Multifunctional Materials through Modular Protein Engineering

*Rebecca L. DiMarco and Sarah C. Heilshorn**

The diversity of potential applications for protein-engineered materials has undergone profound recent expansion through a rapid increase in the library of domains that have been utilized in these materials. Historically, protein-engineered biomaterials have been generated from a handful of peptides that were selected and exploited for their naturally evolved functionalities. In recent years, the scope of the field has drastically expanded to include peptide domains that were designed through computational modeling, identified through high-throughput screening, or repurposed from wild type domains to perform functions distinct from their primary native applications. The strategy of exploiting a diverse library of peptide domains to design modular block copolymers enables the synthesis of multifunctional protein-engineered materials with a range of customizable properties and activities. As the diversity of peptide domains utilized in modular protein engineering continues to expand, a tremendous and ever-growing combinatorial expanse of material functionalities will result.

1. Current State of Protein-Engineered Materials

1.1. Benefits of Protein-Engineered Materials: Biofunctionality and Customizability

Protein-engineered biomaterials consist of peptide building blocks that have been strategically selected on the basis of their individual structural properties or functions.[1,2] These building blocks are combined in ordered, repetitive sequences to form polypeptide materials. Through the modular combination of functionally distinct domains, a virtually limitless frontier of multifunctional materials can be generated with the unique ability to precisely fit the needs of a particular application. Protein-engineered materials offer both predictable biofunctionality and precise tunability, properties that are lacking in many synthetic polymeric materials.[3] Because engineered proteins can be reliably customized, they are ideal candidates for a wide range of biotechnological applications.

Engineered materials combine several of the advantages of natural and synthetic materials.[1,4,5] Natural materials are often chosen for biotechnological applications due to their known bioactivity and biocompatibility; however, these materials must often be used "as harvested," greatly hindering the ability to tune material properties for a specific application. Natural materials are also susceptible to denaturation or degradation as a result of harvesting and post-processing. Furthermore, inherent inconsistencies in batch composition and discrepancies across sources result in the inability to precisely dictate the mechanical and biofunctional properties of most natural materials. Conversely, synthetic materials offer improved control over material composition and resultant properties. Flexibilities afforded by design and synthesis techniques provide for application-based customization of synthetics materials. However, imperfections in synthetic polymerization techniques result in a distribution of product chemistries, and significant property variability can ensue.[6] Additionally, synthetic polymeric materials are generally bio-inert and must be further modified with peptides or proteins to impart biological functionality into the materials.[5] Protein-engineered materials use modular peptide templates to capture the best of both natural and synthetic materials: biofunctionality and customizability. Furthermore, by using recombinant protein synthesis technology, the precision of the cellular machinery can be exploited to reproducibly create an exactly specified material, thus eliminating compositional variation and achieving molecular level reproducibility.[6,7]

R. L. DiMarco
Department of Bioengineering
Stanford University
Dr. S. C. Heilshorn
Department of Materials Science & Engineering
Stanford University
476 Lomita Mall, McCullough Building,
Room 246, Stanford, CA 94305, USA
E-mail: heilshorn@stanford.edu

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1.2. Use of Recombinant Technologies to Generate Protein-Engineered Materials

In recombinant synthesis (**Figure 1**), a host organism (commonly a bacterium such as *Escherichia coli*) is induced to over-express a protein of interest, or more specifically its corresponding amino acid sequence.[1,2] Engineered amino acid sequences can be designed by mimicking naturally occurring proteins, by high-throughput screening of random sequences,

or by computational modeling.[7] Combining multiple amino acid building blocks into a single sequence yields a multidomain, multifunctional polypeptide chain. Once the amino acid sequence of the full polypeptide chain has been designed, a synthetic gene encoding the polypeptide is constructed and inserted into a genetic vector. This vector is then transferred into a host organism that will express the encoded polypeptide target material in culture. The culture products are then collected and purified to isolate the intended protein-engineered material.

1.3. Expansion of the Peptide Library: A Virtually Limitless Frontier

Engineered polypeptides were historically identified for use as biomaterials due to their unique repetitive structural domains.[1,8] For example, collagen,[9,10] elastin,[8,11–13] and silk[9,13–15] have been widely studied as repetitive polypeptide sequences for their structural integrity and resultant mechanical properties. The family of recombinantly synthesized structural proteins continues to grow, as evidenced by the recent development of resilin-like materials.[16–24] In addition to the increasing prevalence of the use of recombinant elastomeric peptides, biomaterial constructs have been generated with an increasing diversity of other peptide functions such as proteolytic degradation,[21,25,26] cell-adhesion,[13,27–29] enzymatic activity,[30] and antimicrobial activity.[31] Traditionally, peptide selection has been based on the notion of harnessing the naturally evolved functionalities of the respective domains within the intended protein-engineered material (**Table 1**).

In contrast to the traditional selection of peptides for their naturally evolved functionalities, recent design strategies employ the notion of utilizing peptide domains for repurposed functionalities (**Table 2**). A repurposed domain is chosen to perform a function that is distinct from its traditional, physiologically evolved role. For example, the environmentally responsive structural transitions of several elastomeric proteins, such as elastin[37,53–57] and resilin,[22–24] have been tailored for the development of stimuli-responsive, "smart," materials, and resilin has been investigated as a reporter molecule due to the inherent fluorescence of its tyrosine content.[23] Additionally, protein assembly has recently been exploited in various applications, such as the use of molecular recognition interactions between leucine-zipper domains to form three-dimensional networks.[30,45,46] As a further example, the self-assembling properties of viral coat proteins have been utilized to facilitate the templated synthesis of inorganic materials, including carbon nanotubes[52] and semiconducting nanowires.[49] Through repurposing of peptide functionalities, the library of functional domains for protein-engineered materials continues to expand without bounds.

As the field progresses, a new era of modular material design is emerging through the combination of multiple, functionally distinct peptide domains. This modular design strategy enables the formation of complex, multifunctional polypeptides (Figure 1). The incredible combinatorial expanse of individual domain building blocks suggests that the resultant diversity of potential multifunctional materials is far reaching. Many biotechnology applications will benefit from this reliably precise and functionally customizable source of biologically relevant materials. Demonstrated applications that will be discussed in this review include targeted drug delivery,[11,37,56,60] templated synthesis of inorganic materials,[41,42] highly resilient tissue engineering scaffolds,[21] dynamically switchable substrates,[35] enzymatically active hydrogels,[30] and injectable cell delivery vehicles.[34]

In this review, we limit our discussion to engineered modular polypeptide materials that are recombinantly expressed using the twenty naturally occurring canonical amino acids, for which standard single letter abbreviations are used throughout the text. The incorporation of non-canonical amino acids provides for an even further expansion of achievable functionalities of engineered materials, and several exceptional reviews exist on this topic.[61–64] In addition, low molecular weight synthetic peptide-based materials will not be discussed here, and the interested reader is directed to several excellent reviews of the field.[65–69]

Dr. Sarah C. Heilshorn is an Assistant Professor of Materials Science and Engineering at Stanford University. She obtained a B.S. in Chemical Engineering from the Georgia Institute of Technology in 1998 and a M.S. and Ph.D. in Chemical Engineering from the California Institute of Technology in 2000 and 2004, respectively. She has worked in the Department of Polymer Science at the Kyoto Institute of Technology as a visiting scientist and in the Department of Molecular and Cell Biology at the University of California, Berkeley as a postdoctoral scholar. The Heilshorn Biomaterials Group at Stanford University studies the design of materials and scaffolds that mimic the micro- and nano-scale order found in nature for applications in energy, tissue engineering, and regenerative medicine.

Rebecca L. DiMarco obtained a B.S. in Materials Science and Engineering with a dual major in Biomedical Engineering from Carnegie Mellon University in 2008. She then completed a year of research at the Nanyang Technological Institute in Singapore as a Fulbright Scholar Award recipient. In 2010, Rebecca earned a M.S. in Bioengineering from Stanford University, where she is currently pursuing a Ph.D.

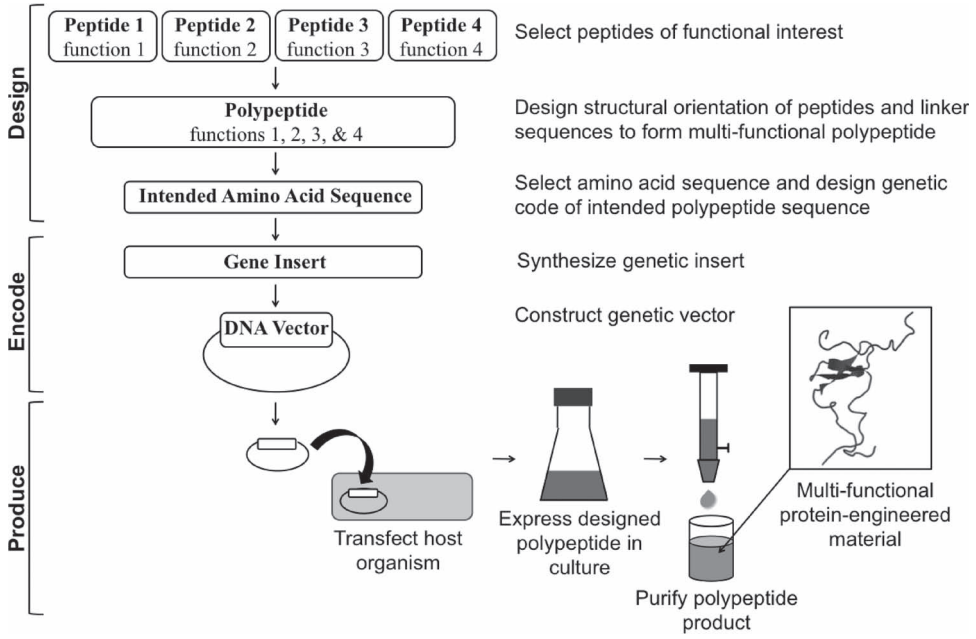

Figure 1. Schematic of key steps involved in the design, encoding, and production of protein-engineered materials. The design process involves the selection of applicable peptides, design of intended structural orientation of peptide domains, and selection of the intended amino acid sequence and encoding genetic sequence. Design steps are followed by the synthesis of a genetic insert and construction of a genetic vector. Genetic constructs are then used in the transfection of a host organism to induce expression of the intended multifunctional polypeptide material. (Protein structure shown is of a WW domain obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank, protein identification 1I5H.)

