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Enzymatic Writing to Soft Films: Potential to Filter, Store, and Analyze Biologically Relevant Chemical Information

*Yi Liu, Eunkyoung Kim, Morgan E. Lee, Boce Zhang, Yossef A. Elabd, Qin Wang, Ian M. White, William E. Bentley, and Gregory F. Payne**

Sensor-based chemical analyses commonly enlist either the molecular recognition capabilities of biology (e.g., enzyme biosensors) or advanced information processing algorithms (e.g., the electronic nose). Here, a hybrid approach is proposed in which an enzyme is used to "filter" chemical information and write this information to a film which then serves as a permanent storage medium that can be 'read' repeatedly, interactively, and by multiple sensor modalities. This approach is demonstrated by analyzing common dietary phenols that are reported to offer health benefits. Specifically, the enzyme tyrosinase is used to convert these phenols into reactive quinones that graft (i.e., write) to a chitosan film. Grafting can be detected by optical, mechanical, and electrochemical sensors. Importantly, grafting confers redox activity to the films and this redox activity can be probed interactively by advanced electrochemical methods that allow the intrinsic redox reactivities to be compared, redox interactions to be identified, and biologically relevant redox activities to be examined. The transfer of chemical and biological information to a film is envisioned to provide broader access to the extensive capabilities offered by sensor technologies and signal processing methodologies.

1. Introduction

There are two broad sensor-based approaches that are commonly used for chemical analysis. The first approach, illustrated at the left in **Scheme 1**, is to couple a molecular recognition element (e.g., enzyme or nucleic acid) with an appropriate signal-transduction mechanism. The classic example of this approach is the detection of glucose by coupling enzymatic recognition with electrochemically based signal transduction.[1,2] The advantage of this approach is that the selectivity of the molecular recognition element simplifies analysis (e.g., by eliminating the need for chemical separations) while the sensor yields a signal that can be readily processed. This approach is well suited for analyses when the problem/question is well posed. For instance, devices for in-home glucose analysis have been widely adopted because the causes and consequences of glucose excursions in the body are reasonably well-understood, and measurement of this single chemical species provides reliable information on a biological condition and indicates the necessary corrective action.

The second broad sensor-based approach to chemical analysis is illustrated at the right in Scheme 1. This approach employs an array of sensors to generate output from a sample and then analyzes this output using pattern recognition software. A classic example of this approach is the artificial or electronic nose that is being investigated as a non-invasive diagnostic tool to detect pulmonary diseases from a "breadth print".[3,4] The advantage of this approach is that it enlists the extensive capabilities of sensor and signal processing technologies to extract information from the sample.[5] The disadvantages of this approach are that relationships between the input and output signals are often subtle and may not provide information of the molecular species or mechanisms being investigated (e.g., the output signals may be correlated to a condition without providing insights on causes, consequences or corrective action). This approach is well-suited to problems/questions that are not well-posed such as when the underlying molecular-level mechanisms and participating chemical species are incompletely understood.

One example of a poorly-posed analytical challenge of considerable current interest involves antioxidant food phenolics. There is extensive evidence that dietary phenols provide health benefits.[6–8] However, the analytical question is poorly posed because: (i) typical sources of dietary phenols (e.g., fruits) contain numerous phenolic compounds and oftentimes it is

Dr. Y. Liu, Dr. E. Kim, Prof. W. E. Bentley, Prof. G. F. Payne
Institute for Bioscience and Biotechnology Research
University of Maryland, College Park, MD 20742, USA
E-mail: gpayne@umd.edu
Dr. E. Kim, M. E. Lee, Prof. I. M. White, Prof. W. E. Bentley, Prof. G. F. Payne
Fischell Department of Bioengineering
University of Maryland, College Park, MD 20742, USA
Dr. B. Zhang, Prof. Q. Wang
Department of Nutrition and Food Science
University of Maryland
College Park, MD 20742, USA
Prof. Y. A. Elabd
Department of Chemical and Biological Engineering
Drexel University
Philadelphia, Pennsylvania, 19104, USA
Prof. I. M. White
Institute for Systems Research
University of Maryland
College Park, MD 20742, USA

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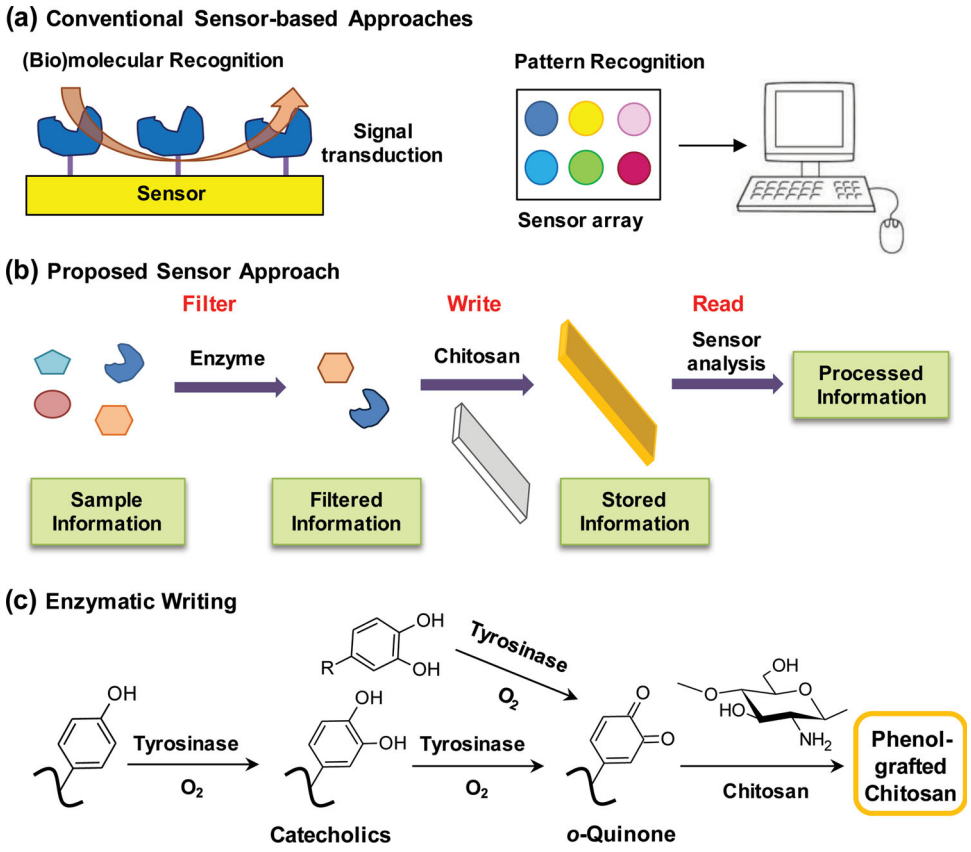


Scheme 1. Schematics of (a) conventional approaches for sensor-based chemical analysis, (b) proposed approach, and (c) enzymatic writing of chemical information to a chitosan film.

not clear if a single compound or combination confers benefit; (ii) dietary sources often contain additional non-phenolic antioxidants (e.g., ascorbic acid) that may have interacting effects; (iii) the health benefits may result from poorly understood and pleiotropic cellular and molecular-level mechanisms, and (iv) endpoint measures of health are difficult to define.[9–13] Because the analytical question is poorly-posed, a variety of chemical methods have been employed for analyzing antioxidant food phenols. Advanced metabolomic methods based on HPLC and mass spectrometry allow individual chemical compounds to be analyzed and this is important for characterizing the composition of foods and beverages.[14,15] Activity-based methods generally attempt to quantify the antioxidant activity (e.g., radical scavenging activity) of a sample by a single value.[16,17] Electrochemical sensor-based methods have also been explored to provide sensitive analytical measurements that may also contain biologically relevant information of antioxidant activity.[18–20]

Here, we propose a hybrid sensor-based approach for analyzing phenolics. Scheme 1 illustrates that the enzyme tyrosinase is used as a molecular recognition element to react with phenolics. Tyrosinase is specific for phenols (vs non-phenols) but reacts with a broad range of phenolics; ideally, the enzyme would "filter information" of phenolic from non-phenolic antioxidants. As illustrated in Scheme 1, tyrosinase generates reactive *o*-quinones which then graft (i.e., write) to a film of the aminopolysaccharide chitosan where the phenolic's information is stored as permanent memory. In this proof-of-concept-study, we demonstrate that the information stored in this chitosan film can be; (i) analyzed using multiple sensor modalities to provide orthogonal information, (ii) analyzed repeatedly, and (iii) analyzed interactively.

