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Specificity of metal ion cross-linking in marine mussel adhesives

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In an effort to understand the formation of marine bioadhesives, mussel protein extracts were cured with various reagents and the enhanced cross-linking ability of Fe^{3+} was found.

Barnacle shells, kelp adhesives, mussel glues, and oyster cements are examples of biological materials found in the oceans.[1,2] A common theme in the synthesis of these materials is the cross-linking of biopolymers to yield a final, hardened matrix.[1–3] To date, the key cross-linking agents responsible for such material construction remain to be identified. For the common blue mussel, *Mytilus edulis*, adhesive production is based upon surface application and cross-linking of proteins to form a plaque which tethers the animal to a substrate.[1–3] These proteins are high in the unusual amino acid 3,4-dihydroxyphenylalanine (DOPA).[1–3] Another interesting property of mussel adhesives is a transition metal content (*e.g.* copper, iron, manganese, and zinc)[4,5] up to 100,000 times that of open ocean waters.[6] However, more specific detail on the bonding in this material is lacking. We are investigating the potential role of metals in cross-linking and biomaterial formation. To this end, we now show that metal ions bring about curing of extracted adhesive proteins with marked specificity.

Owing to the intractability of cured mussel glue, pliable adhesive precursors are collected from the animal prior to surface application and cross-linking. These adhesive precursors are extracted from excised feet of *M. edulis* (pellet of acetone-precipitated protein)[7] and homogenized with water. Soluble proteins isolated from this gel contain high levels of DOPA residues,[7] an essential component to protein cross-linking in mussel glues.[1–3] One gram of the gelatinous protein extract was mixed with 100 μL of various 0.5 M solutions to yield final reagent concentrations of 45 mM. Total reaction time was one hour. Compression and shear properties of the resulting materials were queried by measuring the force required to move a rod through the reaction products at a constant velocity.[8,9] An Instron 5544 Materials Testing System was used for these measurements with a 3.5 mm diameter rod and a penetration rate of 20 mm min^{-1}. The ability of each sample to resist the penetration force was recorded at a final rod extension of 20 mm. Samples were held in plastic microcentrifuge tubes (9 mm inner diameter × 35 mm long). With curing and hardening of the extract, an increased force is required to move a rod through the material at a constant velocity.[8,9] Where commercially available, both the chloride and nitrate salts of a given metal ion were studied. Nitrate salts of Na^+, K^+, Mg^{2+}, Ca^{2+}, Cr^{3+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Al^{3+}, and Ga^{3+} as well as chloride salts of Na^+, K^+, Mg^{2+}, Ca^{2+}, V^{3+}, Cr^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^+, Cu^{2+}, Zn^{2+}, and Al^{3+} were examined. Further investigations were performed with $VOSO_4$, HNO_3, HCl, $HClO_4$, H_2O_2, and *tert*-butyl hydroperoxide (BHP). Data provided for a given reagent are the average of at least four runs.

Results from the penetration tests are summarized in Fig. 1. No significant differences were seen between the chloride and nitrate salts of any specific metal ion. Thus the force values presented for these ions are averages from both salts. A striking observation is that the protein extract containing Fe^{3+} yielded the highest resistance to penetration. Clearly, Fe^{3+} brings about the greatest degree of cross-linking within the extracted adhesive precursor. The majority of metal ions tested do not bring about any curing beyond that of the water control. The level of hardening induced by Ca^{2+}, Cu^{2+}, H_2O_2, and BHP are slightly greater than that of the water control, but not nearly as high as that found for Fe^{3+}. These data indicate that, of the transition metal ions most prevalent in mussel adhesive plaques, Fe^{3+} plays the greatest role in cross-linking.

Given the unique ability of Fe^{3+} to cross-link the adhesive precursor, we investigated the effects of this ion in greater detail. Fig. 2 shows the penetration force *versus* rod extension plots for adhesive extract reacted with $Fe(NO_3)_3$ at various final concentrations. Cross-linking of the adhesive precursor induced by Fe^{3+} occurs in a concentration dependent manner.

