4-15-2013

The Relative Contribution of Calcium, Zinc and Oxidation-Based Cross-Links to the Stiffness of Arion Subfuscus Glue

Andrew M. Smith
Ithaca College, asmith@ithaca.edu

The Journal of Experimental Biology 216, 1475-1483

doi:10.1242/jeb.077149

RESEARCH ARTICLE

The relative contribution of calcium, zinc and oxidation-based cross-links to the stiffness of *Arion subfuscus* glue

M. Braun, M. Menges, F. Opoku and A. M. Smith*

Department of Biology, Ithaca College, Ithaca, NY 14850, USA

*Author for correspondence (asmith@ithaca.edu)

SUMMARY

Metal ions are present in many different biological materials, and are capable of forming strong cross-links in aqueous environments. The relative contribution of different metal-based cross-links was measured in the defensive glue produced by the terrestrial slug *Arion subfuscus.* This glue contains calcium, magnesium, zinc, manganese, iron and copper. These metals are essential to the integrity of the glue and to gel stiffening. Removal of all metals caused at least a 15-fold decrease in the storage modulus of the glue. Selectively disrupting cross-links involving hard Lewis acids such as calcium reduced the stiffness of the glue, while disrupting cross-links involving borderline Lewis acids such as zinc did not. Calcium is the most common cation bound to the glue (40 mmol l^{-1}), and its charge is balanced primarily by sulphate at 82–84 mmol l^{-1}. Thus these ions probably play a primary role in bringing polymers together directly. Imine bonds formed as a result of protein oxidation also contribute substantially to the stiffness of the glue. Disrupting these bonds with hydroxylamine caused a 33% decrease in storage modulus of the glue, while stabilizing them by reduction with sodium borohydride increased the storage modulus by 40%. Thus a combination of metal-based bonds operates in this glue. Most likely, cross-links directly involving calcium play a primary role in bringing together and stabilizing the polymer network, followed by imine bond formation and possible iron coordination.

Key words: adhesion, glue, gel, oxidation, metal, coordinate covalent, gastropod, slug, *Arion subfuscus*.

Received 9 July 2012; Accepted 7 December 2012

INTRODUCTION

Recent work on biological adhesives has identified a number of different cross-linking mechanisms that can contribute to the performance of glues. One theme that has emerged is that there can be multiple types of intermolecular interactions in a single secretion. Barnacle cement (Kamino, 2006; Dickinson et al., 2009) and the mussel byssus (Sagert et al., 2006) are excellent examples of complex adhesive structures that depend on a combination of different polymers. There may be several different cross-linking mechanisms operating within each glue. Furthermore, some interactions contribute to cohesive strength, while others contribute to adhesion. Finally some of the proteins in the glue may be more important in creating a protective coating (Harrington et al., 2010), or fighting degradation (Kamino, 2006). This complexity has made it challenging to create biomimetic adhesives.

The adhesive secretion of the slug *Arion subfuscus* (Draparnaud 1805) is another example of a glue that uses multiple types of interactions. This glue is a defensive secretion that sets within seconds into an elastic, adhesive gel. This is a tough gel, yet it contains more than 95% water. It gains its mechanical strength from a network of proteins and polysaccharides (Smith, 2006). The overall structure is similar to the mucus used in locomotion, except for the presence of specific metal-binding proteins that are present in large quantities and are characteristic of the adhesive (Smith, 2006). The glue also contains a number of different metals. Removing these metals increases the solubility of the glue (Smith et al., 2009), and also blocks the gel-stiffening activity of key proteins (Werneke et al., 2007). Thus multiple cross-linking mechanisms appear to act in the glue; the most important of these appear to be direct metal interactions and metal-catalyzed oxidations of amino acids (Smith, 2006; Smith et al., 2009).

Direct metal cross-links are due to the ability of metals to bind strongly to multiple ligands in an aqueous environment, thus bringing them together. The tubeworm *Phragmatopoma californica*, for example, uses electrostatic interactions between calcium and phosphoproteins to create a structural foam; these interactions become insoluble ionic bonds as the pH shifts upon secretion (Stewart et al., 2004; Sagert et al., 2006). The mussel byssal plaque is held together partly by coordinate covalent bonds where plaque proteins are joined by metals such as calcium and copper (Zhao and Waite, 2006), and similar interactions probably strengthen the byssal threads (Harrington and Waite, 2007), while the varnish coating the thread depends on iron coordination (Harrington et al., 2010). Also, the hardness of Nereid worm jaws depends on multiple histidine-rich proteins chelating zinc *via* coordinate covalent bonds (Lichtenegger et al., 2003; Broomell et al., 2006; Lichtenegger et al., 2008). Similarly, manganese often strengthens arthropod tissues (Lichtenegger et al., 2008).

Protein oxidation by redox active metals like iron and copper is another way that metals can contribute to a glue (Bradshaw et al., 2011). The oxidation of the rare amino acid 3,4-dihydroxyphenylalanine (dopa), has been studied in depth as a cross-linking step in various biomaterials (Sagert et al., 2006). It is also possible, however, to create cross-links by oxidizing the side chains of common amino acids such as lysine. This typically creates carbonyl groups, which would react readily with the primary amines of other lysine side-chains to form a Schiff base, specifically an imine bond (Tanzer, 1973). Bradshaw et al. (Bradshaw et al.,

2011) provide evidence that such bonds are present in *A. subfuscus* glue.

Thus there appear to be oxidatively derived cross-links as well as direct metal interactions in slug glue, but it is not clear what their relative importance is. Furthermore, it is unknown which of the metals contributes most to the direct interactions. In *A. subfuscus* glue, zinc (~1 mmol l^{-1}), and iron, manganese and copper (~0.1 mmol l^{-1} each) are all present in significant quantities, and could participate in direct cross-links (Werneke et al., 2007). There is roughly ten times as much zinc as the other transition metals (Werneke et al., 2007), making it a likely candidate for cross-linking. Calcium is also probably present in significant quantities. The primary adhesive gland cells in another slug, *Ariolimax columbianus*, appear to contain calcium (Luchtel et al., 1984). Verdugo et al. (Verdugo et al., 1987) found that calcium made up over 10% of the dry material (2.5–3.6 mol Ca^{2+} kg^{-1}) in the secretory packets of *A. columbianus*. It is unknown, however, precisely how much calcium is present relative to other metals, and how much each of these metals contributes to cross-linking.

The gelled nature of slug glue provides a unique opportunity to determine the relative importance of different cross-links. It is possible to modify the glue selectively with different reagents and to test the effect of these modifications on glue mechanics using dynamic rheometry. Different treatments can remove specific metals or groups of metals (Smith et al., 2009), and other treatments can break or stabilize imine bonds (Bradshaw et al., 2011). Therefore, the relative effects of different direct metal interactions as well as protein oxidation on glue mechanics can be quantified. In this study, we test the hypothesis that both direct metal interactions and oxidatively derived cross-links contribute to the stiffness of the glue.

MATERIALS AND METHODS

The defensive secretion from the dorsal epithelium of the slug *Arion subfuscus* was used. Slugs were collected locally (Ithaca, NY, USA), brought to the laboratory and sampled within hours of collection. Glue was collected by gently rubbing the dorsal surface with a metal spatula. The slugs typically respond to physical contact such as this by secreting large amounts of the defensive secretion. This emerges as a viscous material that can flow off their back, but sets within less than a minute into a highly adhesive, elastic gel. The gel was collected on the spatula, then placed on ice before storage at −80°C. Slugs were released back into the forest on the same day.

