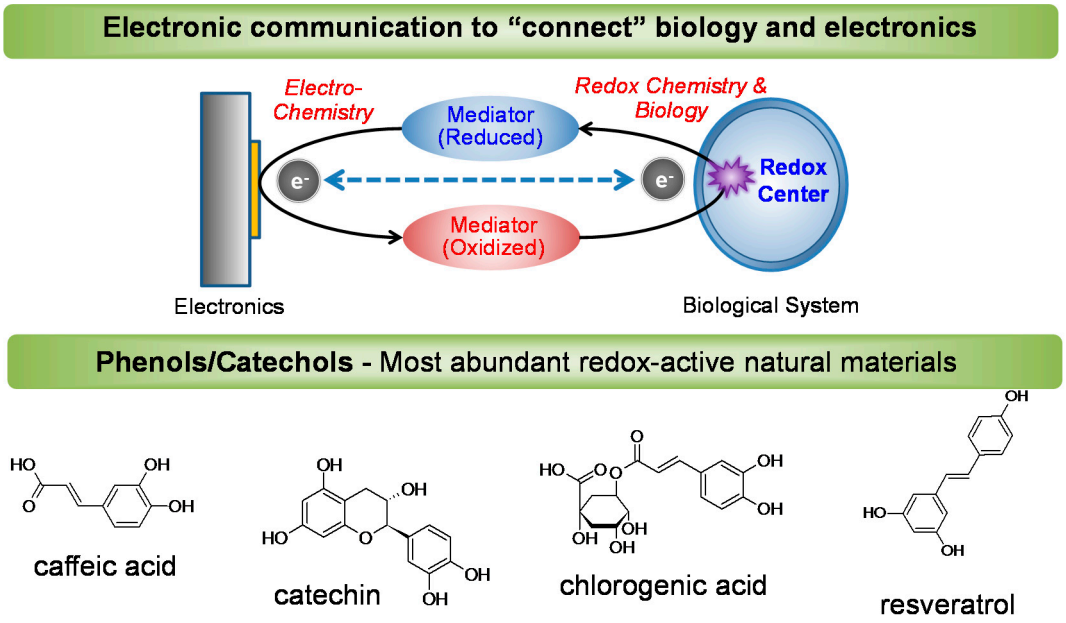


Figure 13. Connecting biology and electronics through electron-based communication relies on coupling electrochemistry with redox-biology. Examples of redox-active phenolic and catecholic natural products.

To build an interface capable of engaging both electronics and biology in electron-based communication, we are examining catecholic compounds. As indicated by the structures in Figure 13, phenols and catechols are common natural products and may represent the most abundant redox-active organics in nature [200]. Phenolic/catecholic based materials are also emerging in importance for technological applications [201–207]. In many cases, natural catechols/polyphenols have been shown to possess redox activities [208–210]. While the redox (*i.e.*, antioxidant) activities of dietary phenols [211,212] attract the most attention, there have been reports of phenolics as potential electronic materials [213–215]. For instance, melanins were reported to be amorphous semiconductors [216,217] and lignin derivatives were reported to improve the performance of conducting polymers [218]. This diverse evidence has motivated us to explore redox-active catechols as a unique means to bridge communication between biology and electronics. Integral to our redox-based approach to bridge bio-device communication is to chemically graft redox-active catecholic compounds to chitosan films.

4.3. Catechol-Chitosan Redox-Capacitor

4.3.1. Oxidative Conjugation of Catechols to Chitosan

Figure 14 illustrates that we graft catechols to chitosan films using either enzymatic [219–222] or electrochemical [223,224] oxidation reactions. Catechol oxidation generates *o*-quinones which are reactive electrophiles that can undergo uncatalyzed reactions with the nucleophilic amines of

chitosan [225–227]. Figure 14 shows that grafting is expected to result in the formation of Schiff-bases or Michael-type adducts [180,228,229] although various additional reactions could occur leading to the generation of grafted catecholic oligomers or the formation of chitosan crosslinks. While the grafted catechols confer the desired redox activities, chitosan is a convenient matrix because chitosan's pH-responsive film-forming properties allow electrodeposition of a film (*i.e.*, electroaddressing), while chitosan's nucleophilic properties allow quinones to be readily grafted.

