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Engineered Protein Hydrogels as Biomimetic Cellular Scaffolds

*Yueming Liu, Aidan E. Gilchrist, and Sarah C. Heilshorn**

The biochemical and biophysical properties of the extracellular matrix (ECM) play a pivotal role in regulating cellular behaviors such as proliferation, migration, and differentiation. Engineered protein-based hydrogels, with highly tunable multifunctional properties, have the potential to replicate key features of the native ECM. Formed by self-assembly or crosslinking, engineered protein-based hydrogels can induce a range of cell behaviors through bioactive and functional domains incorporated into the polymer backbone. Using recombinant techniques, the amino acid sequence of the protein backbone can be designed with precise control over the chain-length, folded structure, and cell-interaction sites. In this review, the modular design of engineered protein-based hydrogels from both a molecular- and network-level perspective are discussed, and summarize recent progress and case studies to highlight the diverse strategies used to construct biomimetic scaffolds. This review focuses on amino acid sequences that form structural blocks, bioactive blocks, and stimuli-responsive blocks designed into the protein backbone for highly precise and tunable control of scaffold properties. Both physical and chemical methods to stabilize dynamic protein networks with defined structure and bioactivity for cell culture applications are discussed. Finally, a discussion of future directions of engineered protein-based hydrogels as biomimetic cellular scaffolds is concluded.

1. Introduction

Protein-based hydrogels are 3D matrices of hydrated, polymeric protein networks.[1] Formed by self-assembly or crosslinking of proteins, these bioactive hydrogels can induce a range of cell behaviors, making them well suited as cellular scaffolds for tissue engineering and regenerative medicine applications.[2,3] Hydrogels to support encapsulated cell culture can be made using either naturally derived biopolymers or synthetic polymers. While the former often include inherent cell-interaction motifs, they can suffer from batch-to-batch variability, the presence of undefined impurities and growth factors, and limited tuning of biochemical and biophysical properties.[4] On the other hand, synthetic polymers have excellent reproducibility and tunability, but they typically require additional modification with peptides or proteins to impart bio-functionality and have potentially toxic and inflammatory by-products after degradation.[5,6] As an alternative approach that leverages the advantages of these two classes of scaffolds, engineered protein-based hydrogels are reproducible, customizable, and can be designed to have inherent bioactivity. Through design of the amino acid sequence of the protein backbone or chemical modification of the amino acid side chains, engineered protein-based hydrogels can have well-defined structures and biomimetic functionality as scaffolds for cell culture.

Using recombinant DNA techniques, engineered proteins can be synthesized with precise control over amino acid sequence, chain length, folded structure, and monodispersity.[7,8] These techniques enable fabrication of reproducible protein hydrogels with defined biological, physical, and chemical properties. Additionally, like block copolymers that are composed of distinct modules of different polymeric blocks, diverse peptide building blocks can be incorporated into the backbone of a single recombinant protein. This modular design approach allows incorporation of multiple peptide building blocks such as bioactive domains (e.g. cell-adhesive motifs) and functional domains (e.g. stimuli-responsive motifs), into one highly tunable design of multifunctional, biomimetic scaffold.

Engineered protein-based hydrogels enable the development of biomimetic environments that mimic essential structural and compositional features of the native extracellular matrix (**ECM**; for reader convenience, a list of all acronyms and abbreviations can be found in Table SI, Supporting Information).[9,10] The ECM presents a complex mixture of instructive cell signaling through biochemical and biomechanical cues. First, the peptide ligands present in the native ECM, including the proteins fibronectin, collagen, elastin, and laminin, among others, can directly bind to cell-surface receptors and induce biochemical signaling.[11]

Y. Liu
Department of Materials Science & Engineering
Stanford University
Stanford, CA 94305, USA

A. E. Gilchrist
Department of Biomedical Engineering
University of California
Davis 451 Health Sciences Dr, GBSF 3315, Davis, CA 95616, USA

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

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/adma.202407794

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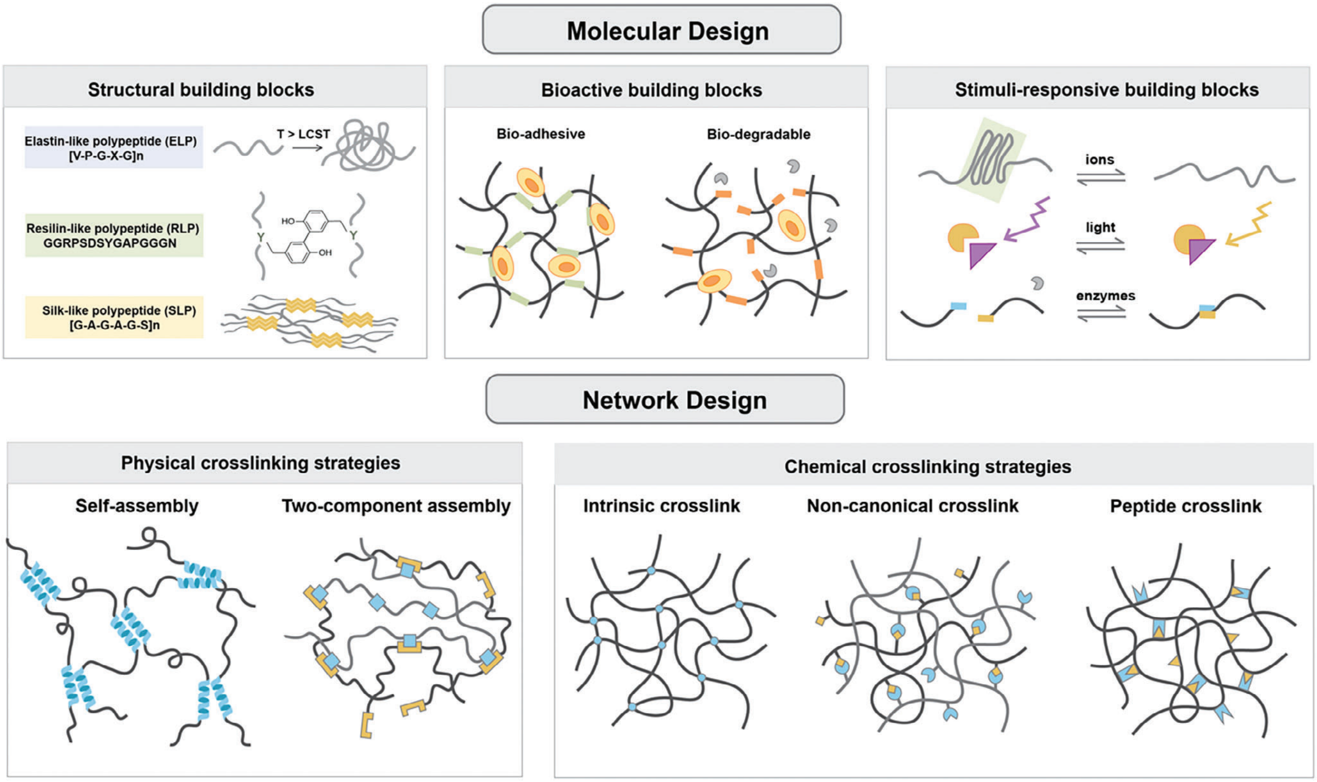

Figure 1. Molecular- and network-level design considerations for biomimetic, engineered protein hydrogels. Molecular-level design of recombinant protein backbones can include structural building blocks (e.g. elastin-like, silk-like, or resilin-like peptide sequences) to provide defined mechanical support and/or controlled chain–chain interactions, bioactive building blocks to interact with cell-surface receptors and cell-secreted proteases, and stimuli-responsive building blocks with on-demand, dynamic properties (e.g. in response to ions, light, or enzymes). Network-level design refers to the many different strategies that can be used to crosslink individual protein chains into a stable hydrogel. Physical crosslinking can occur either through self-interactions or interactions between two (or more) engineered components. Chemical crosslinking can be designed to occur by using the intrinsic chemical reactivity of the side chains of certain amino acid residues, by modifying the recombinant protein to present non-canonical chemical functional groups, or by designing peptide sequences into the recombinant protein backbone that are substrates for enzymatic ligation.

Furthermore, the mechanical properties and microstructure of the ECM are also known to regulate cell behaviors including proliferation, migration, and differentiation.[12–15] Additionally, the ECM is a highly dynamic environment that is constantly remodeled by cell activity during tissue development, disease progression, aging, and tissue regeneration.[16–18] Thus, to design engineered protein-based hydrogels to provide biomimetic instructive signals, there are several critical design criteria that should be considered, including the 1) structural properties, 2) mechanical properties, 3) bioactive properties, and 4) bio-responsive, dynamic properties, each of which are known to significantly impact cell behavior.

Here, we discuss modular block design of engineered protein-based hydrogels for cellular scaffolds, beginning with structural, repetitive blocks that provide mechanical infrastructure (**Figure 1**). We next discuss physicochemical methods to crosslink and stabilize these structural blocks into 3D hydrogel networks. Following this, we describe how bioactive blocks can be designed into the scaffolds to promote cell adhesion and biodegradation. Finally, we report how stimuli-responsive blocks can be incorporated to impart dynamic functionality. In each section, we present several recent case studies of engineered protein-based hydrogels to highlight the diversity of strategies to control each of these four material properties. Hydrogels constructed from short peptides or naturally occurring proteins purified from natural sources (like corn-based zein and bovine collagen) are not discussed, and the interested reader is directed to other excellent timely reviews on these topics.[19–23] We conclude by discussing several possible directions that can be explored for the future development of engineered protein-based hydrogels as biomimetic cellular scaffolds.

2. Molecular-Level Design and Engineering Strategies

2.1. Engineering Structural Domains

In this section, we will discuss incorporation of structural building blocks into the protein backbone. Repeat units of amino acid sequences have been identified in a range of naturally occurring proteins, which provide unique mechanical properties to the protein and often act as spacers between different bioactive domains.[24,25] Here, we focus on structural building blocks derived from elastin, resilin, and silk, which have been widely studied and are frequently used as the backbones of engineered protein-based hydrogels for cellular applications.

