Chapter V

CONTROL OF HYDROGEL VISCOELASTICITY BY SMALL MOLECULE PROTEIN LIGANDS

1. Abstract

The transient association of physical cross-linking domains such as coiled coils in protein hydrogels results in a viscoelastic response to applied stresses. In this chapter, the timescale for the viscoelastic stress relaxation in chemical-physical protein hydrogels was modulated by the addition of small, hydrophobic molecules including vitamin D3 and medium- and long-chain fatty acids. Addition of fatty acids of varying chain lengths increased the characteristic relaxation time of the protein network by 10- to 1000-fold. The relationship between the length of the fatty acid chain and the network relaxation time was shown to be biphasic or bell-shaped, with the 12-carbon ligand lauric acid having the largest effect. The proposed mechanism for the increase in the relaxation time involves binding of the ligands within the hydrophobic pore or channel created by a coiled-coil physical cross-link. Binding of hydrophobic ligands is known to stabilize coiled coils, which would increase the average lifetime of the physical cross-links and result in a slower network relaxation. Exploiting natural and designed protein-ligand interactions represents a new approach to developing hydrogel “formulations” in which the viscoelastic properties of the material can be engineered to meet specific design criteria.

2. Introduction

Protein hydrogels can be regulated by external stimuli including temperature, pH, and ionic strength. External stimuli are often used to trigger a gel-sol transition by causing protein domains to fold or unfold, or by promoting the assembly or disassembly of cross-links between protein chains. This behavior is exploited for the encapsulation and release of cells and biomolecules. Examples include temperature- and pH-induced unfolding of coiled-coiled cross-linkers [1] and temperature-induced aggregation of elastin-like polypeptide domains [2]. External stimuli can also have more subtle effects on network properties such as viscoelastic behavior. In physical gels cross-linked by leucine zipper coiled coils, viscoelastic behavior arises from the transient association of the coiled-coil network junctions. By varying the pH over a narrow range from 7-8, the characteristic relaxation time of the network could be tuned from approximately 100 s to 1000 s [3]. This pH sensitivity arises from changes to the protonation state of glutamic acids at the *e* and *g* positions of the helical heptad repeat, which influences that rate of strand exchange between coils. The relaxation dynamics of a protein hydrogel containing nucleoporin-like polypeptide (NLP) repeats were also tunable by an external stimulus, specifically the addition of 1,6-hexanediol, which disrupts the association of Phe-Gly dipeptides in the NLP domains [4]. Developing new strategies for regulating the viscoelastic behavior of protein hydrogels has important implications for engineering tough elastic materials, tuning the material erosion rate, and controlling the behavior of cells cultured on or within the material.

Protein binding to ions, metals, and organic ligands is crucial for a variety of functions including catalysis, signaling, regulation of protein function, storage, and transport. Several hydrogel designs have exploited these interactions to regulate material properties. Among the most common are hydrogels that respond to environmental calcium ions behavior or cross-linking.

Calcium sensitivity in these materials is mediated by the reversible folding or conformational changes of *β*-roll domains [5, 6] and calmodulin domains [7, 8], respectively. Other protein-ligand interactions that have been exploited in hydrogel networks include the enzyme-substrate binding exhibited by a polymer-grafted adenylate kinase and ATP [9], antibody-antigen binding [10], and binding of a small molecule inhibitor of the *Escherichia coli* DNA gyrase enzyme [11].

The N-terminal fragment (residues 20-83) of the rat cartilage oligomeric matrix protein (COMP) forms a homopentameric coiled coil that has been used extensively to cross-link physical protein hydrogels [12, 13]. In the crystal structure of the pentameric coiled coil [14], the pore or channel is lined primarily by hydrophobic residues with two exceptions: glutamine at position 54 and threonine at position 40. The amide side chains of Gln54 form a hydrogen-bonded ring that binds a chloride ion in the crystal structure. The channel is also occupied by several water molecules, particularly near Thr40. Due to the apolar nature of the other side chains buried within the channel, it was hypothesized that small hydrophobic molecules, rather than water, would bind preferentially and that COMP might play some role in the storage of these molecules *in vivo*. Subsequently, crystal structures were obtained of the COMP coiled coil in complex with several small hydrophobic molecules including vitamin D3 [15], all-*trans*-retinol [16], and several long-chain fatty acids (myristic acid, palmitic acid, stearic acid, and oleic acid) [17]. In the presence of vitamin D3, the folded coiled-coil structure displays a modest increase in thermal stability; a shift of +8 °C in the melting temperature was measured by circular dichroism spectroscopy [16]. Fluorescence spectroscopy studies have also indicated that all-*trans*-retinol and long-chain fatty acids bind within the COMP coiled coil, with dissociation constants varying between 0.4-0.9 μM [16, 17].

