Chapter I

CHEMICAL AND PHYSICAL CROSS-LINKING OF ARTIFICIAL PROTEIN HYDROGELS

1. Abstract

Recombinant artificial proteins contain genetically encoded information specifying their assembly into higher order structures, elastic behavior, and biological or chemical function. The goal of this introductory chapter is to describe the some of the common sequences used to design artificial proteins and how materials are fabricated from these proteins by the formation of physical or covalent interactions between protein chains. The focus of this chapter is cross-linking approaches for artificial protein hydrogels, although some of the interactions described can also be used to prepare films, fibrous materials, and particulate systems. Finally, challenges and future directions are discussed with a focus on the specific areas addressed in this thesis.

2. Introduction

Hydrogels are polymeric or supramolecular networks that absorb large amounts of water without dissolving in the aqueous medium or precipitating into the solid phase. As such, hydrogels require a balance between the forces promoting solvation of the polymer chains and the forces driving their association. While the materials and methods for engineering hydrogels vary widely, their high water content and favorable mechanical properties have attracted significant attention for applications in biomedical engineering as well as consumer products [1, 2].

Two central questions arise in the design and application of hydrogels. The first question concerns the source of the polymers (or other macromolecules) that will be used to construct the hydrogel network. Most polymeric hydrogels can be classified as being prepared from either synthetic or natural polymers. Synthetic polymers for hydrogels are typically carbon-based, although silicone polymers are an important exception. Examples include poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), and poly(acrylic acid) (PAA) and its derivatives [3]. Natural polymers for hydrogels include proteins such as collagen and fibrin and polysaccharides such as agarose and alginate [3]. Recombinant artificial proteins, which are the subject of this chapter, combine many of the desirable features of synthetic and naturally-derived polymers [4, 5]. These features include the ability to engineer chemically-defined polymers with precise sequences and the ability to incorporate biological and chemical activity. Other advantages and disadvantages of artificial proteins are discussed in the next section.

The second question in the design and application of hydrogels concerns how these polymers will be assembled into an extended network. Polymers can be cross-linked by covalent bonds between chains, by noncovalent association of chains through hydrogen bonding, hydrophobic interactions, and ionic bonds, or by the entanglement of long chains in a concentrated

solution. Artificial protein hydrogels are well-suited to chemical and physical cross-linking. Strategies for both methods are discussed in detail in this chapter and throughout this thesis.

3. Artificial Proteins and Protein-Based Materials

3.1 Artificial Proteins

Biosynthesis of artificial proteins offers numerous advantages when compared to synthetic routes to polymers. While synthetic polymers exhibit at least some degree of polydispersity, or variation in chain length, proteins synthesized by the ribosome are monodisperse and have a precise chain length that can vary from tens of monomers in small proteins to over 10,000 monomers in the giant muscle protein titin. Proteins are also stereospecific polymers, composed entirely of L-amino acids. This has important consequences for the higher order structures formed in proteins. Most importantly, however, is that like the chain length and stereochemistry, the sequence of amino acid monomers in proteins is also precisely controlled. The sequence of amino acids determines the folded structure of a protein, which in turn determines protein function. While proteins have evolved to perform an enormous number of tasks in living organisms, they have three primary functions:

(1) catalysis by enzymes
(2) maintaining the (dynamic) structures of cells, tissues, and natural protein-based materials
(3) molecular recognition including interactions with other proteins and biomolecules and binding of organic and inorganic ligands

When designing artificial proteins for materials applications, sequences can be adapted from proteins that occur in nature or they can be designed *de novo*. Currently, there are more than

500,000 protein sequences in the UnitProtKB/Swiss-Prot database [6] and over 120,000 protein structures in the Worldwide Protein Databank [7]. This provides an enormous library of sequences for the design of artificial proteins. Many artificial proteins are inspired by sequences found in natural protein-based materials including silk fibers spun by silkworms and spiders, mussel byssus thread, mineralized shells, and the extracellular matrices of various tissues. Other protein designs incorporate domains with useful properties like extreme thermal stability or responsiveness to specific environmental stimuli. When necessary, protein engineering approaches including directed evolution and rational and computational design can be used to improve artificial protein sequences toward a desired specification. Finally, artificial proteins are a green alternative to synthetic polymers, which is likely to become important as the petroleum-based feedstocks that are the current source of monomers for synthetic polymerization reactions are replaced by renewable, biobased feedstocks.

The disadvantages of artificial proteins must be considered along with the advantages described above. Ribosomal synthesis of proteins is limited to 20 amino acid monomers, although a number of strategies have been developed to engineer organisms that are capable of incorporating noncanonical amino acids during protein synthesis [8, 9]. Biosynthesis of proteins is also restricted in terms of the backbone polymer chemistry and the polymer topology; ribosomes produce exclusively linear polypeptide chains. Again, however, several engineering efforts have made some progress in both areas [10, 11]. In addition to the inherent limitations of the cellular protein synthesis machinery, several technical challenges to producing artificial proteins also exist. Each new polymer sequence requires a new template in the form of a gene encoding the artificial protein. Fortunately, recent advances in gene synthesis as well as advanced cloning techniques have made the generation of genetic templates for artificial proteins easier than ever. Other potential

challenges that are specific to each artificial protein include poor yields and difficulties purifying the target protein from the complex mixture of host proteins and other host macromolecules. Artificial proteins may exhibit poor stability under certain conditions and may be susceptible to degradation by proteases. Finally, the immunological properties of artificial proteins have not been well studied, which may pose challenges as well as opportunities for biomedical applications.

3.2 Design of Artificial Proteins for Protein-Based Materials

The sequences of artificial proteins for protein-based materials are composed of three types of domains (Figure I-1): (1) cross-linking or assembly domains, (2) unstructured elastomeric domains, and (3) biologically or chemically functional domains. Examples of these types of domains are given in Table I-1.

Cross-linking or assembly domains mediate interactions between chains in protein-based materials, giving rise to a polymeric or supramolecular network. They are typically folded and possess higher order (secondary, tertiary, and quaternary) structure that is related to their function. Examples discussed in Section 4 include helical domains that form coiled coils or triple helices, β-motifs including extended β-sheets, β-rolls and WW domains, and multimeric globular proteins. Several types of physical protein hydrogels can be obtained depending on how the cross-linking or assembly domains associate. Cross-linking domains can associate to form well-defined aggregates separated by a hydrophilic spacer (Figure I-2 a). Alternatively, cross-linking domains can assemble in less well ordered micellar phases that are also separated by hydrophilic spacers (Figure I-2 b). Finally, proteins can assemble into extended supramolecular fibers to produce nanofibrous hydrogels (Figure I-2 c).

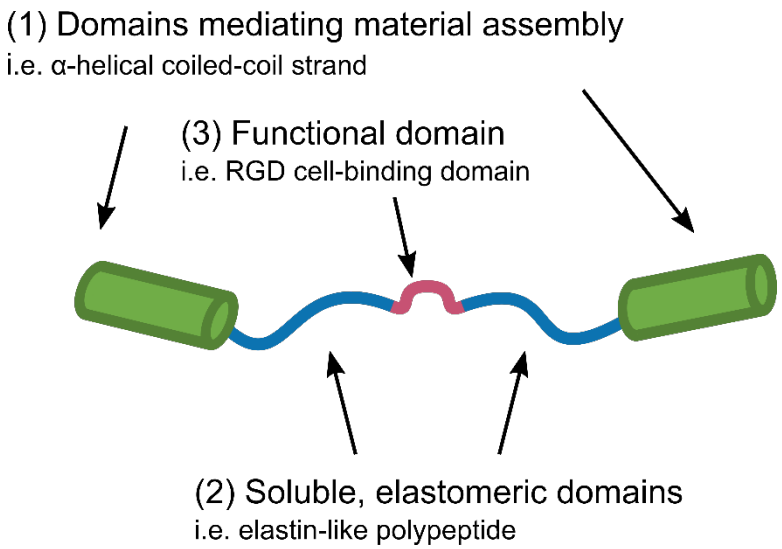


Figure I-1. Design of artificial proteins for protein-based materials. Artificial proteins can include (1) domains mediating the cross-linking or assembly of protein chains by the mechanisms described in Figure I-2, (2) soluble elastomeric domains, and (3) domains encoding biological or chemical function.