Metal and sulphate concentrations

The metal content of the glue was measured using inductively coupled plasma atomic emission spectrometry (Thermo iCAP 6300, Thermo Scientific, West Palm Beach, FL, USA) and also a scanning electron microscope with an energy dispersive spectrometer (SEM-EDS) (VEGA TS 5130MM, Tescan, Cranberry Township, PA, USA), as described previously (Werneke et al., 2007). It should be noted that the calcium values were above the calibrated range for the atomic emission spectrometer. For SEM-EDS, all metals rising significantly above the detection threshold were recorded, as well as phosphorus and sulphur. SEM-EDS spectra were taken from seven samples. The sample area for each measurement was 144×144 μm. The first five samples were scanned in multiple places to determine whether the spectra were consistent. Data were recorded for the last two samples at two different sites, one in the center and one at the periphery. In order to determine how tightly bound the metals were to the gel, atomic emission spectrometry was also performed on samples that had soaked for 72 h in 50 mmol l^{-1} Tris-Cl (pH 8), either with or without 100 mmol l^{-1} EDTA. The integrity of the glue was assessed after soaking, and the undissolved glue was sedimented at 300 ***g*** for 10 min. The pellet was rinsed with distilled, deionized water, resuspended and recentrifuged twice. It was then analyzed in the same way as the other samples. The sulphate content of the glue was measured using the barium rhodizonate colorimetric assay following hydrolysis of polysaccharides as described by Terho and Hartiala (Terho and Hartiala, 1971).

Rheometry

The stiffness of homogenized glue samples after different treatments was measured using dynamic rheometry. The following procedure was used for the different treatments, unless otherwise specified. Freshly thawed samples were immersed in 33 volumes of the treatment or control buffer and incubated overnight at 4°C. The overnight incubation allowed the treatment to act on the native glue, and also swelled and softened the glue, making the homogenization step more consistent. Samples and buffer were homogenized for 1–2 s by a rotor-stator homogenizer (PRO-200, PRO Scientific, Oxford, CT, USA). This was usually sufficient to create a uniform homogenate, though some samples required an additional 1–2 s to ensure complete uniformity. Long homogenizations tended to cause foaming, which interfered with the measurements. Homogenization created uniform material for testing, provided enough material for the rheometer, and ensured thorough mixing of the reagents and sample. Despite being diluted by homogenization with a large volume of buffer, the control gels were still markedly elastic and viscous. Directly after homogenizing, samples were loaded onto a dynamic rheometer (ARES, TA Instruments, New Castle, DE, USA). Time sweeps at 5% strain and 10 rad s^{-1} were performed as described previously (Pawlicki et al., 2004), and the storage modulus was recorded. The storage modulus is a measure of the sample's elastic behavior, and thus stiffness.

In trials involving comparison of one treatment with a control, each sample was cut in half, with one half incubated in the control buffer and the other half in the treatment. Both were otherwise treated identically. These results were compared statistically with a paired Student's *t*-test. In tests involving more than one treatment, samples were not split in this way and a single factor ANOVA with *post hoc* tests was used. For all treatments, eight or ten samples were tested unless otherwise specified. Because there appeared to be seasonal variation among samples, when a paired design was not used, comparisons were only made between samples collected at the same time.

The effect of overall ionic strength and pH on the gels was tested. To test ionic strength, the storage modulus of samples was measured after they had incubated in 20 mmol l^{-1} Tris-Cl (pH 8) with six different concentrations of sodium chloride between 0 and 500 mmol l^{-1}. A larger number of samples with no salt were measured throughout the experiment to check that the samples were consistent, while five samples each were tested at the four higher concentrations. The effect of pH on gel mechanics was tested by measuring the storage modulus after incubating glue samples in buffers with pH values of 4, 5, 6, 7 and 7.75. In all of the pH trials, a citrate–phosphate buffer was used (10–20 mmol l^{-1}). The citrate–phosphate buffer was chosen because it could span the range of pH values used in the other experiments, and also extend more into the acidic range. Acid pH may have significant effects on the hypothesized interactions. Finally, a pH of 7.75 was used instead of 8 because 8 is too near the limit of the useful range for this buffer.

The relative importance of cross-links involving different metal ions was tested by measuring the stiffness of the gels after incubation with buffers that preferentially removed some or all metals. In one

set of trials, samples were incubated in 20 mmol l^{-1} Tris-Cl (pH 8) or the same Tris-Cl buffer with 10 mmol l^{-1} EDTA or with 10 mmol l^{-1} deferoxamine. Deferoxamine has a high affinity for transition metals such as iron, copper and zinc, but much lower affinity for calcium, whereas EDTA has a high affinity for all these metals (Keberle, 1964; Hider et al., 1999; Maclean et al., 2001; Permyakov, 2009). In another set of trials, the effects of phosphate and Tris buffers on gel stiffness were compared (20 mmol l^{-1} sodium phosphate, pH 7.5 *versus* 20 mmol l^{-1} Tris-Cl, pH 7.5). Phosphate will form insoluble complexes with many metals, but it is a particularly good ligand for hard Lewis acids such as Ca^{2+}, Mg^{2+}, Mn^{2+} and Fe^{3+} (Ho, 1975; Lippard and Berg, 1994). Zn^{2+} is borderline in the hard–soft acid categorization, and thus would not coordinate as well with phosphate. To remove Zn^{2+} preferentially, as well as Fe^{2+} and Cu^{2+}, an imidazole buffer was used (200 mmol l^{-1} imidazole, pH 7.5 *versus* 200 mmol l^{-1} Tris-Cl, pH 7.5). Imidazole is a borderline hard–soft ligand that coordinates zinc well (Lippard and Berg, 1994). It is widely used at concentrations of 100 mmol l^{-1} to disrupt Zn–His interactions in immobilized metal affinity chromatography. In addition, the last set of trials was tested with the Tris and imidazole buffers at 20 mmol l^{-1}, to be sure that the high concentrations were not causing non-specific effects. A pH of 7.5 was used for the trials with different buffers because it better matches the working range for all three buffers.

To test the relative importance of oxidatively derived cross-links, gel stiffness was measured after treatments that would either stabilize or competitively disrupt imine bonds. Sodium borohydride was used to reduce and stabilize imine bonds (Bradshaw et al., 2011). Samples were first incubated overnight at 4°C in 20 mmol l^{-1} Tris-Cl (pH 8). Freshly prepared sodium borohydride in 100 mmol l^{-1} sodium hydroxide was then added to a final concentration of 20 mmol l^{-1} $NaBH_4$, 10 mmol l^{-1} NaOH, while the control only received sodium hydroxide. Samples were incubated for 1 h at room temperature, then homogenized and analyzed. To disrupt imine bonds, hydroxylamine was used as in Bradshaw et al. (Bradshaw et al., 2011). This is a potent nucleophile that will compete with lysine for carbonyls that can form imine bonds. Samples and paired controls were incubated overnight at 4°C in 5 mmol l^{-1} sodium phosphate (pH 6) with or without 27 mmol l^{-1} hydroxylamine. The pH was adjusted as necessary with hydrochloric acid. The incubation was carried out at pH 6 because imine bonds are acid labile. For example, the half-life of aromatic imine bonds is much shorter at pH 5 than at pH 7.4 (Xu et al., 2007). Preliminary work also suggested that the modification of proteins in the glue by hydroxylamine occurred more readily at lower pH. The lower pH would help disrupt the bond so that any carbonyls could be modified by hydroxylamine. Because the lower pH can have other effects, after the incubation, Tris-Cl (pH 8) was added to all samples and controls to a final concentration of 50 mmol l^{-1}. The samples were then homogenized and measured with the dynamic rheometer as described above. Litmus paper was used to verify that the final pH was between 7.8 and 8.

