



Recombinant protein polymer-antibody conjugates for applications in nanotechnology and biomedicine

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ABSTRACT

Currently, there are over 100 antibody-based therapeutics on the market for the treatment of various diseases. The increasing importance of antibody treatment is further highlighted by the recent FDA emergency use authorization of certain antibody therapies for COVID-19 treatment. Protein-based materials have gained momentum for antibody delivery due to their biocompatibility, tunable chemistry, monodispersity, and straightforward synthesis and purification. In this review, we discuss progress in engineering the molecular features of protein-based biomaterials, in particular recombinant protein polymers, for introducing novel functionalities and enhancing the delivery properties of antibodies and related binding protein domains.

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1. Introduction

Antibodies [1] and immunoglobulin fragments [2] are rapidly becoming important classes of therapeutic proteins used to treat

a wide range of diseases. However, clinical success of antibody therapeutics has been hindered by poor pharmacokinetics and inherent toxicity [3]. To address these issues, significant effort has been invested into designing strategies to increase circulation half-life, enhance therapeutic activity, and decrease necessary dosages. Polyethylene glycol (PEG) conjugation has been widely employed to increase the apparent molecular weight of antibody products beyond the renal filtration threshold [4,5]. However,

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while PEG is used in a variety of clinically-approved products [6], there is increasing concern over the immunogenicity of PEG [7] as well as its lack of biodegradability, which has been reported to cause cellular vacuolization [8]. Therefore, additional effective and biocompatible delivery strategies that address these deficiencies by modulating solubility, aggregation, degradation, permeability, clearance, and cellular distribution are essential for enhanced therapeutic efficacy and reduced side effects [9].

Recombinant protein-based polymers, including elastin [10], silk [11], resilin [12], collagen [13], XTEN [14], PAS [15], and coiled-coils [16] have the potential to provide these favorable properties and offer unique advantages that stem from the properties of the amino acid side chains and polypeptide backbone (Fig. 1). Collagen-, elastin-, and silk-based biopolymers are biocompatible, giving them increased potential to be immunotolerant. Furthermore, most protein polymers can be easily expressed as a monodisperse product in microbial hosts such as *E. coli* and yeasts, which is ideal for drug delivery applications. The sequence-level control over recombinant protein polymers allows for tunable modulation of physical properties as well as straightforward alteration of the number and location of potential chemical conjugation handles. In addition to improving pharmacokinetics of small antibody-like binders, the self-assembly motifs in protein polymers allow for spontaneous formation of well-defined nanostructures through inter- and intra-polymer interactions such as triple helix formation in collagen, beta-sheet fibrilization in silks, and multimerization of coiled-coil domains [17]. Furthermore, elastin-like and resilin-like polymers exhibit stimuli-responsive phase behavior that can drive the formation of nanoparticles with tunable aspect ratios [18]. These structures further expand the potential application space of antibody-like binders, as multimerization may be employed to enhance avidity or cluster/cross-link cell receptors to stimulate responses such as apoptosis.

In this review, we will discuss therapeutic antibodies, antibody fragments, nanobodies, and other non-immunoglobulin binding

domains and peptides such as antibody binding domains. We will highlight the structural features of the key classes of recombinant protein polymers used to alter the properties of antibodies and antibody-like binders. The applications of each type of antibody-recombinant protein polymer conjugate or fusion will be presented, and we will discuss future outlooks on the direction of the field.

2. Therapeutic antibodies

In nature, our immune systems utilize antibodies to defend us from disease through various mechanisms of action including antibody-dependent cell-mediated cytotoxicity, complement mediated cytotoxicity, and antibody-dependent cell-mediated phagocytosis [19]. These mechanisms have been repurposed in therapeutic areas ranging from cancers, to neurologic diseases, to infectious diseases, to cardiovascular diseases via the intravenous administration of antibodies against particular targets, such as cancer biomarkers [20]. Since the first FDA approved antibody treatment in 1985 [21], over a hundred antibody-based biologics have been approved for clinical use [20,22], with a robust pipeline of additional entities in clinical trials [23]; antibodies continue to be the most rapidly growing protein therapeutic [19]. While only traditional monoclonal antibodies (mAbs) were used initially, there are now a variety of mAb-related products such as bispecifics [24], mAb fragments [1], and nanobodies [25] that have been developed for therapeutic use [26]. These more varied and smaller antibody-type variants increase the flexibility for designing peptide bioconjugates for improved cell targeting and therapeutic efficacy [26].