In Chapter 3, chemical-physical protein hydrogels were described consisting of a triblock artificial protein (EPE) end-linked with a tetrafunctional cross-linker to form a covalent network. The midblock sequence of EPE contains the COMP coiled-coil domain (denoted P), which forms transient noncovalent cross-links within the covalent network. In Chapter 4, several single mutant variants of the P domain were described with respect to their effects on the dynamic properties of protein hydrogels. Networks prepared from EPE variants containing the T40A or Q54A mutation in P exhibited longer characteristic relaxation times than networks prepared from proteins containing the wild-type P domain. This was attributed to an increase in the lifetime of the coiled-coil physical cross-links, and was consistent with the increased thermal stability of these mutant P domains as reported by Gunasekar *et al*. [18]. Given that the binding of small hydrophobic molecules within the channel or pore of P also results in increased thermal stability of the coiled-coil structure, it was reasoned that these ligands might likewise affect the relaxation behavior of protein networks. The data presented in this chapter represent preliminary experiments assessing the effects of vitamin D3 on chemical-physical hydrogels prepared from EPE as well as the effects of various fatty acids containing six to eighteen carbon atoms on chemical-physical hydrogels prepared from EPE L37V.

3. Materials and Methods

3.1 Small Molecule Ligands

Lauric (dodecanoic) acid, myristic (tetradecanoic) acid, palmitic (hexadecanoic) acid, and stearic (octadecanoic) acid were obtained from Fluka. Caproic (hexanoic) acid, capryilic (octanoic) acid, capric (decanoic) acid, and vitamin D3 (cholecalciferol) were obtained from Sigma.

3.2 Protein Synthesis and Characterization

The expression and purification of EPE L37V were performed as described in Chapter 4 and Appendix B. Following reduction with tris(hydroxypropyl) phosphine, the protein solution was desalted with several PD10 columns (GE Healthcare Life Sciences), lyophilized, and stored under argon at -80 °C. The thiol content and protein oligomerization state were characterized by Ellman's assay and non-reducing SDS-PAGE.

3.3 Hydrogel Swelling with Small Molecule Ligands

Hydrogel cross-linking was also performed as described in Chapter 4 and Appendix B. A single large hydrogel was formed by cross-linking 1 mL of 15 wt% EPE L37V with 231 μL of 15 wt% PEG-4VS between glass slides separated by a 1 mm spacer. The cross-linked EPE L37V hydrogel was swollen in decreasing concentrations of guanidinium chloride (6M, 3M, 2M, 1M) in PBS for 3 hr each. The gel was then swollen in PBS containing 0.02% (w/v) sodium azide. For rheological experiments, disks 8 mm in diameter were cut from the larger hydrogel and transferred to a 35 mm dish with 4 mL of PBS containing 0.02% (w/v) NaN_3. To these dishes, the hydrophobic ligands were added as solids (or as liquids in the case of caprylic acid and caproic acid) to a final concentration of approximately 5 mM. Most of the ligands were not soluble at this concentration, so solid particulates and oil-like liquid droplets remained suspended in the buffer. Addition of the fatty acid ligands required the pH of the buffer to be adjusted back to 7.4 with NaOH. EPE gels were prepared in a similar manner and swollen with vitamin D3 (100 μg mL^{-1}, diluted 1:100 from a 10 mg mL^{-1} stock solution in ethanol).

3.4 Rheological Characterization

The EPE L37V gels were swollen in buffer containing a fatty acid ligand for 3 days, rinsed briefly in PBS to remove solids sticking to the surface of the gel, and loaded on the ARES-RFS rheometer as described in the previous chapters and Appendix B. Strain sweep (at 10 rad s^{-1}), frequency sweep (at 1% strain amplitude), and stress relaxation (at 2% strain) experiments were performed at 25 °C. EPE gels were swollen for 7 days with vitamin D3 prior to rheological measurements.

4. Results and Discussion

4.1 Vitamin D3 Increases the Characteristic Relaxation Time of EPE Hydrogels

Motivated in part by the increase in the thermal stability reported by Özbek *et al*. for P coiled coils incubated with vitamin D3 [16], EPE hydrogels cross-linked by PEG-4VS were treated in a similar way. A large excess of vitamin D3 (100 μg mL^{-1}) (Figure V-1 a) was added to PBS buffer containing an EPE hydrogel. Vitamin D3 is not soluble at this concentration, but it was assumed that the solid vitamin D3 was in equilibrium with a saturated solution, and that the vitamin D3 in solution could diffuse into the hydrogel and bind within the coiled-coil cross-linkers (Figure V-1 b). Hydrogels treated in this way were characterized by dynamic oscillatory shear rheology (Figure V-1 c) and stress relaxation experiments (Figure V-1 d). In EPE gels swollen with vitamin D3 solutions, the storage and loss moduli (*G'* and *G"*) curves are shifted to lower frequencies relative to the EPE control without vitamin D3. Likewise, the relaxation function $G(t)$ measured in the stress relaxation experiment is shifted to longer times by two orders of magnitude. This behavior is similar to that of hydrogels prepared from the EPE variants EPE T40A and EPE Q54A described in Chapter 4. The mean relaxation times determined by fitting $G(t)$ with a stretched

exponential model (Equation IV-1 and IV-2) were 107 s for EPE and 10,700 s for EPE with vitamin D3. These results indicate that vitamin D3 binding slows the relaxation of the stress stored in chain segments cross-linked by transient coiled-coil cross-linkers and demonstrate that protein hydrogels can be formulated with small molecule ligands in order to tune their viscoelastic behavior.

