Recombinant protein-based injectable materials for biomedical applications

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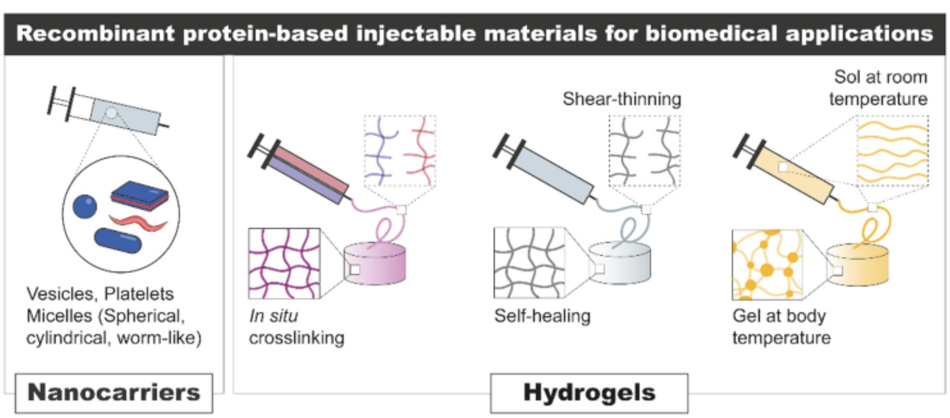
Abstract

Injectable nanocarriers and hydrogels have found widespread use in a variety of biomedical applications such as local and sustained biotherapeutic cargo delivery, and as cell-instructive matrices for tissue engineering. Recent advances in the development and application of recombinant protein-based materials as injectable platforms under physiological conditions have made them useful platforms for the development of nanoparticles and tissue engineering matrices, which are reviewed in this work. Protein-engineered biomaterials are highly customizable, and they provide distinctly tunable rheological properties, encapsulation efficiencies, and delivery profiles. In particular, the key advantages of emerging technologies which harness the stimuli-responsive properties of recombinant polypeptide-based materials are highlighted in this review.

Graphical abstract



Keywords: Injectable hydrogels, Bioinspired materials, Polypeptides, Recombinant proteins, Tissue engineering, Regenerative medicine, Stimuli-responsive polymers, Drug Delivery, Nanocarriers

Contents

1. Introduction

Recombinant synthesis of protein-engineered biomaterials offers unparalleled opportunities for developing highly tunable polymeric materials for a variety of biomedical applications,[1,2] by combining the advantages and overcoming the limitations of natural and synthetic polymers.[3] Natural polymers are biodegradable and biocompatible and possess structural and compositional similarities to the native extracellular matrix (ECM),[4] but since they are often used as harvested, their chemical and physical versatility is limited and the material properties can be affected due to batch-to-batch variability.[3] Synthetic polymers are highly tunable, but they often lack biodegradability, are bioinert, and often require modification with PEG and/or bioactive peptides for improving their performance when deployed in vivo.[3,5] In contrast, genetically-engineered polypeptides, with consensus repeat sequences derived from natural proteins, provide excellent templates for facilitating the modular design of multifunctional materials.[1,3] Molecular cloning allows for monomer-level control over chain length, composition, and stereochemistry;[6] the genetically directed and high-fidelity nature of recombinant synthesis yields chemically diverse materials with monodisperse populations.

The highly customizable and modular nature of this process allows inclusion of a variety of biological and structural motifs for generating biomaterials in which mechanical properties and biological functions can be tuned independently.[3,7,8] Furthermore, protein-based polymers are biologically interactive and cell-instructive but their degradation products are nontoxic for in vivo applications.[9] Another key advantage for the use of protein-based polymers or polypeptides is that multi-stimuli-responsiveness can be encoded by altering their amino acid sequence in a highly precise fashion,[10] which can be harnessed for developing injectable materials to achieve controlled and sustained drug release. The variety of polar and non-polar properties of amino acids in the polypeptide sequences, as well as their ionic character, make protein-based polymers versatile enough to bind to therapeutical agents of hydrophilic and hydrophobic nature as well as polyanionic macromolecules such as DNA or RNA used in gene delivery therapies.[11,12] Hence, recombinant protein-based materials are extremely attractive candidates for developing controlled delivery vehicles for therapeutic agents.

Finding efficient and controlled ways to deliver biotherapeutic agents has become one of the most impactful advances in human health.[13–15] From the pharmacological point of view, oral administration is convenient but some conditions such as dysphagia (difficulty in swallowing) reduce the universal use of this method,[16–18] and passage through the gastrointestinal tract exposes drugs to interactions with intestinal bacteria, pH, and enzymes.[19–21] Parenteral delivery (injection through subcutaneous, intramuscular, or intravenous routes) helps to bypass the instability problem of pharmaceuticals in the gastrointestinal tract.[22,23] but delivery efficacy and patient compliance become challenging when multiple and/or repeated injections are required. Controlled drug delivery from injectable materials offers a solution to such problems by administrating fewer (or even unique) doses of a long-lasting delivery system.[24] Besides, applications such as the treatment of tumors, wounds, and recovery from myocardial infarction or spinal cord surgery require the administration of the therapeutic agent to a specific site, making injectable materials an ideal way of administration.[25–28]

Two of the most studied injectable platforms are nanocarriers and hydrogels. The former, due to their nanoscale nature, are intrinsically injectable as long as the nanoparticles do not aggregate to a significant extent, although hydrogels in contrast need to be carefully designed to provide appropriate materials properties for injectability. Many routes for producing injectable hydrogels in situ under physiological conditions have been developed. Injectability can be achieved by optimizing the gelation kinetics of the hydrogel precursors so they are liquid prior to and during the injection and rapidly form a covalently crosslinked network when delivered.[29,30] Another approach involves using stimuli-responsive materials, such as polypeptides or some synthetic polymers, which can be injected as liquids and quickly form a physically crosslinked hydrogel upon exposure to physiological temperature, pH, or ionic conditions.[31–33] Shear-thinning materials flow under the shear stress applied during injection due to the dissociation of the networks that structure them, then they quickly recover their structural integrity and related mechanical properties upon release of the shear force.[34,35]. This recovery is generally described as self-healing under the scope of injectable materials,[34,35] and for the purposes of this review, should not be misunderstood as the general definition of the repair of a fractured surface or interface.[36–38] Approaches to produce shear-thinning materials can take advantage of dynamic covalent bonds, such as Schiff bases, disulfide bonds, oxime chemistry, and reversible Diels-Alder chemistries, for instance, that permit the network to be formed, broken, and re-formed,[30] self-assembled macromolecules that disassemble under shear stress and recover upon removal of such force,[39–41] or a combination of both.[42,43]

Nanocarriers have found widespread use in clinical settings due to their ability to overcome systemic, microenvironmental, and cellular barriers by improving the solubility, stability, membrane permeability, and circulation time of diverse biotherapeutic cargo.[44] Highly targeted and enhanced delivery is made possible via the strategic incorporation of biorecognition and stimuli-responsive ligands in nanocarriers with complex architectures comprising varying sizes, shapes, and surface features.[44] Hydrogels are crosslinked hydrophilic polymeric networks which have gained popularity as sustained drug delivery depots owing to their ability to protect and deliver drugs to target tissues in an extremely controlled fashion.[45] Sustained and on-demand release of cargo can be achieved by dynamically tuning its affinity to the biopolymer matrix depending on the desired application.[45] Hybrid approaches involving the encapsulation of nanocarriers within increasingly complex hydrogel networks provide spatiotemporal control over release kinetics.[46] When developing these nanocarrier-hydrogel hybrids, triggering responses to multiple orthogonal stimuli such as pH, temperature, redox potential, light, enzymes, and hydrolysis can be harnessed to achieve sequential release of biotherapeutic payload.[47]

To date, the most popular recombinant protein-based biomaterials used in injectable nanocarriers and hydrogels comprise multiple repeats of consensus units of structural proteins such as collagen, elastin, silk, and/or resilin. This is due to the structural integrity and outstanding mechanical properties derived from their naturally evolved functionalities.[3] Furthermore, constructs based on collagen, elastin, and resilin are also of interest due to their stimuli-responsive properties.[3] Other modular constructs based on non-structural proteins have been designed to impart the capacity for hierarchical assembly into nanostructures and hydrogels. These include computationally designed WW-domains, polyproline-rich peptide modules, coiled-coil leucine zipper motifs, and calmodulin (CaM) motifs, where physical interactions such as π-π stacking, electrostatic attraction or repulsion, and hydrophobic interactions commonly drive the assembly.[3,6,48,49] In addition to the phase separation and biorecognition-based physical assembly of nanostructures and hydrogels, chemical crosslinking strategies can be employed to further improve stability via the generation of covalent bonds. Specific amino acid residues can be incorporated along the polypeptide backbone to permit site-specific chemical modification or crosslinking. For biomedical applications, the most widely employed amino acids, among the 20 canonical amino acids, are cysteine, lysine, and tyrosine residues.[6] Chemical diversity can be further expanded via the incorporation of reactive non-canonical amino acids. Azidohomoalanine and homopropargylglycine, for instance, are among the most common non-canonical amino acids due to their selective reaction via copper-catalyzed azide-alkyne cycloaddition (CuAAC);[50] azidohomoalanine can also react with cyclooctynes via strain-promoted azide-alkyne cycloaddition (SPAAC),[51] and *p*-azidophenylalanine has been exploited due to its photoreactive properties[52] and has been also modified via CuAAC.[53] These click chemistry techniques offer an effective orthogonal chemical modification option for polypeptides.[50,54]

There are many excellent reviews in the field covering research advances on polypeptide-based injectable hydrogels and nanocarriers.[55–61] The objective of this review is to complement such work with relevant research from recent years in the field; protein-engineered biomaterials that can be used to develop versatile platforms are highlighted. These platforms improve the responsiveness, homogeneity, and bioactivity of both matrix and drug formulations, which will contribute to the expansion of new drug-, gene-, and cell-based therapies in the future.