RESULTS

Metal and sulphate concentrations

Calcium and sulphur are the most common inorganic components of *A. subfuscus* glue (Fig. 1). In the first five samples tested by SEM-EDS, the calcium concentration was roughly 2% dry weight at all sites. Data were recorded from two sites on the last two samples. One sample had a concentration of 2.7% dry weight in the middle and 2.8% at the periphery, and the other had 6.9% in the middle and 2.0% at the periphery (Fig. 1). The value of 6.9% was the only

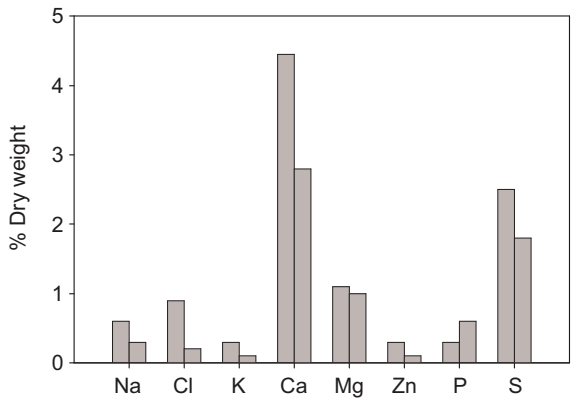

Fig. 1. Elemental composition of *Arion subfuscus* glue by SEM-EDS. Data for two different samples are shown. Each value is the average of two sites on a sample. All metals that were above the resolution limit are shown, as well as sulphur and phosphorus.

measurement substantially different from the rest. The calcium concentration of the glue was confirmed more precisely by the atomic emission spectrometer. The average concentration was 39.6±5.7 mmol l^{-1} (N=9). The concentration of other metals was consistent with previous reports (Werneke et al., 2007). Assuming the glue contains at least 95% water, which is a rough estimate for these materials (Smith, 2006), a calcium concentration of 39.6 mmol l^{-1} corresponds to 3.2% by dry weight. Other notable elements that were detected at relatively high concentrations by SEM-EDS were magnesium and phosphorus.

The SEM elemental analysis found high quantities of sulphur, making up roughly 2% of the dry weight (Fig. 1). The barium rhodizonate assay confirmed the high sulphur content and showed that the sulphur was in the form of sulphate; the two samples of glue tested had 82 and 84 mmol l^{-1} sulphate, respectively. The calibration curve for this assay exactly matched the data published by Terho and Hartiala (Terho and Hartiala, 1971), and the absorbance values were taken from the linear range, where the measurement error was roughly 5%. The sulphate values were consistent with the range predicted from the percentage dry weight of sulphur measured by SEM-EDS, assuming that the glue contains 95% water (Smith, 2006).

Calcium and some other metals were removed from the gel by extended soaking, while iron and copper remained firmly bound. After soaking in neutral buffers, the calcium, manganese and zinc content of the glue dropped to 8, 24 and 29% of the untreated values measured by the atomic emission spectrometer, respectively, while iron and copper were only reduced to 63 and 52% of their original values (N=5). When the glue was soaked in EDTA, almost all of the calcium, manganese and zinc were removed (N=10). These metals fell to 1% of their original level, or to the detection threshold of the instrument in the case of manganese. In contrast, the iron and copper levels stayed at 71 and 45% of their original levels. It is worth noting that EDTA often fragmented the gels into tiny particles, while gels without chelation often remained intact or in several large fragments, only swelling. Even with EDTA, however, a large amount of the particulate material did not dissolve.

The effect of salt concentration and pH on gel stiffness

Increased salt concentration decreased the stiffness of the glue (ANOVA, P=0.004; Fig. 2). The stiffness at 200 and 500 mmol l^{-1} sodium chloride was significantly less than at 0 mmol l^{-1} (*post hoc*

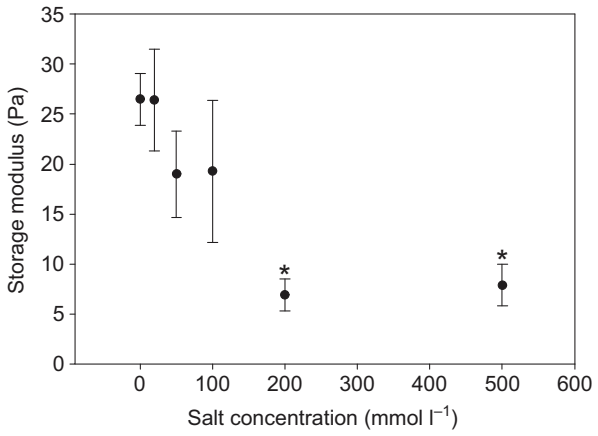

Fig. 2. The effect of salt concentration on glue stiffness. Samples were in 20 mmol l^{-1} Tris-Cl (pH 8) with varying amounts of sodium chloride. Values are means ± s.e.m. (*N*=15, 8, 5, 5, 5, 5). *Significant differences from 0 mmol l^{-1}.

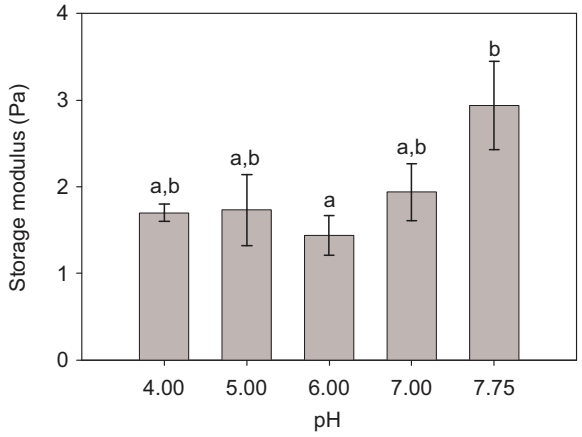

Fig. 3. The effect of pH on the stiffness of *A. subfuscus* glue. All treatments were in citrate–phosphate buffer. Values are means ± s.e.m. (*N*=8 for all). Groups without a letter in common are significantly different.

tests, $P<0.05$). Changes in pH also affected the stiffness of the glue (ANOVA, $P=0.04$; Fig. 3). The stiffness at pH 7.5 was significantly greater than at pH 6 (*post hoc* tests, $P<0.05$), but there were no other significant differences between means. Note that the citrate–phosphate buffer used for these trials weakened the glue irrespective of pH.

The effect of direct metal interactions on gel stiffness

Metal removal affected the stiffness of the glue (ANOVA, $P<0.001$; Fig. 4). Removal of all divalent metals from the glue with the chelating agent EDTA caused a 15-fold decrease in the storage modulus of the glue (*post hoc* test, $P<0.05$). Without metals, the modulus was near the resolution limit of the rheometer. Deferoxamine, which should selectively remove transition metals such as iron and zinc, did not cause a decrease in stiffness. In fact, the average storage modulus of glue samples with deferoxamine was 33% greater than that of control samples (*post hoc* test, $P<0.05$).

