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Chemistry between crosslinks affects the properties of peptide hydrogels

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ABSTRACT

Protein hydrogels were prepared by substituting ovalbumin with different concentrations of ethyl vinyl sulfone (EVS) or acrylic acid (AA) and crosslinking with divinyl sulfone (DVS). Fourier transform-infrared (FT-IR) spectroscopic studies confirmed the addition of EVS, AA, and DVS onto the protein. Swelling was assessed as a function of pH in the range of 2.5 to 9.4 and ionic strength. The elastic modulus of the gels was determined in shear and compression. Stress relaxation was assessed in compression. The substituent highly affected swelling and modulus with both hydrogels displaying non-Gaussian behavior in the range of hydrogel environments studied. Acrylic acid substituted ovalbumin exhibited a decreasing modulus with increasing swelling behaving as a polyelectrolyte with low added salt content. Ethyl vinyl sulfone substituted ovalbumin displayed an increasing modulus with swelling originating in the finite extensibility of the highly swollen chains. AA-substituted ovalbumin showed higher modulus and reduced swelling compared to EVS-substituted ovalbumin because of its ability to hydrogen and ionic bond to other molecules.

1. Introduction

The ability of hydrogels to swell to many times their original volume in water is one interesting property that makes them desirable for biological applications. Hydrogels are used as stimuli-responsive materials [1–3] often termed smart materials [4,5] for drug delivery applications [6–8], 3D scaffolds for cell culture and tissue engineering [9–14], and superabsorbent gels in the sanitary industry [15]. The properties of hydrogels are often tailored to suit the end use. Hydrogels made from synthetic polymers have been used because of their availability and superior swelling properties [16–19]. Hydrogels made of poly(acrylamide), poly(acrylic acid), and poly(methacrylate) are common. However, for the biomedical industry it is desirable to have a hydrogel that is biocompatible and biodegradable. Hence the research focus in recent years has shifted to hydrogels made of natural polymers. Hydrogel synthesis by crosslinking polymers like chitosan [20], dextran [3,7,21], and other polysaccharides [22] has been attempted. Efforts were also made to create hydrogels by crosslinking proteins [6,23–25].

Elastin and collagen based hydrogels have been prominently featured because of their elastic nature [26–30]. Annabi et al. reported the synthesis of porous hydrogels at atmospheric conditions and in the presence of high pressure CO_2 and obtained gels with modulus around 3–18 kPa and swelling ratio of 9–18 (g liquid/g protein) [26]. Leach et al. used α-elastin that swelled up to 250% and had modulus around 90–100 kPa [28]. *De novo* synthesis of proteins or peptides mimicking elastin is also one active research area [31–35]. Wang et al. reported the synthesis of a protein that formed coiled coils and attached it to synthetic polymers to give hybrid hydrogels [34]. Genetically engineered Calmodulin protein that can bind to calcium and form hydrogels was reported by Ehrick et al. [33]. The disadvantages of elastin are that it is scarce, expensive, and difficult to solubilize. In the case of *de novo* synthesis, the yield of the fermentation process is low forcing the product into high priced niche markets.

The mechanical properties of hydrogels are also important in biological applications [26]. For soft materials like hydrogels there are not many established techniques for measuring the mechanical properties. Commonly used mechanical characterization techniques are rheology and unconfined compression [36–38]. New methods for accurate determination of the mechanical properties are being researched [39]. Mathematical models are also being developed to estimate the properties of hydrogels that are difficult to determine experimentally [1,11,40].

The properties of hydrogels are determined by crosslink density and the chemistry between crosslinks. Therefore, it is possible to prepare protein-based hydrogels with specific crosslink density and chemistry between crosslinks by chemically substituting an existing protein. This can be achieved using a simple nucleophilic addition reaction where vinyl groups react with amines and thiols present on the protein [41]. Ovalbumin is chosen as a "model" protein because its structure and sequence have been well characterized and it has a variety of amine and thiol-containing amino acids for chemical

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substitution [42]. Ovalbumin is crosslinked with divinyl sulfone and substituted with acrylic acid, a more hydrophilic substituent, or ethyl vinyl sulfone, a less hydrophilic substituent, to note the effect of chemistry between crosslinks on hydrogel properties. The degree of swelling, static and dynamic modulus, crosslink density, and state of hydrogen bonding are experimentally determined and compared as a function of hydrogel chemistry.

2. Materials and methods

2.1. Materials

Technical grade ovalbumin (UniProt P01012, mol. wt. 49.8 kDa), dithiothrietol (DTT, mol. wt. 154.5 g/mol), divinyl sulfone (DVS, mol. wt. 118.15 g/mol) and acrylic acid (AA, mol. wt. 72.01 g/mol) were purchased from Sigma Aldrich (USA). Dialysis membranes with 3500 g/mol molecular weight cut off and 95% ethyl vinyl sulfone (EVS, mol. wt. 120.17 g/mol) were obtained from VWR (USA).

