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Synergistic effects of metals and oxidants in the curing of marine mussel adhesive

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Abstract Marine mussels produce an impressive adhesive material for affixing themselves to rocks in the turbulent marine environment. This glue is generated by application of proteins to the surface followed by extensive cross-linking to yield the final matrix. Prior studies have shown that simple oxidation or reactivity brought about by metal ions may be key to this protein cross-linking process. Here we have explored protein cross-linking reactivity in which combinations of metals and oxidants may display synergistic effects with respect to adhesive curing. Extracted adhesive proteins were mixed with a series of metals, oxidants, and combinations thereof. In some cases, synergistic curing was observed. For example, we found that iron(II) ions with hydrogen peroxide brought about a greater degree of protein cross-linking than the sum of the individual components. These studies were performed as part of our efforts to provide perspectives on the connections between biology, chemistry, and functional materials.

Introduction

The oceans abound with a fascinating collection of unique materials, ranging from barnacle, tube worm, and oyster cements to the adhesives of sea stars, mussels, and corals. We cannot help but marvel at how these organisms design and generate such functional materials for affixing themselves to underwater surfaces. This material development must account for performance within the turbulent and harsh marine environment. We are curious to see what links exist between small-scale chemical bonds and the resulting macroscopic materials.

Perhaps the two most well studied marine biological materials are barnacle cement [1–6] and mussel adhesive [7–10]. In both cases, the material is produced by the animal depositing a mixture of proteins onto a surface. Cross-linking of the proteins then yields the final, cured matrix. The exact details of the cross-linking process and surface adhesion remain to be determined. In the case of barnacles, the proteins contain cysteine thiols that may become oxidized to disulfides for cross-linking [1–6]. Mussel adhesive proteins contain the unusual amino acid 3,4-dihydroxyphenylalanine (DOPA) [7–10]. These DOPA residues are essential for cross-linking and can comprise high levels (~10–22%) of the total protein amino acid content. Also present in mussel adhesive at remarkably high levels are transition metals such as iron and zinc [11–16].

The majority of studies related to cross-linking of mussel adhesive proteins have focused upon oxidation reactions. Oxidants such as $NaIO_4$ [17–19] and the oxidizing tyrosinase enzyme [18–21] readily react with DOPA-containing proteins, peptides, and synthetic polymers to yield cross-linked products. A recent atomic force microscopy study indicates that DOPA oxidation to the quinone may be responsible for curing the bulk material [22]. Surface adhesive bonding, by contrast, may depend upon the "reduced" (i.e., not oxidized) catechol sidechain of DOPA interacting with the animals' chosen substrate [22].

Our laboratory [23–28] and others [17, 18, 29–32] have been exploring potential roles of metals in the formation of mussel adhesive. Metal ions such as Fe^{3+} have the ability to

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both become chelated by the DOPA sidechain [25] as well as bring about DOPA oxidation [7, 33, 34]. From spectroscopic and reactivity studies, we found that the iron in mussel adhesive may be bound by three DOPA-containing protein strands [26]. Furthermore, these $Fe(DOPA)_3$ centers can react with oxygen to generate protein-based radicals [24, 26]. Such oxidized protein may go on to afford protein–protein coupling for curing or protein-surface bonding for direct adhesion.

We wondered how such chemical and bonding processes may contribute to the actual generation and performance of mussel adhesive. In particular, we were curious to see how well metals or oxidants might be able to bring about cross-linking of mussel adhesive protein. Adhesive precursor proteins were extracted from mussels prior to cross-linking [27, 28]. After precipitation from aqueous solutions by addition of an organic solvent and centrifugation, a slightly viscous protein hydrogel was obtained. We reacted this gel with a collection of potential cross-linking agents [27, 28]. The various resulting matrices were examined for materials properties employing a direct measure of curing [27, 28]. Using an Instron materials testing system, a rod was moved into the gel at constant velocity, all the while recording force (c.f., Fig. 1). This measurement of compressibility and shear properties allows rapid analysis of many samples and provides quantitative comparisons (c.f., Table 1) [27, 28]. We have recently also reported on the rheological characteristics of these hydrogels both before and after cross-linking [23].

We reacted the protein hydrogel with a collection of metals and oxidants [27, 28]. In general, we found that

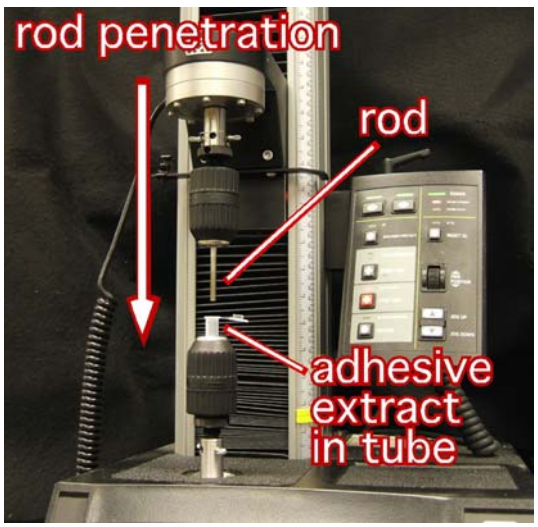

Fig. 1 Photograph of the experimental setup used for direct measurements of curing mussel adhesive extract with various reagents. A rod was run into the protein hydrogel at constant velocity while force was recorded. The sample tube in the photograph is raised for visibility