2.1. Antibodies, antibody fragments, nanobodies, and other affinity binding domains

The immunoglobulin G (IgG) is the most prevalent antibody in the blood and the most commonly used antibody isoform for cancer

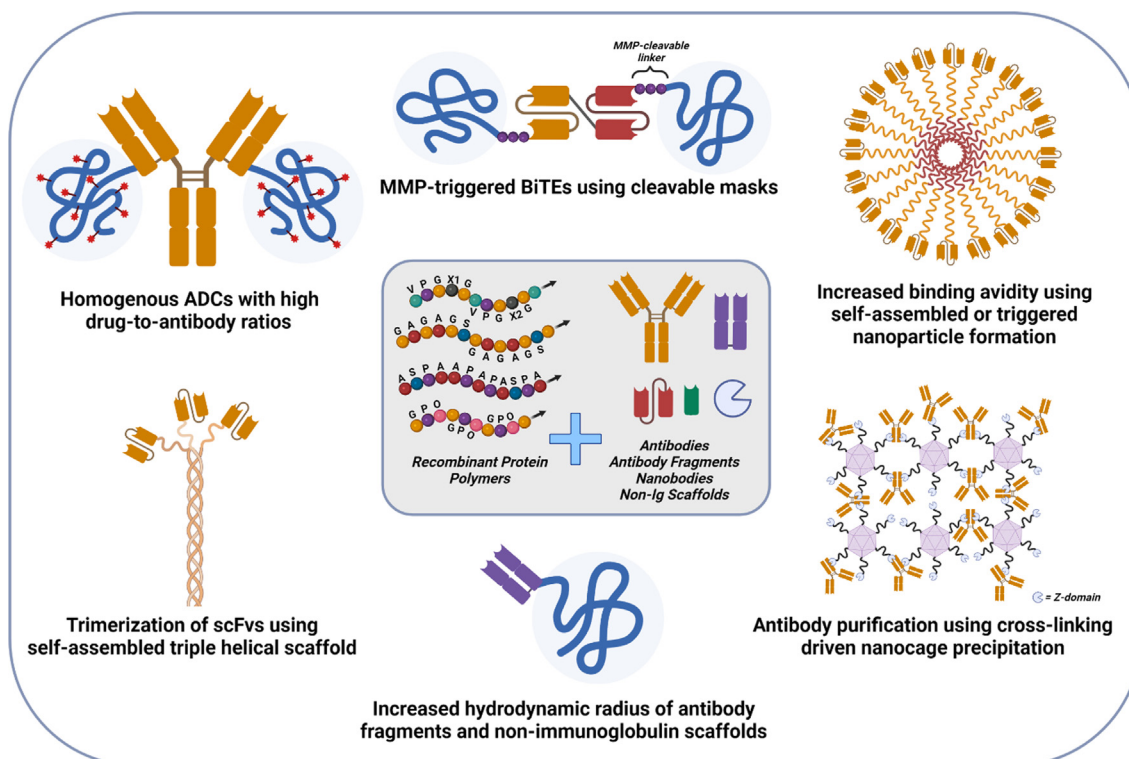


Fig. 1. Recent advances in the use of recombinant protein polymers in conjunction with antibodies, antibody fragments, nanobodies and non-immunoglobulin scaffolds. Created with [BioRender.com](https://www.biorender.com).

therapeutics applications [19,27]. The 150 kDa, Y-shaped structure is made of two heavy chain units and two light chain units held together with disulfide bonds. The heavy chains contain a single variable domain (V_H) followed by three constant regions (C_H1 , C_H2 , C_H3). The heavy chains make up the body of the Y, and are joined by disulfides at the hinge region between the C_H1 and C_H2 . The light chains contain a single variable region (V_L) followed by a single constant region (C_L) and are connected to the arms of the Y through disulfide bonds between the C_L and C_H1 . The arms of the Y, which contain the light chain in combination with the V_H and C_H1 , are denoted the Fab. The V_H and V_L at the tips of the Y arms make up the variable region (Fv), which is responsible for antigen binding. The two Fvs of the antibody provide avidity, which enhances the apparent binding affinity of the full antibody structure [28,29]. The heavy chain C_H2 and C_H3 domains make up the fragment crystallizable (Fc) region, which varies by isotype (1, 2, 3, and 4), and determines the type of effectors recruited for a particular immune response [19]. In biomedical applications, this region often is employed for conjugation to other moieties, such as polymers, so that the Fv domains remain accessible [30].

While the Fc domain provides antibodies with extended half-lives in circulation [28], it can stimulate undesirable immune responses [29]. Additionally, the glycosylation patterns and disulfide bonds in full length antibodies make them incompatible to produce in simpler organisms typically used for production of recombinant polypeptides. Given that the Fc is not necessary for target binding, antibody fragments limited to the Fab and Fv regions can be used directly [31]. The Fab domain can be obtained through proteolytic or chemical cleavage of the IgG, or alternatively, by recombinant expression. Additionally, the disulfide bond can be reduced so that the thiols may be reused for chemical coupling. The Fv is typically recombinantly expressed as a single chain (scFv) with a 15–30 amino acid flexible linker. These fragments can be produced in *E. coli* and yeast [32], and binders to new targets can be selected through directed evolution using phage and yeast display technologies [19].