1.4. Selecting Peptide Domains for Native Versus Repurposed Functionality

Two predominant design strategies have evolved in the field of protein-engineered materials: the selection of peptide domains for their native functionalities and the repurposing of domains to achieve novel functionalities. Exceptional highlights from the field have been selected to illustrate the use of both strategies to design a diverse range of multifunctional, protein-engineered materials through the modular combination of functionally distinct domains.

The first section of this review highlights case studies that demonstrate the strategy of exploiting naturally evolved functionalities, with an emphasis on structural peptide domains. Structural domains were first utilized to form synthetic peptide hydrogels due to their unique mechanical properties; however, with the onset of modular polypeptide design, these structural elements have been combined with a diverse library of bioactive domains to yield multifunctional hydrogels. The second section of this review focuses on the incorporation of repurposed domain functionalities that have been tailored to perform functions distinct from their primary native applications. This portion highlights the tuning of molecular recognition interactions in modular, multifunctional materials to yield biologically functional constructs. Throughout this review, each section begins with a brief discussion of earlier work that has paved the way for the selected case studies in order to provide a historical context for these advances. The review closes with a discussion of the challenges and opportunities for the field of protein-engineered materials.

2. Teaching "old" Peptides New Tricks Through Modular Design

Historically, the primary functionality of protein-engineered materials was derived from the naturally evolved activity of the constituent repeat peptide unit. In particular, the first generation of protein-engineered materials generally consisted of repeat structural units, such as elastin-like or silk-like domains, to generate a construct of known structural integrity and resultant mechanical properties.[1,2] As the field progresses, the functionalities of these structural domains are being extended through recombinant coupling with other peptide domains. This concept of modular polypeptide design allows for the generation of multifunctional materials through the combination of multiple peptide building blocks with diverse functionalities within a congruent polypeptide platform. In particular, the strategy of combining structural and bioactive domains into a single polypeptide chain has recently gained tremendous momentum in the field of protein-engineered materials. This is true for first generation recombinant structural polypeptides, such as elastin-like and silk-like materials, as well as for more recently engineered structural proteins, such as resilin-like materials.

2.1. Multifunctional Elastin-Like Polypeptides

Elastin is a prevalent extracellular matrix protein found in connective tissues. Native elastin is formed through enzymatic crosslinking of tropoelastin precursors at chain

Table 1. Functional peptide domains that have been utilized in protein-engineered materials for their naturally evolved functionalities. Select references are provided.

Utilized for Native Functionality		
Intended Function	Domain	References
Mechanical Properties		
	Elastin-like	[8,11–13]
	Silk-like	[9,13,14]
	Collagen-like	[9,10]
	Resilin-like	[16,19,21]
Cellular Adhesion		
	RGD	[21,27,30,32–35]
	REDV	[28]
	YIGSR	[29]
	IKVAV	[36]
Membrane Transport		
	Tat	[37]
	Bac-7	[38]
	Penetratin	[39]
	MTS	[39]
Enzymatic Activity		
	AdhD	[30]
	OPH	[40]
Mineralization		
	R5	[41]
	Dentin	[42]
Growth Factor Activity		
	VEGF	[43]
	KGF	[44]
Antimicrobial Activity		
	HNP-2,4	[31]
	Hepcidin	[31]
Proteolytic Degradation		
	uPA-sensitive domain	[25]
	tPA-sensitive domain	[25]
	MMP-sensitive domain	[21]
	Fibrin	[26]
Growth Factor Adhesion		
	Heparin-binding domain	[21]

Table 2. Functional peptide domains that have been utilized in protein-engineered materials for repurposed functionalities. Select references are provided.

Repurposed for Novel Functionality		
Intended Function	Domain	References
Network Formation Through Self-Assembly		
	Leucine zippers	[30,45]
Network Formation Through Hetero-Assembly		
	Leucine zippers	[46]
	WW/P-rich combination	[34]
	Calmodulin/calmodulin-binding partner combination	[45,47]
Template for Synthesis of Inorganic Materials		
	Viral proteins	[48–52]
Flexibility		
	Random coils	[30,34,45]
Drug Release		
	GFLG	[37]
Environmental Responsiveness		
	Elastin-like	[37,53–57]
	Resilin-like	[22–24]
	Leucine zippers	[58]
	Calmodulin/Ca^{2+} combination	[45,59]
Fluorescence Signal		
	Resilin-like	[23]

locations containing lysine residues. As its name indicates, elastin displays elastic mechanical properties and is capable of deforming to store substantial amounts of mechanical energy. Due to the material's inherent mechanical elasticity, several elastin-like polypeptides (ELP's) have been designed for use in various biotechnological applications, with prevalent use as biomimetic tissue engineering scaffolds and drug-delivery systems.

ELP's are genetically-engineered modular repeat sequences, typically of the form $(VPGXG)_n$ as modeled after the amino acid sequence of bovine elastin.[11,12,70] Recent investigations of various repeat analogues, including $(VPAVG)_n$ as well as the human form $(VAPGVG)_n$ recently have been reported.[54,71,72] The guest residue, X, and the number of repeat units, *n*, are important free variables for strategic ELP design. By tuning these design parameters, families of "smart," or stimuli-responsive, polymers can be generated. This is because ELP's undergo an inverse phase transition, manifested as environmentally induced changes in structure and solubility.[70] Though ELP's can be designed to respond to several stimuli (such as light,[73] kosmotropic salts,[74,75] and pH[53,57,76]), the most commonly exploited stimulus used to induce this transition to date is temperature.

Thermally responsive ELP's are soluble in an aqueous environment below their critical transition temperature, T_t; however, these materials undergo a phase transition and aggregate in solution when raised above this temperature.[12,70] This phase transition is notably abrupt, typically occurring over a temperature range of 2–3 °C.[37] Another key attribute of this inverse transition is that it is reversible. As long as the protein denaturing temperature is avoided, ELP's can be thermally cycled in and out of solution without biochemical or structural damage. Furthermore, by strategic design of ELP's, T_t can be tuned to fall within the relevant temperature regime for a given application. For example, the temperature-induced phase transition of ELP's has been investigated as a means of harvesting intact cellular sheets that have been grown on the recombinant material

by eliciting this structural transition at temperatures that do not disrupt cell viability.[55] Though this cell harvesting technique was first demonstrated on poly(N-isopropylacrylamide),[77,78] the use of ELP's enables an enhanced control of material functionality over alternative synthetic systems.

In characterizing the effects of guest residue polarity on T_t, it was found that addition of hydrophobic residues lowered T_t, whereas addition of polar residues increased this value due to the tendency for hydrophilic residues to resist aggregation.[79] Additional amino acid sequences can be added to the ends of the repeat ELP segment, resulting in a block copeptide structure, and similar T_t dependence on hydrophilicity was observed for these ELP fusion proteins.[80] Through strategic amino acid selection, the hysteretic relaxation of ELP's can be used to stabilize self-aggregated nanoparticles at temperatures slightly below T_t. ELP nanoparticles thus can be formed within liquid media to encapsulate agents such as growth factors for subsequent delivery.[54,56,72] For example, the hysteretic response of ELP's recently was utilized to enable encapsulation and delivery of active bone morphogenetic proteins (BMP's) for tissue engineering applications. Entrapped BMP2 was found to maintain bioactivity, and a sustained release profile was achieved over two weeks.[56]

Recently, the design of ELP fusion proteins has been expanded to include multi-block-copolymer ELP systems that undergo self-assembly to form a variety of functional nanostructures. For example, diblock ELP copolymers were genetically designed to assemble into micelles over the temperature range between the T_t values of the individual ELP building blocks.[53] This work was continued with the synthesis of a more robust triblock copolymer capable of forming both chemical and physical crosslinks within its network.[81] A multi-block ELP copolymer was also generated by combining thermally-responsive and pH-responsive subunits to demonstrate the ease of using the reversible sol-gel transition of ELP's to generate reproducible surface micropatterns.[57]

For an additional level of functionality, the simplicity of adding residues at the ELP repeat flank positions provides for an ideal means of incorporating bioactive amino acid sequences. An extended domain that includes the cell-adhesive RGD sequence derived from fibronectin was added to ELP-containing culture substrates to view the effects of ligand density on degree of cell adhesion, spreading, and migration.[33] An alternative binding domain, CS5, also was added into ELP's to illustrate the effects of guest residue selection on the resultant accessibility and affinity of the cell-binding domain.[27] Functional domains that allowed for the independent tuning of cellular adhesion, ELP mechanics, and degradation properties also have been reported for use as highly tailored tissue engineering scaffolds.[25] In addition, fusion ELP's have been generated to include growth factor sequences, such as keratinocyte growth factor for wound healing applications.[44] These are just a few examples of the range of functionalities that have been achieved in ELP materials through the use of a modular domain design strategy.