2. Results

2.1. Optical and Mechanical Detection of Enzymatic Writing

Previous studies have shown that quinones generated either enzymatically[21–26] or electrochemically[27,28] can graft to chitosan and this enzymatic writing can alter a chitosan film's optical[29–31] and mechanical[32] properties. To extend these observations to an important dietary phenol and to illustrate the potential for enlisting different sensor modalities for detection, we examined reactions with resveratrol. In initial studies, phosphate buffered solutions containing resveratrol (0.1 mM; pH 7.0) were incubated with tyrosinase (50 U mL^{-1}). The UV-vis spectra of this solution are displayed in **Figure 1**a and show a progressive decrease in the solution's absorbance at

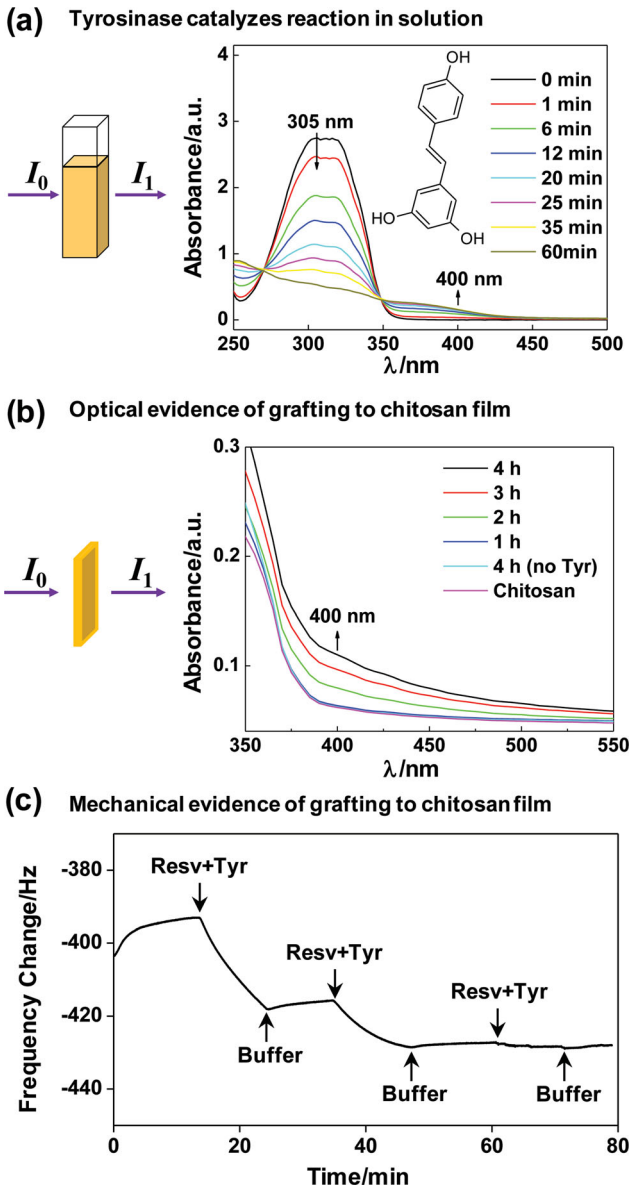


Figure 1. Tyrosinase-catalyzed writing to chitosan film detected by optical and mechanical sensing. (a) In situ UV–vis measurements of a reacting solution containing resveratrol (0.1 mM) and tyrosinase (50 U mL^{-1}). (b) UV-vis spectra for chitosan films incubated with resveratrol (0.12 mM) and tyrosinase (50 U mL^{-1}) for various times. (c) In situ QCM monitoring of frequency changes (i.e., related to added mass) of an electrodeposited chitosan film upon introducing a solution containing 0.05 mM resveratrol and 50 U mL^{-1} tyrosinase (flow rate = 3.7 μL s^{-1}).

305 nm and an increase in absorbance at 400 nm. These UV–vis observations are consistent with the tyrosinase-catalyzed oxidation of resveratrol.[33,34]

To demonstrate that the product generated from the tyrosinase reaction with resveratrol grafts to the chitosan film we performed reactions with resveratrol (0.12 mM) and tyrosinase (50 U mL^{-1}) in the presence of cast chitosan films. At specific times, the films were removed from the reaction solution, rinsed and then the films' UV–vis spectra were measured. Initially, the chitosan films are observed to be visually transparent and Figure 1b shows they have little absorbance at 400 nm. During reaction the film's absorbance at 400 nm increases progressively with incubation time. This observation is consistent with the grafting (i.e., writing) of the tyrosinase reaction product to the chitosan film.[35] Chemical and morphological characterization of resveratrol modified chitosan films are shown in the Supporting Information (Figure S1 and S2).

Further evidence for grafting can be obtained from in situ mechanical measurements using a quartz crystal microbalance (QCM). In this experiment, a thin chitosan film was first electrodeposited onto the gold-coated QCM sensor using a 1% chitosan solution (pH = 5.5) and cathodic conditions (3 A m^{-2}, 30 s).[36] After deposition, the chitosan-coated sensor was dried overnight at room temperature under vacuum. The chitosan-coated gold sensor was then placed in a flow cell, wet with water, and then equilibrated with a buffer solution (20 mM phosphate; pH 7) by passing the buffer through the flow-cell. After equilibration, a solution containing 0.05 mM resveratrol and tyrosinase (50 U mL^{-1}) was introduced into the flow cell to initiate reaction (indicated by the arrow in Figure 1c). The real-time changes in the resonance frequency shown in Figure 1c are consistent with an increasing mass resulting from the grafting of resveratrol to the chitosan film. After 10 min incubation, the reaction solution in the flow cell was exchanged for buffer, and the in situ QCM measurements in Figure 1c indicate that the resonant frequency remained nearly constant indicating no further increase in mass, (i.e., no further resveratrol grafting). Subsequent exchanges in the flow cell with buffer or resveratrol/tyrosinase solutions are consistent with the grafting of tyrosinase-generated reaction product onto the chitosan film.

The results in Figure 1 indicate that; (i) tyrosinase can initiate the grafting of phenols onto the chitosan film, and (ii) this enzymatic-writing can be detected by convenient optical and mechanical detection modalities. We should note that writing to the film may not be a perfect representation of the enzymatic reaction (i.e., some of the enzymatically-filtered information may be altered or lost) because the quinone intermediate can undergo complex and ill-defined non-enzymatic reactions (e.g., oligomerization) that could occur in series or parallel with the chitosan grafting reaction.

