Chapter I. Introduction

1. Background

1.1. Hydrogels

Hydrogels are crosslinked polymer networks that absorb substantial amounts of aqueous solutions. Hydrogels can be divided into two categories based on the chemical or physical nature of the crosslink junctions. Chemically crosslinked networks have permanent junctions, while physical networks have transient junctions that arise from either polymer chain entanglements or physical interactions such as ionic interactions, hydrogen bonds, or hydrophobic interactions[1]. Hydrogels can also be separated into two groups based on their natural or synthetic origins[2]. Hydrogel-forming natural polymers include proteins such as collagen and gelatin, and polysaccharides such as alginate and agarose. Synthetic polymers that form hydrogels are traditionally prepared using chemical polymerization methods. Approaches using genetic engineering and biosynthetic methods to create unique hydrogel materials have been recently reported[3,4]. In biosynthetic methods, predetermined amino acid sequences of artificial proteins are encoded into recombinant DNA, and the target proteins are expressed using host cells such as *E. coli* bacteria. Fidelity of biosynthesis machinery ensures protein products of precisely defined molecular weight, composition, and sequence. The modularity of recombinant DNA technology allows biological determinants such as cell-binding domains and enzyme recognition sites to be incorporated readily. These advantages offered by biosynthetic methodology are not easily realized in chemically synthesized materials.

1.2. Hydrogels as biomaterials

Hydrogels have attracted tremendous research interest over many years, in part for fundamental reasons and in part because of the potential for a wide range of applications. Hydrogels have been successfully used in biomedical fields due to their high water content and the consequent biocompatibility. Successful examples include soft contact lenses[5], wound dressings[6,7], superabsorbents[8-10], and drug-delivery systems[11,12]. The most recent and exciting applications of hydrogels are cell-based therapeutics[1,13] and soft tissue engineering[2]. The biomaterial used to grow the first living, tissue-engineered skin product was a collagen hydrogel[14]. Although the success of skin tissue engineering is encouraging, efforts to engineer other soft tissues have not achieved similar success. The progress in large measure is limited by inappropriate properties of the biomaterials currently available. To elicit desired cell response and coax cells to assemble into functional tissues, the materials that support and contact the cells need to be carefully designed[15,16]. For hydrogels used for cell-based therapeutics and soft tissue engineering, critical design parameters include both physical properties (such as appropriate mechanical strength and integrity) and biological properties (such as nontoxicity and ability to incorporate appropriate biological determinants (cell-binding domains, enzyme recognition sites))[2,17].

1.3. Genetically engineered protein hydrogels assembled through aggregation of leucine zipper domains

The concept of assembling artificial protein hydrogels through naturally occurring protein motifs opens a new approach to creating unique hydrogels. Since the capacity for

self-assembly is encoded in protein sequences, gelation does not require chemical crosslinking reagents, which often compromise material safety in biomedical applications. Artificial protein hydrogels constructed from a rod-coil-rod triblock protein (designated $AC_{10}A$) containing two leucine-zipper endblocks and a soluble random coil midblock have been reported in our laboratory[3] (Figure 1.1). Self-assembly of the leucine-zipper domains provides inter-chain crosslinking and leads to networks that can be switched on and off by controlling pH and temperature. The choice of residues for the leucine zipper domain was based on the residue pattern of the *Jun* oncogene product and a database developed by Lupas *et al*[3,18]. The midblock contains 90 amino acids, and features periodic glutamic acids for solvent retention.

Leucine zippers constitute a subcategory of coiled-coil domains found widely in nature, and play critical roles in functions ranging from muscle contraction[19] to transcriptional control[20]. Coiled-coils are characterized by heptad repeating units designated as *abcdefg*, where the *a* and *d* positions are occupied by hydrophobic residues such as leucine, and the *e* and *g* positions are occupied by charged residues. Each coiled-coil motif folds into an amphiphilic α-helix that places the *a* and *d* residues on a hydrophobic face. Hydrophobic interactions drive them to associate into oligomeric bundles[21]. Among naturally occurring coiled-coils, two-, three-, four-, and five- stranded bundles have been reported[22,23]. Higher order of aggregation has not been found.

