Enzymatic Cross-Linking of a Phenolic Polymer Extracted from the Marine Alga *Fucus serratus*

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We have shown that a phenolic polymer (PP) extracted from *Fucus serratus* can be cross-linked using a vanadium-dependent bromoperoxidase (BPO). The methanol extracted PP was adsorbed to a quartz crystal sensor and the cross-linking was initiated by the addition of BPO, KBr, and H_2O_2. The decreased dissipation upon addition of the cross-linking agents, as measured with the quartz crystal microbalance with dissipation monitoring (QCM-D) method, was interpreted as intramolecular cross-links were formed between different phloroglucinol units in the PP. With surface plasmon resonance, it was shown that no desorption occurred from the sensor surface during the cross-linking. UV/vis spectroscopy verified the results achieved with QCM-D that all components, i.e., BPO, KBr, and H_2O_2, were necessary in order to achieve intramolecular oxidative cross-linking of the polymer.

Introduction

Marine organisms, such as algae, bryozoans, tunicates, mussels, tubeworms, and barnacles, have developed highly specialized adhesive systems to obtain a firm attachment to a variety of substrates in the marine environment. So while we modern chemists struggle to synthesize robust, underwater curable, water-resistant glues, marine invertebrates have used it for millions of years. The precursors of the biopolymers involved in the adhesion are usually water-soluble, which upon curing forms a water unsolvable cross-linked network. A fundamental understanding of the underlying adhesive bond formation and cross-linking mechanisms of these adhesives might lay the groundwork for biologically inspired new synthetic adhesives and coatings for underwater and medical applications as well as new innovative anti-fouling coatings.

The chemical composition and mode of function of the bioadhesive systems used by algae is not fully known. Up to this point, the adhesive system used by swimming spores of the green alga, *Ulva intestinalis* (formerly named *Enteromorpha intestinalis*), during settlement has been most extensively studied.[1−5] The spores secrete an adhesive glycoprotein from Golgi-derived membrane-bounded vesicles. The adhesive material undergoes rapid swelling after the release followed by a fast hardening or cross-linking, and a firm anchorage to the solid substrate is formed. After the curing, considerable forces is needed for the detachment of the spores from both hydrophilic and hydrophobic surfaces.[6−8] After the settling, a continued biosynthesis of the same, or at least a related, adhesive glycoprotein into the developing cell wall of the settled spore takes place. The curing process of both the primary and the secondary adhesive is, yet, still very unclear.

In the case of brown macro-algae adhesion, it has been shown that polyclonal antibodies against the human cell adhesion protein Vitronectin (Vn) recognize a vitronectin-like glycoprotein (Vn-F) secreted exclusively in the cell wall of an elongating rhizoid tip of the zygotes and embryos (*Fucus distichtus*).[9] This observation is interesting due to the fact that the rhizoid cell is highly polar and transports components of the cell wall to its elongating tip, which attaches the algae to the substratum. It was also shown in an adhesive assay that in the presence of the Vn-antibody the rhizoid was not able to adhere to the glass substratum, suggesting that the Vn-like glycoprotein in *F. distichtus* might have a functional role in adhesion. If algal adhesion is promoted via a Vn-like glycoprotein, it displays both similarities and dissimilarities with barnacles and mussels where adhesion is suggested to be purely protein based.[10−14]

In another study on the adhesion of *Fucus gardneri* zygotes, it was shown that the primary adhesion was initially noncovalent in nature based on the loss of adhesive properties in high salt or with chelators.[15] Moreover, the role of phenolic polymers and oxidases as key components in algal adhesion has been speculated on. For example, it has been shown in *Fucus* zygotes that the secretion of phenolic polymers correlated with the attachment process.[16] The secretion started a few hours after fertilization of the egg. Later, after germination, phenolic polymer secretion was localized at the site of attachment.

Phenolic polymers from *Fucus* have been extracted and analyzed with ^1H and ^{13}C nuclear magnetic resonance spectroscopy (NMR). It was found that the polymer extracted

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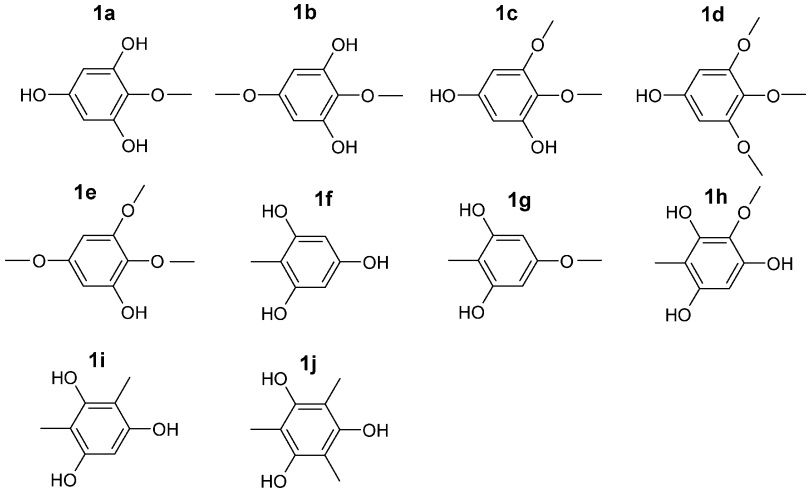