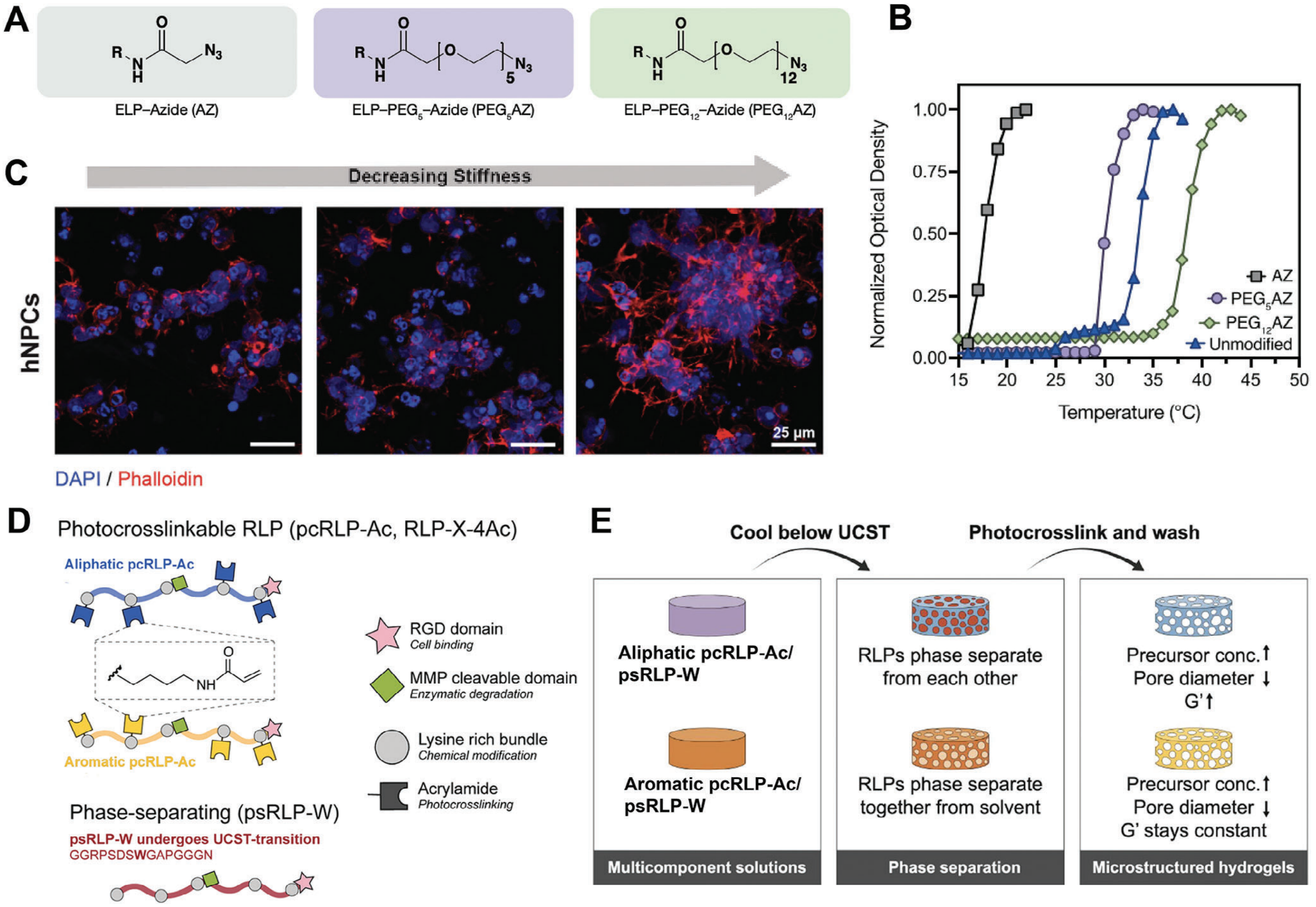

Figure 2. A) Azide side groups used to modify and elastin-like polypeptides (**ELP**), with increasing hydrophilicity from left to right. B) Unmodified ELP and ELPs modified with their respective azide molecules present distinct lower critical solution temperature (**LCST**) behaviors as measured by optical density ($\lambda = 300$ nm). C) Human neural progenitor cells (**hNPCs**) exhibit increased spreading when encapsulated in ELP gels with decreased modulus. Reproduced with permission.[36] Copyright 2023, John Wiley and Sons. D) Resilin-like polypeptides (**RLP**) are recombinant proteins with sequence similarity to native resilin that displays upper critical solution temperature (**UCST**) behavior. Schematic of photocrosslinkable RLPs and phase separating RLP to form microstructured hydrogels. E) Homogeneous gels were formed above the UCST and then cooled to induce phase separation prior to crosslinking into microstructure gels. Adapted with permission.[40] Copyright 2023, American Chemical Society.

2.1.1. Elastin-Like Polypeptides

Elastin, as its name suggests, is a highly elastic protein found in the ECM. The elastin-like polypeptide (**ELP**), derived from elastin, is composed of repeating units of $(VPGXG)_n$, where X is a guest residue consisting of any amino acid except proline.[26] ELPs are intrinsically disordered proteins (**IDP**s) meaning they do not form ordered structures,[27] and can be used as building blocks for recombinant protein-based hydrogels. Importantly, ELPs undergo an inverse temperature phase transition above their lower critical solution temperature (**LCST**), and therefore are soluble in aqueous solution below their transition temperature (T_t) but reversibly separate into a protein- rich coacervate phase above their T_t.[28] LCST behavior of ELPs can be controlled by modifying the protein chain lengths,[29] types of guest residues and side chains,[30] and can be predicted using mathematical models and simulations.[31–33] Thermally active peptides, such as those derived from elastin, typically do not form stable enough protein aggregates on their own to support the formation of a hydrogel for cell culture,[28] thus, these sequences are frequently combined with other motifs to allow for additional physical or chemical crosslinking. While the full ELP amino acid sequence is typically solvent-accessible at temperatures below the T_t, above the T_t, portions of the ELP sequence can be “hidden” within hydrophobic domains, obscuring potential physical or chemical crosslinking sites. This opens up the principle of using sequence-level modulation of the T_t for the design of ELP-based hydrogels with desired mechanical properties, microstructures, and thermos-responsive properties for cell culture.[34,35]

As an example, a library of ELP-based gels was recently designed with varying stiffness as a result of tuning the transition temperature. Specifically, the X guest residue position within the ELP domain was used for chemical conjugation of sides groups with different levels of hydrophobicity (**Figure** 2A).[36] Increasing the lengths of highly hydrophilic polyethylene glycol (**PEG**) within the conjugated side groups altered the overall ELP T_t between 20 and 37 °C (Figure 2B), the temperature range commonly used for working with mammalian cell culture. The elevation in T_t increased the solubility of ELP, and hence the availability of reactive moieties for covalent crosslinking, leading to formation of increasingly stiff gels. In contrast, ELP polymers with lower T_t formed weaker gels with fewer covalent crosslinks due to hydrophobic burying of the reactive moieties. As an example of the importance of crosslinking accessibility and stiffness, human neural progenitor cells (**hNPCs**) exhibited increased neurite outgrowth when cultured within ELP hydrogels with lower T_t and lower stiffness (Figure 2C).

In complementary work, by changing the ELP length and primary sequence, other physical properties, such as a reduced swelling ratio after temperature induced aggregation, can be encoded into the hydrogel backbone.[37] Given that the LCST plays a crucial role in fine-tuning the mechanical properties of ELP scaffolds, predictive computational frameworks have been developed to model the phase transition behavior.[31–33] Using enhanced sampling methods, a virtual library of ELP sequences was created to study the effect of peptide chemistry, chain length, and salt concentration on the structural transitions of ELPs.[38] This library can provide a valuable resource for recombinant protein design for the synthesis of future protein-based hydrogels. In another example, a library of ELP-based hydrogels was designed using a computational model that predicted ELP viscoelasticity and thermal-responsiveness by altering the guest residue, location, and hydrophobicity of the crosslinking blocks.[39] This model enables *de novo* design of different ELP blocks with defined and predictable LCST behaviors for biomimetic cellular scaffolds.

2.1.2. Resilin-Like Polypeptides

Resilin is a structural protein which exists mainly in insect exoskeleton structures and exhibits outstanding resilience and fatigue resistance.[41] Recombinantly expressed resilin-like polypeptides (**RLP**) possess similar properties and functions of native resilin with repeat sequences of $(\mathrm{GGRPSDSYGAPGGGN})_n$.[42] As a structural modular, RLPs are less hydrophobic than ELPs, and have low stiffness while showing high resilience, reversible extensibility and excellent high-frequency-responsiveness, suggesting potential applications for mechanically active tissues like vocal folds, muscles, and cardiovascular tissue.[43–46]

A unique feature of RLPs is their dual-phase transition behavior (**DPTB**), exhibiting both upper critical solution temperature (**UCST**) and LCST at experimentally accessible temperatures. The rich phase behavior is governed by the amino acid sequence and has inspired the generation of libraries of RLPs with defined DPTB.[47,48] As an illustration of the tunability of RLP DPTB behavior, substitution of an aliphatic residue in the RLP domain can eliminate UCST behavior, while an aromatic residue such as tryptophan (W) increases hydrogen bonding and aromatic π–π contacts and induces UCST behavior. By tuning the hydrophobicity of the substitute residue, the T_t can be tuned from 30 to 65 °C. Investigating the impact of these key amino acids on the UCST transition allows for the sequence control of microstructured or continuous hydrogels for cell culture.[49,50]

The phase separation of RLPs has been used in the design of engineered protein scaffolds with precise *de novo* control over microstructure pore size (≈10-50 µm) and micromechanical properties for encapsulated cells.[51,52] In another example, three RLPs sequences were designed to yield one with a UCST behavior and two photocrosslinkable RLPs without a UCST (Figure 2D).[40] The phase-separating RLP is initially combined with the non-phase-separating RLP in a primary solution and cooled below its UCST to form coacervates. The non-phase-separating RLP is then photocrosslinked to form a stable gel. The uncrosslinked, phase-separated RLP serves as a sacrificial template and is subsequently removed, resulting in a microporous hydrogel. This process allows for precise control over pore diameters (ranging from 1.5 to 150 µm) and shear storage moduli (ranging from 0.2 to 5 kPa).[40] Compared to uniform gels, human mesenchymal stem cells (**hMSCs**) exhibited higher viability and elongated morphology on microstructured RLP gels produced using this sacrificial templating method. While much of the recent work has focused on manipulating the UCST behavior of RLPs for designing microstructure of hydrogels, there is an opportunity in the field to leverage the dual-phase behavior and incorporate both the LCST and UCST as design principles for biomimetic scaffolds. An exciting possibility is the design of DPTB of RLPs with sequential phase separation to create hierarchical microstructures for cell culture.

2.1.3. Silk-Like Polypeptides

Silk is a very attractive building component for biomaterials design due to its unique mechanical properties of high strength-to-weight ratios and high elastic moduli of fibers (3–10 GPa).[53] The most commonly used repetitive sequence in silk-like polypeptides (**SLP**s) is based on the hexapeptide motif in silk fibroin $(\mathrm{GAGAGS})_n$ found in silkworms. Unlike ELPs and RLPs, SLPs can self-assemble into a semi-crystalline β-sheet structure, facilitated by hydrophobic interactions and hydrogen bonding. The resulting β-sheet structure gives rise to a microfiber architecture that closely mimics the fiber network in the native ECM. For instance, SLPs derived from a spider silk protein 4RepCT can readily assemble into a foam-like scaffold using an air-water interface fabrication technique.[54] This distinctive microfiber structure promotes cell spreading and proliferation along the aligned fibers, ultimately facilitating the formation of vessel-like structures composed of endothelial cells within the tissue constructs.[54]

SLPs are often fused with other polypeptides to tailor their solubility and functional characteristics, resulting in constructs like silk-elastin-like polypeptides (**SELP**) and silk-resilin-like polypeptides (**SRLP**). Elastin contributes high elasticity and solubility in aqueous environments at lower temperatures, while silk imparts stability through the formation of crystalline β-sheet regions, providing additional strength and rigidity to the material.[55] The LCST behavior of ELP promotes self-assembly of SELP into a metastable microarchitecture, offering a versatile platform for biomimetic 3D cell culture. For example, during the scaffold self-assembly process, the elastin-like domains favor phase separation, while the β sheet silk-like motifs generate an energy barrier that opposes phase separation.[56] Human mesenchymal cells encapsulated in this scaffold were found to selectively distribute within the pores as either individual cells or small clusters of cells.[56] Thus, fine-tuning the thermodynamics and kinetics of phase separation by altering the lengths and primary sequences of the elastin-like and silk-like domains allow precise