2. Injectable nanoparticles and nanocarriers based on genetically engineered polypeptides

Nanocarriers are popular injectable materials for biomedical applications, often regarded as an ideal carrier for therapeutic cargo as they increase drug circulation time and can enhance internalization by cells.[62] Given the routine application of recombinant DNA technology, genetically engineered protein-based polymers have remained popular as materials for these applications due to their low immunogenicity, tunability, and precise control over chemical composition;[9,63] their chemical versatility in particular has made genetically engineered polypeptides useful as injectable nanocarriers.[64] Their ability to phase separate can be exploited for generating a variety of nanostructures with different architectures as shown in **Figure**

1. The design control possible for recombinant protein-based materials has been valuable for these applications because the interplay between nanostructure size, shape, chemical composition, and surface properties determines cellular internalization pathways and resulting therapeutic efficacy.[65–67] While spherical nanoparticles have been widely studied and employed clinically, non-spherical nanostructures are of interest because of their higher aspect ratios that can increase circulation times in vivo, resulting in better clinical outcomes. [68]

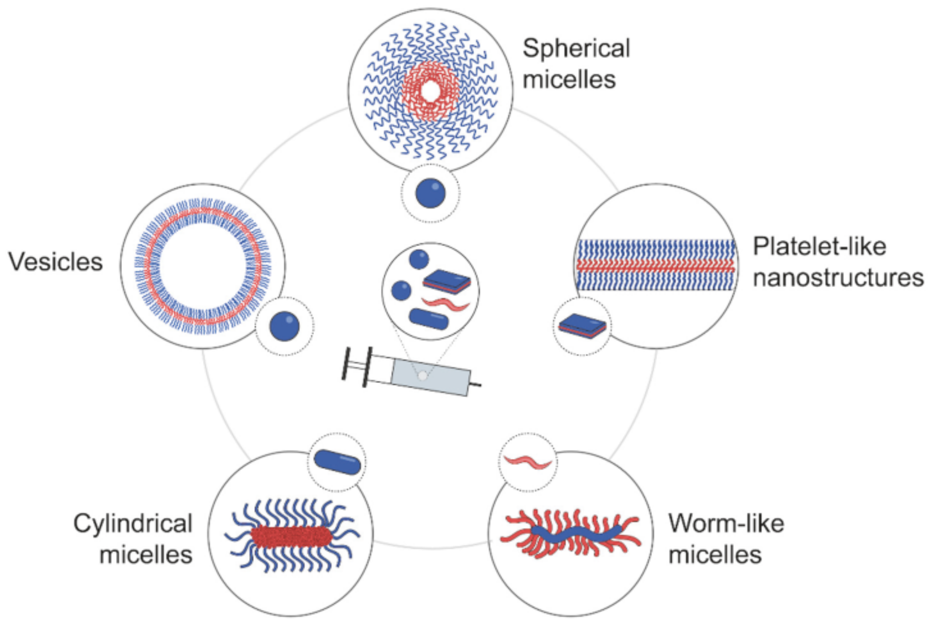


Figure 1. Injectable nanocarriers for delivery of therapeutic cargo. A variety of nanostructures with different architectures can be generated via phase separation of genetically encoded hybrid polypeptides. Red features represent relatively hydrophobic sections and blue features represent hydrophilic regions of the hybrid polypeptide. Morphology illustrations adapted with permission from Saha et al.[69] Copyright 2020, John Wiley & Sons.

2.1. ELP nanostructures

Elastin is an elastomeric protein in the ECM, which provides elasticity and resilience to organs such as skin, cartilage, blood vessels, the heart, and others.[70,71] It serves is function efficiently in mammalian tissues, as it is a stable protein with a half-life of approximately 70 years.[72] The lysine residues of tropoelastin – the precursor to elastin – can be crosslinked by the reaction of the primary amines with desmosine and isodesmosine. This crosslinking and the self-assembly of the hydrophobic domains contribute to tropoelastin folding,[73] and the excellent mechanical properties of native elastin motivated its exploration as a protein-based biomaterial. High purity is crucial for biomaterials, but during the purification of native elastin, contaminations such as calcium-binding microfibrillar components can be hard to remove and trigger immunological responses;[74] these problems were solved by designing elastin-like polypeptides (ELPs). The ELPs mimic natural elastin with proline-rich and/or glycine-rich hydrophobic domains,[75–80] with amino acid sequences most commonly based on the pentapeptide $(VPGXG)_n$ where X is any amino acid except proline.[81–84] Recombinant methods are utilized to modify ELP properties such as chain length and functional groups to introduce the desired interactions with other molecules or mediums.[3,83,85]

ELP nanostructures have also been extensively studied due to their self-assembly properties, which can be engineered by producing ELP-based block 'co-polymers' comprising hydrophilic and hydrophobic blocks. Dai et al. have investigated amphiphilic ELPs synthesized recombinantly and composed of different lengths of hydrophilic ELP units ($VPGVGVPGMG(VPGVG)_2$, indicated as M_1V_3) and hydrophobic ELP units (VPGIG).[86] The hydrophilicity in the sequences could be sequentially increased by oxidizing the methionine residues in the hydrophilic domain. As predicted, the monoblock ELPs and diblock ELPs showed a single lower critical solution temperature (LCST)-like phase transition from unimers to aggregates. The transition temperature (T_t) increased with decreased concentration, decreased chain length, and increased hydrophilicity. However, with the increase of hydrophilic character in the $(M_1V_3)_n$ sequence, a new critical micelle temperature (CMT) was observed, and the amphiphilic ELPs self-assembled into micellar structures between the CMT and T_t. This research highlights the possibility of precisely controlling the phase transition behavior of ELPs by tuning their sequences.[86] Wang et al. performed a systematic analysis of ELP diblock copolymers with 8 different peptide linkers and 15 tumor-homing peptides. From all of the nearly 100 combinations reported, all ELP amphiphiles formed micelle structures, however, the hydrophobic tumor-homing peptides required a more hydrophilic peptide linker to assemble into micelle structures. Positively charged linkers provided a sufficient excess of positive charges to balance the negative charge imposed by the peptide's C-terminus, thus driving the formation of micelles that exhibited more favorable electrostatic binding of multivalent ligands to receptors.[87]

The phase transition behavior of ELPs has been widely used for the formation of nanoparticles.[88,89] For example, Acosta et al. fused antimicrobial peptides (AMPs) to ELP-based amphiphiles. Below the T_t of hydrophobic ELP, the triblock polypeptide (AMP-hydrophilic ELP-hydrophobic ELP) will form nanofibers due to the interactions of AMP. Upon incubating at body temperature, the hydrophobic ELP coacervated and drove AMPs aggregation. Depending on the sequence of AMPs, the nanofibers evolved into spherical aggregates when the AMP is rich in lysine or aggregates with undefined shapes when the AMP is rich in arginine.[90] Similar to this work, Gonzalez-Valdivieso et al. fused multiple bioactive peptides to ELP-based amphiphiles. The recombinantly expressed polypeptide was designed to have an internalization peptide, hydrophilic ELPs, hydrophobic ELPs, an excision sequence that can be activated in the lysosome, a lysosome escape sequence, and a peptidic inhibitor of Akt (Akt-in) which can block phosphorylation and consequent activation of Akt kinase. The nanoparticles provided better internalization, shielded the inhibitor from cellular proteases, and achieved 12 times faster activation and 5 times lower amount of inhibitor compared to the inhibitor alone.[91] Kobatake et al. fused a poly-aspartic acid tail and internalizing RGD (iRDG) to ELPs to generate a nanocarrier for specific delivery of paclitaxel (PTX) to cancer cells. Similar to the ELP-based amphiphiles, poly-aspartic acid was introduced to behave as the hydrophilic portion of amphiphiles, and the charge repulsion of the poly-aspartic acid chains increases the stability of ELP nanoparticles. The hydrophobic PTX was loaded into nanoparticles by incubating PTX with the fusion proteins during particle formation. The nanoparticles displayed iRGD on their surfaces and were able to deliver loaded drugs into cancer cells through integrin binding and internalization via the neuropilin-1 (NRP-1)-dependent pathway, inducing cell death.[92]

Hydrophilic drugs such as metal ions are also able to be delivered by ELP-based nanoparticles via the inclusion in the ELP of specific metal-ion-binding peptide sequences. Pozdniakova et al. designed a recombinant protein comprising a gadolinium ion (Gd^{3+}) binding domain, ELPs, the nucleolin marker F3 peptide, an RGD motif, and a fluorescent protein reporter, for targeted delivery of Gd^{3+} to cancer cells in applications such as binary radiotherapy (BRT) and magnetic resonance imaging (MRI).[93] The recombinant protein nanoparticles exhibited three to four times higher accumulation in selected cancer cell lines than in normal fibroblasts and can remain in tumors for more than 24 hours.[93]

It should be highlighted that the nanoparticle circulation times can be further increased by introducing zwitterionic pairs into the polypeptide sequence. Banskota et al. designed zwitterionic polypeptides (ZIPP) based on ELPs to achieve a longer plasma circulation time;[94] similar to the reported behavior of zwitterionic synthetic polymers, the zwitterionic pair in ELPs enhances their "stealth" behavior and thus increases their plasma residence time. By replacing the glycine-alanine pair with lysine and glutamic acid, these zwitterionic polypeptides were reported to have enhanced pharmacokinetics for both intravenous and subcutaneous administration. The ZIPP that contains lysine-glutamic acid as a dipeptide had a plasma exposure and half-life that was two-fold greater than its uncharged control. The ZIPP-glucagon-like peptide-1(GFP1) could also reduce blood glucose levels for up to 3 days in a diet-induced obesity model of type-2 diabetes in mice after a single subcutaneous injection, which is 70 times longer than the unmodified GFP1.[94]

The building block of ELP-based nanocarriers is not limited to (poly)peptides. Hydrophilic molecules such as DNA also bind to hydrophobic ELPs through linkers. Guo et al. prepared DNA-displaying ELP-based nanoparticles by conjugating single-stranded DNA (ssDNA) with an elastin-like polypeptide (ELP) through a catalytic domain of porcine circovirus type 2 replication initiation protein (pRep).[95] The conjugates can self-assemble into nanoparticles through hydrophobic interactions of the ELPs; by conjugating to the ELP a DNA aptamer that binds to Mucin 1 (MUC1), the nanoparticles show tumor-targeting properties and can be used as an anti-tumor drug carrier. DNA aptamer-displaying nanoparticles encapsulating the anti-cancer drug paclitaxel were able to bind to cells overexpressing MUC1 and induce cell death.[95]

