CONSTRUCTING HYDROGELS FROM ENGINEERED PROTEIN

by

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B.Sc., Nankai University, 2010

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Abstract

Hydrogels are crosslinked polymer networks that absorb large amounts of aqueous solutions. The varieties of hydrogel building blocks include natural polymers, synthetic polymers and genetically engineered proteins. This dissertation will discuss the latest progress of hydrogels constructed from genetically engineered proteins (recombinant proteins), mainly focusing on biorecognition-driven physical hydrogels as well as chemically crosslinked hydrogels. Examples include dynamic hydrogels and designing hydrogels via protein fragments reconstitution.

The first class of studies presented in this dissertation involves the use of molecular level processes to control macroscopic mechanical properties. A novel protein hydrogel is reported showing dynamic mechanical properties based on a redox potential controlled protein folding-unfolding switch, which is constructed from a designed mutually exclusive protein. The changes of the mechanical and physical properties of this hydrogel are fully reversible and can be applied as extracellular matrix to investigate cell response upon varying stiffness.

In addition, another powerful method, metal chelation, is reported to tune the conformational change of mutually exclusive protein, which can stabilize the domain, initiate the folding switch and further affect the mechanical properties of resultant hydrogel.

In the second class of studies, protein fragment reconstitution has been demonstrated as a novel driving force for engineering self-assembling reversible protein hydrogels. Protein fragment reconstitution, also known as fragment complementation, is a self-assembling mechanism by which protein fragments can reconstitute the folded conformation of the native protein when split into two halves. GL5 is a small protein, which is capable of fragment reconstitution spontaneously when split into two halves, G_N and G_C. Using GL5 as a model,
different building blocks are designed to engineer self-assembling, physically crosslinked protein hydrogels. These novel hydrogels show temperature-dependent reversible sol-gel transition, and excellent property against erosion in water. It is anticipated that such fragment reconstitution may offer a general driving force for engineering protein hydrogels from a variety of proteins, expanding the horizon of “bottom-up” approaches in the design principles of biomaterials.
Preface

Portions of this thesis benefited from intellectual and experimental contributions of others.

Chapter 1 has been published as Hongbin Li, [Na Kong], Bryce Laver and Junqiu Liu. Hydrogels constructed from engineered proteins, Small 12 (2015) 973-987. This chapter is incorporated in this thesis with permission of John Wiley and Sons, Copyright (2015). I wrote the manuscript. Dr. Li, Bryce Laver, Dr. Junqiu Liu helped to revise the manuscript.

Chapter 3 has been published as [Na Kong], Qing Peng and Hongbin Li. Rationally Designed Dynamic Protein Hydrogels with Reversibly Tunable Mechanical Properties, Advanced Functional Materials 24 (2014) 7310-7317. This chapter is incorporated in this thesis with permission of John Wiley and Sons, Copyright (2014). Dr. Li conceived the project. Dr. Li and I designed the experiments together. Dr. Peng constructed the gene of mutually exclusive protein. I am responsible for the remaining experiments. I analyzed all the data. I wrote the manuscript together with Dr. Li.

Chapter 4 has been published as [Na Kong] and Hongbin Li. Protein Fragment Reconstitution as a Driving Force for Self-Assembling Reversible Protein Hydrogels, Advanced Functional Materials 25 (2015) 5593-5601. This chapter is incorporated in this thesis with permission of John Wiley and Sons, Copyright (2015). I conceived the project. Dr. Li and I designed the experiments together. I am responsible for the experiments and data analysis. I wrote the manuscript together with Dr. Li.

A version of chapter 5 is in preparation for publication. [Na Kong], Qing Peng and Hongbin Li. Metal induced protein folding switch: a method to dynamically regulate mechanical properties of protein based hydrogel. Dr. Li conceived the project. Dr. Li and I designed the experiments together. Dr. Peng constructed the gene of mutually exclusive protein. I am
responsible for the remaining experiments. I analyzed all the data. I wrote this chapter.

Chapter 6 is the preliminary result of an ongoing study. Dr. Li conceived the project. Dr. Li and I designed the experiments together. I am responsible for all the experiments and data analysis. I wrote this chapter.
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<th>Description</th>
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<tbody>
<tr>
<td>$A$</td>
<td>area of cross-section of specimen</td>
</tr>
<tr>
<td>Ch</td>
<td>channel</td>
</tr>
<tr>
<td>Da</td>
<td>dalton</td>
</tr>
<tr>
<td>$E$</td>
<td>Young’s modulus</td>
</tr>
<tr>
<td>Pa</td>
<td>pascal</td>
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<tr>
<td>M</td>
<td>molar per liter</td>
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<td>nm</td>
<td>nanometer</td>
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<td>mm</td>
<td>millimeter</td>
</tr>
<tr>
<td>$\mu$m</td>
<td>micrometer</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<td>s</td>
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<td>$w$</td>
<td>width</td>
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<tr>
<td>F</td>
<td>folded state</td>
</tr>
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<td>$F$</td>
<td>recorded force</td>
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<tr>
<td>U</td>
<td>unfolded state</td>
</tr>
<tr>
<td>$G'$</td>
<td>storage modulus</td>
</tr>
<tr>
<td>$G''$</td>
<td>loss modulus</td>
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<td>°C</td>
<td>degree celsius</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>tensile stress</td>
</tr>
<tr>
<td>$\varepsilon$</td>
<td>tensile strain</td>
</tr>
<tr>
<td>$\Delta L$</td>
<td>the change of material length</td>
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$L_0$  initial length of material
$E_X$  extension
$dl$  length where the specimen starts to deform
$r$  radius
$r$  swelling ratio
$m_0$  weight of the freshly prepared hydrogel
$m$  weight of the hydrogel after reaching swelling equilibrium
$\theta$  ellipticity
$\theta_{obs}$  observed ellipticity
$\theta_{mrd}$  mean molar ellipticity per residue
$l$  path length
$c$  concentration
$M$  molecular weight
$n$  number of residues
$N$  crosslink density
$F_U$  fraction of unfolded proteins
$m$  the slope of the transition
$[D]$  concentration of the denaturant
$\Delta G_{D-N}^{H,O}$  free energy difference of the protein between native and denatured state in the absence of metal
$\Delta G_{D-N}^{\text{Metal}}$  free energy difference of the protein between native and denatured state in the presence of metal
<table>
<thead>
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<th>Symbol</th>
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<td>$\Delta\Delta G$</td>
<td>free energy difference of the protein between in presence and absence of metal</td>
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<tr>
<td>$R$</td>
<td>gas constant</td>
</tr>
<tr>
<td>$T$</td>
<td>absolute temperature in kelvins</td>
</tr>
<tr>
<td>$[D]_{0.5}$</td>
<td>concentration of denaturant at which 50% of the protein is unfolded</td>
</tr>
<tr>
<td>$\nu$</td>
<td>the volume fraction of rubber in the swollen sample</td>
</tr>
<tr>
<td>$M_c$</td>
<td>molecular mass between crosslinks</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>extension ratio</td>
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<td>$aN$</td>
<td>y-intercept of native section</td>
</tr>
<tr>
<td>$mN$</td>
<td>slope of native section</td>
</tr>
<tr>
<td>$aD$</td>
<td>y-intercept of denatured section</td>
</tr>
<tr>
<td>$mD$</td>
<td>slope of denatured section</td>
</tr>
<tr>
<td>$T_m$</td>
<td>temperature at which 50% of the protein is denatured</td>
</tr>
<tr>
<td>$K_d$</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>$G_N$</td>
<td>N-terminal fragment of GB1</td>
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<tr>
<td>$G_C$</td>
<td>C-terminal fragment of GB1</td>
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<thead>
<tr>
<th>Abbreviation</th>
<th>Explanation</th>
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<tr>
<td>AFM</td>
<td>atomic force microscope</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>CBDs</td>
<td>calmodulin-binding domains</td>
</tr>
<tr>
<td>CnaB2</td>
<td>immunoglobulin-like collagen adhesion domain 2</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ECM</td>
<td>extra-cellular matrix</td>
</tr>
<tr>
<td>E.coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELPs</td>
<td>elastin-like polypeptides</td>
</tr>
<tr>
<td>FTIR</td>
<td>fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>G</td>
<td>GB1</td>
</tr>
<tr>
<td>GB1</td>
<td>the B1 binding domain of protein G from <em>Streptococcus</em></td>
</tr>
<tr>
<td>GdmCl</td>
<td>guanidine hydrogel chloride</td>
</tr>
<tr>
<td>GL5</td>
<td>a loop insertion mutant of GB1 in which five residues (GGGLG) were inserted into the second loop of GB1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
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<tr>
<td>GL5CC</td>
<td>bi-Cysteine mutant of GL5</td>
</tr>
<tr>
<td>GL5HH</td>
<td>bi-Histidine mutant of GL5</td>
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<tr>
<td>hMSCs</td>
<td>human mesenchymal stem cells</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>I27</td>
<td>the 27th Ig domain of human titin</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IPTG</td>
<td>isopropyl-1-β-D-thiogalactoside</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani broth</td>
</tr>
<tr>
<td>L_{ec}</td>
<td>effective chain length between crosslinking points</td>
</tr>
<tr>
<td>MAP2</td>
<td>microtubule-associated protein 2</td>
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<tr>
<td>MEP</td>
<td>mutually exclusive protein</td>
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<tr>
<td>MITCH</td>
<td>mixing-induced, two-component hydrogel</td>
</tr>
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<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEGDA</td>
<td>poly(ethylene glycol) diacrylate</td>
</tr>
<tr>
<td>PPxY</td>
<td>proline-rich peptides</td>
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<tr>
<td>R</td>
<td>resilin</td>
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<tr>
<td>RLP</td>
<td>resilin-like polypeptides</td>
</tr>
<tr>
<td>Ru(II)</td>
<td>ruthenium(II)</td>
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<tr>
<td>[Ru(bpy)₃]²⁺</td>
<td>ruthenium (II) tris-bipyridyl dication</td>
</tr>
<tr>
<td>S-GAG</td>
<td>sulfated glycosaminoglycans</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>THPC</td>
<td>tetrakis (hydroxymethyl) phosphonium chloride</td>
</tr>
<tr>
<td>THPP</td>
<td>β-[tris(hydroxymethyl) phosphino] propionic</td>
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<td>tax interactive protein-1</td>
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<td>TNfn3</td>
<td>the third fibronectin type 3 domain of human tenascin C</td>
</tr>
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<td>TPR</td>
<td>tetratricopeptide repeat</td>
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<td>ubiquitin-like domain</td>
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<td>ultraviolet</td>
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<td>wild type</td>
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<td>two-dimensional</td>
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<td>three-dimensional</td>
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The world is big and salvation lurks around the corner
Chapter 1: Introduction*

1.1 Introduction of recombinant protein based hydrogels

Hydrogels are polymer networks that swell in water but do not dissolve, and have found a wide range of applications, such as super-absorbent materials, contact lenses, tissue engineering scaffolds, drug delivery vehicles, and biosensors. Hydrogel classification is diverse; they can be classified as synthetic and natural, and depending on the crosslinking nature, they can be termed as either chemically or physically crosslinked. Additionally, depending on the nature of chemical bonds, hydrogels can be classified as either degradable or non-degradable. Hydrogels can also be classified according the chemical nature of their building blocks, such as DNA or protein-based hydrogels. Several excellent articles have reviewed various aspects of hydrogels, and interested readers are referred to these articles. Here, we review the latest progress in hydrogels constructed from genetically engineered proteins (recombinant proteins). Other protein-based hydrogels, including those constructed from short peptides, naturally occurring proteins purified from natural sources (like silk proteins and collagens), or protein-polymer hybrid gels, are not discussed here. Interested readers are instead referred to excellent reviews about these specific systems[1-8].

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Hydrogels based on engineered proteins have attracted considerable interest due to the promise they hold in various biomedical applications[4, 6, 9-11]. Protein hydrogels have progressed amazingly over the last two decades, benefiting from significant progress in recombinant DNA technology and protein engineering/design techniques. Compared with synthetic polymer-based hydrogels, recombinant protein-based hydrogels show many unique features and advantages. Recombinant DNA technology allows for the engineering of recombinant proteins with precise control over their sequence, folded structure, chain length, stereochemistry and monodispersity[12], all of which is very difficult to achieve in synthetic polymers. To do this, a gene sequence encoding the target protein is inserted into a genetic vector, which is subsequently transferred to a host organism for over-expression. This exploited cellular machinery can thus reproduce the desired protein, eliminating any compositional variation and creating molecular reproducibility within the expressed protein. This process allows for protein building blocks to be engineered in a modular fashion, incorporating functional and structural domains (such as cell adhesion domains and matrix metalloproteinase sites) to tailor protein properties. In this way, the specific biological, physical and mechanical properties of protein hydrogels necessary for their applications are built in at the protein level. This process allows for better and easier control of the final hydrogel properties than synthetic hydrogels as well as those made from natural materials[9, 11, 13]. Furthermore, protein building blocks can be designed to exhibit structural transitions at the molecular level in response to external stimuli, thus offering endless possibilities towards designing smart, responsive materials at the macroscopic level.

By incorporating signaling sequences, up to entire functional domains into engineered protein
building blocks, protein hydrogels can provide artificial microenvironments that closely mimic key features of the native cellular environment, as well as the extracellular matrix (including high water content and mechanical properties that mimic soft tissues). These properties could facilitate drug delivery, cell proliferation and tissue engineering applications[14, 15]. In this chapter, I present the latest progress in protein hydrogels, focusing on the design and construction of biorecognition-driven physical hydrogels and chemically-crosslinked hydrogels, their biomedical applications, and future perspectives of this fast-evolving field of biomaterials.

1.2 Physically crosslinked protein hydrogels from self-assembling of protein building blocks

Physically crosslinked protein hydrogels are constructed from self-assembling protein building blocks, which create network structures that have transient junctions arising from either protein-polymer chain entanglements or non-covalent physical interactions, such as hydrogen bonds, and ionic or hydrophobic interactions[16]. Protein hydrogels can be largely divided into two categories, depending on the driving forces leading to hydrogelation: 1) self-assembling protein hydrogels based on specific bio-recognitions (like protein-protein/peptide interactions); and 2) protein hydrogels derived from aggregation of polypeptides driven by phase transitions, or the formation of crystalline β-sheets.
1.2.1 Physical protein hydrogels based on biorecognition-driven self-assembly

1.2.1.1 Physical hydrogels driven by the association of coiled-coil domains

Self-assembly is ubiquitous in biology across different length scales from nanometer to millimeter, resulting in a range of biological machineries, being small or complex, that are responsible for all biological processes. Bio-recognition is one of the keys to self-assembly in nature. Scientists have naturally turned to bio-recognition for inspiration towards engineering protein-based self-assembling hydrogels; these efforts, pioneered by the Tirrell’s research group[17], have led to some very successful approaches towards engineering protein-based hydrogels.

Using bio-recognition within coiled-coil motifs is the most widely studied method towards engineering protein hydrogels. The coiled-coil is one of the basic protein folding patterns, and consists of two or more α-helices that wind together to form a superhelix. One important characteristic of coiled-coil motifs is that they share a helical heptad repeat, \((abcdefg)n\), which is comprised of seven polar and non-polar amino acid residues[18, 19](Figure 1.1A). Within this heptad repeat, residues \(a, d, e\) and \(g\) are located at the hydrophobic interface between two α-helices, while residues \(b, c\) and \(f\) are exposed to the outside environment. Thus, changes in the \(a, d, e\) and \(g\) residues will render the association of coiled coil domains responsive to the environment, which may facilitate polypeptide assemblies. Residues \(a\) and \(d\) are especially important as they form the hydrophobic plane along the length of each helix, which is responsible for inter-helical hydrophobic interactions. Since positions \(a\) and \(d\) are often occupied by leucine or other non-polar residues, the motif is also often called a leucine
zipper domain or a leucine zipper. Additionally, charged residues occupy the e and g positions, and charged/neutral residues occupy the b, c and f positions. Side chains of non-polar leucine residues lie in a plane along the length of the α-helix. Hydrophobic effects lead to the formation of dimeric or multimeric helical coiled coils. Indeed, the self-association of coiled-coil domains in multimeric coiled-coils proteins was the first bio-recognition process successfully used as a physical crosslinking mechanism towards engineering self-assembling protein hydrogels[18, 19].

The physical properties of coil-coil domains was utilized by Tirrell’s group, who designed a triblock protein AC_{10}A, in which the leucine zipper A flanks a random-coiled hydrophilic sequence (specifically, [(AG)₃PEG]_{10})[17]. The association of the leucine zipper domain A into dimers or higher order aggregates drives the gelation of the tri-block protein AC_{10}A. The resultant protein hydrogel shows reversible hydrogelation behaviors, where increasing pH or temperature causes dissolution of the coiled coil aggregates, leading to the dissolution of the hydrogel into a viscous solution. This pioneering work demonstrated the potential of recombinant technology and protein engineering when applied towards the rational design of protein hydrogels.
Figure 1.1 Schematics of coiled-coil domain association.
A) Side and top views of a parallel two-stranded coiled-coil constructed from the heptad repeat sequence abcdefg. Positions a and a' represent analogous positions in different helices. When residues at positions a and d are leucines or other non-polar residues, the coiled-coil is called a leucine zipper domain[19]. Copyright 2004, BioMed Central Ltd. B) Schematic of the coiled-coil based hydrogel network. Blue helices represent coiled-coil domains, and green curves represent the spacer domains. The hydrogel is formed through oligomeric self-association of coiled-coil domains (blue helices). By modulating coiled-coil associations, the sol–gel transition of the coiled-coil based hydrogel can be triggered by external stimuli [20]. Copyright 2013, John Wiley and Sons. C) A two component approach to construct hydrogels. CCE and CCK are two complementary leucine zipper sequences (colored in green and blue). Two tandem modular polyprotein sequences containing either three CCK (trifunctional) or two CCE (bifunctional) are used to form hydrogels. The red ellipses represent spacer domain GB1 domains. Gelation occurs upon mixing the two protein solutions, which enables self-assembly of CCK and CCE into coiled-coils[21]. Copyright 2012, American Chemical Society.

How an individual protein blocks affects hydrogel properties can be investigated owing to the modular structure of such triblock proteins. For example, the erosion properties of protein
hydrogels have been successfully improved by tuning the characteristics of leucine zipper domains. More specifically, the original AC\textsubscript{10}A-based hydrogels demonstrated undesired fast erosion when in open solution, which arises due to the association of leucine zipper domains, including the tendency of multidomain protein chains to form intramolecular loops, small aggregation number of the associative domain A, and transient associations\cite{22}. To circumvent this issue, protein engineering has been used to tune the properties of different protein building blocks. By harnessing different coiled-coil domains as end blocks for the triblock protein, the Tirrell group demonstrated that the erosion rate of protein hydrogels could be significantly slowed down. They used a triblock protein PC\textsubscript{10}A carrying dissimilar coiled-coil end domains to engineer hydrogels that eroded 100 times slower than hydrogels built on triblock proteins carrying the same coiled-coil domains (AC\textsubscript{10}A and PC\textsubscript{10}P). This reduced erosion rate is a result of the suppression of looped chains in the hydrogel as P and A tend not to associate with each other\cite{22}. Building on this finding, Li group employed a different approach to improve a protein hydrogel’s erosion properties by tuning the center block. Instead of using the random coiled center block, whose flexibility is largely responsible for the intramolecular loop formation, Cao and Li used the semi-rigid tandem modular protein (GB1)$_8$ (G8) (which consists of eight identical tandem repeats of the small folded globular domain GB1) as the center block\cite{23}. The stiffness of G8 effectively prevents the formation of intramolecular loop formation, and reduces the erosion rate by ~10 times when compared to AG8A-based protein hydrogels\cite{24}.

Over the last decade, leucine zipper domains have become one of the most widely used bio-recognition approaches towards engineering protein hydrogels and protein-polymer
hybrid hydrogels (see review by Kopeck)[6, 25]. Through protein design, unique environmental responsive properties can be engineered into leucine zipper domains, leading to protein hydrogels that can respond to environmental stimuli such as pH, temperature and ionic strength (Figure 1.1B)[17, 22, 25-28]. In addition to creating homo-dimer/oligomers using leucine zipper domains, protein engineering was also used to creating heterodimer formed by complimentary leucine zipper domains (CCE and CCK); such an approach could develop a two-component approach towards engineering reversible protein hydrogels with much improved erosion properties (Figure 1.1C)[21, 29]. Taking advantage of the modular design of the triblock proteins, various functional protein hydrogels have been constructed by incorporating numerous functional protein domains, ranging from cell adhesion domains, fluorescent proteins as well as catalytic enzymes[25, 30-34]. Because of the variety of these functional proteins that can be used as building blocks, various biomedical applications for leucine zipper domain-based hydrogels have been explored[35, 36].