Using buffers that bound hard Lewis acids such as calcium and magnesium decreased the modulus of the glue, while buffers that preferentially bound intermediate Lewis acids such as zinc did not. The phosphate buffer caused a 29% decrease in stiffness compared with the Tris-Cl control (paired Student's *t*-test, $P=0.02$; Fig. 5A). Preferentially interfering with zinc-based cross-links with 200 mmol l^{-1} imidazole did not significantly affect the stiffness ($P=0.08$) (Fig. 5B). Similar results were found for trials using lower concentration buffers, with storage moduli of 9.2 and 12.2 Pa for 20 mmol l^{-1} Tris-Cl and imidazole, respectively, compared with 7.1 and 12.0 Pa for 200 mmol l^{-1} Tris-Cl and imidazole, respectively. At pH 7.5, the results for the controls tended to be more variable, and all moduli for the experiments with different buffers at pH 7.5 were slightly lower than those for previous tests at pH 8. There was some variation in the average stiffness of controls with the same buffer in different sets of experiments, but this appeared to correlate with season-to-season variation among samples. Thus it is important that paired controls from the same sample were used in these experiments. In other experiments where paired controls were not feasible, samples were only compared with other samples taken from the same time period.

The effect of imine bonds on gel stiffness

Treatment with sodium borohydride should stabilize imine bonds, and it caused a 40% increase in stiffness compared with the control without borohydride (paired Student's *t*-test, $P=0.03$; Fig. 6A). The glue was also visibly different; after homogenization and 33-fold dilution, the sodium borohydride-treated samples still had defined boundaries and held their shape. They could be picked up and handled with a thin rod, then dropped on a flat surface so that they spread out, and then peeled off the surface.

Interfering with imine bonds decreased the stiffness of the glue. Modification of the glue with hydroxylamine reduced the stiffness by 33% relative to the controls (paired Student's *t*-test, $P=0.02$) (Fig. 6B). This effect occurred if the modification was carried out at pH 6 to destabilize imine bonds. If the pH was kept at 8 during the incubation, the storage modulus was 12.8±1.4 Pa for the control without hydroxylamine and 13.5±1.7 Pa with hydroxylamine ($P=0.55$, $N=10$).

DISCUSSION

The primary cross-links controlling gel stiffness appear to be direct cross-links involving calcium, supplemented by cross-links formed by the carbonyls of oxidized proteins. Surprisingly, despite its relatively high concentration, zinc does not appear to stiffen the glue. It should be noted that tangling interactions between giant polymers will also probably contribute to the integrity of the glue, as is true for most mucus secretions (Smith, 2006). These tangling interactions might not be apparent in homogenized, diluted samples because of the decrease in polymer overlap.

Direct cross-links between polymers involving calcium

The most important cross-links appear to be direct links involving calcium and possibly magnesium ions. Removing metals with EDTA caused a drastic weakening of the gel. This clearly demonstrates that metals are directly involved in cross-links. It is also consistent with the effect of EDTA on glue solubility (Smith et al., 2009). Calcium and magnesium are the most common metals in the glue by a substantial margin, making them likely candidates for direct metal-based cross-links. Furthermore, the effect of different buffer components on glue stiffness shows that it is probably these metals rather than transition metals that make up these direct links. Preferentially removing iron, zinc and manganese with deferoxamine did not weaken samples. These findings are also consistent with previous work showing that deferoxamine does not improve solubility of the glue once it has set (Werneke et al., 2007). The

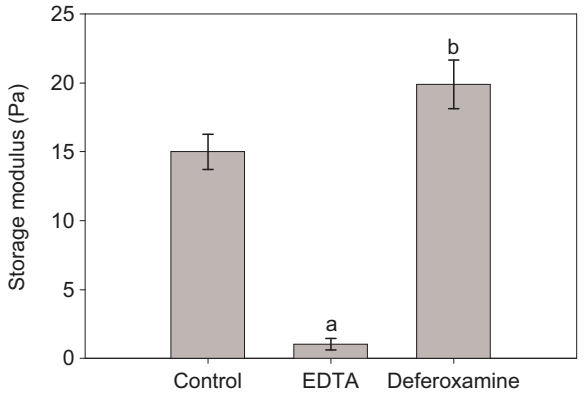

Fig. 4. The effect on glue stiffness of chelating all metals *versus* transition metals. The control contained 20 mmol l^{-1} Tris-Cl buffer (pH 8), while the treatments had either EDTA (10 mmol l^{-1}) or deferoxamine (10 mmol l^{-1}) added. Values are means ± s.e.m. (*N*=8, 12, 8). Letters (a,b) indicate significant differences from control.

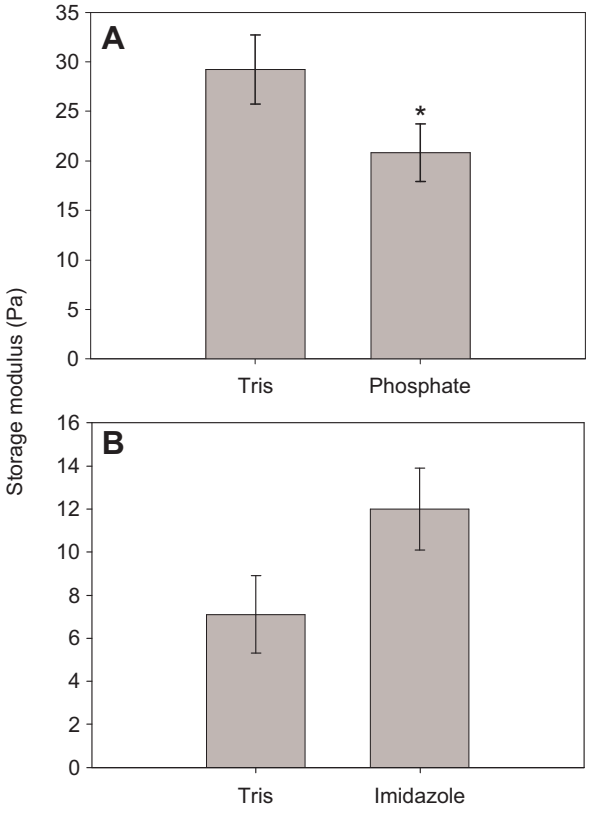

Fig. 5. The effect of different buffer types on the stiffness of *A. subfuscus* glue. (A) 20 mmol l^{-1} Tris-Cl *versus* 20 mmol l^{-1} sodium phosphate (pH 7.5). (B) 200 mmol l^{-1} Tris-Cl *versus* 200 mmol l^{-1} imidazole (pH 7.5). Values are means ± s.e.m. (*N*=8 for all). *Significant difference from control.

results with phosphate buffers were consistent with a role for calcium and magnesium, as phosphate weakened the glue and it is an excellent ligand for hard Lewis acids such as Ca^{2+} and Mg^{2+} (and also Fe^{3+} and Mn^{2+}), but it is not as good a ligand for borderline acids such as Zn^{2+} (Lippard and Berg, 1994). It seems less likely that iron is involved in direct cross-links, given the lack of effect of deferoxamine and the relatively low concentration of iron in the glue. Nevertheless, it cannot be ruled out, because the iron is bound so tightly that chelators could not remove it effectively. Furthermore, even a small amount of iron relative to calcium can dominate the mechanics of a material because of its high affinity for some ligands (Holten-Andersen et al., 2009). In mussel byssus, calcium and iron both form direct cross-links, with calcium being more prevalent, but iron forming stronger interactions (Hwang et al., 2010).

There are a number of possible ligands for calcium and iron in slug glue. The most likely are sulphate, carboxyl and phosphate groups. Sulphated and carboxylated polysaccharides are common in invertebrate mucus (Denny, 1983). The closely related terrestrial slug *Arion ater* secretes a mucus that contains heparan sulphate-like glycosaminoglycans, and it contains roughly 1% sulphate by dry weight (Cottrell et al., 1993). In *A. subfuscus* glue, calcium and sulphate are the predominant ions in the glue, and there is roughly twice as much sulphate as calcium in native glue. Given that the sulphate will carry a single negative charge when linked to a polysaccharide and calcium is divalent, these charges should balance each other. In addition, magnesium and phosphorus are present in significant quantities and these could work in a similar way. The phosphorus could be in the form of phosphate, which is a common functional group added to proteins after translation, as well as a major component of nucleic acids. Phosphate is an excellent ligand for calcium and iron (Lippard and Berg, 1994). Carboxyls are also common on proteins and polysaccharides, and they would also bind calcium and iron. Thus calcium, magnesium and possibly iron may interact with several different types of ligands in the glue. The strength of the interaction will depend on the number and arrangement of ligands as well as their affinity for the metals.