simple oxidants (e.g., H_2O_2, $Na_2S_2O_8$) cross-linked the protein, but only to a limited degree [27]. The most effective reagents were oxidizing metal ions such as Fe^{3+}, Cr^{6+} (in $Cr_2O_7^{2-}$), and Mn^{7+} (in MnO_4^-) [27]. By contrast, simple metal ions (e.g., Na^+, Co^{2+}, Ni^{2+}) did not afford any curing. For oxidizing reagents, no correlation was found between curing and reduction potentials. In general, concentration dependences were found, with higher concentrations of a given reagent generally bringing about more curing. Of the reagents examined that may typically be available to mussels, Fe^{3+} provided the most pronounced cross-linking [28]. Given the high concentrations of iron in mussel adhesive plaques [11–16], we concluded that iron may be the reagent responsible for bringing together DOPA proteins to form this material [26, 28].

In general, metal ions may react with oxidants and reductants to generate reactive species. Perhaps the most well known cases are from metalloenzymes. For example, in the cytochrome $P450_{cam}$ system, a metal ion (iron in a heme) reacts with an oxidant (O_2) and a reductant (electrons) to generate a reactive intermediate capable of oxidizing an unactivated hydrocarbon (camphor) to a product alcohol [35]. Small molecule systems are also well studied in which a metal complex (e.g., Fe EDTA) can react with an oxidant (e.g., H_2O_2) to produce reactive oxygen species (e.g., hydroxyl radical) [36, 37]. Thus metals and oxidants can react in synergistic ways to bring about chemistry unavailable to either component alone. Perhaps both metals and oxidants play a role in the curing of mussel adhesive.

We found oxidized protein, in the form of an organic radical, within fresh adhesive plaques harvested from mussels [26]. Earlier studies showed the presence of oxidase activity in the plaques [37]. Thus oxidation reactions are clearly part of the protein curing process. However, the active oxidant generated by mussels for this cross-linking has not yet been identified. The "usual suspects" list of common biological oxidants include oxidizing enzymes (e.g., tyrosinase) and the most common small molecule oxidizers found in biology such as dioxygen (O_2), hydrogen peroxide (H_2O_2), superoxide (O_2^-), and hydroxyl radicals ($OH^{\cdot}$) [38, 39]. Generally speaking, most protein cross-linking processes require the presence of oxidants [33]. With regard to mussel proteins, nearly all cross-linking studies have focused on oxidation, with periodate (IO_4^-) [17–19] and the tyrosinase enzyme [18–21] comprising the majority of work.

Another interesting issue related to oxidation within this biological material is that of metal ion transport. Prior to internalization by organisms, the majority of environmental iron is in the +3 oxidation state [40–42]. In open ocean water, for example, nearly all iron is particulate and typically in various forms of Fe^{3+} [40–42]. Mussels collect this insoluble iron in their bivalve filtration system [12–14].

The process by which these animals transport this accumulated iron to their adhesive system has not been described in detail. Generally speaking, divalent Fe^{2+} is more soluble and easier to transport than trivalent Fe^{3+} [43]. Thus most organisms accumulate Fe^{3+} and reduce the ion down to Fe^{2+} for actual use [43]. Given our results indicating that Fe^{3+}, rather than Fe^{2+}, appears to be required for mussel protein cross-linking [27, 28], we can envision a scenario in which mussels accumulate Fe^{3+} in their filters, reduce the metal to Fe^{2+} for transport to the byssal adhesive system, then reoxidize to Fe^{3+} for initiating the curing. If such processes are taking place, an oxidant is required for the final $Fe^{2+} \rightarrow Fe^{3+}$ transformation. Here we report on the ability of oxidants to influence the curing of DOPA proteins by metal ions. At this time, we can only speculate as to which oxidants might be pertinent to mussel adhesive. Thus we have focused on two: hydrogen peroxide (H_2O_2) and sodium periodate ($NaIO_4$). Hydrogen peroxide is a thermodynamically strong (E° = 1.78 V H_2O_2/H_2O) [44] but kinetically slow oxidant found in many biological systems. When examining the oxidation of biological molecules, H_2O_2 makes for an obvious choice. Our prior studies showed that H_2O_2 brings about only modest curing of mussel adhesive proteins [27, 28]. We chose $NaIO_4$ for a contrast to H_2O_2. Previously we found that this strong oxidant (E° = 1.59 V IO^{4-}/IO^{3-}) [45] cured mussel adhesive proteins appreciably, to a similar degree as Fe^{3+} [27]. This reagent has also been popular with in vitro studies of mussel adhesive protein cross-linking [17–19]. As will be seen below, synergistic curing effects of metals and oxidants were found. These results may provide insights on how mussels use chemical reactivity to generate their adhesive material.