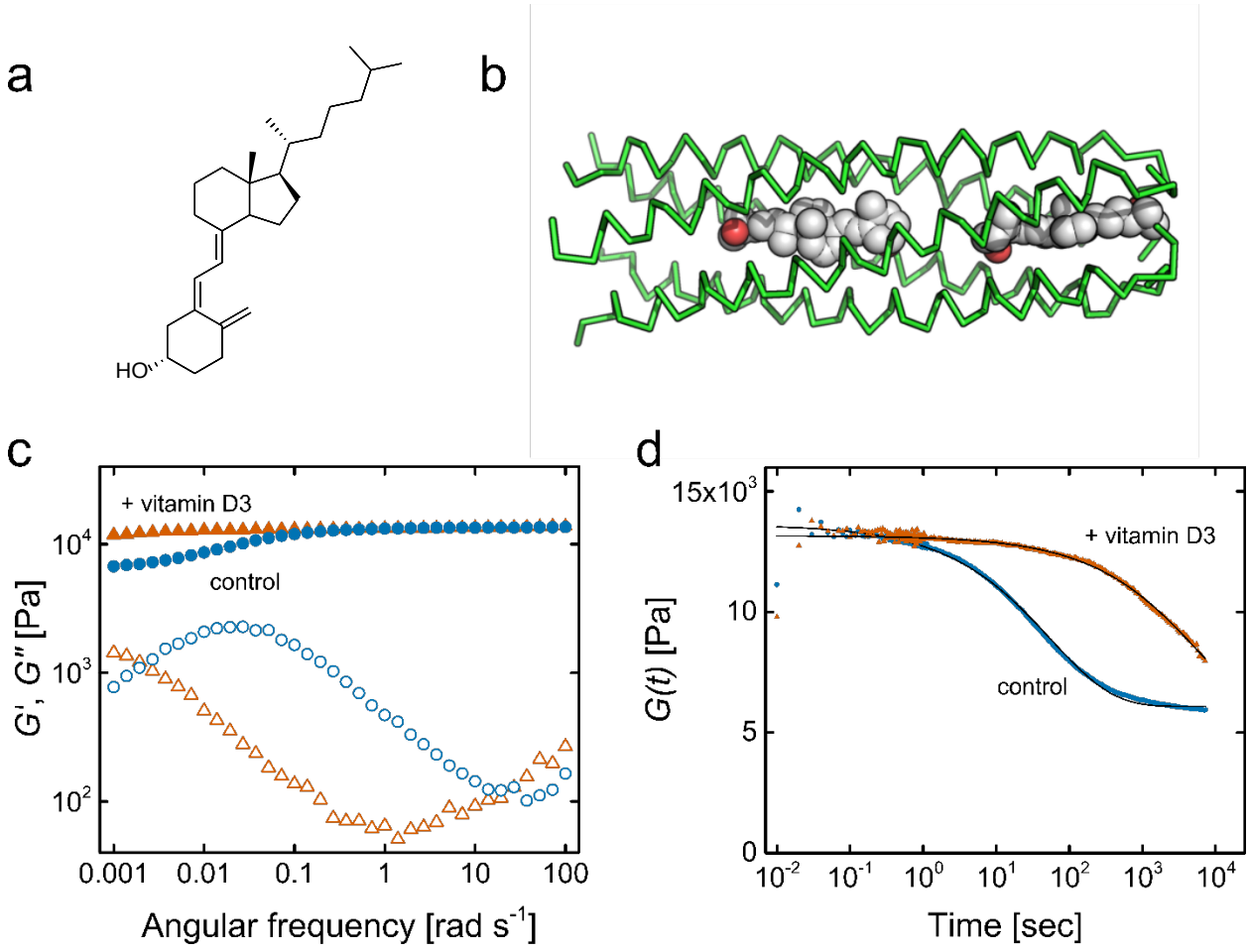


Figure V-1. Effect of vitamin D3 on the viscoelastic behavior of EPE hydrogels. (**a**) Chemical structure of vitamin D3. (**b**) Structure of P coiled coil (green ribbons) with two bound vitamin D3 molecules (white and red spheres). Rendered in PyMol from PDB 1MZ9. (**c**) Frequency sweeps (1% strain amplitude, 25 °C) showing the storage modulus (filled symbols) and loss modulus (open symbols) for EPE gels with vitamin D3 (triangles) and control gels (circles). (**d**) Stress relaxation experiments at 2% strain, 25 °C with and without vitamin D3.