The nature of the interaction between metals such as calcium and their ligands in slug glue is unknown. Calcium may interact electrostatically or by coordinate covalent bonds, with the distance between ligands and their extent of solvation determining the tendency to form one or the other bond type (Lichtenegger et al., 2008). The fact that changing salt concentration reduced the stiffness of the gel suggests that electrostatic interactions play an important role. Sodium chloride concentrations of 200 and 500 mmol l^{-1} decreased the stiffness of the gel significantly. These concentrations should be sufficient to break many electrostatic interactions (Smith et al., 2009). However, changes in salt concentration are also known to affect the spacing between polymers in a gel (Tanaka, 1981). Electrostatic repulsions between charged polymers can maintain the swollen state of the gel. Because of this, some covalently cross-linked gels containing charged polymers can swell or shrink by several orders of magnitude, with changes in salt concentration in the range of 10–100 mmol l^{-1} (Tanaka, 1981). In the experiments on slug glue, it is not clear whether salt causes a loss of stiffness by disrupting calcium-dependent electrostatic cross-links or by blocking electrostatic repulsions between polymers and causing local collapse of the polymers, which would prevent them from interacting. Previous work with slug glue suggested that electrostatic interactions were not involved in cross-linking since the glue was equally insoluble in low- and high-salt buffers (Smith et al., 2009). Nevertheless, mussel byssus plaque proteins form calcium cross-links that generate forces consistent with electrostatic interactions (Hwang et al., 2010). Another complication is that calcium can often promote intermolecular interactions by controlling the shape of individual proteins, putting them in favorable conformations (Maurer and Hohenester, 1997; Li and Graham, 2007). Such intramolecular metal dependence is far more common than metals serving as cross-links between proteins (Zeng et al., 2010). Because of the unusually high amounts of calcium and sulphate in slug glue, however, the

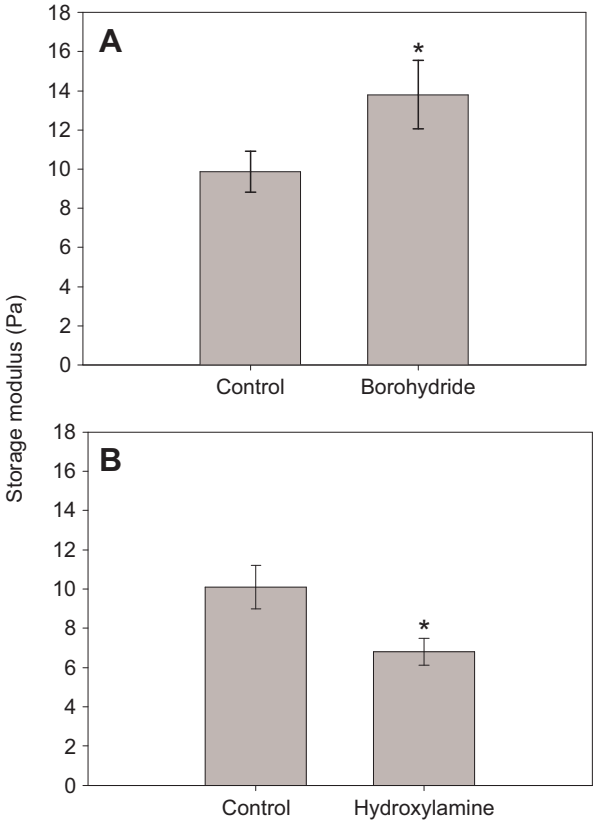

Fig. 6. The effect of stabilizing or disrupting imine bonds on the stiffness of *A. subfuscus* glue. (A) Imine stabilization with sodium borohydride. Both samples contained Tris-Cl and sodium hydroxide, while the treatment also contained 20 mmol l^{-1} sodium borohydride. (B) Imine disruption with hydroxylamine. Both samples contained Tris-Cl (pH 8), while the treatment also has 27 mmol l^{-1} hydroxylamine. Values are means ± s.e.m. (N=10 for all). *Significant differences from control.

latter seems much more likely. Calcium may also form insoluble complexes with sulphate. In fact, calcium and sulphate are the primary components of plaster of Paris. Calcium sulphate is insoluble in water, so when mixed and hydrated, it forms a solid mineral material. Instead of forming a mineral such as plaster, the calcium–sulphate links in slug glue would be scattered along the polymers in the fluid–polymer dominated gel structure. This would not, however, explain the relative ease with which calcium is removed from the gel. The fact that extended soaking in a neutral Tris-Cl buffer removes most of the calcium suggests that the calcium-based cross-links are not insoluble complexes, but instead are either electrostatic or coordinate covalent bonds.

Calcium is known to play a significant role in the extracellular matrix of animal tissues. It normally assists in binding to carbohydrates and phosphates (Maurer and Hohenester, 1997). There are a number of calcium-binding proteins in the extracellular matrix that contribute to its integrity. In most cases the calcium is coordinated by oxygen-containing ligands (Maurer and Hohenester, 1997). Given this, it is significant that matrilin-like proteins have been found in the mucus of the slug *Lehmannia valentiana* (Li and Graham, 2007). Matrilins are calcium-dependent extracellular matrix cross-linkers. The integrity of this slug's mucus was also compromised by aggressive metal extraction with EDTA (Li and Graham, 2007).

It is likely that calcium and sulphate directly control the overall structure of the glue. Because they are the predominant ions, they will almost certainly interact in some way. The calcium would be electrostatically attracted to the sulphate, bridging sulphate groups, or sulphate groups and other calcium-binding groups. Shashoua and Kwart (Shashoua and Kwart, 1959) proposed that calcium forms bridges between sulphates on the polysaccharides and carboxyl groups on proteins. These bridges are unlikely to be solely electrostatic in the glue, as noted previously.

Calcium and sulphate may be common components of many mucus secretions. Grenon and Walker (Grenon and Walker, 1980) report that calcium and magnesium are the primary inorganic ions in limpet mucus (4.5 and 4.62% dry weight, respectively), with sulphate present in roughly twice the quantity (16.8% dry weight). Smith et al. (Smith et al., 1999a) also found elevated calcium, magnesium, iron, phosphate and sulphate in limpet mucus. There was no difference in inorganic content between the adhesive and non-adhesive mucus, however (Smith et al., 1999a). Shashoua and Kwart (Shashoua and Kwart, 1959) report high concentrations of calcium and sulphate in the lubricating mucus of the whelk *Busycon canaliculatum*. Thus high calcium and sulphate do not seem unique to adhesive secretions. It may normally create a structured, cohesive gel that could then serve a lubricating function, or be modified further to become adhesive. In slug glue, oxidative cross-linking seems to work in tandem with calcium–sulphate interactions. The presence of these oxidative cross-links may be what distinguishes lubricating from adhesive mucus, though this has not been investigated. Another possibility is that the lubricating gels depend on calcium and sulphate interactions occurring solely between polysaccharides, but the specific proteins that characterize the glue may interact with calcium in a much more stable way. The proteins may have specific binding regions that allow greater coordination and thus stronger binding to the metals. The addition of such proteins to the lubricating mucus may create stronger cross-links.