Experimental

Protein extractions

Mussel adhesive protein was extracted from the animals according to a literature method [46]. This extraction method relies upon acid precipitation of most biomolecules while leaving the DOPA-containing proteins in solution [46]. Subsequent removal of the proteins from solution, using an organic solvent precipitant, yields a gel containing two of the protein variants, with a relative composition of ~80% Mefp-1 and ~20% Mefp-2 [46]. After precipitation of these DOPA proteins from solution with acetone, centrifugation yielded hydrogel pellets [46]. The pellets were stored in a 4 °C cold box under a blanket of deionized water until used, within 7 days of extraction. Roughly 85 pellets yielded enough sample to complete one set of penetration tests. The pellets were placed in a plastic strainer with 3.5 mm holes. Using a ceramic container with a diameter slightly smaller than the strainer, the pellets were forced through the strainer for homogenization, while chilled with ice. Deionized water was added to the strained pellets by the following steps: strained pellets were transferred to a tared 100 mL beaker, mass was recorded and multiplied by 1/3. This resulting value was the amount of deionized water added in grams to the beaker of strained pellets such that the final amount of water in the mixture was 25% w/w. The strained pellets and water were thoroughly mixed with a spatula to obtain a homogenous mixture. This mixture was then transferred in 10–11 g increments to a 10 mL plastic syringe with a 2 mm opening used to deposit the homogenous material into plastic 2 mL microcentrifuge tubes (9 mm in diameter, 35 mm deep). During the transfer process, the mixture remaining in the beaker was kept on ice and covered with Parafilm until placed in the syringe. The tube was capped and tapped on the bench 6–7 times to eliminate air bubbles and then placed on ice until all of the mixture was dispensed. The sample tubes were covered with aluminum foil and stored in a 4 °C cold box until used, within 7 days of extraction.

Preparation of cross-linking reagent solutions

For all studies discussed below, the final concentration of cross-linkers were 45 mM. This concentration was chosen to be consistent with our prior studies [27, 28] as well as close to that of the natural abundance in mussel adhesive. Total iron in mussel adhesive plaques is approximately one part per thousand [11, 12] or about 20 mM when converted to solution concentrations. To obtain a final cross-linker concentration of 45 mM in the gel, 1 M solutions of the desired reagents were prepared fresh daily. In previous work, 0.5 M solutions were used [27, 28], but experiments discussed below require a 1 M solution to compensate for the dilution that will occur upon addition of two reagents (metal and oxidant) instead of only one. To prepare solutions, the calculated amount of reagent required for a 1 M solution in 5 mL volume was weighed out and placed in a 10 mL volumetric flask. After placing all compounds in individual flasks, 5 mL of deionized water was added and the flask vortexed to aid dissolution. For solutions that did not completely dissolve with mixing alone, mild heat was applied by hotplate for 7–10 min. The heated solutions included $NaIO_4$, $ZnCl_2$, $Mn(OOCCH_3)_3$, CuCl, and TiF_3. All solutions were transferred to 15 × 45 mm screw thread glass vials and capped to prevent any evaporation. For reagents where solubility did not allow a 1 M concentration, saturated solutions were used instead. The saturated solutions included $NaIO_4$, $Mn(OOCCH_3)_3$, CuCl, and TiF_3. All water used in this study was purified to at least 18 MΩ/cm using a Barnstead Nanopure Infinity system. In our

prior report on cross-linking hydrogel extracts, we found the chloride and nitrate salts of a given metal ion yielded identical cross-linking results [27]. Thus only one salt of a given ion was examined here.

Cross-linking reactions

Into each sample tube was dispensed 1.00–1.05 g of the homogenized adhesive protein extract. Then 50 μL of a 1 M non-metal oxidant solution was added followed by a 50 μL solution of a 1 M metal salt. The reagents were added to the sample tube by 100 μL glass syringes. For controls, 100 μL of deionized water was added to the tube. When adding reagent combinations, the non-metal oxidant was consistently added first. In the case of combining a metal and water, the water was added first. Each sample tube was mixed thoroughly with a microspatula for 5–6 s. The spatula was then scraped on the inside rim of the tube to return any mixture that stuck back into the sample tube. Each tube was capped and tapped on the bench 6–7 times to eliminate air bubbles. The sample was then allowed to react at room temperature for one hour.

Direct measurements of curing

A penetration test [47–51] was used to measure the effect of various reagent combinations on cross-linking. A 5 mm diameter rod (blank drill bit) was run into the gel mixtures at constant velocity, all the while measuring force on the rod. Previously we used a 3.5 mm rod, thus data in this current study yields higher penetration force values [27, 28]. The theory behind this mechanical test predicts that linear relationships will be found between penetration depth and force [48, 49]. Some deviations from this expected linearity will be seen below and are a result of inhomogeneity in the samples resulting from cross-linking. The starting protein hydrogel is homogeneous, as are many of the cross-linked products. Extreme levels of cross-linking, however, can yield hardened solids suspended in solutions. Thus the materials no longer behave ideally. We discussed this phenomenon in our recent paper in which we examined the rheological properties of some cross-linked protein gels [23].