4.2 Rheology of EPE L37V Hydrogels Swollen in the Presence of Medium- and Long-Chain Fatty Acids

Because other ligands were also anticipated to stabilize physical cross-links in protein hydrogels, subsequent experiments were performed with a faster relaxing EPE variant so that the effects of these ligands could be observed more easily. As shown in Chapter 4, hydrogels prepared by cross-linking an EPE variant containing a single leucine to valine point mutation at position 37 exhibit a faster relaxation time than hydrogels prepared from EPE. The relaxation times differ by approximately two orders of magnitude (ca. 1 s for EPE L37V versus ca. 100 s for EPE). In frequency sweep experiments with EPE L37V gels, the transition zone between the high and low frequency plateaus in *G'* and the local maximum in *G"* are centered at an angular frequency of approximately 3 rad s^{-1}. Below 0.1 rad s^{-1}, *G'* is nearly constant, indicating that the stress stored between chain segments cross-linked by P L37V has relaxed, and only chain segments between the covalent cross-links remain elastically effective. These observations suggest that the longer relaxation timescales anticipated for hydrogels treated with various hydrophobic ligands should be observable in the dynamic range accessible in frequency sweep experiments (0.001-100 rad s^{-1}). Furthermore, the crystal structures of P in complex with vitamin D3 [15], all-*trans*-retinol [16], and myristic acid, palmitic acid, and stearic acid [17] indicate that the side chain of leucine 37 does not make direct contact with these ligands, with the possible exception of the longest ligand, stearic acid.

The straight chain fatty acids are an attractive set of ligands for engineering the viscoelastic behavior of chemical-physical hydrogels cross-linked by P coiled coils. They are non-toxic and readily available in many different chain lengths. It was anticipated that systematic variation of the fatty acid chain length would be a useful way to investigate the effects of hydrophobic ligands on

EPE L37V hydrogels. While only the long-chain fatty acids (14 to 20 carbon atoms) have been reported previously to bind within P coiled coils, medium-chain fatty acids (6 to 12 carbon atoms) were also considered here. The fatty acids used in this chapter are summarized in Table V-1, which includes the common name, systematic name, structure, and log P value (water-octanol partition coefficient) [19-25].

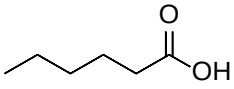
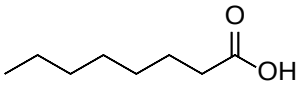
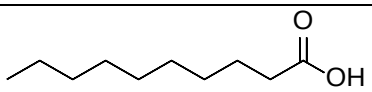
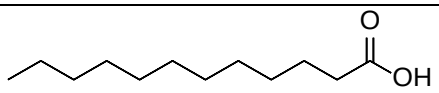
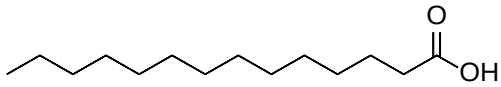
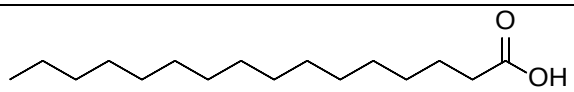
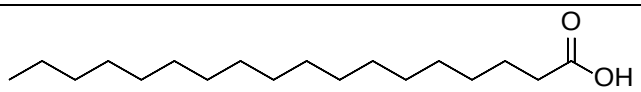
Common Name/ Systematic Name	**Length (# of carbon atoms)**	**Structure**	**Log P**
Caproic acid/ Hexanoic acid	6		1.92
Caprylic acid/ Octanoic acid	8		3.05
Capric acid/ Decanoic acid	10		4.09
Lauric acid/ Dodecanoic acid	12		4.60
Myristic acid/ Tetradecanoic acid	14		6.11
Palmitic acid/ Hexadecanoic acid	16		7.17
Stearic acid/ Octadecanoic acid	18		8.23

Table V-1. Medium- and long-chain fatty acids. The values of log P are from the PubChem Compound Database in refs. [19-25].

EPE L37V hydrogels were swollen in PBS containing 5 mM of the different fatty acids. Similar to vitamin D3, most ligands were not soluble at this concentration and instead formed a suspension of solid particles or oil-like droplets dispersed in the buffer. Binding within the coiled-coil cross-links therefore required a partitioning from the solid or oil phase into solution, diffusion into the hydrogel, and partitioning into the coiled coil. The viscoelastic behavior of the EPE L37V hydrogels was assessed by dynamic oscillatory shear rheology and stress relaxation experiments after 3 days of swelling in the presence of a particular ligand. The frequency sweep experiments are shown in Figure V-2 and the stress relaxation experiments are shown in Figure V-3. For clarity, the data are divided into two plots, one showing fatty acids 6 to 12 carbon atoms in length and the other showing fatty acids 12 to 18 carbon atoms in length. The stress relaxation function, $G(t)$, was fit with a stretched exponential model to determine a mean relaxation time, $\langle\tau\rangle$, as described in Chapters 3 and 4.

