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Tyrosinase-mediated grafting and crosslinking of natural phenols confers functional properties to chitosan

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ABSTRACT

Physics and chemistry underpinned the remarkable advances in materials science that occurred during the 20th century, while biology is poised to provide the scientific underpinning for materials science advances of the 21st century. Biofabrication is the emerging approach to broadly apply biology's materials and mechanisms to create structure and function. Here, we describe one such biofabrication methodology, the use of tyrosinase to graft phenolics to the aminopolysaccharide chitosan. Phenolics are a broad class of abundant natural products and we provide results from a single phenolic reactant, caffeic acid, to illustrate the potential for enzymatically imparting functional properties to chitosan. We show that tyrosinase oxidation mediates the grafting of caffeic acid to chitosan and possibly even results in the covalent crosslinking of chitosan. When this enzymatic reaction is performed in a chitosan solution (pH < 6), it is observed to induce a sol–gel transition. At higher pHs, chitosan forms an insoluble film and this enzymatic reaction can alter the film's mechanical properties. Importantly, caffeic acid grafting also confers redox-activity to the chitosan film, enabling the film to accept, store and donate electrons. The broader efforts to enlist tyrosinase to build macromolecular structures and impart functions are discussed.

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1. Introduction

Biology provides the paradigm for fully integrated and sustainable manufacturing in which renewable starting materials (food) are converted into high performance systems (organisms) that are fully recyclable. Biological processing methods are commonly used to synthesize small molecules and macromolecules for applications in fuels, foods, and pharmaceuticals. We contend that the biological sciences are also poised to make substantial contributions to materials science by providing the scientific underpinnings for the creation of functional materials (especially soft materials) [1]. From a materials standpoint, biology is expert at: (i) controlling structure and conferring function at the nanoscale level (e.g., proteins), (ii) assembling their nano-components into hierarchical structures that can perform complex operations (e.g., energy harvesting by the mitochondria), and (iii) creating materials that can respond to stimuli, heal and resorb. There has been significant progress in understanding the mechanisms by which biology performs these fabrication feats and in some cases these mechanisms can be applied technologically. Thus, we believe biologically-based fabrication (i.e., biofabrication) may provide an emerging paradigm for materials science [2,3].

There are differing definitions for the term "biofabrication". For the purposes of this paper, we use the term biofabrication to mean the use of biological materials and mechanisms to confer structure and function. In general, biofabrication incorporates the varied tools of biotechnology including the templated biosynthesis to produce proteins with precise sequence and size, and engineered functionality. Biofabrication also includes self-assembly for the hierarchical assembly of supramolecular structure through non-covalent bonds. In some cases, biologically-based self-assembly can be triggered by external stimuli or involve molecular recognition (e.g., the assembly of virus particles is often triggered by pH changes). Biofabrication also includes the use of enzymes to build structure (e.g., macromolecular structure) by the addition of covalent bonds.

Here, we describe one biofabrication methodology, the enzymatic grafting of small molecule phenols onto a stimuli-responsive polysaccharide. Phenols are among the most abundant organic materials in nature and include the lignins in plants, the humics

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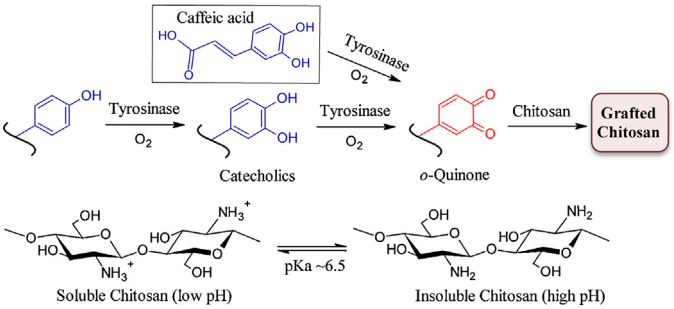


Scheme 1. Tyrosinase-mediated enzymatic grafting of phenolics (upper reactions) and the pH-responsiveness of chitosan (lower reaction).

in soil and the melanin in skin and hair [4]. Small molecule phenols are also abundant in plants (and therefore in our diets) [5–7]. Phenols possess diverse properties and are used in biology to confer mechanical strength (e.g., the dopamine residues of mussel glue [8]), to perform signaling functions (e.g., catecholamine neurotransmitters and salicylic acid plant signaling molecules [9,10]) and facilitate electron transfer (e.g., ubiquinone in the respiratory chain). Phenolic (and especially catecholic) materials are also attracting increasing technological interest because of novel synthesis methods [11–15] and their unique properties [16–22]. Here we present results with a single phenolic, caffeic acid, to illustrate the broad (and largely untapped) potential.

In biology, phenols are often incorporated into materials by enzymes that convert the substrate phenolic into a reactive intermediate. These enzymes include tyrosinases, phenol oxidases, laccases and peroxidases. Often the reactive intermediates that are generated undergo uncatalyzed reactions to create crosslinked networks (e.g., lignin). As illustrated in Scheme 1, the enzyme tyrosinase is capable of reacting with a broad range of low molecular weight phenolics (e.g., caffeic acid) and the phenolic residues of proteins (e.g., the tyrosine residues) [23]. Interestingly, this enzyme can perform two reactions, the hydroxylation of a phenol containing a single aromatic hydroxyl and the oxidation of the resulting catechol to generate an *o*-quinone. The quinone is reactive and can diffuse from the enzyme's active site to undergo uncatalyzed reactions.

As illustrated in Scheme 1, we perform the tyrosinase reaction in the presence of the aminopolysaccharide chitosan to enable the *o*-quinone that is generated to react with and graft to the polysaccharide backbone [24–26]. Chitosan is derived from chitin and is one of the few biologically-derived polymers with rich amine functionality. These amines confer a unique set of properties to chitosan [27]. Chitosan's primary amines confer pH responsiveness; at low pH protonation of the amine makes chitosan a water-soluble cationic polyelectrolyte while at high pH deprotonation removes chitosan's charge and eliminates its water solubility. In addition, the deprotonated primary amine has an unshared electron pair that is nucleophilic and enables chitosan to undergo reactions with electrophilic *o*-quinones.

Specifically, we show that the tyrosinase-mediated grafting of caffeic acid to chitosan confers technologically interesting properties to chitosan. Importantly, these properties are obtained by the enzymatic grafting of components that are safe and routinely ingested.

2. Materials and methods

2.1. Materials

The following materials were purchased from Sigma–Aldrich: chitosan from crab shells (85% deacetylation, and 200 kDa as reported by the supplier), tyrosinase (from mushroom), caffeic acid (Caff), $Ru(NH_3)_6Cl_3$ (Ru^{3+}), 1,1′-ferrocenedimethanol (Fc; an organometallic compound $Fe(C_5H_5)_2$ with a Fe^{2+} center). The gold working electrodes (2 mm diameter) and Ag/AgCl reference electrodes were purchased from CH Instruments, Inc. (Austin, TX). Platinum wire (99.95%) was purchased from Surepure Chemetals Inc. (Florham Park, NJ). Water was de-ionized with Millipore SUPER-Q water system until final resistivity >18 MΩ cm was reached.