Prior investigations of $AC_{10}A$ solutions demonstrate the concept of reversible artificial protein hydrogels and raise important questions regarding their macroscopic properties and the structural and dynamic nature of leucine zipper aggregates. Petka reported a plateau modulus of 200 Pa for a 5% w/v $AC_{10}A$ gel at pH 8.0, determined

using diffusing wave spectroscopy[3]. Systematic studies of the macroscopic material properties of $AC_{10}A$ hydrogels have not been reported. The number of leucine zipper strands in each aggregate was examined by Kennedy with analytical ultracentrifugation and small angle x-ray scattering[24]. Analytical ultracentrifugation studies on solutions of isolated A (less than 1 mM) suggested that this leucine zipper domain formed tetrameric bundles. Data from small angle x-ray scattering studies performed on $AC_{10}A$ solutions at concentrations as high as 7% w/v (ca. 6.3 mM leucine zipper) fit well to a cylindrical model for tetrameric helical bundles with the following dimensions: length 63 Å, radius 13.6 Å, and a 1 Å axial pore. McGrath reported that the leucine zipper strands linked by disulfide bonds formed from C-terminal cysteine residues exhibited identical helical content with their unlinked counterpart, suggesting that the strands in each aggregate oriented parallel in pairs so that the disulfide linkage did not disrupt the secondary structure[25]. However, it has remained unclear whether the four strands in each aggregate all orient parallel exclusively. Kennedy also studied the dynamics of the molecular motions of the leucine zipper A using solid-state NMR spectroscopy[26]. ^{13}C-detected ^{15}N CODEX experiments revealed a correlation time of about 80 ms. Since it is believed that folding and unfolding of coiled-coil domains involve multiple steps[27,28], whether the time scale revealed from NMR studies limits the network relaxation behavior has remained unknown.

1.4. Transient networks

$AC_{10}A$ hydrogels are transient networks, in which network junctions form through physical associations and are not permanent. Therefore these networks retain

internal fluidity due to the finite lifetime of the junctions. In other words, each chain can diffuse through the whole network on a certain time scale. The dynamics of this internal fluidity can be exploited to control the diffusion of large molecules (such as protein drugs) encapsulated in the network. Since the strength of physical associations can be tuned by varying the solution conditions, transient networks are often reversible in response to environmental stimuli such as temperature and pH.

The most extensively studied transient networks are those formed from hydrophobically modified urethane-ethoxylate (HEUR) polymers[29,30]. These polymers have a water-soluble midblock and two hydrophobic associative endgroups (typically hydrocarbon or fluorocarbon groups). These transient networks behave like solids on short time scales: under oscillatory shear, there is a plateau in storage modulus (G') at high frequencies. On time scales longer than a characteristic relaxation time (τ_r), these materials behave like liquids: at low frequencies ($\omega_x<1/\tau_r$), the loss modulus (G'') exceeds G'. This solid-to-liquid transition is called network stress relaxation, which is believed to be controlled by the dynamics of the molecular motions of polymer chains.

Structural and dynamic properties on molecular and microscopic levels account for the viscoelastic behavior and phase behavior of these polymer solutions. The most extensively examined structural property of HEUR solutions is the mean aggregation number of the associative groups. Techniques such as viscometry, fluorescence, dynamic light scattering, NMR, and EPR were used[31]. The aggregation numbers revealed from different techniques were often not consistent, but fell into the range from 20 to 100[31]. Although it was theoretically predicted that the ratio of intermolecular bridges to intramolecular loops was critical in determining material properties[30], it has never been

examined experimentally. Limited efforts have been made to measure the lifetime of the alkyl associative groups in their aggregates on a molecular level[32]. NMR studies revealed the exchange characteristic time for a C_{12} hydrophobe attached on poly(sodium acrylate) to be 14 ms at 25°C[32]. However, Annable reported that transient networks formed from HEUR polymers with C_{12} and C_{20} hydrophobes had a single stress relaxation time of ca. 1 ms and 6 s, respectively, at 25°C[30]. The subject about relaxation behavior of transient network and lifetime of associative groups remains controversial[33] and requires further studies.