A clear trend emerged in the relaxation behavior of EPE L37V hydrogels swollen with fatty acids of different chain lengths. Lauric acid, which is 12 carbon atoms long, had the largest effect on the network relaxation time, increasing τ more than 1000-fold compared to the control EPE L37V gel without fatty acid. When the fatty acid length was increased or decreased by 2 carbon atoms (myristic acid and capric acid, respectively), the effect was smaller than lauric acid but both gels still exhibited relaxation times 100-fold greater than the control gel. The effect was smaller yet for the 8-carbon and 16-carbon fatty acids (caprylic acid and palmitic acid). These gels also appear to have two relaxation timescales; one timescale is approximately 10-fold greater than EPE L37V gels and the other is approximately the same as EPE L37V gels. This is evident in the loss moduli of the two gels, which exhibit a shoulder at the same frequency as the maximum in

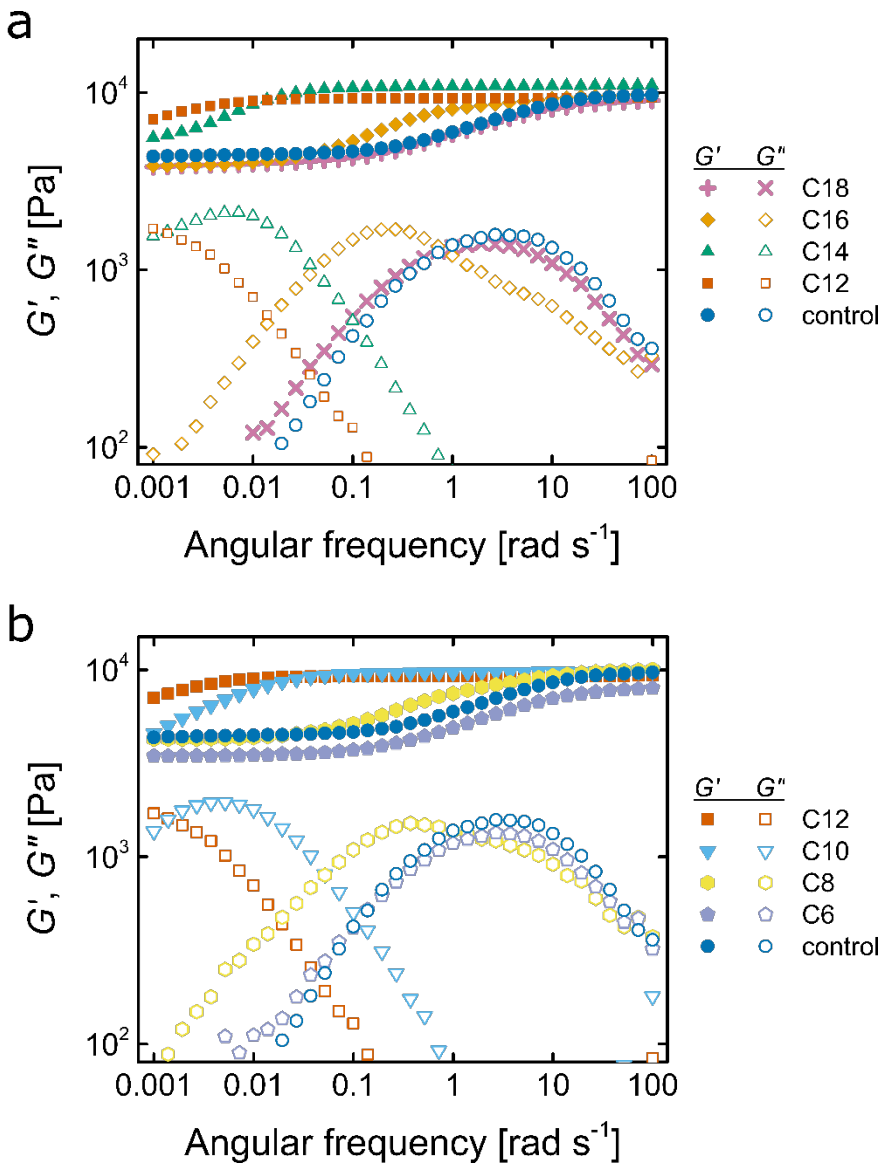


Figure V-2. Frequency sweeps of EPE L37V hydrogels swollen with various fatty acid ligands. (a) Fatty acids 6 to 12 carbon atoms in length. (b) Fatty acids 12 to 18 carbons atoms in length.