2.2. Synthesis of ovalbumin hydrogels

The hydrogels were prepared using Michael type addition reactions. The amines on the protein reacted with the vinyl group of AA, EVS, and DVS. Ovalbumin (5 g) was added to 50 mL of deionized water followed by the addition of 0.017 g of DTT. DTT was used to reduce the cysteine bonds and make the protein soluble in water. The pH of the solution was adjusted to 9.0 using borate buffer. After 30 min, the protein was crosslinked by adding 0.5 g (427 μL) of DVS to the solution and reacting for 24 h at 30 °C. The reacted solution was dialyzed against deionized water for another 24 h. The water was changed every 12 h. Solid disks were prepared by pouring the dialyzed solution into molds with a diameter of 12 mm and depth of 3 mm at regular intervals until a thick gel formed. Solid disks were formed by evaporating the water at ambient conditions.

2.3. Synthesis of AA- and EVS-substituted ovalbumin hydrogels

Ovalbumin was solubilized as above and AA or EVS was added to the solution. The pH dropped to 4.0 after adding AA and was adjusted back to 9.0 using borate buffer. The reaction was run for 24 h at 30 °C and the solution was dialyzed for 24 h again changing the water after 12 h. After dialysis, the pH of the solution was adjusted to 9.0, 0.5 g (427 μL) of DVS was added, and the reaction was run for another 24 h followed by 24 h of dialysis. Solid disks were prepared as described above. Three molar ratios of AA or EVS to ovalbumin (0.2, 0.4 and 0.5) were used. These corresponded to the added volumes of 153 μL, 306 μL, and 383 μL for AA and 265 μL, 530 μL, and 663 μL for EVS. It was found that crosslinking before substituting interfered with the substitution reaction but substituting before crosslinking did not. Substituting first left the protein molecules soluble and spaced far apart allowing for an easy crosslinking second step. Crosslinking first did not allow this to happen. The combined crosslinking and substitution levels were within a 1:1 molar ratio, where it was found that greater than 95% reaction occurred [43,44]. The following abbreviated names were used. DVS-crosslinked ovalbumin hydrogel (OD), ovalbumin substituted with AA and crosslinked with DVS: OAD0.2, OAD0.4, and OAD0.5 (corresponding to the AA:ovalbumin molar ratio 0.2, 0.4, and 0.5, respectively), and ovalbumin substituted with EVS and crosslinked with DVS: OED0.2, OED0.4 and OED0.5.

2.4. Swelling studies

Dry gels were weighed (w_d) and placed in potassium phosphate buffer solution containing penicillin at the corresponding experimental pH. Hydrogels were hydrated for 20 h, which was found to be long enough to reach equilibrium swelling. The hydrated gels were removed from the solution, blotted with Kim wipes to remove excess water, and weighed again (w_h). The equilibrium swelling ratio (Q) was calculated from

$$Q = \frac{V_{eq}}{V_{dry}} \quad (1)$$

where V_{eq} was the hydrated hydrogel volume and V_{dry} was the dry hydrogel volume [45]. The volumes were found from w_d and w_h by using the polymer density of 1.41 g/cm^3, calculated using Ref. [46], and a composite density of a swollen hydrogel.

2.5. Fourier transform-infrared (FT-IR) spectroscopy

A Thermo-Nicolet 6700 FT-IR spectrometer with a Smart Orbit diamond ATR cell was used. Dry hydrogel samples were ground into a fine powder using a mortar and pestle and placed on the ATR crystal. The spectrum was collected over the range of 3400–400 cm^{-1} with a

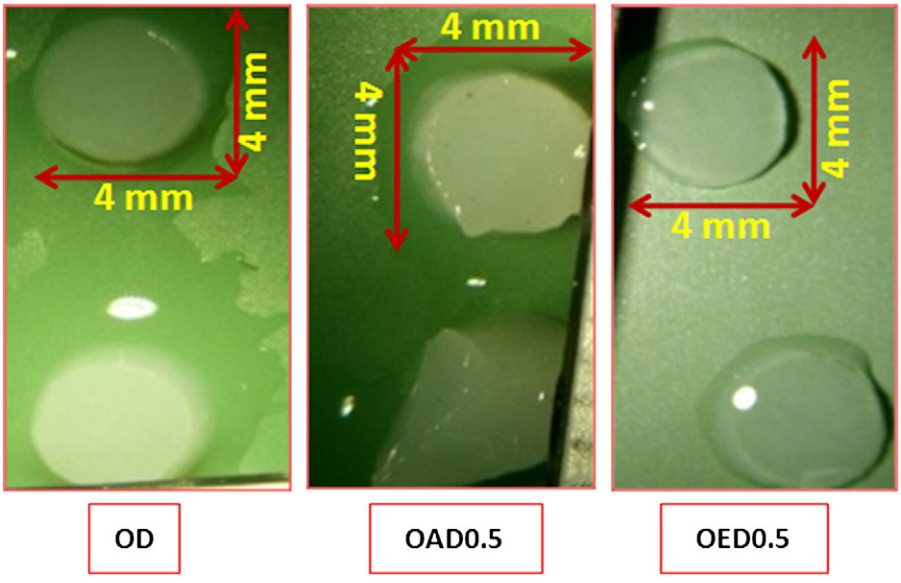

Fig. 1. Photographs of hydrated protein hydrogels.