Protein Engineering in the Development of Functional Hydrogels

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Abstract

Proteins, which are natural heteropolymers, have evolved to exhibit a staggering array of functions and capabilities. As scientists and engineers strive to tackle important challenges in medicine, novel biomaterials continue to be devised, designed, and implemented to help to address critical needs. This review aims to cover the present advances in the use of protein engineering to create new protein and peptide domains that enable the formation of advanced functional hydrogels. Three types of domains are covered in this review: (*a*) the leucine zipper coiled-coil domains, (*b*) the EF-hand domains, and (*c*) the elastin-like polypeptides. In each case, the functionality of these domains is discussed as well as recent advancements in the use of these domains to create novel hydrogel-based biomaterials. As protein engineering is used to both create and improve protein domains, these advances will lead to exciting new biomaterials for use in a variety of applications.

Contents

1. INTRODUCTION

In nature we find myriad examples of consensus amino acid sequences that reliably fold into three-dimensional structures with distinct functions, called protein domains. Researchers have employed a number of different domains to find solutions to challenges in biomedical engineering materials such as scaffolds for tissue engineering, active or reactive drug delivery systems, and in vivo and in vitro biosensors (1–9). These applications often benefit from bioactive, stimuli-responsive, or "smart" hydrogel biomaterials, i.e., the biomaterial undergoes a change in response to the local environment. The event (the action, reaction, or phenomenon) that occurs in response to the environmental change or cue is a result of the structure, and consequent function, of the protein domain or motif incorporated into the biomaterial. For example, β-sheets can form ordered supramolecular structures in response to temperature changes (6, 10, 11); α-helical coiled-coils form pH-dependent physical crosslinks in hydrogel structures (12–14); the calcium-binding protein camodulin undergoes a conformation change upon ligand binding causing macroscopic changes in hydrogel structures (15, 16); elastin-like polypeptides undergo a reversible inverse phase transition to form insoluble β-spiral aggregates (17, 18); and controlled degradation of a hydrogel matrix is possible with the incorporation of protease cleavage sequences (19, 20).

Hydrogel: a three-dimensional physically or covalently crosslinked, hydrated polymer network

Responsive or "smart" material: a material that undergoes a bulk change in response to an environmental cue or a change in environmental conditions

Because the protein domain is the center of the responsive action or "smart" phenomena, manipulation of the domain can be used to control the material properties of the hydrogel. The protein engineering tool set allows for the manipulation of natural and artificial DNA sequences encoding the peptides or proteins of interest, as well as the subsequent biological production of the translated products (21). This methodology is powerful in that it allows for exact control over the identity and sequence of each residue and, consequently, the structural folding patterns of the resultant protein or peptide. Of the generally accessible biological expression systems (yeast, mammalian, and bacterial), heterologous expression in *Escherichia coli* is the most common. Furthermore, the

availability of commercial prepackaged kits for traditional molecular biological experiments and techniques has made this methodology accessible to the nonexpert molecular biologist. Functional peptides of ~100 amino acids can also be produced by solid-phase polypeptide synthesis (22). A schematic of the protein engineering methodology of responsive materials design is presented in **Figure 1**.

The past ten or more years have produced a number of successful examples that use variations of this methodology. As a number of reviews have presented excellent surveys of the broad range of protein-based functional materials (2, 3, 5), we review only the examples that best present the case for protein engineering design of responsive or smart hydrogels. A summary of these examples is

Protein engineering methodology of responsive materials design: the use of proteins, protein domains, or peptide motifs to create hydrogels or other materials that respond to, or interact with, environmental cues