1.2.1.2 Physical hydrogels driven by protein-protein/peptide interactions

Inspired by the work on coiled coil domain driven hydrogels, researchers have explored other specific bio-recognition processes with an eye towards constructing protein hydrogels.

By exploiting specific recognition of proline-rich peptides by the WW domain, Heilshorn and coworkers developed a mixing-induced, two-component hydrogel (MITCH) method of engineering protein hydrogels[37] (Figure 1.2A). In this study, WW domains, which contain conserved tryptophan residues within their consensus sequences, fold into antiparallel β-sheet structures and can bind to proline-rich peptides[38] with a 1:1 stoichiometry. By constructing
multiple repeats of each association domain fused with random-coil hydrophilic spacers, they engineered multifunctional block proteins containing either the WW domain or a proline-rich peptide required for crosslinking. Hydrogels formed readily when the two components were mixed under physiological conditions. Additionally, the mechanical properties of the resultant hydrogels can be readily controlled by tuning the sequences of the two block proteins as well as the stoichiometry of the two components. Due to the transient nature of physical crosslinking in the hydrogel network, these hydrogels are shear-thinning, injectable and self-healing, unique features that enable reproducible encapsulation of multiple cell types. For example, Heilshorn and coworkers showed that neural stem cells could be successful encapsulated by these hydrogels; these encapsulated stem cells can form stable 3D cultures and continue to self-renew, differentiate, and sprout extended neuritis (Figure 1.2A). This result suggests the capability of cells to present different responses in 2D versus 3D culture at range of mechanical properties, as people reported 2D soft culture ($G' = 10\, \text{kPa}$, similar to MITCH) were unable to support cell self-renewal and differentiation[39]. Due to the precise synthetic control and biocompatible nature, MITCH hydrogels may be applicable for testing biomechanical and biochemical effects on 2D and 3D vitro cultures.
Figure 1.2 Examples of protein-protein/peptide interactions.
A) Schematic of the mixing-induced, two-component approach to constructing hydrogels. i. Modular association domains assemble via molecular recognition. Two WW domains, CC43 and a Nedd4.3 variant associate with the same proline peptide, PPxY. ii. Hydrophilic peptides are used as spacers to link multiple repeats of association domains. iii. Mixing component 1 (containing PPxY) with component 2 (containing WW domains) results in gelation. iv. Confocal z-stack projections of encapsulated adult neural stem cells differentiated within C7:P9 and N7:P9 gels after six days of culture (red, glial marker GFAP; green, neuronal marker MAP2; yellow, progenitor marker nestin; blue, nuclei marker DAPI. Scale bars represent 25 μm.)[37]. Copyright 2009, the National Academy of Sciences. B) Representation of Ca\(^{2+}\) ion-responsive two-component hydrogel. One component is constructed with calmodulin, a peptide spacer sequence and a tetrameric leucine zipper. The other component contains two calmodulin-binding domains (CBDs) that are linked through a peptide sequence. Calmodulin can bind to its binding peptides in the presence of Ca\(^{2+}\). Upon mixing of the two components in a Ca\(^{2+}\) buffer, the calmodulin-peptide binding and leucine zipper self-association results in gelation[40]. Copyright 2006, American Chemical Society.
The Regan group reported an ionic-responsive hydrogel based on non-covalent protein–peptide interactions between a 34 amino acid tetratricopeptide repeat (TPR) and the penta-peptide, Asp-Glu-Ser-Val-Asp (DESVD)[41]. To do this, polyproteins containing multiple tandem repeats of the TPR protein separated by spacers were constructed. The penta-peptide was grafted onto four-armed poly(ethylene glycol) (PEG) molecules to serve as crosslinkers. Mixing the TPR protein and four armed PEG crosslinkers decorated with DESVD led to the ready formation of hydrogels. Hydrogelation kinetics was affected by the ionic strength of the surrounding buffer, where the TPR-DESVD interaction was disrupted by a solution of high ionic strength. Thus, these hydrogels exhibit stimuli-responsive assembly and disassembly, dissolving (or gelling) under conditions that weaken (or strengthen) protein-peptide interactions. Along a similar line, Burdick and coworkers used docking and anchoring domains to form the dual-component Dock-and-Lock hydrogel[42, 43]. The RIIa subunit of cAMP-dependent kinase A (the docking domain), can dimerize to process the docking step, and subsequently bind with the A-kinase anchoring protein. Mixing these components results in the almost instant formation of robust physical hydrogels. This Dock and Lock hydrogel has shear-thinning, self-healing and injectable properties, and cytocompatibility and cell-delivery were also subsequently successfully exhibited. In addition, a stiffer hydrogel with a Young’s modulus 10 times greater than the initial hydrogel was engineered when a secondary crosslink was added to the Dock and Lock system[42, 43].

Topp and co-workers also developed a novel protein hydrogel by making use of two different bio-recognitions (specifically, protein-peptide hetero-interactions and coiled coils homo-interactions) (Figure 1.2B)[40]. They designed a triple block tandem protein using
calmodulin (CaM) and tetrameric leucine zipper domains as the termini, a hydrophilic peptide sequence as the spacer, and another triple block polyprotein contained two CaM-binding domains that were linked through a hydrophilic spacer. Gelation occurred upon mixing the two synthetic block proteins. The architecture, sensing behavior, and rheological properties of these biomaterials could be rationally tuned by choosing appropriate components, as well as changing environmental conditions.

Besides the strategy of engineering proteins with tandem repeating units, fusion protein comprising of a binding protein and an oligomeric protein is an alternative powerful way of designing functionality. Fusion proteins can form oligomeric structures, and provide multiple binding sites for crosslinking to occur; for example, Ito and coworkers formed a hydrogel based on the interaction between a recombinant fusion protein and an oligomeric peptide[44]. In this scheme, a tax interactive protein-1 (TIP1) with a PDZ domain was fused to each end of the triangular trimeric CutA protein (yielding CutA-TIP1). The addition of a solution containing four-armed PEG with PDZ domain-recognizable peptides to the CutA-TIP1 construct led to hydrogel formation. Inspired by Ito’s design and the reports of using metal ions to crosslink self-assembled nanofibers for gellations[45], the Yang group designed the tetrameric fusion protein, ubiquitin-like domain (ULD)-TIP1[46]. ULD-TIP1 could enhance interactions between self-assembled nanofibers of Nap-GFFYGGGWRESAI, leading to crosslinking of hydrogel networks. Similar to other protein-based hydrogels obtained through protein–peptide interactions, the mechanical properties of this hydrogel could be manipulated by varying the concentration of the protein building blocks and altering the affinity between protein and peptides[47].
1.2.1.3 Other strategies to construct physical hydrogels from protein self-assembly

Despite the progresses mentioned above, strategies to construct self-assembling protein remain rather limited, limiting the possibility towards the systematic engineering of protein hydrogel properties. In chapter 4, I would like to introduce protein fragment reconstitution, also known as fragment reconstitution, as a novel driving force for engineering self-assembling reversible protein hydrogels. Protein fragment reconstitution, where protein fragments can reconstitute the folded conformation of the native protein when split into two halves, has been used in many basic biophysical studies, including protein folding and theories of protein evolution, as well as in bioassays to study protein-protein interactions in vitro and in vivo. However, protein fragment reconstitution has not been explored for protein hydrogel construction. Using a small protein GL5 as a model protein, which is capable of protein fragment reconstitution to reconstitute the folded GL5 spontaneously, we demonstrate that protein fragment reconstitution can be exploited as an efficient driving force towards engineering physically crosslinking protein hydrogels.

1.2.2 Physical protein hydrogels based on the inverse temperature transition of polypeptides

In addition to bio-recognition, the inverse temperature transition of polypeptides has been widely used to construct protein hydrogels. Elastin-like polypeptides (ELPs) are the best-known sequences that display an inverse temperature transition: at low temperature, ELPs are soluble in water and assume a random coil conformation; the solubility of ELPs decreases with increasing temperature. At the inverse transition temperature, polypeptides
undergo a sharp phase transition leading to aggregation (Figure 1.3A-B). This phase transition is similar to the low critical solution temperature transition of many synthetic polymers, such as poly(N-isopropyl acrylamide)[48, 49]. Moreover, this phase transition is reversible and can be rapidly triggered by shifts in temperature.

Figure 1.3  ELP structure and application.
A) Recurring β-turn and B) β-spiral structure adopted by the poly(VPGVG) upon a rise in temperature above the inverse transition temperature[50, 51]. Figure A, Copyright 1980, American Chemical Society. Figure B, Copyright 1992, Elsevier. C) Cell culture study of ELP hydrogels for cartilaginous tissue repair. Cells in ELP produce an extracellular matrix similar in composition to native cartilage tissue, including sulfated glycosaminoglycans (S-GAG) and collagen. i. Chondrocytes encapsulated with ELP hydrogel after two weeks of culture showed a rounded morphology. ii. Sections stained with toluidine blue indicate the presence of negatively charged S-GAG in the vicinity of the rounded cells. iii. Sections stained with Masson’s trichrome reflect the accumulation of newly synthesized collagenous proteins in the gels[52]. Copyright 2002, American Chemical Society.
Elastin is one of the most abundant elastomeric proteins, and provides tensile strength and elasticity to tissues such as connective tissues, lungs, skin, and blood vessels[53]. The precursor of elastin is tropoelastin, which is comprised of the hydrophobic pentapeptide repeats Val-Pro-Gly-X-Gly (VPGXG, where X is a hydrophobic amino acid) and alanine-rich domains containing lysine[54-56]. Such pentapeptide repeats have been used as the building blocks to construct ELPs polypeptides, (VPGXG)_m (where m typically ranges from 20 to 330). Following pioneering work on ELPs accomplished by Urry and coworkers, ELPs-based protein hydrogels, including their structure-function relationship and potential in biomedical application, have been heavily investigated[56-60]. VPGXG sequences are the most well characterized building blocks within elastin-like polypeptides (ELPs), and have been widely used in hydrogel construction[61-63]. Subsequent studies have shown that substitutions at the 4th residue, X, in the pentapeptide sequence provides a simple and rational way to tune the phase transition temperature. Hydrophobic aggregation and assembly phase transitions alter both the moduli and fracture properties of resultant hydrogels[64]. By designing ELPs with precise genetic encoding, the phase transition temperature, mechanical properties and many other biomaterial features could be tuned accordingly[65]. Due to their excellent cytocompatibility, the potential of ELP-based hydrogels in tissue engineering have been heavily explored. Figure 1.3C shows one such experiment, where Setton and Chilkoti used ELPs to engineer an injectable three-dimensional matrix to encapsulate chondrocytes for cartilaginous tissue repair[52]. In vitro studies showed that encapsulated chondrocytes retain a rounded morphology and a chondrocytic phenotype. Figure 1.3C ii and iii demonstrate the presence of negatively charged S-GAG and accumulated collagen, which are components of
native cartilage tissues. These results show that ELP are suited as extracellular matrix to culture cells producing constituents similar to the native cartilage tissue, such as S-GAG and collagen.

In addition, ELP-fusion proteins with other structural and functional sequences have been engineered (including silk-like sequences), and their biomedical applications, particularly in tissue engineering and drug delivery, have been extensively investigated. One of these proteins is a genetically engineered silk-elastin-like protein polymer, comprising of tandem repeated ELP blocks, GVGVP, and silk-like peptide blocks, GAGAGS[66-68]. The silk-like blocks spontaneously form hydrogen-bonded β-sheet crystals that increase stability, while the insertion of ELP blocks interrupts the crystallinity of silk-like polymers, increasing the flexibility and water solubility of the co-polymers. Studies on the swelling and transport properties of hydrogels formed by the association between silk-elastin-like protein polymers show that these hydrogels have the potential to become matrices for the controlled release of bioactive materials[69, 70].

1.3 Chemically crosslinked protein hydrogels

In addition to physically crosslinked protein hydrogels, chemical crosslinking represents another significant approach towards engineering protein hydrogels. As chemical crosslinking is covalent in nature, gelation of chemical hydrogels is generally more stable than physically crosslinked hydrogels, where chemical crosslinking mainly involves the side chain of residues. To date, a variety of chemical crosslinking approaches have been utilized to form covalently crosslinked protein hydrogels with the aim of achieving the desired
physical, mechanical and functional properties. There are some “hot” residues, whose side chains are especially reactive and often chosen as crosslinking agents to bridge with similar or dissimilar residues. Here, we highlight a few chemical crosslinking strategies that have been recently explored and used to construct protein hydrogels.

1.3.1 Crosslinking via tyrosine residues

Tyrosine plays important roles in the formation of both natural and synthetic biomaterial, by virtue of its phenol functionality. Several amazing naturally occurring biomaterials, including the elastomeric protein resilin found in insects[71], are crosslinked via tyrosine residues in vivo. Over the last few years, the use of a tyrosine-based chemical crosslinking strategy to engineer protein hydrogels has achieved impressive advancements. Several methods for in vitro crosslinking of recombinant proteins have been proposed, involving either the use of peroxidase enzymes (e.g. horseradish peroxidase (HRP)) or photosensitizers (e.g. ruthenium (II) tris-bipyridyl dication [Ru(bpy)]$_2^{2+}$).

1.3.1.1 Ru(II)-catalyzed photochemical crosslinking

Previously, Fancy and Kodadek reported an efficient and rapid photochemical crosslinking reaction to crosslink proteins of interest for protein-protein interaction analysis[72]. This reaction, which crosslinks two tyrosine residues in close proximity to a dityrosine adduct (Figure 1.4A), involves brief photolysis of tris-bipyridyl ruthenium(II) dication with visible light in the presence of the electron acceptor ammoniumpersulfate and the proteins of interest. Although this method was developed as a protein-protein interaction assay, Elvin
and coworkers realized the great value of this efficient photochemical reaction in engineering protein-based biomaterials. They successfully used this photochemical crosslinking reaction to engineer a recombinant pro resilin-based hydrogel that shows resilience exceeds that of a high resilience rubber polybutadiene. Resilin is an elastomeric protein and found in the cuticle of most insects. Resilin demonstrates low stiffness, high strain and efficient energy storage, playing important roles in insect flight and the jumping ability of insects such as fleas[73]. Resilin proteins from insects are crosslinked via enzyme-mediated tyrosine crosslinking. Elvin and coworkers expressed the first exon of the Drosophila CG15920 gene, which comprises 17 repeats of the 15 residue long elastic motif GGRPSDSYGAPGGGN, in *Escherichia coli* (*E. Coli*). The resultant rec1-resilin protein is water-soluble, and assumes a random coil like conformation. Each resilin repeat contains one exposed tyrosine residue that serves as a crosslinking point between different resilin molecules[74]. In the presence of Ru(II) and APS, the aqueous solution rec1-resilin can be crosslinked into a solid hydrogel rapidly (within a few seconds to a few minutes) upon illumination of white light. The hydrogel presents blue fluorescence under ultraviolet light, which is typical of the dityrosine adduct (Figure 1.4B). This material also exhibits higher resilience and longer fatigue resistance time when compared to synthetic, high resilience rubbers[74].
This versatile crosslinking method has become widely used to engineer a variety of protein hydrogels with different properties and biomedical applications, owing to its superb reaction efficiency and speed[75-82]. To highlight this, a few studies are highlighted here to demonstrate the breadth of protein candidates that can be crosslinked into hydrogels using this photochemical method.

As one example of how this photochemical crosslinking strategy has been used, Elvin et al. crosslinked gelatin into an elastic and adhesive hydrogel that can be used as a tissue sealant. After testing that the reagents used in the crosslinking reaction are not toxic at the concentration used, they then applied this novel gelatin-based tissue sealant to a wound in sheep lungs, and found that this tissue sealant not only effectively sealed against blood and air leakage, but also was not cytotoxic and did not produce an inflammatory response[78]. In another study, these gelatin tissue sealants were effectively used in gastrointestinal surgery on
rabbits and colon anastomosis in canines with no evidence of inflammation up to 28 days post-surgery[83]. The superior elastic properties, thermal stability, speed of curing, high tissue adhesive strength, as well as low cost of components of this robust material make it an ideal candidate as a surgical tissue sealant.

Figure 1.5 Folded globular protein as building blocks for protein hydrogels engineering. A) Schematic representation of GRG5RG4R building blocks. G represents GB1 domain and R represents resilin consensus sequence. B) A photograph of a hydrogel ring constructed from GRG5RG4R and the schematic of the network structure. C) Representative stress-strain and stress-relaxation curves of GRG5RG4R-based biomaterials. D) Stress-relaxation curves of GRG5RG4R-based biomaterials at constant strains[84]. Copyright 2010, American Chemical Society.

Using this efficient photochemical crosslinking strategy, Li and coworkers engineered a protein hydrogel that exhibits mechanical properties closely mimicking the passive elastic properties of muscle (Figure 1.5)[85]. This passive elasticity is mediated by the I-band part of
the giant muscle protein titin, which behaves as a complex molecular spring composed of
distinct elastic elements (folded immunoglobulin-like domains as well as unstructured unique
sequences). Using protein engineering, Lv and colleagues engineered artificial elastomeric
proteins comprised of folded GB1 domains and unstructured resilin sequences to mimic the
structure and mechanical properties of I-band titin at the molecular level (Figure 1.5A). The
Ru(II)-mediated photochemical crosslinking strategy enabled us to crosslink an aqueous
solution of these elastomeric proteins into solid hydrogels (Figure 1.5B). Characterizing the
macroscopic mechanical properties of these hydrogels shows that they behave as rubber-like
materials showing high resilience at low strain and as shock-absorber-like materials at high
strain by effectively dissipating energy (Figure 1.5C-D). The energy loss is due to the forced
unfolding of a small number of folded GB1 domains in the hydrogels, similar to that
occurring during muscle overstretching. These properties are comparable to the passive
elastic properties of muscles within their normal working range and such hydrogels represent
a new class of muscle-mimetic biomaterial. By programming the molecular sequence, and
thus nanomechanical properties, of elastomeric proteins at the single-molecule level, this new
approach utilizing folded globular domains with well-characterized nanomechanical
properties opens up a new avenue towards designing protein hydrogels with tailored
mechanical properties at the molecular level. In this vein, Fang and coworkers have
engineered highly elastic and tough protein hydrogels using extremely mechanically labile
proteins as building blocks[81]. The force-induced unfolding of the de novo designed
ferredoxin-like domains is largely responsible for the observed high extensibility and
toughness of the resultant hydrogels.
In addition to these designs, functional motifs such as the RGD cell-binding domain and the TNfn3 domain containing the RGD sequence have been incorporated into protein hydrogels with the aim of exploring their potential applications in tissue engineering. Liu and coworkers incorporated mosquito resilin motifs into a modular protein with RGD cell-binding domains, and found that such protein-based hydrogels supported the adhesion and spread of human mesenchymal stem cells (hMSCs), which are multipotent stromal cells that can differentiate into a variety of cell types. This clearly demonstrates the potential that these hydrogels could be used to direct chondrogenesis of MSCs in cartilage therapies[86]. Lv incorporated the third TNfn3 domain from the extracellular matrix protein tenascin into the resilin-containing modular protein, and used photochemical crosslinking to form hydrogels from this protein solution[79]. The resultant hydrogels supported the adhesion and spread of human fibroblast cells, and demonstrated the potential of using this hydrogel system in constructing artificial biomimetic extracellular matrices.

1.3.1.2 Horseradish peroxidase (HRP) + H$_2$O$_2$

In addition to photo-crosslinking, HRP-H$_2$O$_2$ system was used for protein crosslinking via the formation of dityrosine crosslinked covalent bridges[87]. HRP is a single-chain β-type hemoprotein, which can catalyze the conjugation of phenol and aniline derivatives in the presence of H$_2$O$_2$[88]. In this reaction, HRP promptly combines with H$_2$O$_2$, where the resultant complex can then oxidize hydroxyphenyl groups. HRP is the most commonly used peroxidase in gelation crosslinking, where most previous work has focused on polymer-based hydrogels. Qin et al. recently reported a crosslinked protein expressed from exons 1 and 3 of
resilins from *D. melanogaster*, which formed rubber-like biomaterials when crosslinked via horseradish peroxidase-mediated crosslinking[89]. Gelation of this hydrogel was completed within a few minutes, and no other co-factors were necessary to complete the process. The crosslinked protein was then examined by gel electrophoresis, Fourier transform infrared spectroscopy (FTIR), circular dichroism (CD) and atomic force microscopy (AFM).