The second type of sequence in artificial protein materials includes domains that are unstructured or contain relatively simple structures such as β-turns. In many hydrogel designs, these sequences are required as soluble spacer or linker regions between cross-linking domains in order to prevent the network from precipitating (Figure I-2 a and b). Alternatively, chemical hydrogel networks may be formed by covalent cross-linking of proteins containing these soluble domains, as described in Section 5 (Figure I-2 d). Elastomeric polypeptide domains commonly found in protein hydrogels include elastin-like polypeptides [12, 13], resilin-like polypeptides [13, 14], gelatin-like polypeptides [15], and the alanylglycl-rich nonapeptide repeat, $[AGAGAG(PEG)]_n$ [16].

The final type of sequence in artificial proteins encodes biological or chemical function. This type of sequence includes relatively short cell-binding domains and protease-sensitive

Cross-linking or Assembly Domains	Elastomeric Domains	Functional Domains
Coiled coils and helical bundles	Elastin-like polypeptides	Cell-binding domains
Collagen-like polypeptides	(hydrophilic)	e.g. RGD, REDV, IKVAV
Elastin-like polypeptides (hydrophobic)	Gelatin-like polypeptides	Proteolytic degradation sequences
Silk-like polypeptides	Resilin-like polypeptides	e.g. GPQGIAGQ
WW/Proline-rich domains	Alanylglycl polypeptides	Heparin binding
β-roll domains		Fluorescent proteins
Low complexity sequences		Enzymes
SpyTag/SpyCatcher		Cytokines
Multimeric globular proteins		
e.g. CutA, fluorescent proteins, enzymes		
Calmodulin/CaM binding peptide		

Table I-1. Design of artificial proteins for protein-based materials. Examples of the three types of domains in artificial proteins.

domains as well as much larger domains that can possess enzymatic activity, fluorescence, or growth factor activity. Peptide sequences that promote cell adhesion are included in most artificial protein designs for cellular and tissue engineering applications. The most common cell-binding sequence is the RGD (Arg-Gly-Asp) tripeptide, which is found in a large number of natural proteins including fibronectin, fibrinogen, collagen, vitronectin, and many others [17, 18]. Other cell-adhesion peptides that have been incorporated into artificial proteins include the fibronectin-derived tetrapeptide, REDV (Arg-Glu-Asp-Val) [19], and the laminin-derived pentapeptide, IKVAV (Ile-Lys-Val-Ala-Val) [20]. In addition to promoting cell adhesion, artificial proteins designed for tissue engineering and cell culture applications often include peptide sequences that allow for cell-mediated degradation by secreted or membrane-bound proteases. Degradable peptide sequences incorporated into artificial proteins allow chains to be cleaved by plasmin [21], tissue or urokinase plasminogen activator (tPA, uPA) [22], and matrix metalloproteinases [23].

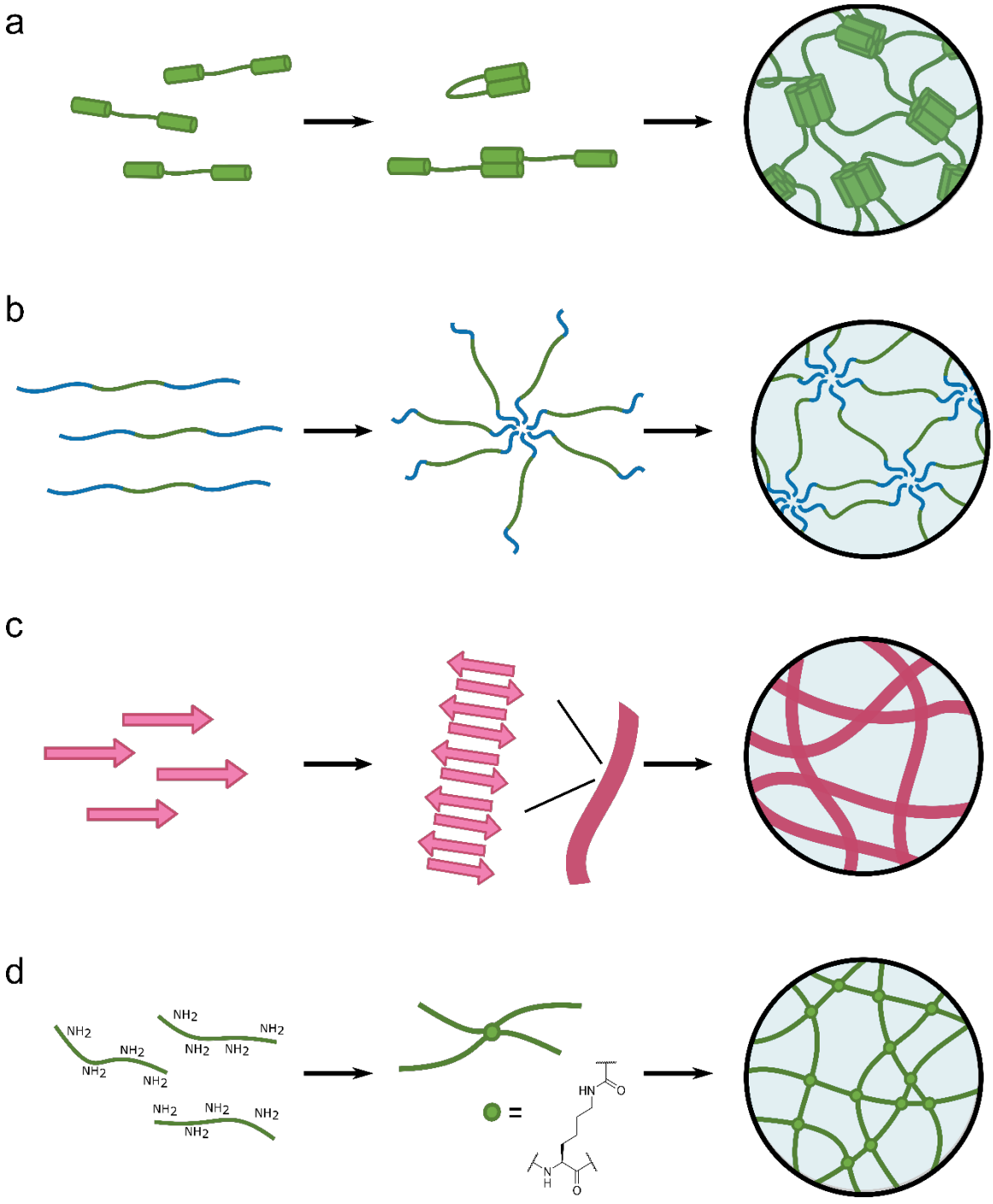


Figure I-2. Cross-linking of protein hydrogels. (**a**) Controlled aggregation of associative domains separated by a soluble elastomeric domain. (**b**) Aggregation of colloidal or micellar domains separated by a soluble elastomeric domain. (**c**) Assembly of proteins into nanofibers that can associate or become entangled to form hydrogels. (**d**) Covalent cross-linking of artificial proteins by reaction of amino acid side chains, such as the ε-amine of lysine.

Recent artificial protein designs have also included heparin-binding domains such as KAAKRPKAAKDKQTK [21, 24]. This sequence contains a high number of positively charged lysine and arginine residues that binding to negatively charged heparin polymers, which in turn sequester a number of different growth factors.

While these three categories are useful for describing artificial protein materials, it is important to note that overlap is both possible and common. By design, many structural cross-linking domains are stimuli responsive and exhibit reversible folding and unfolding based on temperature, pH, and ligand binding. Therefore, domains mediating the cross-linking or assembly of materials under certain conditions may be unstructured or unfolded under other conditions. Similarly, sequences that are primarily characterized as functional domains, such as enzymes encoded within the protein hydrogel backbone, can also contribute to material cross-linking through multimerization of domains on different chains.

