ADVANCED FUNCTIONAL MATERIALS
www.afm-journal.de

Rationally Designed Dynamic Protein Hydrogels with Reversibly Tunable Mechanical Properties

*Na Kong, Qing Peng, and Hongbin Li**

Protein hydrogels have attracted considerable interest due to their potential applications in biomedical engineering. Creating protein hydrogels with dynamic mechanical properties is challenging. Here, the engineering of a novel, rationally designed protein-hydrogel is reported that translates molecular level protein folding-unfolding conformational changes into macroscopic reversibly tunable mechanical properties based on a redox controlled protein folding-unfolding switch. This novel protein folding switch is constructed from a designed mutually exclusive protein. Via oxidation and reduction of an engineered disulfide bond, the protein folding switch can switch its conformation between folded and unfolded states, leading to a drastic change of protein's effective chain length and mechanical compliance. This redox-responsive protein can be readily photochemically crosslinked into solid hydrogels, in which molecular level conformational changes (folding-unfolding) can result in significant macroscopic changes in hydrogel's physical and mechanical properties due to the change of the effective chain length between two crosslinking points in the protein hydrogel network. It is found that when reduced, the hydrogel swells and is mechanically compliant; when oxidized, it swells to a less extent and becomes resilient and stiffer, exhibiting an up to fivefold increase in its Young's modulus. The changes of the mechanical and physical properties of this hydrogel are fully reversible and can be cycled using redox potential. This novel protein hydrogel with dynamic mechanical and physical properties could find numerous applications in material sciences and tissue engineering.

1. Introduction

The ability to rationally design and engineer protein hydrogels with specific properties has attracted considerable attention due to their promise in biomedical engineering applications.[1–5] Protein hydrogels are biologically friendly, and can provide advantageous artificial microenvironments that mimic many aspects of native cellular environment, as well as the extracellular matrix (including high water content and mechanical properties that mimic that of soft tissues). These properties could facilitate drug delivery, cell proliferation and tissue engineering applications.[1,2,5] Most protein-based hydrogels provide a static, bio-mimetic, three-dimensional environment whose properties lack the dynamic, spatiotemporal changes common in cellular and extracellular matrix environments.[6,7] Thus, it is of great interest and importance for applications in basic biological studies as well as tissue engineering to engineer protein-based hydrogels whose properties can be regulated dynamically using external user-defined triggers.

The mechanical properties of hydrogels play important roles in modulating cell-matrix interactions, as well as regulating a variety of biological processes, such as cell proliferation and differentiation.[8–10] Thus, attempting to design hydrogels with tunable mechanical properties has generated considerable research interest.[6] Progress in polymer synthesis has led to novel approaches towards engineering polymer-based hydrogels with mechanical properties that are responsive to external stimuli, such as ligand, light, heat and pH.[11–21] For example, engineered polymer hydrogels can increase or decrease their Young's modulus via photo-mediated crosslinking or photolytic reactions.[13,22] Despite these advances, designing protein-based dynamic hydrogels with tunable mechanical properties have been challenging. Here, we report a novel, rationally designed protein-hydrogel that translates molecular level folding-unfolding conformational changes into macroscopic reversibly tunable mechanical properties.

2. Results and Discussion

2.1. Design Principle of Dynamic Protein Hydrogels with Tunable Mechanical Properties

Hydrogels are water-swollen polymer networks. Their mechanical properties (stress-strain relationships) can be analyzed using the classical statistical theory of rubber elasticity:[23,24]

$$\sigma = NRT\upsilon^{1/3} \cdot \left(1 - \frac{2M_c}{M}\right) \cdot \left(\alpha - \frac{1}{\alpha^2}\right) \quad (1)$$

where σ is the stress, N the crosslink density (equal to ρ/Mc), R the gas constant, T the absolute temperature, υ the volume

N. Kong, Dr. Q. Peng, Prof. Dr. H. Li
Department of Chemistry
The University of British Columbia
2036 Main Mall, Vancouver, BC V6T 1Z1, Canada
Fax: (+1)-604–822–2847
E-mail: Hongbin@chem.ubc.ca

DOI: 10.1002/adfm.201402205

fraction of rubber in the swollen sample, M_c the molecular mass between crosslinks, M the primary molecular mass and α the extension ratio. The Young's modulus of hydrogels is directly related to the crosslinking density N, and inversely related to the chain length between crosslinking points M_c. To modulate the Young's modulus of hydrogels, it is necessary to modulate the chain length between crosslinks and/or the crosslinking density of hydrogels. Previous methods of engineering dynamic polymer hydrogels with tunable mechanical properties have focused on varying the crosslinking density of hydrogels via chemical or physical means.[6,11–21,25] Here, we engineer dynamic protein hydrogels using protein folding-unfolding in a controlled fashion to control the effective chain length between crosslinks, thus controlling the Young's modulus of these hydrogels.

Folding and unfolding is the ultimate conformational change within proteins. Protein unfolding can lead to substantially larger changes in length and compliance than that resulting from ligand binding-induced conformational changes.[15,17,19] For example, single molecule force spectroscopy measurements have shown that the unfolding of a small globular protein such as GB1 and I27, which contains only 56 and 89 residues, can increase the effective protein length by 18 and 28 nm, respectively, and decrease the persistence length of the protein polymer from ~10 nm to 0.4 nm,[26,27] while length change due to ligand-binding induced conformation changes can be only up to 2nm.[15,17,19] Incorporating proteins that can undergo unfolding-folding changes under user-defined triggers will provide an effective means of modulating chain length between crosslinks within hydrogels.

Previously, we used tandem modular proteins as building blocks to engineer novel protein-based hydrogels.[28–33] By chemically denaturing the folded globular domains using chemical denaturants, we showed that it is possible to decrease the Young's modulus of tandem modular protein-based hydrogels.[29,31] However, for any potential biological applications, biologically compatible and mild conditions are required. Here, we propose incorporating protein-folding switches into tandem modular proteins towards constructing protein hydrogels to achieve dynamic mechanical properties regulation.