In addition to traditionally used antibodies, camelid antibodies, which only contain heavy chain fragments, have also become popular homing agents. The variable heavy chain regions (VHH), denoted nanobodies for their small size (15 kDa), can be expressed in high yields in *E. coli*, and they also are easily adaptable for phage display [33–35]. Beyond antibodies and antibody fragments, other protein scaffolds and peptide platforms can be used for binding specific targets [36]. About 20 different proteins have been used as non-immunoglobulin scaffolds. These proteins are typically low molecular weight, single chain molecules that lack disulfide bonds and post-translational modifications so that they are easy to express in *E. coli*. A commonly used scaffold is the affibody, which is a 58 residue protein derived from a fragment of staphylococcal protein A [37]. Affibodies have high solubility and can achieve affinities down to the picomolar range. Non-immunoglobulin scaffolds can even be derived from human sources to lower immune response. Anticalins, derived from human lipocalins, are 160–180 residue α -barrel proteins that have entered clinical development with one molecule, PRS-080#22, going as far as phase IIa for treatment of anemia of chronic kidney disease [38]. The advantage of small homing proteins compared to the 150 kDa IgG is that these smaller proteins can achieve faster cellular internalization and deeper penetration into tumors, and their more rapid clearance is beneficial for imaging and radioactive drug based therapies [33,39]. Peptides, being much smaller than proteins, are less immunogenic than larger protein binders derived from other sources [40]. Both linear and cyclic peptides of varied lengths (~6–20 amino acids) have been selected via phage display for their selective binding to various targets, including 19 biomarkers for cancer [41].

2.2. Antibody binding domains

An alternative strategy for the attachment of antibodies to recombinant polypeptides is by using antibody binding domains (ABDs) that site-specifically bind the Fc and therefore can be used for non-covalent antibody linkage. ABDs originate from pathogenic bacteria, which natively display proteins that shield them from immune responses. Derivatives from Protein A from *Staphylococcus aureus* and Streptococcal protein G are the most commonly used, and these ABDs bind antibodies from a variety of species, although their relative affinities vary [42–44]. This class of ABDs consist of several repeats of a core domain. Each individual domain is capable of binding the Fc between C_H2 and C_H3 [45,46]. For example, Protein A has five triple helix domains (E, D, A, B, and C), each 58 amino acids in length [47]. Domain B has better selectivity for the Fc over the Fab [45–48]. Z-domain, a chemically stable derivative of B domain, is also widely used and can be fused to many recombinant proteins of interest [47,49–51]. Due to their pathogenic origin, these ABDs are primarily used for the purification of antibody therapeutics as well as molecular diagnostics. Peptides [52,53] and non-immunoglobulin scaffolds [54] have also been selected for antibody binding; however, the non-covalent association of these is likely to create issues for in vivo application where native antibodies could compete for binding. Antibody binding peptides directing site-specific covalent coupling to the Fc may hold better promise in translating this strategy to the clinic [55].

3. Recombinant elastin-like polypeptides (ELPs)

Elastin-like polypeptides (ELPs), derived from tropoelastin, were first characterized for their temperature responsive behavior by Urry in the 1980 s [56]. They consist of repeats of the amino acid sequence VPGXG, where X is any “guest” residue except for proline. They exhibit a lower critical solution temperature (LCST) where they shift from a random coil conformation to globule aggregates above their transition temperature (T_t). This reversible transition is a function of ionic strength and concentration of the polymer, and can be further tuned by the identity of the guest residue as well as the length of the polypeptide [57]. Apart from temperature, ELPs can additionally be engineered to transition in response to other stimuli such as pH [58] and the presence of metal ions [59]. This phase transition also allows for simple, non-chromatographic purification of fused or affinity bound protein domains through inverse transition cycling (ITC) [60,61]. ELPs fused to antibody binding domains, for example the Z-domain, have been used for the purification of antibodies [62,63]. Further enhancement in antibody recovery was achieved by conjugating ELP-Z fusions onto protein nanoparticles to enable IgG-triggered cross-linking using a variety of bioconjugation strategies [64,65] as well as the quick and facile detection of antibody titers in media [66].