4. Physical Cross-linking of Protein Hydrogels

Proteins are especially well-suited for preparing physical hydrogel networks. Nature has evolved folded structural motifs that form highly specific noncovalent interactions to mediate the assembly of protein complexes in biological mixtures. Some of these structural motifs have been adopted in the design of cross-linking domains in artificial proteins. Under the appropriate conditions, the cross-linking domains associate with one another to form the junction points that connect an extended polymer network. In order for the network to swell or absorb water rather than precipitate, soluble spacer or linker domains are also required.

The noncovalent interactions between physical cross-linking domains include hydrogen bonding, ionic bonds, hydrophobic interactions, and others. The reversibility of these interactions imparts important properties to physical protein hydrogels. Changes in the pH, temperature, or ionic strength that alter the folding or association of cross-linking domains can trigger a gel-sol transition in protein hydrogels. In this way, it is possible to program the assembly and disassembly of a network in response to environmental stimuli. Reversible cross-linking in protein hydrogels also has implications for the how the material responds when deformed. Physical protein hydrogels are viscoelastic fluids. When stress is applied over a short duration relative to the lifetime of the cross-links, the gel is deformed elastically and recovers its original shape when the stress is removed. When stress is applied over a long time, however, the material flows like a liquid and can be molded into a new shape. Because the protein-protein interactions that are responsible for cross-linking are specified by sequence of amino acids, artificial protein hydrogels offer the exciting prospect of encoding macroscopic material properties such as self-assembly and viscoelasticity at the molecular level.

4.1 Cross-linking based on the Association of Helical Domains

An important example of physical cross-linking in protein hydrogels is the controlled aggregation or multimerization of coiled-coil domains (Figure I-3 a). Coiled coils are composed of two to seven helical strands wrapped around one another to form a supercoil [25]. Self-assembling hydrogels have been prepared from artificial proteins containing coiled-coil domains that serve as physical cross-linkers. Telechelic artificial proteins with coiled-coil endblocks flanking a water-soluble midblock can form hydrogels when one of the endblocks has an

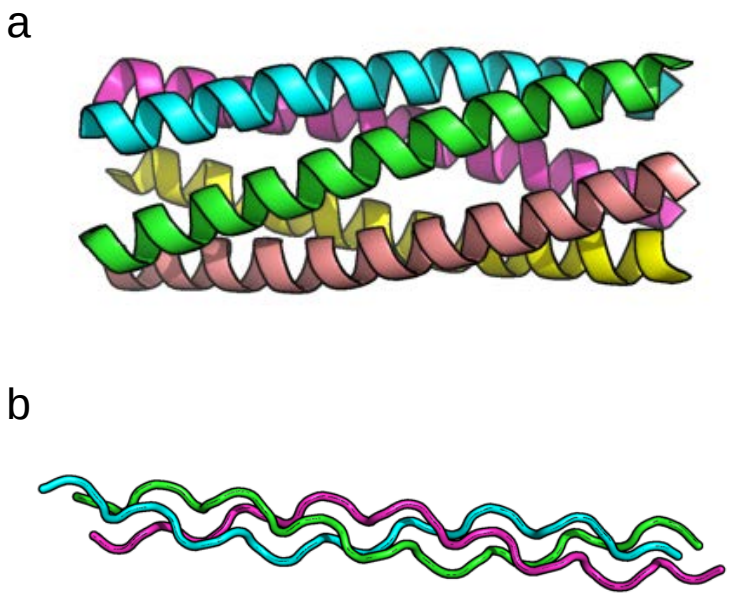


Figure I-3. Physical cross-linking domains based on helical motifs. (**a**) Pentameric coiled coil. (**b**) Collagen-mimetic triple helix. Structures were rendered in PyMol from PDB 1VDF (ref. [123]) and PDB (ref. [124]).

aggregation number greater than or equal to three. The first example of this type of self-assembling protein, denoted ACA, featured designed coiled-coil endblocks A flanking a polyelectrolyte random coil C [26]. The A domain assembles into tetramers under the appropriate conditions, but can be reversibly unfolded by increasing the temperature or the pH. The design of self-assembling protein hydrogels crosslinked by coiled-coil domains has been elaborated in many ways to tune the stability and viscoelastic behavior of coiled-coil gels. Other coiled-coil cross-linking domains have been introduced [27-29] as well as four-helix bundles that do not form a supercoil [30]. Conjugating coiled-coil peptides to synthetic polymers such as pHEMA or PEG results in a hybrid hydrogel where the peptide domains act as cross-linkers and the synthetic polymers act as water-soluble linkers [31, 32].

Physical protein hydrogels have also been designed with cross-linking that is based on the triple helical structure of collagen (Figure I-3 b). Collagen-like cross-linking domains have been incorporated into telechelic, triblock artificial proteins in a design that is analogous to the self-assembling coiled-coiled proteins described above [33, 34]. The collagen-like blocks, denoted T (for triple helix), consist of repeats of the tripeptide sequence (Pro-Gly-Pro) and flank a random coil midblock R (for random coil). The resulting TRT proteins form viscoelastic hydrogels below the melting temperature of the helical blocks. Above the melting temperature, TRT behaves as a viscous liquid. An important feature of the collagen-like block is its well-defined aggregation number of three. This feature enabled the development of a thermodynamic model that accurately predicts the number of network junctions, dangling ends, and looped chains as a function of temperature and protein concentration [33]. Sequence engineering of the endblock triple helical domains has been used to tune hydrogel properties including the gel melting temperature and the network relaxation time [35].

4.2 Cross-linking based on β-motifs

Numerous physical protein hydrogels have been developed with cross-linking domains based on *β*-motifs. Silk-like sequences containing repeats of the hexapeptide GAGAGS were among the earliest recombinant artificial proteins [36]. When combined with elastin-like sequences to form silk-elastin-like proteins (SELPs), the silk domains can aggregate irreversibly to form physical cross-links that are separated by the elastin-like domains, which remain solvated [37]. Nanofibrous hydrogels have also been formed from multiblock artificial proteins containing repeats of the silk-like octapeptide $(AGAGAGEG)_n$ and random coil gelatin-like blocks [38]. Significant efforts have been undertaken to produce recombinant spider silk proteins in a variety

of host organisms. In addition to spinning these recombinant silks into strong fibers, strategies for cross-linking them into hydrogels have also been described [39, 40].

The aggregation of elastin-like polypeptides (ELPs) into a coacervate phase at temperatures above their lower critical solution temperature (LCST) has been exploited in several physical protein hydrogel designs. For example, a triblock elastin-like artificial protein with hydrophobic ELP endblocks flanking a hydrophilic ELP midblock domain exhibits a transition from liquid sol to a hydrogel as the temperature is increased above the LCST of the endblock sequence [41]. Gelation is driven by the phase separation of the hydrophobic ELPs into coacervates that are linked together by the hydrophilic ELP midblock, which by design exhibits a much higher LCST than the endblocks. Physical hydrogels have also been prepared by coacervation of ELPs above their LCST, which causes phase separation into a protein-rich phase that exhibits gel-like properties [42]. More recently, hydrogels were formed by heating concentrated solutions of ELPs composed of repeats of the pentapeptide sequence XPAVG, where X is occupied by Val and Ile at a 4:1 ratio [43]. Hydrogel formation was attributed to an arrested phase transition of the ELPs to create a nanostructured network rather than coacervation.