Figure 1 shows the experimental setup used and the Instron 5544 materials testing system fitted with a 2 N load cell. Protein gel sample remained in the 2 mL plastic tubes and were secured in a chuck grip fastened to the base of the instrument. Similarly, the 5 mm rod was held in place using a chuck grip attached to the load cell. Prior to collecting data, the rod was positioned ~3–4 mm centered above the extract. Each sample was penetrated to a depth of 20 mm at a rate of 20 mm/min. Load data were collected every 0.5 mm. Typical data can be seen below, in Figs. 2–4.

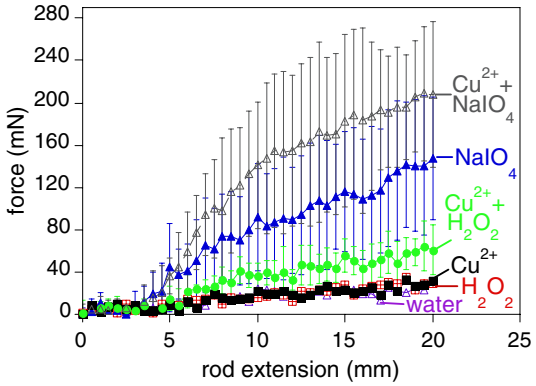

Fig. 2 Extension versus force data for a rod penetrating into a protein hydrogel extracted from marine mussels. The protein gel was reacted with water, Cu^{2+}, H_2O_2, $NaIO_4$, Cu^{2+} + H_2O_2, and Cu^{2+} + $NaIO_4$. Higher force indicates greater cross-linking of the protein. Each trace is averaged from multiple runs. Error bars show one standard deviation for a given data point

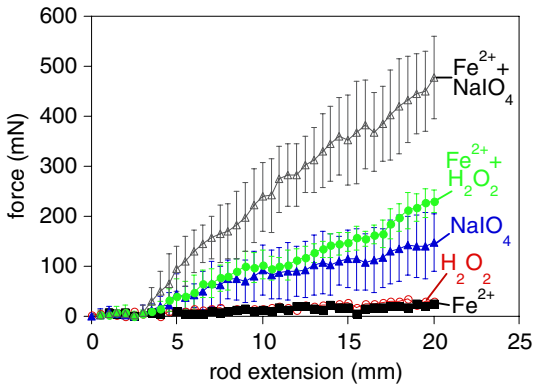

Fig. 3 Penetration plots for the mussel adhesive protein gel reacted with Fe^{2+}, H_2O_2, $NaIO_4$, and combinations thereof. Each trace is averaged from multiple runs and error bars are shown with one standard deviation

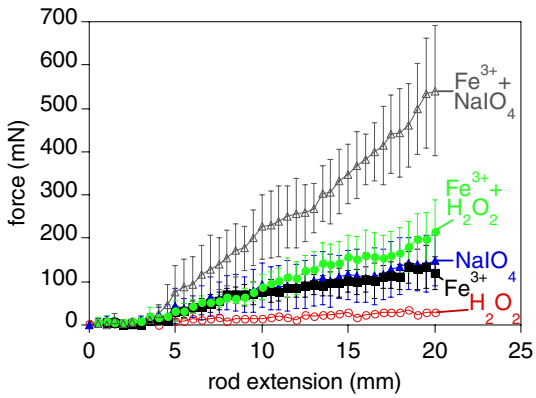

Fig. 4 Penetration plots for the mussel adhesive protein gel reacted with Fe^{3+}, H_2O_2, $NaIO_4$, and combinations thereof. Each trace is averaged from multiple runs and shows error bars of one standard deviation for each point

Table 1 Penetration forces in mN observed for an extract of mussel adhesive proteins mixed with various combinations of reagents

Reagent	Water	*n*	H_2O_2	*n*	$NaIO_4$	*n*
Water	30 ± 12	32	34 ± 20	10	144 ± 57	10
NaCl	31 ± 16	5	19 ± 2	4	113 ± 54	5
$CaCl_2$	22 ± 9	5	38 ± 1	4	126 ± 38	5
$ZnCl_2$	27 ± 12	5	42 ± 12	5	180 ± 42	5
$AlCl_3$	22 ± 15	5	29 ± 9	5	146 ± 51	5
$Ga(NO_3)_3$	23 ± 10	6	35 ± 10	5	322 ± 89	5
$CoCl_2$	28 ± 16	5	24 ± 8	5	111 ± 4	4
TiF_3	24 ± 9	5	27 ± 9	5	211 ± 38	5
CuCl	30 ± 11	5	32 ± 6	5	221 ± 66	5
$CuCl_2$	29 ± 9	5	65 ± 22	5	215 ± 61	5
VCl_3	24 ± 10	6	77 ± 13	4	392 ± 79	4
Na_3VO_4	1201 ± 638	5	741 ± 264	5	1201 ± 619	5
Na_2Cr_2O7	1563 ± 664	5	630 ± 550	5	1859 ± 983	6
$MnCl_2$	26 ± 13	6	41 ± 12	5	626 ± 192	5
$Mn(OOCCH_3)_3$	25 ± 9	5	23 ± 7	5	147 ± 42	5
$KMnO_4$	887 ± 319	6	490 ± 251	5	1646 ± 665	6
$FeCl_2$	28 ± 7	5	222 ± 25	4	478 ± 83	4
$FeCl_3$	134 ± 47	5	213 ± 63	5	560 ± 137	5