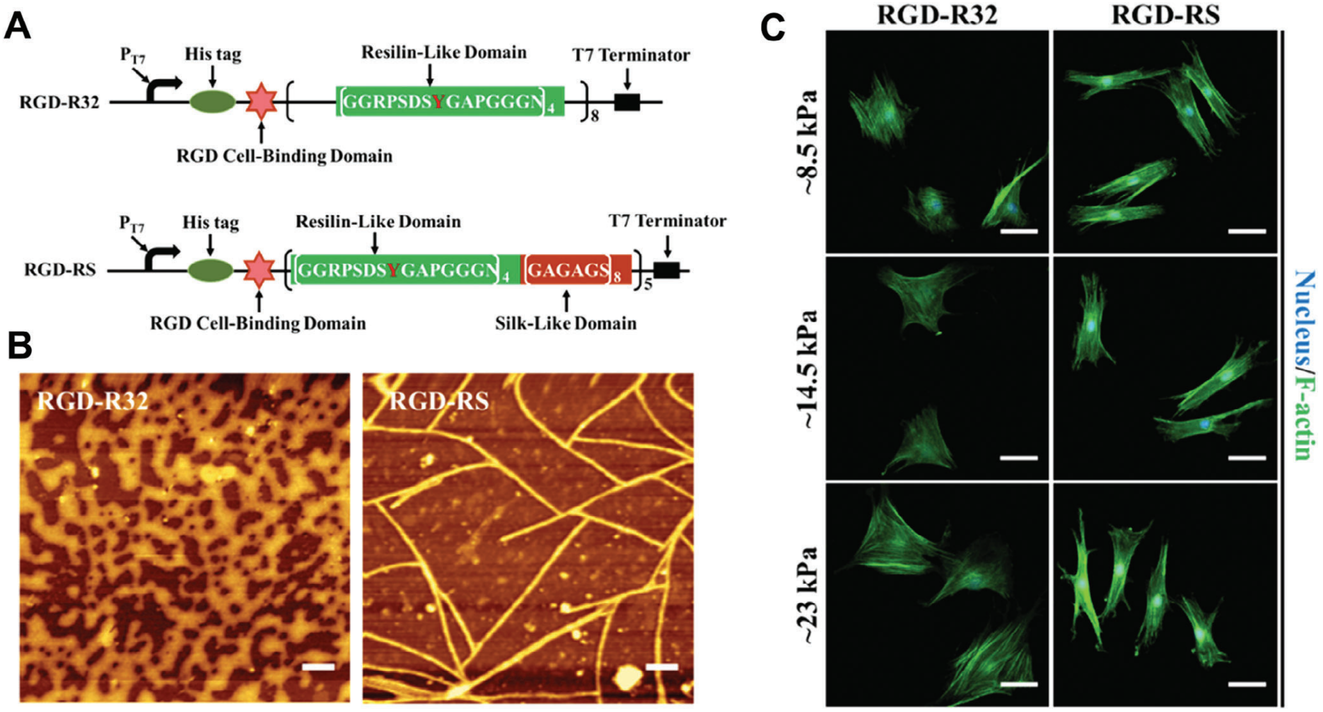

Figure 3. A) Genetic constructs of recombinant RLP (named RGD-R32) and SRLP (named RGD-RS). B) Representative atomic force microscopy images of amorphous aggregates from RGD-R32 and self-assembled nanofibers from RGD-RS. C) Representative confocal images of the bone marrow mesenchymal stromal cells cultured on amorphous (RGD-R32) and fibrous (RGD-RS) hydrogels with different stiffness for 1 day. The cells were labeled to visualize their actin (Phalloidin-FITC, green) and nuclei (DAPI, blue). Scale bar: 50 µm. Reproduced with permission.[58] Copyright 2022, American Chemical Society.

spatiotemporal control over the resulting hydrogel microarchitecture.[56] Expanding on this work, the SELP solution was injected and allowed to gel in situ at body temperature as a potential treatment for spinal cord injury (**SCI**). The SELP scaffold allowed for the infiltration and proliferation of endogenous cells and reduced SCI-associated fibrosis, without inducing further microglia and astroglia reactivity.[57]

Alternatively, SLP can be fused with RLP, which introduces microfiber structure to the otherwise amorphous architecture of RLP hydrogels (**Figure 3**).[58] The amorphous and fibrous hydrogels can be used to study the mechanical and structural cues in cell mechanotransduction. Increasing stiffness of the protein-based hydrogel was found to promote bone marrow mesenchymal stromal cells proliferation and differentiation, and these effects were more pronounced on the fibrous gels. By harnessing the inherent advantages of both intrinsically disordered structural blocks (e.g., ELP, RLP) and self-assembled blocks (e.g., SLP), biomimetic cellular scaffolds can be designed with defined mechanical properties, specific architecture, and tailored functional properties.

2.2. Engineering Crosslinking Strategies

Here we will discuss physicochemical methods to stabilize polypeptide structures and create engineered protein-based hydrogels through the introduction of crosslinks. Crosslinks can be formed through physical or chemical strategies, and careful design of these crosslinking strategies results in hydrogels with improved stability and tunable mechanical properties.

2.2.1. Physical Crosslinking of Protein Hydrogels

Physical crosslinking strategies include self-assembly of a one-component system and molecular- recognition induced assembly of a two-component system. In a one-component system, the protein self-assembles to form a supramolecular structure. In a two-component system, two (or more) proteins assemble through peptide–peptide interactions. These structures are based on weak and reversible interactions such as hydrogen bonds, and physical hydrogels are generally shear-thinning and viscoelastic, with reversible sol–gel phase transitions depending on solvent conditions.

2.2.2. One-Component Coiled-Coil Self-Assembly

The coiled-coil is a fundamental protein self-assembly pattern, formed by the winding together of two or more α-helices to create a superhelix. The coiled-coil motif has a helical repeat of seven amino acid residues, commonly notated as $(abcdefg)_n$. Amino acid positions a and d are typically nonpolar residues that induce interhelical assembly through hydrophobic interactions. Since these positions are often occupied by leucine residues, the motif is also often called a leucine zipper domain.[59] Protein engineering of

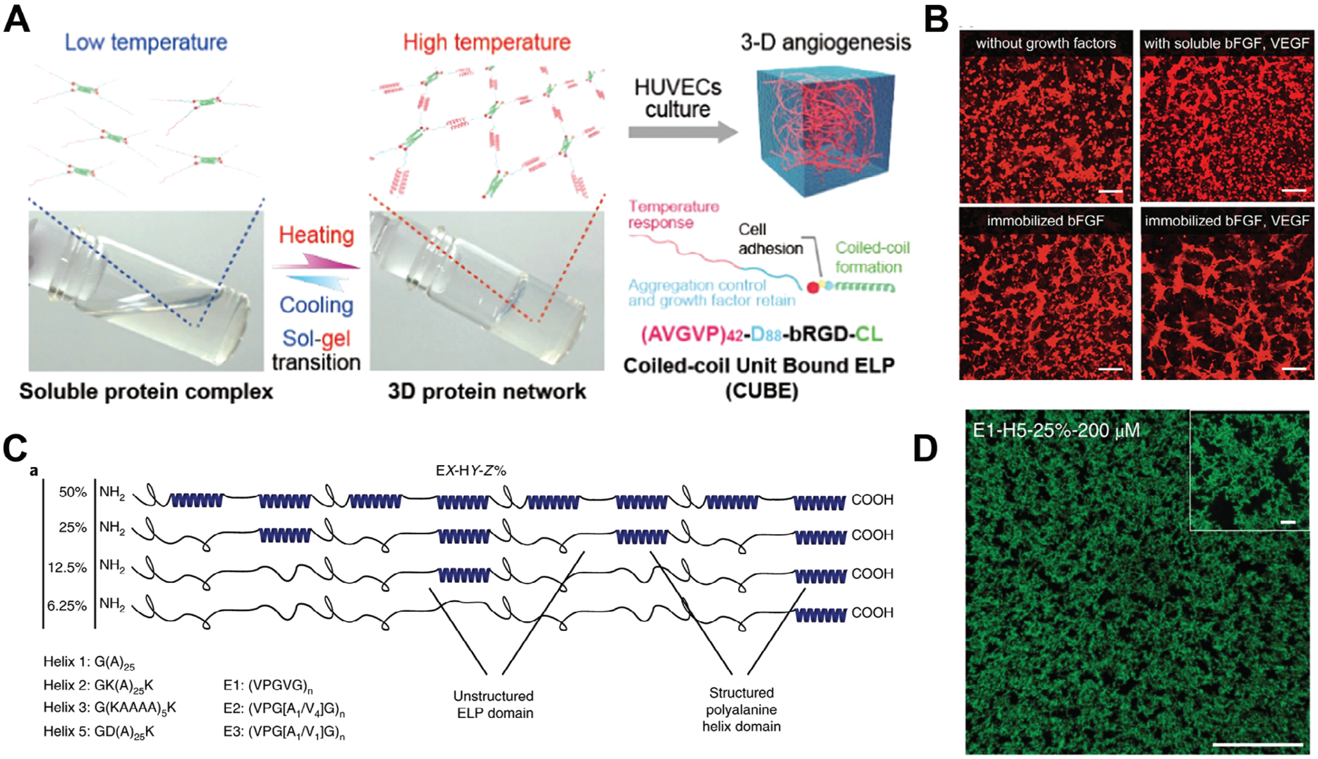

Figure 4. A) Four-armed coiled-coil unit bound ELPs (CUBEs) reversibly form temperature-dependent hydrogels that can immobilize growth factors of angiogenic culture of HUVECs. B) Morphologies of HUVECs (red) cultured in CUBEs with soluble or immobilized basic fibroblast growth factor (**bFGF**) and vascular endothelial growth factor-165 (**VEGF$_{165}$**). Scale bars represent 100 µm. Reproduced with permission.[67] Copyright 2023, American Chemical Society. C) A library of modular, engineered proteins was constructed with three different ELP domains and for polyalanine helices, resulting in different ratios of helical content from 6.25%–50%. D) Hydrogel microstructure (green) of E1-H5-25% demonstrating thermally-responsive porosity. Scale bars are 50 µm for the main image and 10 µm for the inset. Reproduced with permission.[68] Copyright 2018, Springer Nature.

the coiled-coil association in the supramolecular network enables control over the stiffness, viscoelasticity, and erosion rate of hydrogels.

One of the most widely used helices for physically-crosslinked, engineered protein-based hydrogels is based on the leucine zipper domain. A common engineered protein design is a triblock architecture (AC$_{10}$A) consisting of a water-soluble polyelectrolyte domain (C$_{10}$) flanked by "leucine zipper" end blocks (A).[60] that form dimers under appropriate pH and temperature. Hydrogels based on the leucine zipper domain typically have weak mechanical properties and undergo rapid erosion, due to the reversible nature of the crosslinking and defects in the network formation (i.e., intramolecular loops).[61] To overcome these limitations, the protein sequence has been engineered to alter 1) the association/disassociation kinetics of the coiled-coil sequence.[62] 2) the midblock sequence,[63,64] and 3) molecular weight.[65] For example, one end block of AC$_{10}$A can be replaced by a coiled-coil amino acid sequence (P) that does not associate with A, resulting in hydrogels with suppressed intramolecular loops, higher stiffness and a hundredfold slower erosion rate.[61]

To form modular hydrogels, physical crosslinking domains can be combined with structural domains and cell-binding polypeptide sequences to create biomimetic scaffolds. For example, fibronectin- and laminin-derived peptide motifs (RGD and IKVAV, respectively) were include in an engineered protein-based hydrogel that had both ELP structural domains and coiled-coil self-assembly domains to successfully culture pancreatic endocrine-like cells with higher reproducibility compared to a commonly used, naturally derived matrix (Matrigel).[66] In another example, coiled-coil unit bound ELPs (**CUBE**s) were modified with tethered growth factors (fibroblast growth factor and vascular endothelial growth factor) to effectively enhance angiogenic activity of human umbilical cord endothelial cells (**HUVEC**s) (**Figure** 4A,B).[67] In another modular hydrogel design, different ratios of polyalanine helix domain were interspersed with intrinsically disordered, thermally responsive ELP domain (Figure 4C). The resulting hydrogel displayed thermally triggered microstructure formation in the scaffold, which improved in vivo cell recruitment and vascularization (Figure 4D).[68] These examples demonstrate the promise of creating modular hydrogels with multiple peptides to develop biomimetic scaffolds with porous microstructure and viscoelastic mechanics for cell culture.