In an elegant example of functional biomaterials design, self-assembling ELP's are combined with regional hyperthermia to selectively target cancer therapeutics to solid tumors.[82] In these systems, the T_t is tailored to fall above physiologic temperature, yet be achievable by regional hyperthermia. In this manner, ELP's are soluble drug carriers at body temperature and are aggregated drug depots at sites of local heating, thereby selectively exposing the drug to the target tissue. This type of local drug targeting can significantly decrease the occurrence of systemic toxicity by chemotherapeutics. Additionally, inert microcarriers have been found to increase the plasma half-life and overall solubility of several drugs, thereby increasing potential activity time and decreasing the effective dose limit.[11]

Recently, a multi-modular protein was designed to carry and selectively deliver doxorubicin to regions of hyperthermia[37] (**Figure 2**A). Doxorubicin is a topoisomerase II poison and a commonly employed chemotherapeutic agent. The doxorubicin-carrying ELP, termed ELP1, combines multiple distinct functionalities within a single repetitive amino acid sequence to meet the complex demands of this application. ELP1 guest residues and repeat number were chosen to elicit appropriate thermal properties, with an approximate T_t of 40 °C (Figure 2B). Typical hyperthermia treatments can raise tissue from physiologic temperature, 37 °C, to 42 °C, making 40 °C an optimal T_t for hyperthermia triggered release of therapeutics. Amino acids V, G, and A were incorporated into the guest residue positions at a ratio of 5:3:2. The elastin-like pentapeptide was repeated 150 times, yielding a molecular weight of 59.1 kDa. Upon hyperthermic treatment, the thermally-induced inverse phase transition of ELP1 triggered doxorubicin release and caused a profound increase in cellular toxicity[37] (Figure 2C–E). A control system, termed ELP2, was designed to have a T_t of approximately 65 °C by setting the guest residue composition of V, G, and A at a ratio of 1:7:8 and repeating the sequence 160 times. This demonstrates how the phase transition temperature can be dramatically altered on the basis of guest residue configuration and repeat number selection. This control system remained soluble upon hyperthermia treatment, preventing the release of chemotherapeutic agent.

To further increase the functionality of this material, additional domains were incorporated within ELP1 to promote cellular uptake, enzyme-triggered drug release, and the tethering of a doxorubicin derivative. The HIV-1 Tat peptide was incorporated to allow for cell membrane penetration. Tat, a protein known to promote the membrane transport of large molecules, has been utilized as a simplified peptide domain to enhance endocytotic uptake of ELP's.[39,83] A cysteine residue (C) at the carboxyl-terminus was added to allow for chemical attachment of a thiol-reactive doxorubicin derivative (WP936). Finally, a tetrapeptide linker, GFLG, that is susceptible to cleavage by cathepsin lysosomal proteases was added to induce drug release once the polypeptide has entered the intracellular space.[37] This protein-engineered modular design utilizes the distinct functionalities of multiple components to produce a bioactive system with a complex, multi-step pathway of action. ELP1 is just one example of the type of wide-reaching, tunable functionality that can be generated by protein engineering design of materials. This work strongly illustrates the tremendous promise of ELP's for targeted drug delivery applications, and more broadly, the extent of application-specific property control that can be elicited by strategic modular design of polypeptides.

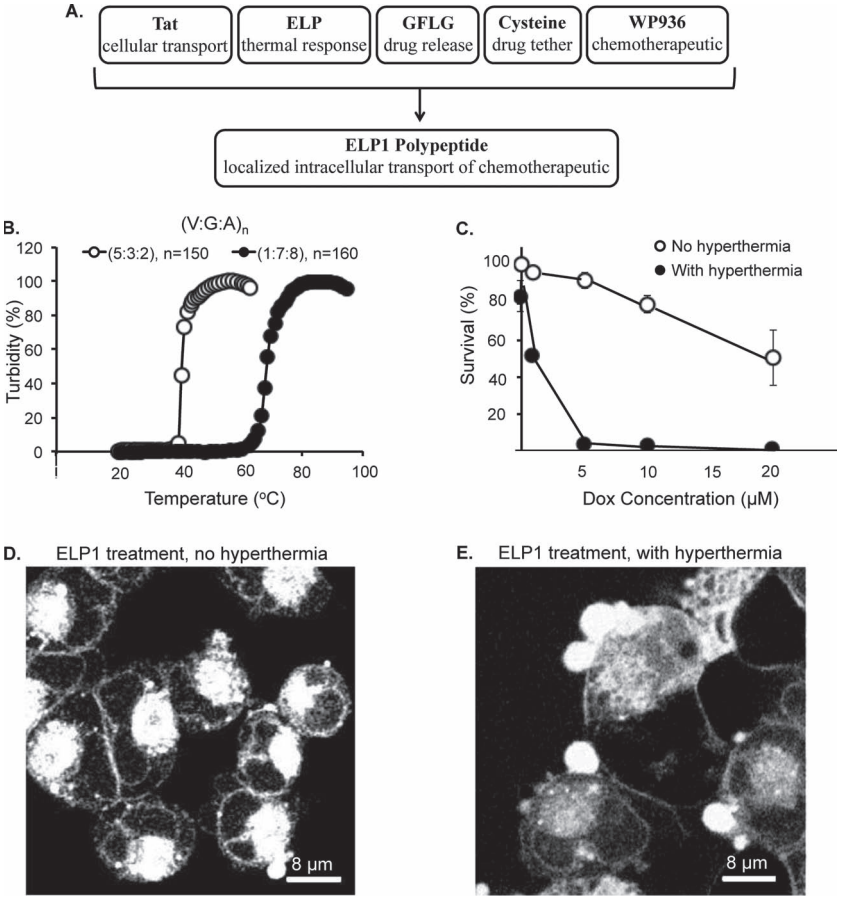

Figure 2. A) Modular design of ELP1 polypeptide utilized for the selective targeting and intracellular delivery of doxorubicin when partnered with local hyperthermia. B) Through design of the amino acid sequence, the temperature at which the ELP1 phase transition occurs can be precisely tuned. C) Co-administration of ELP1 and local hyperthermia, which triggers the phase transition, results in enhanced cytoxicity of treated cells. D) In the absence of hyperthermia, human sarcoma cells treated with ELP1 retain their spread morphology. E) In the presence of hyperthermia and ELP1, apoptotic membrane blebbing is observed, indicative of cell death. Figures 2B–E reprinted with permission from ref. [37]. Copyright 2007 Elsevier.

2.2. Multifunctional Silk-Like Polypeptides

Naturally occurring silk materials, harvested from both silkworms and spiders, have been investigated for use in various biotechnological applications due to their unique mechanical properties. Silks have high tensile strength, toughness, and elasticity while also being lightweight.[9,15] Silk proteins consist of repetitive amino acid sequences that organize into crystalline β-sheet and semi-amorphous regions. Interactions between neighboring β-sheets, predominantly consisting of repeat $(A)_n$, $(GA)_n$, or $(GAS)_n$ units, result in the formation of ordered, crystalline regions that are responsible for the high tensile strength of silk materials. Coexistent non-crystalline regions, typically of the form $(GPGXX)_n$ or $(GGX)_n$, in which X is often glutamine, allow for the high ductility of these materials.[15,84]

Though silkworms can be domesticated easily and have a relatively high protein yield, spiders, which synthesize silk of higher tensile strength, are difficult to domesticate and produce much more sparse amounts of silk protein. One silkworm cocoon can yield up to 900 m of silk fiber, whereas an entire spider web yields only 12 m of fiber.[15,85] Furthermore, harvested silk is subject to the constraints of all natural materials, namely batch-to-batch variability and lack of tunability. Due to these limitations, silk materials formed by recombinant techniques have become increasingly popular in the biomedical industry.

The specific, repetitive amino acid sequence of silk materials highly dictates the secondary structure, self-assembly, and the respective function of each silk variant. Additionally, non-repetitive termini are thought to influence the type of structures formed through self-assembly.[15,86] Silk assembly is also highly influenced by environmental conditions, allowing for micro- and macro-scale tuning of the resultant morphology. Silk proteins have been processed to form biomaterial fibers,[87] films,[88,89] porous sponges,[90,91] and microcapsules,[92] making them suitable for a range of applications. In addition to having tunable mechanical and structural properties, protein-engineered silks can also be functionalized at the genetic level. Similar to elastin, the first bioactive domain to be incorporated into a silk-based polypeptide was the cell-binding sequence, RGD.[32]

An early example of chimeric recombinant structural proteins is the generation of silk-elastin-like proteins (SELP's).

The successful synthesis of recombinant SELP's was reported in 1990,[1] and SELP synthesis, development, and characterization have been heavily continued. Several groups have built upon this seminal early work to generate their own modular SELP designs,[93–98] and an excellent review has been published that describes SELP's for drug delivery applications.[99] Silk and elastin are both structurally and mechanically interesting proteins with vastly different physicochemical properties. Addition of silk to elastin can increase the rate of gelation, whereas addition of elastin to silk has been shown to decrease crystallinity and increase solubility.[1,98,100] Varying the relative amounts of these proteins can therefore be used to generate a range of material properties. One particular SELP is being commercially developed for use in minimally invasive repair of intervertebral disc degeneration.[94] The effects of SELP synthesis parameters on spatial and temporal control of gene delivery have also been investigated.[95]

More recently, domains to induce selective mineralization of hydroxyapatite[42] and silica[41] have been incorporated into recombinant silk materials. Traditional silica synthesis methods require high temperatures and harsh pH. By combining a mineralizable domain with a silk peptide (**Figure 3**A), silica synthesis was achievable under neutral pH and physiologically relevant temperature conditions by the novel polypeptide, RGD15mer+R5. The consensus repeat of the major ampullate spidroin 1 protein (MaSp1) of *Nephila clavipes* spider dragline silk was fused at the C-terminus with the R5 peptide of the diatom *Cylindrotheca fusiformis*. The R5 peptide is known to induce and contribute to the regulation of silica precipitation in diatom skeletal features. A cell-binding domain, RGD, was also incorporated into the design to promote mammalian cell adhesion. Mineralization reactions in the presence of RGD15mer+R5 resulted in the formation of silica microparticles (Figure 3B). Furthermore, heightened control over product size and morphology via processing conditions was demonstrated.[41] Mineralization reactions conducted in the presence of R5 peptide alone resulted in the synthesis of silica particles of 0.5–10 μm diameter. The same reaction conditions carried out in the presence of the fusion chimeric proteins resulted in the formation of particles of 0.5–2 μm diameter. Further alteration to processing conditions resulted in the formation of films and fibers, rather than particles. A continuation of this work demonstrated the potential use of RGD15mer+R5 and subsequently generated silica particles for promoting osteogenic differentiation of human mesenchymal stem cells *in vitro* (Figure 3C–E).[101] This work demonstrates yet another application of protein engineering: the controlled synthesis of inorganic materials for which the industrial synthesis conditions are much harsher than the conditions under which the material is produced biologically. In this example, the self-assembling property of spider silk proteins was coupled with the silica-forming capability of diatom proteins to result in a novel multifunctional material for bone regeneration applications.