Cross-links resulting from protein oxidation

In addition to direct cross-links, there is good evidence that oxidatively derived cross-links contribute substantially to the gel. Bradshaw et al. (Bradshaw et al., 2011) showed that many proteins in *A. subfuscus* glue are heavily oxidized. Furthermore, these oxidized proteins appear to form imine bonds. Most commonly, such bonds result from interaction between the primary amines of lysine side-chains and the carbonyls that are formed by protein oxidation (Tanzer, 1973; Bradshaw et al., 2011). These bonds would be labile unless they are further modified (Tanzer, 1973). They can be reduced by sodium borohydride, resulting in permanent carbon–nitrogen bonds. As expected, sodium borohydride reduction stiffened the glue significantly. In addition, imine bonds are disruptable by potent nucleophiles such as hydroxylamine. The effect of hydroxylamine on the stiffness of the glue is consistent with the hypothesis that imine bonds play a significant role. It is also worth noting that these experiments underestimate the possible role of oxidative cross-linking. The treatments that were used only act on labile imine bonds. If some of these bonds become further modified in the glue to more stable alternatives, as happens in collagen and elastin (Tanzer, 1973), they would not be affected by these treatments. Finally, it is unlikely that the effect of hydroxylamine is non-specific; the concentration of hydroxylamine was relatively low, and hydroxylamine had no effect while the bonds were stable at slightly basic pH, but had a clear effect when the modification was carried out at the lower pH conditions where it is much more likely to disrupt imine bonds.

Schiff bases such as imine bonds are versatile cross-linkers. Imine bonds are one of the few reversible covalent bonds, and their stability depends on a variety of different factors (Belowich and Stoddart, 2012). They have already been used in formalin cross-linked synthetic medical adhesives (Paez and Jorge-Herrero, 2006), and other workers have created useful sealants, adhesives and gels from oxidized polymers that then form Schiff bases with amines on other polymers (Mo et al., 2000; Dawlee et al., 2005; Artzi et al., 2009).

It should be noted that the weakening of the gel at pH values below 7 is consistent with all of the proposed cross-links. An acid environment would improve metal solubility and weaken coordinate covalent bonds by competition between protons and metals for available ligands. Imine bonds are also less stable in an acid environment, because protons catalyze the formation and breakdown of these bonds (Xu et al., 2007). Werneke et al. (Werneke et al., 2007) noted that acid reduced the solubility of the glue rather than helping solubilize it, but this may be due to isoelectric precipitation of the proteins, which are generally acidic (Smith, 2006).

The importance of reversible bonds

The major bonds in slug glue are reversible, which has considerable functional importance. The calcium can be removed relatively easily, which would degrade the long-term performance of the glue. Furthermore, imine bonds are relatively labile. At first glance this would seem to be a substantial disadvantage. Loss of integrity over time is a key problem with glues, especially those exposed to water. There may be a functional reason that more permanent cross-links do not form. Because it appears to be a defensive secretion, the primary functional requirements of slug glue are likely to be a combination of rapid setting with strong initial adhesion and cohesion. Long-term strength is unlikely to be as important as it only needs to deter a predator long enough for the slug to escape. Creating more stable interactions may require more time, and may be unnecessary. There may be a trade-off between speed of setting and long-term strength. Another consideration is that labile bonds can be used to absorb energy. The stiffness of a glue is often not as important as the work of fracture. This is particularly true of gels that are highly deformable. A glue can achieve a high work of fracture by virtue of a high extensibility coupled with a large number of sacrificial bonds that break as the material extends (Smith et al., 1999b; Thompson et al., 2001). This absorbs energy and can make it much more difficult to break a glue. This process contributes a great deal to the performance of mussel byssus (Harrington et al., 2009). Furthermore, reversible bonds such as coordinate covalent bonds can reform once the material is no longer stressed. Coordinate covalent bonds are particularly intriguing in this light, because of their combination of high strength and reversibility (Lee et al., 2006; Lichtenegger et al., 2008; Harrington et al., 2010: Zeng et al., 2010).

Proposed model for glue formation

The results suggest a model that is similar in some ways to what has been proposed for tubeworm cement (Stewart et al., 2004), though with a much more dilute, deformable material. The calcium and sulphate probably interact electrostatically, balancing each other's charge. This could create a coacervate, with the electrostatic attraction bringing together polymers in local regions of high concentration and leaving behind aqueous pockets. This network of fibers would form the primary structure of the glue. Such large fibers have been reported in *A. columbianus* glue (Deyrup-Olsen et al., 1983). Once the fibers are formed, they may be cross-linked further, with imine bonds forming and electrostatic interactions becoming coordinate covalent bonds as the ligands are brought closer together. This general mechanism, involving coacervate formation followed by oxidative cross-linking, has been used with recombinant elastin (Keeley et al., 2002). The bonds created after fiber formation would probably involve proteins, as proteins play an essential role in creating the greater stiffness and viscosity of the glue relative to the normal mucus used in locomotion (Pawlicki et al., 2004). If there are any calcium-binding proteins, they could link the polysaccharides together more firmly through calcium bridges that involve sulphate. The fact that there are several types of cross-links, and a mixture of large protein–polysaccharide complexes and smaller proteins, suggests that the material could behave as a double-network gel, as described by Haque et al. (Haque et al., 2012). In these materials, the combination of sacrificial cross-links forming a relatively rigid network within a more ductile network of polymers gives dramatically improved toughness (Haque et al., 2012).

To function properly, the glue must also be in a secretable form internally, but then change once it is secreted. Oxidation may play an important role, as the proteins do not appear oxidized before secretion, but are strongly oxidized immediately afterwards (Bradshaw et al., 2011). It is likely that the manner of mixing is essential as well. The mucus of terrestrial slugs is secreted in discrete, membrane-bound packets (Deyrup-Olsen et al., 1983). When they are intact, the secretion is watery, but once they rupture, the glue rapidly sets. Mechanical shear or calcium are sufficient to rupture these packets (Deyrup-Olsen et al., 1983). Furthermore, there appear to be two or three primary types of adhesive cells that secrete different components, with one identified as a calcium cell, one secreting mucopolysaccharides and another that secretes a large volume of fluid and some protein (Luchtel et al., 1984; Smith, 2010). The sudden mixing of these moieties could initiate glue formation. Luchtel et al. (Luchtel et al., 1984) did not report the methods they used to detect calcium, and it is surprising that all the mucopolysaccharide-releasing cells are not identified as calcium containing, as there must be shielding charge on the polyanions to keep them condensed (Verdugo et al., 1987). Most histological methods are designed to detect insoluble calcium crystals rather than soluble calcium associated with polymers (Humason, 1979), so it is likely that soluble calcium would not have been detected. In any case, when the different moieties are mixed, there may be a rearrangement as different ligands may replace the original ligands. This would be especially true if any polycationic proteins were mixed with the polysaccharides.