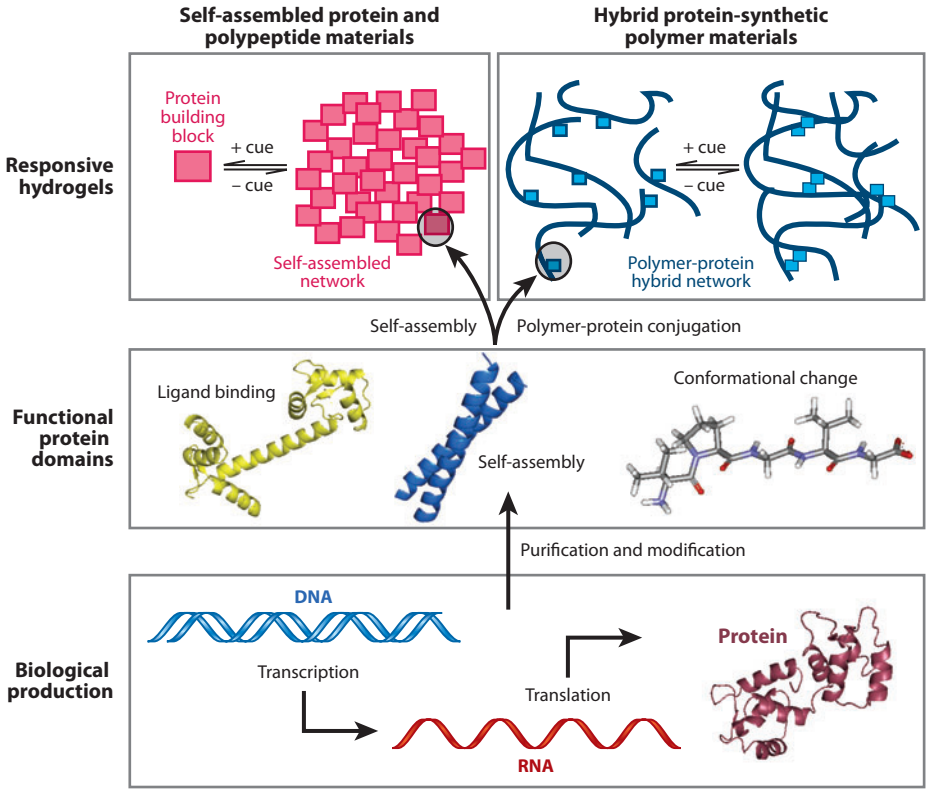

Figure 1

The protein engineering methodology of responsive materials design. Control of the primary amino acid sequence at the DNA level leads to control of the three-dimensional structure and function of the responsive protein domain or peptide motif. Responsive or "smart" materials can be made in one of two manners: first, through self-assembly of protein building block into supramolecular structures; second, by incorporating the responsive protein domain into a hybrid synthetic polymer-protein hydrogel material. In each case the materials are made responsive to an external cue due to the structure and function of the protein domain(s) used in the material. Fine-tuning of the structure and consequent function of the protein domain can be achieved at the DNA level, as mutation to the protein encoding sequence leads to specific changes in the primary amino acid sequence, and the secondary, tertiary, and quaternary structures. The environmental cues are generally pH, temperature, ionic strength, and ligand binding. Calmodulin (1DMO) is shown as an example protein product; calmodulin in extended dumbbell conformation (3CLN) is shown as an example of a ligand binding protein; a two-stranded leucine zipper domain is shown as an example self-assembly domain; and an elastin-like peptide is shown as an example of a conformational change domain.

Table 1 Engineered characteristics and features of the protein domains in responsive hydrogels

Protein domain	Function	Reversibility and responsiveness	Engineering features	Applications and associated references
Leucine zipper	Self-assembly Physical crosslinking	pH Temperature Ionic strength	Hetero- and homo-complexes Oligomerization number Oligomerization orientation Thermal stability Polymer conjugation	Reversible hydrogels (80, 88, 118) Tissue engineering scaffolds (28, 29, 71) Drug delivery (89) Enzymatic hydrogels (114, 115)
Calmodulin	Ligand binding Conformational change	Analyte binding Ligand binding	Ligand binding Analyte binding Polymer conjugation	Drug delivery and biosensor (47, 93) Microfluidic gates and microlenses (26, 27)
Elastin-like peptides	Conformational change Phase change	pH Temperature Ionic strength Hydrophobicity	Disorder to b-turn transition Tunable inverse phase transition Guest residue chemistry	Compliant hydrogels (54, 62, 74) Reversible hydrogels (1, 38, 70) Drug delivery (19, 20, 63, 64)

given in **Table 1**. We focus on the aspects of the protein domains that bring about the desired action and the chemical and physical phenomena that drive the action. In doing so we review not only those works that present responsive materials but also many protein engineering and chemical biology efforts that have led to the understanding of the mechanisms of protein domains and motifs used in the responsive materials. We also highlight protein domains with unrealized potential for hydrogel materials applications.

One of the most widely exploited protein domains is the α-helical coiled-coil or leucine zipper domain. Coiled coils have been extensively studied (23–29) and have been successfully used as physical crosslinks in hybrid hydrogels and supramolecular hydrogel structures (12–14). These engineering efforts have led to the development of hydrogels that structurally respond to changes in pH, temperature, and ionic strength (30–35). Control over the number of strands per bundle, strand identity, and bundle strength is also possible (23, 31, 36, 37). Beta-sheet, β-turn, and elastin-like peptides can also form reversible physical crosslinks that support hydrogel structures (6, 17, 38). Biomaterials that are structurally responsive to light, shear stress, pH, temperature, and ionic strength have been engineered (11, 39–42). In addition to physical crosslinking, the conformational change of elastin-like domains has been exploited to manipulate hydrogel swelling and degradation rate (17, 18). Finally, the ligand and calcium-binding domains of calmodulin have also been extensively studied (43–47) and have proven successful in altering macroscopic properties of hybrid hydrogels in response to binding events (15, 48–50).

Nature is rich with diversity: There are many protein domains and peptide motifs with interesting ligand binding properties, with structures that undergo conformation changes, and that have complex functions yet to be used in responsive materials. Thus far, the protein domains and peptide motifs used in responsive materials have been quite successful in imparting specific and controllable action. Advancements in the three-dimensional architecture of hydrogel structures for tissue engineering applications, advancements in active and passive control of drug delivery, and the development of novel biosensors have been made. We foresee the extension of this work toward the development of multifunctional hydrogels that combine the characteristics of a number of protein domains to create more complex systems that will address biomedical engineering problems yet unresolved.

2. LEUCINE ZIPPER COILED COILS

2.1. Hydrogel Applications

The leucine zipper domains, or simply leucine zippers, are a structural motif commonly found in transcription factors. Much of the pioneering work in elucidating the structure of the motif was focused on the fos and jun oncogenes and the GCN4 transcription factor (23, 24, 36, 37, 51). The helices are characterized by a heptad repeat of the form *abcdefg* where *a* and *d* are leucine, or nonpolar residues, and *e* and *g* are charged residues. The side chains of the nonpolar leucine residues lie in a plane along the length of a helix; the hydrophobic nature of the plane leads to the formation of multistranded coiled coils. The motif's name reflects the predominance of leucine residues at the *a* and *d* positions.

The aggregation or assembly of two or more domains into a coiled-coil bundle is reversible with changes in pH, temperature, and ionic strength. The conditions under which assembly occurs are dependent on the primary sequence. The reversible assembly makes the leucine zipper domain ideal to serve as responsive physical crosslinks in hydrogel structures. Pekta et al. first demonstrated that a supramolecular hydrogel structure assembled from protein subunits could be made responsive to temperature and pH (12). A triblock polypeptide with terminal leucine zipper domains and a central randomly coiled domain self-assembles at neutral pH and temperatures below 40°C. A physically crosslinked hydrogel network forms as the leucine zippers aggregate into tetrameric coiled-coils. Wang et al. first described the use of leucine zipper domains in a hybrid synthetic polymer-protein material (13). The hybrid material undergoes a volume change in response to temperature change as leucine zipper coiled-coils dissociate at high temperature. Since the publication of these two works, a number of studies have shown that the temperature, ionic strength, and pH at which hybrid and protein materials undergo volume change and hydrogel formation can be engineered at the primary sequence level (28–32, 34, 35).