2.2. Optical Information Stored in the Films

In principle, the UV–vis spectrum possesses both qualitative and quantitative information of chemical structure and concentration. To illustrate the capability of storing such optical information in the film, we incubated chitosan films with tyrosinase and various phenolic compounds and then analyzed the films optically by measuring their UV–vis spectra. **Figure 2**a shows that incubation of the films with phenols plus tyrosinase imparts UV–vis absorbance that is absent from the unreacted chitosan film. The most notable observation from Figure 2a is that the spectrum for the film reacted with the flavonoid (catechin) is substantially different from those reacted with the phenylpropanoid (caffeic acid) or the stilbenoid (resveratrol). This observation indicates that for some classes of phenols (e.g.,

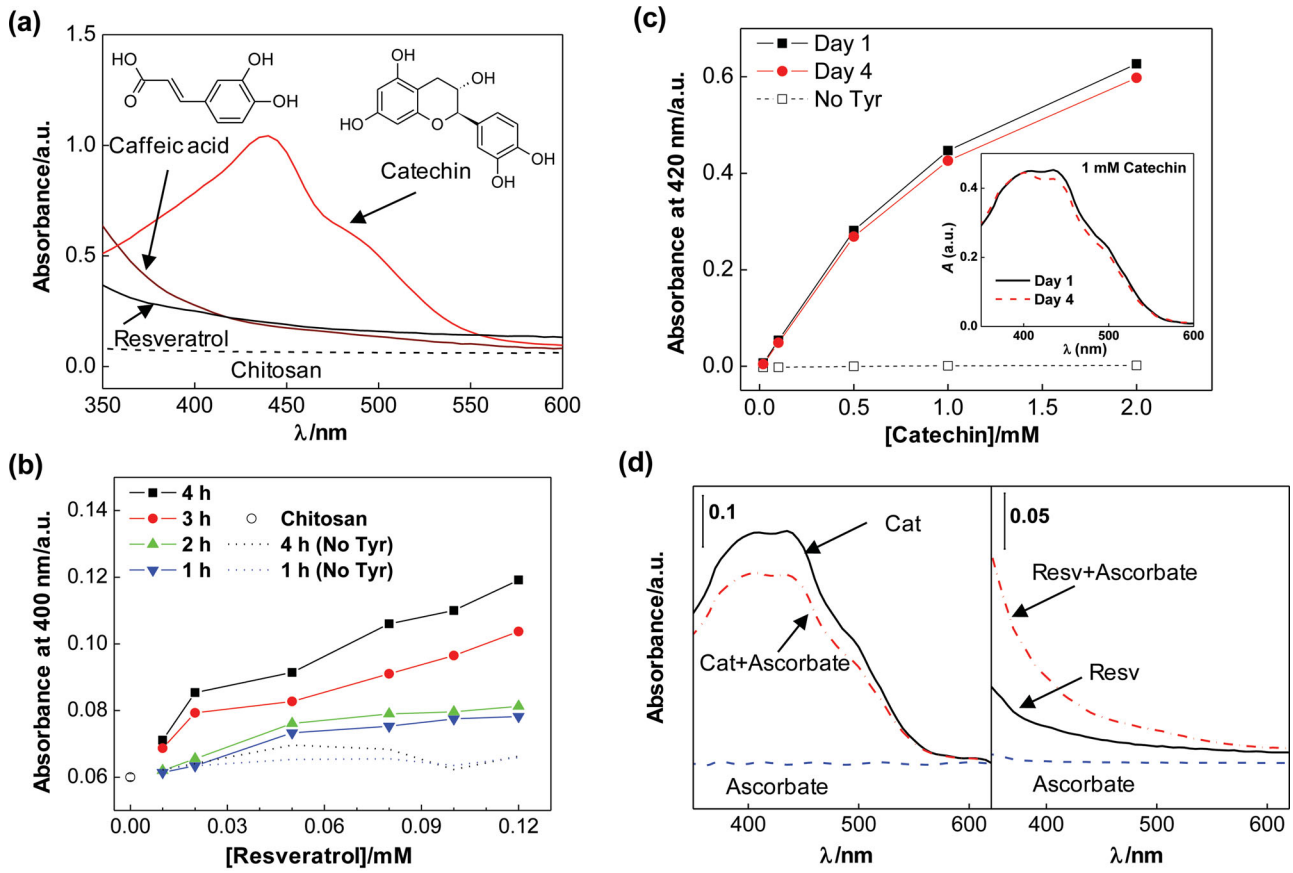


Figure 2. Optical information stored in the chitosan film. (a) Qualitative information (UV–vis spectra) for chitosan films incubated with resveratrol (0.12 mM, 50 U mL^{-1} tyrosinase, 4 h), caffeic acid (0.5 mM, 20 U mL^{-1} tyrosinase, 1 h), or catechin (0.5 mM, 20 U mL^{-1} tyrosinase, 1 h). (b) Quantitative information of resveratrol reaction with tyrosinase (50 U mL^{-1}). (c) Storage and repeatable access of information for chitosan films incubated with catechin and tyrosinase (20 U mL^{-1}). (d) Filtering of information to remove "noise" from the non-phenolic antioxidant ascorbate. [Details of reaction conditions and results for additional phenols are provided in the Supporting Information.]

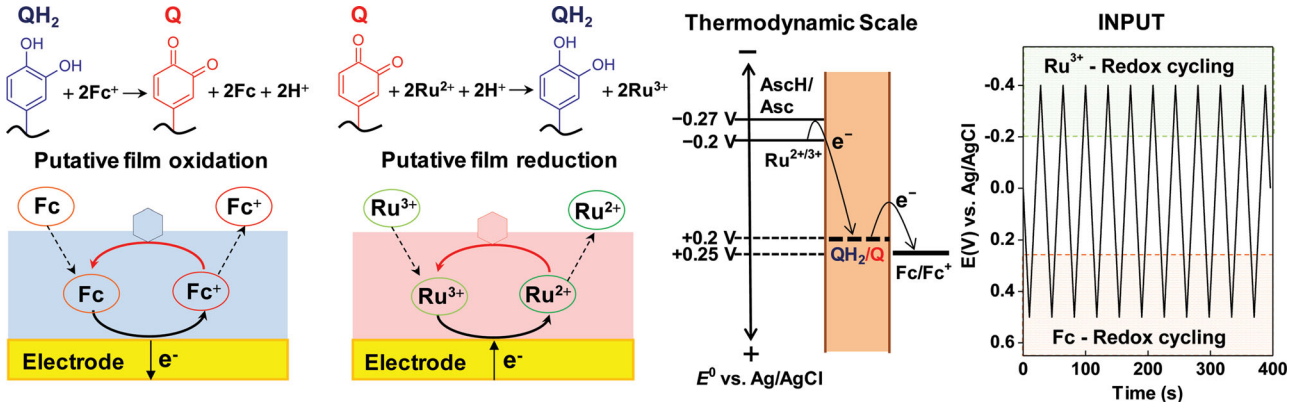
flavonoids) it may be possible to extract qualitative chemical (structural) information from the film's spectrum.

Quantitative or semi-quantitative information of UV-vis spectra typically relates absorbance to concentrations. Figure 2b shows such relationships for films reacted with resveratrol for varying times. Despite the low resveratrol concentrations being studied and the relatively low absorptivity of these films, Figure 2b shows the expected linear relationship between absorbance and the resveratrol concentration in the original sample. Figure 2b also indicates that the sensitivity can be enhanced by altering reactions conditions (e.g., reaction time).