Each value is an average of "*n*" runs and one standard deviation is shown

Reported maximum loads were taken at a final penetration depth of 20 mm. For a given experimental run on a given day, every potential cross-linking reagent or combination of Table 1 was examined. The final force values shown for a given entry in Table 1, below, were averaged from five different runs each performed on five different days. Outlying data points were removed if they failed a Q test at 90% confidence level. Reported errors denote one standard deviation. The number of samples used for each data point is provided in Table 1. When comparing the averaged data in the "Discussion" section, standard *t*-tests were performed on the raw data, thereby yielding probabilities that a given pair of samples showed the same curing.

Results

Curing data

In order to provide a context for the data obtained, we start the discussion with controls. Water added to the adhesive extract showed a penetration force of 30 ± 12 mN. Hydrogen peroxide, alone, did not exhibit curing, with a force of 34 ± 20 mN. On its own, cross-linking was found for $NaIO_4$ at 144 ± 57 mN. Figure 2 shows a penetration test with water, H_2O_2, and $NaIO_4$ each added to separate extracts. As can be seen, there is no appreciable difference between the water control and H_2O_2. Thus Figs. 3 and 4 will not show water traces in the interest of clarity. Below we provide the effects of cross-linking with each of these oxidants in combination with various metal ions. All curing data are shown in Table 1. We will present the data starting with low levels of curing and progress to higher degrees of cross-linking.

Non-redox active metal ions

For the Na^+ ion, from NaCl, no difference was observed for protein cross-linking (31 ± 16 mN) versus the water control (30 ± 12 mN), as is seen in Table 1. Addition of H_2O_2 provided no increase (19 ± 2 mN) and $NaIO_4$ was higher (113 ± 54 mN) but no more so than $NaIO_4$ alone (144 ± 57 mN). Similar results were found for $CaCl_2$ and $ZnCl_2$. Aluminum(III) is often used, along with Ga^{3+}, to mimic the coordination chemistry of Fe^{3+} but without the available redox chemistry. Aluminum(III) alone (22 ± 15 mN) or with H_2O_2 (29 ± 9 mN) showed no curing nor did Al^{3+} with $NaIO_4$ (146 ± 51 mN) beyond that of the individual components. Gallium(III), however, appeared different. Alone Ga^{3+} (23 ± 10 mN) or with H_2O_2 (35 ± 10 mN) exhibited no notable effect. When combined with $NaIO_4$, hardening from Ga^{3+} (322 ± 89 mN) was near double that of the individual components summed together (144 + 23 = 167 mN).

Redox active metal ions

Cobalt has redox activity available, with the Co^{2+} and Co^{3+} ions most common. Results found for Co^{2+} were similar to that of Na^{+}, with Co^{2+} alone (28 ± 16 mN), mixed with H_2O_2 (24 ± 8 mN), or $NaIO_4$ (111 ± 4 mN) no greater than any of the controls. Titanium also has multiple oxidation states available (e.g., 2+, 3+, 4+). For this study we chose a Ti^{3+} salt based upon prior experience showing that TiF_3 is easiest to work with in water, minimizing immediate formation of TiO_2 powder [52]. Titanium(III) alone (24 ± 9 mN) or with H_2O_2 (27 ± 9 mN) did not cure the adhesive extract to any significant degree. With both Ti^{3+} and $NaIO_4$ the observed curing was high (211 ± 38 mN) although still close to the sum of the individual components (24 + 144 = 168 mN).

Given that the copper-containing tyrosinase enzymes can bring about oxidation of substrates such as tyrosine, DOPA, phenols, catechol [53], we were curious to see about potential cross-linking from copper in conjunction with oxidants. Inorganic copper can also catalyze the oxidation of small molecules such as catechol [54]. The Cu^{+} salt CuCl provided data similar to the TiF_3 case-little curing alone (30 ± 11 mN) or with H_2O_2 (32 ± 6 mN). Periodate addition may have provided extra curing (221 ± 66 mN) relative to each reagent on their own (30 + 144 = 174 mN), although the difference was not great. Curing from the Cu^{2+} ion alone (29 ± 9 mN) was insignificant and when combined with $NaIO_4$ (215 ± 61 mN) was high, but not conspicuously so. For Cu^{2+} and H_2O_2 together (65 ± 22 mN), the observed penetration force was no greater than the sum of the components (29 + 34 = 63), but was higher than any of the reagents mentioned so far with added H_2O_2. These data can be seen in both Fig. 2 and Table 1.

