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Mussel-inspired histidine-based transient network metal coordination hydrogels

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Abstract

Transient network hydrogels cross-linked through histidine-divalent cation coordination bonds were studied by conventional rheologic methods using histidine-modified star poly(ethylene glycol) (PEG) polymers. These materials were inspired by the mussel, which is thought to use histidine-metal coordination bonds to impart self-healing properties in the mussel byssal thread. Hydrogel viscoelastic mechanical properties were studied as a function of metal, pH, concentration, and ionic strength. The equilibrium metal-binding constants were determined by dilute solution potentiometric titration of monofunctional histidine-modified methoxy-PEG and were found to be consistent with binding constants of small molecule analogs previously studied. pH-dependent speciation curves were then calculated using the equilibrium constants determined by potentiometric titration, providing insight into the pH dependence of histidine-metal ion coordination and guiding the design of metal coordination hydrogels. Gel relaxation dynamics were found to be uncorrelated with the equilibrium constants measured, but were correlated to the expected coordination bond dissociation rate constants.

Introduction

The marine mussel attaches to intertidal coastal surfaces, such as rocks, via byssal threads that project from the mussel's body and terminate in adhesive plaques. Materials scientists have long been interested in the plaque's water-resistant adhesive chemistry.¹ More recently, interest in the potential mechanical role of coordination bonds in the mussel byssal thread has grown as a result of unique mechanical behavior and composition of this fibrous tissue.^{2–4} When stressed beyond the yield point, the distal byssal thread is able to partially recover its Young's modulus in a completely acellular manner and is thus said to possess self-healing properties.² Waite and coworkers discovered that the byssal thread is composed primarily of specialized collagen proteins (PreCol proteins) that contain domains with a high content of histidine (13–19 %) and sequence homology amongst species.^{5–8} Histidine, an amino acid that is often involved in metal ion coordination in proteins, is hypothesized to bind metal ions such as Zn²⁺ and Cu²⁺ in the byssal thread, whereupon the metal

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coordination bond is thought to break under applied load, serving as an energy dissipating mechanism.⁹ Unlike most covalent bonds whose rupture is generally irreversible, coordinate bonds are capable of reforming after rupture, i.e., functioning as sacrificial bonds. Changes in pH, depletion of solution metal ions, and histidine-specific modifications all have dramatic effects on the mechanical properties of the distal byssal thread, consistent with the proposed role of metal coordination in the mechanical performance of byssal thread.^{2,7} In a mechanical system composed of both covalent and coordination bonds, reversible coordination bond rupture within the byssal threads is hypothesized to spare covalent bonds from irreversible breakage, increase the thread stiffness enthalpically, and impart the thread with self-healing properties. In this work, we focus on studying the rheological properties of transient network hydrogels inspired by the mussel's use of histidine as a reversible cross-linker.

Sacrificial bonds have been shown to be important molecular features in a number of other biological systems including the unfolding of domains within the protein titin,¹⁰ Ca²⁺-mediated sacrificial bonds in bone,^{11,12} and the organic component of nacre.¹³ Theoretical and AFM studies suggest that sacrificial bonds can spare covalent bonds in polymer backbones by breaking reversibly before irreversible covalent bond rupture.^{13,14} Upon rupture, new hidden lengths of polymer are revealed that allow for increased polymer extension. Material stiffness is increased, because the entropic springs that make the polymer backbone are effectively shorter than the total polymer length before sacrificial bond rupture. Thus, sacrificial bonds are thought to enhance material toughness without sacrificing material stiffness. Using cloning techniques, Li and coworkers were able to modulate domain folding and unfolding in proteins that resembled titin, changing it from a molecular shock absorber to an entropic spring in the presence or absence of a modulating protein, respectively.¹⁵ They later went on to incorporate titin-like domains into hydrogels that mimic the passive mechanical properties of muscles, exhibiting stress relaxation and recoverable stress-strain hysteresis.¹⁶ They have also demonstrated the ability to modulate domain stability by genetically engineering metal coordination sites into protein domains and incubating the protein with metal.¹⁷ Guan and coworkers have incorporated modular domains within synthetic polymers that are held together with reversible hydrogen bonds, taking inspiration from titin, which contains 'modular' domains that unfold. They correlated enhanced bulk material mechanical properties with single-molecule force spectroscopy measurements to support their mechanism.^{18–20} Craig and coworkers have investigated organogels held together through coordination bonds between Pt²⁺ and Pd²⁺ pincer metal complexes and poly(4-vinylpyridine) polymers,^{21–27} showing that gel rheological properties are governed primarily by bond dissociation rates and not equilibrium constants.^{22–24} They collapse oscillatory rheometric measurements on to a single master curve by dividing the oscillatory rate by the bond dissociation constant. Using single-molecule force spectroscopy to probe the impact of force on individual coordination bonds,^{25,28} they find bond dissociation to fit generally well with the Bell-Evan's model.^{29,30}