2.2. Sample preparation

Chitosan was dissolved in dilute HCl solution (pH = 5.5) as previously described [28]. Concentrated tyrosinase solution (5 U μL^{-1}) was prepared by dissolving tyrosinase in 20 mM phosphate buffer (pH = 7.0). Caffeic acid solution was prepared by first dissolving caffeic acid in ethanol (200 mM), and then diluting in 20 mM phosphate buffer (pH = 7.0). The solution of electrochemical mediators (Fc and Ru^{3+}) was prepared in phosphate buffer (0.1 M; pH 7.0).

Fabrication and pretreatment of the chip with gold electrodes patterned onto a silicon substrate has been described elsewhere [29,30]. The clean chip (or QCM sensor) was immersed in the 1% chitosan (pH 5.5) solution and connected to the power source (2400 Sourcemeter, Keithley) using alligator clips, and the gold electrode was biased to serve as the cathode (3–4 A m^{-2}) while a platinum wire served as the anode. After electrodeposition, the chitosan-coated electrode was immediately removed from the deposition solution, gently rinsed and soaked with water, and then vacuum-dried at room temperature overnight.

For electrochemical redox studies, the gold working electrode was first cleaned with piranha solution (H_2SO_4:H_2O_2 = 7: 3, v/v) for 15 min and washed thoroughly with DI water, followed by drying under nitrogen stream. The clean electrode was immersed in the 1% chitosan (pH 5.5) solution and cathodically electrodeposited (4 A m^{-2}, 45 s) with a platinum wire serving as the anode. After electrodeposition, the chitosan-coated electrode was immediately removed from the deposition solution, rinsed with DI water and incubated in caffeic acid solution (0.5–5 mM) containing tyrosinase (20 U mL^{-1}) for a predetermined amount of time. The grafting reaction was terminated by rinsing the films extensively with phosphate buffer (pH 7.0).

2.3. Instrumentation

Rheological measurements were performed on a Rheometrics AR2000 stress-controlled rheometer (TA Instruments). A 40 mm diameter plate was used with a solvent trap to prevent drying. Typically, oscillatory strains of 5% (which is within the linear viscoelastic regime) were applied at 0.1 Hz. All measurements were performed at 25 °C.

Chemical analysis was performed with a Jasco 4100 series Fourier Transform Infrared Spectroscopy (FTIR) with an attenuated total reflection (ATR) cell (Jasco Inc., Easton, MO). Morphological analysis of films deposited onto chips was performed using scanning electron microscope (SEM, SU-70, Hitachi, Pleasanton, CA).

The mechanical properties of the electrodeposited chitosan films were probed in situ with a quartz crystal microbalance with dissipation (QCM-D, Q-Sense E1, Glen Burnie, MD). Gold-coated QCM sensor (QSX 301, Bioline Scientific) with electrodeposited chitosan films were placed in a standard flow module where real time monitoring of frequency was performed. The modeling process of collected data was performed using manufacturer-supplied software (Qtools modeling software version 3.0.7.230, Q-Sense).

Electrochemical measurements such as cyclic voltammetry (CV) were carried out with a CHI6273C Electrochemical Analyzer (CH Instruments, Inc., Austin, TX). Measurements were performed using

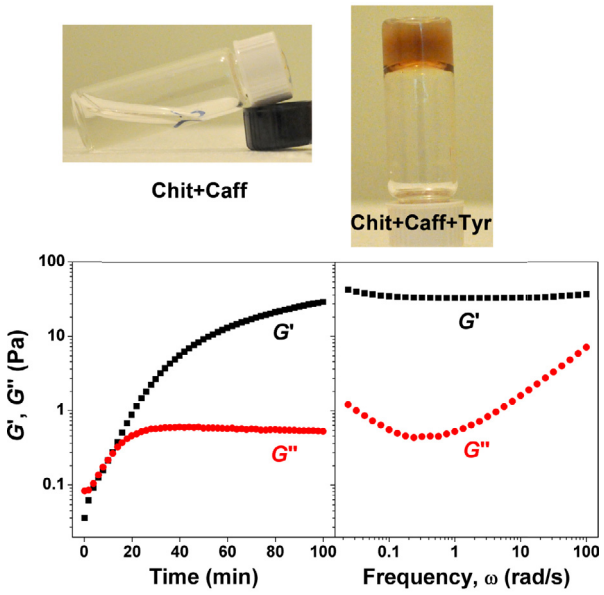


Fig. 1. Photographic and rheological evidence that tyrosinase (50 U mL^{-1}) catalyze a sol–gel transition for a solution of chitosan (0.7%) and caffeic acid (1 mM).

three-electrode configurations with Ag/AgCl as a reference electrode and Pt wire as a counter-electrode [31], while specific experimental conditions are provided in the text and figure captions. The solution of mediators (50 μM Fc and 50 μM Ru^{3+}) was degassed with nitrogen for at least 20 min before CV measurements, and during the CV scans a stream of nitrogen was gently blown over the surface of the solution. Typically, the potential was swept between −0.4 and 0.5 V (vs. Ag/AgCl) at a scan rate of 0.05 V s^{-1}.

3. Results and discussion

3.1. Tyrosinase-mediated gelation

As illustrated in Scheme 1, tyrosinase converts phenols and catechols into reactive *o*-quinones that can undergo uncatalyzed reactions that graft to and crosslink macromolecules. In biology, tyrosinase (or phenoloxidase) mediated crosslinking is believed to be important in the hardening and sealing of the insect cuticle (e.g., often referred to as quinone tanning [32,33]), and also for curing of the mussel's adhesive protein (i.e., the mussel glue) [8].

The tyrosinase-mediated oxidation of caffeic acid in the presence of soluble chitosan chains can also induce gelation (presumably through a crosslinking mechanism [24–26]). This enzymatic gelation is illustrated in Fig. 1 in which a solution of caffeic acid (1 mM) was incubated with chitosan (0.7%, w/v, pH 6) and tyrosinase (50 U mL^{-1}). The photograph in Fig. 1 illustrates that this reaction changes the solution from colorless to brown, and also converts the solution into a gel that can support its own weight. The rheological measurements in Fig. 1 further confirm gelation. In this case, the addition of tyrosinase to the caffeic acid and chitosan solution leads to an increase in the elastic (G') and viscous (G'') moduli with the G' becoming significantly larger than G''.