The stimuli responsive property in ELPs also can be used to create hierarchical structures [67] such as ordered micelles [68] and nanoworms [69]. Recombinant block copolymers with ELPs and resilin-like blocks can self-assemble into spheres, rods, and worm-like morphologies depending on the length of each block [18,70]. Self-assembly into fibrils can be accomplished by combining ELPs with silk-like blocks [71,72]. The LCST also allows for the formation of temperature triggered drug depots which can be subcutaneously injected to provide sustained, local drug release via a sustained surface-to-core dissolution [17,73]. A stealth property also has been applied to ELPs through use of neutral and hydrophilic guest residues [74] as well as by incorporating a zwitterionic amino acid pair into the guest position [75].

3.1. ELPs in antibody drug delivery applications

Antibody fragments, nanobodies, and other small affinity domains have many advantages over antibodies when it comes to therapeutics. They are easier to produce, exhibit faster tissue penetration, and lack the Fc domain, which can lead to undesired immune stimulation [29]. However, their small size leads to rapid clearance and they lack multivalency and thus have less affinity than the full antibody. A nanobody targeting TNF- α was fused to ELP, expressed in transgenic plants, and purified through ITC [76]. This fusion was able to increase half-life of the nanobody 24-fold and prevent septic shock in mice.

To overcome avidity limitations, the temperature responsive property of ELP can be further used to assemble higher order structures that present antibody fragments on the surface at high local concentrations. A PD-1 checkpoint inhibitor scFv fused to a block copolymer consisting of linked hydrophilic and hydrophobic immune tolerant ELP (iTEP) blocks assembled into micelles that demonstrated an order of magnitude stronger binding to PD-1 than either the monomeric scFv or the intact α PD-1 antibody [68]. The PD-L1 blocking efficiency of the micelles was comparable to the antibody. Enhanced avidity also is desirable in cell targeting applications. An ELP micelle displaying nanobodies targeting the epidermal growth factor receptor (EGFR) exhibited better binding to human epidermoid carcinoma cells than the nanobody alone [77]. The self-assembly property of the ELP micelles further allows for the incorporation of other ELP-conjugated moieties providing additional functionality, including the photosensitizer IRDye700DX for photoimmunotherapy applications. This design provides the ability to load a high payload of photosensitizer per molecule of nanobody, where 98 % of the micelle was composed of the photosensitizer-linked ELP and 2 % of the micelle contained the nanobody-linked ELP. The photosensitizer micelle provided a lower EC₅₀ in the carcinoma cells than the singularly labeled nanobody-photosensitizer control, while having similar relative viabilities in the EGFR negative astrocytoma cells.

In addition to the benefits imparted by hierarchical structures for increased avidity, the morphology of hierarchical structures can affect binding and cell uptake, with high-aspect ratio, flexible, worm-like particles exhibit a higher number of accessible binding domains compared to spherical micelles [70]. These types of structures are useful for applications in which cell surface receptor crosslinking is used to induce apoptosis. Worm-like nanoparticles were generated after refolding a CD20-targeting scFv and ELP (A192) fusion [69]. These nanoworms outperformed Rituximab in inhibiting tumor growth in a non-Hodgkin lymphoma xenograft model. Similarly, this nanoworm-mediated receptor clustering strategy was used against CD20, HLA-DR10, CD19, and CD3 targets. The CD3-targeted nanoworm could trigger activation-induced cell death on a Sézary syndrome cell line model [78]. Similar nanostructures were applied to acute myeloid leukemia by targeting CD99 [79] or FMS-like tyrosine kinase 3 (FLT3) [80].

4. Silk and silk-like polypeptides

Silks are fibrous proteins spun by arthropods such as silkworms, spiders, bees, ants, and lacewing insects, for the purpose of protection of pupae during metamorphosis, building webs/nests, capturing prey, and protecting eggs [81]. They are known for their spectacular mechanical properties; for example, *Nephilla clavipes* dragline spider silk, has a high tensile strength while exhibiting up to 30 % stain before breaking, resulting in a more durable material than Kevlar, one of the strongest synthetic fibers [82]. The properties of silkworm and spider silks are a result of the hydrophobic anti-parallel beta-sheet regions that assemble nano

crystallites within the silk fibroin, or spidroin, in combination with the hydrophilic amorphous regions of the silk protein, which provide its extensibility [11]. The most available source of natural silk is the *Bombyx mori* silkworm, with sericulture of large numbers of silkworms first developed in ancient times to enable production of luxury textiles. Antibody fragments and binding domains have been incorporated directly into silkworm fibroin and spun into the cocoons [83–85]. These silk fusion materials can be used for immunoprecipitation [84] or processed into films [85] or coatings for immunosorbent assays [83].