Due to the thermoresponsiveness of ELPs and their ability to load hydrophobic drugs, ELPs can also be used to modify therapeutic-targeting cell receptors to increase targeting and stimuli-responsive behavior. Steinhauf et al. fused ELPs to Rituximab, a clinically used antibody, through the use of a complementary coiled-coil peptide to prove that these new complexes can target CD20 receptors on Raji B cells.[96] The thermoresponsive nature of the ELP domain has the ability to cluster the receptors CD20 receptors, a strategy that has been used to induce cell death for the treatment of non-Hodgkin's lymphoma.[97] By conjugating fluorescent labels to modified and unmodified Rituximab, the Rituximab-ELPs presence could be quantified, showing at least a two-fold increase in mean fluorescent intensities and indicating a stronger binding to the Raj B cells.[96] In related work, Sun et al. chemoselectively conjugated an ELP-based tumor-homing chimeric polypeptide to polypyrrole (PPy) nanoparticles. The ELP was recombinantly fused to the N-terminal fragment of human high-mobility group protein 2 (F3) which can specifically bind to nucleolin expressed on the membrane of tumor cells and endothelial cells[98] and can be internalized into the targeted cells and translocated to the nucleus. The cysteine residues on the C-terminus of ELP-F3 underwent facile thiol-maleimide coupling reaction with PPy nanoparticles. These modified nanoparticles can encapsulate doxorubicin (DOX) through π-π stacking and specifically deliver it to tumor cells.[99] In this research, ELPs performed as a linker to link the F3 peptide to the PPy nanoparticles. The inverse transition cycling (ITC) purification, which utilized the LCST behavior of ELPs, also improved the purification of the F3 peptide.

The collapse of the ELP chain while heating above LCST can be used not only to form nanoparticles, but also to control the release of encapsulated cargo. Ibrahimova et al. grafted lipids to ELPs by chemoselectively alkylating methionine using glycidyl propargyl ether to introduce an aliphatic alkyne group on the side chain.[100] A copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reaction was performed to graft oleyl side chains onto the ELP. The grafted ELPs formed vesicles with ELP membranes and a fatty layer, where the ELP layer remains hydrated upon cooling below the transition temperature and fluorescein-loaded nanoparticles were observed with a burst release of fluorescein upon cooling below the T_t.[100] The same group also conjugated a photosensitizer (peripherally substituted carboxy-Zn(II)-phthalocyanine derivative (TT_1)) to the methionine-containing ELPs through the CuAAC coupling reaction.[101] The conjugates underwent temperature-controlled particle formation to form ELP nanoparticles. With methionine oxidation upon irradiation by red light, the ELPs became more hydrophilic, thus the nanoparticle disassembled and released the loaded cargo. The temperature-controlled particle formation and oxidation-triggered release of nanoparticles can be used to improve the clinical effectiveness of photodynamic therapies.[101]

ELP nanoparticles have also been employed as templating agents for certain cargos that are difficult to self-assemble into nanoparticles, such as lentiviral vectors whose nonspecific delivery has limited their application. Monfort et al. designed a heterogeneous nanoparticle for the targeted delivery of lentiviral particles containing a therapeutic gene.[102] The nanoparticles consisted of lipoprotein receptor repeat 3 (LDLR3) and the keratinocyte growth factor (KGF). The LDLR3 can bind to the lentiviral vector to prevent the non-specific infection of untargeted cell types while the KGF can specifically bind to the cells that overexpress the KGF receptor. The LDLR3 and KGF were fused to ELPs separately and the recombinantly expressed ELP-LDLR3/ELP-GFP molecules assembled into nanoparticles as a result of the LCST behavior of ELP. The heterogeneous nanoparticle was used to deliver the VSV-G pseudotype lentiviral vector encoding a GFP gene to cells overexpressing the KGF receptor.[102]

2.2. SELP nanostructures

A popular strategy to increase the mechanical robustness of ELPs has been to genetically include silk-like polypeptides (SLPs) in ELPs to design silk-elastin-like polypeptides (SELPs) with improved solubility, tunable stiffness, elasticity, and stimuli-responsiveness.[103,104] (**Figure 2**) SELPs have been used for creating thermoresponsive nanogels which arise from the SELPs ability to self-assemble into nanostructured polymeric networks. The presence of SLPs in these polypeptides helps to regulate the stability of the nanostructures due to the formation of beta-sheet structures that create nanogels by physical crosslinking. The thermoresponsiveness comes from the ELP component in these copolymers, which provides the nanogel with swelling and contracting properties in response to temperature variations.[105,106] These SELP nanocarriers have been modified, as in Isaacson et al., in order to make the nanogels susceptible to improved degradation in the presence of solid tumors by the inclusion of a matrix-metalloproteinase (MMP)-2 and MMP-9 sensitive domains. The hydrophilic nature of these degradable domains has a direct effect on the self-assembly capabilities of the nanocarriers. When the domain is located in the middle of the hydrophobic silk-portion of the SELP, the polypeptide loses the ability to self-assemble into nanogels. In contrast, when it is located in the middle of the elastin-portion of the polypeptide, the SELP still self-assembles but has an increase of 7°C (from 51°C to 58°C) in its LCST transition.[107] These experiments demonstrate that any modification to a recombinant polypeptide must be carefully designed in order to have a minimum impact in the material's performance.

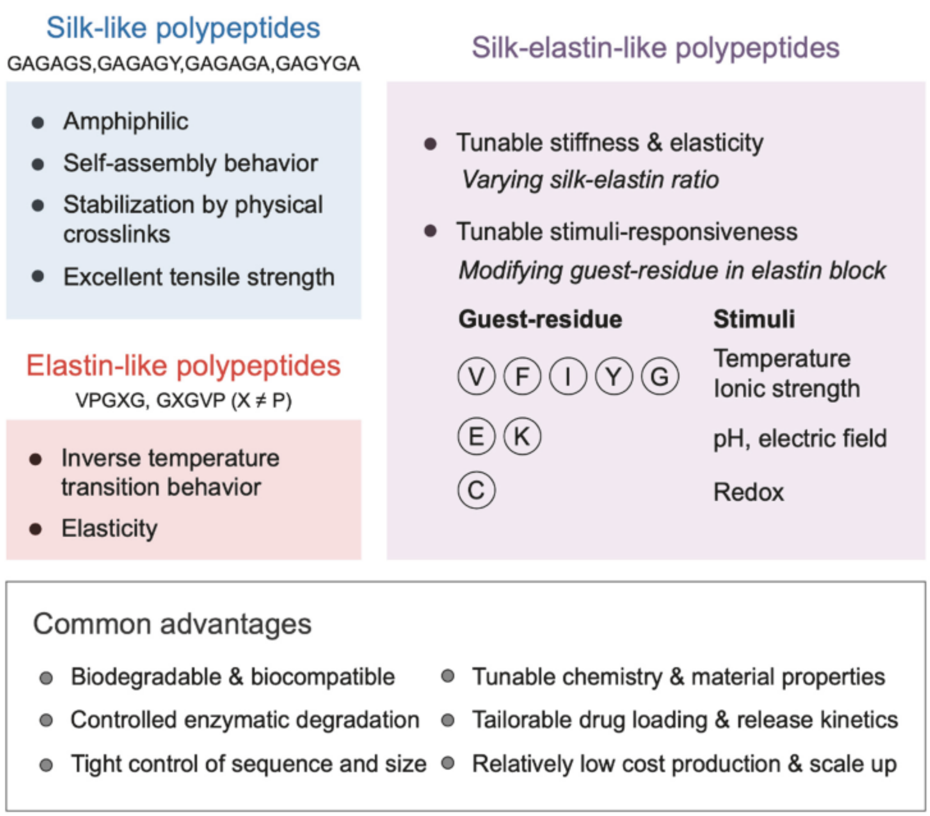


Figure 2. Advantages of silk-like, elastin-like, and silk-elastin-like polypeptides for biomedical applications. SELP-based systems are highly dynamic, tunable, and stimuli-responsive. Adapted with permission.[104] Copyright 2020, Elsevier.

2.3. ERLP nanostructures

Useful elastomeric and phase separation properties are not only prevalent in elastin-based materials but are also present in the elastomeric protein resilin, which was first discovered over 50 years ago in locust wing hinges.[108,109] Resilin is a very efficient elastomer and is important in the flight of fruit flies, jumping of fleas, and sound production in cicadas and moths.[110–112] Resilin of many species of insects consists of a high ratio of hydrophobic residues such as glycine (35–40%) and proline (7–10%);[113–115] natural resilin has a random-coil conformation (intrinsically disordered protein) whose chains are crosslinked chemically through di and tri-tyrosine crosslinks, which are developed by tyrosine oxidation to bond resilin chains in a 3D network. This network stabilizes and controls the flexibility of the material[116,117] and is responsible for the extraordinary elastic properties which restore the elastic force that emerges because of loss in conformational entropy and allows the polypeptide to behave as rubber with an elastic modulus of 0.588 – 0.883 MPa and resilience ranging from 86% to 93%.[118–120]

Derived from the elastomeric insect protein resilin, resilin-like polypeptides (RLPs) have been used to support tissue regeneration in mechanically active tissues such as vocal folds, cartilage, and the cardiovascular system.[121–123] Similar to ELPs, RLPs are intrinsically disordered proteins (IDPs) that can undergo multiple types of stimuli-responsive phase behavior. The putative sequences affording elastomeric properties to native resilins in a variety of insect species (fruit flies, mosquitoes, buffalo flies, and fleas) have been discovered, cloned, and recombinantly expressed successfully. [110] Of these, fruit fly resilin- and mosquito resilin-based constructs, with the canonical amino acid sequences GGRPSDSYGAPGGGN and GAPAQTPSSQY respectively, have been the most widely employed for biomedical applications.[122]