2.2.3. Two-Component, Molecular-Recognition Based Assembly

A complementary approach to forming physically crosslinked hydrogels is to engineer reversible peptide/peptide interactions that can hetero-assemble. One advantage of this two-component strategy is that network assembly occurs spontaneously upon mixing, without the need for pH or temperature modulation that may be detrimental to cells. However, similar to hydrogels

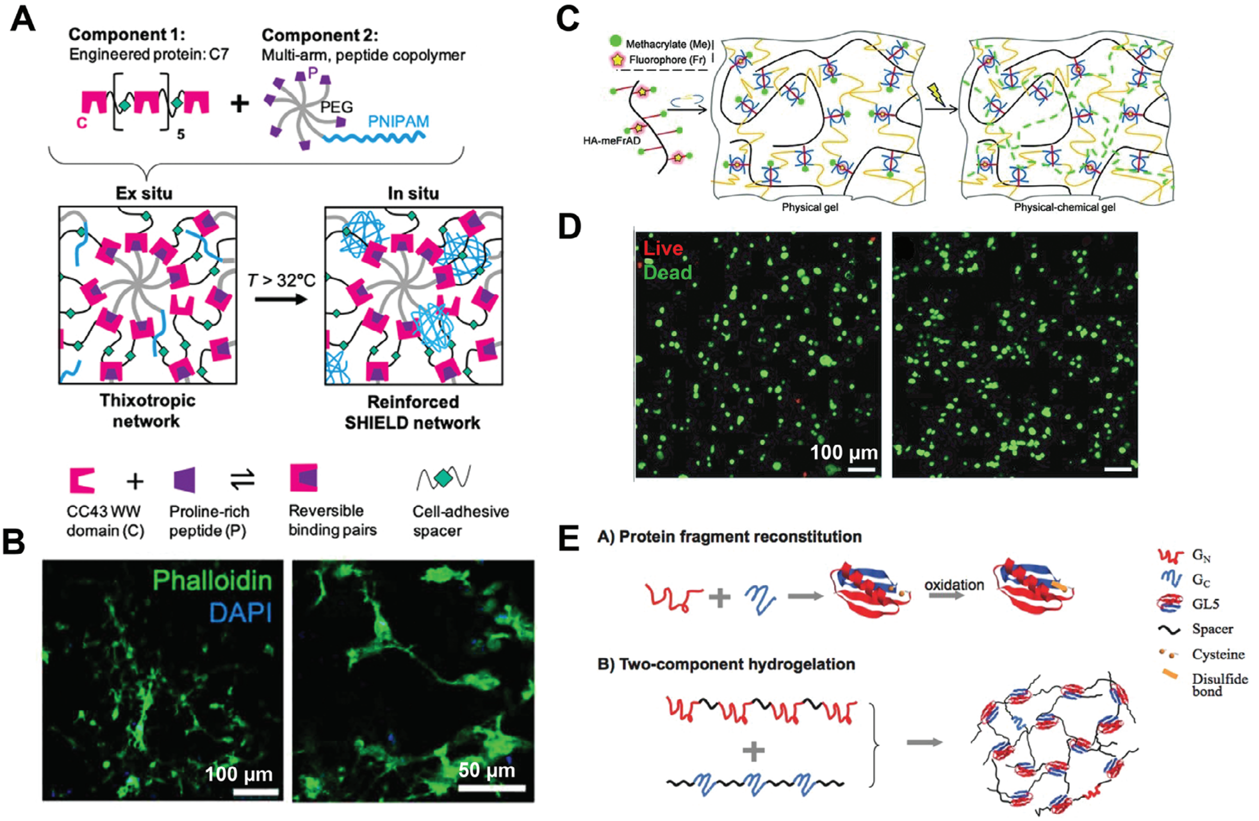

Figure 5. A) Design schematic of SHIELD hydrogel with thermos-responsive polymer reinforcement. Component 1 is C7, an engineered protein composed of seven repeats of CC43 WW domains (C, pink) separated by hydrophilic spacers containing cell-adhesive peptides (green). Component 2 is a multi-arm, peptide copolymer, 8-armed polyethylene glycol (PEG) tethered with proline-rich peptides (P, purple) and a thermo-responsive polymer (PNIPAM).[74] Copyright 2020, American Association for the Advancement of Science. B) Representative fluorescent images of Schwann cell morphology in SHIELD gels after 3 days in culture. Green, F-actin; blue, nuclei. Reproduced under the terms of the CC-BY license.[74] Copyright 2020, American Association for the Advancement of Science. C) Design schematic of DnL hydrogels with light-induced photocrosslinking reinforcement. Dock peptides (blue) and Lock peptides (pink) hetero-assemble to form a network with methacrylate functional groups (green) for secondary covalent crosslinking.[76] Copyright 2013, John Wiley and Sons. D) Mesenchymal cells encapsulated and cultured in DnL gels without (left) and with secondary photocrosslinking (right). Reproduced with permission.[76] Copyright 2013, John Wiley and Sons. E) Design schematic of protein-engineered GL5 hydrogels with disulfide bond reinforcement. G_N (red) and G_C (blue) protein fragments hetero-assemble to form GL5 folded structures that can be reversibly stabilized by formation of disulfide bonds (yellow). Reproduced with permission.[77] Copyright 2015, John Wiley and Sons.

formed through one-component self-assembly strategies, two-component hydrogels still typically suffer from weak mechanical properties and low stability due to high erosion rate. Here, we discuss several representative systems and common strategies to improve mechanical properties.

As an example, a mixing-induced, two-component hydrogel (**MITCH**) was designed with two distinct engineered proteins that hetero-assemble based on interaction between WW domains and proline-rich domains.[69] WW domains fold into antiparallel β-sheet structures and reversibly bind to proline-rich peptides under physiological conditions.[70] The mechanical properties of MITCH can be readily controlled by varying the number of WW and proline-rich domains and tuning the domain association energy through varying amino acid sequence. Due to the transient nature of physical crosslinking, these hydrogels are viscoelastic, and shear-thinning, making them suitable for injectable stem cell transplantation and 3D neural stem cells growth and injectable stem cell transplantation.[71,72] However, the relatively weak and transient peptide-peptide crosslinks resulted in a low modulus (< 50 Pa) and rapid degradation that limits therapeutic applications. To overcome this challenge, two different physical crosslinking mechanisms were designed into a shear-thinning hydrogel for injectable encapsulation and long-term delivery (**SHIELD**).[73,74] To reinforce the network formed by peptide–peptide hetero-assembly, thermally triggered aggregation of poly (N-isopropylacrylamide) (**PNIPAM**) grafted copolymers was designed into the hydrogel (**Figure 5A**). The resulting dual-network hydrogel had significantly improved material stability and cell retention.

As an alternative method to further reinforce and stabilize physically crosslinked hydrogel networks, chemical crosslinks can be introduced as a secondary crosslinking strategy. An example of this design strategy is the Dock-and-Lock (**DnL**) system that forms an injectable, shear-thinning hydrogel using molecular recognition interactions between two components followed by light-induced chemical crosslinking.[75] One of the components is derived from a subunit of cAMP-dependent kinase A that can dimerize (docking step), and the second component is derived from the anchoring domain of A-kinase anchoring protein (AD) (locking step). Mixing these two components results in a physical hydrogel, albeit with low storage modulus and short-term stability. To stabilize the physically-crosslinked hydrogel, covalent crosslinks were induced through light-initiated radical polymerization of methacrylate functional groups (Figure 5B).[76] The secondary crosslinked hydrogel retained the original shear-thinning properties with improved long-term stability, and a tenfold increase in the stiffness compared to the original formulation.

While the modified DnL system used permanent chemical crosslinks, others have incorporated reversible chemical crosslinks into hetero-assembling protein hydrogels. For instance, a modular, two-component protein hydrogel based on the protein GL5 was stabilized through disulfide chemical bonds that can respond to the reduction-oxidation state of the microenvironment. The full-length folded protein GL5 was split into two fragments, G_N and G_C that can spontaneously hetero-assemble into GL5. Multiple repeats of the G_N and G_C fragments were designed into two modular engineered proteins to create a physically crosslinked, two-component hydrogel (Figure 5C).[77] After hetero-assembly, the cysteine residues in the primary amino acid sequence can be oxidized to form disulfide chemical bonds, which improves the thermal stability of the hydrogel and limits its erosion. Because disulfide bonds can be reduced under certain physiological conditions, the hydrogel can be reversibly converted between physically and chemically crosslinked networks. This demonstrates the potential for dynamically tuning the mechanical properties of hydrogel materials in the future.

In another interesting biological application, two-component assembly can be used for growth factor delivery. For example, a protein-based hydrogel was developed via the assembly of VEGF and heparin modified PEG. VEGF dimerizes and binds to heparin, resulting in two-component physical crosslinking of the hydrogel.[78] This material can be used for targeted delivery of growth factors, with VEGF released from the gel increasing the proliferation of VEGF-responsive cell lines.[78] Future work can focus on the *de novo* design of bio-orthogonal, hetero-assembling peptide pairs, which could enlarge the library of potential two-component hydrogel systems. Computational methods to design protein interactions has led to the identification of peptide pairs that can readily form their target heterodimeric complexes upon mixing.[79] This opens up a completely new and accessible library of peptide sequences for novel protein-engineered biomimetic cellular scaffolds with bespoke materials properties.

2.2.4. Chemical Crosslinking of Protein Hydrogels

While physically crosslinked hydrogels are typically shear-thinning and self-healing, they can rapidly erode and tend to have weak mechanical properties. In comparison, chemical crosslinking stabilizes hydrogels by forming covalent bonds between polymer chains, limiting erosion and stiffening the network. A variety of chemical crosslinking approaches has been applied to different protein systems, and we have broadly divided them into three categories: 1) by crosslinking recombinant proteins through the intrinsic chemical reactivity of their amino acid side chains, 2) by modifying recombinant proteins to present non-canonical chemical functional groups, or 3) by designing recombinant proteins to include peptide sequences that are substrates for enzymatic ligation.

2.2.5. Crosslinking Through Intrinsic Reactivity of Canonical Amino Acids

Ionizable amino acid side groups (e.g., the thiol side-chain of cysteine, ϵ-amine of lysine, and the phenol-hydroxyl of tyrosine) provide site-specific targets for cross-linking reactions and hydrogel formation. As described in the previous section, the reaction between two thiol groups present on cysteine residues forms a reversible disulfide bond that can chemically crosslink proteins into a redox-responsive hydrogel.[80] However, this reaction is quite slow at physiological conditions, and the gelation can take several hours.[80] As an alternative to disulfide bonds, a bi-functional crosslinker can be added with terminal vinyl groups.[16] The reaction of vinyl and thiol groups, in a Michael addition reaction, is relatively rapid, leading to faster hydrogel formation. This strategy of crosslinking is often applied to protein-polymer systems to rapidly form stable hydrogels, such as a RLP-PEG biomimetic hydrogel used for fibroblast culture.[81]

The primary amines present on lysine can be chemically crosslinked using well established bifunctional *N*-hydroxysuccinimidyl (**NHS**) ester chemistry. Lysine can be readily incorporated in ELP during recombinant expression, and the amide chemical crosslinks aid in stabilizing the amorphous hydrogel.[82,83] However, the NHS ester is prone to hydrolysis during the crosslinking reaction, potentially resulting in poor conjugation efficiency. As an alternative, β-[tris(hydroxymethyl) phosphino] propionic acid (**THPP**) is a trifunctional crosslinker that reacts with primary and secondary amine groups to form stable covalent crosslinks through a Mannich-type condensation reaction.[84] THPP crosslinked ELP hydrogels showed good cytocompatibility and successful culture of multiple cell types including fibroblasts and cardiomyocytes.[85] In another case, a RLP-based hydrogel with heparin-binding domains rich in lysine (KAAKRPKAAKDKQTK) was rapidly crosslinked with THPP, resulting in tunable shear moduli ranging from 500 to 10 000 Pa.[86] Recently, to overcome the complicated chemical synthesis of THPP, an alternative, commercially-available, trifunctional crosslinker, tetrakis (hydroxymethyl) phosphonium chloride (**THPC**) has been used as an amine crosslinker.[87] The resulting hydrogels showed tunability of storage modulus from 300 to 3000 Pa, and supported the differentiation of embryonic stem cells and neural progenitor cells.[87,88]