Semi-quantitative relationships between the film's stored optical information and the sample's original phenol content were observed for other phenolics as illustrated in Figure 2c for catechin (Figure S3 in the Supporting Information provides results for additional phenols). Also, the insert in Figure 2c shows that the spectra are nearly identical when a film was measured on different days which indicate that the stored information is retained in the films and can be retrieved at later times with reasonably high fidelity.

One of the challenges in detecting phenolics from typical dietary sources (e.g., fruits) is that often these samples contain non-phenolic antioxidants (especially ascorbic acid) that contribute to measurements of antioxidant activities. In principle, tyrosinase should overcome this challenge by recognizing and selectively reacting with the target phenol(s). To demonstrate this selectivity for the tyrosinase-catalyzed grafting step, we incubated chitosan films in tyrosinase solutions containing either a phenol or ascorbate, and then measured the films' UV–vis absorbance. Figure 2d shows that incubation with either catechin or resveratrol imparted significant UV–vis absorbance to the chitosan films while incubation with ascorbate alone resulted in no discernible change in the film's absorbance.

Next, chitosan films were incubated with tyrosinase and solutions containing both a phenol and ascorbate. Figure 2d shows that the films' UV–vis spectra were qualitatively similar when reactions were performed with a phenol alone or a mixture of phenol plus ascorbate. However, there are quantitative differences in the spectra between these films and these differences suggest that ascorbate can sometimes suppress and sometimes accelerate enzymatic-writing. While we did not explore the details of this suppression or acceleration, we offer two possible explanations. A likely explanation for the suppression of catechin's grafting to the chitosan film is that



Scheme 2. Analysis of redox information in the chitosan film. Grafted moieties confer redox-activity although the films remain non-conducting: electron transfer to/from the films only occurs via diffusible mediators. Redox mediators Fc and Ru^{3+} access this information through independent redox-cycling reactions.

the tyrosinase-generated quinone can be reduced by ascorbate and this quinone-reduction competes with and suppresses the rate of quinone-grafting.[37] An explanation for the acceleration of resveratrol's grafting is unclear but potentially involves a decrease in the lag period which is commonly observed for the tyrosinase-catalyzed oxidation of phenols that possess a single aromatic hydroxyl (lags are not typically observed for catecholics that possess *ortho*-dihydroxy substitution).[38–40] (Figure S4 in the Supporting Information provides evidence that ascorbate accelerates the tyrosinase-catalyzed reaction of resveratrol.)

Figure 2 demonstrates that enzymatic-writing imparts information to the chitosan films and this information can be read by optical modalities that in some cases can provide qualitative and quantitative chemical information. The enzyme filters information by removing "noise" associated with the nonphenolic ascorbate to generate a signal that is characteristic of the phenol (i.e., the shape of the UV–vis spectra) although the presence of ascorbate can alter the signal intensity.

2.3. Enzymatic Grafting Imparts Redox Information to Chitosan Films

Recent studies indicate that the grafting of catechols to chitosan films can confer redox-activity to these films (while redox-active, the films are non-conducting and the electrons do not flow in response to an applied potential).[41,42] Typically, the film's redox-activity is measured using electrochemical mediators that can diffuse through the film and engage in redox-cycling reactions that transfer electrons between the film and an underlying electrode. The scheme at the left in **Scheme 2** shows the ferrocene dimethanol mediator (Fc) diffusing through the film, being oxidized at the electrode and then being re-reduced by accepting electrons from the grafted catechol moieties. The scheme at the right shows the $Ru(NH_3)_6Cl_3$ mediator (Ru^{3+}) diffusing through the film, being reduced at the electrode and then being re-oxidized by donating electrons to the grafted quinone moieties. There are four important features of these redox-cycling electron transfer reactions. First, the mediators are required because neither chitosan[43] nor catechol-modified chitosan[41] films are conducting (these films cannot directly exchange electrons with the underlying electrode). Second, the electron transfer reactions are thermodynamically driven as illustrated by the redox-plot in Scheme 2; electrons can only be transferred to the film from mediators that have a more negative redox potential or transferred from the film to mediators that have a more positive redox potential. Third, cyclic potential inputs can be imposed to sequentially engage Ru^{3+} for reductive redox-cycling ($E < -0.2$ V) and Fc for oxidative redox-cycling ($E > +0.25$ V) as illustrated by the input cycle in Scheme 2. Finally, the redox-cycling reactions will amplify output currents and this amplification is expected to depend on the grafted phenolics; thus the amplification should contain information of the redox activity stored in the films.

To demonstrate that tyrosinase-catalyzed modification imparts redox-information to the chitosan film, we prepared films by first electrodepositing chitosan onto a gold electrode (1% chitosan, pH 5.5; 4 A m^{-2}, 45 s) and then incubated the chitosan-coated electrodes in a solution of resveratrol (0.12 mM) and tyrosinase (50 U mL^{-1}) for 3 h. After reaction, the film-coated electrodes were rinsed extensively with water to remove physically bound catechols and then stored in phosphate buffer (pH 7). To measure the film's redox activity, the film-coated electrodes were immersed in a solution containing both the Fc (50 μM) and Ru^{3+} (50 μM) mediators and the potential was swept between –0.4 and 0.5 V (vs Ag/AgCl) at a scan rate of 0.05 V s^{-1}. **Figure 3**a shows that the cyclic voltammogram (CV) for the resveratrol-modified chitosan films has (i) large oxidative currents at 0.27 V which is consistent with the oxidation of the Fc mediator ($E° = 0.25$ V), and (ii) large reducing currents at –0.23 V which is consistent with the reduction of the Ru^{3+} mediators ($E° = -0.2$ V). One control CV in Figure 3a is for the resveratrol-modified chitosan film incubated in the absence of either Fc or Ru^{3+} mediators. This control shows minimal oxidation and reduction currents consistent with the expectation that the resveratrol-modified film is non-conducting.

The second control CV is for an electrode coated with an unmodified chitosan film (the chitosan-coated electrode was not incubated with resveratrol). When this chitosan control was tested in the presence of both the Fc and Ru^{3+} mediators,