Transient network theories have been developed on the basis of early work by Green and Tobolsky[34] . Their theory predicts that a network of n strands per unit volume having junctions that disengage at a rate of $1/\tau$ will have a high-frequency storage modulus given by $G'_\infty=nkT$ and a zero-shear viscosity $\eta(\dot{\gamma}\rightarrow 0)=\tau\ G'_\infty$. Based on Green and Tobolsky's model (the GT model), Jenkins[35], and Tanaka and Edwards[36] developed theories to account for the non-linear rheological behavior of transient networks that arises from changes in network structure and dynamics due to shear forces. Annable[30] recognized discrepancies between experimental data and the predictions of the GT model even in the linear regime. For example, the nonlinear concentration dependence of G'_∞ consistently observed in experiments is not predicted by the GT model. Annable refined Green and Tobolsky's transient network theory by taking into account the topology of the network. In Annable's model, a single chain could adopt either a bridged or a looped configuration (Figure 1.2 (a)), and the ratio of single-chain bridges to single-chain loops was a critical parameter. Dangling ends were neglected based on the strong hydrophobicity of the end groups. The effective network strands could be superbridges

(Figure 1.2 (b)), particularly at low concentrations. More complex topologies such as superloops and remote dangling ends (Figure 1.2 (c)) were ignored due to presumably low probabilities of their occurrence. With increasing concentration, the bridge-to-loop ratio increases due to the decrease in average distance between hydrophobic aggregates; consequently the network topology shifts to shorter superbridges. Annable's model correlated the bridge-to-loop ratio with the mean aggregation number of associative groups, the average dimension of the polymer chains, and the concentration of the solution. This model provided an explanation for the strong increase of viscosity and plateau modulus with concentration. It also pointed out that the stress relaxation time (τ_r) of a transient network was shorter than the lifetime of the associative groups (τ_a) due to the occurrence of superbridges, which were broken at a higher frequency compared to single-chain bridges. With increasing concentration, superbridges become shorter and τ_r increases until the network comprises mainly direct bridges and the relaxation time reaches τ_a.

Annable's model proves valuable in understanding $AC_{10}A$ hydrogels as well. In the context of artificial proteins, the model gives insight into the effects of ionic strength and pH, as well as mid-block length and polymer concentration.

2. Motivation and Objectives

Previous work in our laboratory has proved the concept of forming hydrogels from judiciously designed and genetically engineered proteins through the self-assembly of naturally occurring protein motifs[3]. Biosynthetic methods used to create these materials offer the opportunity to combine the advantages of hydrogels formed from both

chemically synthesized and naturally derived polymers. The molecular weight, sequence, and even higher order structures of these biopolymers can be precisely controlled to levels that remain unsurpassed. Biologically functional moieties such as those enhancing cell adhesion can be readily incorporated. The synthesis process does not involve toxic monomers as chemical polymerization does. In contrast to naturally derived polymers, the structure and properties of genetically engineered biomaterials can be systematically varied, providing engineering flexibility. These artificial protein hydrogels have promise for many biomedical applications.

Control of physical and biological properties of these hydrogels is essential for their biomedical applications. For example, it has been shown that rigidity (storage modulus) of scaffold materials acts as an extracellular signal and plays a critical role in regulating cell adhesion, spreading, migration, and even survival[37-40]. However, the material properties of $AC_{10}A$ hydrogels were not previously optimized for real applications. Their storage moduli are on the order of 100 Pa, lower than the desired modulus for typical soft tissue engineering (on the order of 1000 Pa)[39]. Their rapid dissolution in open systems precludes their use in applications in which materials are surrounded by excess fluids. Understanding of the structure-property relationships in this class of materials is limited. Such understanding would provide molecular design principles to tailor material properties, allowing the materials to be optimized for clinical applications or tuned systematically to address fundamental biological questions. From a fundamental perspective, investigation of the structure-property relationships of these well-defined model systems could provide valuable experimental information to test transient network theories.

The objectives of this research include: (1) understand structure-property relationships of $AC_{10}A$ networks on microscopic and molecular levels. (2) on the basis of the revealed structure-property relationships, rationally design, synthesize, and assemble new artificial protein hydrogels that exhibit desired physical properties, such as greater storage modulus and stability in open systems. (3) examine the toxicity of these artificial proteins, demonstrate the engineering flexibility of incorporating biologically functional moieties, and investigate cellular responses to these moieties. (4) use these materials as model systems to test the validity of transient network theories.