The high affinity of Fe^{3+} for catechol, DOPA, and similar ligands is well established.[10] Ferric ions are also capable of oxidizing catechol to the corresponding quinone.[11,12] In contrast to the ferric ion, Fe^{2+}, Al^{3+}, and Ga^{3+} bring about no protein curing, thereby suggesting an oxidative role for Fe^{3+} in the formation of this adhesive. Data on cross-linking brought about by the oxidants H_2O_2 and BHP, however, suggest that simple oxidation is not suitable for efficient curing. Ferric ions may provide an ideal combination of ligand binding and oxidation to yield protein–iron–protein cross-links.

Recent spectroscopic (*e.g.*, EPR, IR, UV-vis) and reactivity studies performed in our laboratory on glue isolated from live mussels, extracted adhesive protein, and peptide models

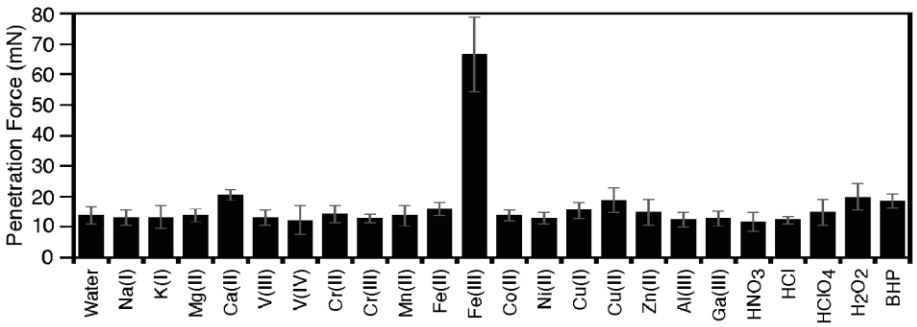

Fig. 1 Average penetration forces found for mussel adhesive precursor extract cured with various reagents. Higher force indicates a greater degree of cross-linking.

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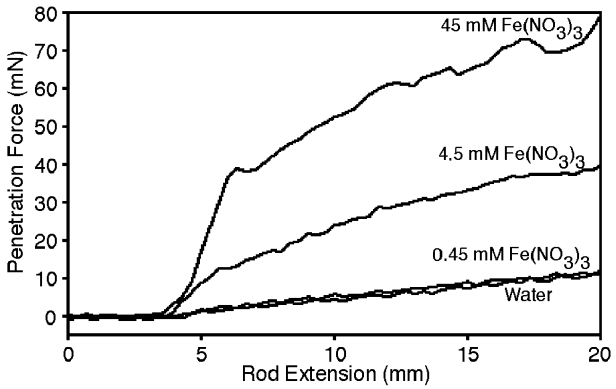

Fig. 2 Penetration force *vs*. extension plots for mussel adhesive precursor extract cured with $Fe(NO_3)_3$ at various concentrations.

implicate DOPA chelation of Fe^{3+} for the genesis of protein–protein interactions.[13] Formation of $Fe(DOPA)_3$ cross-links precede protein oxidation and further curing of mussel glues.[13] When such spectroscopic results are combined with the current materials curing data, a strong case is found for metal ion cross-linking in marine mussel glues. Perhaps the development of a mussel adhesive apparatus, based on protein cross-linking, evolved along with the animal's ability to concentrate oxidizing metals from surrounding waters. Our results demonstrate that Fe^{3+} is likely to be the key reagent used by mussels for adhesive production, as shown in Fig. 3.

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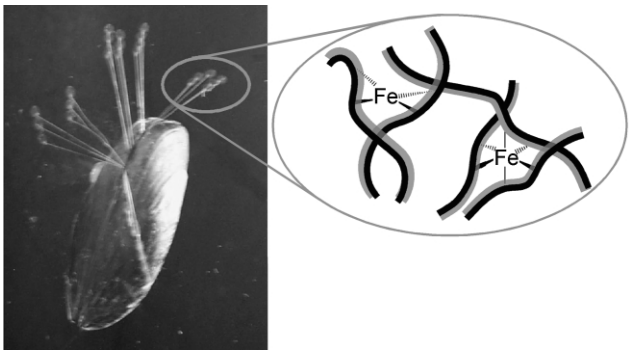

Fig. 3 Proposed iron–protein cross-linking in marine mussel adhesive plaques.

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