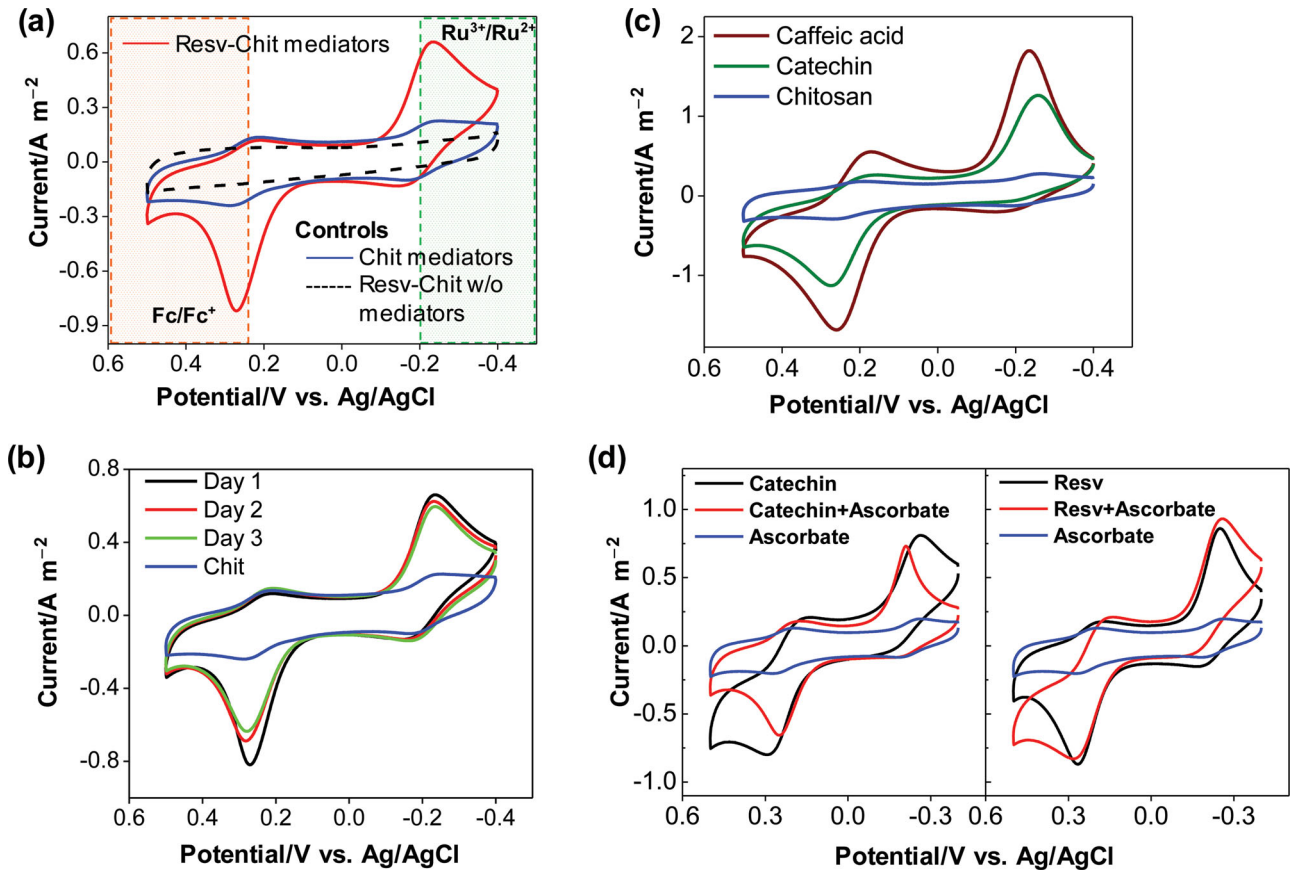


Figure 3. Redox information written into the chitosan film. (a) Redox activity is demonstrated from CVs for chitosan films that had been incubated with resveratrol (0.12 mM) and tyrosinase (50 U mL^{-1}) and then tested with the two mediators Fc (50 μM) and Ru^{3+} (50 μM). (b) Storage of redox information is demonstrated for resveratrol-modified chitosan films that were analyzed on 3 separate days. (c) The possible generalizability that tyrosinase-catalyzed grafting imparts redox information to the chitosan films was examined by performing reactions with caffeic acid (1 mM, 20 U mL^{-1} tyrosinase, 1 h) and catechin (0.5 mM, 20 U mL^{-1} tyrosinase, 20 min). (d) Filtering of redox information to remove "noise" from the non-phenolic antioxidant ascorbate. [Details of reaction conditions are provided in the Supporting Information.]

Figure 3a shows small peaks were observed in the regions of Fc oxidation and Ru^{3+} reduction. This result is consistent with the explanation that these mediators can diffuse through the films to be oxidized and reduced at the electrode. By comparing the CVs for this chitosan control and the resveratrol-modified films it is apparent that there is a substantial amplification of the oxidation and reduction currents. This amplification of the output currents is consistent with the explanation that the grafted resveratrol moieties confer redox-activity to the film and these moieties can engage the mediators in redox-cycling reactions illustrated in Scheme 2.

We next tested three qualitative features of the redox information. First, we examined the stability of the redox information stored in the film by measuring CVs on 3 separate days. The results in Figure 3b show that the output signals (i.e., currents) were repeatable although a small attenuation of the signals was observed. This result is consistent with the optical measurements in Figure 2c and indicates that the stored redox-information can be accessed repeatedly at different times.

Second, we examined the generality of the observation that tyrosinase-catalyzed grafting imparts redox information (i.e., redox activity) to chitosan films. In this experiment, we incubated chitosan-coated electrodes with tyrosinase (20 U mL^{-1}) and various individual phenols and then measured the redox activities of the resulting films. The CVs for these phenol-modified films in Figure 3c show a substantial amplification in the output currents compared to the unmodified chitosan control.

Third, we tested whether the "filtering" function of tyrosinase can be extended from optical measurement modalities (Figure 2d) to electrochemical modalities. For this study, we prepared films by incubating chitosan-coated electrodes and tyrosinase with solutions containing a phenol, ascorbate, or both a phenol plus ascorbate. Consistent with a filtering function of tyrosinase, the CVs in Figure 3d demonstrate that the films incubated with the phenol (either in the presence or absence of ascorbate) possessed redox activity while films incubated with ascorbate alone possessed no redox activity. Interestingly, the shapes of the CV curves differed depending on whether the films were incubated with phenol alone or with phenol plus ascorbate. Specifically, the inclusion of ascorbate during incubation with catechin (a condition observed to suppress enzymatic writing in Figure 2d) changed the CVs from a sigmoidal to peak shape. The opposite was true for inclusion of ascorbate during incubation with

resveratrol (a condition observed to accelerate enzymatic writing in Figure 2d); in this case the CVs changed from peak to sigmoidal shape. Similar differences have been observed elsewhere for CVs of films in which smaller amounts of grafted catechol (peak shape) were compared to those with larger levels of grafted catechol (sigmoidal shape).[41] Thus, the CV results in Figure 3d are consistent with the previous explanation that ascorbate suppresses catechin's enzymatic grafting but accelerates resveratrol's enzymatic grafting.

The results in Figure 3 indicate that: (i) the enzymatic writing of phenolics to the chitosan film imparts redox-activity; (ii) this redox information can be read electrochemically, and (iii) the information obtained from electrochemical reading is consistent with and complementary to the information read from optical modalities. One limitation illustrated in both Figures 2 and 3 is that the rate of the enzymatic reaction varies substantially with the type of phenol and the presence of ascorbate (different incubation times were selected). Thus, a single end-point measurement may not provide the best information. Potentially, end-point measurements could be coupled with dynamic measurements such as those illustrated in Figure 1 to overcome this limitation and to provide richer information.

2.4. Quantification of the Stored Redox Information

Figure 4a illustrates an approach for quantifying the redox-information stored in the films. The current associated with Fc oxidation is integrated with respect to time to determine the oxidative charge transfer ($Q_{Ox} = \int i dt$; which is proportional to the area illustrated in Figure 4a with the scan rate being the proportionality factor). This oxidative charge transfer for the modified film ($Q_{Ox,Modified}$) is compared with that for the unmodified chitosan control ($Q_{Ox,Chitosan}$) to determine the portion of film's oxidative charge transfer that is attributed to the grafted moieties ($Q_{Ox,Phenol}$). The left plot in Figure 4b shows that for the case of resveratrol, the calculated value $Q_{Ox,Phenol}$ increases with incubation time for 3 h. The right plot in Figure 4b shows that $Q_{Ox,Phenol}$ increases systematically with the resveratrol concentration in the incubation solution. Similar results were observed in Figure 4c for catechin although the incubation time needed to be adjusted relative that used for resveratrol. Note that in Figure 2b and c, the optical absorbance increases as a function of phenol concentration and reaction time, indicating that the grafting reactions continues under these conditions. However, Figures 4b and c show that the film's redox acitvities decrease upon extended incubation and/or with concentrated phenols. Presumably, this decrease in redox activity is due to an "over-oxidation" in which further grafting destroys the redox-activity of previously grafted moieties (e.g. quinones). The results in Figure 4 indicate that film's redox activity possesses quantitative information of the phenolic content of the sample.