Our designed protein-folding switch is based upon the so-called mutually exclusive proteins. Mutually exclusive proteins (MEP) are specially designed domain insertion proteins, in which a guest domain with a large distance between its N, C-termini is spliced into a short loop of a host domain.[34–40] The size incompatibility between the guest and host domain allows only one of the two domains to fold at any given time.[34,38,39] In our previous work, we engineered the MEP GL5-I27, in which GL5 serves as the host domain and I27 is guest domain inserted into the second loop of GL5.[37,38] Due to the higher thermodynamic stability of I27, the mutually exclusive protein exists mainly in the GL5(U)-I27(F) conformation, where GL5 is unfolded and I27 is folded. By mutating residues 41 and 43 into cysteines, we reported that GL5CC-I27 can serve as a redox-responsive MEP-based protein folding switch.[37] In the oxidized state, Cys41 and Cys43 can form a disulfide bond, forcing GL5CC-I27 into the GL5CC(F)-I27(U) conformation. Upon reduction, GL5CC-I27 folds into the GL5CC(U)-I27(F) conformation (**Figure 1**A), effectively increase the protein chain length by ~23 nm. If GL5CC-I27 is used as a building block to construct protein hydrogels, dynamically tuning GL5CC-I27's conformation will allow us to effectively change the length of GL5 in the protein network, thus changing the effective length between two crosslinking points and changing the mechanical properties of resultant protein hydrogels (Figure 1B).

2.2. Engineering Elastomeric Proteins for Constructing Dynamic Protein Hydrogels

To engineer protein-based dynamic hydrogels, we constructed the artificial elastomeric protein GB1-R-(GB1-GL5CC-I27-R)$_2$, which incorporates the MEP GL5CC-I27 as a protein folding switch to control the conformation of the host domain GL5CC. For simplicity, we henceforth refer to this polyprotein as GR(G-MEP-R)$_2$. GB1-Resilin based elastomeric proteins have been used to engineer biomaterials that mimics the passive elastic properties of muscles.[29] The incorporation of GB1-R allows us to use the well-developed Ru^{2+}-mediated photocrosslinking strategy to engineer protein-based hydrogels. Figure S1 shows the SDS-PAGE gel of the purified protein.

2.3. Dynamic Protein Hydrogels Based on G-R-(G-MEP-R)$_2$ Show Stimuli-Responsive Physical and Mechanical Properties

We used the well-developed Ru^{2+}-mediated photocrosslinking strategy, which allow the crosslinking of two tyrosine residues in proximity into dityrosine adducts, to engineer protein-based hydrogels.[41] Oxidized G-R-(G-MEP-R)$_2$ was used for hydrogel construction. We found that an aqueous solution of G-R-(G-MEP-R)$_2$ can be readily crosslinked into a solid, transparent hydrogel upon illumination with white light when the protein concentration is higher than 50 mg mL^{-1} (Figure 1C).

In the oxidized state, the dominant conformation for MEP is GL5CC(F)-I27(U), where the host domain GL5CC is folded and the guest domain I27 is unfolded. Thus, G-R-(G-MEP-R)$_2$-based hydrogels should be similar to hydrogels constructed from (G-R)$_4$. **Figure 2**A shows the stress-strain curve of G-R-(G-MEP-R)$_2$-based hydrogels (from a protein concentration of 200 mg mL^{-1}). Indeed, the hydrogel based on oxidized G-R-(G-MEP-R)$_2$ shows a Young's modulus of ~40 kPa, similar to that of (G-R)$_4$ at a similar protein concentration (Figure 2B). The resultant hydrogel is resilient and only shows a small hysteresis between stretching and relaxation cycles. The swelling ratio of the hydrogel is ~20%, similar to that of (G-R)$_4$-based hydrogels.[29]

To check whether the mechanical and physical properties of G-R-(G-MEP-R)$_2$ respond to redox potential, we measured the mechanical and swelling properties of G-R-(G-MEP-R)$_2$-based hydrogels in the presence of 10 mM DTT (Figure 2.A). In the reduced state, the predominant conformation of MEP is GL5CC(U)-I27(F), where the host domain GL5CC is unfolded and the guest domain I27 is folded. In response to the change in MEP conformation, the G-R-(G-MEP-R)$_2$-based hydrogel should become much softer in the presence of DTT. As expected, the Young's modulus of this hydrogel dramatically decreases to ~10 kPa when equilibrated in 10 mM DTT, and the swelling