The dominant *B. mori* hydrophobic region, which forms the strong beta-sheet structures, is comprised of the GAGAGS motif [11]. This motif was shown to increase expression of nanobodies and provide a slight enhancement in thermostability when fused to the C-terminus; increasing copies of the motif further increased transcription levels due to higher mRNA stability [86]. Repeats of this motif have also been used in combination with elastin-like motifs to generate recombinant silk-elastin-like polypeptides (SELPs), which can be expressed in high quantities in *E. coli* [11]. The properties of SELPs can be tuned based on the relative number of repeats of silk and elastin, with silk providing stability and elastin providing stimuli-responsive properties [71]. Similarly, resilin-like polymers can also be used in conjunction with silk motifs to make stimuli responsive materials [87]. These amphiphilic polymers can form fibers [87], films [88], hydrogels [89], and nanoparticles [90] though silk-silk crosslinks. Recombinant major ampullate spider silks have also been constructed from consensus repeat domains and expressed in *E. coli* [81]. These spidroin repeats consist of alternating hydrophobic polyalanine regions and hydrophilic glycine rich regions that are able to form fibers [91], particles [92], coatings [93], gels [94], and films [95].

4.1. Applications of silk-like polypeptides

Given the ability of silk-like domains to form fibrous materials and coatings, antibody-silk biomaterials have been most widely used in various immunoassays. By fusing the silks to antibody binding domains or antibody fragments, functionalized films and coatings can be made for detection of analytes [95]. For example, Z domain fused to spider silk was adsorbed to SiO₂ surfaces [96]. This one-step chemical-free surface biofunctionalization strategy allowed for an 8-fold higher capture of antibodies than the Z-domain chemically attached to the surface. This enhanced capture behavior was due to the site-specific attachment of the Z-domain, which provided a higher number of affinity domains with the optimal orientation for binding. Additionally, the formation of three-dimensional silk fibers provided a higher surface area and binding capacity than non-specific chemical modification of a flat surface. This type of platform can be expanded to micro- and nanoarrays in which spider silk is fused to individual scFvs targeting a range of biomedically-relevant proteins such as VEGF and the complement component C1q, which can be spotted onto the arrays for specific signal detection [97]. The co-localization of affinity domains on surfaces can also be used for applications in cell signaling. Coatings of recombinant spider silk fused to dimeric and tetrameric affibodies targeting VEGF receptor 2 (VEGFR2) were used to induce cell proliferation [93]. The silk-mediated display of affibodies in close proximity allowed for the VEGFR2 to properly dimerize resulting in the activation of its signaling pathways.

Recombinant spider silks can be formulated into particles for drug delivery. These particles can be further fused [92] or conjugated [98] to affinity peptides to target certain cancer models. For example, spider silks have been fused with HER2 targeting peptides for delivery into HER2 positive mouse breast cancer cells [99]. In vitro, the HER2 binding peptide provided a 6-fold higher binding to HER2 positive breast cancer cells than the silk spheres on their

own. When doxorubicin (DOX) was delivered into a mouse orthotopic breast cancer model, the spider silk spheres significantly reduced tumor volume compared to DOX-free controls, while also reducing the side effects of the systemically administered chemotherapy. Spider silk spheres can be further engineered by incorporating functional groups to encapsulate cargo, including DOX binding peptides to prevent premature DOX release [100], polylysine domains for CpG-siRNA [101] or pDNA delivery [102], and metal binding peptides for making iron oxide nanoparticle composites for photothermal therapy [103]. While the use of silk particles functionalized with antibodies and their fragments has not been explored, it should be straightforward to use them in place of binding peptides for cell targeting.

5. Recombinant resilin-like polypeptides

Natural resilin is a rubbery protein with almost perfect elasticity [104]. It is commonly found in the exoskeletons of insects and arthropods and is well-known for having low stiffness, high resilience, and the ability to efficiently store energy [105]. The unique mechanical properties and biodegradability of resilin make it an attractive biomaterial for regenerative medicine and drug delivery applications [106].

Natural resilin is a crosslinked protein with a unique composition of amino acid residues comprising 66 % non-polar groups and 31 % glycyl, resulting in a low isoelectric point and high hydrophilicity [107]. Since the discovery of the resilin gene sequences from *Drosophila melanogaster* in 2011 [108], recombinant resilin-like polypeptides (RLPs) have been produced to mimic the physicochemical and structural properties of natural resilin [12,109]. RLPs are intrinsically disordered polypeptides that contain high concentrations of glycine, and RLPs can be cast into a rubber-like biomaterial with Ru(II)-mediated photo-crosslinking, yielding a material with mechanical properties similar to those of natural resilin. Beyond the unique mechanical properties, RLPs are also responsive to pH and temperature [110], exhibiting dual phase-transition behavior characterized by both lower (LCST) and upper (UCST) critical solution temperatures, the temperatures below which and above which, respectively, the RLP is fully miscible in solution. Because these phase transitions are fully reversible, RLPs have been exploited to provide simple and inexpensive purification of fusion proteins [109]. Moreover, this tunable phase transition feature of RLPs also makes them an attractive biomaterial for therapeutic applications.