### 1.3.2 Crosslinking through lysine

β-[tris(hydroxymethyl) phosphino] propionic acid (THPP) is a trifunctional crosslinker that has been used for engineering protein-based hydrogels. THPP reacts with primary and secondary amine groups to form covalent crosslinks in aqueous solution and at physiological pH. This reaction is fast and cytocompatible, making it possible to use this crosslinking chemistry to engineer biologically compatible hydrogels. By reacting ELP with THPP, Chilkoti *et al.* and Heilshorn *et al.* first engineered ELP hydrogels[90, 91], and subsequently used them to encapsulate various types of cells, including fibroblasts[90], chondrocytes[92], and human embryoid bodies[91]. Recently, the Kiick group used this crosslinking chemistry to engineer resilin-like polypeptide (RLP) hydrogels[93] (Figure 1.6A-B). Inspired by the superb mechanical properties of native resilins, Kiick and coworkers designed the tandem modular protein RLP12, which contains 12 repeats of the resilin consensus sequence from exon 1 of the *D. melanogaster* CG15920 gene as well as functional sequences to impart cell adhesion, proteolytic degradation, and/or heparin immobilization properties. They found that RLP12 can be readily crosslinked using THPP, resulting in protein hydrogels with mechanical properties similar to that of vocal fold tissues; specifically, this hydrogel exhibits
a storage shear moduli of 500 Pa to 10 kPa, Young’s modulus of 15-35 kPa, and storage shear moduli at high frequency of 1000–2000 Pa, as well as facilitating the survival and proliferation of NIH 3T3 fibroblasts in vitro (Figure 1.6C)[94, 95]. These studies demonstrate the potential of using protein materials as an implantable or injectable vocal fold tissue therapy.

Even though THPP is a powerful crosslinker, it is no longer readily available due to its complicated chemical synthesis. To circumvent this problem, Heilshorn and coworkers investigated the possibility of using tetrakis (hydroxymethyl) phosphonium chloride (THPC) as a lysine reactive crosslinker, as THPC and THPP have similar structures. Their results showed that THPC is an amine-reactive crosslinker suitable for engineering protein hydrogels used for 3D cell encapsulation[96] (Figure 1.6D-E).
1.3.3 Crosslinking through cysteine residues via thiol-ene chemistry

Sulfhydryl groups that form disulfide bond crosslinks under oxidized conditions have been used for hydrogel formation. For example, ELPs with periodic cysteine residues were shown...
to undergo self-hydrogelation under physiologically relevant, mild oxidative conditions[97], resulting in the formation of intermolecular disulfide crosslinks and a reversibly crosslinked hydrogel. However, this reaction is slow without the addition of external oxidizers.

In addition to disulfide crosslinking chemistry, thiol-ene chemistry has been explored to engineer protein hydrogels. In these reactions, thiol groups on cysteines react with the vinyl termini of the crosslinker, leading to a Michael addition. This reaction is often used as a crosslinking strategy for protein-polymer hybrid hydrogels[98-100], where the crosslinker is often a soluble polymer with multi-functional termini. In early reports by Hubbell and co-workers, poly(ethylene glycol) (PEG) macromers were used to crosslink recombinant polypeptides through either vinyl sulfone-based Michael addition[101] or photo-initiated polymerization of acrylate groups[102]. For example, Murphy and coworkers reported dynamic hydrogel formation based on the conformational change of calmodulin (CaM) [103]. In this scheme, CaM with two mutated cysteine residues was reacted with a 575 Da poly(ethylene glycol) diacrylate (PEGDA) linker under UV radiation to form a hydrogel. CaM has two distinct conformational states: an apo-state in the absence of a ligand, and a holo-state when a ligand is bound[104]. Therefore, the presence of the ligand can induce a conformational change in CaM, leading to a subsequent change in hydrogel volume.

Kiick and coworkers reported a RLP-based hydrogel using vinyl sulfone-terminated four-arm star PEG as the crosslinker[105]. To do this, high-molecular-weight RLPs containing cysteine residues were crosslinked into elastic hydrogels via a Michael addition reaction with the vinyl sulfone groups on PEG. The biocompatibility of this hydrogel was confirmed when
it was shown to successfully encapsulate human aortic adventitial fibroblast cells. This thiol-ene reaction-mediated gelation is commonly used in many studies. However, a pure protein-based hydrogel has been difficult to achieve via this method, as an additional polymer-based crosslinker is usually necessary to achieve hydrogel formation.

1.3.4 Crosslinking through other residues

Another exciting development in chemically crosslinked hydrogels is the SpyCatcher/SpyTag approach. This approach is especially interesting as interactions between proteins and peptides generally have low thermodynamic stability[106]. Recent studies have explored the immunoglobulin-like collagen adhesion domain 2 (CnaB2), which contains a single isopeptide bond that confers its exceptional stability[107]. When splitting CnaB2 into a peptide tag (SpyTag, 13 amino acid residues) and a protein fragment (SpyCatcher, 138 amino acid residues), researchers discovered that SpyTag and SpyCatcher reconstituted into a complete domain, where the Asp-117 of SpyTag and Lys-31 of SpyCatcher spontaneously form an intermolecular isopeptide bond[108] (Figure 1.7A-B). The reaction is completed in a few minutes, and results in a high yield of the reconstituted domain. This method also provides a simple yet powerful new approach towards engineering protein-based hydrogels for biological applications, as the isopeptides in the reconstituted CnaB2 domain can serve as a covalent crosslinking point between different proteins. Recently, Tirrell et al. exploited this novel SpyTag-SpyCatcher approach to engineer recombinant protein-based hydrogels[109] (Figure 1.7C). By mixing multifunctional SpyCatcher/ELP tandem polyprotein (BB) and multifunctional SpyTag/ELP tandem polyproteins (AAA), covalently crosslinked protein
hydrogels were formed under physiological conditions. The biocompatibility of hydrogels formed in this way was confirmed by encapsulating 3T3 fibroblasts in Spy hydrogels that were built by incorporating cell-binding sites in the multifunctional polyprotein. Decoration of Spy network with fluorescence protein mCherry illustrates the simplicity of incorporating complex folded protein and engineering multifunctional protein hydrogels. The spreading of encapsulated 3T3 fibroblasts in mCherry-Spy hydrogels is presented in Figure 1.7D. Because of the mild conditions used for cell encapsulation, Spy network may be attractive as a scaffold in tissue engineering, regenerative medicine, and cell transplantation. The formation of hydrogels through the SpyCatcher-SpyTag interaction is unique, as no similar protein-peptide interactions with successful chemical crosslinking capability have been discovered to date. The success of this method may be due to the high binding affinity between SpyCatcher-SpyTag, as well as spontaneous isopeptide bond formation.
Figure 1.7 Schematics of Spy network.
A) Isopeptide bond formation between Lys and Asp side chains[107]. B) Representation of SpyCatcher and SpyTag construction from CnaB2[107]. Reactive residues are colored in red. Figure A and B, Copyright 2012, the National Academy of Sciences. C) Schematic of covalently crosslinked hydrogel formed by mixing the trifunctional SpyTag containing protein (AAA) and the bifunctional SpyCatcher containing protein (BB)[109]. D) Confocal z-stack projection of 3T3 fibroblasts encapsulated in the mCherry-Spy network[109]. Channel 1 (Ch1) is the image of the mCherry-Spy network (red) and regions occupied by cells (dark shadows). Channel 2 (Ch2) shows two spreading fibroblast cells (green). Ch1+Ch2 is an overlay of the mCherry-Spy network and cells (Scale bars represent 10µm). Figure C and D, Copyright 2014, the National Academy of Sciences.
1.4 Protein-based hydrogels with dynamic properties

In addition to the crosslinking strategy of protein hydrogel networks, we are also interested in the functionality of biomaterials. By decorating, grafting or naturally incorporating functional agents on or between network chains, desired functionalities can be easily achieved on hydrogels. Previous research used hydrogels as templates that could be decorated with functional groups[110]. These materials were relatively inert materials, which consisted primarily of static systems and those that exhibit simple degradation. The simplicity of these materials may have virtually hindered their application by restricting cellular interactions with environment and preventing uniform extracellular matrix (ECM) formation[110]. However, advances in biopolymer synthesis and processing have led to a new generation of dynamic systems that are able to respond to artificial triggers, as well as biological signals with spatial precision. These systems will open up new avenues for the use of hydrogels as model biological structures and in tissue engineering.

1.4.1 Dynamic degradation and dynamic properties

Currently, hydrogels with dynamic properties either have a dynamic overall phase transition of the system (dynamic degradation), or have stable overall system but dynamic controls over specific properties. Hydrogels with dynamic degradation can undergo a phase transition by either disrupting the protein chain or disconnecting the network through external stimuli, i.e. temperature[111], pH[17], ion concentration[40] (Figure 1.8A). Among protein-based hydrogels, the physical crosslinked protein hydrogel systems usually have a dynamic overall phase transition. Since physical crosslinking is based on intermolecular non-covalent
interactions between residues, any change strong enough to effectively weaken the interactions will result in the collapse of the system. Fortunately, if the damage is not permanent, the system can be recovered by eliminating the damaging factor. For example, ELP-based hydrogel formation is sensitive and reversible to pH and temperature[111]. Specifically designing the building blocks of a hydrogel can achieve fine control over the effective pH or temperature, thereby fulfilling the requirements for applications in drug delivery or tissue engineering.

In addition, other dynamic hydrogels have a stable overall system, but with dynamic and tunable specific properties (Figure 1.8B). Numerous studies have been done on the dynamic physical properties of these hydrogels, particularly on the changes in volume, which are obvious upon direct observation and easy to make measurements. CaM is a well-known protein that can undergo a conformational change upon ligand binding as mentioned above. When CaM is built into a hydrogel network, addition of ligands will drive CaM from an extended to a collapsed conformation and the hydrogel will shrink accordingly[103, 112]. Shrinkage of the hydrogel is proportionally dependent on the content of CaM. As CaM content increases, hydrogels are reported to shrink up to 20% of their original volume[103, 112].
Figure 1.8 Cartoon illustration of dynamic hydrogel.
A) Cartoon illustration of dynamic hydrogel degradation by external stimuli. The degradation could be either reversible or irreversible to external stimuli. B) Cartoon illustration of a hydrogel with specific dynamic properties towards external stimuli, but with a stable overall network. The hydrogel cannot undergo degradation or phase transition. Active agents in the hydrogel network will respond to external stimuli, which affect the hydrogel properties.

Despite volume change being a valuable parameter when investigating dynamic hydrogels, there are many other significant properties that should be considered in a sophisticated hydrogel system. For example, mechanical properties are very important parameters reflecting the stiffness, strength and overall architecture of hydrogels. At the moment, studies on detecting protein hydrogels with dynamic mechanical properties are limited. This may be because of the limited functionalization options and sophistication of hydrogel design and maintenance of protein biofunction.

1.4.2 Methods to dynamically control mechanical properties of hydrogel: goal of the thesis

Currently, there are mainly two ways to design hydrogels with dynamic mechanical properties. The first is by tuning the crosslinking density of a hydrogel network, namely,
increasing or decreasing the amount of crosslinking junctions of a network by a controlled trigger, such as light, redox potential and metal ions. The degradation/enhancement process can be either reversible or irreversible. Photo-initiated breaking or contacting of a network is always irreversible. Photosensitive groups are usually irreversibly reacted after irradiation. On the contrary, junctions based on protein-protein/peptide physical interactions are usually tuned reversibly. When the effective stimuli are removed, proteins can still bind their partner and gelation can be recovered. The second way is via changing the effective chain length between crosslinking points ($L_{ec}$). Unlike changing the crosslinking density, the number of crosslinking joints remains constant, but the junctions are spatially driven closer or farther from each other in response to external stimuli. This means that the effective chain length between crosslinking points are shortened or elongated in a controlled way. Controlling $L_{ec}$ is, in fact, a special way of changing the crosslinking density without affecting the number of junctions, whereas a dense or loose packing of a hydrogel network is related to the crosslinking density.

For recombinant protein-based hydrogels, where proteins are genetically encoded on the polymer chain, an effective way to control $L_{ec}$ is through tuning the protein’s conformational change. Controlling the conformational change of a protein leads to the change in the distance between the N, C-termini of the protein, which is related to the length of the protein and affects $L_{ec}$. In a tremendous amount of protein families, many known globular proteins can undergo a conformational change upon ligand binding, such as CaM and adenylate kinase[103, 112]. The N, C-termini distance of the protein can be changed by $\sim$2 nm. Even though the conformation can be tightly controlled, the change in length of the protein is not
significant enough to make a difference in mechanical properties of the incorporating hydrogel. A powerful way to control a protein’s conformation is through controlling its state of folding. Unfolding of a protein can increase its N, C-termini distance to full length of the primary sequence and make it more compliant than the folded state. However, due to biological concerns, protein unfolding is not the optimal approach, as it always involves harsh environments or denaturants, which dramatically interfere with a protein’s function and that of surrounding species working under physiological conditions.

Another way to control the fold of a protein is by artificially designed domain-insertion motif, i.e., mutually exclusive proteins (MEP). In a typical MEP, a guest domain, which has a long N, C-termini distance, is inserted into one of the loops of a host domain, which is significantly shorter than the N–C distance of the guest[113, 114]. Such size incompatibility leads to a folding tug-of-war between the host and guest domains, where only one domain can be folded at a given time[115]. Folding of the host or guest in MEP is determined by the relative thermodynamic stability of the two domains. The one with the higher thermodynamic stability will remain folded. The deep understanding of MEP enabled and inspired us to apply it to the design of protein-based hydrogels. Dynamically tuning MEP’s conformation allows us to effectively change the length of the host domain in the protein network, thus changing the effective length between two crosslinking points and the mechanical properties of the resultant protein hydrogels. The detailed content will be illustrated in Chapter 3, 5 and 6.

In chapter 3, the domains of MEP can switch its conformation between folded and unfolded states via oxidation and reduction of an engineered disulfide bond, leading to drastic change
of protein’s effective chain length. This redox-responsive protein can be photochemically crosslinked into solid hydrogels. We found that redox-controlled protein folding-unfolding at the molecular level leads to significant macroscopic changes in hydrogel’s physical and mechanical properties in a fully reversible fashion. When reduced, the hydrogel swells and is mechanically compliant. When oxidized, it swells to a lesser extent and becomes resilient and stiffer, exhibiting an up to 5-fold increase in its Young’s modulus. In chapter 5, metal chelation is used as another strategy to control MEP’s conformational change. The mechanical and physical properties of the MEP incorporated hydrogel can be triggered by metal binding. In chapter 6, the redox responsive hydrogel involving MEP is applied on cell spreading studies. We demonstrated that human lung fibroblast cells respond differently to varying hydrogel stiffness.
Chapter 2: Materials and Methods

2.1 Protein engineering

Our desired polyprotein is constructed using standard molecular biology techniques through multiple repeated steps. First, we introduce the restriction sites to the gene of protein of interest by polymer chain reaction (PCR). The typical restriction enzymes used in this thesis are BamHI, BglII and KpnI. The sites on which they act are listed in Table 2.1. BamHI and BglII share the same overhang after digestion.

Table 2.1 Enzymes and restriction sites used in this thesis.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>BamHI</th>
<th>BglII</th>
<th>KpnI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme</td>
<td>5' GGATCC 3'</td>
<td>5' AGATCT 3'</td>
<td>5' GGTACC 3'</td>
</tr>
<tr>
<td>Restriction site</td>
<td>3' CCTAGG 5'</td>
<td>3' TCTAGA 5'</td>
<td>3' CCATGG 5'</td>
</tr>
<tr>
<td>Sticky end</td>
<td>5' G GATCC 3'</td>
<td>5' A GATCT 3'</td>
<td>5' GGTAC C 3'</td>
</tr>
<tr>
<td></td>
<td>3' CCTAG G 5'</td>
<td>3' TCTAG A 5'</td>
<td>3' C CATGG 5'</td>
</tr>
<tr>
<td>Overhang</td>
<td>5'GATC</td>
<td>5'GATC</td>
<td>CATG-3'</td>
</tr>
</tbody>
</table>
Figure 2.1  Engineering the gene of desired polyprotein through multiple standard steps.
As shown in Figure 2.1, in each step, the gene of interest is flanked by BamHI site in the front and BgIII, KpnI sites at the back. The vector, digested by BgIII and KpnI at the back, allows for ligation with an insert gene, digested by BamHI and KpnI. The BgIII/BamHI fusion site formed in this fashion cannot be further digested by either BgIII or BamHI. In each step, the clones containing the correct sequences were confirmed by enzymatic digestion and agarose gel electrophoresis. The representative gel pictures are shown in Figure 2.2.

![Figure 2.2 The representative DNA agarose gel electrophoresis pictures.](image)

The plasmids containing the gene of MEP, G-MEP-R, (G-MEP-R)₂ and GR-(G-MEP-R)₂ are presented. The vector for MEP, G-MEP-R, (G-MEP-R)₂ is pUC19. The vector for GR-(G-MEP-R)₂ is pQE80L. The left lane of each picture is the plasmid digested by BamHI and BgIII restriction enzymes (New England Biolabs). The arrows indicate the correct size of the genes of interest. The bands on top of the gene of interest represent the digested vector. Multiple bands of vector observed in several pictures are possibly due to the incomplete digestion. The right lane of each picture is the 2-log DNA ladder (New England Biolabs) and the corresponding size of each band is shown on the right.

### 2.2 Protein expression

The plasmid containing the gene of interest was then transformed into the *E. coli* strain DH5α competent cell. Cell culture was allowed to grow overnight in 10 mL 2.5%
Luria-Bertani broth (LB) medium and 100 mg/L ampicillin. Overnight culture was then transferred to 1L LB medium and grown at 225 rpm and 37. Protein expression was induced with 1 mM isopropyl-1-β-D-thiogalactoside (IPTG) and further processed for 4 hours. The cells were harvested by centrifugation at 12000 rpm for 10 mins and then frozen at -80 °C. Then, cells were defrosted and lysed by incubation with 1mg/mL lysozyme for 30 mins. The soluble protein was passed through Co²⁺ affinity column and eluted in phosphate buffer saline (PBS) containing 250 mM imidazole. The yield of protein was 30-40 mg per liter of bacterial culture. Purity of the purified proteins is estimated from the SDS-PAGE using AlphaEaseFC software (Figure 2.3, Version 4.0.0, Alpha Innotech, San Leandro, CA 94577). The purified protein was dialyzed against deionized water for two days to remove all the salts, and subsequently lyophilized.

![SDS-PAGE](image)

Figure 2.3 15% SDS-PAGE picture of polyprotein GRG-MEP-RG4R.
2.3 Hydrogel preparation

Preparation of the hydrogel was based on a well-developed Ru(II)(bpy)$_3^{2+}$-mediated photochemical crosslinking strategy[72, 74, 85]. To prepare a 20% (m/v) ring shaped hydrogel, 18 mg lyophilized protein was firstly re-dissolved in 90 µL PBS. Then, 4.5 µL ammonium persulfate (APS, 1M) and 0.9 µL Ru(II)(bpy)$_3^{2+}$ (20 mM) were added to the protein solution with final concentration of 50 mM and 0.2 mM, respectively. The solution 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 200W fiber optic white light source was used to irradiate the sample for 10 mins. The ring sample was then carefully taken out of the mold and stored in PBS. Hydrogels prepared in this way is quite stable, and can be stored for one year with no noticeable erosion.

2.4 Tensile tests

Tensile tests were performed using an Instron-5500R tensometer with a custom-made force gauge. Unless stated otherwise, all samples were tested in PBS at a strain rate of 25 mm/min at a constant temperature of 25 °C. As shown in Figure 2.4, the ring shaped hydrogel is kept in a container filled with PBS. The test specimen is hung with a hook at one end and a bar at the other end. The hook is fixed with the top of the machine frame and connected to a force transducer. The force transducer is driven to estimate the force applied to the specimen during stretching and relaxing cycles. The bar is used to hang the specimen in PBS. It can be moved from its stand in order to put the specimen in. The stand of the bar was attached to the platform, which supported the PBS container. The platform is connected to the strain gauge,
which can accurately move the platform at constant speed to the distance set on the manual controller, while, the PBS container, the bar and its stand, and the specimen will move together. Moving downward will result in the stretching of the specimen and moving upward will result in the relaxation of the specimen. Test results are analyzed using the following protocols.

