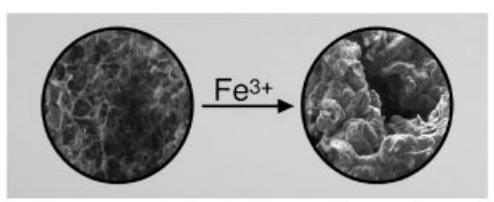
Summary: In an effort to explore new biocompatible substrates for biomedical technologies, we present a structural study on a crosslinked gelatinous protein extracted from marine mussels. Prior studies have shown the importance of iron in protein crosslinking and mussel adhesive formation. Here, the structure and properties of an extracted material were examined both before and after crosslinking with iron. The structures of these protein hydrogels were studied by SEM, SANS, and SAXS. Viscoelasticity was tested by rheological means. The starting gel was found to have a heterogeneous porous structure on a micrometer scale and, surprisingly, a regular structure on the micron to nanometer scale. However disorder, or ''no periodic structure'', was deduced from scattering on nanometer length scales at very high q. Crosslinking with iron condensed the structure on a micrometer level. On nanometer length scales at high q, small angle neutron scattering showed no significant differences between the samples, possibly due to strong heterogeneity. X-ray scattering also confirmed the absence of any defined periodic structure. Partial crosslinking transformed the viscoelastic starting gel into one with more rigid and elastic properties.



Environmental scanning electron micrographs of the protein gel after crosslinking with iron.

Structural Effects of Crosslinking a Biopolymer Hydrogel Derived from Marine Mussel Adhesive Protein

Elena Loizou,[1] *Jaime T. Weisser,*[2] *Avinash Dundigalla,*[1] *Lionel Porcar,*[4] *Gudrun Schmidt,**[1,3] *Jonathan J. Wilker**[2]

[1] Department of Chemistry, Louisiana State University, Baton Rouge, LA 70803, USA
[2] Department of Chemistry, Purdue University, West Lafayette, IN 47907, USA
Fax: +1 765 494 0239; E-mail: wilker@purdue.edu
[3] Department of Biomedical Engineering, Purdue University, West Lafayette, IN 47907, USA
E-mail: gudrun@purdue.edu
[4] National Institute of Standards and Technology, Gaithersburg, MD 20899, USA

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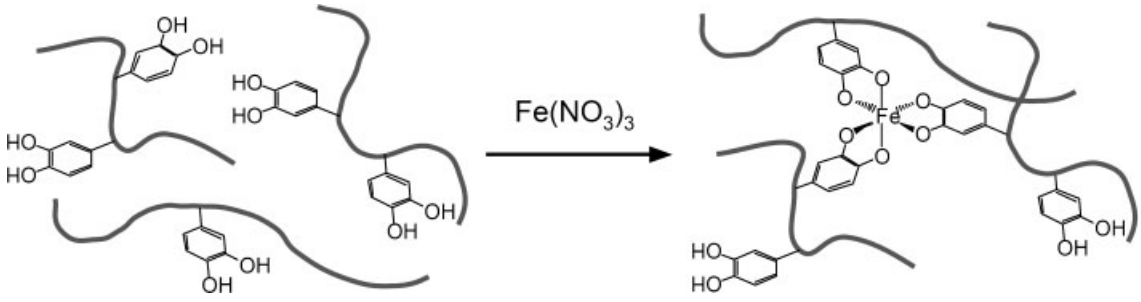
Introduction

Polymeric hydrogel materials are receiving increased attention because of their potential to impact on the development of bioengineering and biomedical applications.[1,2] Tissue engineering and growth, drug delivery formulations and the construction of cell growth scaffolds are examples of developments awaiting the discovery of hydrogels with suitable characteristics.[1,2] Progress in this field has been hampered by a lack of candidate materials with specific properties, such as strength, elasticity, porosity and biocompatibility. Recent attention has turned to the formation of hydrogels based upon peptides, proteins and similar structures.[3,4]

Our efforts in this area have been influenced by the exquisite design incorporated into materials produced by nature. From spider silk to sea shells, biological materials evolve for a specific purpose. These biological systems can provide us with both actual materials for specific needs and inspiration for design efforts. In addition to providing new materials for biomedical devices, studies on biopolymer hydrogels can teach us about the fundamentals of natural material formation. The oceans, for example, contain an array of biomaterials, such as mussel adhesives, barnacle cements and coral reef structures.[5–7] In general, the structural schemes of marine biological materials remain to be described.