Physical crosslinking with leucine zipper coiled-coils has also been used to create cell binding scaffolds. Triblock polypeptides with leucine zipper domains and a central domain containing the cell adhesion ligand, arginine-glycine-aspartic acid (RGD), have been shown to bind cells in a reversible manner (52–54). Triblock polypeptides with antibody binding ability have also been demonstrated (55). In our lab we have used leucine zipper fusions to create enzymatic protein building blocks that self-assemble into enzymatic hydrogels (56, 57). These works combine the self-assembly function of leucine zippers with the catalytic function of an enzyme creating bifunctional building blocks. We have also shown that different building blocks with compatible leucine zipper domains can assemble into multifunctional hydrogel structures (14). The association of α-helices in coiled-coil bundles can be transient (27, 33, 34), i.e., a helix from one bundle can exchange places with a helix from a different bundle. This phenomenon has been used to tailor the rate of erosion of supramolecular triblock polypeptide structures (33).

A number of recent publications have shown the utility of α-helices in fiber formation (58–60). These exciting works have recently been reviewed (61) and are therefore not covered here. In the cases presented in this review, the leucine zipper domain has been used as a physical crosslink, both as the only source of crosslinking in supramolecular protein structures (**Figure 1**, top left) and as a minor source of crosslink density (**Figure 1**, top right). The domain has been used to impart responsive and reversible behavior in hydrogels. The utility of the domain lies in the robustness of the structure and in the long history of investigation into the mechanisms of coiled-coil formation.

2.2. Coiled-Coil Formation, and Oligomerization Number

Leucine zipper domains form supercoils of two or more α-helical strands with a characteristic heptad repeat of *abcdefg*. The natural coiled-coil shape is a left-handed supercoil of two or more right-handed α-helices. The structure of the helices results in 3.5 residues per turn, as opposed to the 3.6 residues per turn common to the α-helical secondary structure. The leucine residues, or isoleucine or valine residues, at positions *a* and *d* form a hydrophobic plane along the length of each helix. Charged residues occupy positions *e* and *g* and charged or neutral residues are commonly found at positions *b*, *c*, and *f* (23, 36). Formation of the coiled-coil structure is driven by the hydrophobic effect as the hydrophobic plane of the *a* and *d* positions buries within the core of the motif. Using the dimer structure shown in **Figure 2** (top, left) as an example, the motif can be envisioned as a ladder with the backbones of the α-helices as the sides, and the sets of *a* and *g* and *d* and *e* residues (along with the *a′* and *g′* and *d′* and *e′*) as the rungs.

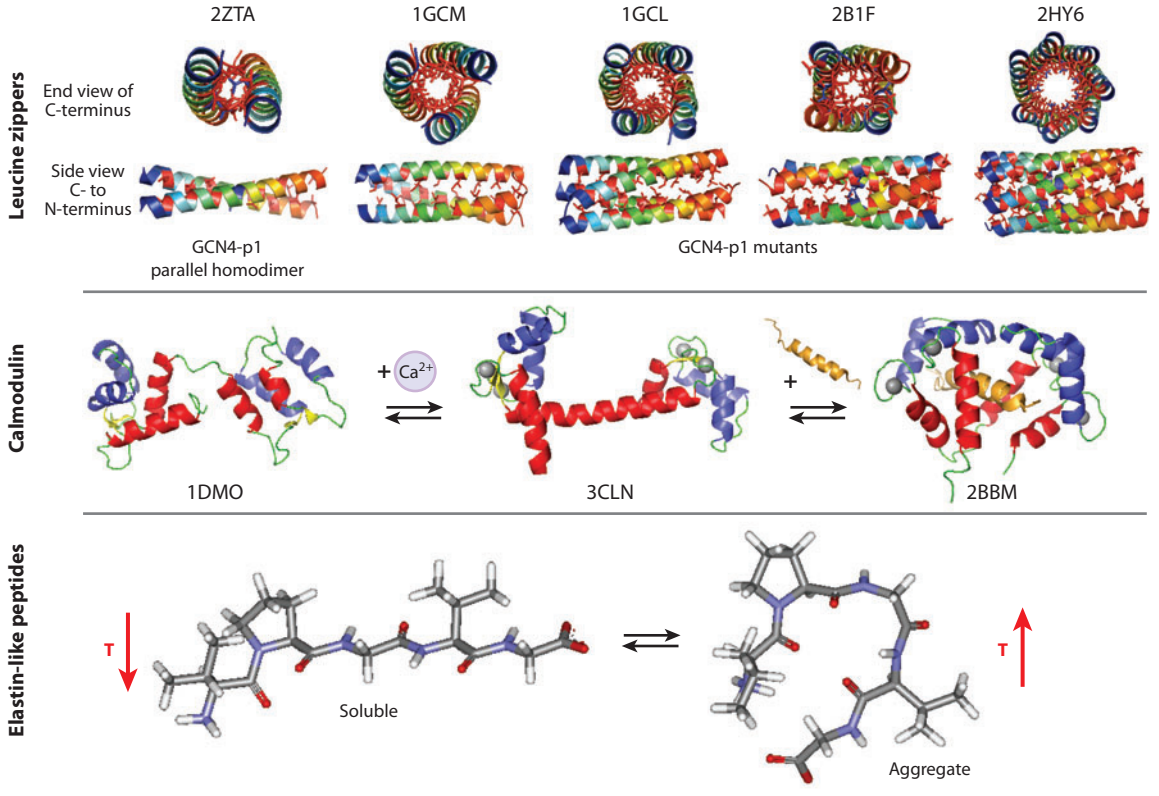

Figure 2

The structure of protein domains and peptide motifs most commonly used in protein and hybrid synthetic polymer-protein–responsive hydrogels. (*Top*) Leucine zipper, coiled-coils from left to right: parallel two (2ZTA), three (1GCM), four (1GCL), antiparallel four (2B1F) and parallel seven (2HY6) member coiled-coils. Protein data bank files are stated in parenthesis. The parallel trimer, tetramer, and heptamer coiled coils are mutants of the GCN4 dimer structure. The series of leucine zipper coiled-coils is shown to demonstrate the variety of structures that are possible through mutation to the primary amino acid sequence of the heptad repeat *abcdefg* of the leucine zipper domain. (*Middle*) The conformational states of the calcium sensor protein calmodulin from left to right: unstructured-globular, nonligand binding (1DMO); extended "dumbbell", bound Ca^{2+} with revealed ligand binding sites (3CLN); globular, bound ligand (2BBM). (*Bottom*) At low temperatures (T), elastin-like peptide is shown as unstructured and water soluble (*left*). Above the inverse phase transition temperature, the elastin-like peptide undergoes a conformational change, taking on a beta-turn structure (*right*).