Other physical cross-linking domains in protein hydrogel designs include β-roll domains [44, 45], WW and proline-rich domains [46, 47], and low complexity sequences [48, 49]. All three of these physical cross-linkers form β-sheet structures. The β-roll cross-linking domain consists of two β-sheets formed by alternating β-strands separated by turns [44, 45]. The folded structure is a flattened helix with a β-sheet on each face of the helix (Figure I-4 a). Folding of this domain from a disordered peptide into a β-roll requires calcium (Ca^{2+}) binding to conserved aspartic acid

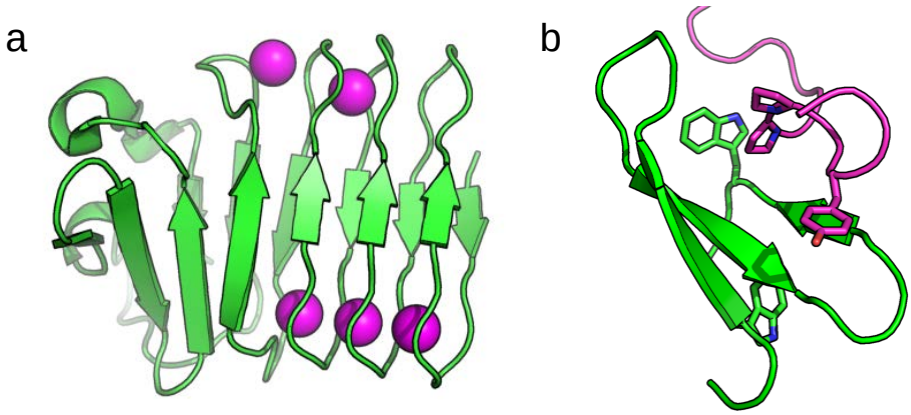


Figure I-4. Physical cross-linking domains based on *β*-motifs. (**a**) *β*-roll domain with Ca^{2+} (magenta spheres). (**b**) WW domain (green) interacting with a proline-rich domain (magenta). The Trp side chains of the WW domain and the Pro and Tyr side chains of the proline-rich domain are shown. Structures were rendered in PyMol from PDB 2Z8X (ref. [125]) and 1I5H (ref. [126]).

residues in the turns. Several solvent-exposed residues on the *β*-sheets were mutated to leucine under the hypothesis that this would increase the hydrophobicity of the exposed surface and lead to the association of the hydrophobic surfaces to form physical cross-links.

Some applications of protein hydrogels, particularly the encapsulation of sensitive cells and biomolecules, would benefit from mild gelation conditions that do not require abrupt changes in temperature, pH, or ionic strength. The simplest hydrogel design that addresses this need consists of two protein solutions that gel upon mixing. This design is accomplished with physical cross-links formed by two different protein domains encoded on separate chains [46, 47]. The first component of the hydrogel is an artificial protein containing WW domains derived from both designed and natural proteins. WW domains consist of approximately 40 amino acids (including two conserved tryptophan residues from which their name is derived) and form antiparallel *β*-sheets (Figure I-4 b). Several artificial proteins were created with 3-8 repeats of the WW domain

separated by soluble polyelectrolyte linkers. The second component is an artificial protein with a sequence consisting of repeats of 3-9 repeats of a proline-rich domain PPxY also separated by soluble linkers. Mixtures of two components with high functionality (7 repeats of the WW domain and 9 repeats of the PPxY domain) formed soft elastic gels (9-50 Pa) through physical cross-linking between WW and PPxY domains (Figure I-4 b).

The β-motif has been exploited widely in fibrous hydrogels consisting of short oligopeptides that form extended β-sheet fibers [50, 51]. Recently, proteins isolated from RNA- and protein-rich subcellular structures known as RNA granules were also observed to form similar fibrous hydrogels [48, 49]. Subsequent analysis showed that many of these proteins contained low complexity (LC) domains that are necessary and sufficient for gelation. Sequencing of an LC domain derived from the RNA-binding protein fused in sarcoma (FUS) revealed repeats of the tripeptide (Gly/Ser)-Tyr-(Gly/Ser), where (Gly/Ser) stands for either glycine or serine. Fusion of green fluorescent protein (GFP) or mCherry fluorescent protein to the LC domain of FUS resulted in fluorescent hydrogels capable of trapping other LC domain proteins with varying affinity. The primary application of hydrogels prepared from LC domains has been *in vitro* experiments to identify how RNA and RNA-regulatory proteins might localize to RNA granules as well as other cellular processes involving LC domains [52-54]. Interestingly, these experiments also revealed the importance of phosphorylation of Ser residues in the LC domain in regulating localization dynamics, offering a potential mechanism for hydrogel assembly and disassembly. Further applications of LC domain hydrogels have not been explored but could be similar to other β-sheet peptide hydrogels.

4.3 Globular Protein Cross-linkers

Well-folded globular protein domains have been incorporated into protein hydrogels to impart chemical or biological activity. Examples include fluorescent proteins to study gel structure and dynamics, enzymes to catalyze desired chemical reactions, and signaling proteins to respond to environmental stimuli. Many globular proteins assemble into multimers or require an interaction with a binding partner to fulfill their function. Therefore, it is possible that globular proteins can also contribute to the cross-linking of networks in addition to their intended function. For example, a chimeric protein was designed in which the A helical domain, which forms coiled coils, was fused to the fluorescent protein dsRed [55]. This artificial protein can assemble into a hydrogel network that contains two types of physical cross-linking. The first is the aggregation of coiled coils and the second is the formation of dsRed tetramers. Similar network formation is possible with a chimera of the A domain fused to an oxidase or a hydrolase enzyme that forms dimers [56, 57].

The calcium-responsive signaling molecule calmodulin (CaM) is another example of a globular protein that has been incorporated into protein hydrogels and protein-polymer hybrid gels [58]. A chimeric protein consisting of the A domain fused to CaM is capable of forming a network in the presence of calcium ions by binding to a bifunctional cross-linker containing two CaM binding peptides (CBP). When Ca^{2+} is removed by chelation, the network is disassembled. Globular domains have the potential to greatly expand the scope of physical cross-linking in protein hydrogels beyond the comparatively simple structural domains such as coiled coils or triple helices. Other globular proteins used in protein and protein-polymer hybrid hydrogels include enzymes and antibody fragments [59, 60].

The CutA protein from the thermophilic archaeon *Pyrococcus horikoshii* forms highly thermostable trimers (Figure I-5) that have been used as physical cross-linkers in protein hydrogels [61-63]. An innovative approach was demonstrated to obtain a two-component system from a single physical cross-linking domain. Two chimeric proteins were designed in which the CutA protein was fused to either the N- or C-terminal fragment of the DnaE split intein from *Nostoc punctiforme*. In solutions of the isolated proteins, trimers form due to the association of the CutA domains but network formation is not possible. Upon mixing of the two proteins, the split intein domains from each protein assemble and undergo trans-splicing to form a new peptide bond. This links the CutA trimers together in a physical hydrogel network. The hydrogel is highly stable to surface erosion, a property that is attributed to the stability of the trimeric cross-linkers. However, the gel is also significantly softer than expected, indicating that most of the cross-links are not elastically effective.

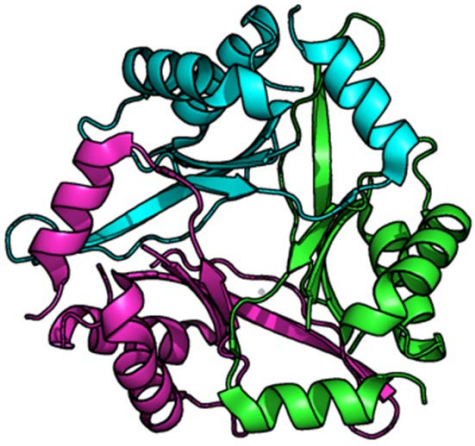


Figure I-5. CutA trimer. The three chains of the CutA trimer are shown in green, blue, and magenta. The structure was rendered in PyMol from PDB 1UMJ (ref. [127]).