Figure 1. Constituent units demonstrated by ^{13}C and ^{1}H NMR in the phenolic polymer extracted from *Fucus vesiculosus.*[17]

from *Fucus vesiculosus* was composed of phloroglucinol units linked by carbon−carbon and ether bonds.[17] The NMR investigation also ruled out the presence of at least 25 potential repeating units of the polymer.[17] The most common (at least 99% of the total number of repeating units in the polymer) are shown in Figure 1. As can be seen, several of the units are able to form branches, which significantly should affect the polymer properties such as crystallinity. The most striking feature of the *F. vesiculosus* polymer was that most of the branches terminated with a bi-hydroxyl or tri-hydroxyl phenol group, which could contribute both to the potential oxidative cross-linking of the polymer and to the adhesive properties of the polymer by the ability to replace water at the interface.[13] Determination of the molecular weight of the extracted phenolic polymers from *Fucus* has been estimated by size exclusion chromatography (SEC). For example, a phenolic polymer purified from *Ascophyllum nodosum* had a molecular-mass range from 320 up to 4×10^5 g/mol.[18] The overall number- and weight-average molecular masses were 2.5×10^3 and 1.3×10^4, respectively.

As discussed above, cross-linking of the water-soluble adhesive polymers must take place rapidly or they will be washed away from the site of action. Most likely, the cross-linking at the interface is dependent on another component. It could be something already present in the water such as ions, and ionic interactions between different polymer chains could take place. It could also be another component secreted simultaneously with the polymer. This second component could be an extra cellular haloperoxidase, such as a vanadium-dependent bromoperoxidase (BPO), that is found in considerable quantities in brown algae.[19,20] Worth noting is that bromide is required for the stalk assembly and the adhesion of the diatom *Achnanthes longipes*, and it could be that haloperoxidases are components in the oxidative cross-linking of adhesive polymers secreted from algae.[21] Hence, in this study, the adsorption and potential enzymatic cross-linking of phenolic polymers extracted from the brown alga *Fucus serratus* were investigated using the quartz crystal microbalance with dissipation monitoring (QCM-D) methodology.[22−24] The QCM technique has grown rapidly the past decade including studies of thin polymeric films to measurements involving micellar systems, self-assembling monolayers and their phase transition behavior, molecularly imprinted polymers, chemical sensors, films formed using the layer-by-layer assembly technique, and biopolymer adsorption.[25]

Experimental Section

Extraction of Phenolic Polymer. The extraction of phenolic polymers was carried out according to the isolation procedure published by Glombitza et al.[26] In summary, *F. serratus* thalli were collected from the shore in the vicinity of Roscoff (Brittany, France). The thalli were freeze-dried, and an extraction in 80% MetOH:H_2O (v/v) was carried out. After the alcohol extraction followed by evaporation, the crude material was dissolved in water and freeze-dried. Preliminary size exclusion chromatography (SEC) investigations of the extracted phenolic polymer indicated at least a bimodal molecular mass distribution with molecular masses ranging from around 3×10^2 up to 2.5×10^4. This rather low molecular mass of our polymer is probably an effect of the extraction method with some degradation and formation of oligomers during the preparation. New extraction protocols are under development, but the interpretation of the results achieved in this study is not affected by the rather low molecular mass of the polymer. Moreover, the purity of the phenolic polymer fraction has not been measured, and it might contain some protein contamination. If the sample contains impurities such as proteins, it will adsorb together with the phenolic polymer and the surface composition will be heterogeneous. It is most unlikely that the proteins are cross-linked or affected by the enzyme used in this study. However, it must be stressed that the proteins might have a steric effect, and by this they may delay or obstruct the cross-linking reaction.

Extraction, Isolation and Purification of Bromoperoxidase (BPO). The procedure to isolate and purify the BPO enzyme has been published elsewhere.[20] Briefly, 30 g of *A. nodosum* thalli were powdered in liquid nitrogen and extracted in a two-phase solution (1 L) containing 17.5% (w/v) K_2HPO_4 and 17.5% (w/v) poly(ethylene glycol, PEG) (M_n = 1550 g mol^{-1}). The two phases were separated by centrifugation at 5000 × g for 15 min, and then 6% (w/v) $MgSO_4.7H_2O$ was added to generate a second phase system.

Then, 10% (w/v) PEG (M_n = 1550 g mol^{-1}) was added to remove phenols and chlorophylls from the bottom phase. A third system was generated for desalting by addition of an equal volume of a solution containing 20% PEG (w/v) and 60% (w/v) NH_2SO_4. Three volumes of acetone were added to the top phase, and after 1 h at −20 °C, the protein extract was pelleted by centrifugation at 10000 × g for 30 min. To reactivate the BPO, the pellet was dissolved and dialyzed overnight against 2 mM $NaVO_3$ in 50 mM Tris-HCl (pH 9.0). The extract was then loaded on a phenyl-Sepharose CL4B hydrophobic interaction column, equilibrated with 30% $(NH_4)SO_4$, 50 mM Tris-HCl, pH 9.0. Proteins were eluted by a decreasing linear gradient down to salt free Tris-HCl buffer. The BPO active fractions were pooled and dialyzed against 50 mM Tris-HCL, pH 9.0 buffer.