Another chemical crosslinking strategy for canonical amino acids is the formation of di-tyrosine crosslinks upon catalysis with a Ru(II)-catalyst or horseradish peroxidase-hydrogen peroxide.[89] The Ru(II)-catalyzed reaction happens rapidly and efficiently under low-energy visible light, making it highly cell compatible.[90] Using this crosslinking strategy, titin-based hydrogels were designed as biomimetic scaffolds with tunable stiffness and energy dissipation to study cell mechanotransduction.[91] Interestingly, retinal pigment epithelial cells seeded on top of the chemically-crosslinked, titin-based hydrogels were more responsive to energy dissipation properties compared to scaffold stiffness, leading to reduced nuclear translocation of yes-associated protein (**YAP**), a marker of mechanosignaling.[91]

2.2.6. Crosslinking Through Non-Canonical Chemical Groups

While the inherent reactivity of amino acids lends itself well to chemical crosslinking, the reactions are often nonspecific and can have off-target effect on the cysteine, lysine, and tyrosine residues present on the surface-proteins of encapsulated cells. Furthermore, there is a large library of cell-compatible crosslinking reactions with a range of tunable kinetic and thermodynamic parameters.[92,93] To access this broader diversity of chemical reactivity, non-canonical chemical groups not present in naturally occurring amino acids have been incorporated into protein hydrogels to achieve more selective and efficient crosslinking.[94–97] For instance, silk protein can be modified with noncanonical vinyl sulfone groups (SilkVS) to create a biomimetic scaffold with site-specific crosslinks. Due to the diverse chemical reactivity of vinyl sulfone groups, three different crosslinking strategies (Michael-type addition, enzymatic crosslinking and photocrosslinking) can be applied to achieve gelation and enable further hydrogel modification (**Figure 6A**).[98] In another example, ELP hydrogels containing tyrosine can be modified with azide and alkyne groups, which then react via the bio-orthogonal strain-promoted azide–alkyne cycloaddition (**SPAAC**) reaction (Figure 6B). Due to the bio-orthogonal nature of the SPAAC cross-linking reaction, gelation of these materials is highly specific, resulting in high viability and phenotypic maintenance of encapsulated cells.[95] To eliminate the additional step of modifying canonical amino acids, noncanonical amino acids can be directly incorporated into the polypeptide backbone during recombinant expression.[99,100] For example, a photoreactive noncanonical amino acid, *para*-azido phenylalanine (*p*AzF), genetically encoded into the ELP backbone, was used to create hydrogel microparticles after UV activation.[101,102]

The native ECM is a viscoelastic environment that can be remodeled by cells as they push and pull on the surrounding biopolymers. Physically-crosslinked hydrogels typically display inherent viscoelastic properties due to the reversibility of their crosslink. However, while chemically crosslinked hydrogels are more stable, the crosslinking strategy often limits or prevents viscous remodeling. To overcome this, reversible, dynamic covalent chemistry (**DCC**) is an emerging crosslinking strategy to design hydrogels with viscoelastic properties for cell culture,[106,107] injectable therapies,[108,109] and bioprinting.[110] For instance, an ELP-hyaluronic acid (**HELP**) hybrid hydrogel was developed using DCC hydrazone crosslinks formed by the reaction of aldehyde and hydrazine functional groups.[111] HELP hydrogels with faster DCC forward and reverse reaction rates displays notably faster stress relaxation rates, which were found to influence the differentiation of neural progenitor cells (Figure 6C), demonstrating the significance of designing viscoelasticity into biomimetic scaffolds.[103]

2.2.7. Crosslinking Through Unique Peptide Sequences

An alternative crosslinking strategy that is particularly well suited to engineered protein materials is the use of unique sequences that serve as substrates for enzymatic crosslinking reactions. This method offers the advantage of chemical crosslinking without requiring further chemical modification or addition of exogenous crosslinker molecules. For example, transglutaminase has been utilized for site specific crosslinking of PEG-peptide hydrogels as well as coupling of cell-adhesive ligands.[112,113] In a more recent demonstration, a covalent crosslink was rapidly and stably introduced into a hydrogel by the interaction of SpyCatcher/SpyTag, a split protein pair derived from the second immunoglobulin-like collagen adhesion domain (CnaB2).[114] The peptide SpyTag can spontaneously react with the protein SpyCatcher to form an intermolecular isopeptide bond between the pair. Using a multi-functional SpyCatcher-ELP polypeptide and a multifunctional SpyTag-ELP polypeptide, a stable ELP-based hydrogel was formed at physiological conditions, crosslinked via the isopeptide bond induced by SpyCatcher/SpyTag.[115]

In further work, a protein-based hydrogel with both chemical and physical crosslinks was designed using SpyCatcher/SpyTag chemical crosslinking and coiled-coil leucine-zipper physical crosslinking.[104] This double network of crosslinks enabled the independent tuning of stress relaxation half-life (1–1000 s) without modifying hydrogel stiffness ($E \approx 2$ kPa), to decouple mechanical regulation events in 3D cell culture (Figure 6D). In another example, *de novo* designed protein building blocks were crosslinked either by SpyCatcher/SpyTag chemical crosslinks or by heterodimer physical crosslinks to study how molecular parameters govern macroscopic viscoelasticity (Figure 6E).[105] Several different molecular parameters, including choice of crosslinker peptides, length of building block peptides, and flexibility of linker peptides were all found to alter the rheological properties of the resulting protein hydrogels, demonstrating the versatility of engineered protein materials.[105]

In addition to the SpyCatcher/SpyTag system, other unique amino acid sequences are being developed that hold the potential for use as crosslinking strategies in biomimetic cellular scaffolds.[116] In a hydrogel system composed of hyaluronic acid (**HA**) and ELP, the network was crosslinked using traditional SpyCatcher/SpyTag; however, they also incorporated SnoopTag/SnoopCatcher sequences that can form a covalent bond through spontaneous transamidation to attach additional protein ligands.[117] In the future, bioinformatics analysis and computational predictions can be used to explore novel peptide sequences to achieve site-specific crosslinking in engineered-protein hydrogels.[118]

2.3. Engineering Cellular Bioactivity

Especially for engineered protein-based hydrogels that will encapsulate cells, a common design element is the inclusion of bioactive peptides that can interact with cell-surface proteins or respond to cell-secreted enzymes. These bioactive peptides are commonly truncated versions of amino acid sequences found in full-length native proteins. Because peptide conformation, and hence peptide binding affinity, is sensitive to the surrounding amino acid sequence, a bioactive peptide will not have the exact same bioactivity as the full-length native protein from which it is

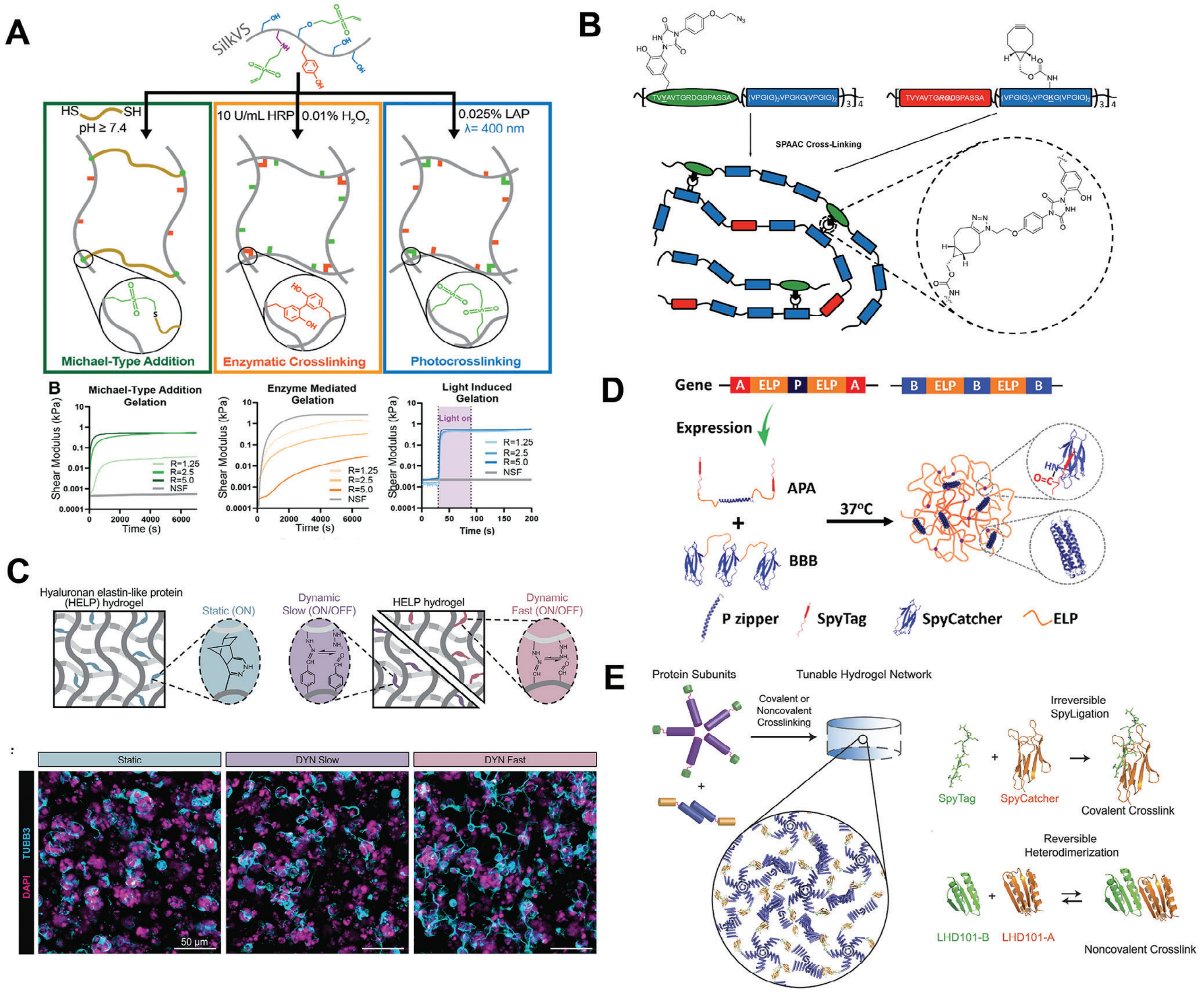

Figure 6. A) A silk protein modified with vinyl sulfone functional groups (SilkVS) has the potential to crosslink via three distinct mechanisms: thiol-ene Michael-type addition (green), enzymatically induced dityrosine formation (orange), and photocrosslinking (blue). The gelation kinetics were tracked using rheometer to monitor shear modulus. Reproduced with permission.[98] Copyright 2023, Elsevier. B) Schematic of an ELP based hydrogel formed by bio-orthogonal cross-linking via a SPAAC reaction. Reproduced with permission.[95] Copyright 2017, American Chemical Society. C) (Top) Schematic of protein hydrogels designed to have static, slow dynamic, or fast dynamic covalent crosslinks. (Bottom) Neural progenitor cells showed enhanced neurite outgrowth in dynamic fast stress–relaxing hydrogels. Reproduced under the terms of the CC-BY license.[103] Copyright 2023, American Association for the Advancement of Science. D) Schematic of the block-copolymer design of two engineered proteins using ELP, SpyTag (A), SpyCatcher (B) and physically assembling P zipper (P) domains. When mixed together, the two proteins form hydrogels with both chemical and physical hydrogels. Reproduced with permission.[104] Copyright 2023, American Chemical Society. E) Schematic of a hydrogel that incorporates *de novo*-designed protein building blocks. The first recombinant protein construct consists of a dimer core (blue) and complementary crosslinking unit (yellow). Hydrogel formation is achieved either by irreversible, covalent crosslinking with SpyCatcher/SpyTag or by reversible, non-covalent crosslinking through heterodimerization of the LHD101A/B pair. Reproduced under the terms of the CC-BY-NC-ND license.[105] Copyright 2024, National Academy of Sciences.

derived. Thus, modification of the adjacent amino acid sequences within an engineered protein can greatly impact the functionality of these bioactive peptides.[119,120] Including bioactive peptides in protein-engineered hydrogels allows for the design of biomimetic scaffolds that can influence a myriad of cell behaviors, including migration, proliferation, and differentiation. In this section, we discuss recent advances in engineering cell-binding domains (i.e. cell-adhesive ligands), cell-degradation domains, and growth factor-derived domains.