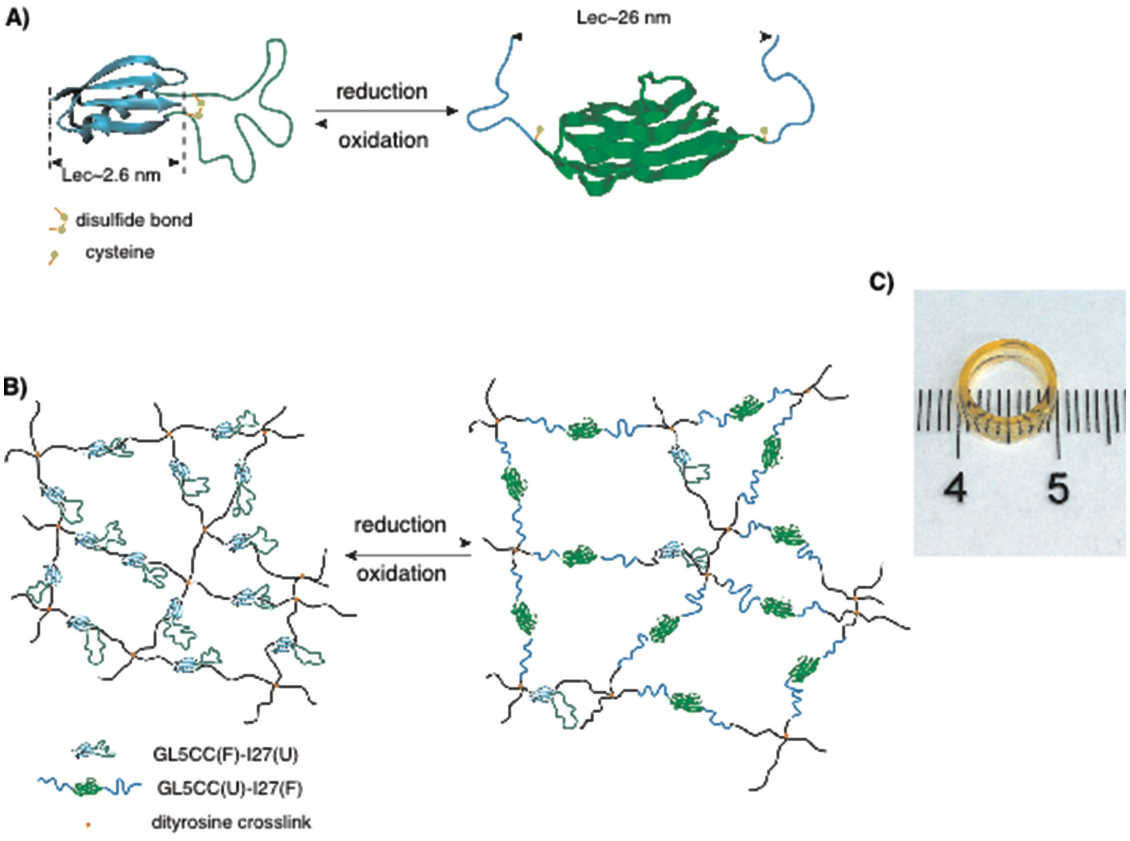

Figure 1. Designed protein folding switches can be used to construct dynamic protein hydrogels. A) Schematic showing the mutually exclusive protein-based, redox-responsive folding switch GL5CC-I27. In response to the redox condition, the host domain GL5CC (colored in cyan) can switch its conformation between folded and unfolded conformations, resulting in two distinct and mutually exclusive conformations for GL5CC-I27. The guest domain I27 is colored in green. Unfolded host and guest domains are colored using the same color coding. Under oxidizing condition, the formation of the disulfide bond in GL5CC makes GL5CC(F)-I27(U) the dominant conformation, where the effective chain length, *Lec*, between the two termini of GL5CC-I27 is only ~2.6 nm; under reducing condition, the reduction of the disulfide bond causes GL5CC(U)-I27(F) to become the dominant conformation, where the unfolded host domain GL5CC significantly increases *Lec* between the two termini to ~26 nm. The two conformations are in dynamic equilibrium and controlled by redox condition. B) Schematic of the three dimensional network of the hydrogels constructed from G-R-(G-MEP-R)$_2$. Under oxidizing condition, the effective chain length between two adjacent crosslinks is short because GL5CC(F)-I27(U) is the dominant conformation in the hydrogel; under reducing condition, the unfolding of GL5CC significantly increases the effective chain length between two neighboring crosslinking points. C) A photograph of a moulded ring constructed from G-R-(G-MEP-R)$_2$ in PBS solution (protein concentration 200 mg mL^{-1}) using Ru(II)(bpy)$_3^{2+}$-mediated photochemical crosslinking strategy. The hydrogel is transparent. The unit of the scale is in cm.

ratio of this reduced G-R-(G-MEP-R)$_2$ hydrogel increases to ~60%. In addition, it is noteworthy that the hysteresis between stretching and relaxation cycles increases significantly in this reduced hydrogel, indicative of a significant decrease in resilience (~55%). Changes in mechanical and physical properties of the G-R-(G-MEP-R)$_2$ hydrogel are fully reversible. After reduction, the hydrogel can be re-oxidized using H_2O_2 to regain similar mechanical and physical properties – specifically, a high Young's modulus, high resilience and low swelling ratio (Figure 2A). These results strongly indicate that hydrogels based on G-R-(G-MEP-R)$_2$ show dynamic mechanical and physical properties that can be regulated via redox potential. Additionally, control experiments showed that mechanical and physical properties of (G-R)$_4$-based hydrogels do not change in response to DTT or H_2O_2 (Figure 1B), suggesting that the property modifications of G-R-(G-MEP-R)$_2$-based hydrogels is due to conformational changes of the MEP in response to redox potential (Figure 2B).

2.4. Dynamic Properties Exhibited by the Hydrogel are Due to the Redox Potential-Controlled MEP Folding Switch

To confirm that dynamic transformations in mechanical and physical properties of G-R-(G-MEP-R)$_2$-based hydrogels are indeed due to conformational changes of the mutually exclusive protein, we carried out kinetic measurements of hydrogel mechanical properties in response to the changes in redox potential and then compared these results to that resulting from conformational changes of MEP as a result of changes in redox potential. **Figure 3**A and Figure S2 show the ellipticity change of GL5CC(F)-I27(U) at 221 nm, which corresponds to the CD signal of the α-helix in the host domain GL5CC as it responds to 10 mM DTT. The gradual increase of the CD signal at 221 nm originates from unfolding of the host domain GL5CC, and the corresponding loss of tertiary and secondary structures. The reduction reaction of the disulfide bond (the thiol-disulfide interchange reaction) proceeds as an overall