Experimentally, catechol-modified chitosan films are fabricated electrochemically by immersing a chitosan-coated electrode in a solution containing the catechol (typically 1–10 mM) and an anodic potential is applied (+0.6 V *vs.* Ag/AgCl for 5 min). The quinones generated at the electrode surface diffuse into the chitosan film where they rapidly react with free amine groups [230]. As suggested from Figure 14, the quinone-chitosan reaction appears to be rapid with grafting occurring as a front such that chitosan regions nearest the electrode react first [224,231].

Enzymatic grafting is typically achieved by immersing the chitosan film into a buffered solution containing the catechol and tyrosinase [232] (note: the electrode is not required for enzymatic fabrication). Again, since quinone-chitosan reactions are rapid, we anticipate grafting proceeds from the solution interface into the film as indicated in Figure 14.

In comparison, tyrosinase-mediated oxidation has the advantage that phenols with a single hydroxyl group can be converted into *o*-quinones that can be conjugated to chitosan (electrochemical oxidation of such phenols often results in free radical generation and less controllable chemistries). A comparative disadvantage is that tyrosinase-mediated oxidation can be slower requiring minutes to hours (*vs*. seconds to minutes for electrochemical oxidative conjugation).

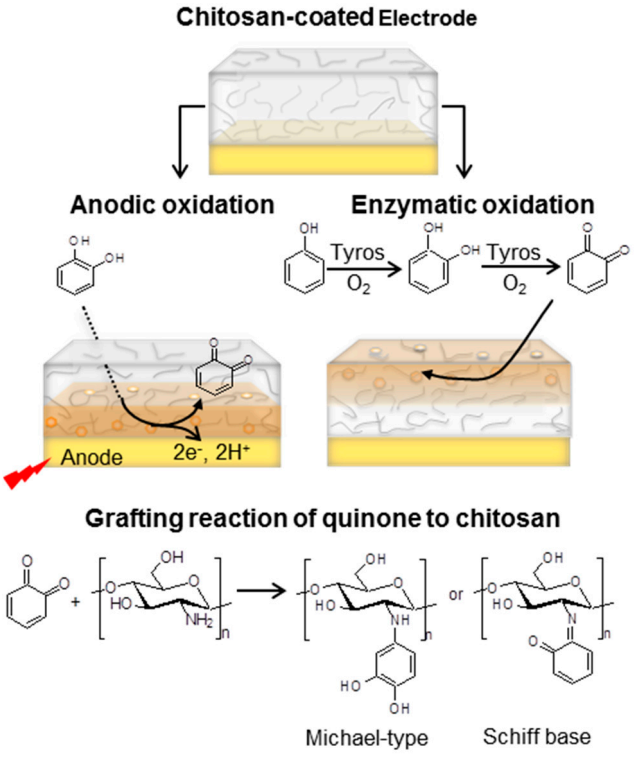


Figure 14. Grafting of catechols to chitosan films using anodic or enzymatic oxidation to generate reactive *o*-quinones that can undergo subsequent uncatalyzed reactions. Reproduced with permission from [233], published by Royal Society of Chemistry, 2014.

4.3.2. Redox-Cycling of the Catechol-Modified Chitosan Films

Our approach of establishing electron-based communication between biology and electronics relies on redox-cycling reactions in the catechol-chitosan film. Redox-cycling in the film is based on two important properties. First, the catechol-chitosan films are *non-conducting*: electrons in the film do not flow in response to an applied electric field and the films cannot exchange electrons directly with the underlying electrode [230]. The observation that the films are non-conducting is not surprising since: (i) the polysaccharide matrix by itself is not redox-active; (ii) the grafted catechol moieties do not appear to form an extended conjugated structure; and (iii) the films are thick (≈1 μm wet and ≈0.3 μm dry thickness) limiting direct contact between the electrode and grafted catechols.