Much of the initial work with the tyrosinase-mediated grafting/crosslinking of phenolics to chitosan examined solution phase reactions either for the in situ gelation (e.g., for adhesive applications) or to generate polymers with unique rheological properties (e.g., associative thickeners). In principle, these studies enlisted phenolics or reactions with phenolics to confer mechanical functionality – a common use for phenolics in biology.

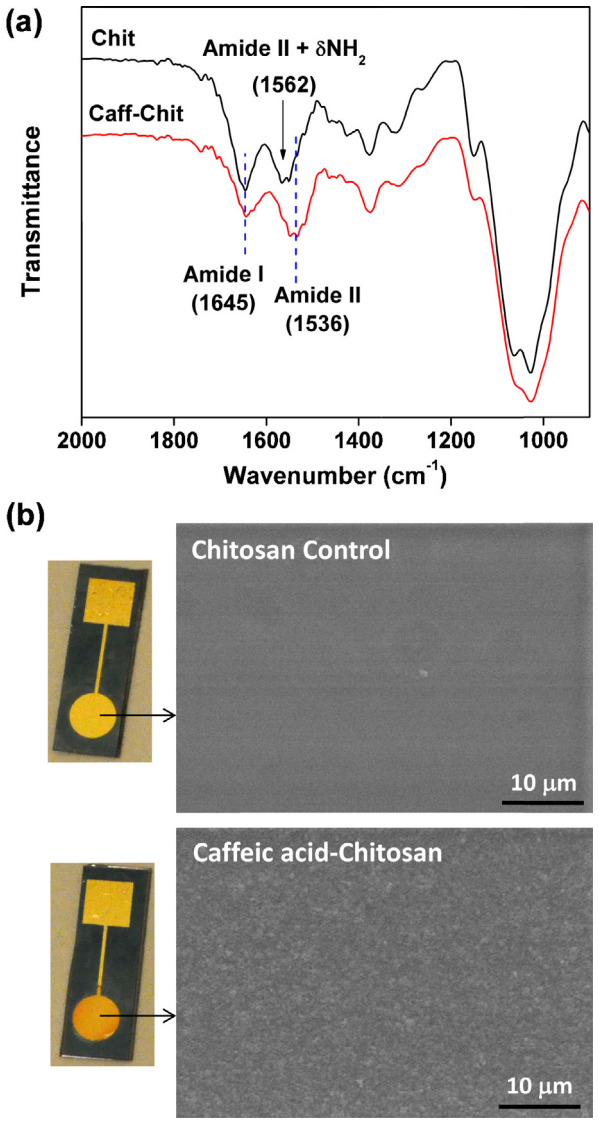


Fig. 2. (a) FTIR spectra of unmodified chitosan and caffeic acid modified chitosan. The N—H bending vibration (δNH_2) of the primary amines decreased in the caffeic acid-chitosan, suggesting that enzymatically oxidized caffeic acid reacts with the primary amine groups of the chitosan. (b) SEM images of unmodified chitosan and caffeic acid-modified chitosan show a relatively smooth surface for the chitosan control film, whereas the surface of the caffeic acid-modified film is rougher.

3.2. Tyrosinase-mediated reactions with chitosan films

As noted in Scheme 1, chitosan is pH-responsive, at low pH it can be dissolved while at higher pH it can form hydrogels, films and fibers. Importantly, chitosan's pK_a is near-neutrality allowing tyrosinase to be used to modify solutions (homogeneous reactions can be performed at pH ≤ 6 as in Fig. 1) or to modify films (heterogeneous reactions can be performed at pH > 6.5).

To illustrate film modification, we prepared caffeic-acid modified films and examined these films by FTIR and SEM. In the FTIR study, a cast chitosan film was incubated with caffeic acid (5 mM) and tyrosinase (20 U mL^{-1}) for 45 min. After reaction, the film was washed extensively with phosphate buffer (pH 7) and sonicated for 15 min to remove the unreacted caffeic acid. This caffeic acid modified chitosan film was then dried and measured with an FTIR instrument. Fig. 2a compares the FTIR spectrum for the caffeic acid modified chitosan with the spectrum for unmodified chitosan. In both spectra, the 1645 cm^{-1} band has been assigned to the amide I vibration [34,35], which originated from the N-acetyl groups of

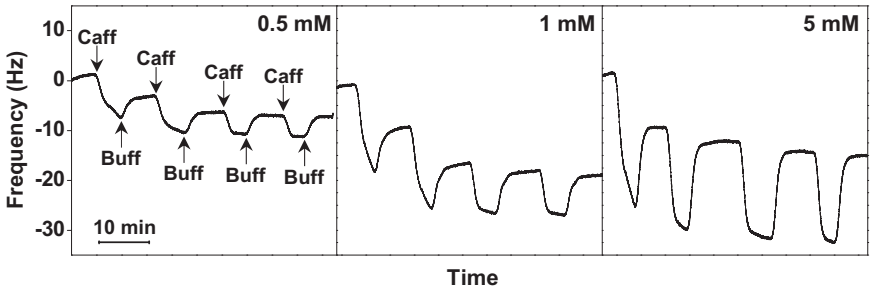


Fig. 3. In situ QCM monitoring of frequency of electrodeposited chitosan films upon introducing solutions containing 0.5, 1, or 5 mM caffeic acid and 20 U mL^{-1} tyrosinase (flow rate = 0.22 mL min^{-1}).

the N-acetylglucosamine residues. (The degree of acetylation of our chitosan was reported by the supplier to be 15%.) For the unmodified chitosan film, the band centered at 1562 cm^{-1} is assigned to overlapping peaks from the amide II and the N—H bending (δNH_2) vibration of the primary amines [36]. For the caffeic acid-modified chitosan film, the amide I and amide II vibrations are present; however, the band in the amide II region shifts from 1562 to 1536 cm^{-1}, indicating the N—H bending vibration of the primary amine decreases. This is consistent with our proposed mechanism that the enzymatically oxidized caffeic acid reacts with the primary amine groups of the chitosan.

For SEM study, films were prepared by first electrodepositing chitosan from solution (1% chitosan, pH 5.5) on a patterned gold-coated silicon chip. Electrodeposition enlists chitosan's pH-responsiveness such that cathodic electrolysis reactions (3 A m^{-2} for 4 min) provide the localized high pH conditions to induce chitosan's sol–gel transition. After deposition, the films were rinsed and then incubated with caffeic acid (5 mM) and tyrosinase (20 U mL^{-1}) for 45 min. The films were rinsed extensively with phosphate buffer to remove unreacted caffeic acid. For comparison, a chitosan coated chip was also prepared in the absence of tyrosinase. As shown by the photographs in Fig. 2b, after caffeic acid modification chitosan film appears to be more brownish than the unmodified chitosan. The SEM images in Fig. 2b show a relatively smooth surface for the unmodified chitosan film, whereas the surface of the caffeic acid-modified film is rougher. These observations are consistent with our previous studies on an electrodeposited chitosan film [29] and a catechol-modified chitosan film prepared by electrochemical oxidization [37].