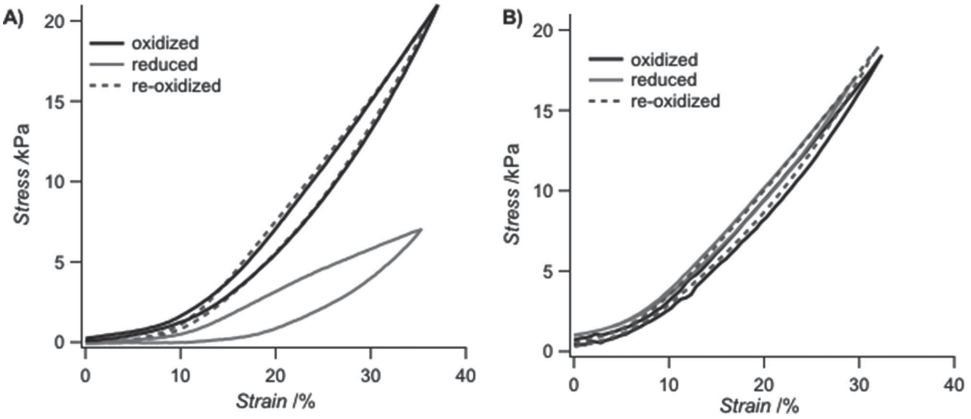

Figure 2. The mechanical properties of G-R-(G-MEP-R)$_2$-based hydrogels are responsive to redox potential. A). Typical stress-strain curves of the G-R-(G-MEP-R)$_2$ hydrogel (200 mg mL^{-1}) in PBS (oxidized), 10 mM DTT (reduced), and 20 mM H_2O_2 (re-oxidized). The G-R-(G-MEP-R)$_2$ hydrogel is stiff and resilient under oxidizing condition, but soft and force-damping under reducing condition. The Young's modulus of the hydrogel is 40 kPa and 10 kPa under oxidizing and reduction conditions, respectively. The mechanical properties of the hydrogels are reversible in response to redox potential. B). The stress-strain curves of (G-R)$_4$ hydrogel in PBS (oxidized), 10 mM DTT (reduced), and 20 mM H_2O_2 (re-oxidized). The mechanical properties of (G-R)$_4$ hydrogel do not change in response to the change of redox condition.

second order reaction: first-order in thiolate and in disulfide.[42] Since DTT concentration is significantly greater than that of MEP, the reaction is a pseudo-first order reaction with a rate constant of 0.09 min^{-1}. Figure 3B and 3C show the change in hydrogel mechanical properties constructed from oxidized MEP in response to 10 mM DTT. It is evident that the Young's modulus of the hydrogel decreases monotonically with time, with an apparent first order rate constant of 0.027 min^{-1}. The close agreement of kinetics resulting from the conformational change of MEP and the change of Young's modulus of hydrogels corroborates that changes in the mechanical and physical properties of hydrogels are indeed due to conformational changes in the mutually exclusive protein. Similarly, the response of the Young's modulus demonstrated by the reduced hydrogel upon reoxidation by 20 mM H_2O_2 is similar to conformational change kinetics[43] of GL5CC(U)-I27(F) upon reoxidation (Figure 3D-F). It is worth noting that reoxidation can occur without addition of additional oxidants, as O_2 in the air is sufficient for this purpose. Air oxidation is a very slow process, taking up to 4 days compared with 30 minutes for H_2O_2 oxidation (Figure S3). This property will be useful if Young's modulus change must occur slowly.

Having confirmed that dynamic changes in mechanical and physical properties of G-R-(G-MEP-R)$_2$-based hydrogels are indeed due to conformational changes within the mutually exclusive protein, we can readily explain the differences in hydrogel mechanical properties between oxidized and reduced states. The high Young's modulus of the hydrogel under oxidizing conditions relates to the shorter effective chain length between two crosslinking points as the host domain GL5CC is folded; the small hysteresis (high resilience) can be readily explained by the high mechanical stability of folded GB1 domain,[26] as only a small number of GB1 domains will unfold at high strain.[29] In contrast, the lower Young's modulus of the hydrogel in the reducing environment is the result of an increase in effective chain length between two crosslinking points as the host domain GL5CC is unfolded. The larger hysteresis is likely due to viscoelastic properties of the unfolded GL5CC domain. PBS buffer is a poor solvent for unfolded GL5CC domains, where unfolded GL5CC are likely to undergo hydrophobic collapse. Stretching the hydrogel will thus lead to the stretching of the unfolded, collapsed protein chain in the poor PBS solvent, leading to a viscoelastic response.

2.5. Physical and Mechanical Properties of the Dynamic Protein Hydrogel can be Further Modulated by Protein Concentration

We further examined the possibility of using protein concentration as a means of controlling physical and mechanical properties of G-R-(G-MEP-R)$_2$-based hydrogels in oxidized and reduced states (**Figure 4**A–C). It is evident that protein concentration has a significant impact on the Young's modulus of oxidized hydrogel as well as resilience and swelling ratio of the reduced hydrogel; while the Young's modulus of the reduced hydrogel as well as the resilience and swelling ratio of oxidized hydrogel are not significantly affected by protein concentration. This result can be readily accounted for by effective crosslink density. The higher the protein concentration, the higher the crosslinking density, causing a more significant effective length change between two crosslinking points in oxidized hydrogel than that in reduced state. It is evident that at higher protein concentrations, the mechanical and physical properties of G-R-(G-MEP-R)$_2$-based hydrogels can be dynamically tuned within a much broader range by a change in redox potential than that at low protein concentrations.