The second important property for electron-based communication is that the catechol-chitosan films are *redox-active*: the films can reversibly accept, store and donate electrons. However, the transfer of electrons to or from the catechol-chitosan film requires soluble mediators as indicated in Figure 15. For instance, soluble mediators that transfer electrons to the film can switch the film from an oxidized state (presumably *o*-quinone moieties designated Q) to a reduced state (presumably catecholic moieties designated QH_2). Alternatively, soluble mediators that transfer electrons from the film switch the reduced moieties of the film (QH_2) to their oxidized state (Q). Importantly, the film's redox-switching requires diffusible mediators to shuttle electrons to/from the film and thus the catechol-chitosan film must be permeable to these mediators.

To switch the catechol–chitosan films between their redox states, we can use electrochemical redox-cycling mechanisms which serve to shuttle electrons between the film and the underlying electrode. This redox-cycling requires mediators that can: (i) diffuse through the film to access both the electrode surface and the bulk solution; (ii) exchange electrons with the underlying electrode; and (iii) exchange electrons with the catechol-chitosan film. A convenient electrochemical mediator for oxidative redox-cycling is ferrocene dimethanol (Fc) which can diffuse through the chitosan film and donate an electron to an electrode that is poised at an oxidative potential (above about +0.25 V *vs.* Ag/AgCl). As illustrated in Figure 15a, the oxidized Fc^+ can either diffuse out of the film into the bulk solution or undergo oxidative redox-cycling within the film by accepting electrons from the film. Oxidative-redox cycling converts Fc^+ back to a reduced Fc state which can again undergo oxidation at the electrode. This Fc-mediated oxidative redox-cycling serves to "discharge" electrons from the film by converting grafted moieties from QH_2 to Q.

Figure 15b illustrates the reductive redox-cycling mechanism with the convenient electrochemical mediator $Ru(NH_3)_6Cl_3$ (Ru^{3+}). In this case, Ru^{3+} accepts electrons at an electrode that is poised at a reducing potential (below about −0.2 V *vs.* Ag/AgCl). Reduced Ru^{2+} can then donate electrons into the film and be oxidized back to its Ru^{3+} state. This reductive redox-cycling serves to "charge" the film with electrons by converting the film's grafted moieties from Q to QH_2.

Figure 15c indicates that redox-reactions are constrained by thermodynamics such that electrons must "flow" from more negative to more positive redox potentials. As a result of this thermodynamic constraint, a single mediator can only transfer electrons in one direction—either to the film or from the film. Importantly, the catechol-chitosan films can be contacted with both mediators simultaneously and the redox-cycling mechanism (either oxidative or reductive) can be independently set by changing the electrode's potential (*i.e.*, voltage).

Overall, Figure 15 shows that the catechol-chitosan film can accept electrons (by reductive redox-cycling), store electrons (as QH_2 moieties), and donate electrons (by oxidative redox-cycling). In essence, this catechol-chitosan film is a redox-capacitor [233]. Importantly, the film's redox-capacity (N_{Film}) is finite with typical values observed to be 20 nmol of exchangeable electrons per cm^2. As an aside, this experimentally observed redox capacity indicates that approximately 5%–10% of the grafted catechols remain redox-active which suggests that a better understanding and control of the grafting chemistry may improve the redox-capacitor's performance.