We are particularly interested in protein-based adhesives used by mussels and other organisms for affixing themselves to surfaces. After application to a substrate such as a rock, the proteins are crosslinked extensively to cure the



Scheme 1. An illustration of the iron dependent crosslinking of proteins in the formation of marine mussel adhesives.

adhesive.[6,8,9] These protein precursors to adhesion contain high levels of the unusual amino acid 3,4-dihydroxyphenylalanine (DOPA).[6,8,9] Recently, our laboratory presented work with live mussels, extracted protein, and peptide models indicated that iron may be the reagent central to this crosslinking process.[10,11] Iron binds to the DOPA residues, thereby bringing together multiple protein strands.[10,12,13] Subsequent protein oxidation and metal reduction then enables protein-protein crosslinking and possibly protein-surface adhesion.[10] A representation of this crosslinking process is presented in Scheme 1.

Such spectroscopic findings led us to wonder about the morphological and rheological implications of metal-induced protein crosslinking. Perhaps this mechanism of material generation could be used to create a new class of polymeric hydrogel material where structure and properties could be controlled. Here we present a study on an extract of marine mussel adhesive precursor proteins. This material resembles a polymeric hydrogel with a gelatinous consistency. We compared the properties of this extract before and after a crosslinking reaction with iron. These materials were studied by scanning electron microscopy (SEM), small angle neutron scattering (SANS), small angle X-ray scattering (SAXS) and rheology. The SEM data provided an insight into the gel structure at a micron level, and the scattering data allowed a complementary description at the nanometer scale. The rheological measurements correlated gel structure with macroscopic physical characteristics. The results presented below show that the crosslinking process induces conspicuous morphological changes as well as imparting enhanced elastic properties. The work is presented to both further basic understanding of structure in biomaterials and to explore the properties of a biopolymer hydrogel in order to hasten the development of biomedical technologies.

Experimental Part

Protein extracts were prepared using a literature method.[14] The SANS experiments required use of deuterated reagents and solvents. Here we provide details of the samples produced for the SANS experiments. Preparation of samples for SEM and rheological measurements was performed in a similar manner, but without the use of deuterated compounds. Excised feet from the blue mussel *Mytilus edulis* (26.04 g) were placed in a chilled (−20 °C) blender with 260 mL of chilled 0.7% perchloric acid (prepared from 1.04 mL of $HClO_4$ in D_2O). This mixture was blended for 1 min and transferred to centrifuge tubes. After centrifugation at 31 000 *g* and 4 °C for 30 min, the supernatant (228 mL) was collected in a glass beaker. The beaker was immersed in an ice bath and placed atop a magnetic stirrer inside a refrigerator (4 °C). Deuterated sulfuric acid (D_2SO_4, 3.82 mL) was added dropwise with stirring. Chilled acetone-d_6 (CD_3COCD_3, 456 mL) was added dropwise over 1.25 h. The solution was then allowed to sit for 30 min without stirring. A precipitate formed and was resuspended by gentle stirring. The mixture was transferred to centrifuge tubes and centrifuged for 30 min at 31 000 *g* and 4 °C. The supernatant was discarded to yield a light beige colored, gelatinous pellet, each of ≈1 g. Multiple pellets were combined and homogenized to provide the extracted adhesive precursor protein gel. Drying experiments showed this extract to be ≈8% solids and ≈92% solvent (e.g., water, acetone).[15] Little of the pellet solids were soluble, so the total concentration of protein or DOPA could not be determined. For soluble DOPA-containing protein, ≈4 mg could be extracted from ≈1 g (≈80 mg solids) of a gelatinous pellet.[14]

The protein extract was examined both with and without the addition of an iron crosslinking agent. Dichromate ($Na_2Cr_2O_7$) was also used as a reference crosslinking agent, to show the rheology effects of a more complete crosslinking versus the partial crosslinking obtained with iron.[15] In a typical experiment to examine the protein gel without the crosslinker, 2.00 g of the extract was placed in a plastic tube. After the addition of 200 μL water (or D_2O), the mixture was homogenized with a small spatula and the tube was tapped on a laboratory bench to settle the gel. For the crosslinked material, 2.00 g of the extract was placed in a plastic tube and 200 μL of a 0.5 M $Fe(NO_3)_3$ solution (or 0.5 M $Fe(NO_3)_3$ in D_2O) was added. The mixture was homogenized and the tube tapped to place the gel at the tube bottom. Changes observed upon addition of the iron salt are described below in the Results section. Analogous reactions were carried out in which a 0.5 M solution of $Na_2Cr_2O_7$ (pH ≈ 4.3) was used.