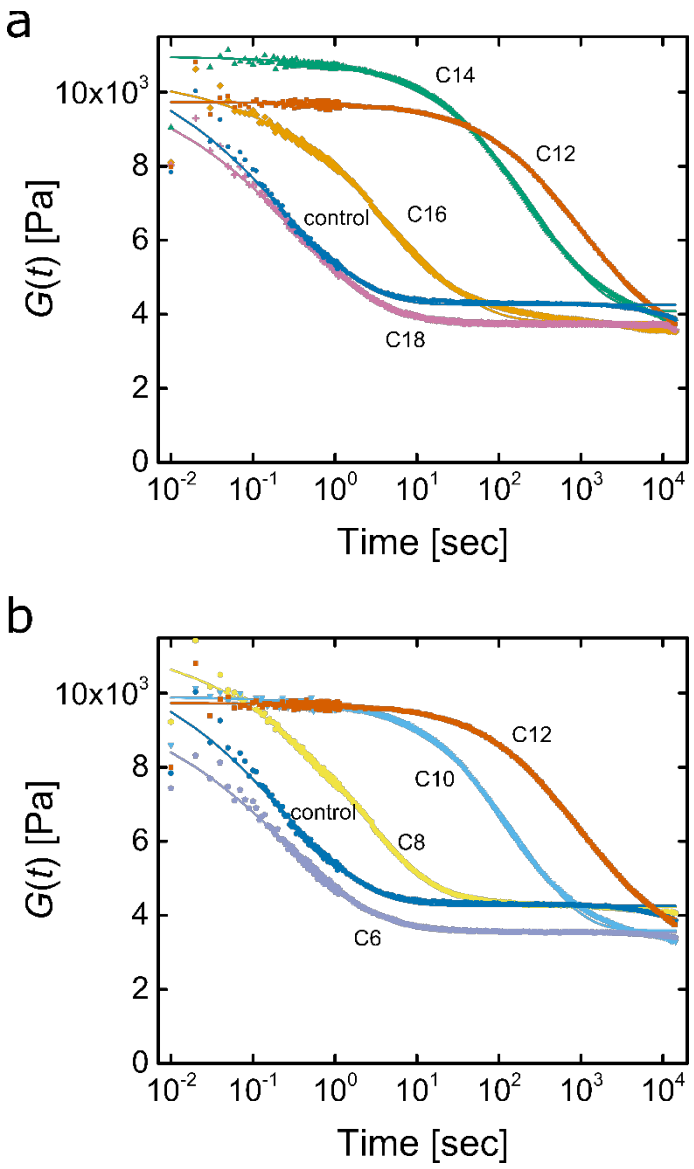


Figure V-3. Stress relaxation of EPE L37V hydrogels swollen with various fatty acid ligands. (a) Fatty acids 6 to 12 carbon atoms in length. (b) Fatty acids 12 to 18 carbons atoms in length. Stretched exponential fits are shown as solid lines.

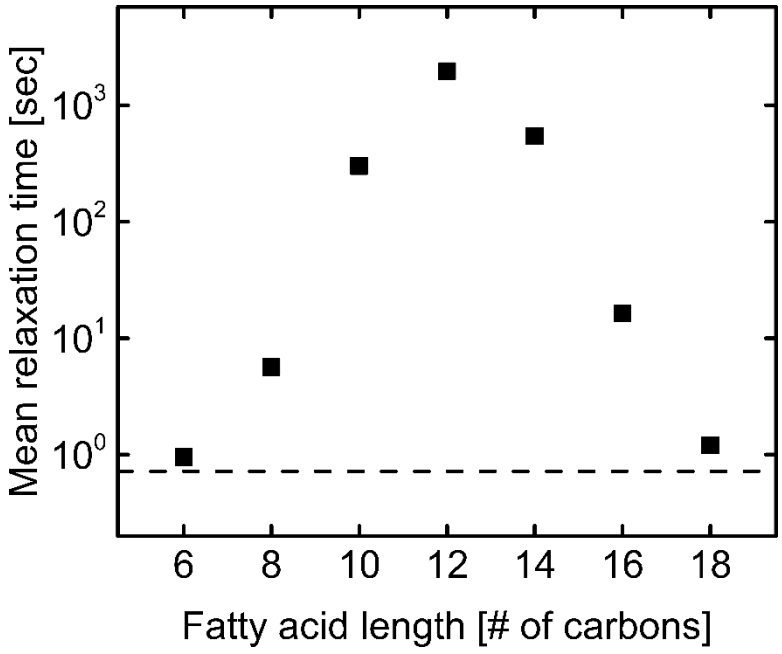


Figure V-4. Plot of mean relaxation time versus fatty acid length. The parameters from the stretched exponential fits in Figure V-3 were used to evaluate the mean relaxation time for each gel. The mean relaxation time of the control EPE L37V gel is shown by the dashed horizontal line.

G" in the control gel. The most likely explanation for this behavior is partial occupancy of the coiled-coil cross-links by these ligands. Finally, hydrogels swollen with 6- and 18-carbon fatty acids (caproic acid and stearic acid) have characteristic relaxation timescales that are similar to the control gels, demonstrating that these compounds do not affect the viscoelastic behavior of EPE L37V gels. Together, these results produce a biphasic or bell-shaped curve describing the relationship between the fatty acid chain length and the characteristic network relaxation time (Figure V-4). The shape of this curve suggests that the lauric acid represents an optimal chain length for binding within the P channel, at least with respect to its effect on the lifetime of the coiled-coil association.

4.3 Proposed Rationale for the Effects of Fatty Acids on EPE L37V Hydrogels

One possible molecular explanation for lauric acid as the optimal ligand length for the P channel involves its length relative to the distance between the polar side chains of Thr40 and Gln54. The crystal structures of P in complex with myristic acid, palmitic acid, and stearic acid indicate that polar carboxylate head groups of these fatty acids interact with the amide side chains of the Gln54 residues [17], which together form a pentameric ring that has been described as an ion trap [14]. Binding of a hydrophobic ligand also appears to displace the water molecules that line the portion of the channel between Gln54 and Thr40 in the *apo*-crystal structure [14]. However, the aliphatic tails of the 14- to 18-carbon fatty acids also extend outside of this region toward the N-terminus and create potentially unfavorable interactions with the Thr40 side chains (Figure V-5). Although the crystal structure of P in complex with lauric acid is not available, it is reasoned that removal of two carbon atoms from the bound myristic acid ligand can provide an estimation of its binding (Figure V-5, top). In this structure, lauric acid is short enough that is does not interact with Thr40. Thus, lauric acid might represent an optimal fatty acid because it maximizes favorable interactions with the apolar side chains of Leu44, Val47, and Leu51 and displaces water from this portion of the channel, but does not have significant interactions with the polar side chain of Thr40. This hypothesis could be tested by mutating Thr40 to an apolar amino acid to determine whether the bell-shaped curve in Figure V-4 shifts toward longer fatty acid chain lengths.