3.3. Mechanical changes upon film modification

Fig. 1 illustrates that the tyrosinase-mediated caffeic-acid reactions alter the mechanical (i.e., rheological) properties of chitosan solutions. We used QCM-D [38–40] to examine if/how caffeic acid reactions alter the mechanical properties of chitosan films. In these studies we electrodeposited a thin film of chitosan onto the quartz crystal sensor (1% chitosan, 3 A m^{-2}, 30 s). The chitosan-coated electrode was rinsed, dried in vacuum overnight, and added to the flow cell and contacted with phosphate buffer (20 mM, pH 7). As indicated in Fig. 3, we then exchanged the solution for buffer containing caffeic acid (0.5 mM) and tyrosinase (20 U mL^{-1}). After this caffeic acid addition, the frequency was observed to decrease substantially, indicating good interaction between caffeic acid solution and chitosan film. After 5 min of reaction, the caffeic acid solution was replaced by phosphate buffer and the film's frequency was observed to initially increase presumably due to desorption of unreacted caffeic acid, but then to stabilize at a value lower than the initial (unreacted) film. This observed frequency decrease is consistent with the enzymatic grafting of caffeic acid to the chitosan film. As indicated in Fig. 3, the reaction and buffer solutions were exchanged four times (total modification time = 20 min) and these experiments were repeated at 2 different caffeic acid concentrations (i.e., 1 mM and 5 mM). In all cases, the results support the conclusion that caffeic acid moieties are covalently attached to the chitosan films.

After the in situ QCM experiments (Fig. 3) were completed (total modification time of 20 min), the resulting caffeic acid-modified chitosan films were vacuum dried overnight and ex situ QCM-D measurements were made as described previously [30]. Briefly, these measurements are analyzed using the manufacturer's software to determine the mass added and elastic modulus (G') of each of the caffeic acid-modified chitosan films. The results from these ex situ QCM-D are summarized in Fig. 4. The tyrosinase-mediated reactions of caffeic acid lead to increases in the dry weight of the films as shown in Fig. 4a. This increase in mass is consistent with a covalent coupling of the caffeic acid moieties to the chitosan film. Fig. 4b shows that the QCM-D measurements indicate that the elastic modulus G' was also increased by the caffeic acid reactions. Finally, the plot in Fig. 4c indicates that the G' increase for the wet film is approximately linear with the amount of caffeic acid added.

These mechanical measurements are consistent with an explanation that caffeic acid can crosslink the chitosan films. Further support for such a crosslinking hypothesis is that the caffeic-acid modified chitosan films no longer dissolve upon contacting with acidic solutions. Previous mass spectrometry measurements with related catecholics suggest that *o*-quinones can generate crosslinks between glucosamine residues [41]. While these varied observations support a conclusion that the tyrosinase-reaction can generate a crosslinked chitosan network, the complexity of the reaction system makes definitive interpretation of the results impossible. Nevertheless, the enzymatic reaction does alter the mechanical properties of the films consistent with the rheological results for solution phase reactions.

3.4. Enzymatic grafting imparts redox-properties to chitosan films

Catecholics (e.g., caffeic acid) are redox-active and among the most abundant antioxidants in our diet. The potential health beneficial effects of dietary phenolics have gained considerable attention. Several recent efforts to graft catecholic onto chitosan (or other biopolymers) have aimed to exploit their antioxidant properties. Interestingly, we recently observed that the catecholics that are grafted to chitosan retain their redox activity. Importantly, while the catecholic-chitosan films are redox-active, they are non-conducting. Specifically, the grafted catechols can exchange electrons with soluble mediators that can diffuse into the film, but the grafted catechols do not exchange electrons directly with a neighboring electrode.

The redox activity of the grafted catecholics enables the caffeic acid-chitosan films to undergo redox-cycling reactions illustrated in Scheme 2. The common electrochemical mediator ferrocene

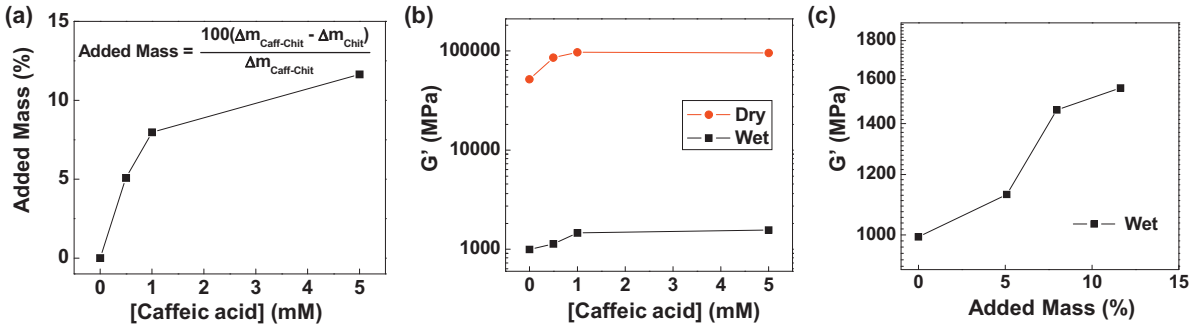


Fig. 4. Ex situ QCM-D measurements of (a) added mass (%) and (b) G' values of caffeic acid-modified chitosan films as a function of caffeic acid concentration used for modification. (c) G' values of wet caffeic acid-modified chitosan films are plotted as a function of the amount of caffeic acid added.

dimethanol (Fc) can be electrochemically oxidized, the oxidized ferrocenium ion (Fc^+) can diffuse into the chitosan film and accept electrons from grafted catechols as illustrated by the left panel in Scheme 2. The Fc-redox-cycling reaction serves to discharge electrons from the caffeic acid-chitosan film by converting reduced QH_2 moieties (presumably catechols) into oxidized Q moieties (presumably *o*-quinones). Film-charging can be performed with the electrochemical mediator $Ru(NH_3)_6Cl_3$ (Ru^{3+}) as illustrated by the center panel in Scheme 2. Electrochemical reduction converts Ru^{3+} to Ru^{2+} which can then transfer its electrons to the grafted Q moieties. The right-most panel in Scheme 2 shows that electron exchange is controlled by thermodynamics with electrons "flowing" from more negative to more positive potentials.