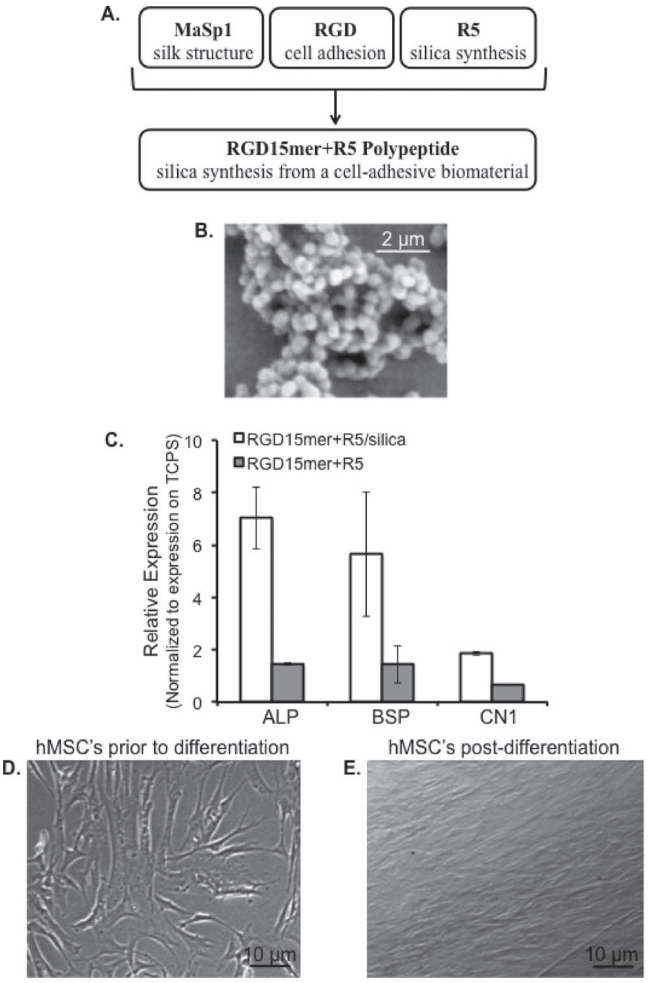

Figure 3. A) Modular design of RGD15mer+R5 polypeptide utilized to achieve silica synthesis under physiological conditions. B) Scanning electron micrograph of spherical silica microparticles catalyzed by RGD15mer+R5. C) The relative expression levels of osteogenic lineage markers alkaline phosphatase (ALP), bone sialoprotein (BSP), and type I collagen (CN1) by human mesenchymal stem cells (hMSC's) on RGD15mer+R5 demonstrate improved osteogenic differentiation in the presence of silica. D, E) hMSC's cultured on silk films mineralized with silica presented morphological changes from D) fibroblast-like into E) closely packed, flattened cells indicative of differentiation into osteogenic lineages. Figure 3B reprinted with permission from ref. [41]. Copyright 2006 Proceedings of the National Academy of Sciences of the United States of America. Figures 3C–E reprinted with permission from ref. [101]. Copyright 2010 American Chemical Society.

2.3 Multifunctional resilin-like polypeptides

In addition to elastin and silk, the family of naturally occurring elastomeric proteins includes polypeptides such as gluten, gliadin, abductin, and resilin.[16] Resilin is a natural extracellular matrix protein found in the cuticle of most insects.[102] Resilin is prevalently found in specialized joints that are involved in sustained high frequency movement, such as those utilized in flight,[103] jumping,[104,105] and sound generation.[106,107] As its name implies, resilin is a very resilient elastomeric material that is capable of recovering from high strain. In fact, this protein is speculated to possibly be the most resilient material known.[23] Resilin also appears to be quite stable in the body; for example, all resilin production occurs during the pupal stage

of *Drosophila melanogaster* development and remains functional for the duration of the insect's life.[16]

Despite the fact that the advantageous mechanical properties of resilin were discovered decades ago,[108] the first successful expression and purification of resilin was a relatively recent development in the field of recombinant structural protein synthesis.[16] Like other elastomeric proteins, native resilin contains a highly repetitive structural domain within its sequence. Recombinant resilin can be generated by expressing all three exons of the resilin gene.[20] The first and third exons correspond to the N-terminus and C-terminus structural domains whereas the second exon has been shown to convey chitin-binding properties.[20] Expression of the full sequence protein often suffers from low yield and the formation of inclusion bodies.[18] Because of this limitation, efforts have focused on the recombinant synthesis of shorter resilin-like sequences that only encode repeats of the structural domains of the native protein.[16–19,21–24]

The first recombinantly synthesized simplified resilin-like polypeptide (RLP) consisted of multiple copies of the first exon of the resilin gene (GGRPSDSYGAPGGGN).[16] This polypeptide is commonly referred to as Rec1-resilin and has been shown to adequately capture the mechanical properties of native resilin despite its condensed sequence. Furthermore, Rec1-resilin has been shown to be highly pH and temperature responsive, demonstrating a dual phase transition behavior characterized by both lower and upper critical solution temperatures.[24] The stimuli responsive, or "smart," behavior of recombinant RLP's has led to the investigation of utilizing such material to functionalize surfaces for use in biosensors. Towards this goal, discrete changes in pH have been shown to elicit rapid, reversible compact-to-brush transitions at a RLP-functionalized interface.[22] Resilin is also naturally fluorescent due to its tyrosine content and has therefore been investigated for use in a reporter system utilizing fluorescently-modified gold nanoparticles.[23]

In addition to the first form of recombinant resilin discussed above, derived from the CG15920 gene of *Drosophila melanogaster*,[16] an alternative RLP has been generated from repeats of the resilin structural domain of *Anopheles gambiae* (AQTPSSQYGAP).[19] Despite the signification variation in amino acid sequences, both repeat polypeptides demonstrate resiliencies of over 90%, a value that is significantly greater than commonly used synthetic rubbers.[16] Though high resiliency is maintained in both simplified RLP's, distinct differences do exist in implied secondary structure,[19] though the root of these differences as well as the notion of how these differences impact material properties merit further attention.

Natural resilin is crosslinked by the enzymatically-assisted interaction of tyrosine residues on neighboring strands to form di- and tri-tyrosine.[110] These covalent bonds form linkages between random coil structures, thus resulting in resilin acting as an entropic spring.[102,108] Enzymatic reactions can be used to crosslink recombinant resilin as well; however, the rapid, uncontrollable rate of these reactions and the accompanying production of oxygen are unfavorable for the synthesis of well-defined, three-dimensional gels.[16] Recombinant resilin can also be crosslinked through the use of copper redox reactions, γ-irradiation, and photo-crosslinking. Photo-crosslinking is often the preferred method, as it can be used to rapidly cast reproducible three-dimensional structures.[16] Cross-linked recombinant RLP's maintain a disordered secondary structure and contain roughly 80% water, rendering them a compliant, yet highly resilient, material.[16,19–21] Such mechanical properties are highly advantageous in cellular culture scaffolds, in particular for use in engineering tissues that are exposed to cyclic strains during either development or routine every day use.[21]

Though the development of recombinant RLP's is relatively recent, interest in these materials is growing rapidly due to the potential advantages of partnering their unique mechanical properties with biologically active domains to yield multifunctional, modular polypeptides. In one recent study, a resilin structural domain was strategically combined with a cell-adhesive domain, a heparin-binding domain, and an enzymatically cleavable domain to form RLP12 (**Figure 4**A).[21] A RGD domain was utilized for its cell-adhesive properties to facilitate cellular attachment to the material surface, and a matrix metalloproteinase (MMP) sensitive domain was incorporated to enable matrix remodeling in response to the secretion of these degradative enzymes. A heparin-binding domain was incorporated in order to achieve non-covalent binding of heparin and the resultant sequestration of growth factors at this domain; therefore, utilization of this domain provides a platform for sustained release of growth factors. Several minor chemical alterations were also made to the polypeptide, including the incorporation of lysine residues outside of the resilin domain to enable gel formation through chemical crosslinking (Figure 4B). This multifunctional, modular RLP12 polypeptide facilitates cellular attachment and subsequent proliferation without causing any significant changes in the conformational properties of RLP's.[21] Three-dimensional crosslinked gels displayed linear stress-strain behavior at up to 200% strain and were able to withstand up to 450% strain prior to rupture. Thin films of RLP12 demonstrated a similar region of linear behavior (Figure 4C) and yielded moduli of 30–60 kPa,[21] a value that is roughly an order of magnitude greater than many other recombinant RLP's previously characterized[16,19] and similar in magnitude to crosslinked recombinant elastin gels.[109] RLP12 enabled the survival and proliferation of fibroblast cells *in vitro* (Figure 4D,E), demonstrating the material's potential as a scaffold for future tissue engineering applications.[21]