Vanadium(III), similarly, did not cure alone (24 ± 10 mN) or with H_2O_2 (77 ± 13 mN) much beyond the sum of each separate reagent (24 + 34 = 58 mN). A mild synergistic effect was observed with V^{3+} and $NaIO_4$ at 392 ± 79 mN. On its own, Na_3VO_4 cured the protein extract substantially (1201 ± 638 mN), consistent with prior observations of V^{5+} being a potent cross-linker [27]. Periodate did not contribute to curing (1201 ± 619) nor did H_2O_2 (741 ± 264). We also reported previously that Cr^{6+}, in the dichromate form $Cr_2O_7^{2-}$, is one of the strongest curing agents we have found to date (1563 ± 664 mN) [27]. Neither H_2O_2 (630 ± 550 mN) nor $NaIO_4$ (1859 ± 983 mN) enhanced the Cr^{6+} reactivity significantly. Indeed, like what was found with V^{5+}, the metal with H_2O_2 actually showed less curing than the sum of the two parts. However, such decreases may be a result of limitations in the methods employed here (vide infra).

We examined the cross-linking afforded by three manganese ions, Mn^{2+}, Mn^{3+}, and Mn^{7+} (Table 1). Starting with Mn^{2+} (26 ± 13 mN) and H_2O_2 combined (41 ± 12 mN) no detectable effect was noted. Pronounced synergistic curing, however, was seen with both Mn^{2+} and $NaIO_4$ together (626 ± 192 mN). By contrast, Mn^{3+} (25 ± 9 mN) did not cure any more effectively when H_2O_2 (23 ± 7 mN) or $NaIO_4$ (147 ± 42 mN) were added, relative to the oxidant-only solutions. As shown in Table 1, Mn^{7+} brought about dramatic curing (887 ± 319 mN). No enhancement was found with H_2O_2 (490 ± 251 mN) but a doubling was seen with Mn^{7+} and $NaIO_4$ (1646 ± 665 mN).

Spectroscopic studies from our laboratory have implicated Fe^{3+} in the curing of mussel adhesive [26]. Thus we were particularly interested to explore possible influences of oxidation upon iron-induced cross-linking of adhesive proteins. Although Fe^{2+} did not yield any curing alone (28 ± 7 mN), in combination with H_2O_2 much greater penetration forces were required (222 ± 25 mN) as can be seen in Fig. 3. Further enhancement of curing was found with Fe^{2+} and $NaIO_4$ (478 ± 83 mN). Alone, Fe^{3+} cured the protein significantly (134 ± 47 mN) and this effect increased to a small degree when combined with H_2O_2 (213 ± 63 mN). Both Fe^{3+} and IO_4^-, together, showed synergistic cross-linking (560 ± 137 mN) greater than that with Fe^{3+} and H_2O_2, presented in Fig. 4. Thus oxidants do enhance the curing ability of iron. Also worth noting is that, of all the metal ions examined here, only Fe^{2+} displayed a dramatic enhancement of curing with H_2O_2 (Fig. 3; Table 1).

Visual observations of curing

The starting gel had a light tan color, a mostly thin and liquidy consistency, with no clumping. To make a food analogy, the material began somewhat like apple sauce. We have reported on the rheological properties of this protein extract, both before and after cross-linking [23]. In general, if the numbers of Table 1 indicate little or no curing (e.g., ~80 mN and less), no changes in color or consistency were found except for added color in the following cases: $CuCl_2$ + H_2O_2 (light brown), $CoCl_2$ + H_2O_2 (slight pink), VCl_3 + H_2O_2 (grey), and Mn^{3+} + $NaIO_4$ (rose). Hydrogen peroxide brought about no observable changes on its own. For $NaIO_4$, alone, the protein gel took on a pinkish-brown color with no conspicuous change in consistency. Likewise, the metal ions and $NaIO_4$ with curing under ~220 mN and lower, along with Ga^{3+} + IO_4^-, were pinkish-brown. Reactions with color changes, but without major viscosity increases were $FeCl_3$ (black), $FeCl_3$ + H_2O_2 (dark brown), $FeCl_2$ + H_2O_2 (dark brown), and TiF_3 + $NaIO_4$ (reddish brown). The latter two samples separated

somewhat, with a darker color solid residing below a lighter colored solution above.

The powerful oxidant $Na_2Cr_2O_7$, alone or with H_2O_2, generated a yellow-tan extremely viscous gel that stuck to the spatulas when handled. Addition of $NaIO_4$ formed an orange-tan solid with a yellow solution above. For $KMnO_4$, alone or with H_2O_2, a colorless solution was found to reside above a black gel containing tan flecks. With $KMnO_4$ and $NaIO_4$, the clear solution was present above a purple, then red solid. In all cases, the increase in viscosity was immediate and extreme. With Na_3VO_4, alone, also a strong oxidant and cross-linker, a brown and then dark green, very viscous and sticky gel was noted with a clear solution above. Similar results were found with Na_3VO_4 + $NaIO_4$ and VCl_3 + $NaIO_4$. A combination Na_3VO_4 and H_2O_2 showed a pink to yellow to green to brown to yellow–brown transformation within 20 s, also producing a viscous gel with a light yellow solution above. Combined with $NaIO_4$, $MnCl_2$ showed a substantial increase in viscosity, a red-brown gel, and a colorless solution above. Both $FeCl_2$ and $FeCl_3$ mixed with $NaIO_4$ yielded viscous yellow–brown–red gels.