3. Organization of the thesis

Chapter 2 focuses on the thermodynamics, structure and rheology of $AC_{10}A$ hydrogels. Their plateau storage moduli are reported as functions of pH, concentration, and ionic strength. Structural features on microscopic and molecular levels—including the aggregation number of the leucine zipper domains, the orientation of the strands in aggregates, the midblock dimensions, and intramolecular association—are also examined. New design principles that emerge from the relationships between the macroscopic mechanical properties of $AC_{10}A$ hydrogels and the structural features of the building blocks are used to design and synthesize new materials that exhibit greater plateau storage moduli. Chapter 3 focuses on the dynamics of $AC_{10}A$ hydrogels. Their network relaxation behavior is correlated with the strand exchange kinetics of the leucine zipper domain. The molecular basis underlying the pH-responsiveness of dynamic properties is also examined. Chapter 4 and 5 present two rationally designed new materials that exhibit improved stability in open systems. Chapter 6 demonstrates the

engineering flexibility of incorporating biologically functional moieties and presents preliminary cell culture studies on artificial protein hydrogels.

4. References

(1) Jen, A. C.; Wake, M. C.; Mikos, A. G. *Biotechnology and Bioengineering* **1996**, *50*, 357-364.

(2) Lee, K. Y.; Mooney, D. J. *Chemical Reviews* **2001**, *101*, 1869-1879.

(3) Petka, W. A.; Harden, J. L.; McGrath, K. P.; Wirtz, D.; Tirrell, D. A. *Science* **1998**, *281*, 389-392.

(4) Wang, C.; Stewart, R. J.; Kopecek, J. *Nature* **1999**, *397*, 417-420.

(5) Compan, V.; Andrio, A.; Lopez-Alemany, A.; Riande, E.; Refojo, M. F. *Biomaterials* **2002**, *23*, 2767-2772.

(6) Azad, A. K.; Sermsintham, N.; Chandrkrachang, S.; Stevens, W. F. *Journal of Biomedical Materials Research Part B-Applied Biomaterials* **2004**, *69B*, 216-222.

(7) Passe, E. R. G.; Blaine, G. *Lancet* **1948**, *255*, 651-651.

(8) Lionetto, F.; Sannino, A.; Mensitieri, G.; Maffezzoli, A. *Macromolecular Symposia* **2003**, *200*, 199-207.

(9) Vashuk, E. V.; Vorobieva, E. V.; Basalyga, II; Krutko, N. P. *Materials Research Innovations* **2001**, *4*, 350-352.

(10) Zohuriaan-Mehr, M. J.; Pourjavadi, A. *Journal of Polymer Materials* **2003**, *20*, 113-120.

(11) Kim, S. W.; Bae, Y. H.; Okano, T. *Pharmaceutical Research* **1992**, *9*, 283-290.

(12) Kim, S. W. *Pharmacy International* **1983**, *4*, 182-182.

(13) Lysaght, M. J.; Aebischer, P. *Scientific American* **1999**, *280*, 76-82.

(14) Parenteau, N. *Scientific American* **1999**, *280*, 83-84.

(15) Lauffenburger, D. A.; Griffith, L. G. *Proceedings of the National Academy of Sciences of the United States of America* **2001**, *98*, 4282-4284.

(16) Hench, L. L.; Polak, J. M. *Science* **2002**, *295*, 1014-+.

(17) Drury, J. L.; Mooney, D. J. *Biomaterials* **2003**, *24*, 4337-4351.

(18) Lupas, A.; Vandyke, M.; Stock, J. *Science* **1991**, *252*, 1162-1164.

(19) Surks, H. K.; Richards, C. T.; Mendelsohn, M. E. *Journal of Biological Chemistry* **2003**, *278*, 51484-51493.

(20) O'Shea, E. K.; Rutkowski, R.; Stafford, W. F.; Kim, P. S. *Science* **1989**, *245*, 646-648.