Elastin-resilin-like polypeptides (ERLPs) are another type of recombinant diblock polypeptides studied in the field of nanocarriers. In contrast to the SELPs where the silk component is responsible for physical crosslinking and the elastin imparts the thermoresponsive behavior, ERLPs have dual thermal responsiveness. This behavior is due to the different phase transition behavior of each of the diblock components. The elastin blocks have a lower critical solution temperature (LCST), while the resilin blocks exhibit an upper critical solution temperature (UCST). Basheer et al. designed ERLPs with LCST<UCST and found that these copolypeptides formed different types of nanostructures depending on the conditions.[124] When the temperature is below the LCST, the copolypeptides assemble into two types of nanostructures: vesicles with ELP in the external and internal layers, and RLP in the middle layer when the ERLP is short (6-12 total repeats) and the ELP to RLP ratio is similar, or micelles with an insoluble core of RLP, and an external layer of ELP when the ERLP is long (36 repeats) and resilin content is double the elastin content. When the temperature is above the LSCT and below the UCST, the ERLPs only aggregate, and when the temperature is above the UCST, the copolypeptides form inverse micelles with an insoluble core being formed by ELP and the external layer comprising RLP. In this last case, the formation of micelles happens independently of the size and ratio of elastin and resilin content (**Figure 3A**).[124] Weitzhandler et al. studied similar ERLP copolypeptides and reported that when the recombinant constructs have 60-100 total repeats and the ELP ratio exceeds two times that of RLP content, no self-assembly occurs.[125] When these same ratios are maintained but the copolypeptide length increases to 120-240 total repeats, the ERLPs form spherical micelles, and when the ratios become similar in length (80-180 total repeats) the constructs exhibit self-assembly into cylindrical micelles.[125]

A challenge with the use of micelles as nanocarriers is that once injected, dilution below their CMC results in the disassembly of the nanostructure into single polypeptide units, which reduces nanoparticle/cargo uptake in cells.[116] Dzuricky et al. have used ERLP constructs and modified them with a terminal fibronectin domain in order to test the uptake by K562 lymphoblasts with variations in the morphologies of the nanocarriers. They found that non-assembled ERLPs are not internalized by cells, while micelles with cylindrical morphology show an uptake five times greater than that of spherical micelles, demonstrating the impact of nanostructure morphology on the intracellular delivery of drugs.[126] Weber et al. modified these recombinant fibronectin-containing ERLPs by adding the unnatural amino acid para-azidophenylalanine to the resilin domain of the copolypeptide, which facilitated crosslinking of the spherical micelles under UV irradiation[127] and cellular uptake of the crosslinked micelles at a concentration 1000-fold lower than their CMC.[127]

2.4. Nanocarriers via engineered peptide-peptide interactions

In addition to nanocarriers formed based on the assembly of structural protein-inspired recombinant polypeptides, polypeptides engineered with specific domains capable of self-assembly, such as coiled-coil sequences, for instance, have been commonly used for designing nanoparticles for drug delivery purposes. Assal et al.[128] synthesized a recombinant single-chain vascular endothelial growth factor (scVEGF) with a coiled-coil forming peptide on the N-terminus named helix A. The complementary interacting pair, helix B, was incorporated into the C-terminus of an ELP sequence followed by a polyaspartic acid domain. When warmed up above the ELP transition temperature (35°C), the helix B-containing ELPs collapse into a nanoparticle with a hydrophobic core and the helix B domain on the surface. The formation of a coiled-coil structure between the helices facilitates the multivalent presentation of VEGFR-binding peptides; paclitaxel-loaded nanoparticles induced cell death in HeLa cells confirming their internalization via binding to the VEGF receptor and the specific delivery of the drug.[128] A more recently reported approach is the use of ZapB, a protein involved in *E. coli* cell division, due to its capacity to self-assemble into large coiled-coil structures.[129] Gil-Garcia and Ventura [130] used a recombinant modular construct based on the ZapB protein as a self-assembly domain, green fluorescent protein (GFP) as an imaging tag, and a Z-domain with high affinity for Immunoglobulins G (IgGs), inspired by the B-domain of *S. aureus* protein A. These constructs form spherical nanoparticles with an average diameter of 500 nm; the preserved native structure of the GFP and Z-domain yields fluorescent particles with the capacity of binding two different types of antibodies (and potentially more) at the same time. The use of the particles was demonstrated to co-localize two different types of cells: T lymphocytes and HeLa cells.[130]

The precise localization of multiple coiled-coil-forming peptides has the potential of creating nanocarriers with controlled morphologies. Yu et al.[131] designed activatable protein nanoparticles (APNPs) relying on coiled-coil dimerization aimed at the delivery of therapeutic peptides, such as the neuroprotective peptide NR2B9c that has difficulty penetrating the cell membrane and melittin, a potent anticancer peptide with cytolytic activity, which cannot be administered at a therapeutically effective dose. Individual APNPs are formed by three unique recombinant polypeptides comprising 5 modules each. A "self" peptide that enhances the circulation of the nanoparticles through inhibition of macrophage-mediated clearance [132] comprises modules 1 and 5, coiled-coil forming peptides are modules 2 and 4, and the central module is the therapeutic cargo. Each module is separated by a flexible spacer and the flanks of the therapeutic motif include enzymatically cleavable sequences such as thrombin and MMP2 sensitive domains. Each of the three full-length polypeptides contains two of six unique coiled-coil interactive sequences labeled from P1 to P6. The only modes of interaction happen between pairs P1-P2, P3-P4, and P5-P6, but their location in each of the three polypeptides follows P1-P5, P2-P3, and P6-P4 respectively (**Figure 3B**). The strategic distribution of these coiled-coil interactive peptides results in nanoparticles formed by a trimer bundle with the cargo therapeutics embedded in the core and with the "self" peptide on the surface. Upon exposure of the particles to enzyme-rich environments, the cargo is detached from the particle and delivered. The addition of target peptides in the N-terminus of the constructs has proved to be effective for accumulation of the particles into targeted tumors upon injection in living mice.[131]

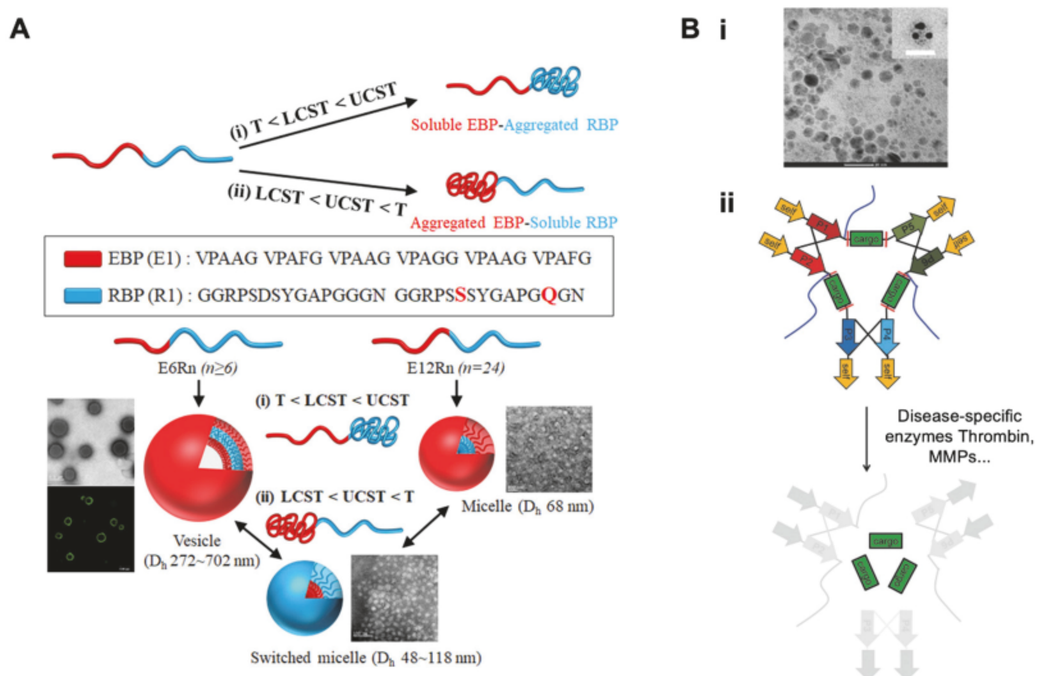


Figure 3. Different nanostructures can be achieved by the careful design of polypeptide constructs. (A) ERLP Molecular design and self-assembly. Nanostructures switch based on the transition temperatures of the construct. Adapted and reprinted with permission from Basheer et al.[124] Copyright 2021, American Chemical Society. (B) i. A representative TEM image of TN-APNPs and close-up views of individual APNPs stained with 5 nm Ni-NTA–nanogold beads (insert) Scale bar: 20 nm. ii. Self-assembly of nanoparticles based on modular polypeptides. The strategical distribution of each coiled-coil interactive sequence allows the formation of a trimer bundle enzymatically cleavable. Adapted and reprinted with permission from Yu et al.[131] Copyright 2018, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

3. Injectable hydrogels based on genetically engineered polypeptides

Genetically engineered polypeptides exploit design cues from natural proteins, which have been extensively explored in the development of injectable hydrogels for biomedical applications. Several polymers derived from the consensus motifs of structural proteins have been successfully produced in a variety of expression hosts in up to gram-scale quantities.[133] Furthermore, the modular nature of the design permits precise control over sequence and molecular weight along with the inclusion of a variety of biological and structural motifs resulting in materials in which mechanical properties and biological functions can be tuned independently.[3,7,8] This simplifies and streamlines synthesis strategies and often obviates the need for the inclusion of peptide-based linkers (produced on the solid phase) to impart cell adhesion and matrix remodeling.

The ability of these materials to undergo multi-stimuli-responsive phase behavior can be harnessed to develop injectable hydrogels for biomedical applications. Phase separation of ELPs or RLPs into coacervates, ordering and oligomerization of helical subunits in coiled-coil-containing polypeptides and collagen, or self-assembly of SELPs and amphiphilic polymers are examples of mechanisms that have been used for this purpose (**Figure 4**). To alter the phase separation behavior of genetically engineered polypeptides, the overall molecular weight, hydrophobicity, and charge can be varied by altering their amino acid composition. Furthermore, the strategic inclusion of select amino acids in specific positions of the polypeptide backbone can be used for site-specific functionalization or tailoring of interactions between the hydrogel matrix and biotherapeutic cargo. A range of such polypeptides, based on the structural proteins elastin and (more recently) resilin, have been widely studied.