Iron(III) salts are only soluble at appreciable concentrations when aqueous solutions become acidic. Dissolution of $Fe(NO_3)_3$

into water at 0.5 M resulted in a solution of pH ≈ 1.5. We have shown that Fe^{3+} binding to DOPA-containing peptides is pH dependent, with binding beginning in acidic solutions, with Fe(peptide) formation observed at pH ≈ 3.5.[16] More basic conditions facilitate greater metal-peptide interactions, with $Fe(peptide)_3$ forming above pH ≈ 7.5.[16] However, our work with whole protein (molecular weight ≈100 000) has shown that such metal-DOPA interactions occur more readily, regardless of pH.[10] For example, when a protein solution at pH ≈ 1.5 was combined with an $Fe(NO_3)_3$ solution at pH ≈ 1.5, the tris $Fe(DOPA)_3$ complex formed immediately[10] and resulted in extensive crosslinking.[11,15] With ≈75–100 DOPA residues per protein,[6,8,9] a strong chelate effect is likely to be at work, resulting in extensive metal-induced crosslinking regardless of solution pH. We have also shown that this chelation, followed by radical generation and protein oxidation, occurs in these acidic solutions in a manner analogous to that in the adhesive plaques of live mussels.[10] For this current study, the high solution pH required to dissolve the Fe^{3+} reagent will not inhibit crosslinking.

Environmental scanning electron microscopy experiments were performed using an FEI Quanta 200 Environmental SEM instrument. The protein samples were freeze-fractured in Freon and placed in the ESEM instrument immediately. The images were taken at a temperature of −10 °C and a pressure of 2 Torr. Relative humidity in the ESEM chamber was maintained at ≈100% using a Peltier stage at −10 °C. These conditions were employed to minimize solvent loss and condensation, and control etching of the sample. Images were obtained within less than 5 min of the sample reaching the chamber. The ESEM images were recorded multiple times and on multiple samples in order to ensure reproducibility. Representative images are shown in Figure 1. ESEM images from fractured samples at 1 °C (fresh sample, no freeze fracture) and 4.9 Torr would show the same structural features but with weak contrast (not shown here). Good fractures or cuts of the soft gel at room temperature were difficult to obtain. The freeze fracture procedure allows a much better fracture of the sample and slight etching is possible, both of which improve contrast.

The SANS measurements were made on the NG7 instrument at the Center for Neutron Research of the National Institute of Standards and Technology, Gaithersburg, Maryland.[17] Data were collected at room temperature, over a q range of ca. 0.003–0.230 $Å^{-1}$. The scattered intensity was corrected for background and parasitic scattering. The data were circularly averaged to provide intensity versus the momentum transfer q, defined as $q = (4\pi/\lambda)\sin(\Theta/2)$, where Θ is the scattering angle and λ defines the incident neutron wavelength.

Protein gel samples were pressed between 1 mm gap size quartz plates for collection of SANS data. Pressing the gels between quartz plates brought about the formation of a few ≈1 mm sized gaps in the material. For this reason, the SANS intensities are given in averaged units rather than absolute units. Deuterated solvent (D_2O, acetone-d_6) filled the small spaces between the protein gel. The starting gel appeared to have slightly more of these gaps than the crosslinked gel. Despite homogenization of the samples, we were unable to avoid the formation of such heterogeneity.

SAXS experiments were performed at the X10A beamline at the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory (BNL). The q range detected by SAXS is complementary to SANS and is shown in Figure 2.