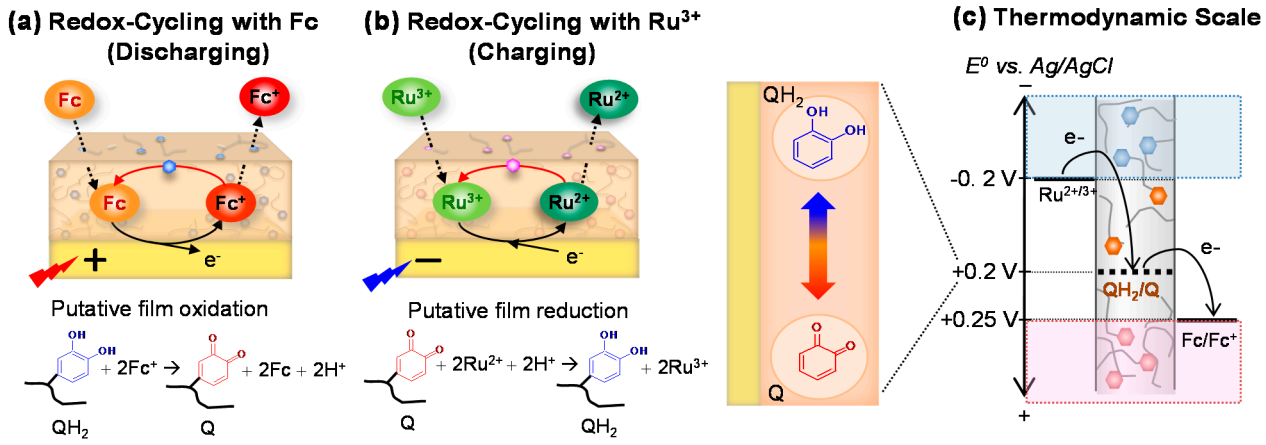


Figure 15. Redox-cycling of the catechol-chitosan redox-capacitor. (**a**) Oxidative redox-cycling can discharge electrons from the film; (**b**) Reductive redox-cycling can charge the films with electrons; (**c**) Thermodynamics controls the direction of electron transfer such that a mediator can only transfer electrons to/from the film in one direction. Reproduced with permission from [233], published by Royal Society of Chemistry, 2014.

4.4. Information Processing Properties of the Redox-Capacitor

While much recent interest in redox-capacitors is focused on energy storage applications, our interest in the catechol-chitosan redox-capacitor is focused on its information processing capabilities [234]. In essence, because the grafted catechol-moieties can reversibly accept, store and donate electrons, they confer six characteristic "molecular electronic" properties to the chitosan film as outlined in Table 5. We believe these properties endow the catechol-chitosan film with an ability to manipulate mediator currents in ways that can provide information of the local environment (e.g., information of localized biological activities).

To date, there have been a handful of studies with the catechol-chitosan redox-capacitor and these studies have generally focused on sensing applications. For instance, the amplification properties of the redox-capacitor could enhance sensitivities for detecting the bacterial virulence factor pyocyanin [235] and the antipsychotic medication clozapine [236], while the combination of amplification and rectification provided signatures of biologically relevant redox-cycling activities (e.g., of the analgesic, acetaminophen) [234,237].

Table 5. Molecular electronic properties of the catechol-chitosan redox-capacitor films.

Property	Details
Switching	Oxidative redox-cycling switches the film to an oxidized (discharged) state while reductive redox-cycling switches the film to a reduced (charged) state
Amplification	Redox-cycling serves to amplify output currents
Partial Rectification	Thermodynamic constraints limit a mediator's redox-cycling to one direction (either oxidative or reductive) and this enhances mediator currents in one direction while inhibiting mediator currents in the other direction (e.g., large oxidative currents and small reductive currents are observed with the Fc mediator, while the opposite is true for the Ru^{3+} mediator)
Gating	Because the catechol-chitosan film is non-conducting, a mediator is required to charge and discharge the film and thus charging/discharging is controlled by the mediator's redox potential (*i.e.,* the mediator's E° serves to gate film charging and discharging)
Steady Oscillating Inputs/Outputs	If oscillating electrode potentials are imposed to sequentially engage oxidative and reductive redox-cycling then oscillating output currents can be generated to yield a pattern that remains nearly steady over time (oscillating inputs and outputs are commonly used in signal processing)
Communicate with Biological Systems	The catechol-chitosan film can accept electrons from common biological reductants (NADPH and ascorbate) and donate electrons to common biological oxidants (e.g., O_2) and thus can "communicate" with biology

We should note that the properties listed in Table 5 are primarily due to the catechol moieties grafted to the chitosan films and not directly related to chitosan's properties. Thus, we will briefly cite two examples to illustrate the potential of this bio-based redox-capacitor for bridging communication across the bio-device interface.