Investigators have studied the physical properties of coordination-based hydrogels, which include pyridyl or bipyridyl-based ligand systems.^{31–37} Interestingly, the swelling properties of these materials tend to reflect the bond inertness (dissociation kinetics) and not the bond energy, e.g., the swelling rate of otherwise identical bipyridyl-branched poly(*N*-alkylacrylamide) gels cross-linked with the following metals is Ru²⁺ < Fe²⁺ < Ni²⁺, consistent with a similar order of bond dissociation rate constants.³⁵ Enhancement in mechanical properties of hydrogel polymer membranes have been seen with the addition of metals to hydrogel membranes with pyridyl and bipyridyl functional groups.³³ CuCl₂ has also been used to induce self-healing of polymer hydrogels that contain Cu²⁺ coordinating groups.³⁸ Hydrogels have been constructed using Ni²⁺-histidine interactions, however these gels' mechanical properties were not investigated and would not likely be mechanically

robust, as in 20 mM sodium phosphate buffer, pH 7.4, the gels dissolved within two hours.³⁹ In addition, hydrogels that incorporate chelators of Ni^{2+} have been used as protein delivery systems for 6 $\times$ histidine tagged proteins.^{40,41} Srivastava creatively embedded agarose hydrogels with poly(vinylimidazole) polymers.⁴² These materials exhibited increased modulus and strength upon addition of Zn^{2+} or Cu^{2+} . The pH of the gel, an important parameter that is expected to dramatically affect the coordination chemistry, unfortunately was unclear in this study. Additionally, dynamic mechanical properties were not investigated, which is where these materials might be expected to show their most important properties. Recently, we have demonstrated that the mechanical properties of catechol-modified PEG hydrogels can be enhanced through Fe^{3+} -catechol coordination cross-linking.⁴³

Hydrogels, while promising biomaterials for their biocompatible properties, are generally weak and brittle. These problems have generally limited the applications of hydrogels to non-structural biomaterials. Double network, sliding network, and clay nanoparticle based hydrogels have all been developed to enhance the mechanical properties of conventional hydrogels.⁴⁴ Interestingly, native biological soft tissues such as heart valves or blood vessels are able to stand up to a lifetime of stress despite being nominally polymer hydrogels, whereas synthetic replacements for these soft tissues have many inherent problems. In the case of small vessel arterial grafts, glutaraldehyde-treated homologous umbilical veins, Dacron, polytetrafluoroethylene (Teflon), and many tissue engineering attempts have been tried and all have the problem of reduced patency and poor matching of mechanical properties.^{45–47} Heart valve replacements in current clinical use are either mechanical (metal) or bioprosthetic (porcine-derived).⁴⁵ While the mechanical valve generally lasts for the life of the patient, anticoagulant therapy is also required throughout the patient's life. The bioprosthetic valve has a 10 year failure rate of approximately 30%, but requires only a short period of anticoagulant therapy.^{45,48} Incorporation of sacrificial coordination bonds into polymer hydrogels and other materials may provide a route to increasing the toughness of medical devices used to repair or replace living tissue.

We believe that the mussel byssal thread provides a model for the development of robust self-healing biomaterials that possess mechanical properties similar to native soft tissues. Herein, we demonstrate that histidine-divalent metal coordination bonds, similar to those found in the mussel byssus, are able to contribute significantly to the viscoelastic mechanical properties of hydrogels cross-linked solely through these sorts of interactions. We study these interactions on monofunctional polymers in order to determine equilibrium binding constants, and then on multifunctional polymers to determine the effect of the interactions on bulk material properties.