Figure 2.4  Schematic cartoon of an Instron-5500R tensometer. One end of the test specimen is hung with a hook, which is fixed with the top of machine frame and connected to force transducer that driven to estimate the force applied to the specimen during stretching and relaxing cycles. The other end of the specimen is hung with a bar, which can be moved from its stand in order to put the specimen in. A platform is used to support the stand of the bar and a PBS container that hydrate the specimen. As the platform is connected to the strain gauge, the platform, together with PBS container, the bar and its stand, and the specimen can be moved up and down at constant speed to the distance set on manual controller. During the movement, the extension of the specimen and the force applied to stretch the specimen will be recorded.
Young’s modulus \((E)\) is a measure of the stiffness of an elastic material. Tensile test is a typical method to detect it. In a tensile test, the specimen will be stretched first and then relaxed. The extension is recorded against the applied force in the force-extension curve. Young’s modulus can be calculated by using the tensile stress \((\sigma)\) divided by the tensile strain \((\varepsilon)\).

\[
E = \frac{\sigma}{\varepsilon}
\]  

(2.1)

The tensile stress is defined as

\[
\sigma = \frac{F}{A}
\]  

(2.2)

where \(F\) is the recorded force and \(A\) is the cross-sectional area of the specimen where force is applied. Since force is generated by both sides of the ring sample when the tensile test is performed, the cross-sectional area equals two times the product of the width \((w)\) and the thickness \((t)\) of specimen,

\[
\sigma = \frac{F}{A} = \frac{F}{2wt}
\]  

(2.3)

The tensile strain is defined as

\[
\varepsilon = \frac{\Delta L}{L_0}
\]  

(2.4)

where \(\Delta L\) is the change of material length, \(L_0\) the initial length of material (Figure 2.5). \(\Delta L\) is twice the difference between the extension \((E_x)\) and the length where the specimen starts to deform \((dL)\),

\[
\Delta L = 2(E_x - dL)
\]  

(2.5)

\(E_x\) can be set manually through manual control. The \(dL\) is measured from force-extension curves and defined as the \(x\)-intercept of linear regression through first linear region of force-extension curves. The \(L_0\) can be calculated as
\[ L_0 = 2 \pi (r + \frac{t}{2}) + 2dL \]  

(2.6)

where \( r \) is the radius of the bar holding the specimen. Thus, tensile strain can be calculated as

\[ \varepsilon = \frac{\Delta L}{L_0} = \frac{2(E_x - dL)}{2 \pi (r + \frac{t}{2}) + 2dL} \]  

(2.7)

Finally, the Young’s modulus can be calculated based on previous procedures.

![Figure 2.5 Schematic cartoon of stretching test specimen on tensometer.](image)

### 2.5 Swelling ratio measurement

The hydrogel swelling ratio \((r)\) is measured by mass difference, calculated between the freshly made gel and the equilibrium gel in different states (e.g. oxidized, reduced and with/without metal binding),

\[ r = \frac{m - m_0}{m_0} \times 100\% \]  

(2.8)

where \( m_0 \) is the weight of the freshly prepared hydrogel and \( m \) is the weight of the hydrogel after reaching swelling equilibrium.
2.6 Circular dichroism (CD) spectroscopy

Circular Dichroism (CD) spectra in the far UV region can probe the secondary structure of proteins. CD signals are reported in term of ellipticity ($\theta$) in degrees. All experiments were recorded on a Jasco-J815 spectrometer in a 1mm path length quartz cuvette flushed with nitrogen gas. For spectrum measurements, the experiments were performed at a scan rate of 50 nm/min in the far UV wavelength from 250 to 190 nm unless stated otherwise. All data were corrected for the buffer contributions. In order to compare the observed data ($\theta_{obs}$) values, we convert them into a normalized value, the mean molar ellipticity per residue ($\theta_{mrd}$). We need to consider path length $l$ (in cm), concentration $c$ (in g/mL), molecular weight $M$ (in g/mol) and number of residues $n$,

$$\theta_{mrd} = \theta_{obs} \cdot \frac{M}{10 \cdot c \cdot l \cdot (n-1)} \left( \frac{\text{deg} \cdot \text{cm}^2}{\text{dmol} \cdot \text{residue}} \right).$$  \hspace{1cm} (2.9)

For kinetics measurement, all experiments are detected in the ‘time course measurement’ mode, which monitors a single wavelength over a period of time.

2.7 Fluorescence spectroscopy for chemical and thermal denaturation

Chemical denaturation experiments were carried out on a Cary Eclipse fluorescence spectrophotometer. Unless stated otherwise, a set of protein solutions was prepared with constant protein concentration. The denaturant, guanidine hydrochloride (GdmCl), was prepared with gradient concentration, ranging from 0 to 4 M. Tryptophan fluorescence of each protein solution was monitored at excitation wavelength 280 nm and emission wavelength 350 nm in ‘simple read’ mode. The chemical denaturation data were normalized
according to standard procedures and fitted to the following equation:

\[
F = \frac{\exp[(m \cdot [D] - \Delta G_{D-N}^{H,O}) / RT]}{1 + \exp[(m \cdot [D] - \Delta G_{D-N}^{H,O}) / RT]}
\]  

(2.10)

where \(F\) is the fraction of unfolded proteins, \(m\) is the slope of the transition, \([D]\) is the concentration of the denaturant, \(\Delta G_{D-N}^{H,O}\) is the thermodynamic stability of the protein in the absence of denaturant between denatured and native states, \(R\) is the gas constant and \(T\) is the absolute temperature in Kelvins. The thermodynamic stability, \(\Delta G_{D-N}^{H,O}\), of the protein can be measured using

\[
\Delta G_{D-N}^{H,O} = m \cdot [D]_{0.5},
\]  

(2.11)

where \([D]_{0.5}\) is the concentration of denaturant at which 50% of the protein is unfolded. When protein is bound to ligand (e.g. metal ions), the difference of thermodynamic stability, can be calculated using

\[
\Delta \Delta G = \Delta G_{D-N}^{Metal} - \Delta G_{D-N}^{H,O}
\]  

(2.12)

Thermal denaturation experiments were carried out on the same fluorescence spectrophotometer and an additional Single Cell Peltier Accessory, which is used to control temperature. The tryptophan fluorescence at 350 nm is monitored with the temperature increasing from 10 to 90 degrees. Raw data were globally fitted using the equation below:

\[
f(T) = \frac{(aN + mN \cdot T) + (aD + mD \cdot T) \cdot \exp(m \cdot (T - T_m) / 0.6)}{1 + \exp(m \cdot (T - T_m) / 0.6)}
\]  

(2.13)

where \(aN\) is the y-intercept of native section, \(mN\) is the slope of native section, \(aD\) is the y-intercept of denatured section, \(mD\) is the slope of denatured section, \(m\) is the slope of transition section, \(T_m\) is the temperature at which 50% of the protein is denatured.
2.8 Stopped-flow fluorescence spectroscopy

Stopped-flow experiments are used to detect fast kinetics of unfolding and folding of protein. The measurements provide accurate kinetics data as manual mixing and transfer are avoided. All experiments were carried out on a BioLogic SFM-4 stopped-flow instrument. Tryptophan fluorescence of sample was monitored at excitation wavelength 280 nm and emission wavelength 350 nm. Three syringes are assembled in the machine allowing automatic and synchronous mixing of three reagents. For unfolding kinetics, the three syringes are filled with protein solution, denaturant and buffer, respectively. Unfolding of the protein can be monitored by initiating the mixing of these reagents. For example, in a typical unfolding kinetics experiment of GB1, 1.4 mg/mL GB1, 7 M GdmCl and PBS were used as original solutions. They are mixed in ratio of 1:4:2 in order to maintain the final concentration of 0.2 mg/mL GB1 and 4 M GdmCl. Buffer was used to adjust the ratio and concentration of protein and denaturant. For folding kinetics, the three syringes were filled with protein fragment A, protein fragment B and buffer. Mixing of A and B will result in the fragment reconstitution. For re-folding kinetics, the three syringes were filled with denatured protein solution, buffer and buffer. In this case, two syringes were filled with buffer in order to sufficiently dilute the denaturant in denatured protein solution, as well as balance the volume of reagent in each syringe. For example, in a typical unfolding kinetics experiment of GB1, 4 mg/mL GB1 in 4 M GdmCl, PBS and PBS were used as original solutions for three syringes. Mixing of the components in a ratio of 1:9.5:9.5 will make the solution with final concentration of 0.2 mg/mL GB1 and 0.2 M GdmCl.
Chapter 3: Dynamic Protein Hydrogels with Reversibly Tunable Mechanical Properties*

3.1 Synopsis

Creating protein hydrogels with dynamic mechanical properties is challenging. Here, we report the engineering of a stimuli-responsive dynamic protein hydrogel with reversibly tunable mechanical properties using a MEP-based folding-unfolding switch. Via oxidation and reduction of an engineered disulfide bond, the protein can switch its conformation between folded and unfolded states, leading to drastic change of protein’s effective chain length. This redox-responsive protein can be readily photochemically crosslinked into solid hydrogels. We found that redox-controlled protein folding-unfolding at the molecular level leads to significant macroscopic changes in hydrogel’s physical and mechanical properties in a fully reversible fashion. When reduced, the hydrogel swells and is mechanically compliant. When oxidized, it swells to a lesser extent and becomes resilient and stiffer, exhibiting an up to 5-fold increase in its Young’s modulus. This novel protein hydrogel with dynamic mechanical and physical properties could find numerous applications in material sciences and tissue engineering.

* A version of Chapter 3 has been published as [Na Kong], Qing Peng and Hongbin Li. Rationally Designed Dynamic Protein Hydrogels with Reversibly Tunable Mechanical Properties, Advanced Functional Materials 24 (2014) 7310-7317. This chapter is adapted with permission of John Wiley and Sons, Copyright (2014).
3.2 Introduction

Protein hydrogels 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[4, 6, 11, 116]. Most protein-based hydrogels provide a static, bio-mimetic, 3D environment whose properties lack the dynamic, spatiotemporal changes common in cellular and extracellular matrix environments[110, 117]. 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.

As mentioned in Chapter 1, 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[118-120]. Thus, attempting to design hydrogels with tunable mechanical properties has generated considerable research interest[110]. 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[103, 121-129]. For example, engineered polymer hydrogels can increase or decrease their Young’s modulus via photo-mediated crosslinking or photolytic reactions[123, 130]. 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. We
designed a mutually exclusive protein-based folding switch to control folding and unfolding of the protein building block in response to changing redox potential[115, 131]. Such conformational changes lead to the change of effective chain length between two crosslinking points and enable one to reversibly and rapidly tune the Young’s modulus and swelling properties of the hydrogel. Our method may open up new avenues towards designing novel dynamic protein hydrogels that are valuable for applications in biological studies and tissue engineering.

3.3 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[132, 133]:

\[
\sigma = NRT\left(1 - \frac{2M_c}{M}\right)\left(\frac{\alpha - 1}{\alpha^2}\right)
\]

(3.1)

where \(\sigma\) is the stress, \(N\) the crosslink density (equal to \(\rho/M_c\)), \(R\) the gas constant, \(T\) the absolute temperature, \(\nu\) the volume fraction of rubber in the swollen sample, \(M_c\) the molecular mass between crosslinks, \(M\) the primary molecular mass and \(\alpha\) 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 [103, 110, 121-129, 134]. 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[23, 103, 125, 127]. For example, single molecule force spectroscopy measurements have shown that the unfolding of a small protein such as GB1, which contains only 56 residues, can increase the effective protein length by 18 nm, and decrease the persistence length of the protein polymer from ~10 nm to 0.4 nm[24], while length change due to ligand-binding induced conformation changes can be only up to 2 nm[103, 125, 127]. 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, Lv et al. used tandem modular proteins as building blocks to engineer novel protein-based hydrogels[79-81, 84, 85]. By chemically denaturing the folded globular domains using chemical denaturants, Li and coworkers showed that it is possible to decrease the Young’s modulus of tandem modular protein-based hydrogels[81, 85]. 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[115, 131, 135]. The size incompatibility between the guest and host domain allows only one of the two domains to fold at any given time[115]. In previous work, Peng 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[115, 131]. 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, it is reported that GL5CC-I27 can serve as a redox-responsive MEP-based protein folding switch[131]. 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 3.1A), effectively increasing the protein chain length by ~18 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 3.1B).
Figure 3.1 Designed protein folding switches constructing dynamic protein hydrogels.
A) Schematic showing the MEP-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, $L_{ec}$, between the two termini of GL5CC-I27 is only $\sim 2.6$ 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 $L_{ec}$ between the two termini to $\sim 26$ 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) using Ru(II)(bpy)$_2^{2+}$-mediated photochemical crosslinking strategy. The hydrogel is transparent. The unit of the scale is in cm.
3.4 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 mimic the passive elastic properties of muscles[85]. The incorporation of GB1-R allows us to use the well-developed Ru$^{2+}$-mediated photocrosslinking strategy to engineer protein-based hydrogels. Figure 3.2 shows the SDS-PAGE gel of the purified protein.

![Figure 3.2](image.png)

Figure 3.2 12% SDS-PAGE picture of purified G-R(G-MEP-R)$_2$. Lane 1: protein molecular weight marker; Lane 2: First elution fraction from Co$^{2+}$ affinity column; Lane 3: Second elution fraction from Co$^{2+}$ affinity column.
3.5 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 allows the crosslinking of two tyrosine residues in proximity into dityrosine adducts, to engineer protein-based hydrogels[72]. 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 (Figure 3.1C).

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 3.3A shows the stress-strain curve of G-R-(G-MEP-R)$_2$-based hydrogels (from a protein concentration of 200 mg/mL). 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 3.3B). 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[85].
The mechanical properties of hydrogels are responsive to redox potential. A). Typical stress-strain curves of the G-R-(G-MEP-R)$_2$ hydrogel (200 mg/mL) in PBS (oxidized), 10 mM DTT (reduced), and 20 mM H$_2$O$_2$ (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$_2$O$_2$ (re-oxidized). The mechanical properties of (G-R)$_4$ hydrogel do not change in response to the change of redox condition.

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 3.3A). 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 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 H₂O₂ to regain similar mechanical and physical properties - specifically, a high Young’s modulus, high resilience and low swelling ratio (Figure 3.3A). 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 H₂O₂ (Figure 3.3B), 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.

### 3.6 Dynamic properties exhibited by the hydrogel are due to the redox potential-controlled MEP folding switch

To further 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 3.4A 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 second order reaction: first-order in thiolate and in disulfide[136]. Since DTT concentration is significantly greater than that of MEP, the reaction is a pseudo-first order reaction with a rate constant of $0.090 \pm 0.004 \text{ min}^{-1}$. Figure 3.4B and C 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 $0.027 \pm 0.002 \text{ min}^{-1}$. 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$_2$O$_2$ is similar to conformational change kinetics[137] of GL5CC(U)-I27(F) upon reoxidation (Figure 3.4 D-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 mins for H$_2$O$_2$ oxidation (Figure 3.5). This property will be useful if Young’s modulus change must occur slowly.
Figure 3.4 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 $0.090 \pm 0.004 \text{ min}^{-1}$. 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, $k = 0.027 \pm 0.002 \text{ min}^{-1}$, was obtained from a single exponential fit (solid line). D) Re-oxidizing kinetics of the MEP GL5CC(U)-I27(F) in 20 mM H$_2$O$_2$ 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 $0.120 \pm 0.003 \text{ min}^{-1}$. E) Stress-strain curves of G-R-(G-MEP-R)$_2$ hydrogel in 20 mM H$_2$O$_2$ 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, $k = 0.083 \pm 0.006 \text{ min}^{-1}$, was obtained from a single exponential fit.
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[24], as only a small number of GB1 domains will unfold at high strain[85]. 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.

Figure 3.5  Stress-strain curves of reduced hydrogels upon air oxidation.
3.7 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 3.6A-C). It is evident that protein concentration has a significant impact on the Young’s modulus in the oxidized state, as well as upon resilience and swelling ratio of the hydrogel in the reduced form, while the Young’s modulus of the hydrogel in the reduced state, as well as the resilience and swelling ratio, are not significantly affected by protein concentration. This result can be readily accounted for by the 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 the oxidized hydrogel than that in the 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.
Figure 3.6  Protein-concentration dependence test.
Protein-concentration dependence of the Young’s modulus A), resilience B), and swelling ratio C) of the G-R-(G-MEP-R)$_2$ hydrogel (n=3). Protein concentration ranges from 50 to 250 mg/mL.
3.8 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 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 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 imparts this novel dynamic protein hydrogel with an advantage over previously reported dynamics 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 3.7A-C). This unique feature will enable new biological experiments that have not been previously possible.

![Graph showing physical and mechanical properties of the G-R-(G-MEP-R)\textsubscript{2} hydrogel](image)

**Figure 3.7** Physical and mechanical properties of the G-R-(G-MEP-R)\textsubscript{2} hydrogel can be cycled reversibly in their oxidizing and reducing state. A) Young’s modulus, B) Swelling ration 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\textsubscript{2}O\textsubscript{2} buffer (re-oxidized state). The sample was allowed to sufficiently reduce for 2 hours and re-oxidize for 1 hour between each measurement.

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. 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.
Chapter 4: Protein Fragment Reconstitution as a Driving Force for Self-Assembling Reversible Protein Hydrogels

4.1 Synopsis

Protein fragment reconstitution, where protein fragments can reconstitute the folded conformation of the native protein when split into two halves, has been used in many basic biophysical studies, including protein folding and theories of protein evolution, as well as in bioassays to study protein-protein interactions in vitro and in vivo. However, protein fragment reconstitution has not been explored for protein hydrogel construction. Using a small protein GL5 as a model protein, which is capable of protein fragment reconstitution to reconstitute the folded GL5 spontaneously, we demonstrate that protein fragment reconstitution can be exploited as an efficient driving force towards engineering physically crosslinking protein hydrogels. When split into two halves, GN and GC, the fragment reconstitution between GN and GC serves as an efficient crosslinking strategy to crosslink GN and GC-containing proteins into self-assembling reversible protein hydrogels via a two-component approach as well as single-component approach. These novel protein hydrogels show temperature-dependent reversible sol-gel transition, as well as excellent properties against erosion in excess water. Since a large number of proteins can undergo protein fragment reconstitution to reconstitute their native folded structures, we anticipate that

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such protein fragment reconstitution may offer a general driving force for engineering protein hydrogels from a variety of proteins, and thus significantly expand the ‘toolbox’ currently available to protein engineers.

4.2 Introduction

In Chapter 1, we have introduced many outstanding strategies for engineering self-assembling protein hydrogels[13, 17, 25-27]. To construct these self-assembling protein-based hydrogels, the most widely used technique involves the self-assembly of coiled-coil leucine zipper domains. These coiled-coil domains are basic folding motifs found in proteins, consisting of two or more α-helices that self-assemble to form an intertwined superhelix. Using this robust assembling strategy, a wide range of protein hydrogels have been engineered, ranging from random coil sequence-based hydrogels, to hydrogels encompassing folded globular domains with diverse functions[17, 21, 23, 26, 27, 32, 138]. Other methodologies towards constructing hydrogels have been subsequently developed, including those based on heterodimeric molecular recognition between protein motifs[25], including growth factor mediated hydrogels[139], ‘Dock-and-Lock’ hydrogels[42, 43], and mixing-induced two-component hydrogels (MITCH)[37]. Despite this progress, strategies to construct self-assembling proteins remain rather limited, limiting the possibility towards the systematic engineering of protein hydrogel properties. Here, we present a novel approach towards engineering self-assembling protein hydrogels based on protein fragment reconstitution (also known as fragment complementation).
Figure 4.1  Schematic showing hydrogelation based on protein fragment reconstitution. A) Formation of reconstituted GL5 from $G_N$ and $G_C$. B) Schematic of fragment reconstitution induced two-component hydrogelation. The two tandem polyproteins, containing either $G_N$ or $G_C$ fragments, serve as multiple functional precursors for hydrogelation. C) Schematic of fragment reconstitution induced single-component hydrogelation. The tandem polyprotein, containing both $G_N$ and $G_C$ fragments, serves as a multi-functional precursor for hydrogelation.

Previously, it was discovered that when some proteins split into two fragments, these fragments can undergo complementation to reconstitute the folded conformation of the native protein, either spontaneously or assisted by helper proteins that can bring the two fragments into proximity[140-145]. The small protein GB1 is a good example of a protein that is capable of undergoing spontaneous complementation when split into two fragments[146-149] (Figure 4.1A). This assisted protein fragment complementation property has been used to
develop assays to probe protein-protein interactions in vitro as well as in living cells[144, 145, 150, 151]. Subsequently, a suite of proteins has been found that demonstrate such fragment complementation behavior, such as ubiquitin, dihydroforlinate reductase, and green fluorescent protein[140, 141, 143, 150-152]. Despite the wide ubiquity of proteins able to undergo fragment complementation and their application in bioassays, surprisingly, use of fragment complementation has not been explored in engineering self-assembling protein hydrogels. For the two split fragments A and B that can undergo fragment complementation to reconstitute the native folded structure, it should be feasible to crosslink multi-functional polymers (S-A)_n and (S-B)_m into a polymer network and form a hydrogel, where S represents spacers, A and B are the two fragments capable of reconstituting, and m and n represent the number of repeating units (Figure 4.1B). Using fragments from a loop elongation variant of GB1 as a model system, we demonstrate a proof-of-principle of using protein fragment complementation as a driving force to engineer self-assembling protein hydrogels. Using different protein designs, we demonstrate both two-component and single component approaches to engineer protein fragment complementation-driven protein hydrogels.