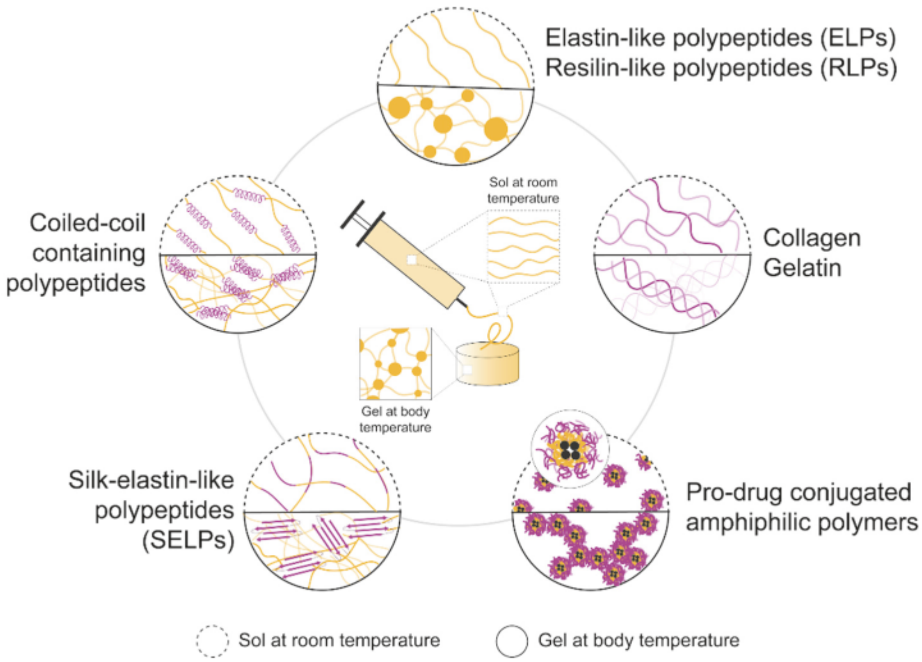


Figure 4. Injectable hydrogels can be produced via stimuli-responsive gelation of liquid precursors. A variety of mechanisms allow for the formation of physically crosslinked hydrogels under physiological conditions. The illustration for pro-drug conjugated amphiphilic polymers was adapted from the illustration of Correa, et al.[134]

3.1. ELP injectable hydrogels

The formation of ELP hydrogels is limited by the concentration, crosslinking density, and molecular weight, and as a result, ELPs mainly form hydrogels only at high concentrations or high molecular weight (> 50 mg/mL; > 22kDa). Increasing the content of intermolecular crosslinks in the sequence reduces the minimum MW necessary for hydrogel formation at a given protein concentration.[135] Various chemical crosslinking mechanisms have been employed for forming ELP hydrogels, including photoinitiation, enzymatic crosslinking, redox reactions of cysteines, and coacervation.[135–140] Nagapudi et al. prepared the ELP sequence $(VPGVG)_4(VPGKG)_{39}$, where the lysine residues were modified to achieve different degrees of functionalization with methacrylate groups for photochemical crosslinking.[141] Photocrosslinking was also used by Zhang et al. to prepare an ELP hydrogel based on the sequence $[[VPGVG]_4\ IPGVG]_{14}$, with the inclusion of cysteine-containing domains (KCTS). When these ELPs were subjected to UV light in the presence of a photoinitiator (Irgacure 2959), a highly elastic hydrogel was formed due to the formation of disulfide crosslinking. Another type of crosslinking was developed in this case, but its nature was not identified; possibly related to the radical crosslinking of moieties (C—H, O—H, or N—H groups) in the ELP sequence due to the excess of photoinitiator.[142] Another type of injectable ELPs were prepared by McHale et al. using enzyme-initiated crosslinking to repair functional cartilage. In this case, lysine and glutamine groups were crosslinked in the presence of transglutaminase.[143]

ELPs and their derivatives are the most popular protein-engineered polymers for use as injectable hydrogels in tissue engineering applications, not only because of their useful mechanical properties upon chemical crosslinking, but owing also to their phase separation.[144,145] ELPs undergo reversible phase separation and form protein-rich coacervates upon warming above their lower critical solution temperature (LCST).[146] The ability of ELPs to retain their stimuli-responsive phase-transition behavior even upon conjugation to peptides or small-molecule drugs has been utilized in the formation of injectable delivery depots for sustained delivery.[147] Liu et al. developed improved ELP-based polymeric brachytherapy carriers via labeling of ELPs with therapeutic radioisotopes. These molecules self-assembled into seed-like depots upon injection within solid tumors and resulted in prolonged intratumoral (i.t.) retention.[148] While increasing the ELP molecular weight led to an increase in tumor retention by improving seed stability, the altered microstructure had a more pronounced effect on the tumor-retention half-life. This was afforded by the formation of aggregated micelles upon increasing the number of tyrosine residues on the C-terminus.[148] Schaal et al. improved this design by developing a strategy for radiation-induced covalent crosslinking to facilitate long-term depot stabilization (over 60 days) via the emission of high-energy beta particles from ^{131}I, for the treatment of solid tumors in prostate and pancreatic cancers.[149] Building on those studies, Kelly et al. developed a combined approach for providing immunotherapy and ^{131}I brachytherapy for the treatment of metastatic breast cancer.[150] (**Figure 5**) An oligolysine tail (ELP-K_{12}) was included in the depot-forming ELP constructs which allowed for electrostatic complexation with a CpG oligodeoxynucleotide (CpG), a toll-like receptor 9 (TLR9) agonist for antitumor immunity.[150] This complexation improved the cellular uptake by two-fold compared to that for free CpG.[150] Furthermore, when injected intratumorally, free CpG was cleared from the tumor in less than 24 h, while the ELP-K_{12}/CpG depot provided sustained release of CpG for 3 weeks.[150]

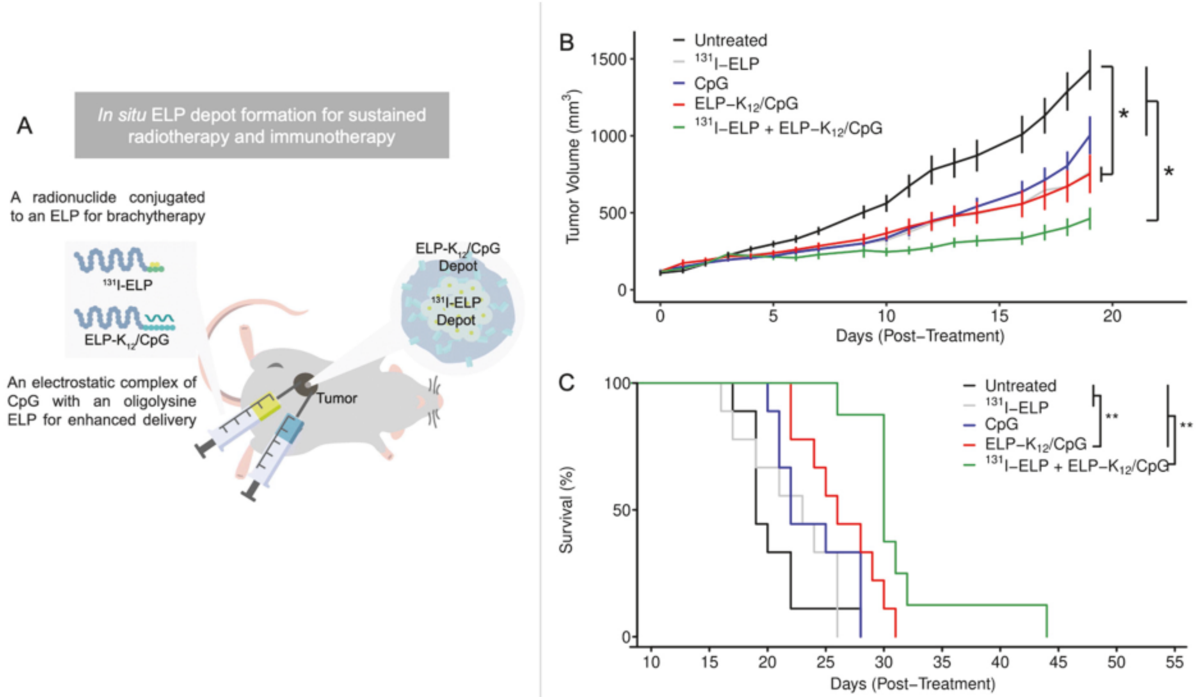


Figure 5. (A) Schematic of combination of ^{131}I-ELP brachytherapy and ELP-K_{12}/CpG immunotherapy. ELP brachytherapy is formulated as a ^{131}I radionuclide conjugated to Tyr residues at the C-terminus of an ELP (^{131}I-ELP), while CpG is electrostatically complexed to a $(Lys)_{12}$ peptide at the C-terminus of an ELP (ELP-K_{12}). Each conjugate is injected i.t. and undergoes an LCST phase transition at body temperature to form ELP depots. The ^{131}I is retained within the tumor because of radiation-induced crosslinking of the ELP and irradiates the tumor from the inside-out, while the electrostatically complexed CpG is released from the depot over time to activate immune signaling within the tumor. (B,C) Combination of ^{131}I-ELP and ELP-K_{12}/CpG significantly inhibits tumor growth and extends mouse survival. (B) Average size of 4 T1 tumors untreated or treated with ^{131}I-ELP monotherapy, CpG monotherapy, ELP-K_{12}/CpG monotherapy, or a combination of ^{131}I-ELP and ELP-K_{12}/ CpG. CpG alone or in complex with ELP-K_{12} was administered i.t. at a dose of 100 µg; for combination therapy ^{131}I-ELP was administered intratumorally one day later at a dose of 122.1 kBq/mm^3. The same radioactivity dose was used for ^{131}I-ELP monotherapy. *$p < 0.05$ (ANOVA, Tukey). (C) Kaplan-Meier survival curves for treatment groups (n = 8–9). **$p < 0.05$ (Mantel-Cox test). Adapted and reprinted with permission from Kelly, et al.[150] Copyright 2022, Elsevier.