Experimental and Methods

4A-PEG-dHis synthesis

In a 50 mL Schlenk flask, 4arm-PEG-amine (3.0 g, 1.2 mmol of amine groups, Mw 10,000 g/mol, Laysan Bio Inc.) was dissolved in 10.0 mL of dichloromethane. 3-(*N*-1-Trityl-imidazol-4-yl)propionic acid (0.69 g, 1.8 mmol, Chem-Impex International, Inc.), PyBop (0.94 g, 1.8 mmol, Novabiochem), and *N,N*-diisopropylethylamine (0.63 mL, 3.6 mmol, DIPEA, Sigma-Aldrich) were added sequentially to PEG solution. Afterwards, 5.0 mL of DMF was added to give a clear solution and this coupling reaction was carried out at 20 °C under an N_2 atmosphere with continuous stirring for 2.0 h. The crude product was purified by precipitation in diethyl ether (300 mL) once and in methanol (300 mL) three times at –20 °C. The PEG polymer was vacuum dried after one additional precipitation in diethyl ether. The obtained polymer (3.0 g) was deprotected by dissolving it in trifluoroacetic acid (60 g)

with triisopropylsilane (1.63 g) and water (1.62 g). Deprotection was carried out at 20 °C for 2 h and purification was performed as described above for the coupling step.

Synthesis of 4A-PEG-His and mPEG-His

4arm-PEG-amine (5.0 g, 2.0 mmol NH² end-group, MW 10k, Laysan Bio Inc.), Boc-His(Trt)-OH (1.49 g, 3.0 mmol, Chem-Impex International, Inc.), and BOP (1.33 g, 3.0 mmol, Novabiochem) were dissolved in ~15 mL dichloromethane (DCM). DIPEA (1.07 mL, 6 mmol, Sigma-Aldrich) was added and the reaction was allowed to proceed for 2 h. The crude product was purified by precipitation in 500 mL ether 1× and 500 mL methanol at -20 °C 3×. The polymer was vacuum dried after 1 additional ether precipitation. A ninhydrin test on 5 mg polymer was negative on the protected product. Protecting groups were removed by a cleavage solution of 95 mL trifluoroacetic acid, 2.5 mL triisopropylsilane and 2.5 mL water for 2 h. The solvent was removed under reduced pressure and purification was performed by redissolving the polymer in a minimal amount of methanol and precipitation in 500 mL ether 3×. mPEG-His was similarly synthesized using mPEG-amine (MW 5k, Laysan Bio).

Potentiometric titration of mPEG-His

Potentiometric titrations were performed using standard methods⁴⁹ with a custom built autotitrator that was controlled using a custom MATLAB program. A Mettler Toledo SevenEasy pH meter was interfaced with a PC via the serial port and pH readings were collected ~1 s⁻¹. An Orion Ross 8220BNWP microelectrode was employed to allow small volume titrations. Temperature was controlled with a heated water circulator at 25 °C. A Ramé-Hart Automated Dispensing System was used to titrate 0.100 M KOH (Ricca Chemical) with a 250 µL syringe, interfaced to the serial port. A stock mPEG-His polymer solution was made by dissolving mPEG-His in 0.100 M KCl to 14.8 mg polymer/mL, and 1.500 mL was pipetted from the stock solution into a glass vial to perform the titration. Metal stock solutions (~100 mM, concentrations determined by ICP-AES) were added to the solutions at a 1:2 metal: polymer ratio. Base was titrated in 1.50 µL increments. The pH was deemed stable when the standard deviation of the pH over the last 60 s was <0.001. These criteria were relaxed for pH greater than 9, due to baseline drift at higher pH.

Computation of polymer metal equilibrium

The second derivative of the mPEG-His titration curve was used to determine the endpoint of the titration and the number of moles of histidine end group/g polymer. Titration curves were fit using the program BEST⁴⁹ by assuming the following reactions could occur, which are described by the equations:

$$\text{HisM} + \text{His} \rightleftharpoons \text{His}_2\text{M} \quad K_2 = \frac{[\text{His}_2\text{M}]}{[\text{HisM}][\text{His}]} \quad (4)$$

where His represents mPEG-His, H_n represents the protonation state, and M represents the metal. Square brackets take the traditional meaning of concentration. Total histidine end-group, His_T and metal, M_T , are assumed conserved:

$$[\text{His}_T] = [\text{His}] + [\text{HisH}] + [\text{HisH}_2] + [\text{HisM}] + 2([\text{His}_2\text{M}]) \quad (5)$$

$$[\text{M}_T] = [\text{M}] + [\text{HisM}] + [\text{His}_2\text{M}] \quad (6)$$

Base additions $>2\times$ histidine end groups were not considered in the model used to determine equilibrium constants.