3. Making the Peptide Work for you: Repurposing of Domain Functionalities

The range of functionalities of protein-engineered materials has rapidly expanded during recent years, and a prominent contributor to this expansion has been the utilization of repurposed peptide domains. A repurposed domain is one that has been chosen to perform a role distinct from its evolved physiological function. Repurposed domains are often chosen on the basis of their unique biochemical, biophysical, or structural properties, which can be exploited to perform an alternative function. For example, self-assembly of leucine-zipper peptides has been exploited to design a range of materials

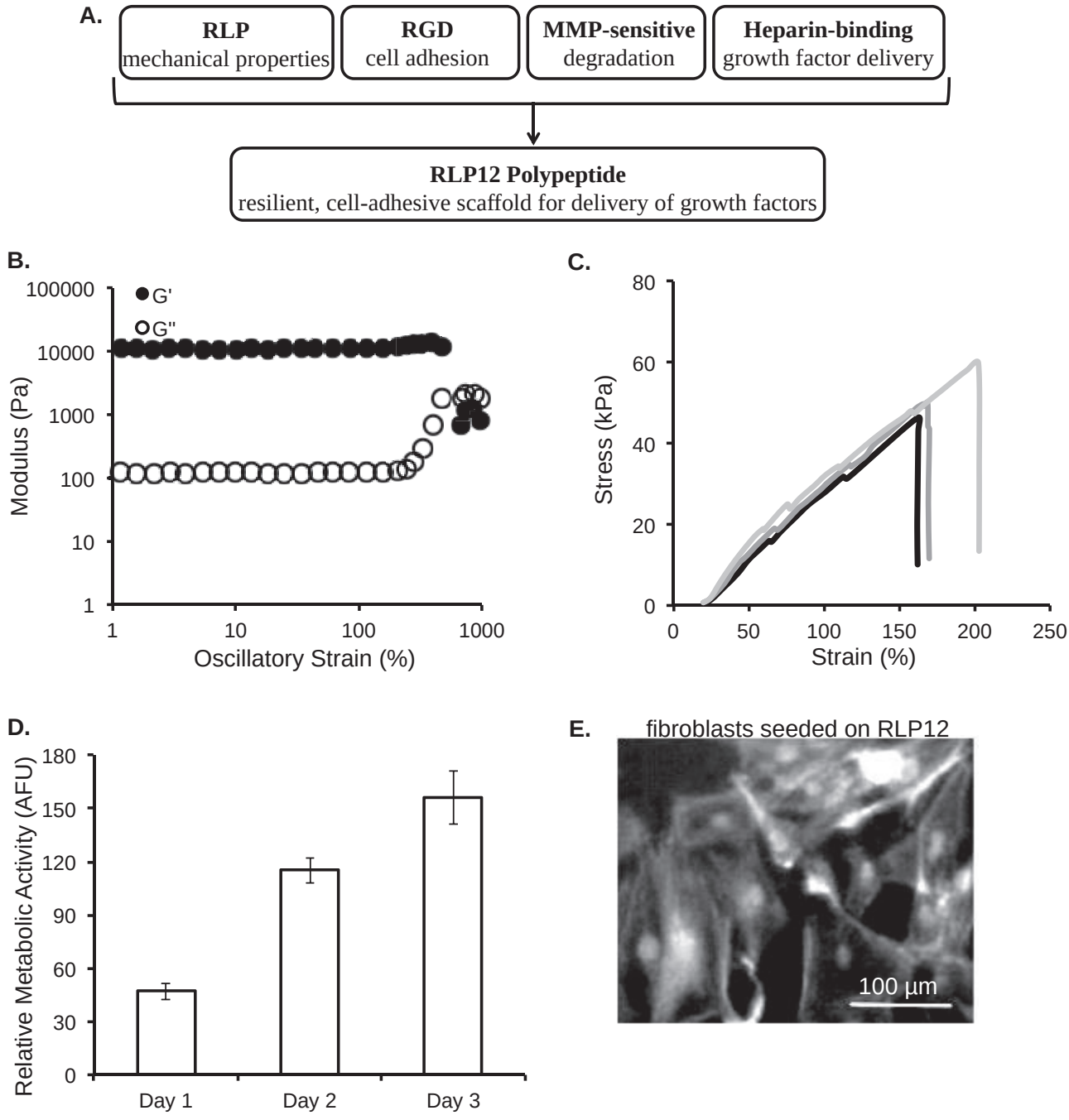

Figure 4. A) Modular design of RLP12 polypeptide, which forms a resilient, cell-adhesive scaffold for the delivery of growth factors. B) Post-crosslinking, the material storage modulus (G′) is greater than the loss modulus (G″), indicating hydrogel formation. C) Tensile testing demonstrates that crosslinked RLP12 thin films reproducibly achieve strains of 150%. D) Proliferation of NIH-3T3 fibroblasts seeded on RLP12, as quantified by an Alamar Blue assay, demonstrates suitability of the material as a culture substrate. E) Dual nuclear and F-actin stains of NIH-3T3 cells demonstrate cell spreading and adhesion on RLP12. Figures 4B–E reproduced by permission of ref. [21]. Copyright 2009, The Royal Society of Chemistry.

with diverse functions such as the dynamic regulation of cell-binding ligand accessibility[35] and the induction of apoptosis in specific cell populations of interest.[111] Leucine-zipper interactions have also been utilized in the generation of virus-like particles[112] and the construction of three-dimensional networks that are environmentally responsive and enzymatically active.[30,40] By creatively exploiting a peptide domain for a repurposed function, the range of complex functionalities of protein-engineered materials is drastically increased.

3.1. Self-Assembly of Multifunctional Polypeptides Through Repurposed Domains

Physical hydrogels have been the focus of a great deal of biomedical research due to their gelation by non-covalent binding. Gelation by the formation of physical interactions rather than chemical covalent crosslinks provides for an environmental rather than chemical-based encapsulation mode for fragile entities including living cells and biological macromolecules. Protein-engineered materials can be designed to undergo physical gelation through the action of hydrogen bonds, electrostatic interactions, or hydrophobic interactions that initiate peptide self-assembly. Using the same recombinant genetic engineering approaches described in the previous sections, modular physical hydrogels can be designed with peptide domains that enable precise control over network interactions along with the potential for incorporation of bioactive sequences to aid in the maintenance and delivery of cells or other encapsulated elements.

Coiled coil segments such as leucine zippers were one of the first repurposed peptide domains to be utilized in protein-engineered materials, and they have continued to be widely explored in the design of pH- and thermally-responsive physical hydrogels.[46,58,113] Recombinant segments are typically of the form $(abcdefg)_n$, for which positions *a* and *d* are occupied by hydrophobic residues, often leucine, and positions *e* and *g* are usually occupied by charged residues. Given the high dissociation constant (K_d) of native leucine-zipper interactions, hydrogels formed through this mechanism undergo erosion in open solutions near physiologic pH.[46] Through protein engineering design, the chain length and flanking amino acid sequences can be altered to gain control over a range of properties, including aggregate structure,[114] stability,[115] aggregation number,[116] and dimerization specificity.[117] The addition of alternative domain regions can also be used to strengthen chain interactions. For example, recombinant techniques were used to add a cysteine residue within the leucine domain, resulting in the formation of disulfide bonds at the leucine-zipper interface.[46] This causes the chains to become covalently linked, resulting in the formation of a more stable structure with tunable pH-responsiveness.

Leucine-zipper domains have been partnered with a range of functional domains to generate complex self-assembling materials. For example, leucine-zippers were combined with calmodulin domains to generate hydrogels that are capable of undergoing selective and reversible self-assembly in the presence of calcium ions.[45] Calmodulin is a relatively small protein that transitions from a collapsed to an extended dumbbell conformational state in the presence of calcium. Moreover, this transition is reversible upon the removal of calcium.[118] Calmoldin is only able to bind to specific binding partners in the extended conformation; therefore, the presence of calcium ions enables transitions between these two conformational states and resultant binding or release of calmodulin-binding ligands. Three different recombinant triblock copolymers were designed by linking together leucine-zipper domains, calmodulin domains, and two distinct calmodulin-binding domain segments: endothelial NO synthase (eNOS) and petunia glutamate decarboxylase (PGD) (**Figure 5**A).[45] In the presence of calcium ions, these materials underwent spontaneous self-assembly and gelation, which was reversible upon chelation of calcium ions. As demonstration of rational hydrogel design at the molecular level, gels formed through use of eNOS calmodulin-binding domains, which bind to calmodulin as a monomer, exhibited a significantly lower viscosity than gels formed through use of PGD domains, which bind to calmodulin as a dimer (Figure 5B). Strategic selection of the combinatorial ratios of these distinct polypeptides facilitated the formation of a reversible network with tunable architecture, sensing behavior, and rheological properties.[45]

Dynamic, stable hydrogels have also been generated by incorporation of genetically tailored calmodulin domains.[47] Tyrosine residues that typically reside on the ends of the dumbbell shape of the protein were replaced with cysteines to allow for crosslinking within a polymer strand containing acrylate groups. These crosslinked gels undergo a gradual, reversible volume change in response to calmodulin-binding ligands.