4.3 Materials and methods

4.3.1 Rheology test

The viscoelastic response of hydrogel samples was determined by measuring the frequency-dependent viscoelastic moduli $G'(f)$ and $G''(f)$ using a stress-controlled rheometer (ARES-G2, TA Instruments; New Castle, DE, USA) over a frequency range of three magnitudes. During frequency sweep experiments, deformation was held constant to 1%,
with approximately 20 μL sample loaded into the rheometer. Measurements were conducted at room temperature using an 8 mm plate–plate geometry with 250 μm of plate separation. To prevent any drying artifacts, oil was applied around the sample.

4.3.2 Scanning electron microscopy (SEM) imaging

Each hydrogel was imaged using a Hitachi S4700 scanning electron microscope. An 8% hydrogel sample was prepared in Eppendorf tubes, and stored at 4 °C for 24 hours to allow the mixture to completely gelate. The samples were then shock-frozen in liquid nitrogen, and quickly transferred to a freeze drier where they were lyophilized for 12 hours. Lyophilized samples were then carefully fractured in liquid nitrogen, and fixed on aluminum stubs. The sample surface was coated by 5 nm of gold prior to SEM measurements.

4.3.3 Erosion rate measurements

The hydrogel erosion rate was measured using previously reported methods[21]. Briefly, 8 mg of GR(G-GN-I27-GC-R)2 protein was dissolved in 100 μL PBS buffer to a concentration of 8%. These protein solutions were then transferred to a cylindrical glass tube with a flat bottom (1.05 cm diameter), and the hydrogel allowed to stand overnight. The hydrogel was then soaked in 2 mL of PBS, and placed on a compact rocker mechanism rotating at 50 rpm. Erosion profiles were determined by measuring the protein content of the supernatant using the absorbance at 280 nm at successive time points using a Nanodrop ultraviolet-visible spectrophotometer; two hydrogel samples were measured, and the average value reported here.
4.4 Results and discussion

4.4.1 GL5 can be reconstituted via fragment complementation from its two fragments 

$G_N$ and $G_C$

To demonstrate the concept of protein fragment reconstitution as a driving force towards engineering protein hydrogels, we used GL5 fragments as a model system. GL5 is a loop elongation mutant of the small protein GB1, which has been studied extensively using protein-folding studies[153]. It was previously shown that when GB1 is split into two fragments at residue 40, the two fragments 1-40 and 41-56 of GB1 can re-assemble into a native-like fold[146-149], with a dissociation constant $K_d$ of $\sim 9 \mu M$[148]. In previous work on mutually exclusive proteins, we found that when GL5 is split into two fragments at residue 42 (residues 1-42 and 43-61) by the insertion of a 89-residue guest domain I27, the two fragments $G_N$ (1-42) and $G_C$ (43-61) can refold to its native structure, despite the fact that $G_N$ (1-42) and $G_C$ (43-61) are separated by 89 residues[115, 131]. Such refolding can be considered as intramolecular fragment reconstitution; additionally, if $G_N$ and $G_C$ can reconstitute into a native-like GL5 structure, fragment reconstitution between $G_N$ and $G_C$ can be used as a driving force to construct protein hydrogels.

We first carried out experiments to confirm that the two fragments $G_N$ and $G_C$ can undergo intermolecular fragment reconstitution to reconstitute the native fold of GL5. We utilized two engineered proteins $G_N$I27w34f and I27w34f-$G_C$; for simplicity, we subsequently refer to these two proteins as $G_N$I27 and I27-$G_C$. 
Figure 4.2 G_N and G_C can reconstitute into a folded structure via fragment reconstitution. Figures 4.2 A-D shows stopped-flow fluorimetry measurements of hydrogel unfolding and folding kinetics. A) Folding kinetics of a 1:1 mixture solution of I27-G_N and I27-G_C (red) in PBS. The experimental data is fitted to a second order rate law, giving a rate constant of $63.98 \pm 0.01 \text{ s}^{-1} \text{M}^{-1}$, which is smaller than that determined by using surface plasmon resonance[147]. For comparison, the data for I27-Gc is shown (in blue). B) Refolding kinetics of GL5 diluted from 4 M GdmCl to 0.2 mM. The rate constant was determined to be $23.63 \pm 0.01 \text{ s}^{-1}$ from a single-exponential fitting of the data. C) Unfolding kinetics of I27-G_N and I27-G_C mixture (red), I27-G_C (blue), I27-G_N (green) in 4 M GdmCl. The black line shows a single-exponential fit, with an unfolding rate constant of $18.47 \pm 0.09 \text{ s}^{-1}$. D) Unfolding kinetics of GL5 in 4 M GdmCl. The black line is a single-exponential fit, with a resultant unfolding rate constant of $39.63 \pm 0.01 \text{ s}^{-1}$. E) Thermal denaturation of the I27-G_N and I27-G_C solution, as monitored by fluorescence. The data is fitted globally, with a $T_{m}$ of 22.6 ± 1.8 °C.
We performed stopped flow fluorimetry experiments using a 1:1 mixture of G_N-I27 and I27-G_C, which is similar in design to stopped flow experiments investigating the unfolding/folding kinetics of the intact GL5 domain. Since I27w34f in both G_N-I27 and I27-G_C does not contain any tryptophan residues, any observed change in tryptophan fluorescence in stopped flow experiments should reflect the behavior of the reconstituted GL5 domain.

Figure 4.2A shows the “folding” (reconstitution) kinetics of 1:1 solutions of G_N-I27 and I27-G_C. Upon mixing at this 1:1 ratio, we observed that tryptophan fluorescence increases as a function of time in a nonlinear fashion, suggesting that some G_N-I27 and I27-G_C reconstitute into the native fold via fragment reconstitution. In contrast, G_N-I27 or I27-G_C alone does not show any change in tryptophan fluorescence under the same conditions. In comparison, Figure 4.2B shows folding kinetics of the intact GL5 domain under the same conditions; the “reconstitution folding” rate of I27-G_N and I27-G_C is significantly slower than that of intact GL5 under similar conditions.

To further confirm the fragment reconstitution of G_N/G_C, we also carried out “unfolding” experiments. Figure 4.2C shows the “unfolding” kinetics of a 1:1 mixture of G_N-I27 and I27-G_C (at a concentration of 10 µM in 4 M GdmCl). We observed that tryptophan fluorescence shows a fast decay as a function of time, a phenomenon similar to that observed in the unfolding of intact GL5 (Figure 4.2D). In contrast, similar experiments on singular G_N-I27 or I27-G_C fragments do not show any change in tryptophan fluorescence. This strongly indicates that some G_N-I27 and I27-G_C reconstituted to the folded state, which gives rise to decaying tryptophan fluorescence under denaturing conditions. By fitting the
fluorescence decay to a first-order rate equation, we measured the unfolding rate constant of the ‘reconstituted’ G\textsubscript{N}/G\textsubscript{C} of 18.47 ± 0.01 s\textsuperscript{-1} in 4 M GdmCl; this rate constant is close to the unfolding rate constant of intact GL5 under the same conditions (specifically, 39.63 ± 0.01 s\textsuperscript{-1}). This close agreement supports that the observed fluorescence decay in G\textsubscript{N}-I\textsubscript{27} and I\textsubscript{27}-G\textsubscript{C} indeed results from unfolding of the reconstituted G\textsubscript{N}/G\textsubscript{C}.

This reconstituted G\textsubscript{N}/G\textsubscript{C} has a melting temperature of 22.6 ± 1.8 °C, indicating that the reconstituted G\textsubscript{N}/G\textsubscript{C} is much less stable than intact GL5 (Figure 4.2E). This result is similar to previous studies on reconstituted GB1 (with a T\textsubscript{m} of 42 °C[148] for reconstituted G\textsubscript{1-40}/G\textsubscript{41-56}, and 25 °C for the reconstituted GB1 mutant GB1QDD[146]). Combined together, these results strongly indicate that G\textsubscript{N} and G\textsubscript{C} can reconstitute into a folded structure similar to the native structure of GL5 via fragment reconstitution, albeit at a lower thermodynamic stability. Thus, the reconstitution of GL5 from G\textsubscript{N}/G\textsubscript{C} shows that it is feasible to use fragment reconstitution as a driving force to engineer self-assembling, temperature-responsive protein hydrogels.

4.4.2 Fragment reconstitution can serve as a driving force to design self-assembling protein hydrogels

Having confirmed that G\textsubscript{N} and G\textsubscript{C} can indeed reconstitute to form a native GB1-like fold, we then endeavored to use fragment reconstitution as a driving force towards engineering protein hydrogels. To demonstrate the feasibility of this design, we employed a two-component approach[37, 79], where two proteins (I\textsubscript{27}-G\textsubscript{N}-I\textsubscript{27})\textsubscript{4} and (I\textsubscript{27}-G\textsubscript{C})\textsubscript{3} were engineered and used as building blocks in hydrogel construction. These two proteins can be considered as tetra-and tri-functional, where the reconstitution between G\textsubscript{N} and G\textsubscript{C} in the two proteins should
result in the crosslinking of (I27-GN-I27)$_4$ and (I27$_3$-GC)$_3$, leading to the formation of a network structure when the protein concentrations are high enough. It is of note that (I27-GN-I27)$_4$ and (I27$_3$-GC)$_3$ contain different number of I27 domains, ensuring that the two proteins are not complementary in structure and will not form dimers after the association of GN and GC fragments[21, 79].

Figure 4.3  Self-assembling protein hydrogels constructed via fragment reconstitution. A) Photographs of an aqueous solution of the protein building blocks. 10% aqueous solution of (I27-GN-I27)$_4$ and (I27$_3$-GC)$_3$ can readily form a hydrogel, while the isolated 10% (I27-GN-I27)$_4$ or 10% (I27$_3$-GC)$_3$ remain solutions. B) Hydrogel formation is temperature-dependent, where the 10% hydrogel turns clear at 40 ºC (left). Upon cooling to room temperature, the hydrogel reforms (right). C) SEM image of the freeze-dried 10% hydrogel of (I27-GN-I27)$_4$ and (I27$_3$-GC)$_3$. The scale bar represents 100 µm. D) The storage modulus $G'$ and loss modulus $G''$ of a 10% hydrogel as a function of angular frequency. The storage modulus is ~ 700 Pa, which is about 10 times greater than loss modulus (~80 Pa). E) The erosion profile of a 100 mg 10% hydrogel containing (I27-GN-I27)$_4$ and (I27$_3$-GC)$_3$, with a surface area of 0.865 cm$^2$ at room temperature. Complete erosion takes about nine days with an erosion rate of ($5.43 \pm 0.01) \times 10^{-2}$ mg cm$^{-2}$ hour$^{-1}$ (n=3)
As expected, we found that mixing aqueous solutions of 10% (I27-GN-I27)4 and 10% (I273-GC)3 readily leads to the formation of a transparent solid hydrogel (Figure 4.3A). By contrast, mixing an aqueous solution of (I27-GN-I27)4 or (I273-GC)3 alone does not form hydrogels, even at concentrations greater than 10%. In addition, hydrogel formation involving (I27-GN-I27)4 and (I273-GC)3 is temperature-dependent; when the temperature is greater than 23°C (the melting temperature of the reconstituted GN/GC), (I27-GN-I27)4 and (I273-GC)3-based hydrogels melt into a viscous solution (Figure 4.3B). Upon cooling, the hydrogel can readily reform, demonstrating the reversible properties of the (I27-GN-I27)4/(I273-GC)3-based hydrogel. These results are consistent with the physical crosslinking nature of the hydrogel, which is mediated by protein fragment reconstitution. Figure 4.3C shows a SEM micrograph of the freeze-dried 10% hydrogel, which displays an interconnected, porous network structure, with a pore size of ~tens micrometers.

To characterize the mechanical properties of the hydrogel, we performed dynamic shear rheology experiments in frequency-sweep mode (Figure 4.3D). The storage modulus G' of the hydrogel is ~800 Pa, significantly larger than the loss modulus G'' (~80 Pa). G' remains largely constant from 0.1 to 10 rad/s at 20 °C, as expected for a stable hydrogel. Additionally, this hydrogel also shows excellent stability in excess water (Figure 4.3E). The erosion of the hydrogel shows a linear mass loss versus time profile, suggesting that erosion occurs at the surface of the hydrogel. Despite the physical crosslinking within the hydrogel structure, the erosion rate is very slow; it takes nine days for the hydrogel to completely erode. This slow erosion of the (I27-GN-I27)4/(I273-GC)3 hydrogel compares favorably with that of protein hydrogels constructed from strong protein-protein interactions (such as leucine...
zipper domains), suggesting that fragment reconstitution provides a strong driving force for hydrogel formation.

4.4.3 From physically crosslinked hydrogels to chemically crosslinked hydrogels

In addition to the temperature responsiveness of the physically crosslinked hydrogel, this self-assembled protein hydrogel exhibits another unique property: the redox-driven conversion of the physically crosslinked form to a chemically crosslinked hydrogel. The $G_N$ and $G_C$ fragments we used within this study each carry one cysteine residue (Cys41 and Cys43); the location of the two cysteine residues should allow for the formation of a disulfide bond in the folded GL5CC form under oxidizing conditions[82]. If this conversion can be accomplished in the hydrogel, the physically crosslinked hydrogel can thus be converted into a chemically crosslinked hydrogel, much improving the hydrogel’s thermal stability.

To test this hypothesis, we thermally denatured a 1:1 mixture of I27-$G_N$ and I27-$G_C$. As shown in Figure 4.2E, the reconstituted $G_N$-$G_C$ has a melting temperature of 22 ºC under reduced conditions. After I27-$G_N$ and I27-$G_C$ reconstituted into a folded domain, we used $H_2O_2$ to oxidize the refolded I27-$G_N$/I27-$G_C$. As shown in Figure 4.4A, two melting transitions are clearly observed in the thermal melting curve of this oxidized sample: the first “unfolding” transition occurs between 10-40 ºC, which is similar to that of reduced $G_N$/$G_C$, and the second unfolding transition occurs between 40-90 ºC, which is similar to that of oxidized GL5CC (Figure 4.5). These transitions correspond to a $T_{m1}$ of 22 ºC and a $T_{m2}$ of 63 ºC, where the first transition can be ascribed to the melting of reconstituted $G_N$/$G_C$ in the reduced state, while the second to the melting of the oxidized, reconstituted $G_N$/$G_C$. This result clearly demonstrates that oxidation can lead to disulfide bond formation within the
reconstituted G\textsubscript{N}/G\textsubscript{C}, and that the oxidized G\textsubscript{N}/G\textsubscript{C} behaves like an intact GL5.

Figure 4.4 The (I27-G\textsubscript{N}-I27)\textsubscript{4} and (I27\textsubscript{3}-G\textsubscript{C})\textsubscript{3}-hydrogel can chemically crosslinked via the formation of disulfide bonds within the reconstituted G\textsubscript{N}/G\textsubscript{C}-domain.
A) Thermal denaturation of the I27-G\textsubscript{N} and I27-G\textsubscript{C} mixture by fluorescence in oxidized states. B) 10\% (I27-G\textsubscript{N}-I27)\textsubscript{4} and 10\% (I27\textsubscript{3}-G\textsubscript{C})\textsubscript{3} hydrogels in the oxidized state show improved thermal stability. Under oxidized conditions (20mM H\textsubscript{2}O\textsubscript{2}), bubbles were observed in the hydrogel due to the production of O\textsubscript{2} (top). The oxidized hydrogel remain clear and gelated at 40 ºC; however, it turns opaque when temperature is higher than 85 ºC. C) SEM image of the 10\% (I27-G\textsubscript{N}-I27)\textsubscript{4} and (I27\textsubscript{3}-G\textsubscript{C})\textsubscript{3}-hydrogel in the oxidized state. The scale bar represents 100 µm. D) The storage modulus G’ and loss modulus G” of the 10\% oxidized hydrogel as a function of angular frequency. The storage modulus increases to ~1000 Pa, compared to the reduced physical hydrogel (~700 Pa); the loss modulus remains at ~80 Pa. E) The oxidized 10\% (I27-G\textsubscript{N}-I27)\textsubscript{4} and (I27\textsubscript{3}-G\textsubscript{C})\textsubscript{3}-hydrogel shows improved stability against erosion. Compared with the reduced hydrogel (which is completely eroded in nine days (Figure 4.3E), 50\% of the oxidized hydrogel remains after nine days, with an erosion rate of (2.17 ± 0.01) × 10\textsuperscript{-2} mg cm\textsuperscript{-2} hour\textsuperscript{-1} (n=3).
Having confirmed the feasibility of oxidizing the reconstituted G\textsubscript{N}/G\textsubscript{C}, we then tested the possibility of converting the (I\textsubscript{27}-G\textsubscript{N}-I\textsubscript{27})\textsubscript{4} and (I\textsubscript{27}-G\textsubscript{C})\textsubscript{3} physical hydrogel into a chemical hydrogel by oxidation. After forming the hydrogels under reducing conditions, we immersed the hydrogel in PBS buffer containing 20 mM H\textsubscript{2}O\textsubscript{2} for 30 mins. The resultant oxidized hydrogel shows much improved thermal stability: this hydrogel remains a transparent gel even when the temperature is raised to 85 °C (Figure 4.4B). At temperature higher than 85 °C, the hydrogel remains a gel but turns opaque, an indication that reconstituted G\textsubscript{N}/G\textsubscript{C} and/or I27 domains in the hydrogel have thermally denatured. This result strongly suggests that following oxidation the hydrogel is converted into a chemically crosslinked hydrogel mediated by the formation of disulfide bonds within the reconstituted GL5 domain. SEM imaging shows that the oxidized hydrogel retains a similar porous structure as the reduced hydrogel (Figure 4.4C).

Rheology measurements show that the storage modulus of the oxidized hydrogel increases slightly, from ~800 to ~1000 Pa, where the loss modulus remained at 80 Pa (Figure 4.4D). This result clearly indicates that oxidation does not introduce additional secondary crosslinks, where only physical crosslinks are converted into chemical ones. Thus, the hydrogel structure remains largely unchanged after oxidation, as do viscoelastic properties of the oxidized hydrogels.
Figure 4.5 Thermal denaturation of GL5CC by fluorescence in oxidized states. Data were fitted globally with a T_m of 57.4 ± 0.6 °C.

The erosion profile of these oxidized hydrogels also demonstrated that they are more stable than reduced ones; for example, reduced hydrogels completely dissolved within nine days, while ~50% of the oxidized hydrogels remained after the same amount of time (Figure 4.4E). This clearly shows that the conversion of physical to chemically crosslinked hydrogels significantly improves their stability in excess water. The erosion profile of the oxidized hydrogel indicates the presence of a sol fraction in the oxidized hydrogel, suggesting that oxidation is not complete. It is of note that the conversion from physical to chemical crosslinking is fully reversible; reducing the hydrogel will revert it back to a physically crosslinked hydrogel. This shows the potential for dynamically tuning the physical/mechanical properties of hydrogel materials in the future.

A similar strategy was developed by Tirrell et al. to enhance the stability of protein hydrogels based on proteins with coiled-coil sequences[27]. By incorporating cysteine residues at specific locations, disulfide bonds can form upon self-association of the coiled coil
sequences[27], leading to improved thermal stability as well as stability against erosion. These results further support that protein engineering can offer additional possibilities for tuning the physical and mechanical properties of hydrogels via the redox potential of their environments.

4.4.4 From two-component to single component hydrogels

The two-component approach described previously offers a convenient way of engineering protein-based hydrogels. In this approach, the two protein fragments must be expressed and purified separately, which could potentially lead to technical challenges. Compared with this two-component approach, a single component approach could solve many of these technical issues. It could be feasible to use the same protein fragment reconstitution principle to construct protein hydrogels from a single protein component if one could incorporate both \( G_N \) and \( G_C \) fragments into one protein, and prevent the reconstitution of \( G_N/G_C \) via intramolecular fragment reconstitution.

In previous experiments, the so-called mutually exclusive protein GL5-I27 was engineered (where I27 is inserted into the loop of the host protein GL5)[82, 115]. GL5-I27 can be considered as \( G_N\text{-I}27\text{-G}_C \); since I27 is thermodynamically more stable than GL5, the thermodynamically stable conformation GL5-I27 is GL5(U)-I27(F), in which GL5 is unfolded and I27 is folded. Thus, in this mutually exclusive protein GL5-I27 (\( G_N\text{-I}27\text{-G}_C \)), intramolecular fragment reconstitution is prevented. In previous work, we constructed chemically crosslinked hydrogels using polyprotein GB1-R-(GB1-GL5CC-I27-R)\(_2\)[82], which can be represented as GR(G-G\(_N\)-I27-G\(_C\)-R)\(_2\). This protein can be considered tetra-functional in terms of crosslinking, where the fragment reconstitution of \( G_N \) and \( G_C \)
from neighboring molecules can reconstitute the folded GL5 domain via domain-swapping. When the protein concentration is sufficiently high, an aqueous solution of GR(G-GN-I27-GC-R)$_2$ should self-assemble to form a physically crosslinked hydrogel (Figure 4.1C).