5.1. Resilin-based materials for biomedical applications

The controllable phase behavior of RLPs has been exploited to generate nanoparticles with tailorable sizes and shapes that are useful as drug delivery vehicles [111]. For example, RLP nanoparticles can be tuned from sizes of ~ 20 nm below the LCST transition temperature to ~ 400 nm above the transition temperature. Hybrid resilin-elastin polypeptides (RLP-ELPs) also have been created, and nanoparticle sizes can be altered by varying the RLP-ELP ratio (Fig. 2A) [112]. The nanostructure morphology can be further controlled by changing the hydrophobicity of the ELP block, resulting in formation of either spherical micelles or worm-like structures when using a more hydrophilic ELP core [18]. Increasing the hydrophobicity of the corona-forming ELP decreases the core repulsion, causing a transition from spherical to cylindrical morphology.

For drug delivery applications, a fibronectin type-III (Fn3)-binding domain that binds the $\alpha v \beta 3$ integrin was successfully fused to the C-terminus of an RLP-ELP diblock copolymer without impacting nanostructure formation [70]. While displaying Fn3 on

spherical nanoparticles improved the binding affinity by 10-fold due to multivalent binding, the worm-like nanostructures exhibited a remarkable 1000-fold increase in affinity, likely due to their flexible morphology and corresponding ability to provide improved multivalent binding onto integrin-decorated surfaces (Fig. 2B). The ability to display fusion protein partners on RLP nanostructures can be extended to other polypeptide/protein modalities such as antibodies to impart selective cellular targeting or therapeutic activity.

In addition to nanoparticles, RLP-based hydrogels have been created [113]. Different functional domains have been inserted within RLPs [114] to provide additional responsiveness to metalloproteinases (MMPs) [115] or intracellular redox conditions [116]. Furthermore, “smart” RLP hydrogels have been designed for cell-selective adhesion by inserting a cell-binding domain into the RLP building block [114]. A wide range of cell-binding moieties have been inserted to recruit different cell types [117–119]. While none of these hydrogels have been used for drug delivery purposes, these examples point to the feasibility of appending antibodies or nanobodies for therapeutic applications.

Resilin has also been combined with other proteins, including elastin, collagen, and silk, to generate hybrid resilin biomaterials with enhanced properties [120]. A chimeric polypeptide comprised of an RLP, ELP, and collagen-like peptide (CLP) in tandem was generated to enable formation of structures including large fibers and fiber bundles with Young's moduli in the range of 0.1–3 MPa [120]. These hybrid RLP biopolymers present a unique opportunity to create recombinant hybrid RLP-antibody/nanobody conjugates with customizable mechanical and targeting properties.

6. Recombinant collagen-like polypeptides (CLPs)

Native human collagen is a highly abundant class of extracellular matrix proteins encompassing 28 subclasses ranging from fibrillar to network-forming collagens. Each subclass is comprised predominantly, if not entirely, of proteins that form a prototypical right-handed triple helical tertiary structure based on the tight-packing of three parallel left-handed polyproline II-type helical polypeptide strands in a one-residue stagger [121]. This tight packing is facilitated by the obligatory repetitive (Gly-X-Y)_n primary sequence of each strand. Buried glycine residues within the center of the helix coupled with the high predominancies for proline in the X position and 4-hydroxyproline in the Y position give rise to both the relatively large average helical rise of about 0.86 nm per tripeptide repeat in proline-rich regions [122], and the small average triple helix diameter of about 1.5 nm [123,124]. Additionally, each subclass of collagen is marked by a characteristic suprastructure that varies between groups of subclasses [125]. Of particular interest in biomaterials applications is the use of fibrillar collagens such as type I collagen, for the creation of biocompatible wound-dressings [126,127] and matrices for drug delivery [128,129]. However, extraction of collagen from animal sources, which is the traditional method for generating collagen-based biomaterials, often generates heterogeneous products and also can contain trace impurities that illicit strong immune responses. [130,131] Therefore, most collagen-based biomaterials have moved towards utilizing either synthetic collagen mimetic peptides [132] or recombinant collagen-like polypeptides (CLPs) [13], of which the latter and its applications will be further discussed in this review.