The differences in the solubility of the various fatty acids in aqueous buffer are also likely to influence how they affect the viscoelastic behavior of EPE L37V networks. As shown in Table V-1, the log P values of the fatty acids range from 1.9 for hexanoic acid to 8.2 for stearic acid. The shorter chain fatty acids may be too water soluble and lack the driving force to enter the

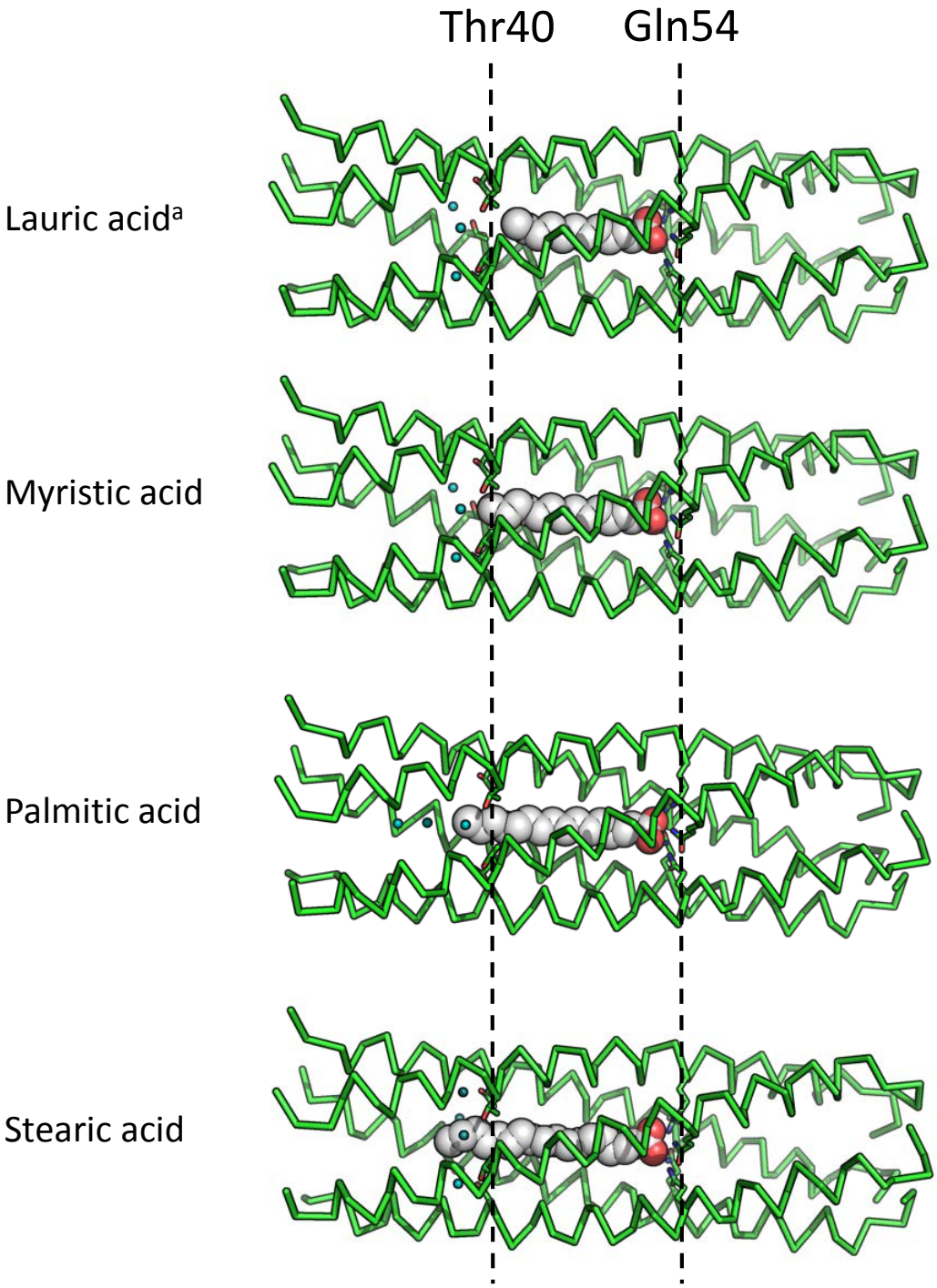


Figure V-5. Fatty acid binding in P coiled coils. The protein chains are shown in green. The fatty acids are shown as spheres (carbon – white, oxygen – red). Water molecules are shown as small teal spheres. The position of Thr40 and Gln54 are denoted by the dashed vertical lines. The structures were rendered in PyMol from PDB 3V2N (myristic acid), 3V2Q (palmitic acid), and 3V2P (stearic acid) reported in ref. [17]. [a]The structure for P with lauric acid was generated by removing 2 carbon atoms from myristic acid in PDB 3V2N.