Cross-Linking of Phenolic Polymer. We have developed a method for the determination of the chemical/physical/enzymatic cross-linking of a variety of biopolymers including marine adhesives based on the surface sensitive quartz crystal microbalance with dissipation monitoring (QCM-D) methodology.[24] The QCM-D technique allows simultaneous time-resolved measuring of the amount of adsorbed mass and the mechanical properties of the adhesives.[22,23] The quartz crystal microbalance used in this study was a Q-Sense D300 (Q-Sense AB, Göteborg, Sweden) with a temperature controlled fluid cell. In summary, the QCM-D technique is an acoustic method where a quartz crystal is set in lateral resonance oscillation with a predefined frequency (f). Simultaneous frequency (f) and dissipation (D) measurements are made by periodically switching on and off the AC-voltage over the crystal. The decay signal is recorded and fitted to an exponentially damped sinusoidal curve. The adsorbed amount (g cm^{-2}) can be calculated from eq 1 by the frequency shift (Δf) using the Saurbrey equation, provided that the mass is evenly distributed, does not slip on the sensor surface, and is sufficiently rigid and/or thin to have negligible internal friction.[27] C_f is the mass sensitive constant (e.g., 17.7 × 10^{-9} g cm^{-2} Hz^{-1}), n_r is the shear wavenumber, $\rho_{biopolymer}$ is the density (g cm^{-3}) of the biopolymer, $\delta_{biopolymer}$ is the thickness of the adsorbed layer, and $m_{bioplymer}$ is the mass per unit area (g cm^{-2}) of the adsorbed biopolymer.

$$-\frac{C_f}{n_r}\Delta f = \rho_{biopolymer}\delta_{biopolymer} = m_{biopolymer} \qquad (1)$$

In addition the decay time (τ) of the sensor crystal is measured and is used to calculate the dissipation (D), eq 2

$$D = \frac{1}{\pi f \tau} \qquad (2)$$

The dissipation gives valuable information about the mechanical properties of the adsorbed layer. A very rigid material will have an increased decay time and, consequently, a low dissipation (D). Vice versa, a viscoelastic/soft material will result in fast damping of the sensor crystal, i.e., a short decay time and higher dissipation. Thus, the cross-linking of a biopolymer can be studied by time-resolved measurements of changes in dissipation.

The experiments were done using the following procedure: (1) establishing a baseline with degassed 0.1 M Na-acetate buffer (pH 5.5, 0.075 M NaCl) for at least 5 min; (2) adding PP 25 μg × mL^{-1} to the sample chamber and observing the adsorption during 20−40 min; (3) washing with pure 0.1 M Na-acetate buffer for 5 min; (4) adding the cross-linking agents, i.e., BPO enzyme (740 units/ml in Na-acetate buffer), H_2O_2 (1 mM in Na-acetate buffer), and KBr (1 mM in Na-acetate buffer) and studying the cross-linking for 20−40 min; (5) washing with Na-acetate buffer. Control experiments were carried out by not adding any of the three components needed for the cross-linking, i.e., leaving out the BPO enzyme, H_2O_2, or KBr. The QCM-D instrument operated at a drive amplitude of 2 V and with automatic adjustment of the sensitivity.

The enzyme activity was measured spectrophotometrically by monitoring at 290 nm the conversion of monochlorodimedone (20 cm^{-1} × mM) into monochlorobromodimedone (0.2 cm^{-1} × mM). Specific activity is expressed in units per milligram of protein, where one unit of bromoperoxidase activity is defined as the amount required for brominating 1 μmol of monochlorodimedone per minute.

Surface Plasmon Resonance (SPR). SPR studies were done as a control to the QCM-D experiments. SPR is an optical method and is therefore not affected by cross-linking or any other reactions that change the mechanical properties of the polymer.[23] There is a straightforward relation for calculating the adsorbed mass based on the change in the angle of incidence as can be seen in eq 3. However, the SPR method is very sensitive to changes in refractive index of the medium sensed by the evanescent wave and care must be taken when analyzing the data

$$\Delta m_{SPR} = C_{SPR}\Delta RU \qquad (3)$$

ΔRU is the measured change in response units (a dimensionless quantity that is proportional to the change in refractive index). C_{SPR} is a calibrated constant, which in the case of protein adsorption has been determined to be 6.5 × 10^{-2} ng cm^{-2} for a large number of proteins.[28]

The SPR measurements were done using a BIAcore 2000 system in a flow cell providing laminar flow, using a flow rate of 20 μL× min^{-1}. A typical experiment was carried out as follows; after a stable baseline was achieved, the phenolic polymer solution (25 μg× mL^{-1}) was injected and the adsorption was studied for 30 min. Adding the cross-linking agents was done in two ways. The reactants were mixed just prior to injection or the reactants were added in a sequential manner starting with BPO followed by injection of a solution containing H_2O_2 and KBr. Gold (Au) sensor surfaces (product nummer: BR-1004−05, BIAcore AB, Sweden) were used for the experiments. The experiments were done at least in triplicate.