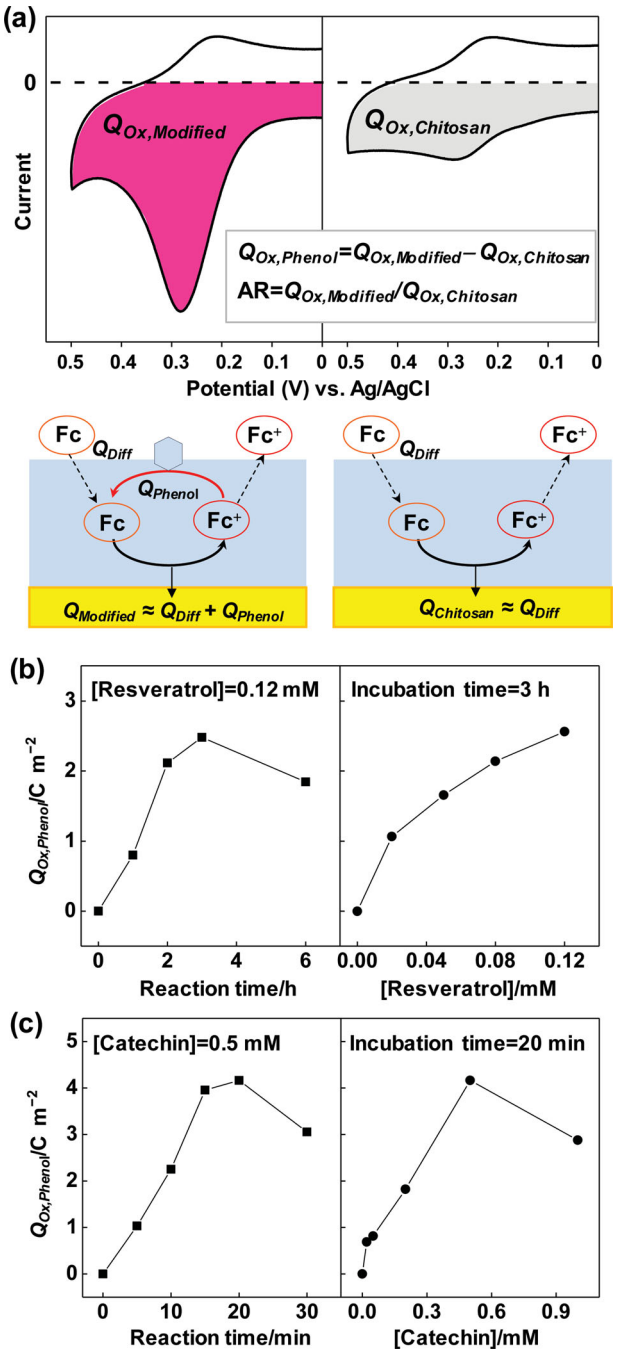


Figure 4. Quantification of stored redox-information. (a) Schematic illustrating the quantitative parameters obtained by integrating the current during the oxidative portion of the CV (Q_{Ox}) and by comparing the phenol-modified films with an unmodified chitosan control. (b) $Q_{Ox,Phenol}$ calculated from the CVs for resveratrol-modified chitosan films as a function of reaction time (left) and resveratrol concentration (right). (c) $Q_{Ox,Phenol}$ calculated from the CVs for catechin-modified chitosan-films as a function of reaction time (left) and catechin concentration (right).

2.5. Interactive Analysis Using Advanced Methodologies

The redox-information stored in the chitosan film can be probed interactively to provide broader information of molecular behavior. To illustrate this potential we performed interactive studies using advanced methodologies adapted from electrochemistry and signal processing.

A common electrochemical approach is to vary scan rate (i.e., the frequency of the potential input) to provide insights of the

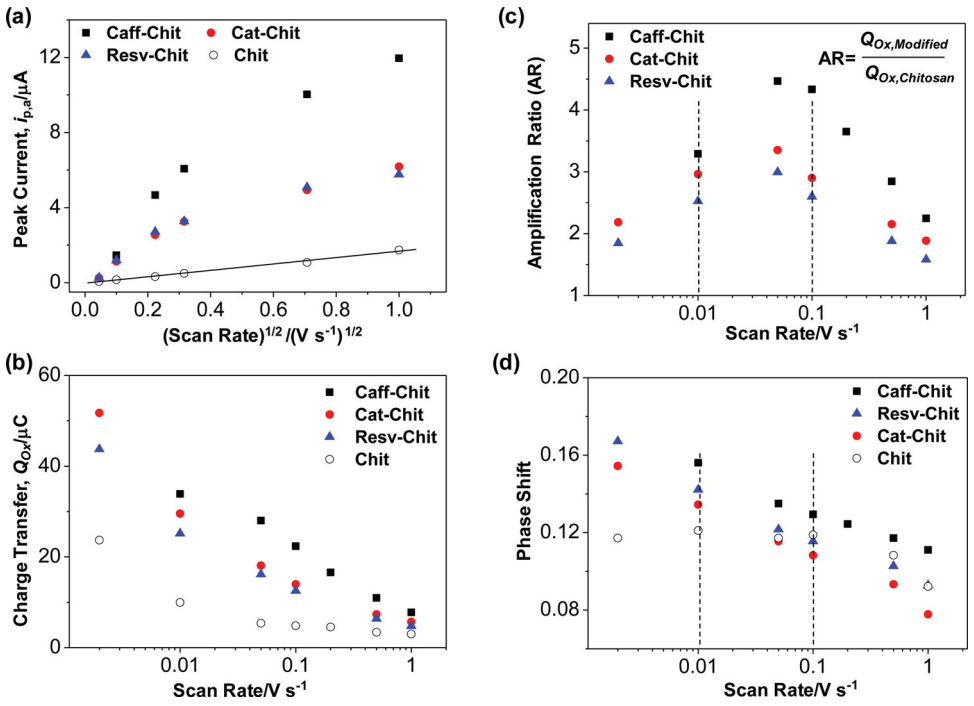


Figure 5. Advanced methodologies to interactively probe the films. (a) The non-linearity between anodic peak current ($i_{p,a}$) and (scan rate)$^{1/2}$ indicates that output current from phenol-modified films is not limited by mediator diffusion from the bulk solution. (b) The charge transfer during the anodic cycle (Q_{Ox}) decreases with increasing scan rate. (c) Bode-type analysis using amplification ratio (AR) and (d) phase shift information indicates that the time constants for electron exchange between the Fc mediator and grafted phenols are similar for all three phenols tested (i.e., they have similar redox-reactivities). [See text and Supporting Information for further details.]

relative rates of electron transfer at the electrode to the diffusion of the redox-active species.[44–47] To apply this methodology, we prepared several chitosan-coated electrodes, enzymatically grafted various phenolics to these films and then tested the film-coated electrodes in solutions containing Fc and Ru^{3+} using varying scan rates (CVs are provided in Figure S5 of the Supporting Information). Often, results are plotted as shown in **Figure 5**a with the peak anodic current ($i_{p,a}$) plotted vs. (scan rate)$^{1/2}$; linearity in this plot is indicative of diffusion-controlled processes. The results in Figure 5a for the unmodified chitosan control show such linearity indicating that diffusion of Fc through the unmodified chitosan film is reversible and controlled by diffusion.[45] Compared to this control, the results in Figure 5a for the phenol-modified films display amplified peak currents and significant deviations from linearity. These observations are consistent with the redox-cycling reactions in the films.