4.5. Enzymatic Charging of the Redox-Capacitor

Initial studies demonstrated that when the catechol-chitosan film was incubated with the common biological reducing agent NADPH (5 mM for 5 min), electrons could be transferred from this reductant to the film (see the original publication for experimental details) [238]. This result suggested that the catechol-chitosan redox-capacitor could be charged by the enzymatic redox-cycling scheme illustrated in Figure 16a. In this scheme the enzyme glucose dehydrogenase (GDH) transfers electrons from glucose to $NADP^+$ after which the electrons are transferred to the catechol-chitosan film. Experimentally catechol-chitosan films were incubated for 15 min with, GDH, $NADP^+$ and varying levels of glucose, and then the number of electrons transferred to the films was measured electrochemically. The experimental results in Figure 16b show that the amount of electrons donated into the film ($N_{Film,Charged}$) varied with the glucose content of the solution (experimental details are provided in the original publication) [239]. These results demonstrate that the catechol-chitosan redox-capacitor can accept electrons from common biological reducing agents. Importantly, the *y* and *x* axes in Figure 16b show an electrochemical output as a function of a biologically-relevant chemical input (glucose concentration). Thus the axes of this plot illustrate the potential of the redox-capacitor to bridge electron-mediated communication between biology and electronics.

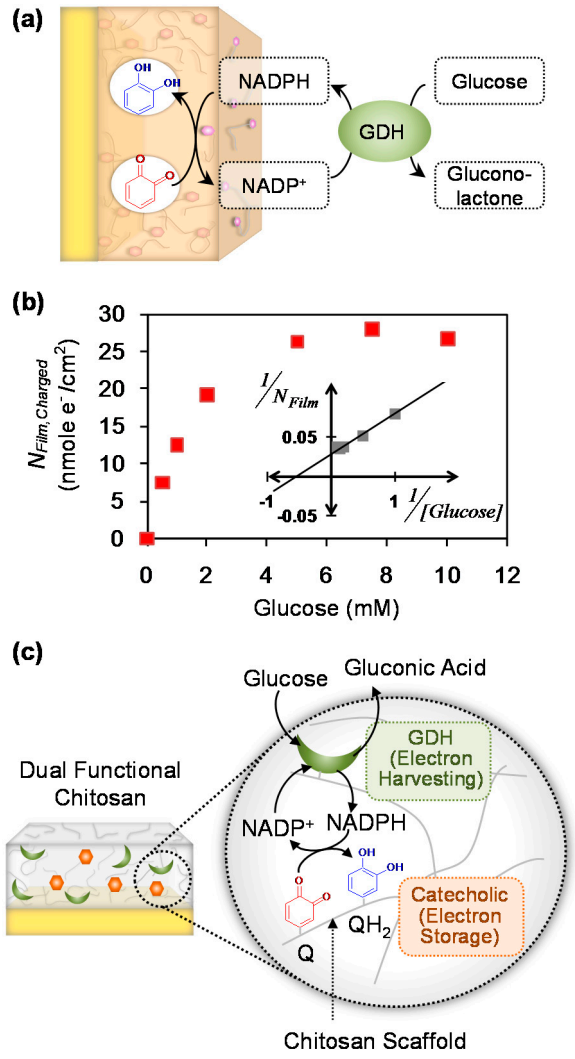


Figure 16. (**a**) Glucose dehydrogenase (GDH) catalyzed redox-cycling to charge the catechol-chitosan film; (**b**) Enzymatic charging is controlled by the electron source glucose (insert shows analogous Lineweaver-Burke plot); (**c**) Schematic illustrating how GDH harvests electrons from glucose and NADPH shuttles them to the grafted catecholic moieties where the electrons are stored. Reproduced with permission from [239] and [232], published by John Wiley and Sons, 2012 and Institute of Physics Publishing, 2013.

In subsequent studies we prepared chitosan films with both the glucose dehydrogenase enzyme and the catechol moieties. As illustrated in Figure 16c, this film has the enzymatic activity to "harvest" electrons from glucose and transfer them to NADPH, and the redox-capacitor activities to accept the electrons from NADPH and store them in the film.

