A Facile Way to Tune Mechanical Properties of Artificial Elastomeric Proteins-Based Hydrogels

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Supporting Information

ABSTRACT: Protein-based hydrogels have attracted considerable interests due to their potential applications in biomedical engineering and material sciences. Using a tandem modular protein (GB1)₈ as building blocks, we have engineered chemically cross-linked hydrogels via a photochemical cross-linking strategy, which is based on the cross-linking of two adjacent tyrosine residues into dityrosine adducts. However, because of the relatively low reactivity of tyrosine residues in GB1, (GB1)₈-based hydrogels exhibit poor mechanical properties. Here, we report a Bolton−Hunter reagent-based, facile method to improve and tune the mechanical properties of such protein-based hydrogels. Using Bolton−Hunter reagent, we can derivatize lysine residues with phenolic functional groups to modulate the phenolic (tyrosine-like) content of (GB1)₈. We show that hydrogels made from derivatized (GB1)₈ with increased phenolic content show significantly improved mechanical properties, including improved Young’s modulus, breaking modulus as well as reduced swelling. These results demonstrate the great potential of this derivatization method in constructing protein-based biomaterials with desired macroscopic mechanical properties.

INTRODUCTION

Elastomeric proteins are present in a wide range of biological systems, where they have evolved precise structures and properties to fulfill their specific biological functions.¹,² They exhibit rubber-like elasticity and can undergo high deformation without rupture under stress. Upon removing the stress, they can recover their original state. The development of single molecule force spectroscopy techniques has enabled the characterization of the mechanical properties and architectural design of these natural elastomeric proteins in great detail.³−⁸ In particular, the single molecule force spectroscopy studies of molecular determinants of the mechanical stability of elastomeric protein domains have yielded some general design principles of natural elastomeric proteins, which have helped guide the design of novel artificial elastomeric proteins with defined and improved nanomechanical properties.⁹−¹⁴ These designed artificial proteins have expanded the toolbox of elastomeric proteins and offered exciting opportunities to exploit them as building blocks for the construction of protein-based biomaterials.¹⁵,¹⁶

Recently, our single molecule force spectroscopy studies revealed that a nonmechanical protein GB1¹⁷ exhibits mechanical properties¹⁸ that are similar to those of the constituting Ig domains of the giant muscle protein titin, a classical natural elastomeric protein.³ Polyprotein (GB1)₈ consisting of eight identical tandem repeats of GB1, shows a unique combination of mechanical features, including high mechanical stability, fast folding kinetics, high folding fidelity, low mechanical fatigue during repeated stretching−relaxation cycles, and ability to fold against residual forces. These fine features make (GB1)₈ polyprotein an ideal artificial elastomeric protein.¹⁹ By combining GB1 with an ideal entropic spring-like elastomeric protein resilin,²⁰ we have constructed artificial elastomeric proteins that mimic the structure and mechanical properties of the muscle protein titin. Using a ruthenium complex-catalyzed photochemical cross-linking method, we successfully cross-linked this mini-titin mimetic protein into hydrogels with three-dimensional network structure that exhibit mechanical properties mimicking those of the passive elastic properties of muscles.¹⁶

The ruthenium complex-catalyzed photochemical method cross-links two adjacent tyrosine residues into a dityrosine adduct.²⁰,²¹ We noted that, although each GB1 domain has three tyrosine residues, the cross-linking of GB1-resilin is mainly through the tyrosine residues in resilin, as biomaterials constructed from (GB1)₈ display poor mechanical properties compared with GB1-resilin-based biomaterials due to lower cross-linking density and poor quality of the three-dimensional network.¹⁶

To improve the mechanical properties of (GB1)₈-based hydrogels and develop a general method to tune the mechanical properties of tandem modular protein-based biomaterials, we...
have employed a lysine derivatization method based on Bolton—Hunter reagent (BH reagent), which has been used to derivatize gelatin and successfully improve the elastic properties of gelatin-based biomaterials. Using this method, we successfully modulated the phenolic (tyrosine-like) content of (GB1)₈. Biomaterials made of derivatized (GB1)₈ show significantly improved mechanical properties, including improved Young’s modulus and breaking modulus, as well as reduced swelling upon increasing the phenolic content. These results demonstrate the great potential of this derivatization method in constructing protein-based biomaterials with desired macroscopic mechanical properties.