Gilroy et al. fused ELPs to the fibroblast growth factor 21 (FGF21) to improve its solubility during expression in *E. coli*.[151] Not only did this result in the improved recombinant production of active FGF21, but also prolonged circulation half-life in vivo.[151] The temporally controlled 'outside-in' dissolution of the subcutaneously injected fusion proteins led to blood glucose control in *ob/ob* mice for up to five days allowing for the development of a weekly dosing regimen. Since PEG has been associated with renal tubular vacuole formation,[152,153] ELP-FGF21 fusions are attractive alternatives to PEGylated FGF21 for improving circulation half-lives owing to their biodegradability and biocompatibility. The incorporation of ELP in cell delivery platforms could improve clinical outcomes for treating osteochondral injuries via mesenchymal stromal cell therapy. Pescador et al. utilized injectable RGD-containing ELP hydrogels as cell carriers to deliver human mesenchymal cells (hMSCs) to critical osteochondral defects in rabbits.[154] The presence of viable and engrafted hMSCs was demonstrated by using immunohistological methods.[154] Magnetic resonance imaging results showed complete tissue regeneration of the injured cartilage after three months.[154] Those results were corroborated by computed tomography assessments which also showed the development of the subchondral bone.[154]

The elastic nature of ELP provides a promising approach to address the challenges associated with therapeutic cargo retention in contracting myocardium tissues. Suhar et al. developed an injectable two-part HELP material comprising aldehyde-functionalized hyaluronic acid (HA) and hydrazine-functionalized ELPs to form dynamically crosslinked hydrogel networks via hydrazone linkages.[155] (**Figure 6**). The authors evaluated hydrogel performance compared to commercial Matrigel by screening the stress relaxation and matrix stiffness of different formulations where the HELP hydrogels resulted in a 10-100-fold stiffness increase and longer relaxation times. In addition, in vitro injectability (failure stress, qualitative injectability), and in vivo retention of fluorescent 10-micron microspheres in contracting healthy rat myocardium was assessed; compared to Matrigel, these approaches supported the retention of double the cargo for up to 7 days post-injection.[155] In another study, Wang et al. investigated the use of the HELP hydrogels as injectable human mesenchymal stem cell carrier.[156] Compared to saline, the shear-thinning properties, afforded by the dynamically coupled matrix, protect cells from being subjected to mechanically damaging shear forces during injection. Furthermore, upon rapid recovery post-injection, secondary network formation, arising due to the LCST of ELP under physiological conditions, led to a tenfold reduction in erosion rate compared to control hydrogels lacking any thermally triggered reinforcement.[156]

While such stem cell therapies are promising for repairing and regenerating infarcted myocardium, certain limitations prevent their widespread use in clinical settings.[157,158] Low cell retention and limited engraftment necessitate excessive cell dosing,[158] and the cost of producing large amounts of cells to match demand is further exacerbated by the stringent requirements for their storage and transportation. Hence, emerging therapeutic modalities are moving towards the delivery of microRNAs (miRNAs) for treating cardiovascular diseases, and recombinant polypeptides have shown promise in such investigations as well. Yang et al. developed an in vivo miRNA delivery system to treat post-MI in a rat ischemia/ reperfusion model.[158] Shear thinning hydrogels were used to encapsulate polymeric nanoparticles comprising therapeutic miRNA for non-invasive delivery to the heart via intramyocardial injections.[158] The incorporation of cell-penetrating peptides (CPPs) allowed for rapid transfection of miRNA in cardiovascular cells.[158] The therapeutic efficacy of delivering genetic cargo was tested in vitro in normoxic and hypoxic conditions by monitoring the proliferation of human embryonic stem cell-derived cardiomyocytes and endothelial cells.[158] In vivo studies in a 3-month ischemia/reperfusion rat model led to restoration in the contractile mobility of damaged myocardium accompanied by smaller scar area and increased angiogenesis resulting in improved vascular function just after a single injection.[158]

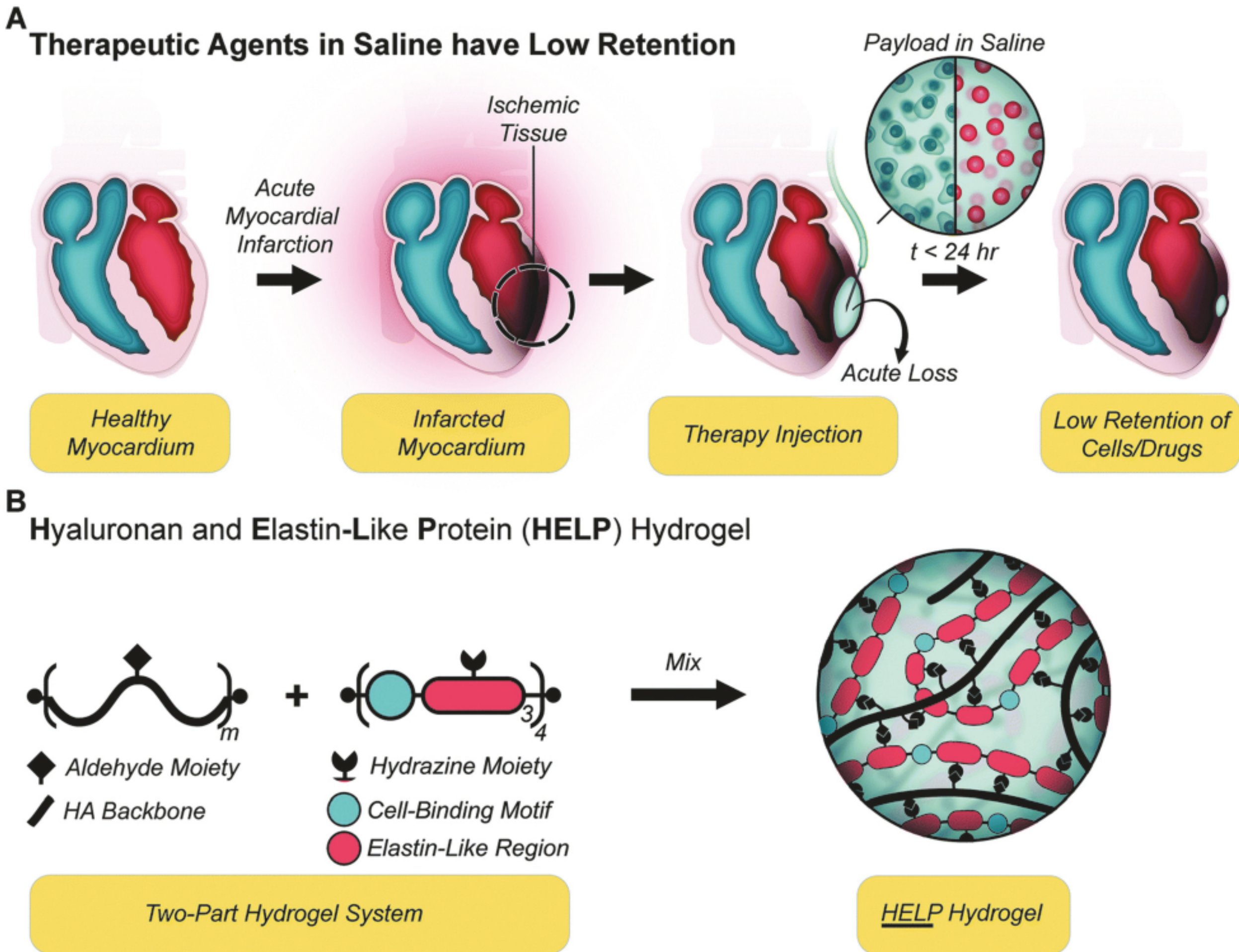


Figure 6. Injectable hydrogel strategy to improve myocardium retention: (A) Schematic of acute reflux following injection of therapeutic agents (cells (blue) and/or drug-loaded particles (red)) suspended in saline. (B) A two-part hydrogel (termed HELP; hyaluronan and elastin-like protein) is designed to improve the retention of therapeutic agents injected into the myocardium. Hyaluronan (also called hyaluronic acid; HA) is modified via oxidation or bio-conjugation reaction to display aldehydes, while the recombinant elastin-like protein (ELP) presents hydrazine moieties allowing formation of hydrazone bonds upon mixing. Reprinted with permission from Suhar et al.[155] Copyright 2022, Royal Society of Chemistry.

Although ELPs are intrinsically disordered proteins that lack any secondary structure, ordered building blocks can be genetically encoded along the polypeptide backbone to harness the propensity of ordered/disordered hybrid materials to self-assemble into hierarchically ordered structures. Drawing inspiration from tropoelastin, Roberts et al. doped polyalanine alpha-helical motifs along ELP chains to construct partially ordered polypeptides (POPs) which formed injectable porous scaffolds for tissue regeneration.[159] Upon heating above their LCST, ELPs form homogenous coacervates.[144] In contrast, POPs formed physically crosslinked viscoelastic fractal networks. Interestingly, the authors observed pronounced hysteresis between aggregation and dissolution temperatures which could be precisely tuned by altering the composition and degree of polyalanine doping.[159] In vivo studies in mice showed that the porosity of the POPs allowed for cell proliferation, vascularization, and integration in the subcutaneous space, and the POP depots did not exhibit any reduction in dimensions even after 21 days.[159]

The thermoresponsive properties of ELPs have been harnessed to develop other sophisticated block copolymeric constructs with precise control over inter- and intramolecular chain dynamics. Sing et al. created highly defined triblock copolymers by fusing ELPs with distinct transition temperatures to each end of a self-associating coiled-coil domain.[160] The resulting materials formed networks via the association of the mid-block coiled-coil linker. The network was further reinforced by micellar aggregation above the T_t of the ELPs. Linear and non-linear rheology was employed, along with transient scattering behavior under shear, to investigate the effects of molecular level changes to the end-blocks on the macroscopic deformation behavior of the resulting hydrogels.[160] Results illustrated the impact of minor changes to the thermoresponsive end blocks; just a simple substitution of the glycine with an alanine residue at the third position of the canonical motif XPGVG, resulted in a dramatic increase in the toughness and extension of the resulting hydrogels by two orders of magnitude and one order of magnitude, respectively.[160] In another study, Dai et al. synthesized an ABC block copolymer comprising a synthetic poly(trimethylene carbonate) (PTMC) block (A) and a thermosensitive diblock (BC) of distinct ELPs.[161] At concentrations greater than 4% w/v, the hydrophobicity and incompatibility of the A and C blocks led to the formation of rapidly switchable, thermoresponsive 3D porous hydrogel networks. The proposed mechanism for hydrogel formation was reportedly mimetic to the behavior of tropoelastin; micellar structures formed coacervates above the T_t, and the resulting globules organized and merged into a porous network. The PTMC domains stabilized the network via physical crosslinking. Lee et al. developed multi stimuli-responsive ABA-type triblock copolymers by fusing ligand-responsive calmodulin domains (B) to the thermoresponsive ELPs (A).[162] The resulting dynamic hydrogels underwent conformational changes in response to ligands such as calcium ions which offered orthogonal control over rheological and mechanical properties.[162]