UV/vis Spectroscopy. Aqueous solutions of PP exhibit a relatively nondescript UV/Vis spectrum dominated by "end-absorption", but a small absorption characteristic of aromatic nuclei can be found at c.a. 268 nm.[18] The UV/vis spectra (660−220 nm) of the PP solution were measured at room temperature with a DU 7400 UV/Vis spectrometer (Beckman, USA). A minor shoulder in the spectra was found at 275 nm (data not shown). To study the cross-linking reaction, PP was dissolved in 0.1 M acetate buffer pH 5.5, 0.075 M

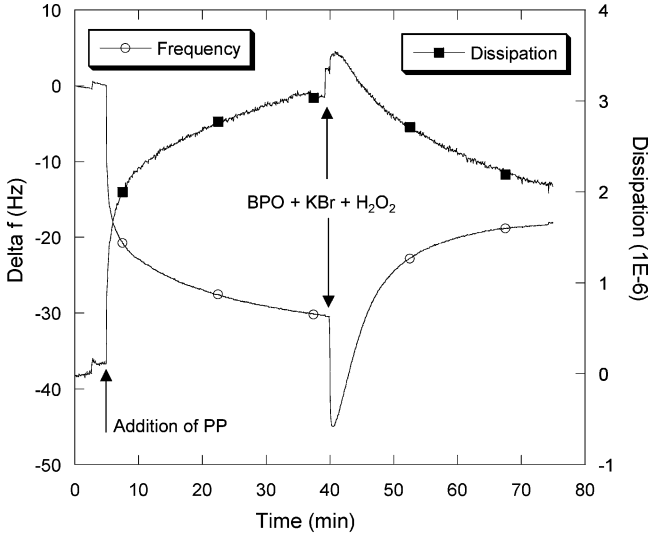


Figure 2. Representative QCM-D experiment showing the shift in frequency (1st *y* axis) and dissipation (2nd *y* axis) during the adsorption and the enzymatic cross-linking of phenolic polymer extracted from *Fucus serratus*.

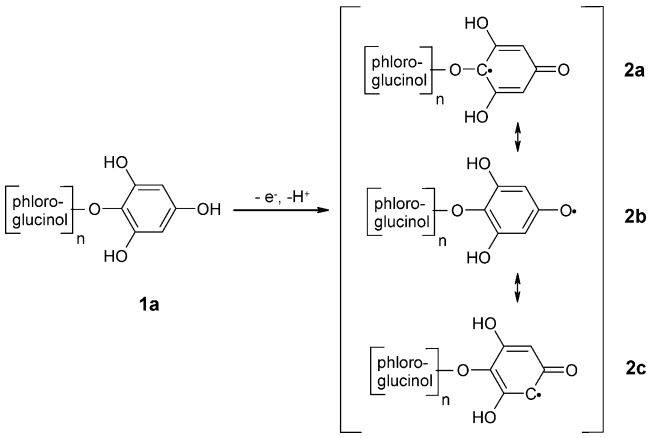
NaCl at a concentration of 25 μg/mL. BPO and KBr or H_2O_2 and KBr or BPO and KBr and H_2O_2 was added and the shift in absorbance (%) at 275 nm as a function of time was measured. PP, BPO, KBr, and H_2O_2 were all used so that the final concentrations were the same as used in the QCM-D experiments.

Size Exclusion Chromatography (SEC). The phenolic polymers were analyzed with size exclusion chromatography using 0.1 M acetate buffer, 0.075 M NaCl as eluent. For the analysis, four serially connected columns were used: gel G5000 WXL, G4000 WXL, G3000 WXL, and G1000 WXL, all provided by TosoHaas, Germany. A UV detector (PL-LC1200, Polymer Laboratories, UK), a refractive index detector (RI-71, Shodex, Japan), and a viscometric detector (Viscotek H502, Viscoptek Corp., USA) were used for detection. The PP samples were filtered using regenerated cellulose membranes (pore-size 0.45 μm) before injection. 100 μL of solution were injected onto the columns, and the eluent flow rate was set to 0.4 mL/min at 60 °C. Molar masses were calculated from the viscosity and RI signals by constructing a universal calibration curve using Pullulan standards (Shodex, Japan).

Results and Discussion

A typical cross-linking experiment is shown in Figure 2. In this experiment, a gold-coated quartz crystal sensor was used, and after 5 min of having a stable baseline, the adsorption of PP was studied for 30 min. As expected, the frequency decreased during adsorption and the adsorbed mass after 30 min of adsorption was calculated to be 180 ng$\times$ cm^{-2} using eq 1. Observe that it includes the water hydrodynamically coupled to the adsorbed film.[23] The rather broad molecular mass distribution of our polymer is affecting the adsorption in the following way. Low molecular mass polymers with their fast diffusion are first adsorbed. During the time of the measurement, the high molecular mass polymers, with their slower diffusion rate to the surface but with stronger interaction, will replace the low molecular mass polymers. This general phenomenon, better known as the Vroman effect,[29] leads to an enrichment of the high molecular mass components after extended adsorption. Thus, we can assume that at the time when the cross-linking agents are added to the reaction chamber we have a surface mainly composed of the high molecular mass phenolic polymers.