The X-ray crystal structures shown in **Figure 2** are a set of examples of coiled-coils demonstrating the diversity of natural and engineered structures. The series is also historical, in that the discovery or design is from earliest (left) to most recent (right). The dimer structure (left) is that of the GCN4 leucine zipper domain (the final 33 C-terminal residues of the full GCN4 protein) and was first solved by Kim and colleagues (51). The heptamer structure (right) is an engineered seven-helix bundle designed by Liu and coworkers (62). Parallel (**Figure 2**, top, third from the left) and antiparallel (**Figure 2**, top, second from the right) orientations are also possible (63).

Alber and colleagues demonstrate that dimeric, trimeric, and tetrameric coiled-coil configurations are possible by altering the packing characteristics of the hydrophobic residues at positions *a* and *d*. The identity of the *a* and *d* residues drives the formation of different quaternary structures owing to the destabilizing effects of isoleucine and valine in specific packing arrangements (37). As can be seen in the end view of each example, the center of the core forms a channel. At 1.0 to 1.3 Å in diameter, the channel in tetra-, tri-, and dimers is too small to allow water to enter the hydrophobic core. Coiled-coils with five (64) and seven (62) (**Figure 2**, top, right) strands have also been demonstrated.

The homodimer forms a shallow left-handed superhelix with parallel strands. The structure shows the typical core packing as well as the presence of salt bridges between the charged side chains at positions *e* and *g′*, and *g* and *e′*. The asparagine side chain visible in the core of the structure forms hydrogen bonds with its opposing partner. Despite a destabilization of helical and coiled-coil structure, asparagines are commonly found in native coiled-coil structures to promote specific dimerization (24, 51).

2.3. Engineering Coiled-Coil Stability and Specificity

Substantial effort has been devoted to understanding the specificity of homo- and heterodimerization (25, 27, 65). A seminal work by O'Shea et al. demonstrates that homo- and heterodimerization is driven not by stabilizing salt bridges of the *e* and *g* residues but by the destabilizing effect of homodimers with similar charge (24). In a detailed study of the length of the charged side chains at *e* and *g*, Ryan & Kennan demonstrate that strand exchange can occur in a dimer pair where a more stabilizing strand replaces a less stable strand (27). They do not conclude that the salt bridges have an overall stabilizing effect, but that preference is made toward the more stable dimer pair. In fact, it is has been shown that some salt bridges are destabilizing.

A number of works have explored leucine zipper coiled-coil temperature stability as a function of domain length. Su et al. present evidence of a three-heptad repeat minimum for dimer formation, and also demonstrate that temperature stability increases with increasing length (66). Kopecek and coworkers show that the correlation between temperature stability and domain length is also observed with triblock polypeptides with terminal leucine zipper domains (30); however, coiled-coil stability does not necessarily translate into hydrogel stability as the coiled-coil melting temperature, as measured by circular dichroism spectroscopy, is greater than the sol-gel transition temperature of protein hydrogels assembled from triblock polypeptides (12, 32).

2.4. Underexplored Helical Structures—Heme-Binding Helical Bundles

There are many helical motifs and domains that have yet to be used or that have only recently been used in responsive materials design. The assembly of long fibers from the association of designed α-helices (58–61) is such an example. One possibility with great potential yet to be explored is the incorporation of heme-binding helical bundles into responsive protein and hybrid hydrogels. An example of a heme-binding helical bundle is shown in **Figure 3**. Dutton and coworkers have

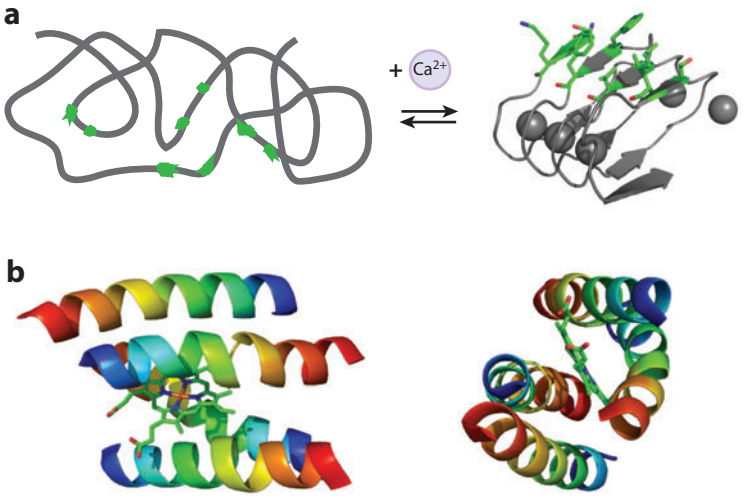

Figure 3

New domains for responsive hydrogels. These protein domains have not yet been used to create responsive hydrogels. The structures are shown here to demonstrate the potential of these domains in engineering new responsive materials. (*a*) Beta-roll domain (2Z8X); (*b*) a heme binding helix bundle (3EH5).

designed four-helix bundles with controlled heme binding (67, 68), and have recently demonstrated the design and engineering of a dioxygen transport protein (69). Shu et al. have successfully constructed peptide-polymer conjugates with PEG (polyethylene glycol) polymer chains selectively bound to the exterior side chains of a trimeric heme-binding domain (70). These works, in combination with the development of self-assembled and responsive hydrogels, could lead to new and exciting avenues of protein-based functional materials design and engineering.