2.3.1. Cell-Binding Domains

Cells bind and adhere to a variety of ECM proteins including fibronectin, laminin, and collagen. Several short peptide motifs have been identified within native, full-sequence ECM proteins that present ligands for direct binding to cell-surface receptors.[144] Incorporating these peptide ligands into the polypeptide backbone is a common strategy to impart cell-adhesion functionally into engineered protein-based hydrogels

(**Table** 1; peptides presented in this table are the minimally active sequences known to impart bioactivity, and variations of these motifs with extended sequences are frequently used). Cell-adhesive motifs have been identified from many of the major protein components of the ECM, including laminin (e.g. IKVAV, YIGSR, PPFLMLLKGSTR)[124–127] and collagen (e.g. DGEA, GFOGER, GEFYFYDLRLKGDK).[128,129] The most widely used and studied cell-adhesive peptide is the RGD motif, which is found in fibronectin as well as several other ECM proteins.[145] While the RGD tripeptide alone is often sufficient to induce cell-adhesion, inclusion of additional flanking amino acids is frequently used to enhance cell binding.[146] RGD bioactivity can be further increased by the co-presence of PHSRN, fibronectin-derived motif known as the synergy site that cannot induce binding on its own but can significantly enhance integrin binding affinity when presented at a specific distance from an RGD peptide.[147] Alternatively, RGD bioactivity can be enhanced through incorporation of other cell-adhesive sequences found in different ECM proteins. For example, a library of ELP-based protein hydrogels was designed incorporating cell-adhesive ligands from fibronectin (RGD), laminin (IKVAV, YIGSR), collagen (DGEA), and tenascin-C (PLAEIDGIELTY and VFDNFVL).[132] Through combinatorial experiments, it was shown that RGD with PLAEIDGIELTY significantly increased neurite outgrowth from embryonic dorsal root ganglion compared to RGD-only gels at equivalent ligand concentrations (**Figure** 7A).[132]

Importantly, one of the advantages of engineered protein-based hydrogels is the ability to decouple biochemical and mechanical properties, to parse out the individual contributions to cellular function. This is achieved through modular protein design, where the crosslinking and structural domains are distinct from the cell-adhesive bioactive domains. In one instance, a family of hydrogels with increasing RGD ligand concentration (0, 0.5, 0.75, and 1mᴍ) and tunable mechanics was used to evaluate the individual effect of cell-adhesion and matrix stiffness on the growth of human intestinal organoids.[148] In another example, the cell-binding domains were sequentially added and subtracted on-demand using a reversible protein fragment reconstitution, similar to that employed by two-component hetero-assembly crosslinking.[149] Ligands decorated with an amino acid sequence can bind to the hydrogel through stable peptide interactions (Figure 7B). This interaction can then be reversed through the addition of a reduction agent. While this strategy can be used to study cellular response to matrix factors at specific time intervals, it can also be leveraged for enzymatic free recovery of adherent cells. Human lung fibroblasts cultured on the surface of an RGD-decorated peptide-hydrogel were reversibly released with high viability upon removal of the cell binding motif.[149] This opens a new strategy of designing on-demand control of biochemical ligands in protein-based hydrogels with independent tuning of chemical and physical properties.

Many other cell-binding motifs have been reported and are described in previous reviews.[150] New bioactive domains are still being discovered and explored. As cellular response to mixtures of cell-binding motifs are often non-addictive and non-intuitive.[151] there is immense potential to combine multiple cell-binding motifs to build up complexity within engineered protein hydrogels.

2.3.2. Cell-Induced Degradation Domains

Cells remodel their extracellular environment through deposition of ECM components and secretion of enzymes that modify the ECM, such as crosslinking enzymes and degradation enzymes. Proteolytic enzymes (i.e. proteases) are of particular importance for engineered protein hydrogels, as they enable the biodegradation of the materials through cleavage of peptide bonds, which are present in all protein-based hydrogels. Many different types of proteases exist, and they have differing catalytic efficiency depending on the primary protein sequence and differing expression levels throughout the body.[152] Thus, the rate of proteolytic degradation for a protein-engineered gel will depend on its amino acid sequence and which active proteases are present. For example, native elastin is resistant to most proteases except elastase.[153] so inherent degradability of ELP-based hydrogels is relatively slow in most tissues. This opens up opportunities to control the cell-induced degradation rate by including amino acid sequences that are specifically targeted by certain proteases. Incorporation of peptide domains that are substrates for proteolytic activity into an engineered protein hydrogel (Table 1) is a common strategy to design materials that enable cell-mediated remodeling. Matrix metalloproteinases (**MMPs**) are a large family of proteases that can degrade a wide variety of ECM proteins including collagen, elastin, fibronectin, and laminin. Peptide motifs that are sensitive to MMP-mediated degradation have been incorporated into protein hydrogel applications.[154] and polymer-protein hybrid systems.[155] For example, MMP degradable domains can be incorporated into an ELP hydrogel for myocardial infarction applications.[156] Including an MMP-degradable sequence into an ELP hydrogel permitted stromal cell invasion and endothelial cell sprouting in vitro, and showed less fibrosis and more angiogenesis when delivered in an animal model.[156]

Besides MMP sensitive sequences, other degradable domains have been designed into protein chains (Table 1) for a variety of applications, including studies of how matrix-degradation mediates cell maturation.[96] or spatiotemporal control of angiogenesis.[135] For example, ELP hydrogels have been designed with three different sequences sensitive to urokinase plasminogen activator (**uPA**) to enable tailored degradation kinetics for neurite outgrowth.[128] Furthermore, these different ELP sequences were combined together to create composite hydrogels with distinct regions that respond differentially to enzymes, resulting in 3D patterned hydrogels with control of spatial and sequential degradation.[137] In a more recent work, a coaxial elastin-like recombinamer (**ELR**) tubular construct was designed with inner and outer layers that displayed faster and slower proteolytic degradation rates, respectively (Figure 7C).[135] When conjugated with the VEGF-mimetic QK peptide in the inner region, the scaffold promoted sequential cell invasion and migration, which occurred first in the fast-degrading layer and spread to the slow-degrading layer (Figure 7D).[135] This opens future opportunities in designing engineered protein hydrogels with tunable degradation rates for guiding spatiotemporal vascularization and studying multistep cellular development.

Table 1. Bioactive motifs incorporated in engineered protein hydrogels.

Category	Bioactive peptide motifs	Origins	Functions	References
Cell adhesive peptides	RGD	Fibronectin	Cell adhesion	[66, 115]
	PHSRN	Fibronectin	Cell adhesion	[121]
	EEIQIGHI-PREDVDYHLYPG (CS5 domain)	Fibronectin	Cell adhesion	[122, 123]
	IKVAV	Laminin (alpha1)	Cell adhesion, neurite growth and nerve regeneration	[124,125]
	YISGR	Laminin (beta 1)	Cell adhesion	[126, 127]
	PPFLMLLKGSTR	Laminin (alpha 3)	Cell adhesion	[66, 121]
	GFOGER	Collagen	Cell adhesion and osteogenesis	[128]
	DGEA	Collagen I	Cell adhesion and osteogenesis	[129]
	DWVIPPISCPENEKGPFPKNL VQIKSNRDK	E-cadherin	Cell-cell adhesion	[130, 131]
	HAVDI	N-cadherin	Cell-cell adhesion	[130, 131]
	PLAEIDGIELTY	Tenascin-C	Cell adhesion	[132]
	VFDNFVL	Tenascin-C	Cell adhesion	[132]
Cleavable peptides	GPQGIWGQ	Collagen	Matrix metalloproteinase (MMP) cleavable	[133, 134]
	GTAR, TSHR, DRIR	Fibronectin	Urokinase plasminogen activator (uPa) cleavable	[96, 124, 135, 136]
	FFSR, SILR, DNRR	Fibronectin	Tissue plasminogen activator (tPa) cleavable	[137]
Growth factor derived peptides	KLTWQELQLKYKGIGGG (QK)	VEGF	Promote endothelial cell adhesion and angiogenesis	[124, 138–140]
	NCKHQCTCIDGAVGCIPLCP (V2)	Heparin binding protein	Supports $\alpha_v\beta_3$ mediated endothelial cell adhesion	[141]
	KIPKASSVPTELSAISTLYL	Bone morphogenetic protein 2 (BMP-2)	Promote osteogenic differentiation	[142]
	KPSSAPTQLN	BMP-7	Promote bone and cartilage formation	[143]

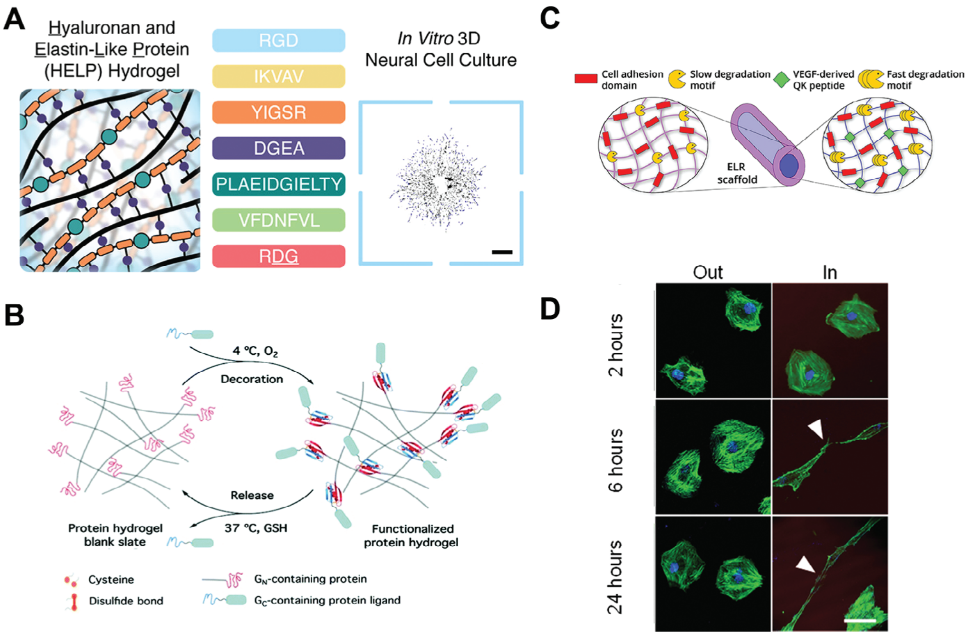

Figure 7. A) Schematic of hyaluronan and elastin-like protein (HELP) hydrogel (left). A library of engineered ELPs with different cell-adhesive peptide ligands were incorporated into the HELP hydrogels (middle). A representative digital quantification of neurite out-growth in a HELP hydrogel with RGD peptides (right). Reproduced with permission.[132] Copyright 2023, American Chemical Society. B) Schematic of a hydrogel design that can be decorated with a protein ligand using assembly and disulfide bonding between G_N and G_C protein fragments. Decoration occurs under oxidizing conditions, while ligand release occurs under reducing conditions through the addition of glutathione (GSH). Reproduced with permission.[149] Copyright 2019, Royal Society of Chemistry. C) Design of a coaxial binary ELR tubular scaffold with fast and slow degradation kinetics. The inner layer is fast degrading and conjugated with the VEGF-mimetic peptide (QK), while the outer layer is slow-degrading. D) Morphology of endothelial cells cultured on top of the ELR hydrogels with slow-degrading kinetics (Out) or fast-degrading kinetics with the QK peptide (In) over time. Reproduced with permission.[135] Copyright 2021, Elsevier.