4.6. Accessing Global, Systems-Level Redox Information

The results in Figure 16b illustrate the use of the redox-capacitor when a reasonably well-defined sequence of biochemical events is used to convert a biochemical input (*i.e.*, glucose) into a readily detected electrical output (*i.e.*, currents). However, we envision broader possibilities: to probe complex biological systems to obtain less well-defined biological inputs that are interpreted from the observed electrical outputs. In essence, we envision the use of information processing methodologies to

investigate redox-biology through reverse engineering. Our approach is analogous to the use of electrocardiograms and electroencephalograms to observe heart and brain waves as a means of understanding the function of complex biological systems (e.g., the heart and brain). Thus, we are attempting to couple the capabilities of electrochemistry for rapid, real time and *in situ* measurement with the power of information processing to discern subtle patterns (*i.e.*, signatures) that reveal important interactions in redox-biology [233].

Our initial experimental approach is schematically illustrated in Figure 17a. In this example, an oscillating potential input is imposed through the catechol-chitosan redox-capacitor that is in contact with a complex biological milieu (a bacterial suspension as illustrated in Figure 17a). Diffusible redox-active chemical species "sample" the localized redox-environment in the milieu. These mediators then exchange electrons with the redox-capacitor as a means of "transmitting" the sample's redox-information to the film. The electrode "reads" this information through a sequence of oscillating input potentials that generate complex oscillating output currents. Our goal is to process the information contained in these output signals to extract biologically-relevant redox-information. Importantly, the redox-capacitor serves to amplify, rectify and gate the output currents in consistent ways to facilitate information extraction. While this approach may seem abstract or implausible, it is important to note that standard communication technology rely on oscillating electromagnetic waves to transmit information. Thus, we are simply trying to enlist the capabilities of signal processing to extract biological redox information.

In some cases, the biological system under investigation may generate its own diffusible mediators while in other cases exogenous mediators may need to be added. In an initial study with a culture of *Escherichia coli*, we added two biological mediators shown in Figure 17b. For reductive redox-cycling, we added pyocyanin which is produced by the bacteria *Pseudomonas aeruginosa* and reported to be both a virulence factor and a signaling molecule [240–243]. Pyocyanin is a well-known biological redox-cycler and has been investigated as an electron shuttle for microbial fuel cells [244–246]. As suggested in Figure 17c, pyocyanin readily accepts electrons from cells and can donate them to O_2 or the redox-capacitor (thereby charging the film). Thus, pyocyanin is able to sample the localized redox environment. For oxidative-redox-cycling we used acetosyringone (AS) which is a plant signaling molecule [247,248] that can be oxidized enzymatically or electrochemically and can accept electrons from the redox-capacitor and thereby discharge the film [238].

As an initial test of this concept, we exposed the redox-capacitor to four different conditions: with or without bacteria and with air or N_2. Figure 17d shows the input output curves for these four conditions. As can be seen, the output can be expressed as either an output current (i) or an integrated charge transfer ($Q = \int i dt$) but in both cases, the output charge transfer is markedly different for these four different conditions. Using a more detailed quantitative analysis of the individual signals it was possible to discern trends that could distinguish the four conditions (see original paper for details) [249]. While we are encouraged by these initial results, there is much that is needed to effectively integrate electrochemistry with redox-biology to bridge the bio-device communication gap.