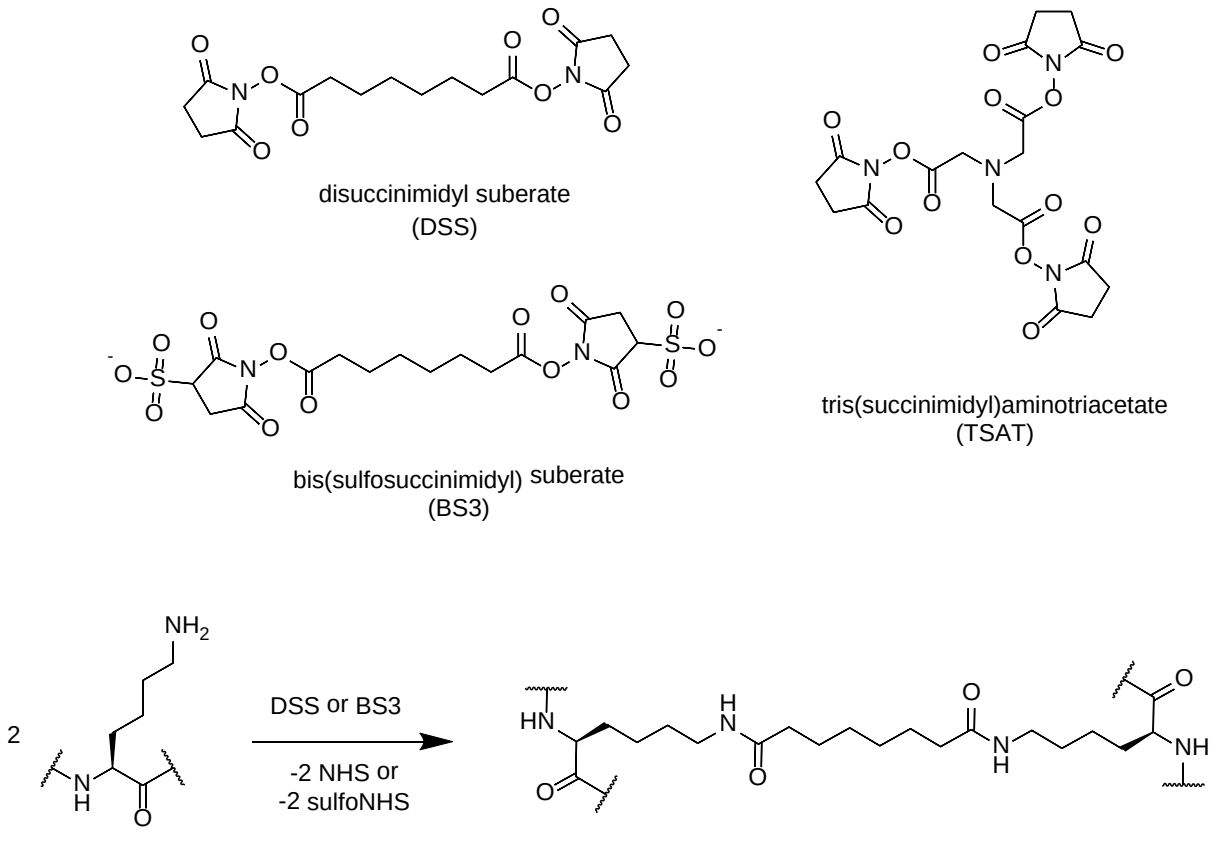
5. Chemical Cross-linking of Protein Hydrogels

Protein networks can be prepared by covalent or chemical cross-linking of protein chains. This typically involves reactions linking together amino acid side chains but in principal can also include reactions of the protein backbone or termini. In contrast to the transient and often weak noncovalent association of physical network junctions, chemical cross-links are strong and usually permanent. The chemical reactions employed for protein cross-linking into hydrogel networks closely resembles the vast set of bioconjuagation reactions developed to label proteins with fluorescent dyes and affinity probes and to form intramolecular and intermolecular cross-links for protein structural studies [64]. A key advantage of artificial proteins is that the density and location of these cross-linking residues can be controlled through the design of the protein sequence. Cross-linking is often accomplished by small molecules with two or more functional groups capable of reacting with the artificial proteins. Due to concerns over the toxicity and poor solubility of some small molecule cross-linkers, alternative cross-linking strategies have been developed including photo-chemical cross-linking, bioorthogonal cross-linking, enzymatic cross-linking, and cross-linking by macromolecules such as end-functionalized poly(ethylene glycol).

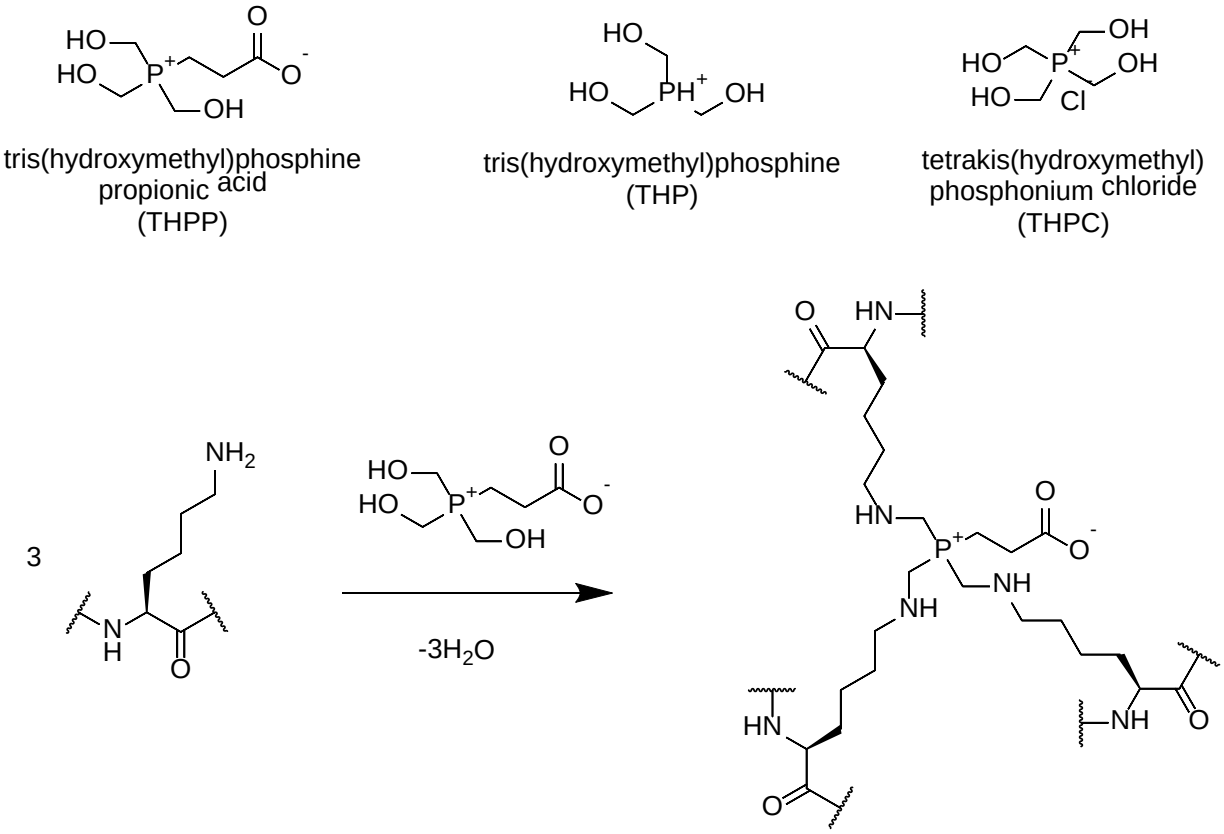
5.1 Cross-linking Reactions Involving Lysine

The ε-amine of lysine is mildly nucleophilic with a typical pK_a of 9-10 and is a common target for bioconjugation reactions. The bifunctional cross-linkers disuccinimidyl suberate (DSS) and bis(sulfosuccinimidyl) suberate (BS3) [65, 66] and the trifunctional cross-linker tris-succinimidyl aminotriacetate (TSAT) [67] contain either two or three succinimidyl esters that are capable of reacting with amines to form amide bonds (Scheme I-1). Reaction of the ester groups

on DSS, BS3, and TSAT with lysine residues on different protein chains creates a covalent cross-link. More recently, hydroxymethyl phosphine and phosphonium (HMP) compounds (Scheme I-2) have emerged as popular cross-linking reagents for artificial protein hydrogels due to their high solubility in aqueous buffer, rapid gelation kinetics, and commercial availability. Examples of these reagents include β-[tris(hydroxymethyl)phosphino] propionic acid (THPP) [68, 69], tris(hydroxymethyl) phosphine (THP) [70], and tetrakis(hydroxymethyl) phosphoninium chloride (THPC) [71]. Other cross-linking reagents targeting primarily lysine residues include diisocyantates [72], glutaraldehyde [73-75], and the natural product genipin [76]. Elastin-like artificial proteins and resilin-like artificial proteins containing lysine residues have been cross-linked with these reagents.