2.3.3. Growth Factor-Derived Domains

Cell-secreted growth factors play an important role in guiding cell migration, proliferation, and morphogenesis, and several peptides derived from growth factors have been identified and designed into protein hydrogel design (Table 1). These growth factor-derived domains can be either bioconjugated onto an already expressed protein or directly encoded into the polypeptide backbone. In the first strategy, physical or chemical conjugation is used to tether the growth factor-derived peptides into the protein system.[124,149] For example, a VEGF-mimetic peptide QK has been covalently immobilized onto ELP hydrogels using THPC crosslinking chemistry.[138] The bioactive hydrogel with tethered QK peptides enhanced proliferation of HUVEC cells and 3D outgrowth of HUVEC spheroids compared to simply dosing the medium with the same concentration of soluble QK peptide. While bioconjugation reactions are quite versatile, site selectivity and the degree of functionalization can be difficult to control, and the peptide activity can be negatively impacted by the chemical reaction and steric bulk of appended chemical groups. The second strategy for incorporating growth factor-derived peptides can overcome these problems by genetically engineering the sequence of desired peptides directly into the protein design. Peptides derived from BMP-2 and BMP-7 have been incorporated into the backbones of short self-assembly peptides and RLP polypeptides, respectively.[142,143] Both systems demonstrated bioactivity by enhancing cell differentiation. Importantly, to maintain the bioactivity and bioavailability of growth factor-derived peptide sequences, the method of crosslinking should be carefully selected to avoid off-target chemical reactions and maintain peptide flexibility.[157]

2.4. Engineering Stimuli-Responsive Properties

The native ECM is a highly dynamic and heterogeneous environment that is continuously being remodeled by resident cells. These remodeling events occur both in healthy and diseased tissues, for example, to promote healthy wound healing and during abnormal tissue morphogenesis, such as cancer progression.[158] These remodeling events include both changes in biochemical content and biomechanical properties of the matrix. Thus, inspired by the native matrix, dynamic engineered protein-based hydrogels have been designed to be capable of responding to specific triggers such as temperature, light, and enzymatic activity.

2.4.1. Temperature-Responsive Materials

Changes in temperature can be used as an environmental trigger for bio-responsive hydrogel design.[36,159–161] One of the most common building blocks for temperature-responsive protein hydrogels is ELP, which can be engineered to self-assemble into protein-rich aggregates at biologically-relevant

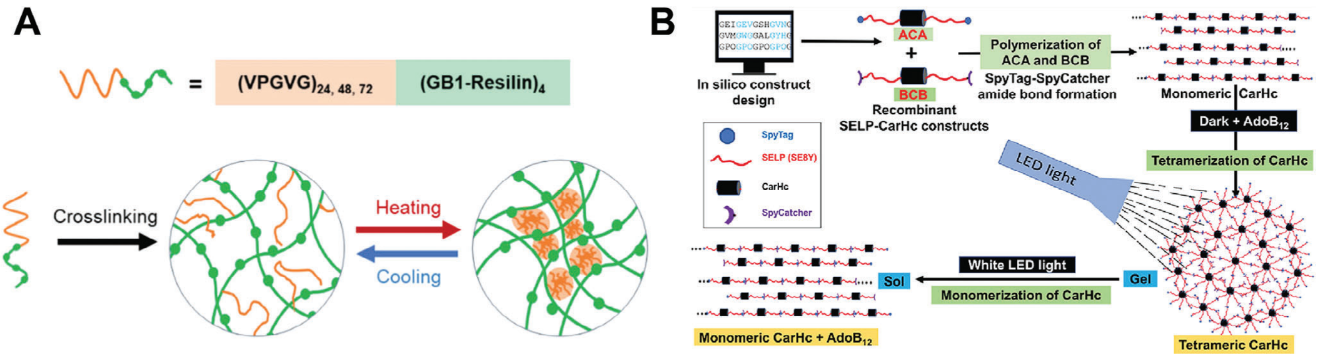

Figure 8. A) Thermal-responsive hydrogels were created using elastin-like domains, resilin-like domains, and a globular GB1 domain. Upon heating, the elastin-like domains underwent thermal aggregation to reversibly strengthen the hydrogel. Reproduced with permission.[37] Copyright 2023, American Chemical Society. B) Schematic of light responsive SELP sequence design. The proteins are polymerized through SpyTag-SpyCatcher chemistry and assembled into a hydrogel network through light sensitive AdoB$_{12}$ and CarH$_C$ domain binding. Reproduced with permission.[163] Copyright 2021, Elsevier.

temperatures.[37,162] For example, a thermally-responsive block-copolymer was designed to reversibly stiffen on demand by combining temperature-sensitive ELP peptides with a structural globular protein domain (**GB1**) and unstructured RLP peptides.[37] By increasing the temperature over ELP's transition temperature, the ELP side chains underwent aggregation, leading to a reversible secondary physical crosslinking and increasing the material storage modulus (**Figure** 8A). Importantly, by changing the ELP chain length and the relative concentrations of each of the peptide (ELP, GB1, and RLP), the transition temperature and the resulting hydrogel mechanical properties were tuned.[37] This enables the design of dynamic protein hydrogels with on-demand changes in stiffness and network structure for tissue engineering and mechanobiology applications.

2.4.2. Light-Responsive Materials

Light-responsive hydrogels are of particular interest because their material properties can be spatiotemporally tuned in real time with minimal disruption to encapsulated cells. Identification of proteins sequences that are responsive to light has led to the development of engineered protein-based hydrogels with on-demand, light-responsive properties. For example, hydrogels with switchable mechanical properties have been designed using protein domains that undergo reversible, photo-induced conformational changes, including CarH$_C$,[163,164] Dronpa,[165] and UVR8 domains.[166] These light-induced, dynamic changes in hydrogels mechanics have been used to provide spatiotemporal control of cell migration rate.[165] In another study, a photo-responsive SELP hydrogel was designed as a cytocompatible scaffold for reversible encapsulation and release of cells. The SELP was genetically encoded with SpyTag/SpyCatcher protein domains flanking a light-sensitive CarHc domain (Figure 8B).[163] The SpyTag/SpyCatcher sequences enabled synthesis of long polymer chains that could be crosslinked into a gel through interaction of CarHc with AdoB12, a biologically active version of vitamin B12. The resulting hydrogel was stable in the dark environment of the cell culture incubator; however, upon stimulation with light, the CarHc reverts back to monomeric form, resulting in loss of gelation.[163] This material provided a useful platform for cell culture, as the encapsulated cells could be easily recovered from the gel at the desired time point by simply stimulating the system with light. In addition to dynamic 3D culture matrices, light-responsive hydrogels can also be used as injectable materials with viscoelastic properties. Recently, a light-responsive protein interaction between light-oxygen-voltage-sensing domain 2 (**LOV2**) and its binding partner ZDark1 (zdk1), named as LOV-TRAP, was used as a non-covalent crosslinker.[167] In another notable protein engineering example, a blue light inducible SpyTag system (named BLISS) was designed with photo-kinetic control over the SpyCatcher/SpyTag enzymatic reaction.[168] In the future, complex hydrogels with light-responsive, non-covalent crosslinks maybe useful as stable cellular scaffolds to study cell responses to viscoelastic matrix properties.

2.4.3. Enzyme-Responsive Materials

Recently, engineered protein-based hydrogels have been designed to modulate their properties in response to enzymes that are not typically found in mammalian systems.[169] While we previously discussed the design of hydrogels that respond to cell-secreted enzymes, such as proteases (Section 2.3.2), these new enzyme-responsive materials require the addition of an exogenous enzyme. This allows for complex hydrogel design that can be triggered on-demand in a cell-orthogonal manner. In particular, sortase A (**SrtA**) is a gram-positive bacterial transpeptidase that natively cleaves a pentapeptide motif present in bacteria that is not commonly found in mammalian proteins. Importantly, because the peptide motif recognized by SrtA is uncommon in mammalian cells, SrtA-mediated degradation is cytocompatible.[169–171] Many different variants of SrtA enzyme have been engineered to have improved stability and selectivity for different peptide substrates.[170] In one example, different sortase-recognized peptides (e.g. LPETG, LAXTG, and LPXSG) were incorporated into a hydrogel to achieve sequential degradation and cell release from a complex, trilayered, biomimetic scaffold (**Figure** 9A).[172] This allowed for retrieval of cells for phenotypic analysis from different regions of the gel, and preserving spatial information (Figure 9B). In another example, enzyme responsive properties were designed into peptide

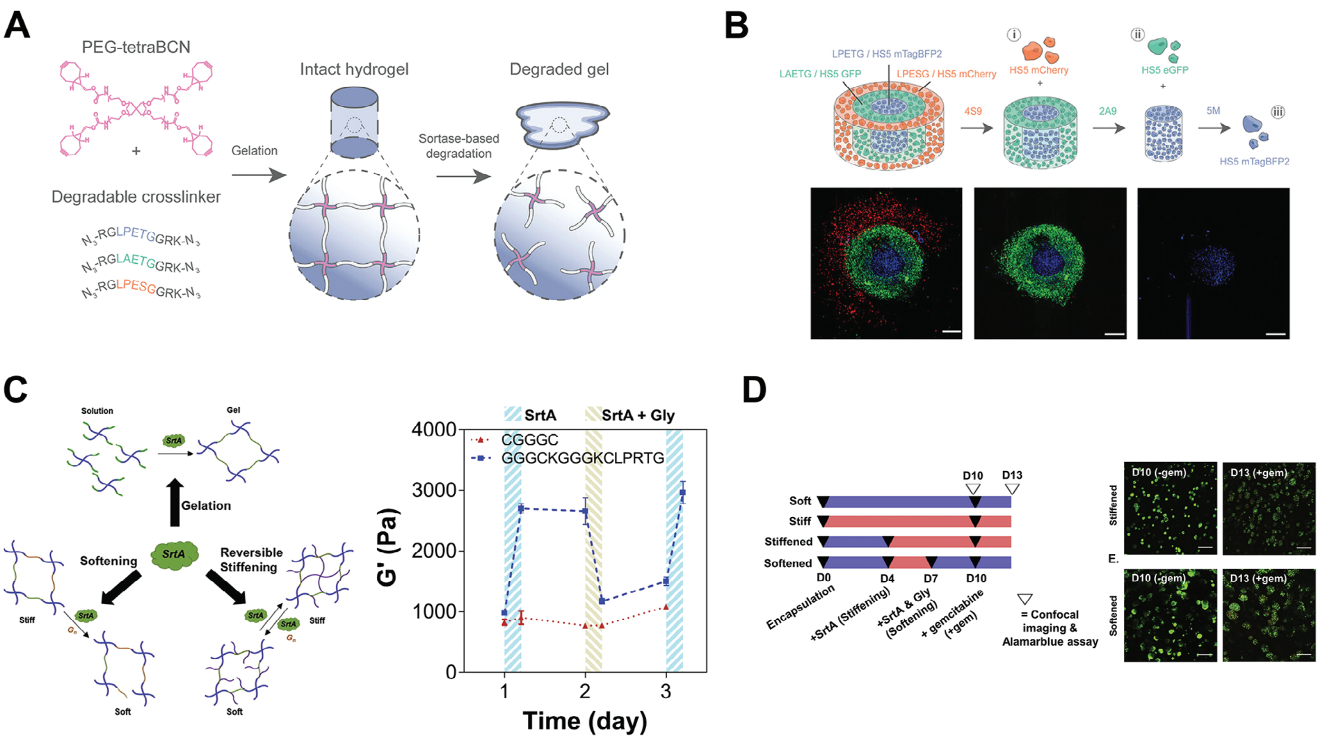

Figure 9. A) Enzyme-responsive hydrogels are formed by mixing PEG-tetraBCN with azide-flanked peptide crosslinkers that include sortase (SrtA) substrate sequences. Upon exposure to exogenous, substrate-matched SrtA, the gel becomes degraded.[172] B) Spatiotemporally controlled release of HS5 human stromal cells from complex trilayered materials was achieved through multiplexed, sortase-based degradation. Reproduced with permission.[172] Copyright 2023, John Wiley and Sons. C) SrtA-sensitive peptides can be designed into hydrogels to form reversible stiffening and softening biomimetic scaffolds upon application of SrtA or SrtA with a repetitive glycine (Gly) peptide, respectively.[174] D) Cancer spheroids showed higher cell viability after gemcitabine treatment in stiffened gels, while lower cell viability in softened gels. Reproduced with permission.[174] Copyright 2020, Elsevier.