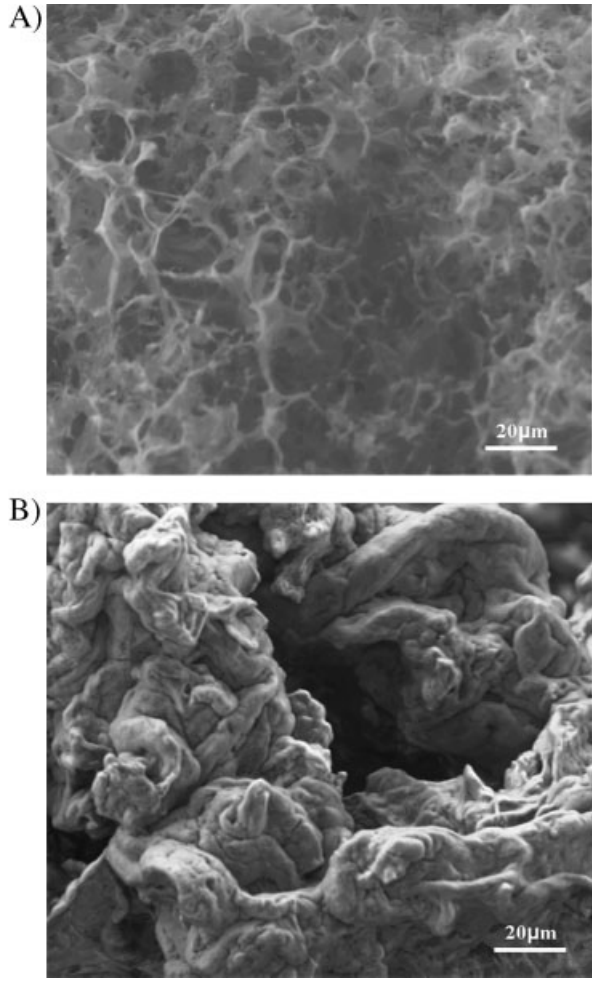


Figure 1. Environmental scanning electron micrographs (ESEM) of (A) the protein gel and (B) the protein gel after crosslinking with iron. The scale bar shown indicates a length of 20 μm.

Frequency sweep and strain sweep experiments were performed on a TA Instruments ARES rheometer with a parallel plate geometry. Both tests were performed at room temperature and on fresh samples. A 35 mm diameter quartz parallel plate geometry was employed with a 1 mm gap distance. The protein gels were cut and pressed between the two plates and allowed to relax for ca. 30 min prior to beginning data collection. This method was usually sufficient to zero normal forces. Multiple samples were used to collect the rheological data, thereby ensuring reproducibility. We obtained good reproducibility of data from the iron crosslinked gel because this sample was only partially crosslinked and was thus easy to press between the plates. The more crosslinked dichromate reference gel had to be cut precisely to fit the shear cell. Modulus measurements obtained before and after data collection indicated no change in the equilibrium structure of the materials within a relative error of ≈9%.

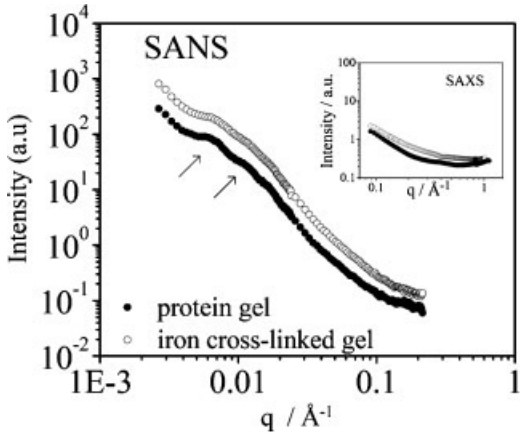


Figure 2. Small angle neutron scattering (SANS) data for the pure protein gel and the gel after crosslinking with iron. For the protein gel, shoulders can be observed at q and $2q$ corresponding to 0.006 and 0.012 $Å^{-1}$. Insert: SAXS intensity versus q for the protein and iron crosslinked gel. Intensity is reported in arbitrary units.

Results

Qualitative Observations of Crosslinking

The starting, non-crosslinked gel extract was of a beige color with a pasty consistency. Addition of water brought about no visible changes. By contrast, addition of the iron solution induced immediate and conspicuous changes. The gel took on a medium-dark brown color from iron addition. This color change was more than that expected from a simple combination of the beige gel and the orange colored iron solution. Mixing of the iron and gel with a spatula led this combination to take on a thicker consistency than the control gel without iron. The iron-containing gel adhered to the mixing spatula more than the gel to which only water was added. Iron also gave a slightly heterogeneous, ''lumpy'' texture to the sample. No conspicuous volume change was observed upon crosslinking beyond the addition of the iron or water solutions. When the crosslinked material was pressed between quartz plates, some separation of solvent from the gel was observed. These qualitative observations on crosslinking are consistent with our earlier reports in which the addition of iron to the gel was found to increase the resistance to compression.[11,15] Crosslinking of the protein with dichromate led to a rubbery and elastic material. Significant contraction and local volume change were observed.