3. CAMODULIN AND THE EF-HAND DOMAIN

3.1. Hydrogel Applications

The EF-hand motif is a calcium-binding domain common to many calcium-binding proteins and calcium sensor proteins that are essential for intracellular signal transduction. The domain is characterized by a helix-loop-helix structure that binds Ca^{2+} in a cooperative manner (44, 47). The prototypical calcium sensor protein is calmodulin (CaM). When the four EF-hand domains of CaM bind cystolic Ca^{2+} the protein undergoes a conformational change to reveal a ligand binding site. CaM can bind many different ligands, but the target is often an enzyme that becomes activated. In this way, CaM facilitates the transduction of a cystolic Ca^{2+} signal into a biochemical action (46).

The structure of CaM is such that it undergoes two distinct conformational changes. Upon calcium binding the unstructured-globular CaM takes on an extended dumbbell shape (**Figure 2**, center). The extended state is an activated state, as a ligand binding site is revealed. A second conformational change occurs upon ligand binding as the extended dumbbell shape collapses around a peptide ligand (**Figure 2**, center right). Structures of all three states are shown in **Figure 2** (center). The conformational change of CaM upon Ca^{2+} and ligand binding is substantial. The distance between the globular ends of CaM in the extended state is approximately 50 Å (71). The distance is reduced to approximately 15 Å upon ligand binding (72). The mechanical action in response to biological cues is ideal for engineering responsive hydrogels. Two excellent examples

PEG: polyethylene glycol

CaM: calmodulin

of the protein engineering methodology of responsive materials design are those from Murphy and coworkers (16, 49, 50, 73) and from Ehrick et al. (15, 48).

Ehrick et al. first describe an acrylamide hydrogel with CaM and a phenothiazine peptide ligand covalently bound to the hydrogel network (15). In the presence of Ca^{2+}, CaM binds the immobilized phenothiazine ligand, thus adding to the crosslink density of the hydrogel. Chelation of Ca^{2+} from the EF-hand domains of CaM inhibits ligand binding and results in an increase in hydrogel volume as the physical crosslinks formed by CaM-phenothiazine interactions are disrupted. Swelling is reversible with the subsequent addition of calcium. This mechanism is schematically represented in **Figure 1** (top, right). An increase in hydrogel volume also occurs in response to soluble CaM-binding ligand cues. In the presence of a soluble ligand that binds with higher affinity than the immobilized phenothiazine, CaM-phenothiazine interactions are replaced with CaM-soluble ligand interactions, resulting in an increase in volume.

EF-hand: a Ca^{2+} binding domain

The Murphy group has also produced CaM-based responsive hydrogels. In their approach, CaM is modified with diacrylated poly(ethylene glycol) (PEGDA) to produce a triblock PEGDA-CaM-PEGDA prepolymer solution. Hydrogels responsive to Ca^{2+} and CaM-ligand binding are made upon UV crosslinking of PEGDA-CaM-PEGDA monomers in the presence of photoinitiator. The mechanical action of the bound CaM in response to binding events can be modulated by tuning the PEGDA chain length. With PEGDA of MW 575, Sui et al. produce a hydrogel that undergoes an 80% change in volume in response to binding the antipsychotic drug trifluoperazine in the presence of Ca^{2+} (49). The volume change is reversible, losing only minimal dynamic range after 10 swelling/deswelling cycles.

One key to incorporating the CaM domain into both the acrylate and photocurable PEGDA hydrogels is the mutation of two threionine residues, one at each end of the extended dumbbell conformation. Mutation of the threionines to cysteines (T34C and T110C) does not disrupt Ca^{2+} or ligand binding and allows for selective modification of the cysteine residues. The thiol side chains of the cysteine residues are available for modification by Michael-type addition to acrylate groups (16), by reaction with *N*-succinimidylacrylate (NHS) chemistries (15) or by other thiol-specific chemistries.

The mechanical action and conformational change of the calmodulin domain are functionalities that are much different in character from the functionalities of the leucine zipper coiled coils. These functionalities, mechanical action and conformational change, have been shown to be adaptable to hydrogels to produce a high degree of swelling and volume control, characteristics that make these systems amenable to applications such as active drug release (50), biosensing (49), microlenses, (48) and microfluidic gates (15).

3.2. Conformational Change, and Calcium and Ligand Binding

The EF-hand domain takes its name from the E and F helices first described in the crystal structure of parvalbumin, a calcium-binding protein of the CaM superfamily (74). Much has been written about the structure and function of the EF-hand domain because of the importance of calcium signaling in intracellular processes. Here we review the mechanism of calcium and ligand binding to provide sufficient background for a detailed understanding of the responsive behavior of the systems described above. A more complete description of the structure and function of the EF-hand domain can be found in a recent review by Gifford et al. (75).

Typically, as is the case for CaM, EF-hand domains are found in pairs. Each pair interacts to form globular domains, each with two EF-hand domains, that cooperatively bind Ca^{2+}. In CaM, a flexible linker joins two pairs of EF-hand domains to make a 16.7-kDa protein with a pI of 4.6. Apo-CaM is globular and the loops adjoining the helices of each EF-hand domain are exposed

(**Figure 2**, center, left). Each loop provides the bulk of the Ca^{2+} ligands, as five of the seven ligands per ion are donated from the residues within a flexible loop. Tight Ca^{2+} binding is a result of the high number of acidic residues that donate oxygen ligands to the bound ions. Binding affinity can be altered through mutations to the 12-residue consensus loop adjoining the helical domains of the motif (47, 76). Ca^{2+} binding causes a change in orientation of the helices in each EF-hand domain; the repositioning of the helices in turn causes a conformational change in each pair of EF-hand domains. Detailed aspects of the cooperative nature of Ca^{2+} binding and the spatial repositioning of the helix-loop-helix structure are reviewed in References 44, 46, and 75.