3.2. SELP injectable hydrogels

The approach of combining the desirable properties of ELPs and SLPs, as explained above, has been successfully employed for creating injectable therapies, as the SELPs undergo a sol-gel transition under physiological conditions.[163] The physical crosslinking in the SLP domains creates stable depots without necessitating chemical or radiation-induced crosslinking.[144] Early reports used SELP scaffolds for encapsulation and chondrogenic differentiation of human mesenchymal stem cells (hMSCs) for 28 days to support cartilage tissue engineering efforts.[164] More recently, Cipriani et al. designed SELPs with RGD domains and pre-annealed them at 37°C for up to 48h. This facilitates both the formation of β-sheet structures in the SLP regions for improved mechanical properties and rapid formation of ECM-mimicking fibrillar structure upon injection even when surrounded by the synovial fluid environment. Chondrocytes were injected and cultured in an ex vivo cartilage tissue platform for 4 weeks, after which de novo ECM and hyaline cartilage formation was reported, along with a remarkable lack of fibrocartilage immunohistochemical markers.[165]

Different applications such as cell storage at low temperatures and injectability at physiological conditions can also be achieved via similar approaches by combining the ELP and

SLP transition temperatures in the same SELP construct. Song et al. fused the alpha-helical C-terminal domains (CTD) of major ampullate spidroin 1, derived from *Nephila clavipes* spiders, to ELPs of various lengths to achieve globular protein constructs with dual thermosensitive gelation.[166] The ELPs were chosen for their ability to undergo LCST-type transitions at high temperatures, whereas the CTD afforded UCST-like behavior via intermolecular hydrogen bonding at low temperatures. Due to their ability to undergo dual phase behavior at distinct temperatures, the utility of these materials for mammalian cell storage and injectable delivery was demonstrated for two model cell lines, murine fibroblast line L929 and rat bone marrow-derived mesenchymal stem cells (BMSCs). Cells mixed with the SELP and stored in the SELP gels (at 4°C or 10°C for up to two weeks) had 66% viability after 2 weeks compared to 16% viability in phosphate-buffered saline. Cells could be harvested at room temperature by triggering the gel-sol transition, and LCST-triggered gelation at 37°C allowed for injectable delivery of encapsulated cells.[166]

In addition to cell delivery, the injectability and sol-gel transition of SELPs have been harnessed for delivery of a variety of therapeutics[167,168] such as chemotherapy agents (doxorubicin and sorafenib),[169] glycosaminoglycans,[170–172] nucleic acids,[173,174] and viruses[175,176] Øyvind et al. used SELPs as bioresorbable embolic materials to block local blood flow to tumors for transarterial chemoembolization (TACE), for treating hepatocellular carcinoma.[167] SELPs were liquid when injected, which allowed them to penetrate the porcine vasculature more effectively compared to the clinically approved microparticle agent Embosphere.[167] In a similar study, Jensen et al. harnessed the sol-gel transition of SELPs to deliver indocyanine green (ICG) as a pre-surgery liquid embolic agent. This decreased intraoperative bleeding, reduced damage to surrounding tissues, and facilitated endoscopically visualized tumor margins to improve gross tumor resection.[177] The addition of indocyanine green improved both the gelation kinetics due to an increase of osmolarity and the viscosity of SELPs due to the ICG divalent anionic character which creates ionic bridges between lysine residues in the ELP block. Furthermore, using a biocompatible polypeptide to deliver ICG via SELPs carrier hydrogels was tolerated better by human umbilical vein endothelial cells (HUVECs) in vitro.

Instead of relying on the passive sol-gel transition of SELPs, Narayan et al. synthesized photo-responsive SELPs for spatiotemporal control over assembly and disassembly. This was facilitated by the binding of adenosylcobalamin ($AdoB_{12}$) to the C-terminus of bacterial carotenoid synthesis protein (CarHc).[178] SpyTag and SpyCatcher chemistry was used to construct SELP-CarHc protein polymers. In the dark, $AdoB_{12}$ and CarHc tetramerized to form a hydrogel network which could be triggered to disassemble and undergo gel-sol transition upon exposure to white light.[178] This precise spatiotemporal control over hydrogel dissociation was used to deliver encapsulated murine fibroblast cells on demand from three-dimensional cultures.[178] In related studies, Fonseca et al. recombinantly synthesized SELPs fused to two different fluorescent proteins to develop injectable hydrogels comprising Förster Resonance Energy Transfer (FRET) pairs, a green *Aequorea coerulescens* enhanced green fluorescent protein (AcEGFP) and the near-infrared eqFP650.[179] Physical crosslinking between two neighboring SELP molecules led to hydrogel formation that also underwent FRET upon self-assembly. Such hydrogels capable of undergoing FRET due to their molecular self-association could be useful for introducing traceability in tissue engineering scaffolds.

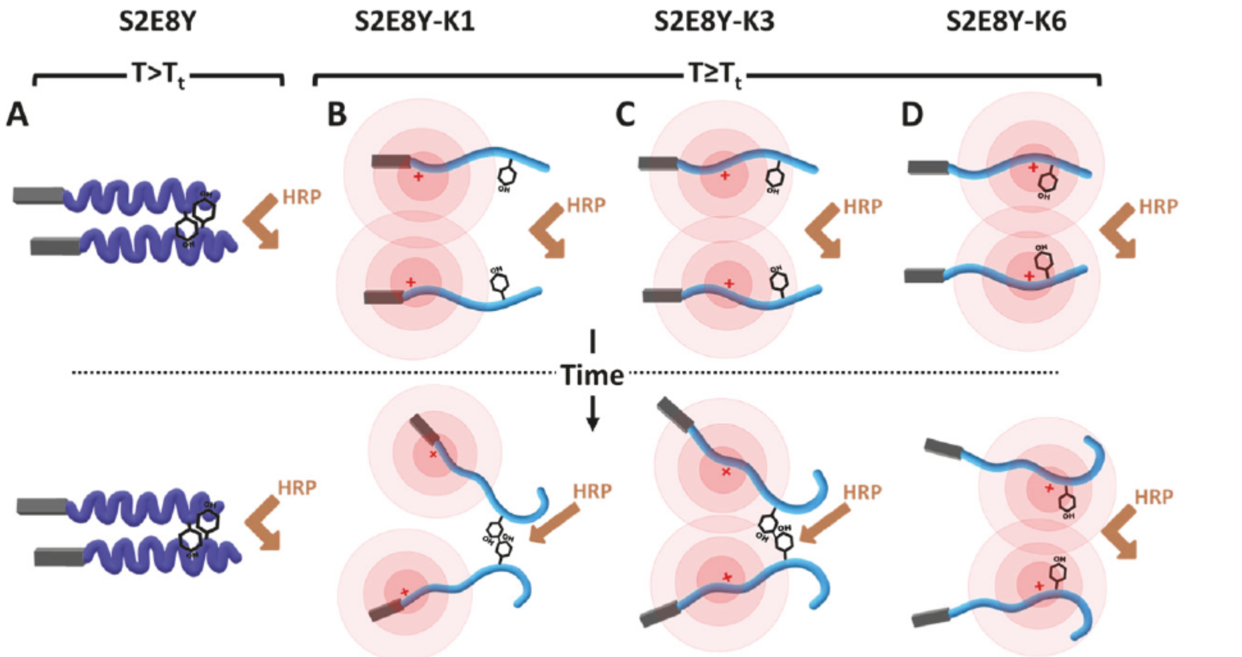


Figure 7. Schematic representation of the effect of positive charges on the HRP-mediated cross-linking of the four SELPs used in this study: (A) S2E8Y, with a T_t far below 37 °C, completely phase-transitioned and formed hydrophobic clusters that prevent HRP from accessing crosslinked tyrosines. For S2E8Y-K1 (B) and S2E8Y-K3 (C), the presence of positively charged lysines raises the T_t of these SELPs to 35 °C, preventing full phase transition. With time, the electrostatic repulsion between lysines and the molecular flexibility of the SELPs further expose tyrosines for cross-linking. For (D) S2E8Y-K6, the repulsion between positively charged residues and their proximity to tyrosines prevent HRP-mediated crosslinks. Reprinted with permission from Obeso et al.[180] Copyright 2022, American Chemical Society.

Obeso et al. utilized the activation of horseradish peroxidase (HRP) in the presence of hydrogen peroxide to form enzymatic crosslinks under physiological conditions in tyrosine-enriched SELPs.[180] The incorporation of lysine residues along the backbone not only increased the T_t of the SELPs closer to physiological temperatures but also disrupted the π-π stacking of the aromatic tyrosine residues. Furthermore, precisely positioning the lysine residues relative to the tyrosine residues allowed control over the formation of dityrosine bonds.[180] **Figure 7** illustrates that the electrostatic repulsion between lysine residues in the primary sequence, when closely positioned to the tyrosine residues, yielded hydrogels with low shear storage moduli (G' ~100s Pa).[180] When lysines were positioned further from the tyrosines in the SELP, crosslinked hydrogels exhibited G' values that were an order of magnitude higher,[180] while positioning the lysine furthest from the tyrosine (toward the N-terminus of the SELP) yielded the stiffest hydrogels (two orders of magnitude higher G'). These latter hydrogels underwent rapid gelation in situ due to the increased π-π stacking, leading to the formation of more enzymatic dityrosine crosslinks.[180]

3.3. RLP injectable hydrogels

Fewer reports of injectable RLP hydrogelss exist compared to ELPs, SELPs, and their derivatives. Li et al. investigated the feasibility of subcutaneous injection of RLPs and their rapid in situ gelation via Mannich-type condensation reaction in wild-type mice models.[181] King et al. improved the elastomeric properties of HA-based biopolymers by fabricating hybrid hydrogels via physically or covalently integrating RLPs to mimic the mechanical properties of the extracellular matrix of the lamina propria. Both formulations triggered minimal inflammation upon injection into rabbit vocal folds and were shown to be safe for up to 3 weeks in the airway.[182]

In contrast to ELPs, RLPs have been shown to undergo dual phase behavior, i.e., both upper critical solution temperature (UCST) transition and lower critical solution temperature (LCST) transition upon cooling below and heating above the respective transition temperatures.[183] This ability has uniquely positioned them to be used as models for investigating sequence determinants governing the phase behavior of intrinsically disordered proteins for the formation of membraneless organelles.[184–186] Hence, in recent years, research efforts have shifted focus on identifying key interactions between specific amino acids via heuristic approaches.[63,187] The insights obtained from these studies have been employed for developing advanced drug delivery carriers[188] and microstructured hydrogels.[122,123,189] A variety of RLPs have been explored for the development of stimuli-responsive[190] and microstructured hybrid hydrogels for drug delivery and for use as cell-instructive tissue engineering scaffolds via photopolymerization of multicomponent systems undergoing liquid-liquid phase separation.[191–194] Opportunities remain to integrate these existing formulations for developing injectable platforms for biomedical applications.

