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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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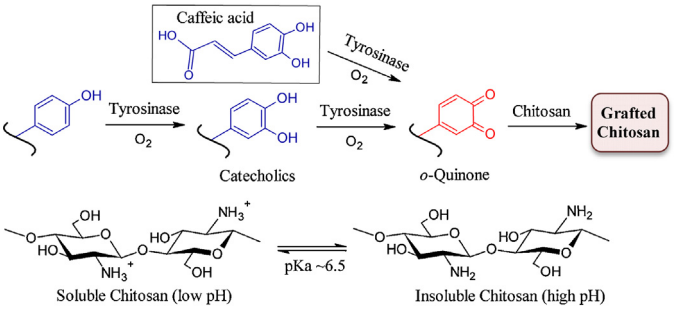

Scheme 1. Tyrosinase-mediated enzymatic grafting of phenolics (upper reactions) and the pH-responsiveness of chitosan (lower reaction).

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