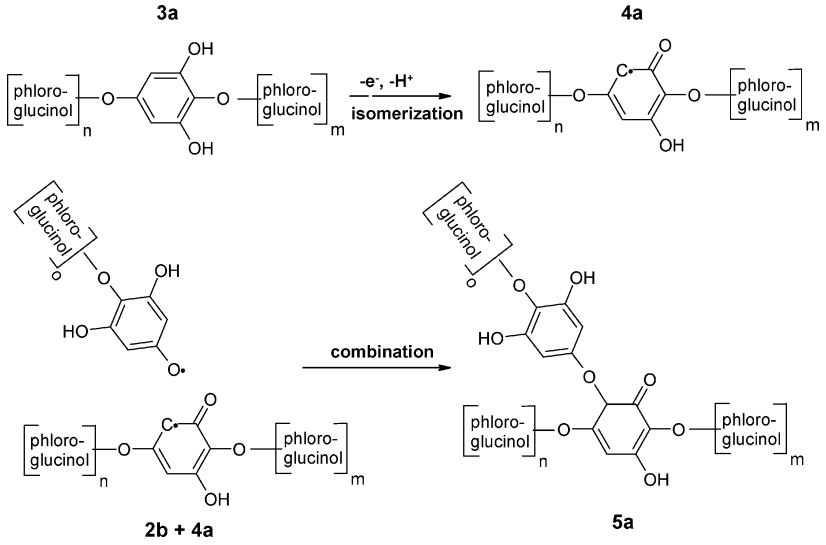
Scheme 1. Oxidation and Isomerization of Phloroglucinol Units as Suggested by Oudgenoeg and Co-workers[31]



Simultaneously, the dissipation increases during the adsorption as can be seen in Figure 2. After the adsorption and wash, the cross-linking agents BPO, H_2O_2, and KBr were added. Upon the addition of the cross-linking agents, we first observed a sharp drop in frequency and the opposite, a sharp rise in the dissipation. This was interpreted as the enzyme binding to the polymer film. The fast attachment of the enzyme to the substrate is notable. In this context, it can be mentioned that horseradish peroxidase forms unusually stable adducts with substituted phenols.[30] This strong enzyme−substrate binding could be one way of controlling the extent of cross-linking of the biopolymer gel. The enzyme will be immobilized to the substrate and subsequent cross-linking can only occur in close proximity to the enzyme. Similar speculations were brought up when cross-linking an adhesive protein extracted from blue mussel.[24] Hence, this mechanism of controlling the degree of cross-linking of biopolymer gels might be general. Furthermore, as discussed by Vreeland et al. if the enzyme is incorporated in a polysaccharide network, it can easily be mistaken for a glycoprotein and lead to false conclusions regarding the components involved and mechanisms of adhesion.[16]

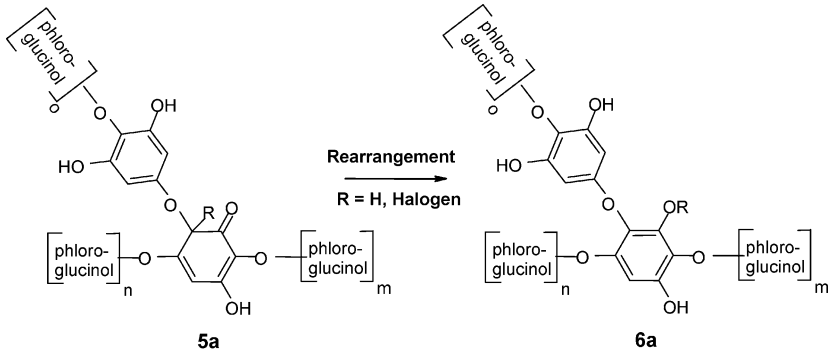
About a minute after the addition of BPO, KBr, and H_2O_2, we observed a gradual decrease in dissipation. Note that the frequency increased concomitantly with the decrease in dissipation during the cross-linking. This suggests that the cross-linking reaction is accompanied by the loss of a reaction product, loss of the water binding capacity of the film or both. It has been shown that the oxidative cross-linking of phenolics involves a one-electron oxidation and subsequent deprotonation,[31] as schematically illustrated in Scheme 1. The formed phenoxyl radical species can combine with other radical species forming a covalent cross-link as shown in Scheme 2.[32] It has also been shown that the dimerization and rearrangement or fragmentation of phenoxyls is stimulated if the phenols were substituted with a

Scheme 2. Putative Intramolecular Cross-linking Mechanism of Phloroglucinols[a]

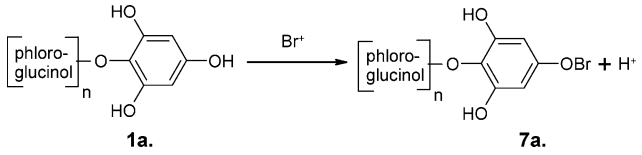


[a] Reaction Scheme Adopted from Eickhoff et al.[33]

Scheme 3. Putative One-electron Intramolecular Cross-linking Mechanism of Halogenated Phloroglucinols



Scheme 4. Putative Two-electron Halogenation Mechanism of Phloroglucinols[34]



halogen, as schematically shown in Scheme 3.[33] Based on these reaction schemes, both loss of the water binding capacity and losses of reaction products are possible. It can also be noted that de-chlorination was observed with mass spectroscopy during the oxidation of 2,4-dichlorophenol with horseradish peroxidase.[30] A speculative cross-linking mechanism could thus start with the bromination of the phenols (as has been suggested as the main task for bromoperoxidases), followed by the oxidation and combination, and finally, the rearrangement of HBr. However, it should be noted that the bromination of the phenols might take place via a two-electron oxidation model as suggested by de Boer and Wever.[34] Oxidized bromine species (Br^+) generated by the enzyme reacts with the nucleophilic phloroglucinol repeating unit according to Scheme 4. Which model holds for the cross-linking in our study could probably be investigated with electron scavengers or with electron spin resonance studies (ESR) but such studies are out of the scope of this paper.