Calculation of polymer-metal speciation curves

Using the equilibrium constants determined by the titration study, metal-polymer equilibrium was determined as a function of pH by solving the set of nonlinear equations listed above by custom written MATLAB software. Polymer end-group (60 mM) and metal (30 mM) concentrations were representative of the conditions used in gel formation.

Gel formation technique and rheological characterization

The gel formation technique was modeled after that developed by Holten-Anderson et al.,⁴ using 4A-PEG-His at several concentrations. Final buffer concentration was 100 mM for all buffered solutions (pH 5, 6–7, and 8–9 were buffered with acetate, bis-tris, and tris, respectively). 4A-PEG-His was first dissolved in water, then buffer was added from a 600 mM stock, followed by addition of ~100 mM metal chloride stock (exact concentration determined by ICP-AES) to achieve a molar ratio of $0.5\times$ relative to the histidine end-group, and finally 1.00 M NaOH was added to the polymer solution to give the desired pH as determined by dilute solution potentiometric titrations of the polymers. At each step, the polymers were mixed vigorously to homogeneity. The amount of water added to each polymer solution was calculated to give the final polymer concentration. NaOH was added because the buffer concentration alone was insufficient to buffer the solution at high polymer concentrations. Gels were centrifuged to remove any air bubbles before loading them on the rheometer. Rheometric characterization was performed on an Anton Paar MCR-300 equipped with a Peltier hood and evaporation blocker using a CP 25-2 cone and plate fixture (25 mm diameter, 2° cone angle). Experiments were performed at 10 °C, 25 °C, and 37 °C. Frequency sweeps were performed at 5 % strain from 100 to 0.1 rad/s. For gels with long relaxation times (4A-PEG-His + $0.5\times\text{Ni}^{2+}$), frequency sweeps were performed at 100 Pa constant shear stress from 100 to 0.1 rad/s, as constant stress measurements were found to give better instrumental performance in these cases. Strain sweeps from 0.1 to 10 % at 10 rad/s were used to confirm that polymer solutions were measured in the linear viscoelastic range. Step-strain measurements were performed by applying a 10 % strain and following the stress relaxation of the material over 200 s, varying the data collection rate logarithmically. 4A-PEG-dHis gels were similarly formed by adding $0.25\times$ or $0.5\times$ metal and $2\times$ NaOH relative to the end-group (although buffers were not used in the experiments presented). Exact control over pH was not attempted in this case.

Results and Discussion

4-arm and monofunctional histidine-modified PEG polymers were synthesized as simple mimics of the histidine-rich regions of mussel byssal thread collagen. Using these simple

model systems, we hypothesized that we could better understand the impact of the histidine-rich regions of byssal thread collagen on byssal thread mechanical properties and gain insight into how sacrificial histidine-metal coordination bonds can be used to improve the mechanical properties of bulk soft materials. Conventional rheology was used to study the mechanical responses of these hydrogel materials, and model monofunctional PEG-histidine polymers were used to determine metal-polymer binding constants through potentiometric titration.

4A-PEG-dHis coordination materials

A 4-arm PEG derivatized with deamino-histidine, 4A-PEG-dHis (Chart 1), was synthesized as a mimic for the histidine-rich region of PreCol proteins. We believed that this structure was both synthetically easily accessible and would resemble the coordinating groups believed to be responsible for the self-healing properties of the byssal thread. Each end-group has a single imidazole functionality, structurally identical to the histidine side chains that interact with divalent metal cations in a native protein. A single metal ion is able to coordinate up to 4 imidazoles, with $\log(K) = 1.4 - 4.2$ for Zn^{2+} , Cu^{2+} , Co^{2+} , and Ni^{2+} for the small molecule analog, imidazole ($\text{pK}_a = 7.11$).⁵⁰ We focused on metal:end-group ratios of 1:2 and 1:4, as 1:2 should provide 1 crosslink/polymer end-group and 1:4 should provide the maximum number of end-groups/metal ion. The polymer was synthesized as an acid salt, so an equivalent amount of base (determined by potentiometric titration) was added to the polymer solution to promote the metal-imidazole interaction. Figure 1 shows photographs of the polymer solutions at 1:2 and 1:4 metal:end-group ratios at the final polymer concentration. Upon addition of base to the solutions, a gel-like material was formed near the pipette tip. However, upon mixing the solution to homogeneity, the materials became free-flowing solutions. The color changes were consistent with metal-imidazole coordination bonds, though these interactions did not appear to be sufficient to induce gel-like properties.