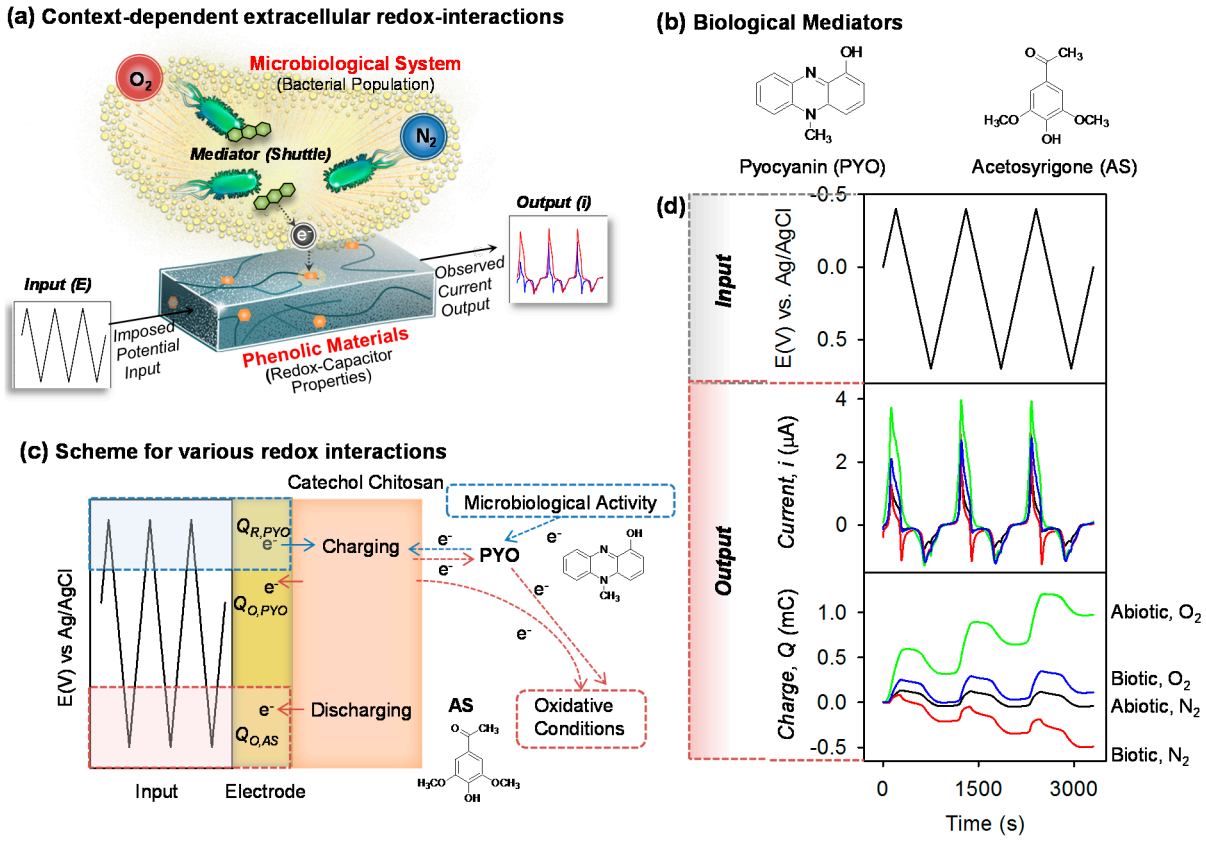


Figure 17. Acquiring global redox-information using the catechol-chitosan redox-capacitor. (**a**) Schematic illustrating how diffusible mediators transfer electrons between biological milieu and the redox-capacitor while cyclic electrical inputs and outputs read this information; (**b**) Biological mediators used for electron exchange; (**c**) redox-interactions of pyocyanin; (**d**) Input/output curves generated from four conditions. Reproduced with permission from [249], published by American Chemical Society, 2013.

Our long term goal of bridging biological and electronic communication may be easier to explain by way of analogy. Electrocardiograms (EKGs) use electrodes to measure ionic currents associated with the beating heart and the measured "waves" have been correlated to the physiological and pathological functioning of this complex organ. Typically, these measurements are made over the course of several minutes using externally-applied electrodes. However, such measurements can be performed by implanted electrodes to allow continuous real-time detection of abnormalities for at-risk patients. These continuous measurements are in a convenient form for data analysis and wireless communication to inform the patient and health-care providers, and these measurements can be further used to direct corrective actions (e.g., defibrillation). Analogous electrode measurements are also used to collect information from ionic current flows in the brain (electroencephalogram; EEG). Thus, electrode measurements of ionic current flow have provided a critical window into the function of neural and neuromuscular systems and these tools have enhanced both our fundamental understanding of medicine and our clinical capabilities to improve the quality of life. Our long-term hope is that we can also enlist similar electrode measurements to provide global measurements of complex biological phenomena. What is different in our case is that we are enlisting electrodes to assess "electron" (*vs*. ion) flow and much less is known about redox-biology. Our long-term goal is to

access and interpret redox-information and especially to correlate such redox measurements to important but ill-defined biological phenomena such as oxidative stress and redox homeostasis.