Figure 4.6 GR(G-GN-I27-GC-R)$_2$ hydrogel constructed via fragment reconstitution. A) The GR(G-GN-I27-GC-R)$_2$ aqueous solution can form hydrogels at a concentration as low as 8% (m/v). Photographs of GR(G-GN-I27-GC-R)$_2$ at concentrations ranging from 6% to 10% are shown. B) Hydrogel formation is temperature-dependent. The 8% GR(G-GN-I27-GC-R)$_2$ solution forms hydrogel at room temperature, turning to a clear solution at 40 ºC. Upon cooling, the hydrogel can readily reform. C) SEM image of the 8% GR(G-GN-I27-GC-R)$_2$ hydrogel. The scale bar represents 100 µm. D) The storage modulus $G'$ and loss modulus $G''$ of the 10% hydrogel as a function of angular frequency. E) The erosion profile of 100 mg 8% hydrogel with a surface area of 0.865 cm$^2$ at room temperature. Complete erosion takes about 10 days, with an erosion rate of $(4.74 \pm 0.01) \times 10^{-2}$ mg cm$^{-2}$ hour$^{-1}$ (n=3).
Indeed, we found that a 8% aqueous solution of \( \text{GR(G-GN-I27-GC-R)}_2 \) can readily form a transparent hydrogel within 30 mins. In contrast, a 6% aqueous solution of \( \text{GR(G-GN-I27-GC-R)}_2 \) remains a clear viscous solution even after 24 hrs (Figure 4.6A). As expected, \( \text{GR(G-GN-I27-GC-R)}_2 \)-based hydrogelation is temperature-dependent; when the temperature is greater than \( T_m \), \( \text{GR(G-GN-I27-GC-R)}_2 \)-based hydrogels melt into a viscous solution (Figure 4.6B). Upon cooling, the hydrogel can readily reform, demonstrating the reversible nature of the \( \text{GR(G-GN-I27-GC-R)}_2 \) hydrogel formation. These results are consistent with the physical crosslinking nature of the hydrogel formed via domain-swapping reconstitution.

Figure 4.6C shows a SEM photograph of a freeze-dried 8% \( \text{GR(G-GN-I27-GC-R)}_2 \) hydrogel. The hydrogel shows a more porous structure than hydrogels made using the two-component approach, with a pore size ranging between tens to 100 micrometers. This hydrogel has a storage modulus of 800 Pa to 2.3 kPa from 0.1 to 10 rad/s at 20 °C (Figure 4.6D).

The hydrogel displays superb stability in excess water, and Figure 4.6E shows its erosion profile. Complete erosion takes \( \sim 10 \) days, resulting in an erosion rate of \( 4.74 \times 10^{-2} \) mg cm\(^{-2}\) hour\(^{-1}\). This erosion rate is \( \sim 6 \) times slower than previously engineered protein hydrogels, which were based on coiled-coil sequences. It is likely that the greater degree of physical crosslinking conferred by tetra-functional polyproteins used to construct these protein hydrogels is responsible for their slow erosion rate.
4.5 Conclusions

Using $G_N$ and $G_C$ proteins as a model system, here we demonstrate that protein fragment reconstitution can be used as a driving force towards engineering protein-based, reversible, self-assembling hydrogels. Using different protein designs, we constructed protein hydrogels using both two-component as well as single component designs. These self-assembling protein hydrogels are physical hydrogels in nature, and thus show a temperature-dependent sol-gel transition; additionally, they exhibit superb stability against erosion. These properties are comparable to or better than those reported previously for other protein hydrogels. Given the large number of proteins that can reconstitute into their native fold via domain reconstitution,[150, 154-163] we anticipate that such reconstitution may offer a general driving force for engineering protein hydrogels from a variety of proteins; this significantly expands the ‘toolbox’ currently available to protein engineers. We anticipate that the concept we demonstrated here can be readily implemented in a variety of proteins to construct protein hydrogels with diverse functionalities that are necessary for potential applications in tissue engineering and regenerative medicine. Furthermore, systematic protein engineering efforts will also help tune reconstitution efficiency and binding affinity, thus offering new strategies to tune the mechanical and physical properties of protein hydrogels for a range of applications in biomedical engineering.
Chapter 5: Metal Induced Protein Folding Switch: a Method to Dynamically Regulate Mechanical Properties of Protein Based Hydrogel

5.1 Synopsis

In this chapter, we would like to introduce another method to regulate the mechanical properties of protein hydrogels. Creating a metal chelation site onto the surface of a protein is one of the most popular approaches for protein stabilization. By engineering a bi-histidine (bi-His) motif, which consists of two histidines in a configuration allowing the binding of a bivalent metal ion (such as Ni$^{2+}$), onto the surface of a protein domain, the engineered metal chelation site can be easily introduced. By introducing the bi-His motif (metal chelation site) in the host domain of MEP, GL5HH-I27, we can now control the conformational state of the host domain through mutually exclusive folding via a simple external stimulus. When GL5HH-I27 is involved in the building block of hydrogels, the effective chain length between two crosslinking points can be readily modulated by controlling the equilibrium conformation of the MEP via metal chelation, resulting in the dynamic modulation of hydrogel mechanical properties. That is, the conformational change of MEP at the microscopic level is translated into mechanical and physical motions at the macroscopic level. The hydrogel change from a soft, yet resilient state in the absence of Ni$^{2+}$ to a stiff, force damping state in presence of Ni$^{2+}$.
5.2 Introduction

By incorporating redox responsive MEP GL5CC-I27 into hydrogel network, we reported in Chapter 3 that mechanical properties of the hydrogel can be dynamically and reversibly regulated by controlling the equilibrium conformation of MEP through a redox potential[82]. The folding or unfolding of host domain GL5CC controlled through redox potential can change the $L_{eq}$ of hydrogel network, which significantly regulates the Young’s modulus of the hydrogel[82]. In addition to redox potential, another way was mentioned to regulate MEP’s conformation. In order to efficiently regulate the equilibrium conformation of a MEP, Loh and coworkers engineered MEP barnase–ubiquitin[164]. By carefully verifying the length of the linker coupling the domains, the final conformation can be precisely and step-wisely regulated in purpose. However, the equilibrium conformation of the MEP designed in this way is coded by its gene sequence and will not response to the external stimulus.

Here we report a new approach to regulate the equilibrium conformation of a mutually exclusive protein, which contains an artificial metal chelation site on the host domain. Metal chelation will stabilize the thermodynamically less stable host domain and induce the folding switch of MEP. The protein hydrogels containing the MEP are reported to present stepwise increasing Young’s modulus with addition of metal ions.

5.3 Design principle of the mutually exclusive protein with a metal chelation site

The principle of our approach is to harness the metal chelation interaction to selectively
stabilize the host domain, reverse the thermodynamic hierarchy of the two domains in a mutually exclusive protein and ultimately, control the folding of the guest and host domains and dynamically regulate the mechanical properties of protein hydrogel[165, 166]. By introducing an engineered metal chelation site into the structure of the host domain which is thermodynamically less stable, the thermodynamic stability of the host domain could be regulated upon binding of divalent cations[165-168]. In previous work, a mutually exclusive protein GL5-I27 was designed [115, 131], in which a 27th Ig domain from the giant muscle protein titin was inserted into the host domain GL5, a loop insertion mutant of the first IgG binding domain of protein G (GB1)[169]. Using stopped-flow spectrofluorimetry and single molecule atomic force microscopy techniques, it is reported the first direct proof of the tug-of-war during the folding of a mutually exclusive protein. Further more, formation of a disulfide bond, formed by 2 mutated cysteine residues on insertion loop of host GL5, can be used to tune the folding between GL5CC-I27. By controlling redox potential, the folding between GL5CC and I27 can be tuned accordingly.

To efficiently regulate the equilibrium conformation of the mutually exclusive protein, a simple stimulation mechanism is necessary. Such an easy strategy should be able to control the relative thermodynamic stability of the guest domain and host domain through a simple stimulus, so that the final conformation of the MEP can be easily monitored. Amongst many smart and effective strategies to regulate the thermodynamic stability of proteins that have been presented, introducing an artificially designed metal chelation site onto the surface of a protein is one of the most popular approaches for protein stabilization[170-174]. By engineering a bi-histidine (bi-His) motif, which consists of two histidines in a configuration
allowing the binding of a bivalent metal ion (such as Ni\textsuperscript{2+}), onto the surface of a protein domain, the engineered metal chelation site can be easily introduced. Given that the divalent metal ion strongly preferred to bind to the native state instead of the denatured state, the protein can thus be stabilized. Based on these results, Li and coworkers has engineered a series of bi-histidine mutants of GB1\textsuperscript{[168]}. It has been proven that both the thermodynamic and the mechanical stability of those GB1 bi-histidine mutants can be significantly enhanced upon the binding of Ni\textsuperscript{2+}. Therefore, we attempted to involve a bi-His motif into the host domain GL5 by enrolling the metal chelation interaction as the external stimulus to thermodynamically stabilize the GL5 domain, and eventually, reverse the thermodynamic hierarchy of the host domain GL5 and guest domain I27. If successful, the equilibrium conformation of the GL5HH-I27 with bi-His motif will be controlled by adding/depleting of the bivalent metal ions in the environment. In the mean while, when GL5HH-I27 with bi-His motif is constructed into a protein hydrogel, the presences of metal ions cannot only sequentially regulate the folding switch between host and guest domain, but also lead to the change of \textit{L}_{ec} of the hydrogel network, and finally lead to a change of mechanical properties of the hydrogel. In principle, this strategy could be used into the regulation of many different protein machineries.
Figure 5.1 The schematic of the engineered mutually exclusive proteins with the bi-His metal chelation site in GL5 domain.

A) Construction of the designed MEP GL5HH-I27. Guest domain I27 (grey) is inserted into the second loop of the host protein GL5HH (green). The mutated histidine residues 32 and 36 are labeled in orange. According to previous results, the residue pair 32&36, when mutated into histidine, can readily form a bi-His motif and bind to a bivalent metal ion[168]. Moreover, locations of the residues are very close to the insertion site of I27, which can effectively guarantee protein conformational change are indeed due to metal chelation. B) Schematic showing the MEP folding switch GL5HH-I27. Unfolded host and guest domains are colored using the same color coding. The host domain GL5HH can switch its conformation between folded and unfolded conformations in response to metal chelation, resulting in two distinct and mutually exclusive conformations. In Ni^{2+}-free state, since I27 is thermodynamically more stable, GL5HH(U)-I27(F) is the dominant conformation, where the effective chain length between the two termini of GL5HH-I27 is 26 nm; in presence of Ni^{2+}, metal chelation makes GL5HH(F)-I27(U) the dominant conformation, where the folded host domain GL5HH significantly decreases Lec between the two termini to 2.6 nm. The two conformations are in dynamic equilibrium and controlled by metal chelation.

5.4 Designing mutually exclusive protein GL5HH-I27

Figure 5.1 shows our design of the mutually exclusive proteins with the bi-Histidine binding motif. According to previous results, the residue pair 32&36, when mutated into histidine, can readily form a bi-His motif and bind to a bivalent metal ion[168]. With the presence of a bivalent metal ion (such as Ni^{2+}), the formation of the metal chelation between H32&36 and a bivalent metal ion will drive the equilibrium towards GL5HH(F)-I27(U), and GL5HH
should remain folded; without the presence of bivalent metal ion, there will be no metal chelation to stabilize the GL5HH domain and the folding of the thermodynamically more stable I27 will forcibly unfold GL5HH, shifting the equilibrium towards GL5HH(U)-I27(F) conformation. In this way, the conformation of GL5HH-I27 can be modulated by the concentration of bivalent metal ions.

5.5 The engineered bi-His motif in GL5HH-I27 can efficiently bind to the Ni$^{2+}$

To establish that the engineered bi-His motif in GL5HH-I27 can efficiently bind to the bivalent metal ions and efficiently shift the conformation from GL5HH(U)-I27(F) to GL5HH(F)-I27(U), we carried out CD spectroscopy detection to characterize the stabilization effect of the metal chelation on GL5HH-I27. Figure 5.2A shows the CD spectra of GL5HH-I27 with gradual increasing of Ni$^{2+}$. The CD spectra of MEP with varying concentration of Ni$^{2+}$ are different from that of stand alone GL5HH and I27, and are a convolution of the CD signal of GL5HH and I27 domains in the MEP. It is evident that the addition of Ni$^{2+}$ ion in the MEP results in a large secondary structure change. The Ni$^{2+}$-free CD spectrum of GL5HH-I27 presents one minimum at 206 nm, which is the characteristic peak of combined folded I27 and unfolded GL5HH. While with 12.8 mM Ni$^{2+}$, the spectrum presents two minima at 208 and 220 nm, which are characteristic peaks for folded GL5HH and unfolded I27. These results suggest that the addition of Ni$^{2+}$ ion leads to the folding of GL5HH and unfolding of I27, and thus converting the MEP from GL5HH(U)-I27(F) to GL5HH(F)-I27(U).
Figure 5.2 Measurement of metal binding by CD spectrum and Tryptophan fluorescence chemical denaturation.
A) CD spectrum of GL5HH-I27 in the presence of different concentrations of Ni$^{2+}$. B) Standard GdmCl denaturation curves of GL5HH-I27 in the presence of different concentrations of Ni$^{2+}$. The solid lines are fits to the experimental data obtained by using Equation (2.10). C) Changes in the thermodynamic stability of GL5HH-I27 upon addition of Ni$^{2+}$. The data fitted as reported gives a dissociation constant $K_d$ 870 µM for the binding of Ni$^{2+}$ ion[175].

In addition, equilibrium chemical denaturation studies on tryptophan fluorescence were taken to demonstrate the presence of Ni$^{2+}$ ion could successfully stabilize host domain GL5HH.
Since I27 does not contain tryptophan residues, the tryptophan fluorescence measured from GL5HH-I27 solely reflects the chemical unfolding process of the host protein GL5HH[115]. As shown in Figure 5.2B, the presence of Ni$^{2+}$ obviously increased the thermodynamic stability of GL5HH, confirming the metal chelation capability of GL5HH-I27. Changes in the thermodynamic stability of GL5HH upon binding of Ni$^{2+}$ were plotted against the concentration of Ni$^{2+}$ added to the system, as shown in Figure 5.2C. We fitted the data as reported and measured a dissociation constant $K_d$ of 870 µM for the binding of Ni$^{2+}$ to GL5HH, which is similar to that measured on GB1 bi-His mutant, 260 µM[175]. Our results clearly indicate that the engineered bi-His motif in mutually exclusive protein GL5HH-I27 can effectively bind to the Ni$^{2+}$. The binding of the Ni$^{2+}$ to the metal chelation site can obviously enhance the thermodynamic stability of the host protein GL5HH in the mutually exclusive GL5HH-I27.

5.6 Designing protein hydrogel with GL5HH-I27

To engineer protein-based dynamic hydrogels, we constructed artificial elastomeric protein GB1-R-(GB1-GL5HH-I27-R)$_2$ (hereinafter G-R-(G-MEP-R)$_2$) which incorporates the mutually exclusive protein GL5HH-I27 as a protein folding switch to switch the conformation of the host domain GL5HH (Figure 5.3A). GB1-Reslin (G-R) based elastomeric proteins have been used to engineer biomaterials to mimic the passive elastic properties of muscles[85]. The incorporation of GB1 and Resilin allows us to use the well-developed Ru$^{2+}$-mediated photocrosslinking strategy which converts accessible tyrosine residues react into dityrosine between soluble proteins. Tuning GL5HH-I27’s conformation
with addition of Ni\textsuperscript{2+} ion will allow us to effectively change the length of GL5HH in the protein network, thus changing the effective length between two crosslinking points and changing the mechanical properties of resultant protein hydrogels. We found that an aqueous solution of G-R-(G-MEP-R)\textsubscript{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 (Figure 5.3B).

Figure 5.3 Schematic of the three-dimensional hydrogel network constructed from G-R-(G-MEP-R)\textsubscript{2}.
A) In Ni\textsuperscript{2+}-free state, the effective chain length between two adjacent crosslinks is long because GL5HH(U)-I27(F) is the dominant conformation in the hydrogel; in presence of Ni\textsuperscript{2+}, metal chelation induced folding of GL5HH significantly decreases the effective chain length between two neighboring crosslinking points, leading to the deswelling of hydrogel.
B) A photograph of a ring shaped transparent hydrogel constructed from G-R-(G-MEP-R)\textsubscript{2} (120 mg/mL) in Tris buffer using Ru(II)(bpy)\textsubscript{3}\textsuperscript{2+}-mediated photochemical crosslinking strategy. The unit of the scale in picture is cm.

5.7 Metal chelation regulates the mechanical properties of protein hydrogel

To investigate the influence of bi-His motif triggered folding switch behavior on the mechanical properties of hydrogel, we carried out the tensile test on G-R(G-MEP-R)\textsubscript{2}
hydrogel (Figure 5.4A). A ring-shaped sample (120 mg/mL) was stretched to 30% strain in Tris buffer (Ni²⁺-free) and then relaxed back to zero strain, with Young’s modulus calculated to be 5 kPa. In Ni²⁺-free state, the predominant conformation of MEP is GL5HH(U)-I27(F), where the host domain GL5HH is unfolded and the guest domain I27 is folded. Since host domain GL5HH is unfolded under non-denaturing condition, stretching of it cause the force damping response, which lead to energy hysteresis between stretching and relaxation cycles. Swelling ratio of the hydrogel in the Ni²⁺-free state is ~5%.

Hydrogel was subsequently treated with 10 mM Ni²⁺. In the presence of Ni²⁺, the predominant conformation of MEP is GL5HH(F)-I27(U), where the host domain GL5HH is folded and the guest domain I27 is unfolded. In response to the change in MEP conformation, the G-R-(G-MEP-R)₂-based hydrogel should become much stiffer in the presence of Ni²⁺. As expected, after 3 hours to arrive equilibrium, hydrogel was tested again. It was found that the hydrogel deswelled to ~70% of its original size and Young’s modulus increased to 13 kPa. In addition, it is noteworthy that the hysteresis of GR(G-MEP-R)₂ hydrogel between stretching and relaxation cycles increased significantly in presence of Ni²⁺, indicative of a significant increase in energy dissipation. In Ni²⁺-free state, the conformation of MEP will be GL5HH(U)-I27(F). As mentioned before, energy is mainly consumed on stretching of unfolded GL5HH, and only a small fraction of folded I27 will be unfolded. While in presence of Ni²⁺, the conformation of MEP will be GL5HH(F)-I27(U). Stretching of both folded GL5HH and unfolded I27 domain will lead to accumulation of hysteresis. This is because the unfolded I27 is trapped in the loop of GL5HH domain. Stretching force will be firstly applied to the folded host domain GL5HH, and subsequently applied on the unfolded guest domain
I27. Then, the energy will be consumed on unfolding of the GL5HH and force damping response of I27. Therefore, energy hysteresis is higher in presence of Ni$^{2+}$ than that in absence of Ni$^{2+}$. Changes in mechanical and physical properties of the G-R-(G-MEP-R)$_2$ hydrogel are fully reversible. When EDTA was added in the system to remove Ni$^{2+}$, hydrogel regained similar mechanical and physical properties as Ni$^{2+}$-free state - specifically, a low Young’s modulus, high resilience and low swelling ratio (Figure 5.4A). These results strongly indicate that hydrogels based on G-R-(G-MEP-R)$_2$ present reversible mechanical and physical properties that can be regulated via metal chelation. On the contrary, control experiments showed that mechanical and physical properties of (G-R)$_4$-based hydrogels do not change in response to metal chelation (Figure 5.4B), suggesting that the property modifications of G-R-(G-MEP-R)$_2$-based hydrogels are due to conformational changes of the MEP in response to metal chelation. Hydrogels are highly elastic and can be stretched to 120% of its original length until breaking (Figure 5.4C).