Discussion

Comparisons with prior data

The general trends of curing reported here agree with our earlier data [27, 28]. For example, H_2O_2 cross-linking is minimal and that of IO_4^- and Fe^{3+} are appreciable. Some specific changes, however, could be noted in that reagents such as H_2O_2 showed higher force values here (34 ± 20 mN) than earlier (20 ± 4 mN). These differences can be attributed to incremental improvements we made to our test procedure. The most conspicuous change was use of a 5 mm penetration rod here versus a 3.5 mm rod previously. Thus the force data tend to now be higher. Other changes in the procedure worth noting include extracting the protein from mussel feet collected in a different season and a slightly different method of mixing reagents into the gel extract. Previously we massed the pellets, strained them to homogeneity, and added water for normalization of volumes based upon the pre-strained mass. Here we avoided variations from loss in the strainer by using the post-strained mass for all calculations [27].

The only significant observed variation from our previous work is that of Mn^{3+} alone. Here curing was not observed for this ion, but previously it was. Earlier we noted a similar darkening and thickening of the sample for both $Mn(OOCCH_3)_3$ and $KMnO_4$. As will be discussed below, Mn^{2+} + $NaIO_4$ curing may be a result of initial oxidation to Mn^{7+}. Beyond possible Mn^{7+} impurities in the earlier starting material, at this time we do not have a satisfactory explanation for previously observed Mn^{3+} cross-linking. Also earlier we noted that Ca^{2+} (21 ± 1 mN) cured slightly versus the water control (14 ± 3). Here Ca^{2+} (22 ± 9 mN) was not notably different from the water control (30 ± 12 mN). In both cases the differences were small. According to a Student *t*-test, there is a 21.5% probability of the Ca^{2+} and water control results being the same.

Potential curing synergy between metals and oxidants

The V^{3+} ion with IO_4^- (392 ± 79 mN) cured significantly more than either component alone (24 ± 10 and 144 ± 57 mN), thereby indicating the presence of synergistic curing. Only a 1.5% probability exists that V^{3+} with IO_4^- cured to the same degree as IO_4^- alone, according to a Student *t*-test. Hydrogen peroxide did not provide an analogous effect with V^{3+}. By contrast, the V^{5+} ion did not enhance curing when H_2O_2 or $NaIO_4$ were added. However, V^{5+} curing was very pronounced (1201 ± 638 mN). As noted above, the extreme cross-linking yielded separation between the solution and solid, thereby making measurements of the homogeneous gel difficult. Thus a limit of our method may be found here. Were enhanced cross-linking to occur by addition of an oxidant, such reactivity may not be observable. This separation of solid and solution also became problematic when we measured the rheological properties of protein gels cross-linked at the extreme limits [23]. The current lack of observed curing enhancement with oxidant addition to V^{5+} does not necessarily mean such an enhancement does not exist.

Curing observed from VCl_3 + $NaIO_4$ may be a result of partial $V^{3+} \rightarrow V^{5+}$ oxidation brought about by the periodate. The resulting V^{5+} could then cross-link in a manner analogous to that shown for Na_3VO_4 alone. Prior work showed no reactivity from V^{4+}. Oxidation of V^{3+} to V^{5+} is thermodynamically uphill by ~1.3 V, based upon the sum of reduction potentials for the VO^{2+}/V^{3+} and $HV_2O_5^-/HV_2O_7^{3-}$ couples [44, 55]. Reduction of IO_4^- occurs at a comparable potential, at 1.59 V for IO_4^-/IO_3^- [45], thereby indicating such $V^{3+} \rightarrow V^{5+}$ oxidations are feasible. The degree of cross-linking from V^{3+} and $NaIO_4$ (392 ± 79 mN) was less than that of V^{5+} alone (1201 ± 638 mN, same as V^{3+} and $NaIO_4$ at 4.5%) indicating that if such metal-based oxidation took place, the reaction did not go to completion. Alternatively, a more complex cross-linking mechanism, possibly incorporating enzyme catalysis principles, could be at play. We have tested the ability of oxidizing enzymes to cure this adhesive protein abstract [27]. The lack of observed hardening with enzymes may have been a result of the enzymes having

poor access to substrate when suspended in the viscous gelatinous extract [27].