(21) O'Shea, E. K.; Klemm, J. D.; Kim, P. S.; Alber, T. *Science* **1991**, *254*, 539-544.

(22) Harbury, P. B.; Zhang, T.; Kim, P. S.; Alber, T. *Science* **1993**, *262*, 1401-1407.

(23) Malashkevich, V. N.; Kammerer, R. A.; Efimov, V. P.; Schulthess, T.; Engel, J. *Science* **1996**, *274*, 761-765.

(24) Kennedy, S. B.; Ph.D. Dissertation; University of Massachusetts Amherst: Amherst, MA 2001.

(25) McGrath, K. P.; Butler, M. M.; DiGirolamo, C. M.; Kaplan, D. L.; Petka, W. A.; Laue, T. M. *Journal of Bioactive and Compatible Polymers* **2000**, *15*, 334-356.

(26) Kennedy, S. B.; deAzevedo, E. R.; Petka, W. A.; Russell, T. P.; Tirrell, D. A.; Hong, M. *Macromolecules* **2001**, *34*, 8675-8685.

(27) Zhu, H.; Celinski, S. A.; Scholtz, J. M.; Hu, J. C. *Protein Science* **2001**, *10*, 24-33.

(28) Wendt, H.; Berger, C.; Baici, A.; Thomas, R. M.; Bosshard, H. R. *Biochemistry* **1995**, *34*, 4097-4107.

(29) Serero, Y.; Aznar, R.; Porte, G.; Berret, J. F.; Calvet, D.; Collet, A.; Viguier, M. *Physical Review Letters* **1998**, *81*, 5584-5587.

(30) Annable, T.; Buscall, R.; Ettelaie, R.; Whittlestone, D. *Journal of Rheology* **1993**, *37*, 695-726.

(31) Pham, Q. T.; Russel, W. B.; Thibeault, J. C.; Lau, W. *Macromolecules* **1999**, *32*, 2996-3005.

(32) Petit-Agnely, F.; Iliopoulos, I. *Journal of Physical Chemistry B* **1999**, *103*, 4803-4808.

(33) Ng, W. K.; Tam, K. C.; Jenkins, R. D. *Journal of Rheology* **2000**, *44*, 137-147.

(34) Green, M. S.; Tobolsky, A. V. *Journal of Chemical Physics* **1946**, *14*, 80-92.

(35) Jenkins, R. D.; Ph.D. Dissertation; Lehigh University, 1990.

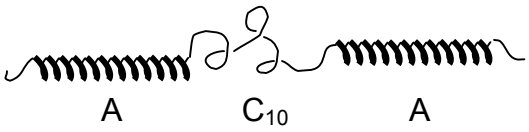
(36) Tanaka, F.; Edwards, S. F. *Journal of Non-Newtonian Fluid Mechanics* **1992**, *43*, 247-271.

(37) Lo, C. M.; Wang, H. B.; Dembo, M.; Wang, Y. L. *Biophysical Journal* **2000**, *79*, 144-152.

(38) Wong, J. Y.; Velasco, A.; Rajagopalan, P.; Pham, Q. *Langmuir* **2003**, *19*, 1908-1913.