Signal processing theories also employ periodic inputs of varying frequencies and one such analysis involves the creation of Bode plots in which 2 output parameters are plotted as a semi-logarithmic function of input frequency. To apply this framework, we used the anodic charge transfer illustrated in Figure 4a. Figure 5b shows that Q_{Ox} decreases with scan rate for all film-coated electrodes. Thus, although currents increase at faster scan rates (Figure 5a), the time that these currents are drawn is reduced such that the integral ($Q_{Ox} = \int i dt$) decreases with scan rate (Figure 5b).

One output parameter for Bode analysis measures the output amplitude. For this parameter, we calculated the amplification ratio (AR) as the ratio of $Q_{Ox,Modified}$ for the phenol-modified film to $Q_{Ox,Chitosan}$ for the control chitosan film as illustrated in Figure 4a. The plot in Figure 5c shows the AR increases and then decreases with increasing scan rate. The scheme in Figure 4a is helpful in interpreting this result as it illustrates that $Q_{Ox,Modified}$ has two contributions, from diffusion of the Fc mediator from the bulk and from the redox-cycling in the film. In contrast, $Q_{Ox,Chitosan}$ has only one contribution, from the diffusion of Fc from the bulk.

In the regime of very low scan rates, when the total charge transfer is large (Figure 5b), the contribution from the redox-cycling is quantitatively comparable to (or less than) the transfer by Fc diffusion from the bulk; thus, the modified and unmodified films have comparable Q_{Ox} values and AR approaches 1. As the scan rate is increased in the low frequency regime, Fc diffusion from the bulk contributes less to the observed charge transfer (Figure 5b shows total Q_{Ox} decreases with scan rate) and the redox-cycling becomes relatively more important; thus greater amplification is observed for the phenol-modified films. Finally, in the regime of very large scan rates, the Fc mediator has little time to diffuse such that both diffusion from the bulk and redox-cycling in the film become small (both Q and AR become small). The results in Figure 5c indicate that the transition from the low to high frequency regime occurs near 0.05 V s^{-1} for films prepared from all three phenols (catechin, caffeic acid and resveratrol). This observation suggests that the grafted moieties from these three phenolics have comparable rates for exchanging electrons with the Fc mediator (i.e., this analysis suggests there are no differences in the intrinsic redox-reactivities of these moieties).

A second Bode-type representation compares the phase-change between the input and output signals. We estimate the phase by comparing how the potential for peak anodic current changes with the input potential scan rate (the calculational procedure is described in Figure S6 of the Supporting Information). Figure 5d shows this estimate of phase shift for the three phenol-modified and the unmodified chitosan films. While this phase shift parameter is less intuitive, there are two important points. First, the phenol-modified and control chitosan films behave similarly at high frequencies but their behavior deviates at low frequencies and the regime-shift occurs near the same frequencies (0.01–0.1 V s^{-1}) as observed in Figure 5c. Second, all three of the phenol-modified films appear to behave similarly.

Figure 5 illustrates that redox information stored in the film can be analyzed by advanced methodologies from electrochemistry and signal processing, and these analyses should enhance the ability to extract redox-information. In particular, the Bode analysis in Figures 5c and d suggests that the moieties grafted from catechin, caffeic acid and resveratrol have similar reactivities. The observation that two common antioxidant phenolics in red wine (catechin and resveratrol) have similar redox-reactivities may seem surprising given resveratrol's unusual biological activities[48] (vs catechin). However, this observation is consistent with current explanations that resveratrol's benefits may be due to a mechanism that is independent of redox.[49] Thus, access to advanced information processing (i.e., Bode analysis) may allow hypotheses of mechanisms-of-action to be tested simply and rapidly.

2.6. Interactive Analysis to Discern Redox Interactions

The results in Figure 5 indicate that the stored redox information in the film can be probed using standard electrochemical redox mediators (i.e., Fc and Ru^{3+}) to understand intrinsic redox-activities of the grafted catechols. We next examined the ability of the grafted moieites to undergo redox-interactions with the common biological redox-active component ascorbate. The redox scale in Scheme 2 indicates that it is thermodynamically favorable for ascorbate to donate electrons to grafted catecholic moieties (i.e., to "charge" the film with electrons). To experimentally test this possibility, we prepared chitosan-coated electrodes and enzymatically grafted resveratrol to the chitosan films. After the tyrosinase-mediated grafting, the films were removed from the reaction solution and washed extensively (i.e., the following measurements aimed to assess the activities of the grafted catecholic moieties). One film-coated electrode was then incubated for 5 minutes in a solution containing 5 mM ascorbate (pH 7) after which this film-coated electrode was transferred into an Fc (50 μM) containing solution and cyclic voltammograms (CVs) were collected by sweeping between 0 and +0.5 V. These CV conditions allow the catecholic moieties to be oxidized but not reduced (i.e., the CV conditions allow film to be discharged but not charged).

Figure 6a shows significant oxidation peaks for the first CV cycle, however subsequent CV cycles show progressively smaller oxidation peaks. This observation is consistent with an explanation that the film had been charged by the initial ascorbate treatment and it is progressively discharged by Fc during the oxidation steps. To test whether the film can be re-charged, the film-coated electrode was removed from the Fc-solution after the 10th cycle and re-incubated with ascorbate

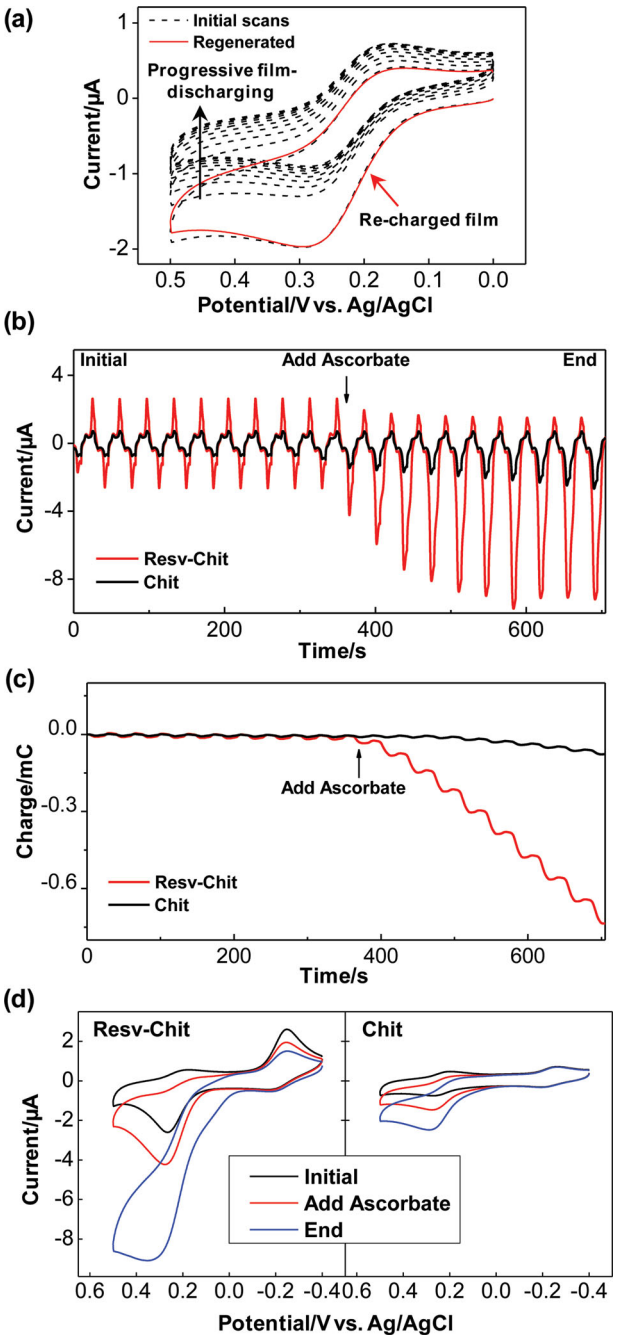


Figure 6. Interactive probing to discern redox-interactions between ascorbate and phenolics. (a) Sequential CVs of resveratrol modified chitosan in Fc (50 μM) show progressive discharging of the films (dotted curves) but they can be re-charged by incubation with ascorbic acid (5 mM; 5 min). (b) Current outputs for the chitosan film and resveratrol-modified film in a solution containing both Fc and Ru^{3+} and imposed cyclic voltages (Scheme 4). Ascorbic acid (final concentration 2 mM) was added at 350 s. (c) Charge transfer outputs calculated from (b). (d) CVs at three different time points; before ascorbate addition, immediately after ascorbate addition, and 350 s after ascorbate addition.