While the imidazole-metal coordination bond of 4A-PEG-dHis should be similar to the native mussel proteins, there are several key differences to point out. First, sacrificial coordination bonds most likely exist within a rigid, insoluble collagen network in the mussel byssal thread, whereas in our synthetic polymer mimics the polymer chains cross-linked by coordination bonds are highly hydrated. Second, there is a defined primary structure of the native proteins, which may allow for coordination motifs to form, potentially inducing secondary, tertiary, or quaternary structure in native proteins. Zinc fingers highlight the importance of primary structure in protein-metal interactions, as the binding constant has been measured to be on the order of 10^{11} in model zinc finger peptides.⁵¹ In the mussel thread collagen, histidine is often found in -HGH- or -HAH- motifs, which may enable similar secondary structure effects as well as allow chelate types of interactions between nearby histidine residues.

Formation of 4A-PEG-His coordination gels

In order to produce materials with relaxation times accessible by conventional oscillatory rheometry, we synthesized 4-arm-PEG derivatized with histidine, 4A-PEG-His (Chart 2). A key difference in 4A-PEG-His lies in the presence of the α -amine, which we hypothesized would increase gel relaxation times, as the kinetic dissociation constant should be decreased compared to 4A-PEG-dHis gels due to contributions of the α -amine toward metal coordination. Studies with the small molecule analog, histidine methylester, indicate that the polymer end groups should coordinate divalent metals through both the imidazole nitrogen and α -amine, with a $\log(K)$ on the order of twice that of imidazole for the metals studied.⁵⁰ Based on small molecule studies, we hypothesized that this would allow a single metal ion to bridge two polymer arms (Scheme 1). Using dilute solution titrations of 4A-PEG-His,

hydrogels were formed at pH ~7 at the stoichiometric ratio of 2:1 histidine polymer endgroup:metal. At pH 7, titration curves for all metal-polymer solutions are near their inflection point. Using an ideal solution analysis, as presented later, we demonstrate that all metal-ligand complexes should predominantly be the bis metal complex at this pH. Dramatic changes in solution physical properties were seen after increasing the pH of the metal-polymer solution to pH 7. Photographs of gel solutions are shown in Figure 2 with their colors being consistent with coordination of the respective metals. Based on potentiometric titration data, adjustment of the pH to 7 corresponded to ~2× base to end-group (complete deprotonation of the synthesized acid salt). When manipulated with a spatula, the Zn^{2+} gel was viscous and easily deformable, the Cu^{2+} gel was more viscous but of a similar quality to the Zn^{2+} gel, the Co^{2+} gel was even more viscous and if manipulated rapidly would undergo brittle failure, and the Ni^{2+} gel underwent brittle failure with small deformations. Brittle failure however, was repaired almost instantly for the Co^{2+} gel and over a timescale of minutes for the Ni^{2+} gel. Because these materials are cross-linked by reversible coordination interactions and are rheologically viscoelastic liquids, it is not surprising that these materials self-heal after brittle failure. Healing of the materials requires coordination bond breakage and reformation. The gel with the most kinetically inert metal (longest bond-dissociation rate), Ni^{2+} , should require the longest time for self-repair, which was observed. Ni^{2+} gels are quantitatively shown to relax the slowest by rheometry in the section to follow. Quantitative exploration of the non-linear properties of these materials will be reserved for a future publication.