Having confirmed that transformations in mechanical and physical properties of G-R-(G-MEP-R)$_2$-based hydrogels are indeed due to metal chelation induced conformational changes of the MEP, we carried out measurements of hydrogel mechanical properties in response to increasing concentration of Ni$^{2+}$ (Figure 5.5A-B). Since thermodynamic stability of GL5HH in MEP can be regulated stepwisely upon binding of gradient addition of Ni$^{2+}$ ion, it is possible that the properties of hydrogel composed by MEP can be tuned as well. As expected, with Ni$^{2+}$ ion treated from 0 to 10 mM, Young’s modulus of the hydrogel increases monotonically from 5 to 13 kPa, and the resilience decreases from 85% to 60%. Every treatment of Ni$^{2+}$ ion takes about 3 hours to achieve equilibrium. This result fully confirmed
that gradual and stepwise regulation of hydrogel is achieved through metal chelation.

Figure 5.4  The mechanical properties of hydrogels are responsive to metal chelation.
A). Typical stress-strain curves of the G-R-(G-MEP-R)_2 hydrogel (120 mg/mL) in Tris (Ni^{2+}-free state), 10 mM Ni^{2+} (in presence of Ni^{2+}), and 20 mM EDTA. The G-R-(G-MEP-R)_2 hydrogel is resilient and soft, with Young’s modulus of 5 kPa in Ni^{2+}-free state; but force-damping and stiff, with Young’s modulus of 13 kPa under in presence of Ni^{2+}. The mechanical properties of the hydrogels are reversible in response to metal chelation. B). The stress-strain curves of (G-R)_4 hydrogel in Tris (Ni^{2+}-free state), 10 mM Ni^{2+} (in presence of Ni^{2+}), and 20 mM EDTA. The mechanical properties of (G-R)_4 hydrogel do not change in response to metal presence. C) The G-R-(G-MEP-R)_2 hydrogel can be stretched to 120% of its original length until breaking.
Figure 5.5 Stepwise control of hydrogel mechanical properties through metal chelation. A) Stress-strain curves of G-R-(G-MEP-R)$_2$ hydrogel as a function of increased concentration of Ni$^{2+}$ ion. The hydrogel shows gradual increase in Young’s modulus and gradual decrease in resilience with gradient treatment of Ni$^{2+}$ ion. B) Young’s modulus and resilience derived from stress-strain curves in Figure 5.5A.

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 absence and presence of Ni$^{2+}$ (Figure 5.6A-C). It is evident that protein concentration has a more significant impact in presence of Ni$^{2+}$ state on the Young’s modulus, resilience and swelling ratio of the hydrogel, than that in Ni$^{2+}$-free state. This result can be readily accounted for effective crosslink density. The higher the protein concentration means the higher
crosslinking density, causing a more significant effective length change between two crosslinking points of hydrogel in presence of Ni$^{2+}$ than that in Ni$^{2+}$-free state. At higher protein concentrations, the mechanical and physical properties of G-R-(G-MEP-R)$_2$-based hydrogels can be tuned within a much broader range by a change in metal chelation than that at low protein concentration.

Figure 5.6  Protein-concentration dependence of the Young’s modulus (A), resilience (B), and deswelling ratio (C) of the G-R-(G-MEP-R)$_2$ hydrogel (n>2).
5.8 Conclusion

Our results on the bi-His mutant of the mutually exclusive protein clearly indicate that by introducing the bi-His motif (metal chelation site) in the host domain, we can now control the conformational state of the host domain through mutually exclusive folding via a simple external stimulus. In Ni$^{2+}$-free state, majority of the mutually exclusive protein is GL5HH(U)-I27(F); in presence of Ni$^{2+}$, the majority of the mutually exclusive protein is GL5HH(F)-I27(U). By controlling the folded state of the MEP via metal chelation, the effective chain length between two crosslinking points can be readily modulated, resulting in the dynamic modulation of hydrogel mechanical properties. That is, the conformational change of MEP in microscopic level is translated into mechanical and physical motions at the macroscopic level. The hydrogel is regulated from a soft, yet resilient state to a stiff, force damping state.

The designed hydrogel has several unique characteristics. First of all, the regulation of mechanical and physical properties of hydrogel is based on changes of effective chain length between crosslinking points. Previously, most of dynamic hydrogels are regulated through changes of crosslinking density, which is introducing or removing crosslinking joints via external stimuli. However, some of these methods involve permanent changes or harsh treatments to hydrogel, which may restrict the applications of the materials. By changing the chain length between crosslinking points, alterations in the mechanical and physical properties of this hydrogel are fully reversible. Metal ions can be easily added to the hydrogel system and be removed by addition of EDTA. This unique feature will make our hydrogel a
competitive candidate when reversibility and mild condition are seriously concerned.

Another feature of our protein hydrogel system is the mechanical/physical hydrogel properties can be tuned in a stepwise fashion. By gradually treating the hydrogel with metal ion, mechanical and physical properties of hydrogel can be precisely regulated step by step. When combining the changes on metal concentration and protein concentration, the hydrogel can be regulated over an even broader range. Comparing with simple "on-off" control mode, the broad but precise control of hydrogel will benefit plenty of applications on biological and artificial tissue engineering studies.

In addition, the dynamic mechanical and physical properties of hydrogel are regulated through conformational change of host domain in MEP. The host domain is split into two halves by guest domain. It is possible that the fragments of host domain not only undergo intramolecular folding/unfolding, but also undergo intermolecular reconstitution. However, the intermolecular reconstitution is less possible to happen, whereas strong binding between fragments and desired low temperature are necessary. Base on the result reported before, the intermolecular interaction has a melting temperature of 22 °C, which is lower than our working temperature, 25 °C. The relative high temperature can effectively inhibit intermolecular binding. In addition, intermolecular interaction is a very slow process comparing to photo induced chemical crosslinking through Tyrosine (less than one minute). The interactions among polyprotein sequences are dramatically restricted after chemical crosslinking and gelation.

Overall, we have endeavored to engineer the MEP based hydrogel with dynamically tuned
mechanical and physical properties through metal chelation. The property changes of hydrogel are regulated through conformational change of host domain in MEP. We anticipate that improved design and engineering approaches will allow us to further expand the dynamic range of mechanical properties and improve the mechanical strength. Metal chelation is a common method to stabilize protein, therefore, we hope our design can be used universally to engineer more MEP and to create smart protein hydrogels with unique mechanical and physical properties. The hydrogel can be potentially applied on tissue engineering and drug delivery vehicle studies in future.
Chapter 6: Protein Hydrogels with Varying Stiffness Directing Different Cellular Responses

6.1 Synopsis

Protein-based hydrogels can be applied as an extracellular matrix that is capable of supporting cell viability and function. People have known that matrix elasticity has a profound influence on the behaviour of anchorage-dependent cells. Hydrogels with different stiffness are powerful materials for studying cell responses to substrates with varying stiffness. In this chapter, we construct TNfn3-MEP-resilin based hydrogel. TNfn3 provides RGD sequence to bind cells. Resilin provides tyrosine residues to crosslink. Particularly, the MEP GL5CC-I27, is used as a third building block to regulate mechanical properties of hydrogels responding to redox potential. In oxidized condition, hydrogels are stiffer and the cells (e.g. human lung fibroblast) prefer to spread out; while in reduced condition, hydrogels are softer and the cells remain round and do not spread out. The presented preliminary date provides an opportunity to investigate the mechanical effects in cellular responses.

6.2 Introduction

Protein-based hydrogels not only retain the biocompatibility of their protein components, but also gain unique features from the hydrogel network, such as mechanical and structural properties, which can be made to resemble many natural tissues. As a consequence of these unique properties, a variety of protein-based hydrogels have been developed for use as scaffolds capable of supporting cell viability and function for tissue engineering applications.
Tissue engineering hydrogels are being designed to mimic the ability of natural, native extracellular matrix (ECM) components[176]. In living tissue, ECM helps regulate cell dynamics, such as cell proliferation, differentiation and migration, with specific molecular cues. Variations in arrangement and composition of the ECM macromolecules give rise to an amazing diversity of forms and functions within different types of tissues[18]. Analysis of ECM macromolecules has identified a variety of active sequences that are responsible for distinct functions, providing molecular sequences that can be adopted for inclusion in biomimetic hydrogel designs. One of the most well known cell adhesion and ECM motifs employed in the preparation of synthetic tissue engineering scaffolds is arginine–glycine–aspartic acid (RGD) tripeptide. RGD is a common cell binding domain found in many natural proteins in the ECM and blood (e.g., fibronectin, vitronectin, osteopontin, collagens)[177]. It can be recognized by over 20 known integrins and promotes cell adhesion, spreading, and growth. Correspondingly, scientists have incorporated RGD into hydrogels and surfaces, on which desired cells are able to bind, spread, and proliferate[178]. For various hydrogel designing purposes, the RGD sequence can either be directly incorporated into the polyprotein sequence or be incorporated through naturally RGD-containing proteins[79, 109, 179].

Tenascin-C is a tandem modular ECM protein with important roles in regulating cell-matrix interactions. The third fibronectin type III domain of tenascin-C (TNfn3) is a RGD-containing domain with 90 amino acid residues. Previously, Lv et al. used TNfn3 to construct TNfn3-resilin (F-R) based hydrogels, FRF₄RF₄R. Resilin is used to provide tyrosine residues to crosslink through the well-established Ru(II) induced photo crosslinking. Their
results demonstrated that a polyprotein incorporating TNfn3 and resilin could be readily photo-crosslinked into elastic hydrogels with Young’s modulus of ~ 20 kPa[79]. None of the components involved in hydrogel formation were cytotoxic in vitro at the levels tested. Human lung fibroblast cells could successfully grow and spread on the surface of TNfn3-resilin hydrogels. However, the FRF4RF4R hydrogel can provide only a simple system that lack flexibility, the different performance of cells at varying mechanical ranges is hard to observe on the hydrogel. Therefore, a biocompatible hydrogel with dynamic control on mechanical properties are needed.

The mechanical rigidity is an important factor that affecting material applications. The stiffness of ECM in different tissues within the body varies significantly. For example, the elastic modulus of bone tissue can be as stiff as 18 GPa[180], while that of brain tissue can be as soft as 0.1 kPa[118-120]. Correspondingly, recent studies have demonstrated that matrix elasticity has a profound influence on the behavior of anchorage-dependent cells[181, 182]. Successful interactions between cells and their substrates, therefore, require control over the mechanical properties, so that biophysically friendly microenvironments can be created.

In this study, we would like to construct TNfn3-MEP-resilin based hydrogel, (F4-MEP-R)2. As mentioned before, TNfn3 will provide RGD sequence to bind cell and resilin will provide tyrosine to crosslink. Particularly, MEP will be used as a third building block to respond to redox potential. Based on the result from Chapter 3, the conformational change of GL5CC-I27 will affect the mechanical properties of hydrogel. We expect the mechanical properties of hydrogel will be controlled dynamically and the cell behavior will be different under different condition on hydrogel surface. Under the oxidized condition, hydrogel will be
stiff and cells will spread and extend; under reduced condition, hydrogel will be soft and compliant, and cells will remain round shape on the hydrogel surface (Figure 6.1).

![Schematics of cell behavior on hydrogel surface in different redox potential.](image)

Figure 6.1  Schematics of cell behavior on hydrogel surface in different redox potential. In oxidized condition, hydrogel will be stiff, where cells (e.g. human lung fibroblast) prefer to spread out. In reduced condition, hydrogel will be soft, where cells will remain round and do not spread out.

6.3 Materials and methods

The cell spreading assay.

Hydrogels were firstly washed with DMEM medium to get rid of unconsumed crosslinking reagents. Then, 2000 HFL1 cells were seeded on the top of (F₄-MEP-R)₂ hydrogel at 37 degree for 48 hours, with FRF₄RF₄R hydrogel serving as controls. Samples were washed twice with sterilized PBS (pH 7.2) to remove non-adherent cells. Throughout the progress, reduced hydrogels were treated with additional 10 mM Glutathione (GSH), which is a reductant generally used for cell studies. To evaluate cell morphology, adherent cells were stained with Alexa Fluor 488 phalloidin and DAPI following the manufacturer’s protocol. Briefly, cells were fixed with 3.7% formaldehyde solution in PBS for 10 mins at room
temperature. Fixed cells were immobilized by incubating in a 0.1% Triton X-100 solution in PBS for 5 mins. Samples were then incubated with phalloidin for 20 mins and DAPI for 5 mins. After staining, samples were washed with PBS prior to imaging using a fluorescence microscope (Nikon, Eclipse Ti). Images were taken at ×10 magnifications.

6.4 Dynamic protein hydrogels based on (F₄-MEP-R)₂ show stimuli-responsive mechanical properties

To investigate the influence of disulfide bond triggered folding switch behavior on mechanical properties of hydrogel, we used the well-developed Ru²⁺-mediated photocrosslinking strategy to crosslink protein and carried out the tensile tests on (F₄-MEP-R)₂ hydrogel (Figure 6.2A) as well as TNfn3-resilin hydrogel, FRF₄RF₄R (control, Figure 6.2B)[72]. In oxidized state, ring-shape sample (F₄-MEP-R)₂ (70 mg/mL) was stretched to 40% strain in PBS and then relaxed back to zero strain with a Young’s Modulus of 11 kPa (Figure 6.2A), similar to that of FRF₄RF₄R (Figure 6.2B). The resultant hydrogel is resilient (78%) and only shows a small hysteresis between stretching and relaxation cycles. In the oxidized state, the predominant conformation of MEP will be GL5CC(F)-I27(U). Similar to that discussed in Chapter 3, the hysteresis between stretching and relaxation cycles comes from unfolding of folded TNfn3 at high strain[82].

Hydrogel was subsequently treated with 10 mM DTT. In the reduced state, the predominant conformation of MEP will change from GL5CC(F)-I27(U) to GL5CC(U)-I27(F), in which the host domain GL5CC is unfolded and the guest domain I27 is folded. In response to the change of the conformation of MEP, the (F₄-MEP-R)₂-based hydrogels in the presence of
DTT should become much softer. Indeed, during stretching, an obvious force reduction was observed. Young’s modulus deceased from 11 kPa to 4 kPa. While, without MEP in the hydrogel, the stress-strain curves of FRF₄RF₄R hydrogel in reduced and oxidized state superposed with each other and there were no similar observations on modulus changes. It is clear that the mechanical difference between reduced and oxidized state of our hydrogel is based on triggered folding switches within MEP. Interestingly, the hysteresis in the stress-strain curves of (F₄-MEP-R)₂ hydrogel in reduced state remains similar to oxidized state, which is different from GR(G-GL5CC-I27-R)₂ hydrogel, in which hysteresis is higher in reduced state than in oxidized state (Chapter 3). In reduced GR(G-MEP-R)₂, the hysteresis mainly comes from the viscoelastic response of unfolded GL5CC of MEP and only a very small number of GB1 is unfolded. However, it is worth mentioning that TNfn3 domain is mechanically more labile than GB1, more fraction of TNfn3 domain will be unfolded even under lower force[183]. Therefore, in reduced state, unfolding of TNfn3 domain in (F₄-MEP-R)₂ will contribute more to hysteresis than that of GB1 in GR(G-MEP-R)₂, which leads to similar hysteresis of (F₄-MEP-R)₂ in reduced and oxidized state.
Figure 6.2  Tensile tests of hydrogels in oxidized and reduced condition. A). Typical stress-strain curves of the (F₄-MEP-R)₂ hydrogel (70 mg/mL) in PBS (oxidized, red) and 10 mM DTT (reduced, green). The Young’s modulus of the hydrogel is 11 kPa and 4 kPa under oxidizing and reduction conditions, respectively. B). The stress-strain curves of FRF₄RF₄R hydrogel (70 mg/mL) in PBS (oxidized) and 10 mM DTT (reduced). The Young’s modulus of FRF₄RF₄R hydrogel is 14 kPa and do not change in response to the change of redox condition.

6.5 Morphology of (F₄-MEP-R)₂ hydrogels

After confirmed the redox response of (F₄-MEP-R)₂ hydrogel in tensile test, we used SEM to directly visualize the morphology of (F₄-MEP-R)₂ hydrogel (Figure 6.3). The pore size of the reduced sample is bigger than the oxidized ones. This is consistent with swelling ratios, where the hydrogel swells from 7.6% in oxidized state to 38.1% in reduced state. This result further confirmed that (F₄-MEP-R)₂ hydrogel can be triggered by redox potential. When engineered for tissue engineering, pores of hydrogels need to be open and evenly distributed in order to allow nutrition cycling, metabolism, and uniform growth of cells. From the SEM image, (F₄-MEP-R)₂ hydrogels demonstrate interconnected open pores, which allowing them to retain water and nutrients, and diffuse metabolites. (F₄-MEP-R)₂ hydrogels with these properties are suitable for cell adherence, growth and proliferation.
Figure 6.3 SEM images and hydrogel pictures (70 mg/mL) reveal the formation of a microporous network structure. The scale bar represents 50 μm.

### 6.6 (F₄-MEP-R)₂ hydrogel supports cell spreading

TNfn3 is a protein containing RGD peptide sequence that can interact with integrin within many mammalian cell lines[177]. We have proved that TNfn3-containing hydrogels are suitable for supporting cell proliferation[79]. To further investigate the cytocompatible properties of (F₄-MEP-R)₂ hydrogel, we tested the cell spreading properties on hydrogel by fluorescence imaging. Fibroblast cells were cultured for 24 hrs and substantially fixed on (F₄-MEP-R)₂ hydrogel surface. Fibroblast cytoskeletal protein actin was fluorescently stained using Alexa Fluor 488-conjugated phalloidin and the nuclei DNA was fluorescently stained using DAPI. Both oxidized and reduced hydrogels are investigated, and FRF₄RF₄R hydrogels are used as control sample.
Figure 6.4  Morphology of fibroblasts grown on 70 mg/mL (F₄-MEP-R)₂ and FRF₄RF₄R hydrogel surfaces in oxidized and reduced condition. Actin filaments were stained with Alexa Fluor 488 Phalloidin (Invitrogen) and Nuclei DNA were stained with DAPI. The scale bar represents 10 μm.

As shown in fluorescence image, human fibroblasts grew well on the oxidized (F₄-MEP-R)₂ hydrogel, exhibiting the prototypical well-spread morphology expected for adherent fibroblasts[184, 185]. In contrast, cells cultured on reduced (F₄-MEP-R)₂ hydrogel mostly remained rounded morphology and did not spread (Figure 6.4). While, for control sample FRF₄RF₄R, human fibroblasts attached well, proliferated and spread out in both oxidized and reduced condition. Imaging analysis demonstrated that cells cultured on oxidized (F₄-MEP-R)₂ hydrogel surfaces have an average cell length of ~16 μm, while cells on reduced (F₄-MEP-R)₂ hydrogel surface are ~7 μm long (Figure 6.5A-B). These experiments
clearly indicate that (F₄-MEP-R)₂ hydrogel can support cell adhesion and cell spreading. Addition of reductant does not affect the cytocompatibility of hydrogels. The difference in stiffness of oxidized and reduced hydrogel results in the different cell behaviour.

![Cell length distribution](image)

**Figure 6.5** Cell length distribution. (A) Cells grown on oxidized (F₄-MEP-R)₂ hydrogel surfaces have an average cell length of ~16 μm. (B) Cells grown on reduced (F₄-MEP-R)₂ hydrogel surfaces have an average cell length of ~7 μm.

### 6.7 Discussion

In natural ECM, mechanical properties play important roles in cell adhesion and spreading. Previously, researchers reported fibroblasts have a maximal spread area on a modulus of greater than 10 kPa[181, 182]. Our results are consistent with these findings: for oxidized hydrogels with modulus at 11 kPa, cells attach and spread very well; for the reduced
hydrogels with modulus at 4 kPa, cells remain round morphology and do not spread.

Different crosslinking strategies in recombinant protein-based systems provide possibilities of materials preparation with a wide variety of stiffness. For hydrogel with determined crosslinking strategy, regulation of crosslinking density and effective chain length between crosslinking points are two accessible options for controlling mechanical properties of materials as mentioned in Chapter 1. Since alternations in crosslinking density usually simultaneously change density of bioactive ligands presented in the matrix, regulation of effective chain length between crosslinking points is a particularly powerful way to provide a single variable system when mechanical effects are evaluated. MEP is a powerful motif that can be used as a building block to regulate effective chain length between crosslinking points on protein hydrogel network. Due to the cytocompatible nature of the polyprotein, MEP-based hydrogel can be used to mimic the natural ECM to support cell growth and spreading. More importantly, the mechanical properties of hydrogel can be controlled dynamically, leading to the different behavior of cultured cells.