Cellular concentrations of Ca^{2+} range from 10^{-7}–10^{-8} M in an inactive state and up to 10^{-5}–10^{-6} M in an active state. The binding K_d of EF-hand proteins ranges from 10^{-4} to 10^{-9} M (47), but at low concentrations ($\sim 10^{-7}$ M), the EF-hand domains of CaM are inactive and do not bind Ca^{2+}. CaM is active when the Ca^{2+} concentration reaches $\sim 10^{-6}$ M. The conformation change that occurs from apo-CaM to CaM with the binding of four Ca^{2+} ions results in a structural change from globular to extended states (**Figure 2**, center, middle). A hydrophobic patch is revealed in the extended, or dumbbell, state as four previously buried methionine residues within each pair of EF-hand domains are exposed. The hydrophobic patch is the site of ligand binding. The linker joining the globular ends of the extended conformation is highly flexible. Upon ligand binding a second conformational change occurs, as the flexible linker joining the globular ends of the extended conformation collapses around the ligand (45, 77) (**Figure 2**, middle, right).

3.3. Calmodulin Ligands

Calcium-binding proteins with EF-hand domains such as calbindin D_{9K} and parvalbumin are calcium buffers, i.e., the EF-hand domains of calcium buffer proteins act to maintain the intracellular calcium concentration and bind calcium without transmitting a signal in response to a change in calcium concentration. The exposure of the ligand binding site in the extended conformation of CaM results in the eventual transmission of the calcium-binding event to a ligand binding event. The structure of the flexible linker that contains the ligand binding site is such that it allows for the binding of many different ligands. Although binding is promiscuous, it is strong (K_d of 10^{-7} to 10^{-11} M^{-1}) (44, 46). The diversity of ligands that bind to CaM speaks to the broad importance of the protein in intracellular signaling (46).

Despite little sequence homology in CaM ligands, the structures are often similar. Generally, CaM-ligand sequences are predicted to fold into an amphiphilic helical structure (44, 46), and the amino acid sequence of peptide ligands is highly regular. CaM ligands are classified by the position of the hydrophobic residues. The 1–10 type has hydrophobic residues at positions 1 and 10, the 1–14 type has hydrophobic residues at positions 1 and 14, and so on for 1–16, and the 1–10 subclass 1-5-10 (44). An active and growing list of CaM ligands can be found at **http://calcium.uhnres.utoronto.ca/ctdb**. Combined, the regularity of sequence pattern and the diversity of potential ligand provide a powerful advantage to CaM-based biosensors.

3.4. Underexplored Conformational Changing Domains—β Rolls

The beta roll domain is a calcium-binding motif (**Figure 3*a***) common to proteins secreted through the bacterial Type 1 Secretion System (T1SS) (78). Two parallel beta sheets connected by calcium-binding turns form a parallel beta helix (79). The beta roll sequence is comprised of tandem repeats of GGXGXDXUX, where X can be any residue and U is a hydrophobic amino acid (80). GGXGXD forms the calcium-binding turn and XUX forms a short β-strand, completing one half of a beta helix turn. Calcium is coordinated by aspartate amino acids (D) and the glycine amino

acids (G) allow turns to form above and below the calcium ions. NMR and biophysical data suggest the beta roll is largely unstructured in the absence of calcium (81). The beta roll likely acts as a calcium-induced structural switch preventing premature folding of proteins utilizing the T1SS (82). Once in the extracellular environment, beta roll formation prevents diffusion back through the outer membrane pore.

The beta roll from *Serratia marcescens* serralysin was used to modulate the distance between 6-phospho-β-galactosidase (PGAL) proteins to demonstrate the possibility of using responsive proteins to tune material pore size. Electron micrograph data show that chelating calcium with EDTA induced 3–13-nm changes in PGAL separation (83). Theoretically, the repeating structure of the beta roll should make possible engineering efforts to control protein separation by simply adding more beta roll repeats to the desired constructs.

Studies of the calcium-responsiveness of the isolated beta roll have proven difficult, requiring either folding aids, such as PEG (84), or the inclusion of natural non-beta roll flanking sequences (81). Recent work in our lab has demonstrated that nonnative globular proteins are also capable of enabling calcium-responsive behavior (85, 86). This finding suggests that beta rolls may find utility as calcium-responsive elements in protein-based hydrogel systems.

4. ELASTIN AND ELASTIN-LIKE PEPTIDES

4.1. Hydrogel Applications

ELP: elastin-like peptide

Engineering of elastin-like peptides (ELPs) has matured greatly over the past 30 years, and ELPs are among the best characterized proteins used for tissue engineering and drug delivery applications. ELPs are 5 to 1500 amino acids in length, generally made from the pentamer consensus sequence, VPGXG, where X is any amino acid. ELPs exist in disordered conformations below their transition temperatures and form more ordered β-turns above their transition temperatures (**Figure 2**, bottom). Above the transition temperature, ELPs form reversible proteinaceous networks comprised of repetitive β-turns forming a β-spiral. The combination of ELPs' inherent elasticity and the inverse phase transition has led to the development of different materials applications including synthetic-ELP hydrid hydrogels with reversible swelling, inverse phase transition hybrid materials, and other switchable interfaces.

ELP-cell interactions have been well studied. Some ELP technology may be considered mature in the tissue engineering and responsive biomaterials fields. ELPs are generally incorporated into polymer-based tissue engineering scaffolds for their desirable mechanical properties. Other tissue engineering applications for ELPs involve the genetic manipulation of ELPs for favorable crosslinking chemistry and the use of the inverse phase transition of ELPs to create a reversible physically crosslinked network. In each application, bacterial expression of ELPs enables exquisite control of ELP mechanical, chemical, and materials properties. Furthermore, bacterial expression offers ELP monodispersity that is otherwise unattainable with synthetic approaches.