SEM

Figure 1(A) shows a typical SEM image of the starting protein gel, after freezing and fracturing. Many SEM images were inspected and representative ones are shown. The image in Figure 1(A) displays a porous and heterogeneous structure for this material. The pores are of a somewhat consistent size, typically in the ≈15–20 μm range. The wall thickness, between the pores, is of the order of a micron, as roughly measured and averaged from several SEM images. Although not observed directly, these pores are possibly filled with the solvent (water, acetone) used to extract and precipitate this protein-based gel.

The addition of iron, and the subsequent crosslinking, brought about conspicuous textural and morphological changes. The hydrogel superstructure is shown in Figure 1(B) which indicates the clear contrast between the crosslinked protein gels versus the sample without iron (Figure 1(A)). The iron-containing gel formed a more compact and rigid structure. The material now appears to contain large aggregates and interconnected domains. The small pores that were visible before (Figure 1(A)) are no longer observed. Instead the dark areas in Figure 1(B) suggest the presence of much larger pores. The addition of iron may yield greater contrast in the SEM image, relative to the starting gel without iron. The observed morphological changes appear consistent with our previous studies, indicating that iron crosslinks the adhesive precursor proteins and brings about setting of the material.[11,15] Clearly the crosslinking induced structural changes are at a micron scale. As discussed in the following section, the major changes observed by SEM on a micron length scale are not reflected on the nm length scale when monitored by scattering techniques. The strong heterogeneity in the protein and the aggregation after crosslinking may be reasons why the effects of crosslinking are not visible in scattering experiments.

Scattering Techniques

Complementary to the micron level details shown by SEM, scattering techniques such as SANS and SAXS provide structural information on a nanometer length scale. Figure 2 shows SANS data for the protein gel before and after crosslinking. The general appearance of both spectra is, overall, quite similar. For the protein gel weak shoulders can be observed at q and at $2q$ corresponding to 0.006 and 0.012 $Å^{-1}$. This result is characteristic of a regular structure with nanometer scale periodicity. If the shoulder at ≈0.006 $Å^{-1}$ came from a first order interparticle spacing between regular (possibly lamella-like) structures, then the characteristic dimension of this feature would be $d_{SANS} = 2\pi/q_{max}$ ≈ 100 nm. Such a size does not correlate to individual proteins for which a ≈10–20 nm size may be anticipated.[13] The shape of the scattering curve and position of the maxima were generally similar for the protein gel both before and after crosslinking. However, the shoulders were somewhat more exaggerated for the starting gel, prior to crosslinking. Iron did not appear to bring about overall contraction or network expansion on the nanometer level detected by SANS. However, the strong heterogeneity in the protein was increased

even more by crosslinking, which probably comes from increased aggregation. Heterogeneity and aggregation may be reasons why the effects of crosslinking are not visible in the scattering experiments.

At $q > 0.01$ Å^{-1}, SANS and SAXS data shown in Figure 2 follow the same trend and suggest the absence of a "defined structure." This lack of correlation length observed over approximately 2 decades could perhaps indicate a disordered structure. Deviations from any linear I versus q relation in our data, which would have been characteristic for real fractals, may be due to density fluctuations of pores (voids) in a certain q range. A region with a slope of ca. -2.6 could be imagined over one decade in q, between 0.01–0.1 reciprocal Angstroms. A region with a slope of ca. -1.2 was observed below 0.1 reciprocal Angstroms, characteristic of rod-like structures on the nanoscale. For rigid objects such as rod-like systems, a fractal dimension of 1 is expected.[18]

Although understanding this natural gel may be important for developing synthetic mimics, the overall disorder, heterogeneity and aggregation observed make characterization difficult. Nevertheless, similar to other mesoporous materials studied in the past, disorder can be a desirable property.[19]

Rheology Studies

The viscoelastic properties of these protein gels were explored by frequency sweep and strain sweep experiments. Gels were pressed between two quartz plates for an oscillatory shear experiment. Storage and loss moduli G' and G'' were measured as a function of frequency. Strains of both 0.1% and 10% were used. Brittleness in the sample made the use of strains above 10% difficult, due to the sample pushing out of the cell.