Scheme I-1: Cross-linking of Lys with succinimidyl ester compounds.



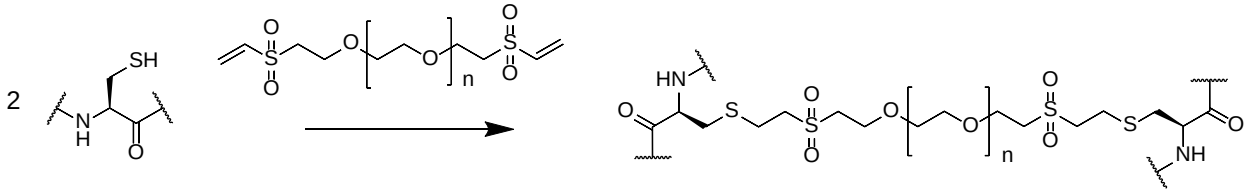
Scheme I-2. Cross-linking of Lys with hydroxymethyl phosphine compounds.

5.2 Cross-linking Reactions Involving Cysteine

Cysteine is an attractive target for cross-linking artificial proteins. Its thiol side chain is nucleophilic with a typical pK_a of 8-9 and can undergo Michael-type conjugate addition to electrophiles including maleimides, vinyl sulfones, and acrylates or displacement reactions with haloacetyls and benzyl halides. This set of reactions is used widely for both bioconjugation and protein cross-linking. Hydrogels have been formed from artificial proteins by cross-linking cysteine residues by Michael-type conjugate addition reactions. These reactions are frequently performed with macromolecular cross-linkers such as PEG-divinyl sulfone and 4-arm PEG tetra

vinyl sulfone (Scheme I-3). Examples include hydrogels prepared from artificial proteins based on fibrinogen- and collagen-like sequences [23, 77], elastin-like artificial proteins (Chapter 2 in this thesis), and resilin-like artificial proteins [78].

An alternative approach to cross-linking artificial protein hydrogels takes advantage of the oxidation of cysteine to form cystine disulfide cross-links between protein chains. Elastin-like artificial proteins with the guest residue occupied periodically by cysteine were oxidized by the addition of hydrogen peroxide, forming a covalent protein network [79]. Disulfide bonds were also used to stabilize physical protein gels cross-linked by leucine zipper coiled coils [80]. In these materials, an oxidant was not required because the reacting cysteine residues were brought into proximity at the coiled-coil interface. The placement of the cysteine residues also favored the antiparallel orientation of coiled coils, preventing loop formation and stabilizing the hydrogels against surface erosion.



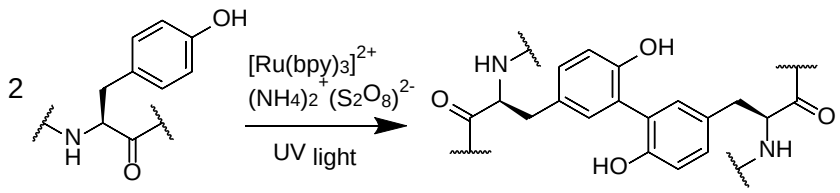
Scheme I-3. Michael-type conjugate addition of Cys and PEG-divinyl sulfone.

5.3 Photo Cross-linking

Cross-linking of polymeric materials including protein hydrogels by irradiation with visible or ultraviolet (UV) light is a powerful method for spatial and temporal control over network formation. One of the earliest methods for cross-linking recombinant elastin-like artificial proteins was the use of γ-irradiation [81-84]. The dose of irradiation could be used to control the cross-

linking density in ELP networks and tune their mechanical properties and swelling behavior. For UV and visible light photo cross-linking, artificial proteins have been modified at lysine and cysteine residues with photo-reactive functional groups including acrylates [21, 85], norbornenes [86], and diazirines [87].

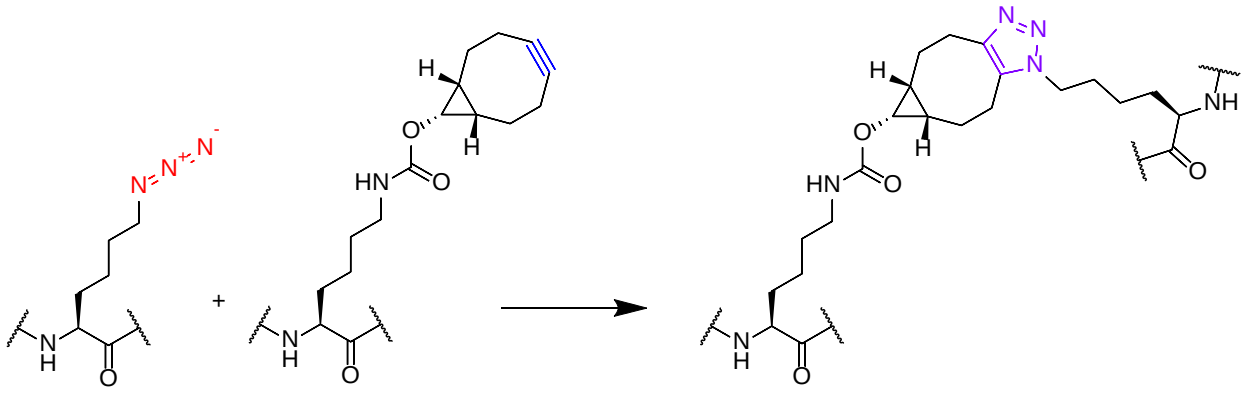
Aromatic amino acid side chains are also potential targets for photo cross-linking. Several artificial proteins including recombinant resilin [88], resilin-like proteins [89-92], and silk-like proteins [40, 93, 94] have been cross-linked by dityrosine formation after irradiation with visible light in the presence of tris(bipyridine)ruthenium(II), $[Ru(bpy)_3]^{2+}$ and ammonium persulfate (Scheme I-4). Elastin-like artificial proteins with phenylalanine guest residues replaced by a noncanonical Phe analog, 4-azidophenylalanine, were cross-linked into films by UV irradiation [95-97]. The mechanical properties of the films could be tuned by controlling the level of Phe replacement during protein expression. A similar strategy would likely be possible using other photoreactive noncanoncial amino acids such as the diazirine analogs of methionine, leucine, and lysine [98, 99].



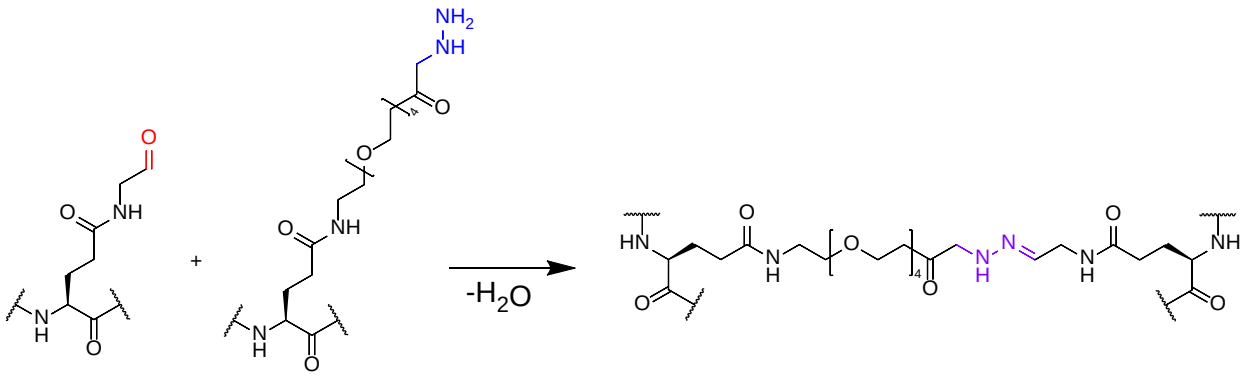
Scheme I-4. Photo cross-linking of Tyr to dityrosine.