ELPs are most often employed for their mechanical properties. ELPs with lysine residues periodically used as the guest residue were chemically crosslinked to form hydrogels with reversible swelling properties (87), which are able to form without detriment to implanted cell viability (88). Conjunctival epithelial cells can be proliferated on ELP substrates (89). By creating blocks of lysine-containing hydrophobic ELPs, and blocks containing aliphatic and hydrophilic ELPs, the mechanical properties of the hydrogel can be tuned (87). Chemical crosslinking takes advantage of the lysine residues, and hydrogel crosslink density can be controlled via the lysine molar fraction. Because ELPs can be genetically encoded, other responsive domains can be incorporated into the hydrogel using standard molecular biology (17, 18, 90). ELP was injected into osteochondral

defects and crosslinked in situ, showing increased infiltration over three months (91). Using an ELP hydrogel with urokinase plasminogen activator cleavage sites and RGD cell adhesion sites, PC-12 cells were able to grow, differentiate, and exhibited extensive neurite outgrowth (92). The ELP inverse phase transition was used to create a cell sheet; and after confluence, the temperature was dropped below the transition temperature and the sheet was detached (93). In drug delivery applications, ELPs are used for their acervation properties, where upon inverse phase transition, soluble ELPs form an aggregate. Using local hyperthermia, the ELP phase transition was used to target ELPs to solid tumors (94). ELPs have also been used in creating switchable interfaces (95). ELP was fused to Interleukin 1 Receptor antagonist (IL1-Ra) to create a persistent "depot" of IL1-Ra. Proteolytic cleavage released soluble IL1-Ra. ELPs have been used to control the attachment of cells in cell-based biochips (96). Hyperthermophilic targeting of an anticancer lactoferrin L12 domain to a Tat-ELP caused a 30-fold increase in cytotoxicity of pancreatic adenocarcinoma cells in vitro (97, 98). In situ depot formation of ELP aggregates encapsulating antibiotics has prolonged release properties with first-order time constants ranging from days to weeks, opening up the possibility of using ELPs to prolong drug delivery (99).

4.2. Conformational Change, and Inverse Phase Transition

There are several excellent reviews on ELP conformational change and inverse phase transition (90, 100–102). We cover these topics only in sufficient detail to understand how ELP inverse phase transition can be used in tissue engineering and drug delivery applications, and general trends observed in the engineering of ELP properties. ELPs are frequently composed of repeating pentamers with the sequence VPGXG, where X is known as the guest residue. The guest residue can be any amino acid except Pro. The prototypical ELP contains many repeats of the VPGVG unit, denoted $(VPGVG)_n$. At lower temperatures, this biopolymer is soluble in aqueous environments, and forms a crosslinked network above its inverse transition temperature (103). Structural information for ELPs is limited because the crosslinked ELP is insoluble. Various NMR, X-ray, CD, and FTIR studies have shown that in its soluble form, ELPs are mostly disordered, whereas crosslinked ELPs are higher in β-turns and form β-spiral structures (104–106). The inverse temperature transition (T_t) is named as such because typically proteins lose structure upon increasing temperature. On the contrary, ELPs become more structured. ELPs are believed to be hydrated below T_t, and increasing temperature causes dehydration and β-turn formation within each ELP repeat (101, 107, 108). Entropy increases associated with the liberation of structured water of hydrophobic hydration drive ELP structure formation. The dehydrated, structured ELP then aggregates in order to bury more hydrophobic regions and minimize hydration, forming a physically crosslinked network. This inverse temperature transition is reversible (109). Here, increasing temperature drives ELP conformational change that results in an aggregate reversibly crosslinked network. In addition to thermal responsiveness, crosslinking can occur via any mechanism by which the hydrophobicity of the ELP can be increased (110). The prominent means to enhance hydrophobicity are increasing chain length, ionic strength, and pH. In each of these cases, the enhanced hydrophobicity acts to effectively lower the T_t, thereby allowing isothermal phase transitioning (111).

4.3. Responsiveness Tuning: Temperature, Ionic Strength, pH, Chain Length, Fusion

ELPs are highly desired biomaterials because they can be genetically encoded through recursive directional ligation, allowing precise control of chemical composition as well as a degree of monodispersity not possible using synthetic methods (112). Furthermore, the conditions under

which ELPs can undergo inverse phase transition are predictable and therefore can be engineered. Depending on the application, a variety of methods are available for tuning responsiveness. The most obvious method is to change the identity and molar fraction of the guest residue. Methods to predict the effect of the guest residue and guest residue molar fraction are extensively covered elsewhere (113). The effect of chain length and ELP concentration on phase transition has been described previously (114). Increasing concentration and increasing chain length both decrease T_t. Longer ELPs have a weaker dependence on concentration. Increasing ionic strength has the effect of enhancing hydrophobicity and decreasing T_t (101). Changing pH likewise changes the ionization of certain amino acid side chains, causing a change in hydrophobicity and T_t (110).

Fusing ELPs to other proteins allows the ELP to make the protein responsive to stimulus (115, 116). However, the surface hydrophobicity of the fused protein likewise affects the inverse phase transition of the ELP (117). Since surface hydrophobicity can tune the inverse phase transition, fusion to allosteric calmodulin, whose surface hydrophobicity changes upon calcium and ligand binding, has been used to modulate inverse phase transition of a calmodulin-ELP fusion (118). This concept should be able to extend to ELP fusions with other responsive protein domains. Should protein domains and protein-ligand pairs exist, be discovered, or designed that change surface hydrophobicity upon binding a target biomolecule of interest, then fusing an ELP to this protein domain may make possible specific target-driven hydrogel formation.