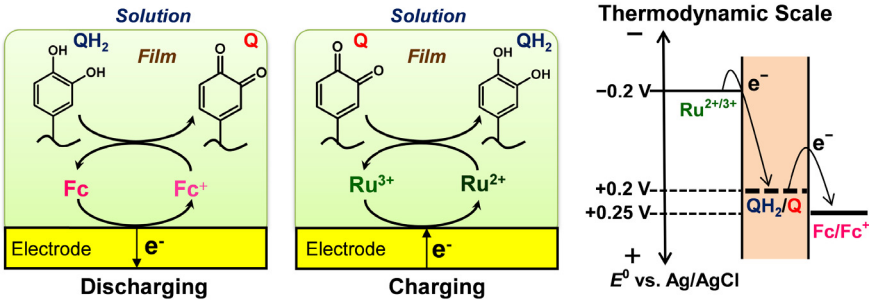
The redox activity of the caffeic acid-chitosan film is illustrated in Fig. 5a. In this experiment, the film-coated electrode was incubated with both the Fc and Ru^{3+} mediators (50 μM each). When the potential was cycled to positive potentials (greater than about 0.25 V vs Ag/AgCl), Fc oxidation occurred and the redox-cycling in the film served to discharge the film. When the potential was cycled to negative potentials (less than about −0.2 V), Ru^{3+} was reduced and the subsequent redox-cycling in the film served to charge the film. These redox-cycling reactions in the caffeic acid-chitosan film have the effect of amplifying the mediator currents compared to a control electrode coated with an unmodified chitosan film (chitosan is not redox active).

Fig. 5b illustrates a quantitative approach to characterize this amplification. Specifically, the oxidative current is integrated with respect to time to generate the charge transfer (Q) during the Fc oxidation portion of the cyclic voltammagram (CV). Fig. 5c shows that the amplification ratio (AR) increases with the extent of caffeic acid added to the tyrosinase reaction mixture. Previous studies have shown that grafting correlates to the amount of phenolic added and thus the film's redox activity can be systematically controlled by experimental conditions [4,37].

4. Discussion

Over 20 years ago it was observed that enzymatically oxidized low molecular weight phenols could graft to chitosan [42,43]. Grafting occurs because of the somewhat unique nucleophilic activity of chitosan (few natural polymers exist with such high amine content). Early studies focused on environmental applications to remove phenolics from waste waters and process streams [42–51]. The observation that enzymatic grafting also altered the properties of the chitosan [52,53] suggested that the diverse functionalities of natural phenolics could be accessed for materials synthesis. Many of the initial materials science efforts focused on solution properties – for in situ gelation or to generate water-soluble polymers with interesting solubility or rheological properties [54]. Later studies considered grafting low molecular weight phenolics to chitosan (or other biopolymers) to confer antioxidant, antimicrobial and/or mechanical properties to particles, films and fibers [55–60]. The ability of *o*-quinones to crosslink chitosan indicates their potential for coupling macromolecules; either coupling proteins to chitosan [61] or coupling proteins to other proteins [62,63].

Recently, we observed that quinone grafting confers redox-activity to chitosan films: these films can accept, store and donate electrons [31,37]. The compatibility of these redox-active films suggests opportunities for sensing and detection in biological systems [64,65]. Further, the ability of these films to "harvest" electrons from biology suggests potential applications in bioelectronics [66,67]. Interestingly, we have observed a variety of phenolics can be grafted to chitosan either electrochemically [31,37,64–66] or enzymatically (i.e., through tyrosinase-mediated reactions) [67,68] and these grafted phenolic moieties confer redox activity to the films. Tyrosinase offers advantages for the grafting of phenols that possess a single OH group (e.g., resveratrol) [68], since these compounds are typically difficult to oxidize electrochemically. Electrochemical grafting of catechols offers the benefit of greater



Scheme 2. Schematics show that the soluble redox mediators Fc and Ru^{3+} can access the caffeic acid redox-centers of the film for "discharging" or "charging" through independent redox-cycling reactions.

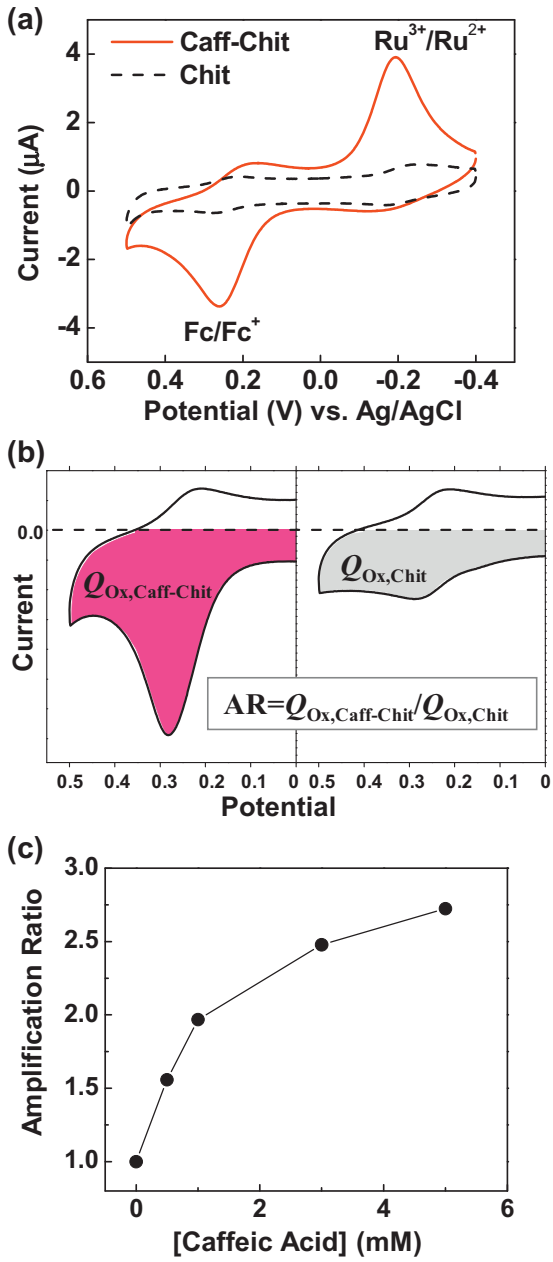


Fig. 5. (a) Redox-activity is demonstrated from CVs for chitosan films that had been incubated with caffeic acid (5 mM) and tyrosinase (20 U mL^{-1}) and then tested with the two mediators Fc (50 μM) and Ru^{3+} (50 μM). (b) Schematic illustrating the calculation of amplification ratio (AR). (c) Quantification shows AR increases as a function of caffeic acid concentration used during tyrosinase-mediated grafting.

control because oxidation can be quantitatively controlled by the anodic charge-transfer (Q). To date we have been unable to generate films with significantly different redox-potentials (i.e., E^0) presumably because the films contain similar grafted catecholic moieties.