hydrophobic channel. Conversely, the longer chain fatty acids are poorly soluble in water and may not be capable of dissolving sufficiently and diffusing into the hydrogel. This would explain the apparent lack of an effect of stearic acid on the relaxation time of the EPE L37V network. Lauric acid, myristic acid, and capric acid might represent an ideal balance between dissolution of the ligand in the aqueous media and partitioning into the hydrophobic channel of the coiled-coil. However, differences in solubility alone cannot explain the biphasic relationship between fatty acid chain length and the relaxation time. For example, it does not provide an adequate explanation for why networks swollen in the presence of myristic acid and palmitic acid have shorter relaxation times than networks swollen in the presence of lauric acid. If water solubility the fatty acids was the only determinant of their effect on the network relaxation time, then it is likely that once the longer fatty acids were bound within the channel, the coils would be much more difficult to dissociate and these networks would exhibit longer relaxation times.

MacFarlane *et al*. also reported a biphasic relationship between the fatty acid chain length and the equilibrium dissociation constant (K_d) for the binding of various fatty acids and the P coiled coil [17]. In their experiment, however, stearic acid had the lowest value of K_d (0.44 μM), indicating it was bound most tightly. The dissociation constant increased as the fatty acid length was decreased to 14 or 16 carbon atoms or increased to 20 carbon atoms, indicating weaker affinity. This is somewhat surprising given that stearic acid does not seem to affect the viscoelastic behavior of EPE L37V hydrogels, and it is unclear whether it is even bound within the physical cross-linking domains of the gels. It is possible that stearic acid is bound in the hydrogels, but that tight ligand binding is not necessarily correlated with the network relaxation time. Alternatively, the discrepancy may arise from other differences between the experiment described here and that by MacFarlane *et al*., including differences in the protein concentration and the protein sequence.

The measurements of K_d were obtained at a much lower protein concentration (100 nM) than the typical protein concentration in a hydrogel (approximately 2 mM), which could be important given the poor solubility of stearic acid. Constraining the P domain within a covalent network is almost certain to affect the association energy and dynamics of P coiled coils, and may influence the energetics of ligand binding as well. Finally, although the COMP coiled coil investigated by MacFarlane *et al*. did not contain the L37V mutation used in this chapter, this is likely not the source of the discrepancy because the same trend in the relaxation time was observed for wild-type EPE gels swollen with fatty acids (data not shown).

5. Future Experiments

Experiments are underway to determine whether the various ligands described in this chapter are actually bound within the coiled-coil cross-linkers in EPE and EPE L37V hydrogels. Several hydrophobic compounds including all-*trans*-retinol [16], *cis*-parinaric acid [17], and curcumin [18] have been reported to undergo an increase in fluorescence when bound within the hydrophobic channel of P. This allows for the spectroscopic detection of binding, either by direct detection of one of these fluorescent ligands or by a competition assay between a fluorescent ligand and a non-fluorescent ligand. Extraction of the hydrophobic ligands into organic solvent and detection by high-performance liquid chromatography can also be performed to measure their concentration within the hydrogel.

The kinetics of ligand diffusion into the hydrogels will also be evaluated. The experiments with fatty acids were conducted at a single time point after 3 days of swelling, which is clearly sufficient to observe the effect for some of the ligands. However, the larger, less soluble ligands

such as stearic acid or palmitic acid may require longer swelling times to diffuse into the hydrogel and partition into the coiled coils.

6. Conclusions

Small hydrophobic molecules including vitamin D3 and medium- and long-chain fatty acids were used to engineer the relaxation timescales of protein networks cross-linked by the association of helical domains derived from cartilage oligomeric matrix protein. Using saturated, straight-chain fatty acids varying in length from six to eighteen carbon atoms, the relaxation time of chemical-physical hydrogel networks could be increased 10- to 1000-fold. While the mechanism is still not clear, it is proposed to involve the binding of these ligands within the hydrophobic channel of the P coiled coil, which has been demonstrated previously by crystallization of protein-ligand complexes and by fluorescence spectroscopy. Binding of ligands within the channel stabilizes the coiled coil and is expected to increase the lifetime of the physical cross-links within the hydrogel network. The optimal fatty acid ligand for increasing the network relaxation time was lauric acid, with networks swollen in the presence of shorter and longer fatty acids exhibiting smaller increases in the relaxation time. Further fine-tuning of the relaxation time would likely be possible with odd-numbered saturated fatty acids, unsaturated fatty acids, branched fatty acids, and fatty acids containing heteroatoms. In addition to engineering the viscoelasticity of protein-based materials, the capacity of P coiled coils to bind hydrophobic compounds could also be exploited for drug delivery applications. Incorporating these domains within a hydrogel would provide a method for the local, sustained delivery of therapeutics. This work represents the initial steps toward a new approach to materials design in which different

“formulations” of protein hydrogels and small molecule ligands can be used to engineer material properties for specific applications.

7. References

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