In addition to structural chimeric proteins, such as silk-elastin and leucine-zipper-elastin,[119] biofunctional fusion proteins can be generated by combining structural domains with bioactive domains. This modular, multi-domain strategy was utilized to achieve the dynamic presentation and accessibility of cell-binding ligands.[35] In this clever model, molecular recognition between complimentary leucine domains was used to either expose a cell-adhesive RGD domain or shield it with a conjugated polyethylene glycol (PEG) strand. Two distinct recombinant leucine-zipper domains (**Figure 6**A) were synthesized such that heterodimerization was strongly preferred over homodimerization. One leucine-zipper domain, strand A, was immobilized onto a gold surface through the addition of a terminal cysteine residue. The opposite terminus of strand A contained the cell-adhesive RGD domain to facilitate cell culture on the otherwise inert gold surface. By adding a solution of a complimentary leucine-zipper, strand B, heterodimerization occurs. Strand B was modified through the addition of a terminal PEG strand, and if the PEG strand is large enough (larger than 10 kDa), the RGD domain is thus shielded and inaccessible.[35] Furthermore, by adding in an excess of soluble strand A, strand B is removed from the surface and the RGD domain is re-exposed (Figure 6B–E); therefore, a non-adhesive surface can thus be transitioned into a surface that is conducive to cellular attachment. This procedure is reversible; adding strand B back in to the culture media under gentle agitation re-establishes interactions between strand B and immobilized strand A and results in RGD shielding and subsequent cell detachment. The strategic combination of multiple protein-engineered components enables the dynamic switching between a cell-adhesive and a non-adhesive surface without the undesirable exposure of cells to soluble binding domains.[120–122] This is an elegant approach to mimicking the temporal changes that occur within the extracellular matrix of tissue under normal physiological conditions such as development. Furthermore, such dynamic signaling shows tremendous potential for utilizing immobilized signaling molecules to guide cellular behavior *in vitro*.

In another elegant example, the molecular recognition capabilities of coiled coil leucine-zipper domains were recently partnered with antibody recognition to induce death in cancerous B cells.[111] Two complementary leucine-zipper domains were generated to favor antiparallel heterodimerization upon mixing. The first leucine-zipper is presented as a fusion with

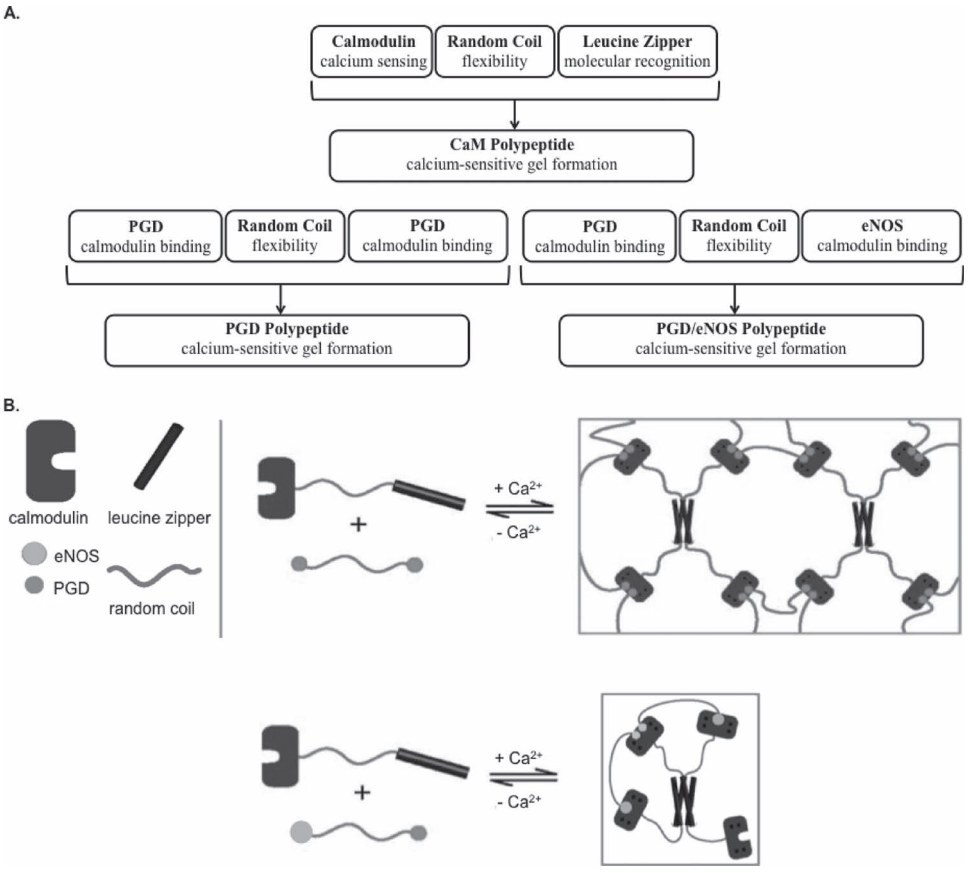

Figure 5. A) Modular design of self-assembling polypeptides that form reversible, calcium-sensitive hydrogels through molecular recognition interactions. B) Schematic of the underlying molecular recognition interactions that lead to formation of a 3D network with tunable viscosity. Figure 5B reprinted with permission from ref. [45]. Copyright 2006 American Chemical Society.

an antibody fragment that specifically binds to CD20, a cell surface receptor expressed on the majority of Non-Hodgkin's lymphoma (NHL) as well as normal B cells. By exposing cells to this engineered protein, biorecognition facilitates binding to CD20 receptors and decoration of the cell surface with leucine-zippers. Cells are then exposed to a copolymer (N-(2-hydroxypropyl)methacrylamide, HPMA) that has been grafted with multiple copies of a second leucine-zipper motif. These leucine-zippers preferentially bind to the complementary strands already decorating the cell surface. This effectively crosslinks the CD20 receptors, an event that causes apoptosis (*i.e.,* cell death).[123–126] Furthermore, because CD20 is not present on the surface of stem cells or mature plasma cells, healthy B cell counts are thought to be able to reach full restoration following treatment.[111,127] This potential therapy for Non-Hodgkin's lymphoma was tested *in vivo* and resulted in enhanced long-term survival in a murine model.[111] This clever design introduces a novel technique of utilizing molecular recognition to specifically target and induce apoptosis in a desired cell population without the need for any toxic or otherwise harsh low molecular weight drugs.

In yet another example, the modular combination of leucine-zipper domains was used to generate bioactive hydrogels capable of enzymatic activity as well as self-assembly.[30] In this modular chimeric design, termed HS-AdhD-H polypeptide, incorporation of the aldo-keto reductase (AKR) domain lends oxidative and reductive capabilities to a hydrogel formed by the self-assembly of leucine-zipper domains (**Figure 7**A,B). An alcohol dehydrogenase (AdhD) from *Pyrococcus furiosus* was chosen to serve as the enzymatic domain due to its AKR activity. This enzyme domain previously has been shown to catalyze the oxidation of secondary alcohols and the reduction of ketones under basic and acidic conditions, respectively.[128] The AdhD enzyme must first bind a cofactor in order to interact with substrate molecules. More specifically, AdhD demonstrates a strong preference for nicotinamide adenine dinucleotide (NAD) cofactors. In this chimeric protein, a homology model of the AdhD domain was fused with a random coil for chain flexibility while leucine-zipper domains were placed at both termini to allow folding into the α-helical secondary structure with minimal effects on the kinetic parameters of the active enzyme domain.[30] Measured dissociation and Michaelis-Menten constants reflect an increased affinity for NAD+ and a slightly decreased affinity for NADH compared to wild-type enzyme; however, these alterations in cofactor binding do not alter enzyme activity, for the turnover numbers of both oxidative and reductive activities were maintained by the polypeptide over a range of temperatures (Figure 7C). Furthermore,

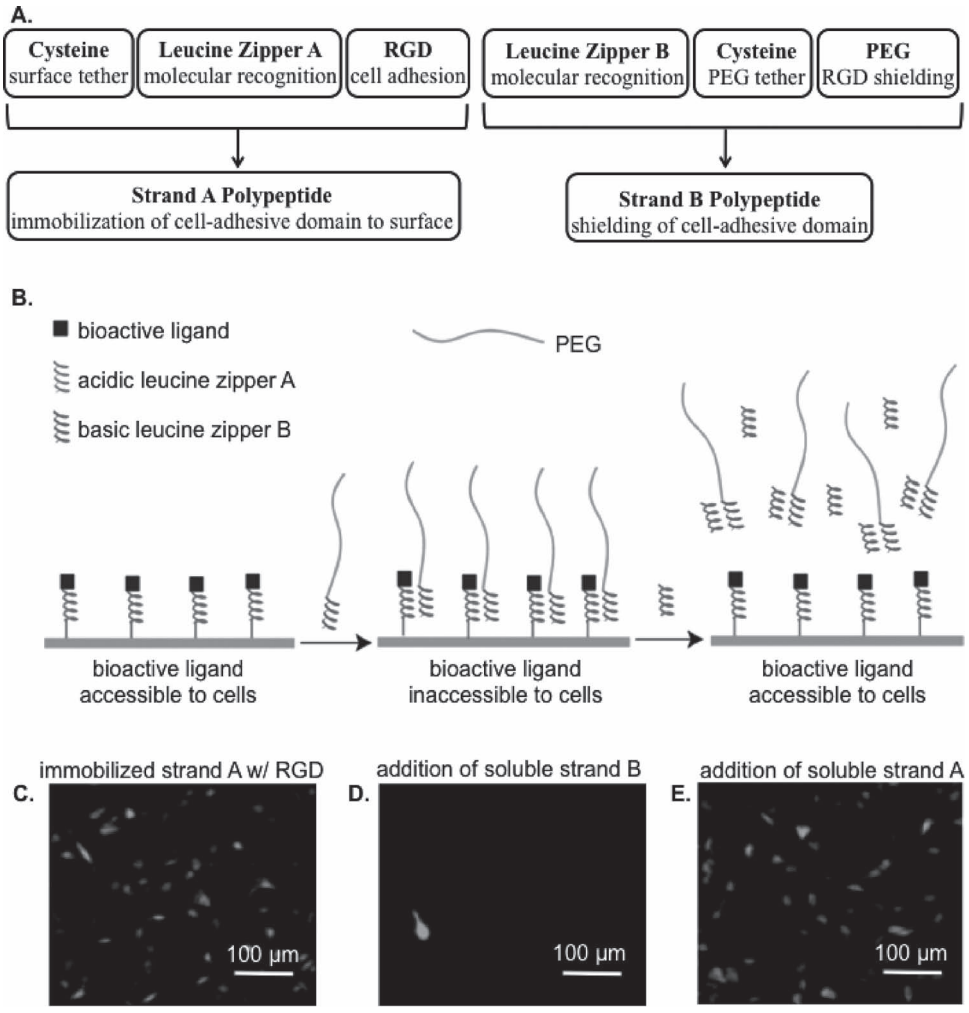

Figure 6. A) Modular design of two leucine-zipper polypeptides utilized to achieve reversible switching between cell-adhesive and non-adhesive surface functionalization. B) Schematic of the dynamic control mechanism. (Left) Strand A polypeptides are grafted to a substrate to create a cell-adhesive coating. (Middle) Assembly between Strand A and Strand B polypeptides renders the RGD ligand inaccessible to cells. (Right) The Strand B polypeptide is removed through competitive binding with soluble leucine-zipper A peptides. C–E) Fluorescence microscopy of NIH 3T3 fibroblasts seeded onto the surfaces schematically represented above. Figures 6B–E reprinted with permission from ref. [35]. Copyright 2010 American Chemical Society.