3. Conclusion

Using a designed mutually exclusive protein-based folding switch, we have engineered the first dynamic protein-hydrogels with tunable mechanical and physical properties. These hydrogels can mimic the dynamic changes that are common in cellular environments and the extracellular matrix. By controlling the folded state of the mutually exclusive protein via the redox

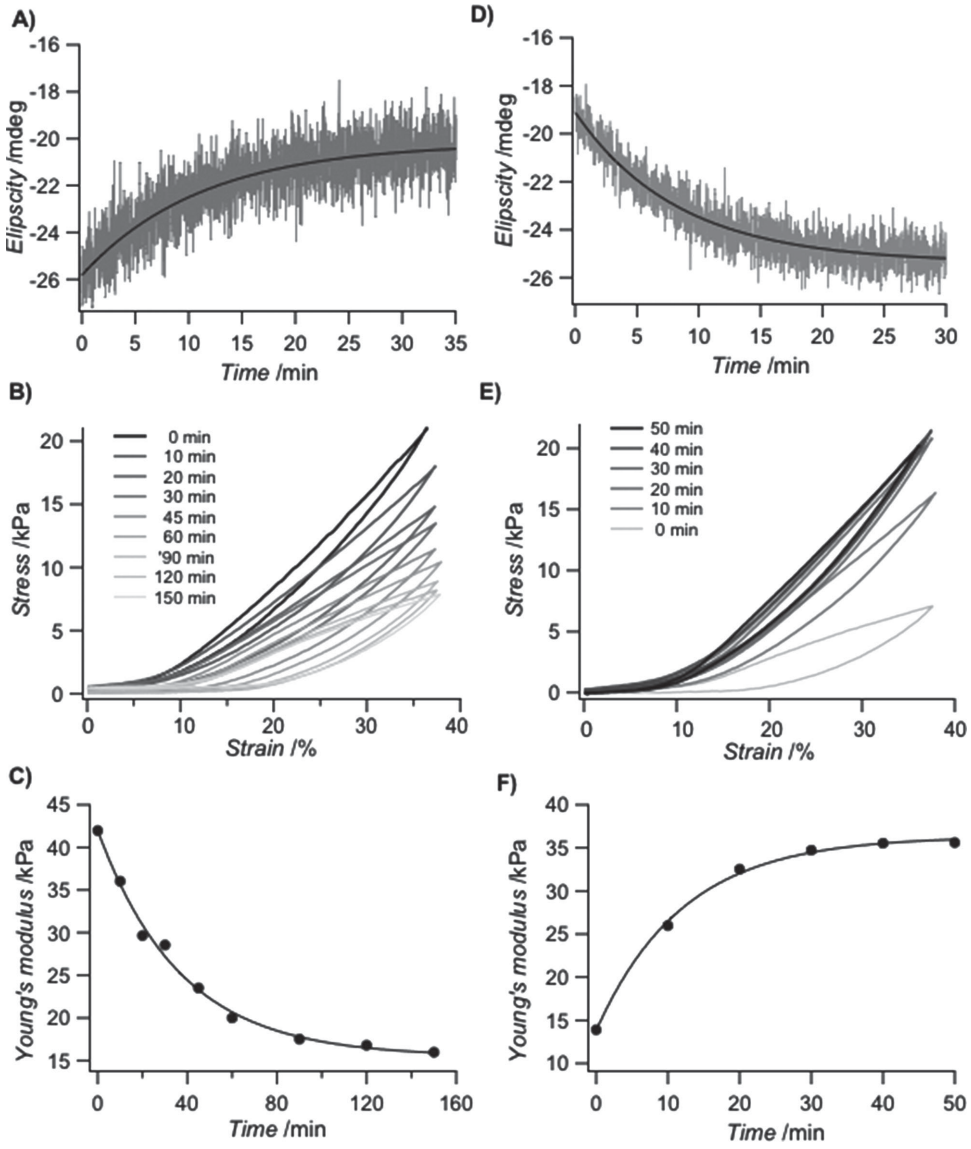

Figure 3. Dynamic mechanical properties are directly correlated with conformational changes of the protein folding switch. A) Reducing kinetics of the MEP GL5CC(F)-I27(U) as monitored by ellipticity at 221 nm in 10 mM DTT. The increase of ellipticity at 221 nm indicates the unfolding of the host domain GL5CC under reducing condition. Single exponential fit (black line) to the experimental data (in grey) measures a pseudo first-order rate constant of 0.09 min^{-1}. B) Stress-strain curves of oxidized G-R-(G-MEP-R)$_2$ hydrogel as a function of time in 10 mM DTT. The hydrogel shows gradual decrease in Young's modulus and resilience with reduction time. C). Young's modulus derived from stress-strain curves in B) as plotted as a function of reduction time. The rate constant, k = 0.027 min^{-1}, was obtained from a single exponential fit (solid line). D) Re-oxidizing kinetics of the MEP GL5CC(U)-I27(F) in 20 mM H_2O_2 monitored by ellipticity at 221 nm by CD spectrometry. The decrease of ellipticity at 221 nm indicates the folding of the host domain GL5CC under oxidizing condition. Single exponential fit (black line) to the experimental data (in grey) measures a pseudo first-order rate constant of 0.12 min^{-1}. E) Stress-strain curves of G-R-(G-MEP-R)$_2$ hydrogel in 20 mM H_2O_2 as a function of the reaction time. The hydrogel shows gradual increase in Young's modulus and resilience with oxidation time. F) Young's modulus derived from each of stress-strain curves from (E) plotted as a function of time. The rate constant, k = 0.083 min^{-1}, was obtained from a single exponential fit.

potential, the effective chain length between two crosslinking points can be readily modulated, resulting in the dynamic modulation of hydrogel mechanical properties. This novel protein hydrogel can be tuned via redox potential between a soft, force-damping hydrogel to a stiff, yet elastic hydrogel. This protein hydrogel platform enables the translation of protein folding-unfolding conformational changes at the molecular level into macroscopic reversibly tunable mechanical properties, bridging molecular events that occur at the single molecule level with macroscopic properties of biomaterials. Our work demonstrates the great potential and feasibility of molecular level engineering of biomaterials with precise mechanical properties.