5. Conclusions

Chitosan possesses a unique set of properties that enable it to serve as an interface material for the assembly of biological components (especially proteins) into electronic devices (*i.e.*, the left-side of our vision in Figure 1). Specifically, chitosan's pH-responsive film-forming properties enable cathodic electrodeposition while its ability to undergo partial oxidation enables anodic deposition. These electrodeposition mechanisms are particularly important because they allow convenient and controllable device-imposed electrical signals to guide assembly from aqueous solution to an electrode address as illustrated in Figure 1. Importantly, these electrodeposition mechanisms are simple, rapid, require no reagents, and yield films that can be removed allowing re-use of the electronics. Chitosan also possesses properties that enable proteins to be assembled to the chitosan using facile chemical, biochemical (enzymatic), electrochemical (electroclick) and specific non-covalent (avidin-biotin and metal chelation) mechanisms. Of course chitosan is not a panacea and has limitations. In fact, we favor alternative stimuli-responsive biopolymer matrices (e.g., alginate, agarose or gelatin) for the assembly of cells at electrode addresses. One troublesome feature of chitosan that we have not been able to fully understand or overcome is the non-specific binding of proteins which has precluded our use of chitosan as a matrix for immunoassays. Additional issues that will be critical for practical applications are the repeatability of film fabrication and the long term stability of the biofunctionalized chitosan films.

Chitosan's ability to be electrodeposited and then modified by quinones has been integral to enabling us to fabricate a bio-based redox-capacitor. We believe this redox-capacitor offers unique redox-properties that we hope to employ to bridge communication across the bio-device interface (*i.e.*, the right-side of our vision in Figure 1). These communication studies are just beginning and we are essentially trying to couple electrochemistry with information processing methodologies to acquire and interpret information of redox biology. If we can succeed at this step, the longer term goal will be to impose electrical inputs to actuate biological redox-based responses. Much remains to be learned not only in terms of our methodologies but also in terms of the fundamental biological interactions in redox biology.

We conclude this review with a final thought: that despite decades of study by our groups (and many more) chitosan remains an intriguing biopolymer with many unanswered questions. We contend that many of chitosan's properties are understood at only a superficial level and that many important questions remain. In many cases, questions span length scales from the individual chain conformation to the 3D network structure. Answering these questions will require a deeper understanding of the attractive and repulsive interaction mechanisms that are responsible for chitosan's solubility and gelation, as well as its sensitivity to environmental conditions (e.g., pH and salt). Answers to these questions will provide a deeper understanding of chitosan's structure–property–function relations and this knowledge will provide greater opportunities to enlist chitosan to solve current and future technological problems. Further, understanding the mechanisms by which chitosan interacts with biology will enable a deeper appreciation of chitosan's unique biological properties (e.g., antimicrobial

activity and the induction of plant defense responses) [250]. Thus, chitosan remains a biopolymer worthy of continued investigation!

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Author Contributions

This review describes results from a long term multi-investigator collaboration. Eunkyoung Kim and Gregory F. Payne were jointly responsible for the redox capacitor studies; Yuan Xiong and Xiaowen Shi were jointly responsible for the chitosan multilayer studies; Yi Cheng and Gary Rubloff were jointly responsible for the microfluidic studies; Hsuan-Chen Wu and Yi Liu were jointly responsible for the enzymatic conjugation studies; Hadar Ben-Yoav and Reza Ghodssi were jointly responsible for biosensor studies; Brain Morrow and Jana Shen were jointly responsible for the macromolecular interpretation of chitosan's properties. William E. Bentley, Reza Ghodssi, Gregory F. Payne, Gary W. Rubloff and Jana Shen are responsible for the conceptualization of many of the advances described in this paper. Gregory F. Payne wrote the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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