Figure 3(A) shows results from a frequency sweep obtained for the protein gel prior to crosslinking. The storage modulus G' of this protein gel was conspicuously dependent upon strain, with 0.1% strain yielding significantly higher G' values than 10% strain. The storage modulus G' was found to be consistently greater than the

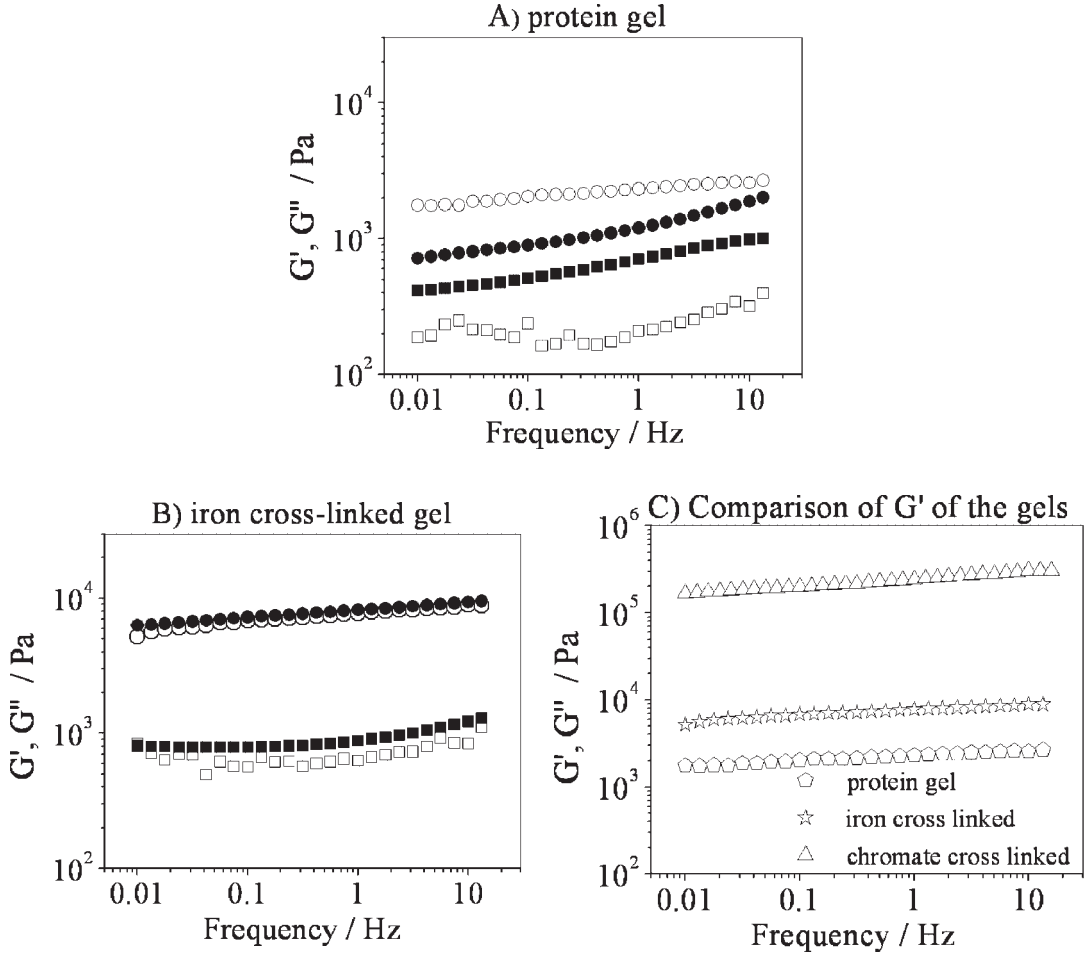


Figure 3. Frequency sweep data for (A) the protein gel and (B) the protein gel after iron-induced crosslinking. After crosslinking, the gel is much stiffer and does not flow when the vial is inverted. G' and G'' are provided at both 0.1% strain and 10% strain. (C) Comparison of G' of the protein gel and the iron and chromate crosslinked protein gels at strain 0.1%.

loss modulus G''. The moduli also displayed frequency dependence, with higher frequencies increasing the observed modulus. A power law relationship appeared to be present with a 0.1 exponent for G' at 10% strain and a 0.06 exponent for G' at 0.1% strain. Data obtained between 0.1–10% strain gave exponents between 0.1 and 0.06 (data not shown). Taken together, these data indicate a ''low rigidity'' of the viscoelastic gel and suggest partial and imperfect crosslinking. A more perfect crosslinked network would have an expected exponent of near zero.