no noticeable change was found between substrate affinities in either oxidation or reduction reactions in comparing fused to native AdhD.[30] The customizability of the HS-AdhD-H modular design was further demonstrated through the replacement of the AdhD domain with the enzyme organophosphate hydrolase (OPH), which acts to hydrolyze organophosphate neurotoxins.[40] Similar to results with the AdhD domain, this OPH-leucine zipper fusion also demonstrated self-assembly into a three dimensional hydrogel network and preservation of enzymatic activity (Figure 7D).[40] This strategy to sequester catalytic domains within a solid material without compromising enzymatic activity may find wide-spread use in a variety of applications, including the development of engineered metabolons as well as mimics of cellular metabolic pathways in which enzymes are colocalized.

In addition to the design of modular leucine-zipper proteins for use in hydrogel self-assembly, leucine-zipper fusion proteins are also being explored for use in nanoparticle synthesis. In one example aimed at illuminating the mechanisms of retrovirus assembly, chimeric proteins have been formed that in which the nucleocapsid domain of human immunodeficiency virus (HIV) type-1 Gag protein is replaced with leucine-zipper domains.[112] Mammalian expression of Gag protein alone is suggested to facilitate the assembly of virus-like particles in the presence of nucleic acids. When Gag nucleocapsid is replaced with trimerizing leucine zippers, assembly can be induced in the presence of inositol phosphates, a condition that does not support wild-type assembly.[112] In a second example, leucine-zipper domains were fused to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), resulting in the formation of a self-assembling, chimeric protein with improved ease of production and purification as well as a prolonged *in vivo* half-life compared to previous recombinant TRAIL products.[60] This chimeric protein displayed specificity

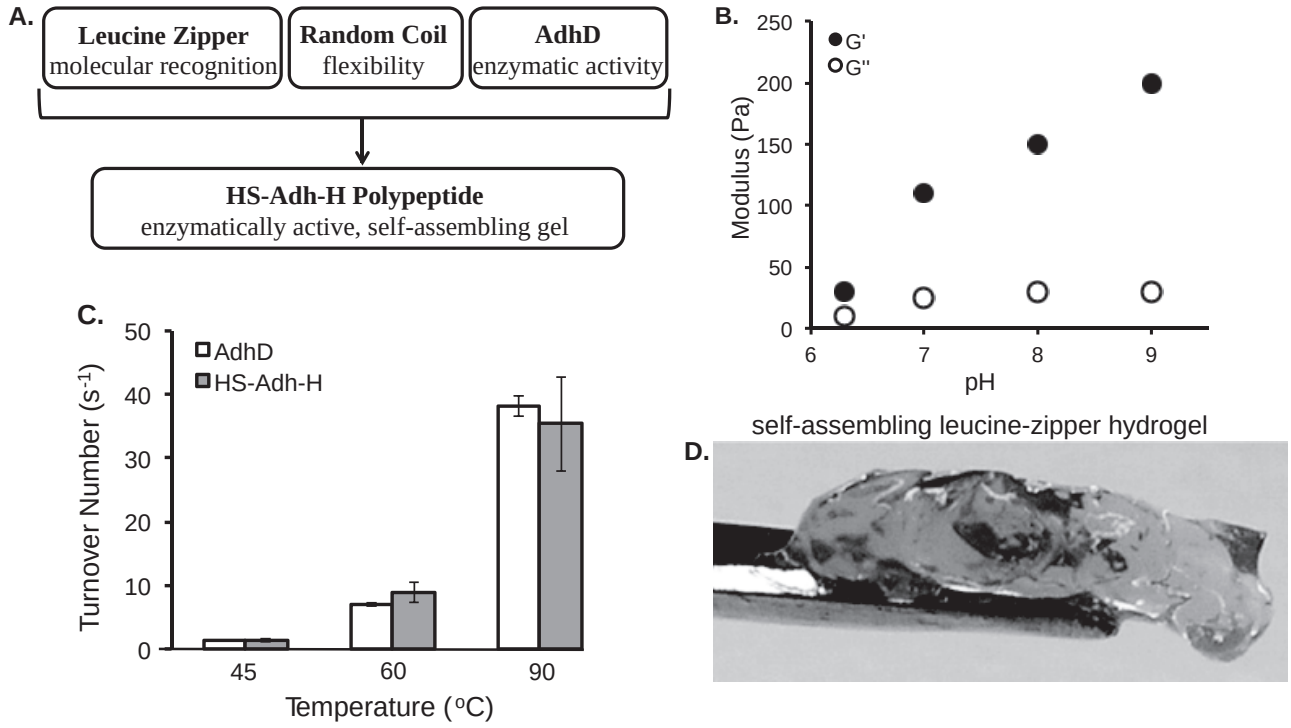

Figure 7. A) Modular design of HS-AdhD-H polypeptide utilized to achieve immobilized enzymatic activity within a hydrogel. B) The storage (G′) and loss (G″) moduli of self-assembled HS-AdhD-H hydrogels indicate pH sensitivity due to changes in the molecular recognition interactions between leucine-zipper domains. C) Hydrogels maintain enzymatic activity comparable to the native AdhD enzyme over a range of temperatures. D) Image of a bulk leucine-zipper hydrogel where the AdhD domain was replaced with a neurotoxin hydrolyzing enzyme, OPH, demonstrating flexibility of the modular design. Figures 7B,C reprinted with permission from ref. [30]. Copyright 2009, Elsevier. Figure 7D reprinted with permission from ref. [40]. Copyright 2010, Elsevier.

of activity both *in vitro* and *in vivo*, showing great promise for future employment as a cancer therapeutic.

When considered together, these examples highlight the immense range of functionalities that can result from strategically repurposing a single type of peptide domain for use in modular, engineered protein materials.

3.2. Exploring Beyond the Library of Naturally Evolved-Domains to Design Multifunctional Polypeptides:

While the previous case studies describe protein-engineered materials constructed from naturally evolved peptide domains with occasional site-specific mutations, target amino acid sequences can also be identified through high-throughput screening or computational modeling approaches. The use of directed evolution, high-throughput screening, and computational modeling allow for the restrictions of naturally occurring amino acid sequences to be relaxed. Natural protein evolution is thought to occur with the guidance of functional rather than structural influences.[129] By this reasoning, non-native amino acid sequences are beginning to be explored to achieve precise, intricate, three-dimensional structures that are often otherwise unavailable. In one example, high-throughput screening was used to identify various non-native sequences for protease degradation.[130] Incorporation of these protease-sensitive domains into ELP's yielded a family of cell-adhesive polypeptides with tunable degradation rates spanning two orders of magnitude despite having 97% sequence homology.[25] Constructing a composite ELP gel with isolated regions of fast-degrading protein within a matrix of slow-degrading protein enabled the selective removal of the sacrificial regions to reveal three-dimensional patterned void structures within the gel.[131] This strategy was also utilized to incorporate soluble factors into regions of varying protease sensitivity within a composite gel, resulting in the tailored release of multiple factors with distinct spatial and temporal control.[131]

Many computational approaches have been developed in effort to model the structure and stability of peptides that contain non-native amino acid sequences. For example, evolution-based statistical coupling analysis (SCA) was used to evaluate sequence conservation in effort to predict non-native amino acid sequences that would fold to form a WW domain type of structure.[129] The WW domain is a naturally evolved peptide fold characterized by an anti-parallel, triple-stranded beta-sheet that can hetero-assemble with a proline-rich (P-rich) peptide binding partner.[132] Out of ten experimentally synthesized, properly folded artificial sequences, six demonstrated selective binding to the group I P-rich peptides (PPXY), whereas four demonstrated selective binding to group III peptides (PPR).[129]

These studies demonstrated that a surprisingly small amount of amino acid sequence information is needed to facilitate molecular recognition in this system. For example, a mere eight resides were shown to comprise the co-evolving cluster that governs group I binding specificity.[129]

One of these computationally-predicted sequences recently was utilized to design a hetero-assembling, polypeptide hydrogel.[34] Seven repeats of the computationally-predicted WW domain were linked by random coil peptides to form a soluble block copolypeptide (C7). When a solution of C7 was exposed to a second engineered protein containing nine repeats of a P-rich peptide (P9), the two components hetero-assembled due to the specific binding of C and P domains (**Figure 8**A,B). Through basic polymer physics considerations, the number of