This novel protein-based material platform is distinct from previously reported dynamic polymer or polymer/protein hybrid hydrogels, as control of its mechanical and physical properties is modulated by a change in chain length between crosslinking points instead of a change in crosslinking density. This distinct feature entails this novel dynamic protein

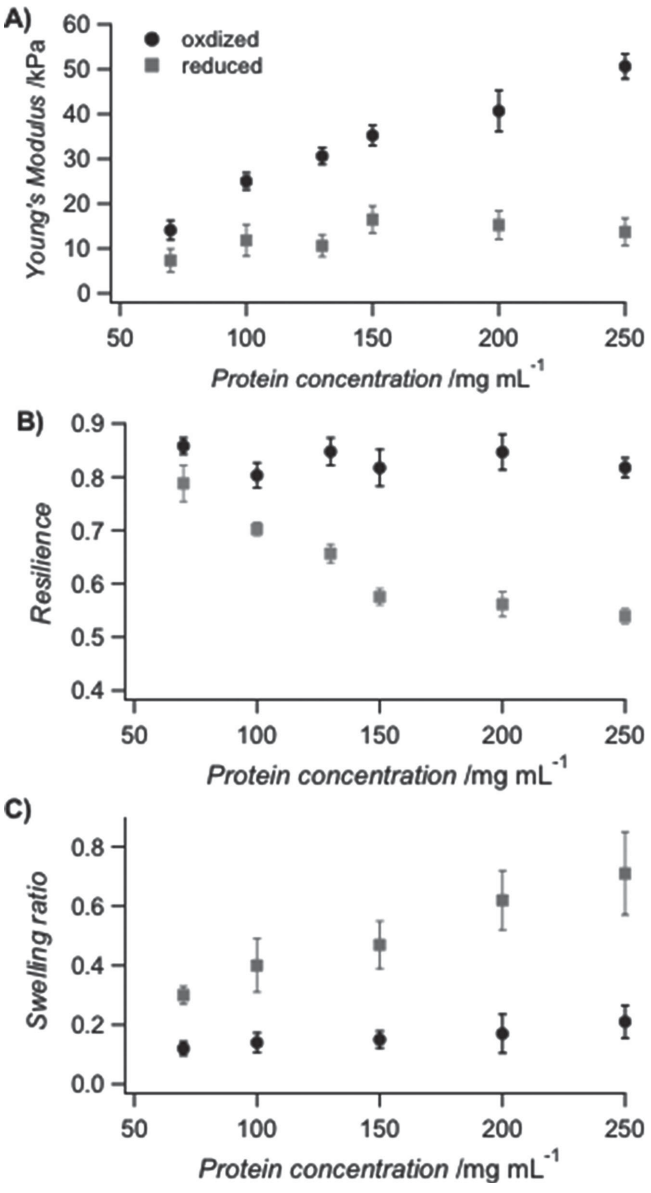

Figure 4. Protein-concentration dependence of A) the Young's modulus, B) resilience, and C) swelling ratio of the G-R-(G-MEP-R)$_2$ hydrogel. Protein concentration ranges from 70 to 250 mg mL^{-1}.

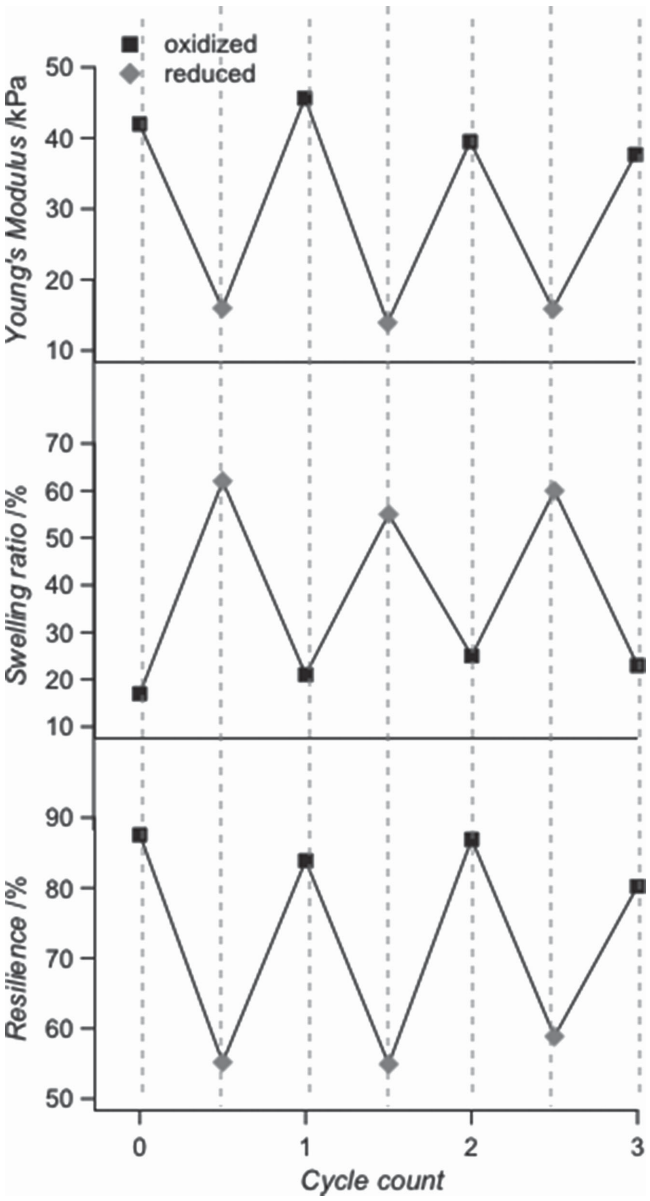

Figure 5. Physical and mechanical properties of the G-R-(G-MEP-R)$_2$ hydrogel can be cycled reversibly in their oxidizing and reducing state. A) Young's modulus, B) swelling ratio and C) resilience. Within each cycle, the sample was repeatedly tested in PBS buffer (oxidized state), 10 mM DTT (reduced state) and 20 mM H_2O_2 buffer (re-oxidized state). The sample was allowed to sufficiently reduce for 2 hours and re-oxidize for 1 hour between each measurement.

hydrogel an advantage over previously reported dynamic hydrogels: specifically, the ability for reversible dynamic modulation. Photolytic or photocrosslinking methods lead to permanent, non-reversible changes in the mechanical properties of the hydrogel. By changing the chain length between crosslinking points, alterations in the mechanical and physical properties of this hydrogel are fully reversible, and can be cycled between two states over multiple cycles (**Figure 5**A–C, S4). This unique feature will enable new biological experiments that have not been possible.