For the manganese ions, Mn^{7+} exhibited curing at the high extreme, characteristic of oxidizing metal ions. No additional curing was noted with H_2O_2 or $NaIO_4$ although that from Mn^{7+}, alone, may be approaching the limit of what is observable. Manganese(III) did not cure beyond the appropriate control reactions. By contrast, Mn^{2+} provided some interesting results. Alone (26 ± 13 mN) or with H_2O_2 (41 ± 12 mN) Mn^{2+} induced no cross-linking. An obvious synergistic effect was detected when $NaIO_4$ was added (626 ± 192 mN), with only a 0.7% probability of this hardening being the same as $NaIO_4$ alone. With no curing for Mn^{3+} or Mn^{4+} ions, oxidation of Mn^{2+} to Mn^{3+} or Mn^{4+} cannot be involved. Oxidation of Mn^{2+} all the way up to Mn^{7+}, at –1.51 V, is thermodynamically possible [44] with a strong 1.59 V (for IO_4^-/IO_3^-) oxidant such as $NaIO_4$ [45]. Thus the curing seen for Mn^{2+} + $NaIO_4$ may simply be a result of partial oxidation to Mn^{7+}. However, a lack of added curing for Mn^{3+} with $NaIO_4$ implies that this ion was not brought to Mn^{7+}. A more complex interplay between metal, oxidant, and protein may well be at work here.

Perhaps the most unexpected observation found is that of enhanced cross-linking for Ga^{3+} (23 ± 10 mN alone) with added IO_4^- (322 ± 89 mN). This apparent enhanced curing of Ga^{3+} and IO_4^- has a 10.2% probability of being the same as only IO_4^-. The Ga^{3+} ion is inert to redox chemistry except under extreme conditions. Typically, when such an ion binds to oxidizable ligands such as thiolates, electron density is removed from the ligand and placed onto the metal ion, thereby making the ligand more difficult to oxidize. Thus we might expect no major enhancement of Ga^{3+}-induced curing with an added oxidant. A contrasting case may occur when a redox active metal ion such as Fe^{3+} binds to a redox active ligand. Metal reduction and ligand oxidation can take place. If such oxidation of the ligand takes place within the framework of a DOPA protein, for example, cross-linking may then begin. At this time we have no suitable explanation for the Ga^{3+} + $NaIO_4$ result.

In terms of potential synergistic curing when both a metal and oxidant are present, Fe^{2+} provides a nice example. Figure 3 shows that the combination of $FeCl_2$ and H_2O_2 at 222 ± 25 mN at 20 mm extension is clearly greater than the simple sum of Fe^{2+} (28 ± 7 mN) and H_2O_2 (34 ± 20 mN). The likelihood of overlap between these values is only 0.1%. Similarly Fe^{2+} and $NaIO_4$ at 478 ± 83 mN is enhanced significantly over only Fe^{2+} and $NaIO_4$ summed (28 + 144 = 172 mN), with only a 0.6% probability of the values being coincident. These oxidants may start by bringing about an $Fe^{2+} \rightarrow Fe^{3+}$ reaction. We know that Fe^{3+} is an effective cross-linker (Fig. 4; Table 1). Indeed, the cross-linking of Fe^{3+} + H_2O_2 (213 ± 63 mN) is nearly identical to that of Fe^{2+} + H_2O_2 (222 ± 25 mN), with a 33.6% chance of the values being identical. Also similar, at 93.4% probability, are Fe^{3+} with $NaIO_4$ (560 ± 137 mN) and Fe^{2+} with $NaIO_4$ (478 ± 83 mN), shown in Fig. 4. However, the high degree of curing found with Fe^{3+} and $NaIO_4$ cannot be easily attributed to simple oxidation of the metal center. Thus a synergistic effect of protein cross-linking appears to be present.

Although Fe^{3+}, alone, may be a more effective curing agent than Fe^{2+}, the aqueous solubility of Fe^{2+} is generally greater. Iron in marine environments is readily available at ~10 parts per billion [56], predominantly existing as Fe^{3+} and predominantly as insoluble, particulate species [40–42]. Mussels are known to collect iron from sea water with their bivalve filtration system, accumulating high concentrations in their filter [12, 13]. How this particulate iron transfers to the adhesion system is not known. If the animal were to internalize the metal for transport, reduction to more soluble Fe^{2+} may be required [57]. Our results here show that extrusion of only this Fe^{2+} along with the adhesive proteins would not bring about curing and adhesive production. If mussels apply oxidants along with the Fe^{2+} and protein, oxidation to Fe^{3+} could then result, thereby initiating the adhesive curing process. Such a mechanism is only speculation at this stage. However, we [26] and others [37] have found oxidative activity in adhesive plaques.

Conclusions

Mussels are among the rich collection of marine species that use clever and unique chemistry for the construction of macroscopic structures. The results shown above are presented as part of our efforts to describe the links between biology, chemistry, and materials. Combinations of metals and oxidants do appear to exhibit synergy with respect to adhesive curing. We find that the effects of some metal-induced cross-linking is enhanced with added oxidants to degrees greater than the sum of the individual components. Metal concentrations in mussel adhesive are quite high and, perhaps, can generate all the cross-linking needed by the animal. Metal-induced cross-linking by metals such as iron requires the oxidized ion (i.e., Fe^{3+} and not Fe^{2+}). However the reduced forms are often easier to transport in biological contexts [57]. Perhaps oxidants enhance the curing ability of metal ions by generating sufficient concentrations of the particular ion most needed for materials generation. Oxidants could be in the form of small molecules such as H_2O_2 or even enzymes. These results raise the possibility that mussels may use a combination of metals and oxidants to generate their impressive adhesive material.

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