5.4 Bioorthogonal Cross-linking

Applications that require the encapsulation of cells or biomolecules or the incorporation of functional protein domains within the hydrogel backbone impose significant limitations on the use of chemical cross-linking methods. The hydrogel protein and cargo proteins are composed of the same 20 amino acids, leading to inevitable side reactions between the cross-linker and the cargo. In the case of cell encapsulation, these side reactions may prove toxic to the cells. Likewise, side reactions with encapsulated biomolecules may interfere with their performance by inhibiting diffusion or by irreversibly binding to an active site. These challenges may potentially be met by advances in the field of bioorthogonal chemistry [100] as well as efforts to expand the genetic code beyond the 20 proteinogenic amino acids. Recently, the copper-catalyzed azide-alkyne cycloaddition (CuAAC), strain-promoted azide-alkyne cycloaddition (SPAAC) (Scheme I-5), and hydrazone formation (Scheme I-6) reactions have been used to cross-link artificial protein hydrogels [101-103]. In each example, the bioorthogonal functional groups were installed on recombinant elastin-like artificial proteins after purification by modification of Lys or Glu residues. Rather than functionalize artificial proteins post-translationally, bioorthogonal groups could instead be introduced into proteins during translation using various methods for the residue-specific [8] or site-specific [9] incorporation of an appropriate noncanonical amino acid.



Scheme I-5: Strain-promoted azide-alkyne cycloaddition.



Scheme I-6: Hydrazone formation.

5.5 Enzymatic Cross-linking

Enzymes are also used catalyze the formation of covalent cross-links between artificial proteins to form hydrogel networks. In particular, tissue transglutaminase (tTG) has been used to form intermolecular isopeptide bonds between glutamine and lysine residues on different protein

chains. Artificial proteins cross-linked by this method include elastin-like proteins with guest residues occupied by Gln or Lys [104], recombinant tropoelastin [105], and a pair of designed artificial proteins containing repeat domains rich in Lys and Gln [106]. A highly specific "enzymatic" cross-linking strategy has recently been developed using isopeptide bond formation between the split protein domains SpyTag and SpyCatcher derived from the FbaB protein of *Streptococcus pyogenes* (Figure I-6) [107]. Using this reaction, networks were formed between a pair of elastin-like artificial proteins containing either three repeats of the SpyTag domain or two repeats of the SpyCatcher domain [108]. The highly specific nature of the SpyTag-SpyCatcher covalent bond enabled the formation of networks from multiblock ELP sequences containing a functional LIF (leukemia inhibitory factor) cytokine for the encapsulation of mouse embryonic stem cells.

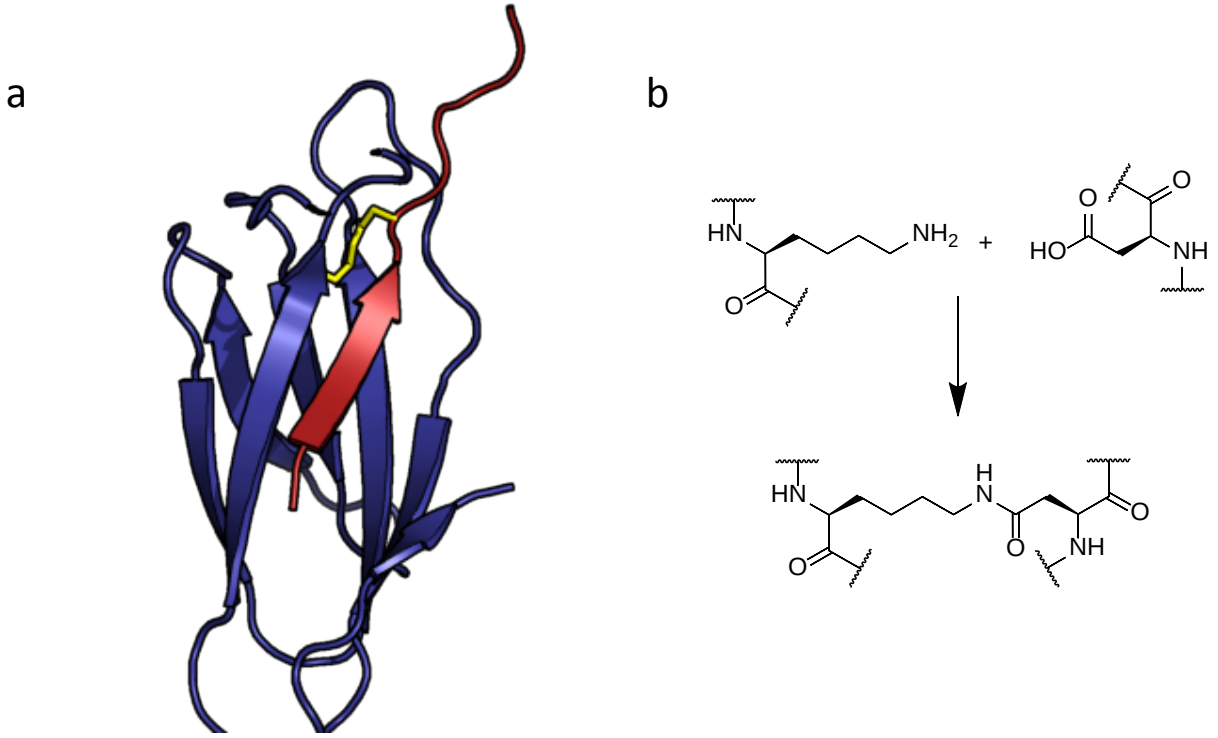


Figure I-6. SpyTag-SpyCatcher cross-linking. (**a**) Reconstitution of the split SpyTag (red) and SpyCatcher (blue) protein domains results in a spontaneous isopeptide bond (yellow) between Lys31 and Asp117 (**b**). The structure was rendered in PyMol from PDB 4MLI (ref. [128]).

6. Current Challenges Addressed by New Cross-linking Strategies

6.1 Dynamic, Viscoelastic Hydrogels

A major advancement in cell and tissue engineering was the recognition that the physical environment in which a cell resides greatly influences cellular behaviors such as proliferation and apoptosis, spreading and migration, and differentiation [109]. This has had a profound effect on the design of hydrogels and other biomaterials, with significant attention now given to engineering materials with the appropriate compliance or stiffness for a particular application. More recently, investigators have begun to develop a similar appreciation for the role of the viscoelastic or time-dependent mechanical properties of the cellular environment [110-112]. For example, fibroblast cells cultured on alginate hydrogels capable of stress relaxation exhibited enhanced spreading when compared to cells cultured on purely elastic substrates [111]. Similarly, stress-relaxing hydrogels can also influence the differentiation of encapsulated mesenchymal stem cells into adipose and osteogenic lineages [110, 112]. The behavior of cells and tissues in viscoelastic environments is particularly relevant for physical protein hydrogels containing transient network junctions. Epithelial cells cultured within a physical protein hydrogel cross-linked by coiled coils were capable of forming multicellular acinar structures [32]. This behavior was attributed to the migration of individual cells through the transiently cross-linked matrix. In contrast, when the same gel was formed and then covalently cross-linked, formation of acinar structures did not occur. Beyond this study, however, the relationship between the dynamic properties of protein hydrogels and cellular behavior has not been investigated. Further progress in this area will require new strategies to engineer the dynamic properties of materials in the same way that the modulus or stiffness is currently controlled.