6.8 Conclusion

Herein, we demonstrate a novel cytocompatible material that without changing the structure of the biopolymer backbone and crosslinks, or initial biopolymer concentration, the mechanical properties can be dynamically controlled by environmental stimulus, that is, the redox potential. Porous morphology of hydrogels is detected by SEM. Behavior of human fibroblast cells cultured on hydrogel surface under oxidized and reduced condition are investigated by fluorescence imaging. The presented preliminary data provides an
opportunity to investigate the mechanical effects on cellular responses. Further experiments are needed to confirm the cytocompatibility of hydrogels, such as MTT assay and live/dead assay. Additional measurements are still necessary to detect the perturbation of other hydrogel properties to cell behaviors in different redox potential.
Chapter 7: Conclusions and Outlook

7.1 Conclusions

This dissertation mainly discusses the construction of engineered protein hydrogels and their applications on biological studies. The work starts from an introduction of engineered protein hydrogels depending on different crosslinking nature. In Chapter 1, numerous milestone studies in physical crosslinking hydrogels are reviewed, including the developments based on bio-recognition association and inverse phase transition. Another section describes the chemical crosslinking systems that involve crosslinking on side chains of tyrosine, lysine and cysteine residues. The last section of the chapter presents various approaches on designing protein hydrogel with dynamic system, such as controlling crosslinking density and controlling effective chain length between crosslinking points.

The second chapter contains experimental methods and material preparation. Detailed protocols, instruments and data analysis are included.

Chapter 3 describes redox-responsive dynamic protein hydrogels with reversible mechanical properties. This is achieved by incorporation of mutually exclusive protein as a building block. Through oxidation and reduction of an engineered disulfide bond, the protein can switch its conformation between folded and unfolded state, leading to dramatic change of effective chain length between hydrogel crosslinking points. In reduced state, hydrogel is high swelling and soft; while in oxidized state, hydrogel is lesser swelling and stiffened by as much as 5 times.
Chapter 4 describes the use of protein fragment reconstitution as a driving force for engineering physically crosslinking protein hydrogel. Two construction designs are described, including a two-component hydrogel that gelates by mixing and has the ability to further chemically crosslinked by disulfide bond formation, as well as a single-component hydrogel that self-assembles and undergoes reversible phase transition under thermal control. Chapter 5 continues the study on designing hydrogels with dynamic mechanical properties. Metal chelation is used as the external stimulus to regulate mechanical properties of hydrogel. Two histidine residues are introduced to the host domain of MEP, allowing metal ions to control conformational state of MEP. Through changing the effective chain length between crosslinking points, the absence and presence of Ni\(^{2+}\) ion leads to stiffness, resilience and volume changes in the resultant hydrogel.

Finally, Chapter 6 demonstrates the different cell response to hydrogels with varying stiffness. The redox responsive hydrogels are used as substrates to support cell growing on surface. The preliminary result showed changes in cell spreading and proliferation between oxidized and reduced condition. This study establishes the potential application of hydrogels for controlling cell behavior.

### 7.2 Outlook

#### 7.2.1 Progress of designing recombinant protein based hydrogels

Over the last two decades, the field of recombinant protein-based hydrogels has made significant progress. Many innovative systems have been designed and developed to form hydrogels that have the capacity to advance applications in diverse fields, ranging from basic
biological studies, tissue engineering, to drug delivery and novel therapies. Through protein engineering, each system can be tailored to achieve desired properties, including the conditions under which hydrogelation occurs, as well as mechanical and degradation properties, which are crucial for their intended applications. It can be safely anticipated that the field will continue to advance at a fast pace, and new protein hydrogels will be developed to enable novel applications within a range of fields.

Table 7.1  Physical gelation strategies.

<table>
<thead>
<tr>
<th>Type of Association</th>
<th>Recombinant Protein Sequence and Network</th>
<th>Number of Sequences</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homo-association</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>Leucine zipper[23], elastin [64, 190] Elastin and silk[66-70]</td>
</tr>
<tr>
<td>Hetero-association</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single and match</td>
<td></td>
<td>2 and above</td>
<td>Leucine zipper[24], dock and lock[42, 43, 147], CaM and ligand[191], growth factor mediation[139], MITCH[37], TPR + DESVD[41], cutA-TIP1(PDZ)+PDZ binding domain[44]</td>
</tr>
<tr>
<td>Dual and match</td>
<td></td>
<td>2 and above</td>
<td>CaM and leucine zipper[40]</td>
</tr>
<tr>
<td>Self and match</td>
<td></td>
<td>1</td>
<td>Mutually exclusive protein-based fragment reconstitution[186]</td>
</tr>
</tbody>
</table>
7.2.2 Designing recombinant protein based physical hydrogels

Given the progress in protein engineering (both in experimental and de novo design), bio-conjugate chemistry as well as cell biology, exciting opportunities are present in the field of engineered protein hydrogels. For physical protein hydrogel, bio-recognition will continue to be a major driving force in innovation (Table 7.1). Depending on different types of bio-recognition, building blocks and protein sequences of hydrogel network could be designed accordingly. Bio-recognition is ubiquitous in biology, thus offering potentially unlimited candidates to use as a driving force to engineer physically crosslinked protein hydrogels, provided that bio-recognition is or can be arranged into multivalent. Compared with the vast space of bio-recognition, the methods reported so far barely scratched the tip of an iceberg, where many bio-recognitions that are abundant in nature have not been explored. For example, protein fragments reconstitution has been studied extensively in biophysics and used as assay for protein-protein interaction detection[140, 141, 144, 147-149]. We have just started to explore this new bio-recognition for engineering protein hydrogels[186] . We anticipate that novel and more efficient bio-recognition will be exploited and designed to create protein hydrogels with desired chemical, physical and functional properties. In particular, tuning the binding affinity of the bio-recognition process will subsequently tune the physical properties of the hydrogel; specifically, a weak binding affinity may lead to transient crosslinks, which could be important for engineering shear-thinning hydrogels; on the other hand, tight binding may result in physical hydrogels that could display properties similar to those of chemical hydrogels. Moreover, unique properties of the bio-recognition process will likely result in hydrogels with novel properties and functions. For example, most
protein-protein interactions belong to the so-called slip bond, where the lifetime of the protein-protein interaction decreases when subject to a stretching force. Some protein-protein interactions belong to the so-called catch-bond[187-189], where the lifetime increases with applied stretching forces. Using such catch-bond bio-recognition, protein hydrogels could display unusual rheological properties at different levels of deformation.

7.2.3 Designing recombinant protein based chemical hydrogels

In terms of chemical protein hydrogels, developing novel crosslinking chemistries remains an important task. Ru(II)-mediated photochemical crosslinking and SpyCatcher-SpyTag schemes are excellent examples that have made significant impacts in the field of protein hydrogels. Optimizing such novel crosslinking chemistries and discovering new avenues will help engineer protein hydrogels for various biomedical applications. In this area, the incorporation of non-natural amino acid into proteins will likely offer a new path towards efficient and well-controlled crosslinking chemistry[192]. Developing this method into a more robust technique for constructing protein hydrogels will require more efforts and will likely introduce more efficient crosslinking chemistry, such as click chemistry, into the toolbox researchers can use to engineer protein hydrogels.

7.2.4 Designing recombinant protein based dynamic hydrogels

Another area of great potential interest is dynamic protein hydrogels. Hydrogels have been regarded as “blank slates” that could be decorated with functional groups[110]. Traditional hydrogels are relatively inert and static, providing a static, biomimetic environment. Such hydrogels lack the dynamic, spatiotemporal changes common in cellular and extracellular
matrix environments. In addition, the mechanical properties of hydrogel substrates play important roles in modulating cell-matrices, as well as directing stem cell lineage differentiation[119, 120]. Developing hydrogels with dynamically tunable mechanical properties will not only better mimic the dynamic extracellular matrix environment, but also enable important mechano-biology studies, such as how mechanical cues direct stem cell specification. Although some significant progress has been made in polymer-based dynamic hydrogels[110, 123], engineering protein-based dynamic hydrogels has just begun[82]. Combining intrinsic biologically functional entities with dynamic mechanical properties into protein hydrogels will likely enable the study of mechano-biology systems in environments that closely mimic those in nature.

As we mentioned in previous chapters, controlling protein’s conformational change is a powerful method to regulate mechanical properties of protein-based hydrogels. The engineered protein-folding switch, MEP, is a newly developed method that can be applied in hydrogel network to create dynamic properties[113]. The mutated disulfide bond engineered on MEP can control equilibrium conformation of that through redox potential, thus changing the effective length between crosslinking points of network and changing the mechanical properties of resultant protein hydrogels. In addition, Ni\(^{2+}\) chelation can be used as another method to control equilibrium conformation of MEP. Except disulfide bond trigger and metal chelation, in our case, antibody binding will be another potential method to trigger MEP’s conformation, as GB1 is a known IgG antibody binding protein. Moreover, in term of other strategies on protein conformational change, fragment reconstitution might be an interesting candidate to regulate protein conformation, where fragment’s conformation can be triggered
by addition of the complimentary fragment.

7.2.5 Applying recombinant protein based hydrogels as ECM

Hydrogels are widely applied to tissue engineering studies that mimicking ECM to investigate cell growth, proliferation and differentiation. Mechanical properties play important roles in regulating cell-matrix interactions. People have found that stiffness of substrates affects cell morphology, cytoskeletal structure, and adhesion[181]. Fibroblast cells were found to present an abrupt change in spreading that occurs at a stiffness range from 1 to 10 kPa[182]. Since the hydrogels engineered with MEP can provide a hydrogel substrate with dynamic mechanical properties, introducing MEP into TNfn3-resilin hydrogel can not only satisfy the dynamic stiffness request but also provide a cytocompatible environment (Chapter 6).

In addition to 2D surface cell culturing, numerous studies are highlighting the importance of 3D cell encapsulations, whereas 3D culture substrates are able to more accurately mimic natural ECMS and cells behave more natively when cultured in 3D environments. The materials used for 3D cell encapsulations are designed to mimic many critical matrix properties such as mechanical rigidity and cell adhesion. It has been confirmed that precisely tuning these properties will influence cell behavior, including morphology, migration, differentiation, interaction with neighboring cells and intracellular signaling[10, 193]. As mentioned above, the SpyCatcher and SpyTag engineered chemical hydrogel are successfully applied for 3D cell encapsulation[109]. In addition, the MITCH system was also tested for its capabilities to encapsulate multiple cell types within 3D hydrogels of tunable viscoelasticity[37]. The transient, non-covalent crosslinks of MITCH allow network
disassemble under reasonable shear forces during injection and re-assemble after force removal[194]. These features make the hydrogel as an ideal injectable cell delivery vehicle. Based on different chemical or physical crosslinking strategies, recombinant protein hydrogels are suitable candidates to provide variable properties and features to satisfy material requirements. In our case, the photo-crosslinked chemical hydrogel could be a good candidate for 3D cell encapsulation with only one limitation that the catalyst APS needs to decrease to biocompatible level (~5 mM) and ensure crosslinking. We are currently testing the minimal necessary amount of APS for crosslinking. Crosslinking could be successfully completed at the expected level with no problem. For the fragment reconstitution based physical hydrogel, inoculation temperature of 3D cell culture (37 degree) is relatively too high to maintain fragment reconstitution. However, the secondary disulfide bond chemical crosslinking is powerful to stabilize the fragment competition and support cell growth. Overall, recombinant protein based hydrogel is a promising biomaterial. Many innovative systems have been engineered for biological, mechanical and physiological applications. Every system has its unique preparing conditions, degradation rates, hydrogel strength and elasticity, which are precisely designed for a desired use and application. Understanding the nature of crosslinking in hydrogel networks is essential for determining the properties of materials and in considering the material for a particular application.
References

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Appendix: Protein and DNA sequence

A.1  wild type GB1

Protein:
MDTYKLILNGKTLGETTTEAVDAATAEKFQYANDNGVDGEWTYDDATKTFTVTE

cDNA:
ATGGACACC\color{red}{TACAAACTGATCCTGAACGGTAAAACCCTGAAGGTGAAACCA}CCACCGAAGCTGTAGACGCTGCTACTGCAGAAAAAGTTTTCAAACAGTACGC
TAACGACAACGGTGTCAAGCTACGACGCTACCAAAACC\color{red}{TTCACGGTTACCGAA}

A.2  GL5

Protein:
MDTYKLILNGKTLGETTTEAVDAATAEKFQYANDNGV\color{red}{GGGLGDGEWTYDDATKTFVTET}

cDNA:
ATGGACACC\color{red}{TACAAACTGATCCTGAACGGTAAAACCCTGAAGGTGAAACCACCGAAGCTGTAGACGCTGCTACTGCAGAAAAAGTTTTCAAACAGTACGCTAACGACAACGGTGTCAAGCTACGACGCTACCAAAACCTTCACGGTTACCGAA
A.3 I27F

Protein:

\[
\text{LIEVEKPLYGVEVFVGETAHFEIELSEPVDHGQFKLKGPQPLAACSPDCEIEDGKKHELHNCQLGMTGEVFSAANTKS}
\]

\[
\text{AANLKVKE}
\]

cDNA:

\[
\text{CTAATAGAAGTGGAACGCCTCTGTACGGAGTAGAGGTGGTGTGGTTGGACAG}
\]

\[
\text{CAGCCCACTTTGAATTAACCTTGAAACCTGATGGTCACCGCCAGTTTAAAG}
\]

\[
\text{CTGAAAGGACAGCCTTTGGACGCTTTCCCTGACTGTGAACATTTGAGGATG}
\]

\[
\text{GAAAGAGCATATTTCTGACACCTTCATCAACTGCAGCAGCTTGGTAATGAGGAGA}
\]

\[
\text{GGTTTCCCTCCAGGCTGCTAATACCAAATCTCGACGCAAATCTGGAAGGTGAC}
\]

\[
\text{GAATTG}
\]

A.4 GL5-I27F

Protein:

\[
\text{MTIYKLNLNGLKKTGETTTEAVDAATAEKVFKQYANDNGVGGGLGGLIEVEKPLYGV}
\]

\[
\text{EVVFGETAHFEIELSEPVDHGQFKLKGPQPLAASPDCEIEDGKKHELHNCQ}
\]

\[
\text{LGMTGEVFSAANTKSAAANLKVKE}
\]

\[
\text{LDGEWTRYDDATKFTTVTE}
\]

where the sequence in italic is from the host domain GL5, and the sequence in bold is from the guest domain I27F. The junction between GL5 and I27F is Leu--Gly resulted from the enzyme restriction site.

cDNA:

\[
\text{ATGGACACCTACAAACTGATCTGAACGGTAAAAACCCTGAAAGGTGAAACCC}
\]

\[
\text{ACCACCAGCTGTAGACGCTTGACTGGAGAAAAGTTTCTAAAAACAGGACG}
\]

\[
\text{CTAACSAGCAACGGTGTCCGTGCGGACTCGGGCTAATAGAAGGTGGAAAGCC}
\]

\[
\text{CTCTGTACGGAGTAGGTTGGTGGTGAAACAGCCCACCTGGAATTGAC}
\]

134
ACTTTCTGAACCTGATGTTCACGGCCAGTTTAGCTGAAAGGACACACCTTTG
GCAGCTTCCCTGACTGTGAAATCATTTGAGGATGAAAGAAGCATATTCTGA
TCCTTCTACAATCTGACGAGGTATGACACAGGAGAGGTCTTCCAGGCTGCT
AATACCAAAATCTGCAGGCAATCTGAAAGTGAAGAATTGCACCTAGCGCTACC
GAATGGACCTACGACGACGCTACCAAAACCTTCACGGTTACCGAA

A.5 GL5CC-I27F

Protein:

MDTYKLILNGKTLKGETTTEAVDAATAEKFQKYANDNGVCGGLGLIEVEKPLYGV
EVFVGTAHFEIELSEPVDHGQFKLKGQPLAASPDCEIIEDEGKKHILILHCQ
LGMTGEVSFQAANTKSAANLKVKECGDGWYDDATKFTFTVE

cDNA:

ATGGACACCTACAAACTGATCTCAAGGCTAAAACCTGAAAGGTGAAACC
ACCACCGAAGCTGAGCCTGCTACTCGAGAAAGATTTACACAGTCAGC
CTAACGAGCAACCGTGTCGGTTTGCTGAGGCTAATAGAAAGTGAAAGAAGC
CTCTGTACGGAGATGTTTGTTGTGTGAAACAGGCACTTTGAAATTTGA
ACTTTCTGAAACCTGATCTCAGACGCGTATTAAAGCTGAAAGACGCCTTTT
GCAGCTTCCCTGACTGTGAAATCATTTGAGGATGAAAGAAGCATATTCTGA
TCCTTCTACAATCTGACGAGGTATGACACAGGAGAGGTCTTCCAGGCTGCT
AATACCAAAATCTGCAGGCAATCTGAAAGTGAAGAATTGCACCTAGCGCTACC
GAATGGACCTACGACGACGCTACCAAAACCTTCACGGTTACCGAA

A.6 GN-I27F

Protein:

MDTYKLILNGKTLKGETTTEAVDAATAEKFQKYANDNGVCGGLGLIEVEKPLYGV
EVFVGTAHFEIELSEPVDHGQFKLKGQPLAASPDCEIIEDEGKKHILILHCQ
LGMTGEVFQAA ANT KSAANLKVKE

cDNA:
ATGGACACCTAC AA ACTGAT CCTGAACG GTAAAACCCTGAAAGGTGAAACC
ACCACC GAAGCT GTAGACGCTG CACTG CAG AAAGATTTTCAAACAGTACG
CTAACGACAACGGGTGTCGTTGG ACTCGGGCTAATAGAAGTGGAAAAGC
CTCTGTACGGAGTAGG GTTTGTGTTGGTGAACAC AGCCCAACTTTGAAATTGA
ACTTTCTGAAACCTGATGTTCACGGCCAGTTT AAGCTGAAAGGACAGCCTTTG
GCAGCTTCCCCTGACT GTGA AAATCATTGAGGATG GAAAGAGCATAT TT CTA
TCCTCATAACTGT CAGCTGGGTACCTGC C ATTTCCAGG CTGCT AATACCAAATCTGCAGCCAAATCTGAAAGTGAAGAATTGA

A.7 I27F-GC

Protein:
LIEVEKPLYGVEVFVGA TAHI SELSEP DVIHGQFKLGQPLAASPDCEIIEDG
KKHILILHNCQLGM TGEVFQAA NT KSAANLKVKE CGDGEWTYDDATKTFT

VTE

cDNA:
CTAATAGAAGTGAA AAAAGCCCTC TGATCGGAGTAGG GTTTGTGTGTTGGTGA
CAGCCCAC TTTGAA ATTTG AAACCT GCACGTCGACTGCG GAATTG
CTGAAAGGACAGC CTGGCAGACTC CTGACTGGA AATCATTGAGGATG
GAAAGAACCATATTCTGATCCTTCATAACTGTCAGCTGGGTATTGACAAGGAGA
GGTTGCCTTCCAGGCTGAATACCAAATCTGCAGCCAATCTGAAAGTGA
GAATTTGTCGGGCAGCTGGAATGACGCTACGACGCTACCAAAACCTTCACG
CGTTACC GAA
A.8 GL5HH-I27F

Protein:

\[ \text{MDTYKLILNGKTLGETTEAVDAATEKVKHYANHNGVGGGLGLIEVEKPLYGV} \]
\[ \text{EVFVGETAHFEIELSEPDPHVQFGKLKGQPLAASPDCEIIEGDKKHILILHNCQ} \]
\[ \text{LGMTGEVSFQAANTKSAANLKVKELEGDGEWTYDDATKFTTVTE} \]

cDNA:

\[ \text{ATGGACACCTACAAACTGTCTCTGAAACGTTGAAACCTGAAAGGTGAAACC} \]
\[ \text{ACCACCGAAGCTGTAGACGCTGCTACTGCAGAAAGTTTCAAACATTAACG} \]
\[ \text{CTAACCAACCGGTGTCCGTTGCGGACTCTGGGCTAATAGAAGTGGAAAAGC} \]
\[ \text{CTCTGTACGGAGTAGAGGTGGTTTTGTGGTGAAACAGCCCACTTTTGAAATTGA} \]
\[ \text{ACTTTTCTGAACCTTGATGTTTACGCCAGGGAAAGCTGAAGACAGCCTTTG} \]
\[ \text{GCAGCTTTCCGACTGTGAAATCATTGAGGATGGAAGAAGACATATTCTTA} \]
\[ \text{TCCTTCATAACTGTCAGCTGCTGATTGAGAGGTTCCTCTTCCAGGCTGCT} \]
\[ \text{AATACCAATCTGCAGCCAATCTGAAAGTGAAGAATTTGCTCGGGGACGTTG} \]
\[ \text{GAATGGACCTACGACGACGCTACCAAAACCTTCACGGTTACCCGAA} \]

A.9 Resilin

Protein:

\[ \text{GGRPSDSYGAPGGGN} \]

cDNA:

\[ \text{GGCGGTGTCGCGCAGCCGTAGCTATGGTGCCCGCGGGTGGCAAC} \]