Our results also indicate that all components are necessary in order to achieve cross-linking as can be seen in Figure 3.

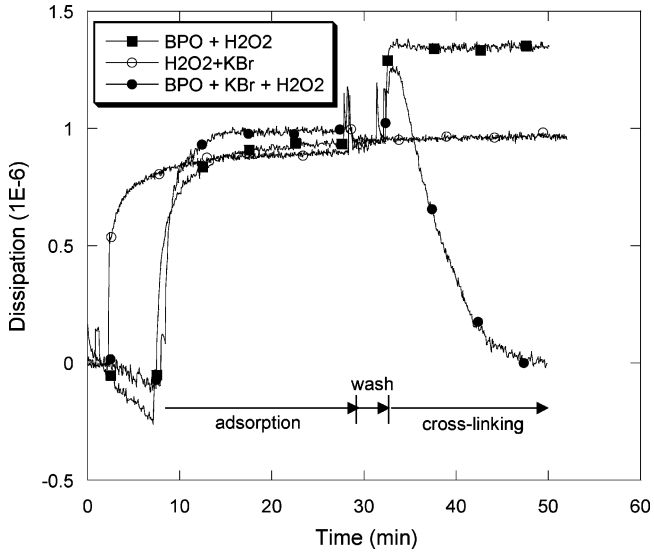


Figure 3. Representative QCM-D experiments showing the shift in dissipation during the adsorption and cross-linking of phenolic polymer. Shown are the experiments when BPO and H_2O_2 (filled squares) and H_2O_2 and KBr (open circles) were used and no cross-linking was observed. Shown is also the control experiment when all three components, i.e., BPO, H_2O_2, and KBr (filled circles) were used and we observed a gradual decrease in dissipation interpreted as intramolecular cross-links were formed.

In these experiments, the pure gold surface of the quartz crystal sensor was coated with a cross-linked phenolic

polymer film. The purpose was to study the adsorption of "polymer to polymer". The adsorption kinetics was in this case much faster than the adsorption kinetics measured using gold (Au) as the substrate (Figure 2). As can be seen in Figure 3, a decrease in dissipation occurred only when all of the components were added to the measurement chamber. If KBr was left out, we observed a fast increase in dissipation, but this was not followed by the gradual decrease after a few minutes. Hence, no cross-linking takes place and we only observed the binding of the BPO enzyme to the polymer film. As mentioned above, bromide was required for the adhesion of the diatom, *A. longipes*.[21] The diatom cells showed no differences in growth or EPS secretion with different bromide concentrations, but the ability to adhere to the substratum was lost at low bromide concentrations. As suggested by the results from this study, bromide is required for the formation of intermolecular cross-links. Consequently, if the cross-linking reaction of the diatom adhesive material was altered at low bromide concentrations, the ability to withstand shear and tension forces should be lost, and as a result, no adhesion could be observed. When only H_2O_2 and KBr were added to the polymer, we observed no changes in dissipation or frequency, Figure 3. It should be stressed that oxidation of the phenolic polymer probably takes place in this experiment but that is not reflected in any changes in dissipation or frequency. Finally, not shown in Figure 3 was the experiment when H_2O_2 was left out from the cross-linking reaction (only BPO and KBr were added to the sample chamber). That experiment resembled the experiment when KBr was left out from the reaction. Thus, we only observed a sharp increase in dissipation with no gradual decrease, and it was concluded that no cross-linking takes place. Each experiment was run in at least triplicate, but representative runs are shown in Figure 3.

It could be noted that the shift in dissipation (or motional resistance) upon cross-linking in our study resembles the results obtained in a study where the QCM technique was used to study the enzymatic polymerization of self-assembled aggregates of the decyl ester of tyrosine by the use of horseradish peroxidase (HRP) and H_2O_2.[35] The QCM technique in that study was able to detect the more mechanically robust polymerized aggregates.