Another unique feature of our dynamic protein hydrogel system is the dynamic range over which mechanical/physical hydrogel properties can be tuned. By combining changes in redox potential with varying protein concentrations, one can tune the mechanical and physical properties of the protein hydrogel over a broad range. In addition, the mechanical properties of hydrogels can be precisely controlled -by the redox environment- in a continuous fashion between two extreme values by controlling reaction time. We anticipate that improved design and engineering approaches will allow us to further expand the dynamic range of mechanical properties that can be realized within a single protein hydrogel system. These unique properties will not only open up new avenues and opportunities for a range of experiments in biological and tissue engineering

studies, such as investigating cellular responses to dynamic changes to the extracellular matrix during cell differentiation, but also lead to new generation of smart biomaterials that may find applications in microfluidics and drug delivery systems.

Furthermore, the concept demonstrated here will also find applications beyond protein-based hydrogels. Proteins that undergo conformational changes upon protein-ligand interactions, such as calmodulin and adenylate kinase, have been used to trigger volume change of smart hybrid hydrogels.[15,17,19]. Compared to length changes resulting from ligand binding-induced conformational changes (which can be up to 2 nm), the length change of the mutually exclusive protein-based folding switch is significantly greater (~18 nm). Hence, we anticipate that the engineered mutually exclusive protein-based folding switch can serve as crosslinkers to provide new approaches to engineering smart polymer-protein hybrid hydrogels for diverse applications.

4. Experimental Section

Protein Engineering: Polyprotein GB1-R-(GB1-GL5CC-I27-R)$_2$ (GR(G-MEP-R)$_2$) was constructed using standard molecular biology techniques (for simplicity, we henceforth refer to it as GR(G-MEP-R)$_2$). The gene for the mutually exclusive protein GL5-I27F was constructed as previously described,[37,38] where the double-point mutation, 41C and 43C, within GL5CC-I27F was engineered via site-directed mutagenesis. The DNA sequence of resilin, flanked with 5' BamHI and 3' BglII and KpnI restriction sites, was synthesized by polymerase chain reaction (PCR). Digestion of resilin with BamHI and KpnI resulted in overhanging "sticky ends" whose sequence corresponded to that of the pQE80L-GB1 vector digested with BglII and KpnI. The sticky-ended resilin insert was subsequently ligated into the digested pQE80L-GB1 vector to form pQE 80L-GR. Sticky ended GB1, GL5CC-I27F and resilin genes were subsequently cloned into the pQE80L-GR vector to form pQE 80L-GR(G-MEP-R)$_2$ in a similar manner. The plasmid pQE80L-GR(G-MEP-R)$_2$ was then transformed into the *Escherichia coli* strain DH5α. Protein expression was induced with 1 mM isopropyl-1-β-D-thiogalactoside (IPTG); the soluble protein was purified by Co^{2+} affinity column. The yield of GR(G-MEP-R)$_2$ was 30–40 mg per liter of bacteria culture; the purified protein was dialyzed against deionized water for two days to remove all the salts, and subsequently lyophilized.

Hydrogel Preparation: Preparation of the hydrogel was based on a well-developed Ru(II)(bpy)$_3^{2+}$-mediated photochemical crosslinking strategy.[29,41,44] The lyophilized protein was re-dissolved in phosphate saline buffer (PBS), and the protein solution in ammonium persulfate (APS, 50 mM) and Ru(II)(bpy)$_3^{2+}$ (0.2 mM) was quickly transferred to a custom-made plexiglass mold containing a ring-shaped slot (d_{in} = 8 mm, d_{out} = 10 mm, h = 3 mm). A 200 W fiber optic white light source was used to irradiate the sample for 10 min at a height of 10 cm. The ring sample was then carefully taken out of the mold and stored in PBS buffer. Hydrogels prepared in this way is quite stable, and can be stored for one year with no noticeable erosion.

Swelling Ratio Measurement: The hydrogel swelling ratio is measured by mass difference, calculated between the freshly made gel and the equilibrium gel in oxidized, reduced and re-oxidized states. 10 mM DTT and 20 mM H_2O_2 were used to create reduced or re-oxidized conditions, and the swelling ratio was calculated by r = (m-m_0)/m_0*100%, where m_0 is the weight of the freshly prepared hydrogel and m is the weight of the hydrogel after reaching swelling equilibrium.

Tensile Test: Tensile tests were performed using an Instron-5500R tensometer with a custom-made force gauge.[29] The ring shaped hydrogel was fixed at one end and stretched by a hook from the other end. The hydrogel stored in PBS was stretched to the given length at a strain rate of 25 mm/min at a constant temperature of 20 °C. The local slope at 15% strain on the stress-strain curve was taken as the Young's modulus at 15% strain. For tensile tests under a reducing condition, 10 mM dithioltheitol (DTT) in PBS was used. For reoxidizing the hydrogel, the hydrogel was transferred to PBS buffer containing 10 mM H_2O_2. To monitor the reducing and reoxidizing kinetics in the hydrogel, stretching-relaxation measurements were carried out as a function of the reaction time.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The authors thank Dr. J. Fang and Dr. S. Lv for their technical assistance and helpful discussions. This work was supported by the Natural Sciences and Engineering Research Council of Canada, Canada Foundation for Innovation, Canadian Institutes for Health Research and Canada Research Chair program.