The expression of fully functional recombinant mammalian collagen requires the implementation of multiple post-translational modification systems. Prolyl 4-hydroxylase, which is exogenous to bacterial and yeast expression systems, is required for the hydroxylation of proline residues throughout the triple helix. [133] This post-translational modification at the Y amino acid posi-

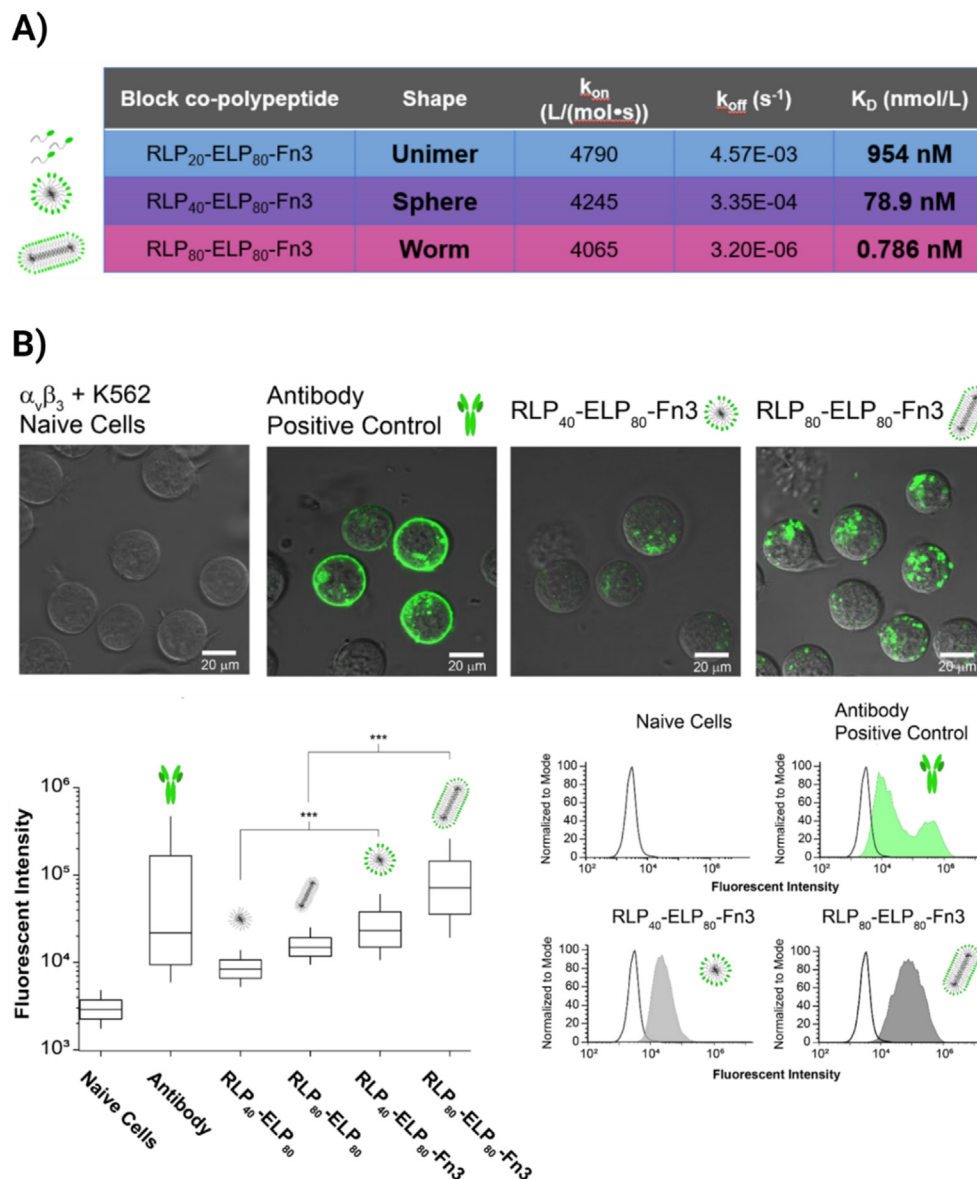


Fig. 2. Shape-dependent avidity effects on binding kinetics and in vitro cellular uptake using recombinant resilin-elastin hybrid polypeptides of different segment lengths with a C-terminal $\alpha_v\beta_3$ integrin-targeting fibronectin type III (Fn3) domain. (A) Binding kinetics and dissociation constants of the monomeric Fn3 domain compared to Fn3-decorated spherical and wormlike micelles. All measurements done using surface plasmon resonance. (B) Representative confocal micrographs and flow cytometry data for delivery of 10 μ M of the Alexa 488 dye-labeled monomeric Fn3 domain and Fn3-decorated spherical and wormlike micelles to K562 cells transfected with the $\alpha_v\beta_3$ integrin for 2.5 h. Adapted with permission from [70].

tion enhances the thermostability of the triple helix through stereoelectronic and steric effects, and is necessary to elevate the triple helix melting temperature above body temperature as needed for biomedical applications [134,135]. Lysine hydroxylation, also endogenous only to mammalian systems, is used for both glycosylation and cross-linking fibrils in collagen to increase tensile strength [136]. Recombinant expression of mammalian collagens has therefore employed either recombinant mammalian systems or non-mammalian systems (E. coli, yeast, plants) in which prolyl 4-hydroxylases are co-expressed. Mammalian expression systems precisely and completely hydroxylate all the proper prolines, but produce relatively low recombinant collagen yields, precluding applications that require high quantities of product [137]. Non-mammalian systems often yield products with either incomplete proline hydroxylation or non-selective proline hydroxylation patterns [138–140]. Therefore, most applications of recombinant

collagens utilize only small sections of human collagen sequences, or alternatively, employ collagen-like sequences from bacterial sources.