3.4. Injectable hydrogels from engineered peptide-peptide interactions

As discussed above, various associating peptide domains, such as those based on coiled-coil interactions, offer an alternative to induce physical crosslinking of polypeptides. Fernandez-Colino et al.[49] synthesized a recombinant elastin-like polypeptide (ELP) with coiled-coil domains in the middle of the sequence and on the C-terminus. The ELP-only control shows an LCST-like transition between 12°C-18°C. Rheological experiments at body temperature (37°C), show that at 15 wt% the polypeptide solution forms an unstable hydrogel that redissolves in a few minutes, while the inclusion of coiled-coil domains stabilizes the hydrogel for up to one hour, with complete stabilization of the hydrogel upon the inclusion of cysteine residues that can form disulfide bonds. The fact that these recombinamers are unable to form a hydrogel below the ELP transition temperature (5°C), denotes the cooperative interplay of the ELP domain with the coiled-coil.[49] A similar approach was studied by Mizuguchi et al.[195] in which the ELP sequence was also linked to a coiled-coil peptide domain rich in leucine to form crosslinked hydrogels. The inclusion of a sialoprotein-derived RGD domain resulted in the adhesion of HUVECs making these polypeptides suitable for scaffolds for 3D angiogenesis.[195] Similar to the work by Fernandez-Colino et al.[49] these polypeptides crosslink above the ELP transition temperature (20°C in this case) in just a few second;[195] the rapid gelation of these materials at body temperature makes them excellent candidates as injectable hydrogels.

Sun et al. designed other hetero-coiled-coil protein-based hydrogels aiming to create an injectable material.[196] Two different block polypeptides ACA and BCBCB were recombinantly synthesized, where domains A and B are five-repeat leucine zipper coiled-coil domains rich in either glutamic acid or lysine, respectively. Domain C is a four or five repeat of an immunoglobulin binding domain. Electrostatic repulsions prevent the homo-oligomeric interactions between A-A or B-B blocks, allowing only hetero-oligomeric interactions between the A and B domains and enabling hydrogel properties to be tuned not only by the total concentration but also by the A:B stoichiometric ratio. Step-strain rheometric studies from 1% to 300% strain, as well as injections through a 24-gauge needle, demonstrate the injectability and fast recovery of these hydrogels based on the dynamics and association kinetics of the glutamic acid/lysine complexes.[196]

Coiled-coil polypeptides have also been used to generate fluorescent injectable hydrogels enriched with quantum dots, which find application in enhancing the emission signal in biomedical imaging. Yang et al.[197] report a $PC_{10}A$ coiled-coil polypeptide, where P and A are an associative hydrophobic domain and a coiled-coil domain, respectively, and C10 is ten repeats of a hydrophilic random coil spacer midblock. Oil-soluble CdSe/ZnS quantum dots were added to a 0.1% w/w $PC_{10}A$ solution to form loaded nanogels and these were incorporated into a $PC_{10}A$ matrix to form a fluorescent hydrogel. The hydrophobic nature of the P and A domains allows interaction with and stabilization of the oil-soluble quantum dots, as well as the incorporation of the quantum dots into the hydrogel matrix, resulting in an increase of fluorescence up to 20% compared with the use of glutathione ligands. Rheological measurements show the decrease and recovery of the storage modulus upon step-strain cycles from 1% to 500% strain, highlighting their injectable properties which are confirmed using a 26-gauge needle. These injectable fluorescent hydrogels have potential applications in bioimaging and tumor therapy.[197]

Coiled-coil interactions, while widely employed, are not the only non-structural peptides used to promote hydrogel formation. Specific protein-peptide binding interactions have also been employed. Inspired by the interaction of the Tax-interacting protein-1 (TIP-1) with its peptide ligand NISYRRESAI, Zhang et al.[198] synthesized recombinantly a diblock construct composed of a ubiquitin-like domain (ULD), from the special AT-rich sequence binding protein 1 (SATB1), and TIP-1, connected by a hexaglycine segment. The ULD spontaneously forms a tetramer with TIP-1 termini on the surface; this tetramer was used to crosslink fibers formed by the self-assembly of a short peptide with the sequence Nap-GFFYGGGWRESAI (Nap = naphthalene). The N-terminal Nap-GFFY self assembles into long fibers with the WRESAI ligand exposed to the surface. Addition of 0.25-1.5% of the ULD-TIP-1 tetramer (relative to the short peptide) results in crosslinking of the fibers to yield weak hydrogels with a shear storage modulus (G') ranging from ~50 to 500 Pa; the hydrogels recover after application of 50% strain, indicating their injectable properties.[198] Xu et al.[199] produced hydrogels based on the crosslinking of an equimolar ratio of the ULD-TIP-1 tetramer with another recombinant tetramer-forming sequence ULD-GGGWRESAI, to yield weak hydrogels with a shear storage modulus of ~6 Pa (2 wt%). The hydrogels proved to be non-cytotoxic to human colonic epithelial cells (HCEC) and L929 cells in 2D cultures with viabilities above 90% in hydrogel concentrations ranging from 0.5 to 16 mg/mL, proving the low cytotoxicity of the materials.[199]

Cai et al.[200] studied similar injectable hydrogels based on interactions between a tryptophan-containing (WW) and a proline-rich peptide (PP). The mechanical properties of the resulting shear-thinning hydrogels for injectable encapsulation and long-term delivery (SHIELD) were expanded to greater shear storage moduli by employing a two-stage gelation mechanism. The first stage comprises weak peptide interactions between a heptameric repeat of the WW domain and a PNIPAM-modified 8-arm PEG with ~6 arms equipped with the PP domain (G' ~ 10-50 Pa); the thermal phase transition of the PNIPAM component increases the G' of the hydrogels (to ~ 1kPa for hydrogels containing 4 wt% PNIPAM) after their initial formation via the WW-PP interactions. These SHIELD were suitable for 3D encapsulation and secretome regulation of human adipose-derived stem cells (hASCs), as well as injection of cell-loaded hydrogels into healthy rats through a 28-gauge needle.[200] Similarly, Marquardt et al.[201] used SHIELD to prevent Schwann cell loss during spinal cord injury therapy. Different cell-adhesive domains in the WW peptide were tested, including two laminin adhesive domains (IKVAV and YIGSR), a fibronectin adhesive domain (RGD), and a scrambled RDG domain as control. The inclusion of the different peptide domains had no significant effect on the mechanical properties of the hydrogels, which were shown to protect cells from damage during injection, with the inclusion of adhesion domains contributing to improved cell morphology and migration.[201]

4. Perspectives and conclusions

Inspired by the load-bearing, biologically active, tunable properties of structural proteins, recombinant polypeptides have remained an attractive focus for the generation of injectable materials with high degrees of chemical, biological, and physical tuning. Polypeptides produced recombinantly combine the chemical versatility of synthetic polymers, i.e. they can have hydrophobic, hydrophilic, positively charged, and negatively charged moieties all in the same chain, and the biocompatibility and biodegradability of natural polymers. Furthermore, the high-fidelity and genetically directed nature of their synthesis allow for highly site-specific and precise sequence modifications depending on the desired material properties.

On-demand release of cargo is one of the most attractive opportunities in the drug delivery field, with a high potential for injectable hydrogels and nanocarriers made from stimuli-responsive polypeptides. Thermoresponsiveness, for example, can be exploited not only for self-assembly and injection but also for on-demand release, triggered either by an external stimulus or by a localized change in temperature due to immunological responses or elevated rates of tissue remodeling. Polypeptide-based materials have significant advantages for developing multipurpose materials capable of loading two or more drugs[130] at a time with the possibility of delivering them selectively under specific triggers. The phase separation behavior in the polypeptides presented here has potential application for the generation of condensed droplets capable of altering the optical properties of the materials, which may offer new sensing platforms. Additionally, accurate manipulation of properties/triggers, via the use of microrobots, could be leveraged in the formation of new patterning methods and localized drug delivery mechanisms. The continued progression of cell- and gene-based therapies also points to continuing needs in producing highly defined, selectively modifiable matrices that can be used to increase throughput, viability, and homogeneity; polypeptide-based materials are likely to be among the approaches that contribute biomedical advances in these areas.

Many opportunities exist to expand the use and utility of recombinant polypeptide materials. Although some recombinant polypeptides, such as ELPs, have been developed to a point where high yields can be consistently achieved,[88,202] there are still constructs that, to date, suffer from relatively low yield and high synthesis costs which have prevented their widespread application;[7,203] collaborative efforts with synthetic biologists will prove to be essential in expanding current expression yields and host machineries. In addition, the scientific community is still working on generating the rules that dictate how the amino acid composition impacts the thermoresponsive properties of polypeptides. As the studies of sequence determinants that govern phase transition properties and binding capabilities of polypeptides continue evolving, along with improved polypeptide expression techniques, it remains likely that genetically engineered protein-based polymers will provide significant benefits for modifying encapsulation and release of therapeutic agents, with great promise for generating a wide variety of injectable carriers finely tuned for their cargo and therapeutic action.

Notes

The authors declare no competing financial interest.

Acknowledgments

Related work in the author's laboratories has been supported in full or in part by funding from the National Science Foundation (DMR-1609544; DMR-2004796; DMR-0239744; DMR-2011824; DMR-2004890) and the National Institutes of Health (P20 RR017716; RO1 DC011377). The contents of this manuscript are solely the responsibility of the authors and do not necessarily reflect the views of the funding agencies.

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