(5 mM for 5 min). The subsequent CV for this re-charged film is shown in red in Figure 6a and indicates that the Fc oxidation peak for this re-charged film is similar to that for the initially-charged film. Recharging can be performed multiple times (see Figure S7 in Supporting Information). These results indicate that the grafted catecholic moieties can interact with and accept electrons from ascorbate which is common component in foods and beverages. The implication from this result is that the redox state of catecholics (Q vs QH_2) can be readily switched through redox interactions and this suggests that the phenolic's biological activity (pro- vs. anti-oxidant) may be context-dependent.

The above suggestions are not new and several reports indicate that; catecholics can interact with other redox active components in foods,[11,50] and that their pro- and antioxidant activities may be context-dependent.[51,52] We believe that isolating such phenolic components within a film and probing them electrochemically provides a simple means to probe redox interactions and context dependency. To illustrate this possibility, we immersed the resveratrol-modified film-coated electrode in a solution containing both Fc and Ru^{3+} and imposed cyclic voltages that allowed the film to be sequentially charged (at negative potentials) and discharged (at positive potentials) by the redox-cycling reactions illustrated in Scheme 2. Figure 6b shows that for the first 350 s, the current output for the control chitosan film and the resveratrol-modified film were different but both attained "steady" output. The integration of the current ($Q = \int i dt$) is shown in Figure 6c and indicates that for both films there was little (if any) net charge transfer during the initial 350 s (under these conditions, Fc oxidation is balanced by Ru^{3+} reduction). At 350 s, ascorbate (2 mM final concentration) was injected into the mediator solution without changing the periodic input potential. As illustrated in Figures 6b and c, the addition of ascorbate dramatically perturbed the output current and charge transfer for the resveratrol-modified film with a smaller perturbation observed for the control chitosan film. Figure 6d shows the CVs at three different time points (before ascorbate addition, immediately after ascorbate addition, and 350 s after ascorbate addition).

The results in Figure 6 illustrate that the redox-information stored in the chitosan film can be probed interactively by adding components (e.g., ascorbate) to determine if they undergo redox interactions with the film. Such a test provides a simple and rapid means to identify interactions and probe these interactions repeatedly under different contexts (e.g., different pH).

3. Discussion

In this proof-of-concept study, we report that common dietary phenolics can be enzymatically grafted to a hydrogel film. This concept integrates the previously-known ideas that tyrosinase catalyzes the oxidation of phenolics,[20,29,53–56] and the *o*-quinone oxidation products covalently graft (i.e., write) to chitosan.[21–24] A new observation in this study is that the grafted phenolic/catecholic moieties confer redox activity to these films. Potentially this observation is important from a materials science standpoint. Phenolic/catecholic films offer diverse functional properties[29,57–66] and much recent technological interest has been generated by the discovery of a simple method to produce dopamine-based films.[67–71] The results from the current study suggest an alternative fabrication approach to generate phenolic/catecholic films with redox-active properties. In addition to technological interests, the chitosan films with grafted phenolic/catecholic moieties may provide a convenient experimental model of natural phenolic materials.[72] In particular, melanins,[73,74] humics[75,76] and lignins[77] have all been reported (or suggested) to have redox active properties, yet clarifying these activities and their biological relevance has been challenging due to the complexity of these materials.[78]

In this study, we view the enzymatic grafting of quinones as a means of imparting information into the chitosan film. Quinone grafting to chitosan alters the film's optical,[31] mechanical,[28] and redox[42] properties enabling various sensing modalitites to be engaged to "read" this information. Most previous studies focused on optical analysis (e.g., UV–vis analysis) of the chitosan films[29] while the results from the current study suggest that redox-measurements may provide complementary information. Our results indicate that both the optical and redox-information are stored in the film and can be read repeatedly at different times. Further, the redox-information can be read using various electrochemical measurement methods and the resulting data is in a convenient format for analyses by signal processing methods (e.g., the Bode-type analysis in Figure 5). Finally, the redox-information can be probed interactively which may provide opportunities to identify redox-interactions among dietary phenolics and other redox-active components in the diet (e.g., ascorbate in Figure 6).

Potentially, the redox-information stored in the chitosan film may assist in analyzing dietary antioxidant phenolics. Conventional approaches to analyze antioxidant phenols have significant limitations. Instrument-intensive metabolomic-type analysis generate high-content data sets[14,15] but do not provide insights on the components or mechanisms that confer benefit. Conventional antioxidant assays generate a single value that is useful for comparative purposes[16,17] but the physiological relevance of this measurement can be uncertain. Potentially, enzymatic writing may have more physiological relevance since tyrosinase may mimic the oxidative metabolism of phenolics (e.g., resveratrol is metabolized through catecholic species)[79,80] and the quinone grafting reaction may mimic an important biological effect of dietary phenolics (i.e., conjugation of phenolics to biomolecules such as DNA).[12,81] In addition, the redox activities of phenolic moieties that are grafted to the chitosan matrix may mimic fiber-bound phenolics. A large fraction (≈50%) of the total dietary antioxidants (mainly polyphenolics) traverse the small intestine bound to dietary fiber[82,83] and thus are inaccessible for direct-contact or uptake by cells; yet the gut is considered a major site of action of antioxidant phenols.[84,85] Possibly, the redox-activity of the grafted moieties may mimic important activities of these fiber-bound phenolics.

In summary, the information stored in the chitosan film can be probed through multiple modalities, the redox-information can be analyzed by advanced information processing methodologies, and the redox-information may have physiological relevance. Thus, the proposed hybrid analytical approach may offer significant advantages over conventional antioxidant assays although it is unlikely to have the discriminating power

to replace instrument-intensive metabolomic measurements when the composition of a complex mixture is required. Thus, we anticipate the hybrid analytical approach may provide a powerful complement to existing methods.

4. Conclusions

This proof-of-concept study demonstrates a hybrid approach that offers the potential to couple the capabilities of molecular recognition to "filter" information with the power of sensor and signal-processing technologies to "analyze" information. Specifically, we demonstrate that the sample's information can be enzymatically "written" into a thin film and this stored information can be analyzed; (i) repeatedly, (ii) interactively, and (iii) by multiple modalities. We anticipate that this approach will be particularly applicable for ill-posed problems such as analysis of the health benefits of dietary phenols. Importantly, the stored information can be probed in ways that may provide insights on molecular mechanisms and context-dependent activities (i.e., redox activities). Finally, these studies revealed that grafted phenolics possess redox-activity which may be a significant observation with respect to the biological activities of dietary phenols.

5. Experimental Section

Details of materials and experimental methods are provided in the Supporting Information.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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