Importantly, tyrosinase's substrate range is not limited to low molecular weight phenolics but this enzyme can oxidize macromolecular substrates (either synthetic or natural) [69,70] provided a phenolic moiety is present and accessible [23,71–74]. Tyrosine residues provide such phenolic moieties and thus tyrosinase has been used for grafting peptides [75–77] and proteins with open structures (e.g., gelatin [78,79], collagen [80,81], and silk fibroin [82,83]). Often, the tyrosine residues of globular proteins are not accessible for enzymatic oxidation. If genetic approaches can be employed, then the gene for a protein-of-interest can be engineered with a short fusion tag to provide accessible tyrosine residues to enable tyrosinase-mediated grafting to chitosan [84–87]. This enzymatic grafting provides a relatively simple and benign means to generate protein–chitosan conjugates. Depending on the extent of grafting, these protein–chitosan conjugates can sometimes retain chitosan's pH-responsive solubility and film-forming abilities [85].

More broadly, the studies with tyrosinase-mediated grafting suggest the greater potential for enlisting enzymes to build structure and confer function to materials [81,88–110].

5. Conclusions

Enzymes are highly selective catalysts that function under mild aqueous conditions and have therefore attracted attention for the environmentally-friendly synthesis of chemicals. There are fewer enzymes known that react with polymeric substrates to build macromolecular structure and confer materials function. One example is the tyrosinase-mediated grafting of phenolics to the aminopolysaccharide chitosan. This enzyme enables access to the diverse array of phenolics that are abundant in nature and provides a simple means to functionalize chitosan. Using a single phenolic substrate, caffeic acid, we demonstrate that tyrosinase-mediated grafting can induce a sol–gel transition, and can increase the modulus and confer redox-properties of thin chitosan films. As more enzymes are studied for building macromolecular structure and more functional properties are explored, we anticipate that enzymatic methods could become an integral method for materials synthesis.

Acknowledgements

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References

[1] G.F. Payne, E. Kim, Y. Cheng, H.C. Wu, R. Ghodssi, G.W. Rubloff, S.R. Raghavan, J.N. Culver, W.E. Bentley, Soft Matter 9 (2013) 6019–6032.
[2] Y. Liu, E. Kim, R. Ghodssi, G.W. Rubloff, J.N. Culver, W.E. Bentley, G.F. Payne, Biofabrication 2 (2010) 022002.
[3] Y. Cheng, X.L. Luo, G.F. Payne, G.W. Rubloff, J. Mater. Chem. 22 (2012) 7659–7666.
[4] K.M. Gray, E. Kim, L.Q. Wu, Y. Liu, W.E. Bentley, G.F. Payne, Soft Matter 7 (2011) 9601–9615.
[5] S. Quideau, D. Deffieux, C. Douat-Casassus, L. Pouysegu, Angew. Chem. Int. Ed. Engl. 50 (2011) 586–621.
[6] P.-G. Pietta, J. Nat. Prod. 63 (2000) 1035–1042.
[7] A. Scalbert, G. Williamson, J. Nutr. 130 (2000) 2073S–2085S.
[8] L.A. Burzio, J.H. Waite, Biochemistry 39 (2000) 11147–11153.
[9] S.W. Park, E. Kaimoyo, D. Kumar, S. Mosher, D.F. Klessig, Science 318 (2007) 113–116.
[10] K. Tsuda, M. Sato, J. Glazebrook, J.D. Cohen, F. Katagiri, Plant J. 53 (2008) 763–775.
[11] H. Lee, S.M. Dellatore, W.M. Miller, P.B. Messersmith, Science 318 (2007) 426–430.
[12] H. Lee, J. Rho, P.B. Messersmith, Adv. Mater. 21 (2009) 431–434.
[13] C.J. Ochs, T. Hong, G.K. Such, J. Cui, A. Postma, F. Caruso, Chem. Mater. 23 (2011) 3141–3143.
[14] A. Postma, Y. Yan, Y. Wang, A.N. Zelikin, E. Tjipto, F. Caruso, Chem. Mater. 21 (2009) 3042–3044.
[15] H. Ejima, J.J. Richardson, K. Liang, J.P. Best, M.P. van Koeverden, G.K. Such, J. Cui, F. Caruso, Science 341 (2013) 154–157.
[16] E. Faure, C. Falentin-Daudre, C. Jerome, J. Lyskawa, D. Fournier, P. Woisel, C. Detrembleur, Prog. Polym. Sci. 38 (2013) 236–270.
[17] R. Jain, A. Agarwal, P.R. Kierski, M.J. Schurr, C.J. Murphy, J.F. McAnulty, N.L. Abbott, Biomaterials 34 (2013) 340–352.
[18] J. Sedo, J. Saiz-Poseu, F. Busque, D. Ruiz-Molina, Adv. Mater. 25 (2013) 653–701.