To check the specificity of the enzyme, the phenolic polymer extracted from *F. serratus* was replaced with a layer of mefp-1, which is an adhesive protein extracted from the blue mussel.[36] In previous studies, we have demonstrated the cross-linking of mefp-1.[23,24] The dominant features of mefp-1, which is found in both adhesive and nonadhesive tissue in the mussel, is a tandemly repeated decapeptide: AKPSYPPTYK, with up to 80 repeats and a variety of posttranslational modifications.[14] The modifications include the hydroxylation of proline to 4-hydroxyproline (P), 3,4-dihydroxyproline (P), and tyrosine to 3,4-dihydroxyphenyl-L-alanine, L-DOPA, (Y).[37] The modification of the decapeptide is putatively responsible for the cross-linking since it has been shown that cross-links are formed via the oxidation of L-DOPA to o-quinone, followed by the formation of di-DOPA residues.[38,39] This reaction mechanism resembles the one proposed in the case of the phenolic polymers used in this study, and the possibility to cross-link mefp-1 with the use of BPO, H_2O_2, and KBr was investigated. As can be seen in Figure 4, mefp-1 and the phenolic polymer displayed similar adsorption kinetics to the pure gold surface. When BPO, H_2O_2, and KBr were added, fast enzyme adsorption to both the phenolic and the mefp-1 layer was observed, but in the case of mefp-1, this was not followed by the gradual increase in frequency as was observed with the phenolic polymer film. Such discrepancies are difficult to elucidate, but one possible explanation could be that in the case of mefp-1 only one out of five units can form cross-links (20 mol % DOPA) but in the case of PP every repeating unit is able to form a covalent cross-link (Figure 1). The changes in mechanical properties during the cross-linking of mefp-1 are thus not detectable with the QCM-D technique on a hydrophilic substrate.[24]

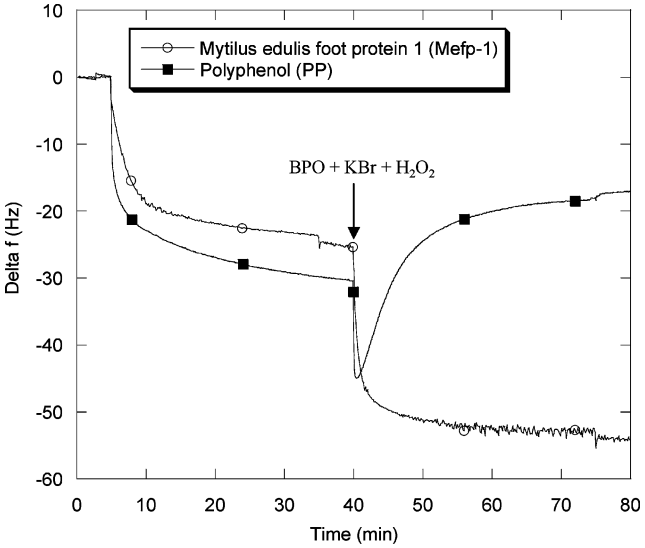


Figure 4. Representative QCM-D experiments showing the shift in frequency when BPO, H_2O_2 and KBr were added to a film of adsorbed mefp-1 (open circles) and to a film of adsorbed phenolic polymer (filled squares).

By using an optical method that just measured the "optical mass" and not the acoustic mass, which includes coupled water, we were able to rule out that no extensive desorption of organic material occurred during the cross-linking reaction. In Figure 5, the time vs dissipation is compared against the time vs optical surface plasmon resonance (SPR) detector signal when the cross-linking components were added. The SPR signal showed a sharp increase followed by a relatively stable signal when the cross-linking components were added. This can be compared with the major decrease in dissipation measured during the cross-linking reaction. Thus, no desorption of polymer or enzyme occurred during the cross-linking reaction. It should be noted that the SPR detector signal showed a minor decrease during the cross-linking reaction. We believe that is a result of the increased density of the polymer network as it cross-links. Increased density might affect the refractive index of the polymer film and thereby affect the calculated mass according to eq 3.

To further understand the cross-linking mechanism, UV/vis spectroscopy experiments were carried out and compared with the QCM-D and SPR results. The scan from 600 to 200 nm of a polymer solution (25 $\mu g \times mL^{-1}$) revealed a

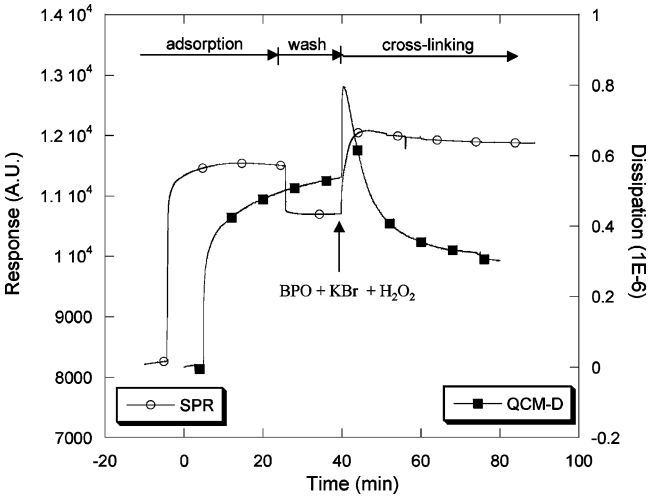


Figure 5. Shown is the shift in Surface Plasmon Resonance (SPR) detector signal (open circles, 1st *y* axis) during the adsorption and cross-linking of phenolic polymer. Also shown is the shift in dissipation during the adsorption and cross-linking (filled squares, 2nd *y* axis).