Possible roles for zinc

Given the relatively high concentration of zinc in the glue, it is striking that zinc does not appear to contribute to the stiffness of the glue. Inhibiting zinc-based interactions failed to weaken the glue, and possibly even increased its stiffness. The deferoxamine-treated samples were significantly stiffer than the controls. It is also intriguing that the imidazole-treated samples appeared similarly stiffer, though this was not significant. It is known that zinc may inhibit oxidative cross-linking (Powell, 2000). It has similar ligand-binding characteristics to Fe^{2+} and Cu^{2+}, and can displace them from their ligands. Replacing a redox active metal such as iron with one that has far less activity would inhibit site-specific oxidation reactions (Chevion, 1988; Berlett and Stadtman, 1997; Powell, 2000). In addition, zinc can bind to sulfhydryls and thus protect them from oxidation, which could impact their activity (Powell, 2000). Another possibility is that there might be matrix metalloproteinases in the glue. These are the primary enzymes involved in modifying the extracellular matrix, and they are found in all organisms. They commonly use zinc as a cofactor and

hydrolyze a variety of extracellular matrix proteins (Nagase and Woessner, 1999; Nagase et al., 2006; Ra and Parks, 2007). If such enzymes are active in slug glue, zinc chelation would block their activity and thus block glue degradation. There is evidence for zinc metalloproteases in barnacle cement (Dougherty, 1996; Dougherty, 1997), but these and other enzymes have been hypothesized to be involved in proteolytic processing that activates cross-linking rather than weakening the glue (Dougherty, 1996; Dougherty, 1997; Dickinson et al., 2009). If indeed zinc is acting as an antioxidant or as a cofactor in a metalloprotease that degrades the glue, it would be an interesting way to modulate the mechanics of the glue. This provides an added layer of complexity, though it is not clear how it would be advantageous. Finally, it is possible that zinc plays a role in adhesion rather than cohesion. Adhesiveness is a crucial feature of slug glue that has not yet been studied.

Conclusions

Overall, these results confirm the functional significance of direct metal-based cross-links and oxidatively based cross-links. Furthermore, they provide evidence that other interactions are involved that may not directly increase stiffness, but clearly play a role in gel mechanics. Because of the ease of manipulation of this experimental system, this approach will probably prove valuable in further hypothesis testing on gelled biomaterials.

LIST OF ABBREVIATIONS

EDS	energy dispersive spectroscopy
EDTA	ethylenediaminetetraacetic acid
$NaBH_4$	sodium borohydride
SEM	scanning electron microscope

ACKNOWLEDGEMENTS

Work with the atomic emission spectrometer and scanning electron microscope was carried out while visiting the laboratory of J. H. Waite.

AUTHOR CONTRIBUTIONS

M.B. conducted many of the experiments, contributed to experimental design and interpretation of the findings, and helped draft the article. F.O. and M.M. carried out several of the experiments and contributed to the design, interpretation and analysis. A.M.S. proposed the research idea, was involved in the design, execution and interpretation of the experiments, and helped draft and revise the article.

COMPETING INTERESTS

No competing interests declared.

FUNDING

This work was supported by a summer research grant from Ithaca College.

REFERENCES

Artzi, N., Shazly, T., Baker, A. B., Bon, A. and Edelman, E. R. (2009). Aldehyde-amine chemistry enables modulated biosealants with tissue-specific adhesion. *Adv. Mater.* **21**, 3399-3403.

Belowich, M. E. and Stoddart, J. F. (2012). Dynamic imine chemistry. *Chem. Soc. Rev.* **41**, 2003-2024.

Berlett, B. S. and Stadtman, E. R. (1997). Protein oxidation in aging, disease, and oxidative stress. *J. Biol. Chem.* **272**, 20313-20316.

Bradshaw, A., Salt, M., Bell, A., Zeitler, M., Litra, N. and Smith, A. M. (2011). Cross-linking by protein oxidation in the rapidly setting gel-based glues of slugs. *J. Exp. Biol.* **214**, 1699-1706.

Broomell, C. C., Mattoni, M. A., Zok, F. W. and Waite, J. H. (2006). Critical role of zinc in hardening of *Nereis* jaws. *J. Exp. Biol.* **209**, 3219-3225.

Chevion, M. (1988). A site-specific mechanism for free radical induced biological damage: the essential role of redox-active transition metals. *Free Radic. Biol. Med.* **5**, 27-37.

Cottrell, J. M., Henderson, I. F., Pickett, J. A. and Wright, D. J. (1993). Evidence for glycosaminoglycans as a major component of trail mucus from the terrestrial slug, *Arion ater* L. *Comp. Biochem. Physiol.* **104B**, 455-468.

Dawlee, S., Sugandhi, A., Balakrishnan, B., Labarre, D. and Jayakrishnan, A. (2005). Oxidized chondroitin sulfate-cross-linked gelatin matrixes: a new class of hydrogels. *Biomacromolecules* **6**, 2040-2048.

Denny, M. W. (1983). Molecular biomechanics of molluscan mucous secretions. In *The Mollusca*. Vol. 1 (ed. K. Wilbur, K. Simkiss and P. W. Hochachka), pp. 431-465. New York, NY: Academic Press.

Deyrup-Olsen, I., Luchtel, D. L. and Martin, A. W. (1983). Components of mucus of terrestrial slugs (Gastropoda). *Am. J. Physiol.* **245**, R448-R452.

Dickinson, G. H., Vega, I. E., Wahl, K. J., Orihuela, B., Beyley, V., Rodriguez, E. N., Everett, R. K., Bonaventura, J. and Rittschof, D. (2009). Barnacle cement: a polymerization model based on evolutionary concepts. *J. Exp. Biol.* **212**, 3499-3510.

Dougherty, W. J. (1996). Zinc metalloprotease activity in the cement precursor secretion of the barnacle, *Chthamalus fragilis* Darwin. *Tissue Cell* **28**, 439-447.

Dougherty, W. J. (1997). Carboxypeptidase activity of the zinc metalloprotease in the cement precursor secretion of the barnacle, *Chthamalus fragilis* Darwin. *Comp. Biochem. Physiol.* **117**, 565-570.

Grenon, J. F. and Walker, G. (1980). Biomechanical and rheological properties of the pedal mucus of the limpet, *Patella vulgata* L. *Comp. Biochem. Physiol.* **66B**, 451-458.

Haque, M. A., Kurokawa, T. and Gong, J. P. (2012). Super tough double network hydrogels and their application as biomaterials. *Polymer* **53**, 1805-1822.

Harrington, M. J. and Waite, J. H. (2007). Holdfast heroics: comparing the molecular and mechanical properties of *Mytilus californianus* byssal threads. *J. Exp. Biol.* **210**, 4307-4318.

Harrington, M. J., Gupta, H. S., Fratzl, P. and Waite, J. H. (2009). Collagen insulated from tensile damage by domains that unfold reversibly: *in situ* X-ray investigation of mechanical yield and damage repair in the mussel byssus. *J. Struct. Biol.* **167**, 47-54.

Harrington, M. J., Masic, A., Holten-Andersen, N., Waite, J. H. and Fratzl, P. (2010). Iron-clad fibers: a metal-based biological strategy for hard flexible coatings. *Science* **328**, 216-220.

Hider, R. C., Bittel, D. and Andrews, G. K. (1999). Competition between iron(III)-selective chelators and zinc-finger domains for zinc(II). *Biochem. Pharmacol.* **57**, 1031-1035.

Ho, T. L. (1975). Hard soft acids bases (HSAB) principle and organic chemistry. *Chem. Rev.* **75**, 1-20.

Holten-Andersen, N., Mates, T. E., Toprak, M. S., Stucky, G. D., Zok, F. W. and Waite, J. H. (2009). Metals and the integrity of a biological coating: the cuticle of mussel byssus. *Langmuir* **25**, 3323-3326.

Humason, G. L. (1979). *Animal Tissue Techniques*. San Francisco, CA: W. H. Freeman.

Hwang, D. S., Zeng, H., Masic, A., Harrington, M. J., Israelachvili, J. N. and Waite, J. H. (2010). Protein- and metal-dependent interactions of a prominent protein in mussel adhesive plaques. *J. Biol. Chem.* **285**, 25850-25858.