[19] P. Meredith, B.J. Powell, J. Riesz, S.P. Nighswander-Rempel, M.R. Pederson, E.G. Moore, Soft Matter 2 (2006) 37–44.
[20] J.P. Bothma, J. de Boor, U. Divakar, P.E. Schwenn, P. Meredith, Adv. Mater. 20 (2008) 3539–3542.
[21] M. d'Ischia, A. Napolitano, A. Pezzella, P. Meredith, T. Sarna, Angew. Chem. Int. Ed. Engl. 48 (2009) 3914–3921.
[22] A.B. Mostert, B.J. Powell, F.L. Pratt, G.R. Hanson, T. Sarna, I.R. Gentle, P. Meredith, Proc. Natl. Acad. Sci. U.S.A. 109 (2012) 8943–8947.
[23] G. Faccio, K. Kruus, M. Saloheimo, L. Thony-Meyer, Process Biochem. 47 (2012) 1749–1760.
[24] C. Muzzarelli, R.A.A. Muzzarelli, Trends Glycosci. Glycotechnol. 14 (2002) 223–229.
[25] R.A.A. Muzzarelli, G. Littarrua, C. Muzzarelli, G. Tosi, Carbohydr. Polym. 53 (2003) 109–115.
[26] R.S. Alkasir, M. Ornatska, S. Andreescu, Anal. Chem. 84 (2012) 9729–9737.
[27] G.F. Payne, S.R. Raghavan, Soft Matter 3 (2007) 521–527.
[28] L.Q. Wu, H.M. Yi, S. Li, G.W. Rubloff, W.E. Bentley, R. Ghodssi, G.F. Payne, Langmuir 19 (2003) 519–524.
[29] L.Q. Wu, A.P. Gadre, H.M. Yi, M.J. Kastantin, G.W. Rubloff, W.E. Bentley, G.F. Payne, R. Ghodssi, Langmuir 18 (2002) 8620–8625.
[30] Y. Liu, B. Zhang, K.M. Gray, Y. Cheng, E. Kim, G.W. Rubloff, W.E. Bentley, Q. Wang, G.F. Payne, Soft Matter 9 (2013) 2703–2710.
[31] E. Kim, Y. Liu, X.-W. Shi, X. Yang, W.E. Bentley, G.F. Payne, Adv. Funct. Mater. 20 (2010) 2683–2694.
[32] R.J. Suderman, N.T. Dittmer, M.R. Kanost, K.J. Kramer, Insect Biochem. Mol. Biol. 36 (2006) 353–365.
[33] S.O. Andersen, Insect Biochem. Mol. Biol. 40 (2010) 166–178.
[34] J.D. Goodrich, W.T. Winter, Biomacromolecules 8 (2007) 252–257.
[35] G. Lawrie, I. Keen, B. Drew, A. Chandler-Temple, L. Rintoul, P. Fredericks, L. Grondahl, Biomacromolecules 8 (2007) 2533–2541.
[36] R. Valentin, B. Bonelli, E. Garrone, F. Di Renzo, F. Quignard, Biomacromolecules 8 (2007) 3646–3650.
[37] E. Kim, Y. Liu, C.J. Baker, R. Owens, S. Xiao, W.E. Bentley, G.F. Payne, Biomacromolecules 12 (2011) 880–888.
[38] M. Rodahl, F. Hook, A. Krozer, P. Brzezinski, B. Kasemo, Rev. Sci. Instrum. 66 (1995) 3924–3930.
[39] F. Hook, B. Kasemo, T. Nylander, C. Fant, K. Sott, H. Elwing, Anal. Chem. 73 (2001) 5796–5804.
[40] E.F. Irwin, J.E. Ho, S.R. Kane, K.E. Healy, Langmuir 21 (2005) 5529–5536.
[41] L.Q. Wu, M.K. McDermott, C. Zhu, R. Ghodssi, G.E. Payne, Adv. Funct. Mater. 16 (2006) 1967–1974.
[42] W.Q. Sun, G.F. Payne, M. Moas, J.H. Chu, K.K. Wallace, Biotechnol. Prog. 8 (1992) 179–186.
[43] K. Tatsumi, H. Ichikawa, S. Wada, Water Sci. Technol. 30 (1994) 79–86.
[44] Y. Kimura, M. Yamamoto, R. Shimazaki, A. Kashiwada, K. Matsuda, K. Yamada, J. Appl. Polym. Sci. 124 (2012) 796–804.
[45] H. Ganjidoust, K. Tatsumi, S. Wada, M. Kawase, Water Sci. Technol. 34 (1996) 151–159.
[46] S. Wada, H. Ichikawa, K. Tatsumi, Biotechnol. Bioeng. 45 (1995) 304–309.
[47] K. Yamada, T. Inoue, Y. Akiba, A. Kashiwada, K. Matsuda, M. Hirata, Biosci. Biotechnol. Biochem. 70 (2006) 2467–2475.
[48] K. Yamada, Y. Akiba, T. Shibuya, A. Kashiwada, K. Matsuda, M. Hirata, Biotechnol. Prog. 21 (2005) 823–829.
[49] G.F. Payne, W.Q. Sun, Appl. Environ. Microbiol. 60 (1994) 397–401.
[50] W. Edwards, W.D. Leukes, P.D. Rose, S.G. Burton, Enzyme Microb. Technol. 25 (1999) 769–773.
[51] W. Edwards, R. Bownes, W.D. Leukes, E.P. Jacobs, R. Sanderson, P.D. Rose, S.G. Burton, Enzyme Microb. Technol. 24 (1999) 209–217.
[52] G. Kumar, J.F. Bristow, P.J. Smith, G.F. Payne, Polymer 41 (2000) 2157–2168.
[53] G. Kumar, P.J. Smith, G.F. Payne, Biotechnol. Bioeng. 63 (1999) 154–165.
[54] L.Q. Wu, H.D. Embree, B.M. Balgley, P.J. Smith, G.F. Payne, Environ. Sci. Technol. 36 (2002) 3446–3454.
[55] L. Fras-Zemljic, V. Kokol, D. Cakara, Text. Res. J. 81 (2011) 1532–1540.
[56] M. Bozic, S. Gorgieva, V. Kokol, Carbohydr. Polym. 89 (2012) 854–864.
[57] M. Bozic, S. Gorgieva, V. Kokol, Carbohydr. Polym. 87 (2012) 2388–2398.
[58] J. Vartiainen, M. Ratto, R. Lantto, K. Nattinen, E. Hurme, Packaging Technol. Sci. 21 (2008) 317–328.
[59] A. Aljawish, I. Chevalot, B. Piffaut, C. Rondeau-Mouro, M. Girardin, J. Jasniewski, J. Scher, L. Muniglia, Carbohydr. Polym. 87 (2012) 537–544.
[60] K.M.G. Hossain, M.D. Gonzalez, A.R. Juan, T. Tzanov, Enzyme Microb. Technol. 46 (2010) 326–330.
[61] T.H. Chen, R. Vazquez-Duhalt, C.F. Wu, W.E. Bentley, G.F. Payne, Biomacromolecules 2 (2001) 456–462.
[62] A. Rollett, B. Thallinger, A. Ohradanova-Repic, C. Machacek, E. Walenta, A. Cavaco-Paulo, R. Birner-Gruenberger, J.G. Bogner-Strauss, H. Stockinger, G.M. Guebitz, RSC Adv. 3 (2013) 1460–1467.
[63] M. Fairhead, L. Thony-Meyer, J. Biotechnol. 150 (2010) 546–551.
[64] E. Kim, T. Gordonov, Y. Liu, W.E. Bentley, G.F. Payne, ACS Chem. Biol. 8 (2013) 716–724.
[65] E. Kim, T. Gordonov, W.E. Bentley, G.F. Payne, Anal. Chem. 85 (2013) 2102–2108.