Rheometry of 4A-PEG-His gels

As shown in Figure 2, 4A-PEG-His gels were homogeneous and yielded colors consistent with the metal studied. However, the rate of self-repair, $\text{Zn}^{2+} > \text{Cu}^{2+} > \text{Co}^{2+} > \text{Ni}^{2+}$, does not appear to be correlated with the energy of the bonds, as the small molecule analog histidine methylester binding constants are $\text{Zn}^{2+} < \text{Ni}^{2+} < \text{Cu}^{2+}$ (Co^{2+} not reported although it can be expected to be similar to Zn^{2+} based on the Irving-Williams series).⁵⁰ To more quantitatively understand these observations, we studied these materials by rheometry. Figure 3 shows oscillatory rheometry of these materials in the linear viscoelastic region at 10 °C, 25 °C, and 37 °C. For a Maxwell liquid, the relaxation time of the material is the inverse of the crossover frequency of G' and G'' . The relaxation spectra for these materials, while not explicitly fitted to a Maxwell model, demonstrate a qualitative pattern that is very close to Maxwell-like relaxation. If the inverse of the crossover frequency is taken as an estimate of the relaxation time of the material (note that the material may possess a spectrum of relaxation times), then the relaxation times of the gels follow the order $\text{Ni}^{2+} > \text{Co}^{2+} > \text{Cu}^{2+} > \text{Zn}^{2+}$. As would be expected, crossover frequencies increased at higher temperatures. Relaxation time was found to correlate with what is known about divalent metal kinetics, with the Ni^{2+} gel relaxing most slowly of all the metals studied. For the metals studied here, the water exchange rate follows the order $\text{Ni}^{2+} < \text{Co}^{2+} < \text{Zn}^{2+} < \text{Cu}^{2+}$.^{52,53} The rate-limiting step for coordination of such ions is usually the dissociation step, which is generally not influenced by the incoming ligand. While the rate of dissociation is in general different for each ligand, the order of dissociation generally follows the same pattern. Similar to our mechanical results, a previous study demonstrated faster dissociation kinetics of Cu^{2+} -ethylenediamine compared with the Ni^{2+} analog, despite the higher Cu^{2+} equilibrium constant.⁵⁴ Thus, the long relaxation times seen in the Ni^{2+} gels relative to the other metals studied is consistent with what is known about divalent metal-ligand dissociation kinetics. The remainder of this work characterizes the response of Ni^{2+} gels due to the easy accessibility on the rheometer time scale.

The concentration dependence of the 4A-PEG-His + 0.5× Ni^{2+} gel was investigated next, for 4A-PEG-His concentrations of 10–150 mg/mL. As shown in Figure 4, as concentration is

decreased, both the relaxation time and the modulus decrease. The decrease in modulus is expected, as the modulus should be proportional to cross-linking density. The decrease in relaxation may be due to increased chain stretching at lower concentrations (longer end-to-end distances between cross-links), influencing the relaxation dynamics of the coordination bonds. At 10 mg/mL 4A-PEG-His, gels did not form.

The effect of changing the ionic strength is shown in Figure 5. Upon addition of 100 mM NaCl, only a very subtle change in the frequency sweep is seen. As the solution already contains 100 mM buffer and metal counter ions, this change in ionic strength may not be extremely significant. However, it does show that small changes in ionic strength will not significantly affect the relaxation dynamics.

Figure 6 shows the effect of pH on gel relaxation in a step strain measurement. Gel relaxation was found to follow the order pH 7 > pH 8 > pH 6 > pH 9. Attempts to form gels at pH 5 only resulted in viscous liquids. Interestingly, pH 7 appears to be an optimum for gel/coordination bond stability. Above and below this pH, certain mechanisms exist for decreasing the bond relaxation time. At low pH, a higher proton concentration could destabilize the chelate-type histidine-metal coordination bond through protonation of a released end (imidazole or α -amine) of the histidine ligand.⁵³ At high pH, a hydroxide ion can act as a nucleophile and attack the metal to destabilize the histidine-metal bond. While we cannot rule out a role for the buffer in the relaxation dynamics, changes in proton and hydroxide concentration seem more likely to be responsible for the observations. Frequency sweeps were also performed, and showed results consistent with this behavior.

Determination of mPEG-His metal binding constants by potentiometric titration

In order to explore the impact that histidine conjugation to PEG might have on the metal-ligand binding constant and the pK_as of the end-group, we prepared mPEG-His (Chart 3), which was synthesized as an acid salt. Figure 7 shows the titration of mPEG-His with and without the presence of divalent metal ions. Using an ideal solution model, pK_as of 5.33 and 7.46 were determined for the imidazole and amine protons, respectively (Table 1). These values are consistent with literature pK_as (5.35 and 7.30) of histidine methylester, a small molecule analog of the polymer conjugate.⁵⁵ As shown Scheme 1, the interaction of the metal with the histidine end-group requires the deprotonation of both the imidazole nitrogen and the α -amine. The addition of divalent metal ions at the stoichiometric ratio resulted in significant stabilization of the deprotonated state of histidine end-groups as seen by the titration curves. Consistent with the hypothesized binding motif, inflection points for all metal solutions are seen at 2 $\times$ base relative to histidine end-group. An ideal solution equilibrium model, as outlined in the Experimental section, was constructed in order to determine the equilibrium binding constants of the metal-histidine interactions from the titration curves. As would be predicted from the literature,⁵⁰ the order of strength of binding constants was found to be $\text{Cu}^{2+} > \text{Ni}^{2+} > \text{Zn}^{2+} \approx \text{Co}^{2+}$ (Table 1).