Protein hydrogels cross-linked by the association of coiled-coil domains are an attractive platform for engineering dynamic materials. Viscoelastic behavior in coiled-coil gels arises from the transient association of the physical cross-links. Shen *et al*. established the relationship between the network relaxation time of coiled-coils gels, as measured by creep rheology experiments, and the rate of exchange of helical strands between coiled coils, as measured by a fluorescence dequenching assay [113]. Coiled coils derived from natural proteins or designed *de novo* exhibit characteristic strand exchange times ranging from approximately 1 second [114] to more than 10,000 seconds [115], suggesting that this dynamic range might also be accessible to engineer the relaxation timescales in viscoelastic hydrogels cross-linked by coiled coils.

In this thesis, the dynamic properties of protein networks containing chemical cross-links and physical cross-links are investigated in detail. Chapter 3 describes the time-dependent mechanical responses of artificial protein hydrogels cross-linked by covalent thioether bonds, by physical association of coiled coils, and by both interactions. These materials can be described as elastic solids, viscoelastic liquids, and viscoelastic solids, respectively. In Chapter 4, variation of the protein sequence is explored as a strategy to engineer the characteristic timescale of stress relaxation in chemical-physical protein networks. It is shown that single point mutations within the physical cross-linking domain can be used to tune the relaxation timescale over five orders of magnitude. In Chapter 5, the relaxation dynamics of chemical-physical protein hydrogels are tuned by addition of external stimuli, specifically small hydrophobic ligands including vitamin D3 and fatty acids that bind within the coiled-coil physical cross-links. By selection of different ligands, it is possible to increase the network relaxation time by 10- to 1000-fold.

6.2 Enhanced Mechanical Properties: Toughness, Strength, and Extensibility

The high water content and elasticity that make hydrogels attractive materials for biomedical engineering applications also result in potentially weak and brittle gels. Several strategies have been proposed for developing tougher hydrogels that can withstand higher strains and stress prior to fracture [116, 117]. Three of these strategies that are well-suited to protein hydrogels are discussed here.

One strategy is to prepare more homogenous hydrogel networks by cross-linking macromolecalar precursors with highly efficient reactions. For example, hydrogels prepared from azide and alkyne end-functionalized poly(ethylene glycol) (PEG) polymers cross-linked by the CuAAC reaction could be extended up to 15 times their original length and sustain a maximum true stress of more than 2 MPa prior to breaking [118]. In comparison, hydrogels formed by photochemical polymerization of PEG-diacrylate, which is known to generate heterogeneous network structures consisting of densely cross-linked regions connected by long chains, fractured at extensions of less than 2 times their original length and sustained maximum stresses of less than 0.2 MPa. Similarly, step-growth PEG networks cross-linked by the Michael-type addition of 4-arm PEG thiol star polymers and PEG-diacrylate were tougher and more extensible than chain-growth networks [119]. The monodispersity of artificial proteins makes them well-suited to this approach, assuming that efficient cross-linking strategies can be developed and that the gelation process results in a homogeneous network structure. Chapter 2 of this thesis describes the formation of a step-growth hydrogel network consisting of a telechelic artificial protein, ERE, and a 4-arm PEG star polymer functionalized with vinyl sulfone on each arm (PEG-4VS) (Figure I-7 a). Tensile experiments with ERE networks are discussed in Chapter 6.

Other emerging strategies for toughening hydrogels rely on various energy-dissipating mechanisms to prevent the stress concentrated near network defects or fracture zones from propagating and resulting in material failure [117, 120, 121]. Hydrogels cross-linked by a combination of permanent covalent cross-links and reversible physical cross-links have been proposed for this purpose. The covalent cross-links maintain the shape and elasticity of the network while the sacrificial physical cross-links can be broken prior to the rupture of polymer chains. As will be described in Chapter 3, hydrogels prepared by end-linking the telechelic artificial protein EPE with PEG-4VS contain both covalent thioether cross-links and physical coiled-coil cross-links (Figure I-7 b). The transient coiled-coil cross-links are expected to dissociate by either thermal or mechanical forces. Tensile experiments with EPE networks, including a comparison to covalent ERE networks, are discussed in Chapter 6.

A final mechanism for hydrogel toughening that is also applicable to protein networks is termed domain transformation [117]. In this strategy, a compact folded domain is stretched and unfolded, dissipating energy and increasing the chain contour length in the process. Protein hydrogels have been prepared with folded structures including the GB1 immunoglobulin-binding domain of streptococcal protein G [89], and FL, a *de novo* designed protein domain with ferrodoxin-like structure [90]. The GB1 domain is mechanically strong, with unfolding forces of 200 pN as measured by single molecule force spectroscopy. As a result, it is difficult to unfold within a hydrogel. In contrast, the FL domain unfolds easily at forces of approximately 10 pN. When cross-linked in a covalent hydrogel network, a fraction of the FL domains appear to unfold easily under forces generated as chains stretch during swelling. While not discussed in this thesis, a variant of the ERE protein containing the full-length tenth type III domain of fibronectin (^{10}FnIII) has also been synthesized and cross-linked with PEG-4VS (Figure I-7 c). These networks may

display enhanced mechanical properties based on the unfolding of ^{10}FnIII, which exhibits moderate unfolding forces of 75-100 pN in single molecule experiments [122].

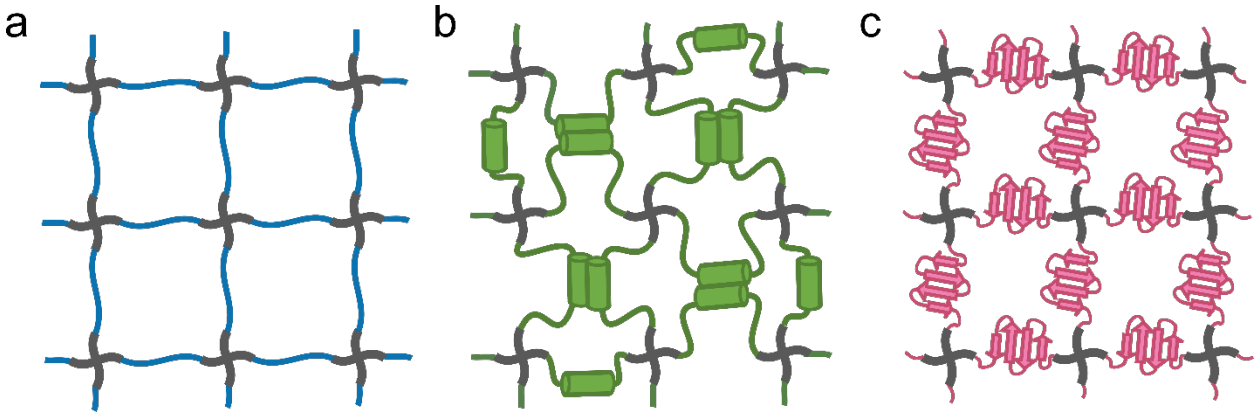


Figure I-7. Hydrogel toughening strategies. (**a**) Network prepared from macromer precursors ERE and PEG-4VS. (**b**) Network containing thioether covalent cross-links between EPE and PEG-4VS and physical cross-links between the midblock domains of EPE. (**c**) Network containing a folded protein domain that can potentially be unfolded by mechanical force.

7. Conclusions

The sequences of artificial proteins contain domains specifying physical or chemical cross-linking, elastic behavior, and biological or chemical function. Physical cross-links are formed by the association of helical domains, β-motif domains, and globular protein domains. Chemical cross-links are formed by the reaction of side chain functional groups with small molecule or macromolecular cross-linkers, by photochemical reactions, and by enzymatic reactions. New approaches to chemical and physical cross-linking of protein hydrogels have the potential to address current challenges in materials design, including engineering dynamic materials and enhancing hydrogel toughness.

8. References

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