[66] E. Kim, Y. Liu, W.E. Bentley, G.F. Payne, Adv. Funct. Mater. 22 (2012) 1409–1416.
[67] B.D. Liba, E. Kim, A.N. Martin, Y. Liu, W.E. Bentley, G.F. Payne, Biofabrication 5 (2013) 015008.
[68] Y. Liu, E. Kim, M.E. Lee, B. Zhang, Y.A. Elabd, Q. Wang, I.M. White, W.E. Bentley, G.F. Payne, Adv. Funct. Mater. (2013), http://dx.doi.org/10.1002/adfm.201301434.
[69] R.A.A. Muzzarelli, P. Ilari, W.S. Xia, M. Pinotti, M. Tomasetti, Carbohydr. Polym. 24 (1994) 295–300.
[70] L.H. Shao, G. Kumar, J.L. Lenhart, P.J. Smith, G.F. Payne, Enzyme Microb. Technol. 25 (1999) 660–668.
[71] T. Heck, G. Faccio, M. Richter, L. Thony-Meyer, Appl. Microbiol. Biotechnol. 97 (2013) 461–475.
[72] M.L. Mattinen, R. Lantto, E. Selinheimo, K. Kruus, J. Buchert, J. Biotechnol. 133 (2008) 395–402.
[73] R. Partanen, M. Torkkeli, M. Hellman, P. Permi, R. Serimaa, J. Buchert, M.L. Mattinen, Enzyme Microb. Technol. 49 (2011) 131–138.
[74] D. Ercili-Cura, R. Partanen, F. Husband, M. Ridout, A. Macierzanka, M. Lille, H. Boer, R. Lantto, J. Buchert, A.R. Mackie, Food Hydrocolloids 28 (2012) 1–9.
[75] A. Demolliens, C. Boucher, Y. Durocher, M. Jolicoeur, M.D. Buschmann, G. De Crescenzo, Bioconjug. Chem. 19 (2008) 1849–1854.
[76] A. Anghileri, R. Lantto, K. Kruus, C. Arosio, G. Freddi, J. Biotechnol. 127 (2007) 508–519.
[77] C.M. Aberg, T.H. Chen, A. Olumide, S.R. Raghavan, G.F. Payne, J. Agric. Food Chem. 52 (2004) 788–793.
[78] T. Chen, H.D. Embree, E.M. Brown, M.M. Taylor, G.F. Payne, Biomaterials 24 (2003) 2831–2841.
[79] T. Chen, H.D. Embree, L.Q. Wu, G.F. Payne, Biopolymers 64 (2002) 292–302.
[80] S. Jus, V. Kokol, G.M. Guebitz, J. Biomater. Sci.-Polym. Ed. 20 (2009) 253–269.
[81] S. Jus, I. Stachel, W. Schloegl, M. Pretzler, W. Friess, M. Meyer, R. Birner-Gruenberger, G.M. Guebitz, Mater. Sci. Eng. C: Mater. Biol. Appl. 31 (2011) 1068–1077.
[82] G.D. Kang, K.H. Lee, C.S. Ki, J.H. Nahm, Y.H. Park, Macromol. Res. 12 (2004) 534–539.
[83] G. Freddi, A. Anghileri, S. Sampaio, J. Buchert, P. Monti, P. Taddei, J. Biotechnol. 125 (2006) 281–294.
[84] M.J.C. Long, L. Hedstrom, ChemBioChem 13 (2012) 1818–1825.
[85] T.H. Chen, D.A. Small, L.Q. Wu, G.W. Rubloff, R. Ghodssi, R. Vazquez-Duhalt, W.E. Bentley, G.F. Payne, Langmuir 19 (2003) 9382–9386.
[86] A.T. Lewandowski, D.A. Small, T.H. Chen, G.F. Payne, W.E. Bentley, Biotechnol. Bioeng. 93 (2006) 1207–1215.
[87] A.T. Lewandowski, H.M. Yi, X.L. Luo, G.F. Payne, R. Ghodssi, G.W. Rubloff, W.E. Bentley, Biotechnol. Bioeng. 99 (2008) 499–507.
[88] L.S.M. Teixeira, J. Feijen, C.A. van Blitterswijk, P.J. Dijkstra, M. Karperien, Biomaterials 33 (2012) 1281–1290.
[89] K. Kuwahara, J.Y. Fang, Z. Yang, B. Han, Tissue Eng. Part A 17 (2011) 2955–2964.
[90] X.H. Yang, Y. Liu, G.F. Payne, J. Adhes. 85 (2009) 576–589.
[91] E.H. Acero, D. Ribitsch, R.D. Rodriguez, A. Dellacher, S. Zitzenbacher, A. Marold, K.J. Greimel, M. Schroeder, A. Kandelbauer, S. Heumann, G.S. Nyanhongo, H. Schwab, G.M. Guebitz, J. Mol. Catal. B: Enzym. 79 (2012) 54–60.
[92] S.R. Ahmed, A.T. Lutes, T.A. Barbari, J. Membr. Sci. 282 (2006) 311–321.
[93] S. Jus, I. Stachel, M. Fairhead, M. Meyer, L. Thony-Meyer, G.M. Guebitz, Biocatal. Biotransform. 30 (2012) 86–95.
[94] G.S. Nyanhongo, E.N. Prasetyo, E.H. Acero, G.M. Guebitz, Chem. Eng. Technol. 35 (2012) 1359–1372.
[95] F. Hollmann, I. Arends, Polymers 4 (2012) 759–793.
[96] A. Rajagopalan, W. Kroutil, Mater. Today 14 (2011) 144–152.
[97] A. Paananen, D. Ercili-Cura, M. Saloheimo, R. Lantto, M.B. Linder, Soft Matter 9 (2013) 1612–1619.
[98] T. Kudanga, G.S. Nyanhongo, G.M. Guebitz, S. Burton, Enzyme Microb. Technol. 48 (2011) 195–208.
[99] C. Silva, T. Matama, S. Kim, J. Padrao, E.N. Prasetyo, T. Kudanga, G.S. Nyanhongo, G.M. Guebitz, M. Casal, A. Cavaco-Paulo, React. Funct. Polym. 71 (2011) 713–720.
[100] X.H. Yang, X.W. Shi, Y. Liu, W.E. Bentley, G.F. Payne, Langmuir 25 (2009) 338–344.
[101] N. Kamiya, S. Doi, J. Tominaga, H. Ichinose, M. Goto, Biomacromolecules 6 (2005) 35–38.
[102] T. Tanaka, N. Kamiya, T. Nagamune, Bioconjug. Chem. 15 (2004) 491–497.
[103] T. Tanaka, N. Kamiya, T. Nagamune, FEBS Lett. 579 (2005) 2092–2096.
[104] J. Tominaga, Y. Kemori, Y. Tanaka, T. Maruyama, N. Kamiya, M. Goto, Chem. Commun. (2007) 401–403.
[105] S. Sakai, K. Hirose, K. Taguchi, Y. Ogushi, K. Kawakami, Biomaterials 30 (2009) 3371–3377.
[106] S. Sakai, T. Matsuyama, K. Hirose, K. Kawakami, Biomacromolecules 11 (2010) 1370–1375.
[107] R. Parthasarathy, S. Subramanian, E.T. Boder, Bioconjug. Chem. 18 (2007) 469–476.
[108] K. Minamihata, M. Goto, N. Kamiya, Bioconjug. Chem. 22 (2011) 74–81.
[109] K. Minamihata, M. Goto, N. Kamiya, Bioconjug. Chem. 22 (2011) 2332–2338.
[110] R.S. Stayner, D.J. Min, P.F. Kiser, R.J. Stewart, Bioconjug. Chem. 16 (2005) 1617–1623.