These results demonstrate that conjugation of histidine to the polymer does not appear to significantly alter metal-histidine coordination bonds as has been studied using small molecules. Interestingly, the Ni^{2+} titration is the only titration that follows the same pathway as the titration of mPEG-His without metal above 2 $\times$ equivalents of base. This indicates that no significant side products, such as metal hydroxides or metal-stabilized amide deprotonation, are formed after coordination of the histidine end-group by Ni^{2+} . In addition, the titration curves for all of the other metals provide good evidence that both the imidazole nitrogen and the α -amine are participating in the coordination bond. If the amide nitrogen deprotonated to form a coordination bond with the metal, the deprotonation of the α -amine would still be expected to show up on the titration curve. From the titration without the metal, the pK_a of this proton is 7.46. Therefore it is unlikely that the amide nitrogen

participates in coordination bond formation at base additions less than $2\times$. At base additions greater than $2\times$, the amide nitrogen can deprotonate to form coordination bonds or bound water can hydrolyze. This is seen in the curve of Cu^{2+} , Co^{2+} , and Zn^{2+} , as the pH after $2\times$ base falls below the metal-free titration curve. Cu^{2+} is well known to have the ability to deprotonate amide nitrogens in peptides and this could be occurring in the titration of mPEG-His.^{50,56} At $3\times$ base addition to a 4A-PEG-His + $0.5\times \text{Cu}^{2+}$ gel, we observed that the blue gel turned to a dark violet and the resulting gel was much more rigid. Although this was not further investigated, it is hypothesized that this transition resulted from amide deprotonation and coordination, producing a more stable and longer-lived coordination bond.

Equilibrium thermodynamic predictions

In order to determine the effect that pH would have on metal cross-linked hydrogels, we used an ideal solution equilibrium model of metal-histidine interactions to calculate speciation curves of 2:1 histidine polymer endgroup:metal solutions. This ratio should provide the maximum cross-linking density for a hydrogel formed through metal-histidine coordination bonds. Shown in Figure 8 are speciation curves for $0.5\times \text{Zn}^{2+}$, Cu^{2+} , Co^{2+} , and Ni^{2+} relative to histidine polymer end-groups, calculated based on the equilibrium constants listed in Table 1. The His^2M species is the most important feature of the curves, as it is the only species that should have a significant influence on the mechanical properties of a coordination cross-linked hydrogel. Using this ideal solution calculation, the His^2M species reaches 90 % of the total histidine polymer end-group concentration at pH 6.70, 5.23, 7.19, and 5.83 for Zn^{2+} , Cu^{2+} , Co^{2+} , and Ni^{2+} , respectively. While we recognize that a hydrogel is not an ideal solution in any sense, these values provide some basic guidance as to the pH at which coordination cross-linking should occur in the gels studied. In this respect it is interesting to note that our findings predict that Zn^{2+} and other transition metals found in mussel byssal threads are favored to form mechanically active His^2M species with His-rich byssal collagens at the typical pH range of seawater (7.5–8.5).

Conclusions

In this work, the relaxation properties of deaminohistidine and histidine-modified PEG polymers were studied by conventional oscillatory rheometry. Only 4A-PEG-His relaxed slowly enough to capture by conventional oscillatory rheology. 4A-PEG-His forms a chelate type interaction with divalent metals, compared to 4A-PEG-dHis, which only forms one coordination bond per polymer end-group. Chelate formation (and therefore lower kinetic dissociation constant) explains the relatively larger relaxation time of the 4A-PEG-His polymers. Using potentiometric titration, the hypothesized binding mechanism of the histidine end-group was confirmed, although the amide nitrogen may deprotonate at higher pHs than studied.