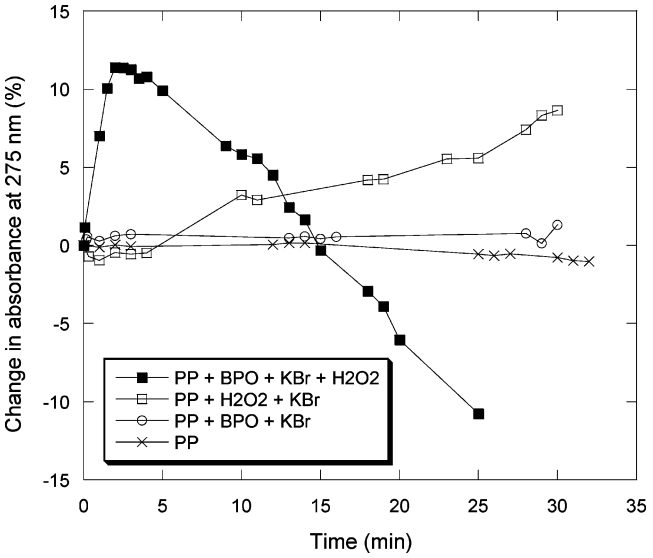


Figure 6. Shown is the change in absorbance (%) at 275 nm of a 25 μg$\times$ mL^{-1} solution of phenolic polymer as a function of time after the addition of; BPO, H_2O_2, and KBr (filled squares), H_2O_2 and KBr (open squares), and BPO and KBr (open circles). Also shown is the control experiment when no components were added to the phenolic polymer solution (crossed lines).

small absorbance peak/shoulder at 275 nm (spectra not shown), which was attributed to the aromatic nuclei present in the polymer. In Figure 6, the change in absorbance (%) at 275 nm is shown as a function of time for a few different combinations of reactants. First it can be noted that we observed a slow and steady increase in absorbance when H_2O_2 and KBr were added to the polymer solution. This can be compared with the dissipation signal that showed no changes when H_2O_2 and KBr were added (Figure 3). Thus, based on the UV/vis data, it can be concluded that PP undergoes oxidation when exposed for H_2O_2 and KBr but that is not resulting in any extensive cross-linking. Again, it was only when all components were added that any remarkable change in the absorbance at 275 nm was observed as can be seen in Figure 6. The change in absorbance at 275 nm showed similar behavior as the dissipation signal, i.e., first there was a rapid increase in absorbance that was followed by a gradual decrease in absorbance. The UV/vis experiments were repeated at least five times, and comparable results were achieved each time. Based on the UV/vis results, we are not able to rule out or propose any new mechanism of cross-linking, but it can be used as a control experiment to the QCM-D results. The most apparent difference between the two studies is that QCM-D is measuring reactions occurring at the surface but the UV/vis experiments are measuring differences occurring in solution. Thus, one explanation for the decreased UV/vis signal during cross-linking could be that when the molecular mass of the cross-linked phenolic polymer increases it will not be soluble in the buffer and precipitates. Phase separation occurs when the Gibbs free energy of mixing, $\Delta G^{mix} = \Delta H^{mix} - T\Delta S^{mix}$, is positive, and consequently, a homogeneous solution is obtained when ΔG is negative. The free energy of mixing (ΔG^{mix}) consists of one enthalpic (ΔH^{mix}) and one entropic ($T\Delta S^{mix}$) component. Flory−Huggins theory of polymer miscibility predicts that polymeric solutions are getting less miscible as the molecular mass of the components increases. Thus, during polymerization or cross-linking, the molecular mass of the components increases, and phase-separation/precipitation might occur even though the components were miscible from the start.

In this paper, we have shown the enzymatic cross-linking of a phenolic polymer extracted from *Fucus serratus*. This result strengthens the previously proposed hypothesis that phenolic polymers could be one of the components of the bioadhesive system of brown algae.[16] It should be stressed that we have not performed any studies of the adhesive properties of the phenolic polymer used in this study, but controlling the cross-linking reaction is crucial for the potential commercial use. The design idea of using components from nature when making an adhesive that can form bonds to a variety of substrates in wet and in high ionic strength environments is not new. To date, the mussel adhesive has been most mimicked and several papers discussing the preparation or synthesis, the adhesive properties, together with the cross-linking and potential use of such adhesives have been published.[40−47] The major drawback with the extraction of adhesive proteins directly from the mussel is the cumbersome procedure and the low yield of material. Furthermore, the post-translational changes of tyrosine to L-DOPA will affect the production of adhesive mimics. Therefore, synthetic routes from low molecular mass components or a considerable, easy extracted source of adhesive material are necessary. Algae can be found and harvested in large quantities but if it can be used as a raw material for a commercial bioadhesive is still very doubtful; however, the idea is very inspiring.

Conclusions

In this paper, we have demonstrated the enzymatic cross-linking of a phenolic polymer extracted from *F. serratus*. The enzyme required for the cross-linking was a vanadium-dependent haloperoxidase, i.e., bromoperoxidase (BPO), extracted from a related species *A. nodosum*. The cross-

linking was initiated by adding the BPO enzyme, KBr, and H_2O_2 to a thin layer of adsorbed phenolic polymer. It was also shown that all of the components were needed for the cross-linking to occur. Practically, this work has demonstrated the feasibility to use a phenolic polymer as a potential component of a water curable and water resistant sealant or coating.

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References and Notes

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