Frequency sweep data for the iron crosslinked gel are provided in Figure 3(B). In stark contrast to the protein gel prior to crosslinking, addition of iron and crosslinking brought about strain-independent behavior. The observed G' modulus was nearly identical for both 0.1% and 10% strains. Moduli G' and G'' showed little frequency dependence over the frequency range measured. It is however possible that the crosslinking shifted the onset of the non-linear regime to higher strains. G' was seen to be significantly greater than G''. Crosslinking provided an elastic G' value of ≈5 000 Pa, approximately three times higher than that found for the non-crosslinked material (Figure 3(C)). The chromate reference sample showed an even higher G' of 150 000 Pa due to stronger crosslinking. When experiments were repeated with protein from new mussels, results could be reproduced within a relative error of 9%. From these data we conclude that this crosslinked material exhibits higher rigidity, especially when compared to the starting gel. Thus the iron affords enhanced storage moduli to the material. While to the eye there is no visible change in volume of the iron crosslinked gel as compared to the protein gel, the reference chromate crosslinked gel showed visible shrinkage which also contributes to the higher moduli. Moduli at 10% strain could not be measured due to wall slip at the cell-sample interface and fracturing of the material.

Strain dependencies of the storage and loss moduli at 1 Hz for both the starting gel and crosslinked material are shown in Figure 4. For both materials, large differences were observed between the elastic G' and the loss G'' moduli, indicating viscoelastic properties. The protein gel prior to addition of iron displayed a small viscoelastic ''plateau'' and a rapid decrease in modulus starting at ≈3% strain (Figure 4(A)). These non-linear effects indicate that the protein gel starts to flow at higher strains. This is possible because the iron crosslinked protein is only partially crosslinked, thus allowing domains of crosslinked gel to slide past each other. This effect can also be visualized with optical microscopy. At a strain of 38%, a reproducible crossover point between G' and G'' was observed (Figure 4(A)). At strains much higher than 40%, fracturing and disruption of the gel structure was possible. By contrast, the modulus of the crosslinked gel did not change significantly as a function of deformation (Figure 4(B)). This property is characteristic of crosslinked networks. The addition of iron affords a more rigid and solid-like structure with a relatively constant elasticity.

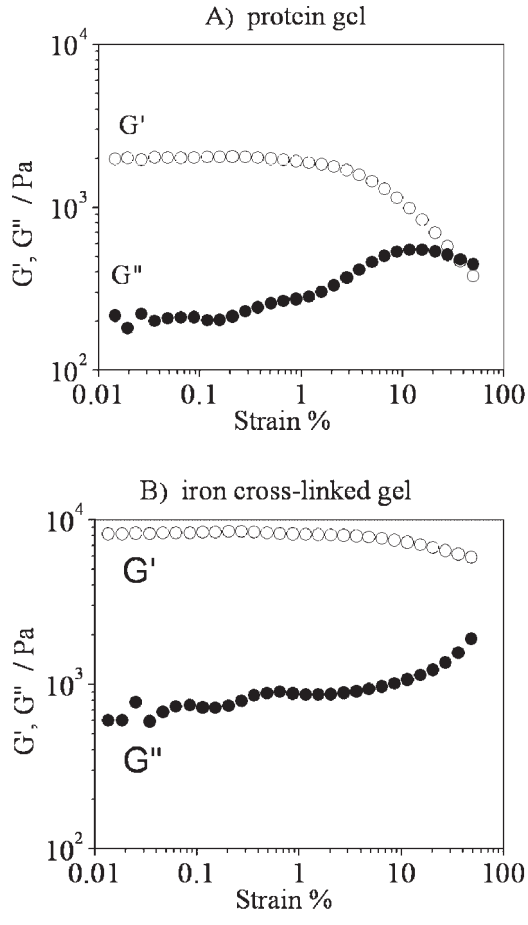


Figure 4. Strain sweep measurements of (A) the starting protein gel and (B) the iron crosslinked gel. The materials moduli G' and G'' are shown at a constant frequency of 1 Hz. The moduli are strongly dependent on the crosslinking agent. In addition to the crosslinking agent, the high modulus of the reference chromate crosslinked gel is due to contraction of the gel during the crosslinking and thus due to higher protein concentration.