Received: July 3, 2014
Revised: August 14, 2014
Published online: September 17, 2014

[1] R. Langer, D. A. Tirrell, *Nature* **2004**, *428*, 487.
[2] K. Y. Lee, D. J. Mooney, *Chem. Rev.* **2001**, *101*, 1869.
[3] M. P. Lutolf, J. A. Hubbell, *Nat. Biotechnol.* **2005**, *23*, 47.
[4] J. Kopecek, *Biomaterials* **2007**, *28*, 5185.
[5] J. Kopecek, J. Yang, *Angew. Chem. Int. Ed. Engl.* **2012**, *51*, 7396.
[6] J. A. Burdick, W. L. Murphy, *Nat. Commun.* **2012**, *3*, 1269.
[7] M. W. Tibbitt, K. S. Anseth, *Sci.Transl. Med.* **2012**, *4*, 160ps124.
[8] D. E. Discher, P. Janmey, Y. L. Wang, *Science* **2005**, *310*, 1139.
[9] D. E. Discher, D. J. Mooney, P. W. Zandstra, *Science* **2009**, *324*, 1673.
[10] A. J. Engler, S. Sen, H. L. Sweeney, D. E. Discher, *Cell* **2006**, *126*, 677.
[11] M. Guvendiren, J. A. Burdick, *Nat. Commun.* **2012**, *3*, 792.
[12] W. J. King, M. W. Toepke, W. L. Murphy, *Acta Biomater.* **2011**, *7*, 975.
[13] A. M. Kloxin, A. M. Kasko, C. N. Salinas, K. S. Anseth, *Science* **2009**, *324*, 59.
[14] T. Miyata, N. Asami, T. Uragami, *Nature* **1999**, *399*, 766.
[15] Z. Sui, W. J. King, W. L. Murphy, *Adv. Mater.* **2007**, *19*, 3377.
[16] Z. J. Sui, W. J. King, W. L. Murphy, *Adv. Funct. Mater.* **2008**, *18*, 1824.
[17] W. Yuan, J. Yang, P. Kopeckova, J. Kopecek, *J. Am. Chem. Soc.* **2008**, *130*, 15760.
[18] C. R. Nuttelman, S. M. Henry, K. S. Anseth, *Biomaterials* **2002**, *23*, 3617.
[19] J. D. Ehrick, S. K. Deo, T. W. Browning, L. G. Bachas, M. J. Madou, S. Daunert, *Nat. Mater.* **2005**, *4*, 298.
[20] M. W. Tibbitt, A. M. Kloxin, L. A. Sawicki, K. S. Anseth, *Macromolecules* **2013**, *46*, 2785.
[21] F. Lin, J. Y. Yu, W. Tang, J. K. Zheng, A. Defante, K. Guo, C. Wesdemiotis, M. L. Becker, *Biomacromolecules* **2013**, *14*, 3749.
[22] M. T. Frey, Y. L. Wang, *Soft Matter* **2009**, *5*, 1918.
[23] B. B. Aaron, J. M. Gosline, *Nature* **1980**, *287*, 865.
[24] L. R. G. Treloar, *The Physics of Rubber Elasticity*, Clarendon Press, Oxford, **1975**.
[25] J. L. Drury, D. J. Mooney, *Biomaterials* **2003**, *24*, 4337.
[26] Y. Cao, H. B. Li, *Nat. Mater.* **2007**, *6*, 109.

[27] H. Li, W. A. Linke, A. F. Oberhauser, M. Carrion-Vazquez, J. G. Kerkvliet, H. Lu, P. E. Marszalek, J. M. Fernandez, *Nature* **2002**, *418*, 998.
[28] Y. Cao, H. Li, *Chem. Commun.* **2008**, 4144.
[29] S. Lv, D. M. Dudek, Y. Cao, M. M. Balamurali, J. Gosline, H. Li, *Nature* **2010**, *465*, 69.
[30] S. S. Lv, T. J. Bu, J. Kayser, A. Bausch, H. B. Li, *Acta Biomater.* **2013**, *9*, 6481.
[31] J. Fang, A. Mehlich, N. Koga, J. Q. Huang, R. Koga, X. Y. Gao, C. G. Hu, C. Jin, M. Rief, J. Kast, D. Baker, H. B. C. Li, *Nat. Commun.* **2013**, *4*, 10.
[32] H. B. Li, Y. Cao, *Acc. Chem. Res.* **2010**, *43*, 1331.
[33] J. Fang, H. Li, *Langmuir* **2012**, *28*, 8260.
[34] T. L. Radley, A. I. Markowska, B. T. Bettinger, J. H. Ha, S. N. Loh, *J. Mol. Biol.* **2003**, *332*, 529.
[35] T. A. Cutler, S. N. Loh, *J. Mol. Biol.* **2007**, *371*, 308.
[36] T. A. Cutler, B. M. Mills, D. J. Lubin, L. T. Chong, S. N. Loh, *J. Mol. Biol.* **2009**, *386*, 854.
[37] Q. Peng, N. Kong, H. C. E. Wang, H. B. Li, *Protein Sci.* **2012**, *21*, 1222.
[38] Q. Peng, H. Li, *J. Am. Chem. Soc.* **2009**, *131*, 13347.
[39] M. M. Stratton, S. N. Loh, *Protein Sci.* **2011**, *20*, 19.
[40] J. H. Ha, S. N. Loh, *Chemistry* **2012**, *18*, 7984.
[41] D. A. Fancy, T. Kodadek, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 6020.
[42] G. M. Whitesides, J. E. Lilburn, R. P. Szajewski, *J. Org. Chem.* **1977**, *42*, 332.
[43] J. W. Chu, B. L. Trout, *J. Am. Chem. Soc.* **2004**, *126*, 900.
[44] C. M. Elvin, A. G. Carr, M. G. Huson, J. M. Maxwell, R. D. Pearson, T. Vuocolo, N. E. Liyou, D. C. C. Wong, D. J. Merritt, N. E. Dixon, *Nature* **2005**, *437*, 999.