5. CONCLUSION

Increasingly, environmentally responsive or smart hydrogels are finding use in biomedical applications such as active drug delivery, biosensing, and tissue engineering. The technologies that create a change in bulk hydrogel properties in response to the environmental cue are often protein domains or peptide motifs. Here we present evidence in support of the argument for the design of responsive hydrogel materials by a protein engineering methodology. The structure and function of proteins, protein domains, and peptide motifs are encoded at the genetic level. The codon sequence within a gene encodes a primary amino acid sequence. The secondary and tertiary protein structures are dictated by the primary sequence. Function is a consequence of structure. Mutation of the DNA sequence leads to changes in the function(s) of the expressed protein, protein domain, or peptide motif. Assembled, or self-assembled, supramolecular protein structures and polymer-protein conjugates that derive responsive behavior from the protein units in the hydrogel structure are susceptible to the same control—from the genetic level to responsive function. This methodology has been put forth as a means of macromolecule design (21); responsive materials design is an extension of the broader concept of engineering the biological production of useful macromolecules.

The examples presented here, leucine zipper coiled-coil domains, the calcium-binding protein calmodulin, and elastin-like peptides, are excellent illustrations of the protein engineering methodology of responsive materials design. These are not the only examples, as amphiphilic peptides (119), β-sheets peptides (120), and stimulus-responsive peptides (121, 122) have also been used to create protein-based hydrogels.

The leucine zipper domain is, for materials design purposes, a reversible self-assembly domain. Hydrophobic forces drive the assembly of coiled-coil bundles as the hydrophobic planes along the length of the α-helices are buried. The mutations required to engineer responsive behavior have been mapped by the extensive literature that collectively describes the workings of the leucine zipper coiled coil. Leucine zipper coiled-coil assembly, in terms of aggregation number (37, 62), strand orientation (63), strand specificity (24, 27), and temperature stability (66), can be tuned.

The mechanical action of the CaM protein is unique to the protein domains and motifs that have thus far been used in responsive materials. Ligand binding in the presence of Ca^{2+} causes a collapse of the extended state and reduces the end-to-end distance by approximately 300%. In capturing the effect of the conformational change, hybrid synthetic polymer-protein hydrogels have been demonstrated with exquisite volume control (48, 49).

The elastin-like pentamer consensus sequence, VPGXG, has proven to be a highly useful motif. Engineering of biotechnologies using ELP repeats has matured beyond that of using either leucine zippers or calmodulin. The inverse phase transition of ELP-based hydrogels and the temperature-induced conformational change have been used to engineer responsive hydrogels for application in drug delivery (94, 95, 97, 98) and in tissue engineering (91–93). ELP technologies are also at the forefront of multifunctionailty (118).

The responsive hydrogel works presented here are, in our view, some of those that best exemplify the protein engineering methodology of responsive materials design. As protein-based materials design is multidisciplinary in nature, we review many protein engineering and biological chemistry works in describing the relevant protein structures and functions. For protein-based functional materials to move forward in a significant and meaningful way, a deep understanding of the structure and function of the relevant domains is essential. In our view, there exist many underexplored designs of materials based on the proteins, protein domains, and peptide motifs discussed above. New designs and new materials incorporating domains yet unexplored will provide new solutions to complex biomedical engineering problems.

SUMMARY POINTS

1. The protein engineering methodology of responsive materials design uses the central dogma of molecular biology to produce proteins, protein domains, and peptide motifs that are used to create responsive protein or protein-polymer conjugate hydrogels.
2. Leucine zipper domains can be used as reversible physical crosslinks in self-assembled supramolecular protein hydrogels and in hybrid polymer-protein hydrogels. The mutations required for tuning the oligomerization number, strand orientation, temperature stability, and oligomerization specificity are known and have been mapped during the course of investigating the leucine zipper coiled coils.
3. The conformational change that the calcium sensor-protein calmodulin undergoes upon ligand binding can be harnessed to generate a finely tuned and reversible change in hydrogel volume. Hybrid polymer-calmodulin hydrogels sensitive to calmodulin-ligand binding can be used as simple and effective biosensors.
4. Elastin-like peptides undergo an inverse phase transition that allows triggered supramolecular assembly of biocompatible elastomeric structures for tissue engineering and drug delivery applications. Increased temperature, ionic strength, and hydrophobicity can trigger soluble unstructured ELPs to undergo a conformational change to form dehydrated beta-turns and reversible aggregation. The genetic manipulation of ELPs allows one to tune the inverse phase transition, the crosslinking chemistry, and to create chimeric fusions with enhanced materials properties.
5. The structure-function relationships of the leucine zipper domain, calmodulin protein, and elastin-like peptides have been well studied. The mutations leading to changes in function have been mapped throughout the course of investigating the mechanisms of oligomerization, ligand binding, and phase transitions.

6. Compatible physical crosslinking domains such as leucine zippers can be used to create supramolecular structures from a variety of differently functional building blocks, resulting in multifunctional hydrogels.
7. Many domains with interesting and useful functions have yet to be incorporated into responsive hydrogel materials. For example, the calcium-binding β-roll domain might prove useful as a conformational change unit that is responsive to ion binding; and heme-binding helix bundles might prove useful in engineering responsive hydrogels with oxygen binding properties.

FUTURE ISSUES

1. The multidisciplinary field of protein-based responsive hydrogels is still in its infancy. There exist many possible protein engineering-based solutions to complex biomedical engineering problems such as active drug delivery, tissue engineering, and biosensing.
2. Natural systems such as biochemical pathways and organelles are highly multifunctional. Mimicking such systems may require merging many different protein domains and functional proteins into multifunctional hydrogel structures.
3. There are many potential applications for hydrogel materials that can elicit a bulk change in response to a surface phenomenon or surface binding event. We foresee highly interesting and useful research into systems that can translate a specific biological surface event into a change in bulk mechanical property.
4. Many medical problems require in vivo solutions. We anticipate that the next evolution of responsive hydrogels will begin to address in vivo compatibility.

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33. Exemplifies the protein engineering methodology of materials design.

48. This work is an excellent example of the fine control afforded by capturing the mechanical action of the calmodulin protein.

49. The engineering of a photocurable hydrogel with conjugated calmodulin could potentially be useful for many different applications.

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Errata

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