**MATERIALS AND METHODS**

**Materials.** Chemicals, including metal-complex tris(2,2′-bipyridyl)dichlororuthenium(II) hexahydrate ([Ru(bpy)₃]²⁺Cl₂·6H₂O), N-succinimidyl-3-[4-hydroxyphenyl]propionate (BH reagent), N,N,N-dimethylformamide (DMF), ammonium persulphate (APS), potassium dihydrogen phosphate, sodium phosphate dibasic anhydrous, potassium chloride, sodium chloride, sodium bicarbonate, sodium dihydrogen phosphate, sodium borate, sodium hydroxide, and hydrochloric acid were purchased from Sigma-Aldrich and Fisher Scientific.

**Protein Engineering.** The gene encoding (GB1)₈ polyprotein was constructed as described and its full amino acid sequence is shown in Figure S1 (Supporting Information). The polyprotein (GB1)₈ was overexpressed in Escherichia coli (E. coli) DH5α strain and purified using Co²⁺-affinity chromatography following published protocols.

The yield of the (GB1)₈ polyprotein was about 60 mg per liter of culture. The purity of the purified protein is above 90%, as estimated from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using AlphaEaseFC software (Version 4.0.0, Alpha Innotech Corporation, San Leandro, CA). The purified protein was then dialyzed against deionized water for 24 h to remove all the salt from elution buffer. During dialysis, water was changed every 6 h. The protein was then lyophilized after dialysis.

**Derivatization of (GB1)₈ by BH Reagent.** To derivatize (GB1)₈ polyprotein using BH reagent, the lyophilized (GB1)₈ polyprotein was redissolved in 100 mg/mL sodium borate buffer (SB buffer) to a concentration of 100 mg/mL (pH 8.5). In a typical (GB1)₈ polyprotein derivatization experiment, 100 mg of polyprotein was used to react with BH reagent (1 M in DMF) in 1 mL SB buffer at a given molar ratio polyprotein/BH reagent (1:1, 1:8, or 1:15) at 37 °C for 1 h. After reaction, the reaction mixture was extensively dialyzed against deionized water at 4 °C, to remove small molecule reactants and salt, and then lyophilized.

**Preparation of Hydrogels.** (GB1)₈- and derivatized (GB1)₈-based hydrogels were prepared by following the well established protocol. A typical cross-linking reaction mixture contains 200 mg/mL of polyprotein, ~200 μM [Ru(bpy)₃]²⁺Cl₂ and 50 mM APS in PBS buffer. The trapped air bubbles can be removed by centrifugation at 12 000 rpm for 3 min. The solution was immediately cast into a custom-made plexiglass mold with inner diameter of 8 mm, outer diameter of 10 mm, and height of 3 mm. The sample was then irradiated for 10 min using a 200 W fiber optical white light source at room temperature. The irradiation was 10 cm away from the mold. The ring-shaped biomaterial was then taken out from the mold and stored in PBS buffer containing 0.05% (w/v) sodium azide.

**Quantification of Dityrosine and Dityrosine-Like Cross-Links in the Hydrogel.** The presence of dityrosine and dityrosine-like compounds in the (GB1)₈-based biomaterials after photochemical cross-linking reaction was demonstrated by fluorescence test after acid hydrolysis. These compounds have particular emission at 410 nm when excited at 315 nm. For quantification of the dityrosine and dityrosine-like compounds generated in these (GB1)₈-based biomaterials, dityrosine was prepared as a standard compound by one step oxidation from t-tyrosine. After purification by HPLC, it was characterized by ESI-MS and fluorescence spectroscopy. Typically, a hydrogel sample (~2 mg) reacts with 1 mL HCl (6 N) in a sealed 1.5 mL centrifuge tube in the metal heat block at 105 °C for 2 h to make sure full hydrolysis of amide bonds. Then, 100 μL of acid hydrolysis product was transferred into a new 1.5 mL centrifuge tube and neutralized by NaOH (5 M). Next, 100 mM Na₂CO₃—NaHCO₃ buffer (pH 9.9) was added to the tube to 1 mL final volume. Fluorescence spectra of all the samples were measured. According to the fluorescence—concentration standard curve of dityrosine (Figure S3, Supporting Information), the yield of dityrosine and dityrosine-like products in the hydrogel was then quantified.