While it would be difficult to put forth our findings as definitive proof that the mussel uses histidine-metal coordination bonds to enhance its mechanical performance, we can say that such interactions are mechanically active and have the potential to significantly alter dynamic material behavior. This is especially true in the case of byssal threads where shock-absorbing viscoelastic properties are important. If one accepts the hypothesis that the mussel uses histidine-metal coordination bonds to enhance the byssal thread's mechanical properties, then one must wonder about the time scale of relaxation of the metal-histidine bonds of the thread. Is the rate of breakage correlated in any way with the average wave velocity or periodicity? If the coordination bonds relax too slowly relative to the timescale of loading, then the thread would behave like an elastic solid and no energy would be dissipated through the breakage of coordination bonds. On the other hand, if the

coordination bonds relax too quickly, then those bonds are just insignificant friction elements.

The implications of these mechanochemical considerations extend beyond the understanding of mussel byssal mechanics, as the history of inorganic chemistry is rich and full of fundamental thermodynamic and kinetics studies of solution phase reactions, including those of interactions between divalent metals and imidazole or histidine. However, the impact of mechanical force on coordination bonds is only beginning to be appreciated, with very few studies in existence. Craig and coworkers have looked at organogels and provided a plausible explanation for the relaxation properties observed in these gels, correlating it to the dissociation rate constant of their metal pincer complexes.^{25,28} Our study of histidine-based coordination hydrogels shows similar relaxation behavior. The kinetically more inert Ni²⁺-histidine gel relaxes more slowly than the thermodynamically stable Cu²⁺ coordination hydrogel. A full understanding of how force and force rate influences different types of interactions will be important for the developing field of mechanochemistry.

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Figure 1.

Visual appearance of solutions of 4A-PEG-dHis (150 mg/mL) with Zn²⁺, Cu²⁺, Co²⁺, and Ni²⁺ at (a) 0.25× and (b) 0.5× metal relative to polymer endgroup.

Figure 2.

Photographs of 4A-PEG-His gels with 0.5× divalent metal/ polymer endgroup. From left to right gels correspond to Zn^{2+} (clear), Cu^{2+} (blue), Co^{2+} (yellow), and Ni^{2+} (purple).

Figure 3.

Rheological characterization of 4A-PEG-His coordination gels with 0.5× divalent metal/polymer end-group at pH 7 and (A) 10 °C, (B) 25 °C, and (C) 37 °C. Frequency sweep of gel with storage (G') and loss (G'') moduli, reported as solid and open symbols, respectively.

Figure 4.
Effect of concentration at pH 8 of 4A-PEG-His + $0.5 \times \text{Ni}^{2+}$ gels.

Figure 5.
Effect of changing ionic strength for 4A-PEG-His + 0.5xNi²⁺, 100 mg/mL, pH 8 gel.

Figure 6. Effect of pH on gel relaxation for 4A-PEG-His + $0.5 \times \text{Ni}^{2+}$, 100 mg/mL gel subjected to a 10 % step strain.

Figure 7.

Potentiometric titration of mPEG-His with and without divalent metal added at 0.5 $\times$ metal/polymer endgroup. Titrations were performed in 0.100 M KCl with 0.100 M KOH at 14.8 mg polymer/mL at 25 $^{\circ}\text{C}$. KOH addition of 1 $\times$ and 2 $\times$ relative to polymer endgroup is marked. Every other data point was removed to increase plot clarity.

Figure 8.

Ideal solution equilibrium predictions for pH-dependence of metal polymer equilibrium at ideal stoichiometric ratio of 2:1 histidine endgroup:metal for Zn^{2+} , Cu^{2+} , Co^{2+} , and Ni^{2+} . Curves were calculated based on equilibrium parameters reported in Table 1. Concentration of polymer endgroup is reflective of approximate concentration in gels (60 mM).

Scheme 1.
Hypothesized cross-linking reaction

Chart 1.
4A-PEG-dHis

Chart 2.
4A-PEG-His

Chart 3.
mPEG-His

Table 1

Equilibrium constants for mPEG-His determined by potentiometric titration.

	pK_a^1	pK_a^2	σ_{fit}
mPEG-His	7.46	5.33	0.019
<i>Metal</i>	<i>Log(K₁)</i>	<i>Log(K₂)</i>	<i>σ_{fit}</i>
ZnCl ₂	4.76	4.33	0.016
CuCl ₂	8.47	6.07	0.014
CoCl ₂	4.99	3.94	0.015
NiCl ₂	6.64	5.24	0.008