Kamino, K. (2006). Barnacle underwater attachment. In *Biological Adhesives* (ed. A. M. Smith and J. A. Callow), pp. 145-166. Berlin: Springer.

Keberle, H. (1964). The biochemistry of desferrioxamine and its relation to iron metabolism. *Ann. N. Y. Acad. Sci.* **119**, 758-768.

Keeley, F. W., Bellingham, C. M. and Woodhouse, K. A. (2002). Elastin as a self-organizing biomaterial: use of recombinantly expressed human elastin polypeptides as a model for investigations of structure and self-assembly of elastin. *Philos. Trans. R. Soc. B* **357**, 185-189.

Lee, H., Scherer, N. F. and Messersmith, P. B. (2006). Single-molecule mechanics of mussel adhesion. *Proc. Natl. Acad. Sci. USA* **103**, 12999-13003.

Li, D. and Graham, L. D. (2007). Epidermal secretions of terrestrial flatworms and slugs: *Lehmannia valentiana* mucus contains matrilin-like proteins. *Comp. Biochem. Physiol.* **148B**, 231-244.

Lichtenegger, H. C., Schöberl, T., Ruokolainen, J. T., Cross, J. O., Heald, S. M., Birkedal, H., Waite, J. H. and Stucky, G. D. (2003). Zinc and mechanical prowess in the jaws of *Nereis*, a marine worm. *Proc. Natl. Acad. Sci. USA* **100**, 9144-9149.

Lichtenegger, H. C., Birkedal, H. and Waite, J. H. (2008). Heavy metals in the jaws of invertebrates. In *Biomineralization: From Nature to Application (Metal Ions in Life Sciences)*, Vol. 4 (ed. A. Sigel, H. Sigel and R. K. O. Sigel), pp. 295-325. Chichester, UK: John Wiley.

Lippard, S. L. and Berg, J. M. (1994). *Principles of Bioinorganic Chemistry*. Mill Valley, CA: University Science Books.

Luchtel, D. L., Martin, A. W. and Deyrup-Olsen, I. (1984). The channel cell of the terrestrial slug *Ariolimax columbianus* (Stylommatophora, Arionidae). *Cell Tissue Res.* **235**, 143-151.

Maclean, K. H., Cleveland, J. L. and Porter, J. B. (2001). Cellular zinc content is a major determinant of iron chelator-induced apoptosis of thymocytes. *Blood* **98**, 3831-3839.

Maurer, P. and Hohenester, E. (1997). Structural and functional aspects of calcium binding in extracellular matrix proteins. *Matrix Biol.* **15**, 569-580.

Mo, X., Iwata, H., Matsuda, S. and Ikada, Y. (2000). Soft tissue adhesive composed of modified gelatin and polysaccharides. *J. Biomater. Sci. Polym. Ed.* **11**, 341-351.

Nagase, H. and Woessner, J. F., Jr (1999). Matrix metalloproteinases. *J. Biol. Chem.* **274**, 21491-21494.

Nagase, H., Visse, R. and Murphy, G. (2006). Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc. Res.* **69**, 562-573.

Paez, J. M. G. and Jorge-Herrero, E. (2006). Biological adhesives. In *Modified Fibers with Medical and Specialty Applications* (ed. J. V. Edwards, G. Buschle-Diller and S. C. Goheen), pp. 145-158. Dordrecht: Springer.

Pawlicki, J. M., Pease, L. B., Pierce, C. M., Startz, T. P., Zhang, Y. and Smith, A. M. (2004). The effect of molluscan glue proteins on gel mechanics. *J. Exp. Biol.* **207**, 1127-1135.

Permyakov, E. (2009). *Metalloproteomics*. Hoboken, NJ: John Wiley & Sons.

Powell, S. R. (2000). The antioxidant properties of zinc. *J. Nutr.* **130**, 1447-1454.

Ra, H.-J. and Parks, W. C. (2007). Control of matrix metalloproteinase catalytic activity. *Matrix Biol.* **26**, 587-596.

Sagert, J., Sun, C. and Waite, J. H. (2006). Chemical subtleties of mussel and polychaete holdfasts. In *Biological Adhesives* (ed. A. M. Smith and J. A. Callow), pp. 125-143. Berlin: Springer.

Shashoua, V. E. and Kwart, H. (1959). The structure and constitution of mucus substances. II. The chemical constitution of Busycon mucus. *J. Am. Chem. Soc.* **81**, 2899-2905.
Smith, A. M. (2006). The biochemistry and mechanics of gastropod adhesive gels. In *Biological Adhesives* (ed. A. M. Smith and J. A. Callow), pp. 167-182. Berlin: Springer.
Smith, A. M. (2010). Gastropod secretory glands and adhesive gels. In *Biological Adhesive Systems: From Nature to Technical and Medical Application* (ed. J. V. Byern and I. Grunwald), pp. 41-51. Berlin: Springer.
Smith, A. M., Quick, T. J. and St. Peter, R. L. (1999a). Differences in the composition of adhesive and non-adhesive mucus from the limpet *Lottia limatula*. *Biol. Bull.* **196**, 34-44.
Smith, B. L., Schaffer, T. E., Viani, M., Thompson, J. B., Frederick, N. A., Kindt, J., Belcher, A., Stucky, G. D., Morse, D. E. and Hansma, P. K. (1999b). Molecular mechanistic origin of the toughness of natural adhesives, fibres and composites. *Nature* **399**, 761-763.
Smith, A. M., Robinson, T. M., Salt, M. D., Hamilton, K. S., Silvia, B. E. and Blasiak, R. (2009). Robust cross-links in molluscan adhesive gels: testing for contributions from hydrophobic and electrostatic interactions. *Comp. Biochem. Physiol. B* **152**, 110-117.
Stewart, R. J., Weaver, J. C., Morse, D. E. and Waite, J. H. (2004). The tube cement of *Phragmatopoma californica*: a solid foam. *J. Exp. Biol.* **207**, 4727-4734.
Tanaka, T. (1981). Gels. *Sci. Am.* **244**, 124-138.
Tanzer, M. L. (1973). Cross-linking of collagen. *Science* **180**, 561-566.
Terho, T. T. and Hartiala, K. (1971). Method for determination of the sulfate content of glycosaminoglycans. *Anal. Biochem.* **41**, 471-476.
Thompson, J. B., Kindt, J. H., Drake, B., Hansma, H. G., Morse, D. E. and Hansma, P. K. (2001). Bone indentation recovery time correlates with bond reforming time. *Nature* **414**, 773-776.
Verdugo, P., Deyrup-Olsen, I., Aitken, M., Villalon, M. and Johnson, D. (1987). Molecular mechanism of mucin secretion: I. The role of intragranular charge shielding. *J. Dent. Res.* **66**, 506-508.
Werneke, S. W., Swann, C., Farquharson, L. A., Hamilton, K. S. and Smith, A. M. (2007). The role of metals in molluscan adhesive gels. *J. Exp. Biol.* **210**, 2137-2145.
Xu, S., Luo, Y. and Haag, R. (2007). Water-soluble pH-responsive dendritic core-shell nanocarriers for polar dyes based on poly(ethylene imine). *Macromol. Biosci.* **7**, 968-974.
Zeng, H., Hwang, D. S., Israelachvili, J. N. and Waite, J. H. (2010). Strong reversible Fe^{3+}-mediated bridging between dopa-containing protein films in water. *Proc. Natl. Acad. Sci. USA* **107**, 12850-12853.
Zhao, H. and Waite, J. H. (2006). Proteins in load-bearing junctions: the histidine-rich metal-binding protein of mussel byssus. *Biochemistry* **45**, 14223-14231.