**Tensile Test and Swelling Measurement.** The tensile tests were performed using an Instron-5500R tensometer with a custom-made force gage. The ring-shaped biomaterial was stretched at an extension rate of 50 mm/min in PBS at 20 °C. Every sample was tested at 4 different strains in which 3 cycles were performed. For the swelling measurement, the ring-shaped biomaterials were weighted immediately after being taken out of the mold, and the weight was recorded as Wᵢ. The rings were then incubated in PBS, respectively, at room temperature. After 24 h, the rings were blotted onto tissue paper to remove excess buffer and then weighted as Wᵢ. The swelling ratio was calculated according to the formula: swelling ratio (%) = [(Wᵢ − W₀)/W₀] × 100%.

**RESULTS AND DISCUSSION**

**Derivatization of (GB1)₈ by BH Reagent.** GB1 contains three tyrosine residues, two of which are surface accessible and can be potentially cross-linked into dityrosine. However, the reactivity of these two tyrosine residues is not high, leading to (GB1)₈ hydrogels with poor mechanical properties. To improve the mechanical properties of (GB1)₈ hydrogels, it is imperative to increase the cross-linking density and improve the quality of the three-dimensional network structure of (GB1)₈ hydrogels. To achieve this goal, we employed the BH derivatization method.

BH reagent has been widely used to label proteins with acylation reaction. The exposed amino groups in the lysine residues of a protein, which are strongly nucleophilic in the
sodium borate buffer (pH 9.5), attack the ester carbonyl group of BH reagent to generate the new amide bond, resulting in lysine residues derivatized with 4-hydroxylphenyl propionyl group (Figure 1). This functional group will undergo a tyrosine-like photochemical cross-linking reaction catalyzed by ruthenium complex to form dityrosine-like adducts. This method has been used to derivatize gelatin from different sources, and the resultant gelatins can be cross-linked into a highly elastic tissue sealant by photopolymerization.23

Each GB1 domain contains six lysine residues; thus, it is possible to use BH reagent to derivatize (GB1)₈ to significantly increase the phenolic content of the polyprotein so that the cross-linking density of the hydrogels can be significantly improved, as the phenolic functional group is highly accessible to solvent. Here, we use BH reagent to derivatize GB1 domains to increase the phenolic content of (GB1)₈ and improve the mechanical properties of the resultant hydrogel (Figure 2).

To systematically investigate the effect of lysine derivatization on the mechanical properties of the resultant hydrogels, we chose three different molar ratios between (GB1)₈ and BH reagent, 1:1, 1:8, and 1:15, to perform the protein modification reaction. The products of (GB1)₈ derivatized by different amounts of BH reagent were labeled as (GB1)₈-Rₓ₁, (GB1)₈-Rₓ₂, and (GB1)₈-Rₓ₃. We used matrix assisted laser desorption ionization-mass spectrometry (MALDI-MS) to estimate the number of lysine residues in the (GB1)₈ polyproteins that were derivatized with 3-(4-hydroxyphenyl)-propionyl group at the ε-amino group. The result is shown in Table S1 (Supporting Information). On average, one, five, and ten out of 48 lysine residues of (GB1)₈ were modified in the (GB1)₈-Rₓ₁, (GB1)₈-Rₓ₂, and (GB1)₈-Rₓ₃, respectively.

We found that the 20% aqueous solution of (GB1)₈ can be rapidly (~10 s) cross-linked into solid and transparent hydrogels using a ruthenium-catalyzed photochemical method upon white light illumination. The presence of dityrosine and dityrosine-like cross-links in the (GB1)₈-based biomaterials after the photochemical cross-linking reaction was evident from their characteristic blue fluorescence upon ultraviolet illumination (Figure 3A). On the basis of the fluorescence standard curve of purified dityrosine compound (Figure S3, Supporting Information), we quantified the yield of dityrosine and dityrosine-like products in the hydrogels after acid hydrolysis of the hydrogel. The result indicates that the cross-linking density indeed increases with the number of lysine residues being derivatized by BH reagent under the same cross-linking condition (Figure 3B).

Mechanical Properties of (GB1)₈-Based Hydrogels Are Improved with the Increase of Cross-Linking Density. Having characterized the cross-linking density of the (GB1)₈-based hydrogels, we measured the macroscopic mechanical properties of the hydrogel to probe the relationship between the cross-linking density and mechanical properties of hydrogels. Figure 4 shows typical stress–strain curves of the
hydrogels constructed from 200 mg/mL aqueous solution of (GB1)$_h$ with different derivatization level.