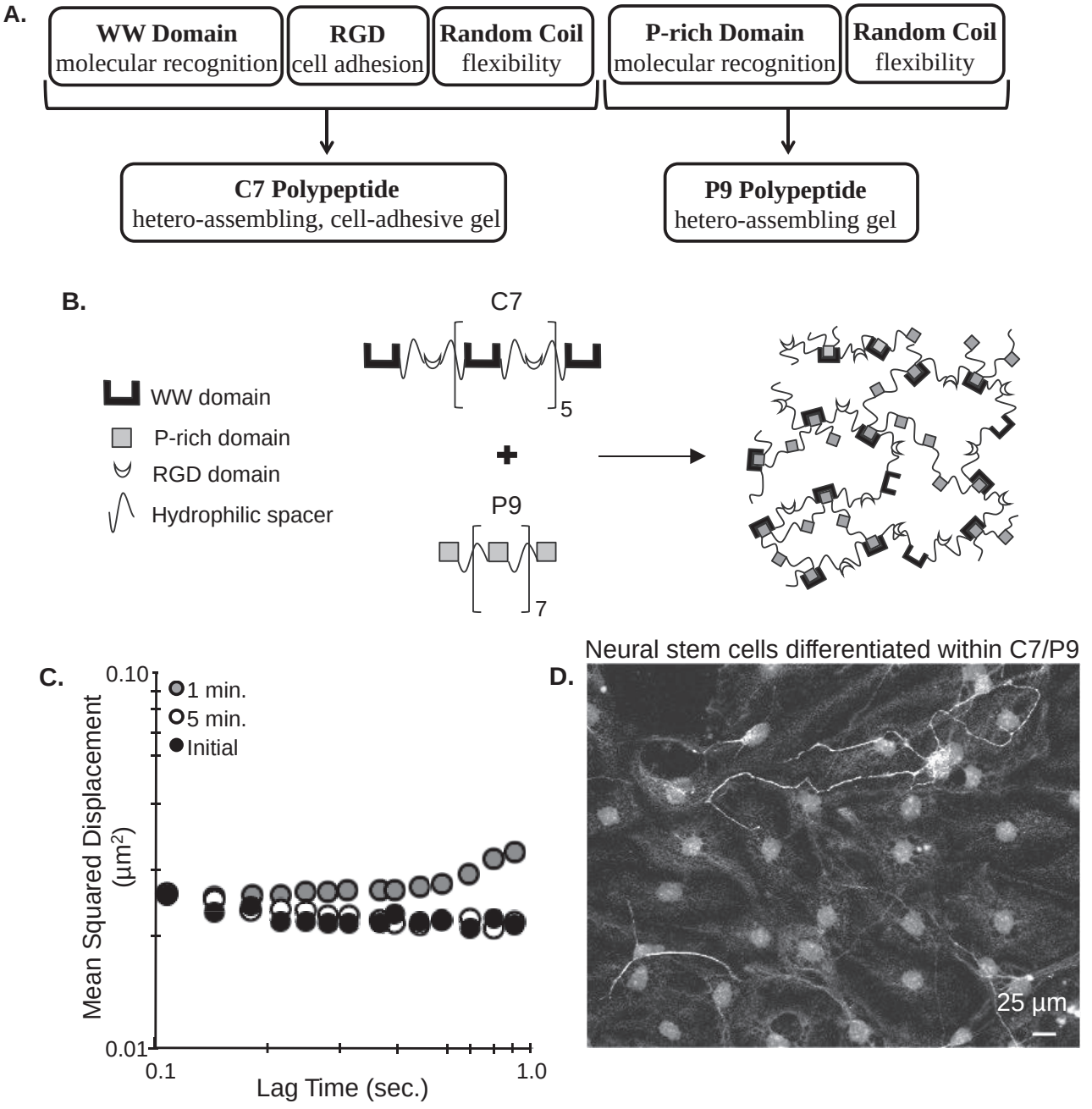

Figure 8. A) Modular design of C7 and P9 polypeptides that form physical crosslinked hydrogels for the encapsulation of fragile biological entities. B) Schematic of the hetero-assembly mechanism. C) Micro-rheology characterization (the mean squared displacement of embedded tracer particles as a function of observation lag time) reveals that C7/P9 hydrogels fully recover their mechanical properties within 5 minutes after being shear-thinned through a syringe needle. D) Fluorescent micrograph of neural stem cells differentiated within C7/P9 hydrogels demonstrates scaffold compatibility with neuronal cell types. Figures 8B–D reprinted with permission from ref. [34]. Copyright 2009, Proceedings of the National Academy of Sciences of the United Stated of America.

repeating C and P domains and the lengths of the interspersed random coils were designed to cause the rapid formation of a hydrogel in response to C and P binding. This two-component, hetero-assembling system, in which the individual components are incapable of undergoing self-assembly, enabled physical gelation to occur simply upon mixing two solutions together for fifteen seconds.[34] This is a particular advantage when encapsulating cells or biomolecules for therapeutic delivery applications, because no chemical crosslinkers or large changes in pH or temperature are needed to induce gelation. Thus, the sensitive biological cargo can be encapsulated without exposure to any detrimental conditions that may irreversibly affect bioactivity.[34]

In addition, this gelation strategy enables the straight-forward tailoring of gel viscoeslastic properties through rational design of the modular protein at the molecular level.[133] By altering the thermal equilibrium binding constant (K_b) between the P-rich and WW domains, the number of interacting domain repeat units, as well as the relative compositional ratio of P-rich and WW domains, a high degree of control is elicited over the resultant mechanical properties.[34,133] Due to the purely physical interactions between protein chains, the C7/P9 hydrogel is a viscoelastic, shear-thinning material that will reestablish its gel structure once the acting shear forces have been removed (Figure 8C). Therefore, this material is an ideal candidate for use as an injectable scaffold for minimally invasive cell transplantation.[34] To impart cell-adhesion functionality, the RGD cell-binding domain was designed into the C7/P9 hydrogel, resulting in the successful three-dimensional proliferation and differentiation of encapsulated neural stem cells (Figure 8D).[34]

4. Into the Future: Challenges and Opportunities For Protein-Engineered Materials

The use of protein engineering techniques to generate multifunctional materials, while showing great promise, is not without its challenges. For example, sequence selection is a complicated process due to the possibility of long range amino acid residue interactions and the effects that these interactions may have on protein structure and function.[2] To optimize yield and decrease the probability of unwanted downstream interactions, short sequences ranging from 5 to 50 amino acids in length are generally used as individual domains.[7] These sequences must be designed to have the appropriate level of structural flexibility or rigidity to allow for all intended interactions to occur. To increase flexibility, random coil linkers can be added to the sequences, while to increase rigidity, functional domains can be fused with α-helical structures.[2] In addition, protein solubility and response to stimuli such as temperature, salt concentration, and pH must be thoroughly considered and characterized.[6]

Once an amino acid sequence has been selected, it must be translated into a series of DNA-based codons which are then translated by a recombinant host to produce the genetically-engineered protein. The large-scale, commercial production of recombinant proteins is now common place in the pharmaceutical industry; therefore, there is a great deal of knowledge about scaling up protein expression from the bench-top to a production facility. Nevertheless, each step in the process presents its own set of challenges, beginning with selection of the codon sequence. Codon optimization as a design step has yet to be thoroughly explored and optimized in the field of protein-engineered materials.[7] The preferred codon to specify a particular amino acid varies from organism to organism, and often designers choose the most prevalent codons in their host organism of choice when constructing an engineered plasmid. However, for highly repetitive amino acid sequences, as is often the case in recombinant protein-engineered materials, a diversity of codons must be selected to decrease the risk of genetic recombination that can occur during gene replication in the host system. In addition, it has been suggested that codon diversity can improve protein yield by preventing depletion of codon-specific enzymes.[7] Furthermore, genetic sequences must be examined for potential binding sites for transcriptional and translational regulators that could jeopardize *in vivo* synthesis of a full-length construct.[2]

A wide range of host organisms has been used to generate recombinant proteins. Selection of a host organism often involves a trade-off between the potential yield and the complexity of the intended protein. *Escherichia coli* is a commonly preferred host organism due to its low cost and high rate of replication; however, this system lacks the natural ability to complete post-translational modifications due to the absence of an intracellular Golgi body.[7] This means that complex proteins, such as those requiring glycosylation, must either be synthesized in a more complex and lower yielding host organism, such as yeast, insect, or mammalian cells, or significant organism engineering must be performed on the bacterial host. Furthermore, while optimizing yield may seem like an ideal intention, overexpression of proteins can result in the formation of unfolded, denatured aggregates of nonfunctional protein material.[7] The point at which expression results in the formation of aggregates or inclusion bodies varies based on the amino acid sequence and the host organism selected. The chosen host system also impacts the steps required in product purification due to the range of contaminants that may be present. The possible presence of contaminants, downstream interactions, genetic alterations, and denaturation that may occur during production all result in the requirement of extensive characterization of recombinant products.[6] These materials must be biophysically, structurally, and biochemically analyzed to verify their properties. In addition, genetic sequencing should routinely be completed to screen for genetic mutations.

When completed successfully, protein engineering generates materials that possess both the inherent biofunctionality of naturally derived materials and the tunability of synthetics. Moreover, through the use of protein engineering, molecular precision is enforced at the genetic level, resulting in the generation of materials with unparalleled regularity and molecular specificity. A hallmark of these materials is the modular combination of multiple domains to design multifunctional block copolymers. As our characterization and understanding of naturally evolved domains continue to expand, the scope of functionalities designed into protein-engineered materials also will greatly increase, both by mimicking wild-type functions and by repurposing domains to perform new functions. Furthermore,

the library of potential peptide domains is expanding even more rapidly through advances in computational modeling, high-throughput screening, and directed evolution studies. As a consequence, the complex functionalities that can result from clever and strategic protein design are virtually limitless. While the use of protein engineering in materials design is only beginning to gain momentum, this precise design strategy represents an unparalleled potential to create new multifunctional materials tailored for a wide range of applications.

Acknowledgements

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