crosslinkers to achieve hydrogels with reversible control of matrix stiffening and softening.[173,174] The hydrogels stiffened by incubating with enzyme and softened by incubating with SrtA and a short-repeating glycine peptide (Figure 9C).[174] As an in vitro model of disease progression, it was found that cancer spheroids showed higher chemoresistance when cultured in a dynamic stiffening hydrogel, compared to a dynamic softening hydrogel (Figure 9D). While most examples to date have used SrtA enzymes to modulate the mechanics of synthetic polymer hydrogels using peptide crosslinkers, similar types of systems have immense potential to create protein-engineered hydrogels with on-demand control of both biochemical and biomechanical properties.

The above sections described innovative hydrogel designs that used engineered protein to respond to changes in temperature, light stimulation, or presence of exogenously-added enzymes. A number of other potential stimuli can also be harnessed to induce protein conformational changes to dynamically modulate biomechanical and biochemical properties of engineered-protein hydrogels. For example, protein domains that alternate between an extended, flexible structure and a folded, rigid structure upon exposure to reducing and oxidizing conditions, respectively, was used to create hydrogels that reversible soften and stiffen.[175,176] Similarly, many protein domains undergo large structural changes in response to binding or ions, and these types of domains have been incorporated into several clever hydrogel designs.[151] Another possibility is the used of protein building blocks that physically associate in response to environmental changes, such as shifts in ionic strength or pH.[177,178] Charged protein domains can also alter their alignment and movement in response to applied electric fields, which has been used to fabricate protein-based hydrogels with dynamic, gradient properties.[179,180] Looking ahead, we expect the library of stimuli-responsive peptide domains used in engineered-protein hydrogels to continue to expand. The broad functional diversity of peptide domains that have evolved or been designed is expansive, including both conductive and magnetic proteins.[181,182] As new stimuli-responsive peptides are introduced in to protein-engineered designs, we expect that on-demand modulation of material properties will be leverage in new and exciting ways for biomimetic cellular scaffolds.

3. Conclusion and Future Directions

Here we surveyed the peptide modules that have been used in engineered protein-based hydrogels to control the structural, mechanical, bioactive, and bio-responsive properties of precisely defined and cytocompatible biomimetic scaffolds. The design of engineered protein-based hydrogels can be thought of as occurring on two distinct levels: the molecular-design level where the primary amino acid sequence is specified, and the network-design level where crosslinking strategy and gelation conditions are selected. Looking ahead, there are exciting opportunities to expand the diversity of engineered-protein materials at both design levels.

At the molecular-design level, structural building blocks of repetitive amino acid sequences can be engineered into peptide backbones to provide tailored mechanical properties. Designing structural domains with temperature-dependent phase separation or self-assembly properties allows for the creation of finely tuned microstructures for biomimetic cellular scaffolds. As the native ECM typically includes ordered fibers, future work may combine both micro- and macro-level strategies to design fibrous, anisotropic hydrogels for more defined cell spreading, alignment, and migration. On the microscopic level, currently, many of the well-studied structural domains (e.g. ELP and RLP) are intrinsically disordered polypeptides and form uniform and amorphous hydrogels without microfiber structures. While some polypeptides (e.g. SLP and collagen-like polypeptides) can self-assemble into fibrous structures, the resulting hydrogels are often weak due to physical crosslinks and lack higher-order alignment.[183] Multiple self-assembled polypeptide sequences, including *de novo* designed sequences, can be fused together to form nanofibers in biomimetic scaffolds to provide micro-level structural architecture.[184] At the macroscopic level, long-range order can be introduced into hydrogels through development of novel fabrication and processing strategies, including stretching, extrusion, electrospinning, or electric field induced polymer alignment.[185]

As discussed above, engineered-protein hydrogels can be designed to have specific cell interactions through the incorporation of bioactive domains that contain cell-adhesive, cell-degradation, or growth factor-derived motifs. While many different bioactive sequences have been used in engineered protein hydrogels, the combined effects of two or more ligands have not been well-studied. In contrast, the native ECM is a complex mixture of multiple matrix proteins and growth factors that cooperatively provide instructions to local cells. Both mutual and cooperative interactions between integrin and growth factor receptor signaling contribute to cellular response.[186] In engineered biomaterials, the interactions between multiple bioactive peptides is frequently found to be non-additive, resulting in non-intuitive cellular responses.[187] The flexible sequence design and bioconjugation potential of engineered-protein hydrogels provides an opportunity to build modular in vitro platforms with multiple biochemical cues for detailed cell signaling studies of the crosstalk between multiple bioactive sequences.

While bioactive domains respond to cell-mediated interactions, biomimetic scaffolds with on-demand, dynamic properties can be designed to respond to external stimuli. Within this review, we discussed stimuli-responsive building blocks that respond to temperature, light, and exogenous enzymes. These user-defined stimuli can be used to trigger changes in the material properties on demand, making these hydrogels useful for evaluating cell-matrix dynamics. Currently the field has established multiple methods of inducing one-time, irreversible changes to hydrogels properties. However, further development of methods to reversibly alter hydrogel properties are needed to recapitulate the dynamic nature of the ECM.[188] For example, peptide-polymer hybrid hydrogels have been developed with reversible stiffening and softening properties under multiple light stimuli via photoisomerization of an azobenzene-containing peptide crosslinker.[189] Future work could incorporate such novel, stimuli-responsive sequences into engineered-proteins to achieve user-defined control of substrate modulus without altering the matrix composition or network connectivity. In addition to dynamic tunability of biomechanical properties, dynamic physicochemical and biochemical cues, such as reversible caging/uncaging of ligands, could also be introduced in engineered-protein hydrogels.[190] While most photoactive hydrogel designs are limited by the penetration depth of light, other stimuli, such as ultrasound, could be used to trigger engineered-protein hydrogels across a broad range of in vitro and in vivo applications.[191]

At the network design level, physical and chemical crosslinking strategies are used to stabilize hydrogel structures and engineer macromolecular mechanical properties. Physical crosslinking of the engineered-protein network through reversible peptide assembly, imparts intrinsic shear-thinning properties and a reversible sol–gel phase transition, enabling development of injectable matrices, micro-structured architectures, and viscoelastic cell substrates. To address the common challenges of limited gel stability and weak mechanical properties for these materials, future work may design peptide assembly domains with increased association affinities to stabilize the hydrogel.[192] As an alternative approach, entanglement of polypeptide chains can increase the number of physical crosslinks and create stiff and tough materials that have properties close to cartilage.[193,194] Future work can expand upon this idea to design high molecular weight polypeptide sequences that introduce entanglements.

Several chemical crosslinking strategies are commonly used for protein-engineered hydrogels, including crosslinking between intrinsic amino acid residues in the peptide backbone, crosslinking through modification of the peptide backbone with non-canonical chemical functional groups, and crosslinking by enzymatic ligation of specific peptide sequences. However, despite these advances, there is a continuing need for additional bioorthogonal, chemical crosslinking methods that are highly site-specific, efficient, and cytocompatible, in order to stabilize biomimetic scaffolds without interfering with bioactive domains or encapsulated cells.[188,195] These bioorthogonal approaches might include use of unnatural amino acids or development of non-natural ligation enzymes that react with unique peptide sequences. In addition, current chemical crosslinking strategies create static, covalent crosslinks that limit cell-mediated remodeling of the microenvironment. Although a library of dynamic covalent chemistries have been developed and applied to synthetic polymer systems,[196] very few of them have been applied to engineered-protein hydrogels.

The integration of computational models holds great promise for engineered protein design, offering valuable insights for the advancement and development of biomimetic cellular scaffolds.[197,198] A growing number of hydrogels have been designed that include peptide modules that were first evaluated computationally.[69,105,199] For example, multiscale computational modeling has been used in the *de novo* design of alpha helices and identification of ELP sequences that have the greatest molecular stability and prolonged self-assembly.[200] The resulting ELP variants exhibited tunable self-assembled structures and phase behavior, which opened up new opportunities for this programmable thermoresponsive matrix in biological applications. Future directions may also involve expanding the library of peptide building blocks to include more *de novo*-designed sequences,[201] thus enabling the creation of highly tunable hydrogels and functional properties.[202] Furthermore, mesoscale models of hydrogel network structures offer an approach to predict dynamic material properties,[203,204] facilitating the careful control and often decoupling of matrix scaffold parameters such as stiffness,[107] viscoelasticity,[205] degradation rate,[15] mesh size, and ligand density.[206] This multifaceted approach has far-reaching applications, from modeling disease progressions to developing translational therapies.[207] While significant progress has already been made, there remains an abundance of exciting opportunities in designing engineered protein hydrogels with enhanced controllability, adaptability, and biomimetic properties for cellular scaffold applications.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Y.L. performed writing – original draft and review & editing. A.E.G performed writing – review & editing. S.C.H. performed supervision, funding acquisition, writing – review & editing.

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Yueming Liu is a Ph.D. candidate in the Department of Materials Science and Engineering at Stanford University. She received her B.S. degree in polymer science and engineering from Zhejiang University in 2020. Her research interests are in the design of tunable biomaterials for 3D organoid generation. Her current work focuses on the design of an in vitro biomaterial platform to study non-alcoholic fatty liver disease progression.

Aidan E. Gilchrist is an assistant professor in the Biomedical Engineering Department at the University of California, Davis. His lab uses biomaterial approaches to identify underlying biological mechanisms that contribute to normal and abnormal tissue development, particularly in the context of stem cell aging. He earned his Ph.D. in Materials Science and Engineering at the University of Illinois at Urbana-Champaign and was a postdoctoral scholar at Stanford University. During his graduate and postdoc training periods, he has work on a broad range of protein-based gels for stem cell and organoid culture and growth.

Sarah C. Heilshorn is a professor and associate chair in the Materials Science and Engineering Department at Stanford University, where she also serves as the director of the Geballe Laboratory for Advanced Materials. Her research team integrates concepts from materials science and protein engineering to design bioinspired materials for regenerative medicine, organoid culture, and bioprinting. She is a fervent supporter of diversifying the scientific research community. She is a fellow of the American Institute for Medical and Biological Engineering and the Royal Society of Chemistry and serves on the Board of Directors for the Materials Research Society and the International Society for Biofabrication.