As shown in Figure 5A, the hydrogel constructed from unmodified (GB1)$_h$ is mechanically weak, exhibiting a Young’s modulus of $\sim 14$ kPa at 15% strain, and typically breaks at a strain $\sim 58\%$. After derivatization, Young’s modulus of (GB1)$_h$-R$_x1$-based biomaterials at 15% strain increased to $\sim 25$ kPa, even only one out of 48 lysine residues was derivatized in the (GB1)$_h$ polyprotein, suggesting that these modified phenolic groups are highly reactive during the photochemical cross-linking reaction and efficient to form cross-linking points. When four more lysine residues were modified, the Young’s modulus increased to more than 50 kPa at 15% strain. It is interesting to note that further doubling of the lysine derivatization level (with five more lysine residues) only led to a slight increase of the Young’s modulus ($62.9 \pm 2.1$ kPa for (GB1)$_h$-R$_x3$-based biomaterials). This behavior is possibly due to the formation of more intramolecular, rather than intermolecular, dityrosine cross-links, as intramolecular cross-links will not contribute to the improvement of mechanical stability of hydrogels.

Moreover, other aspects of the mechanical properties, such as breaking modulus, breaking stress, and breaking strain, of hydrogels also showed significant improvement when the cross-linking density increases (Figure 5B–D). The breaking stress and breaking modulus were also improved by $\sim 10$-fold in the derivatized (GB1)$_h$-based biomaterial (GB1)$_h$-R$_x3$ compared with the (GB1)$_h$-based one, while the breaking strain only shows a slight increase. These results suggest that the increase of cross-linking density improves the quality of the three-dimensional network and makes (GB1)$_h$ hydrogels stronger without sacrificing extensibility.

It is of note that the apparent hysteresis between the stretching–relaxation traces also shows dramatic increase when the cross-linking density increases. In our previous study, the hysteresis observed in the GB1-resilin-based biomaterials is largely attributed to the unfolding of a small number of GB1 domains. The observed increase of hysteresis upon increasing the cross-linking density of hydrogels is consistent with the picture of domain unfolding. Upon increasing the cross-linking density, the stress (or force) per (GB1)$_h$ polyprotein chain increases. As a consequence, the probability of forced unfolding of GB1 domains also increases, leading to the increased hysteresis at the same strain.

Swelling of the Hydrogel Is Suppressed by the Increase of the Cross-Linking Density. The underivatized (GB1)$_h$-based hydrogel swelled by approximately 60% of their initial volumes in PBS buffer in 24 h (Figure 6). Upon

Figure 4. Representative stress–strain curves of (GB1)$_h$- and BH reagent derivatized (GB1)$_h$-based biomaterials.

Figure 5. Mechanical properties of (GB1)$_h$ and derivatized (GB1)$_h$-based materials can be modulated by adjusting the lysine-modification level of (GB1)$_h$. (Experimental data is presented as average ± standard deviation.)
increasing the cross-linking density, the swelling of the modified (GB1)₈-based hydrogel is suppressed significantly. For example, the swelling ratio of (GB1)₈-Rx3-based hydrogel biomaterial decreased by ∼2/3 compared with that of the original (GB1)₈ hydrogel.

■ DISCUSSION

Developing new generations of protein-based biomaterials with tailored and well-defined mechanical properties has become increasingly important. The development of the ruthenium-catalyzed photochemical cross-linking strategy has opened new avenues toward engineering elastomeric biomaterials using native as well as recombinant proteins as building blocks. For example, Elvin and co-workers have pioneered the use of such strategies to engineer highly elastic and resilient biomaterials based on resilin, whose resilience is much improved over synthetic rubber materials. In our recent work, we engineered biomaterials using titin-mimetic artificial elastomeric proteins that exhibit mechanical properties that mimic the passive elastic properties of muscles. Researchers are also exploring potential biomedical applications of such new materials. For example, tissue sealants of much improved mechanical properties will make these hydrogels suitable for diverse applications, including biomedical applications. For proteins having a limited number of tyrosine residues for photochemical cross-linking, the method based on BH reagent provides a convenient way to utilize more proteins as building blocks for engineering protein-based biomaterials. We anticipate that this method will allow the use of a much wider range of proteins for engineering protein-based hydrogels for different applications.

■ ASSOCIATED CONTENT

 Supporting Information
The amino acid sequence of (GB1)₈ polypeptide; a table listing the molecular weight of (GB1)₈ and derivatized (GB1)₈ determined via MALDI-MS; fluorescence spectrum of dityrosine and standard fluorescence–concentration curve of dityrosine. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
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■ REFERENCES