Discussion

Chemical studies performed on marine mussel adhesives indicated that the material forms by iron-induced crosslinking of proteins.[10,11] Three DOPA-containing protein strands bind one iron to provide a nucleus for the crosslinking process.[10] Subsequent reaction of the $Fe(DOPA)_3$ complex with molecular oxygen (O_2) then yields an organic radical in the protein.[10] Reactivity of this radical species may lead to further protein-protein crosslinks and the formation of protein-surface bonds. With up to 100 DOPA residues per protein[20] and high levels of iron,[21,22] the mussel adhesive apparatus is poised for extensive crosslinking and biomaterial formation.

Previous studies in our laboratory showed that this crosslinking brought about by iron was concentration dependent.[11,15] Higher concentrations of iron yielded more curing of the material.[11,15] The 0.5 M iron solutions used here provide an upper limit for what can be used practically (e.g., solubility limitations). Once mixed into the protein gel, the final iron concentration is 45×10^{-3} M. Calculating an equivalent solution concentration of iron in mussel adhesive plaques from the available data[21,22] provides a similar number at $\approx 30 \times 10^{-3}$ M. Thus the iron level employed here for crosslinking approximates to that used by mussels. Nevertheless, on a qualitative basis, our rheology data suggest that only partial crosslinking occurs within an imperfect network.

Intact mussel adhesive plaques, produced by live animals, appear to be amorphous materials rather than crystalline solids (unpublished results). Although further crosslinking may be possible, as evidenced here by the addition of dichromate to the proteins, the animals bring about plenty of crosslinking to adhere quite well. In general, adhesion can be viewed as a balance between surface interactions and bulk-bulk bonding.[23] Too many material-surface interactions may come at the expense of bulk-bulk bonding, thereby yielding a monolayer of material but no adhesion of the bulk material. Conversely, too many bulk-bulk interactions could generate a hardened material with no adhesive characteristics. If mussels were to crosslink their proteins too much, such as found here with dichromate, adhesion might be minimal. At the same time, a lack of crosslinking, shown here with the starting protein gel, might not permit sufficient adhesion. Iron could provide a ''just right'' degree of crosslinking, somewhere between too little and too much, thereby allowing mussels to affix themselves to a variety of surfaces.

Conclusion

The experiments described here provide micron and nanometer scale structural insights into this protein gel before and after curing. SEM images of the starting extract showed a porous structure with features on a micron scale. Conspicuous changes were encountered on the addition of iron. The somewhat regular small pores of the starting gel were transformed to larger aggregates in a more compact and rigid matrix typically observed for partially crosslinked gels. The SANS data provided nanometer scale insights, a complement to the micrometer sized features seen by SEM. In contrast to the micrometer level structure, little structural change was found on a nanometer level before versus after crosslinking. The general feature size observed by SANS ($\approx$100 nm) suggests periodic structure. Qualitative interpretation of these results indicates that the crosslinking process only partially destroys any regular structure. At high q, SANS and SAXS data followed the same trend and showed the absence of ''periodic defined structure''.

Rheology measurements showed significant differences in the gel properties brought about by crosslinking. The addition of iron created a more rigid and solid-like elastic material. Such materials property changes appear consistent with the significant morphological effects of crosslinking observed by ESEM. Thus we are led to believe that the morphological and material property changes, such as stiffness, are brought about by chemical crosslinking. Taken together, these data suggest partial and imperfect crosslinking. Partial crosslinking may be a result of reactive radicals trapped within a solid matrix, thereby not able to access other protein chains for further coupling.

We have begun exploring the biomedical potential of these materials. Preliminary experiments show that this protein gel has desirable elastic moduli and adhesive properties for connecting skin.[24] The biological origin, porosity, strength and elasticity of this material make it a good candidate for future efforts in developing synthetic cell growth scaffolds, drug delivery systems and surgical adhesives. That said, the irregular structure and morphology of an extracted biological material may have limitations. Thus synthetic compounds might be best for such biomedical applications. In order to design these new materials, we will take cues from nature. We hope that the characterization work presented here will provide inspiration for those designing new classes of synthetic crosslinking gels.

Notes

The identification of any commercial or trade name does not imply endorsement or recommendation by the National Institute of Standards and Technology.

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