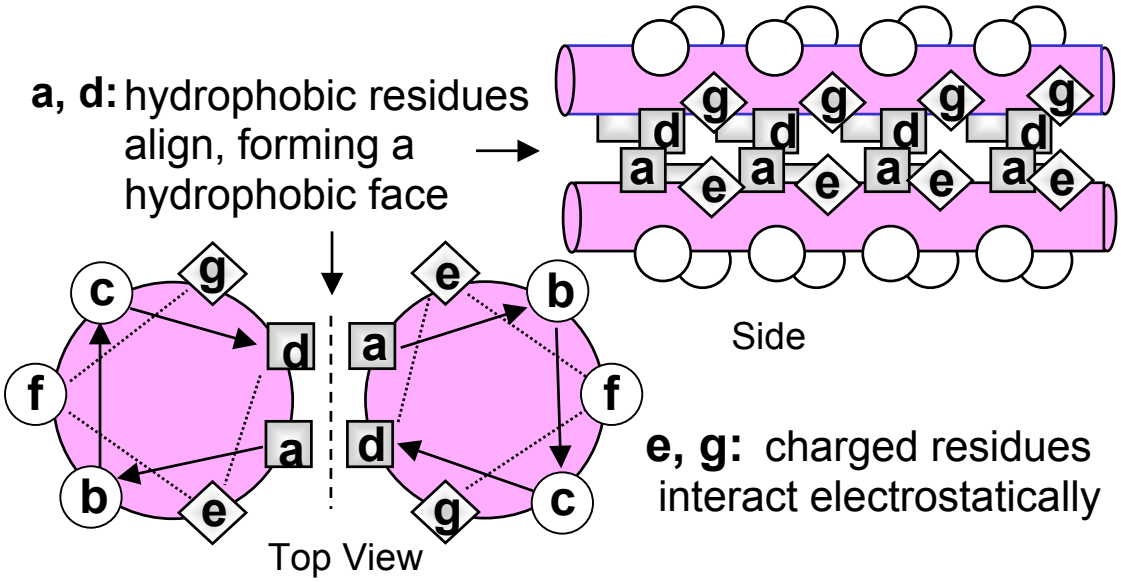
(39) Engler, A.; Bacakova, L.; Newman, C.; Hategan, A.; Griffin, M.; Dischery, D. *Biophysical Journal* **2004**, *86*, 617-628.

(40) Geiger, B.; Bershadsky, A. *Current Opinion in Cell Biology* **2001**, *13*, 584-592.



Leucine zipper A	**SGDLENE VAQLERE VRSLEDE AAELEQK VSRLKNE IEDLKAE**
mid-block C_{10}	**$[AGAGAGPEG]_{10}$** (E: glutamic acid, its pKa is 4.4 for an isolated residue)

(a)



(b)

Figure 1.1. The structure and sequence of the artificial protein $AC_{10}A$. (a) the amino acid sequences of the domains; (b) schematic representation of a leucine zipper as a coiled coil.

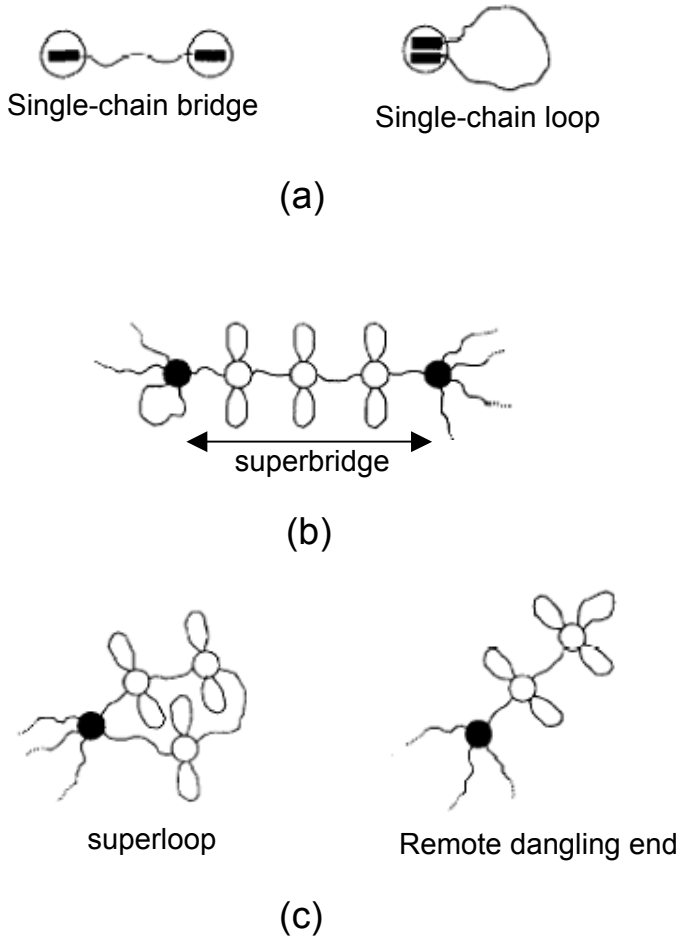


Figure 1.2. Topologies in a transient network. (a) single-chain bridge and single-chain loop; (b) superbridge; (c) superloop and remote dangling end.