Bacterial CLPs contain the same repetitive (Gly-X-Y)_n consensus primary sequence as mammalian collagen, but are stabilized by a high percentage of polar and charged residues in the X and Y positions, as well as a large fraction of prolines in the X position, to compensate for the lack of hydroxyproline [13]. With these high percentages of polar and charged residues, many bacterial collagen-like proteins are able to maintain their triple helical structures at temperatures comparable to those of recombinant mammalian collagen polypeptides [141]. Bacterial CLPs also contain additional terminal non-collagen domains including a terminal globular variable domain (V-domain), which facilitates refolding following heat denaturation of the triple helix [142], and a terminal cell wall anchoring domain. This ability to re-fold the triple helix

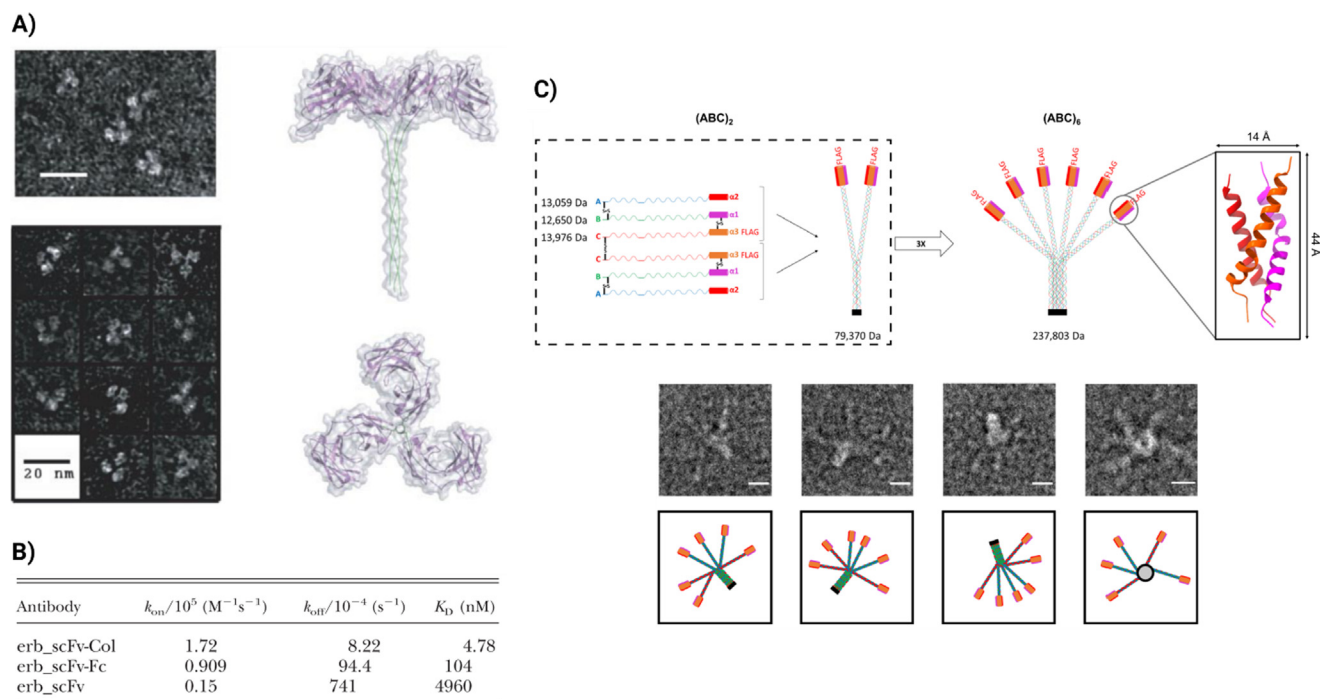


Fig. 3. Application of recombinant collagen-like polypeptides as scaffolds for multimeric clustered protein presentation. (A) Transmission electron micrographs of the collabody constructed from the genetic fusion of a (GPP)₁₀ CLP to a scFv for the human epidermal growth factor receptor's extracellular domain, connected by a short flexible linker. Scale bars are all 20 nm. (B) Binding kinetics and dissociation constants of the trimeric collabody compared to a dimeric scFv–Fc domain fusion and a monomeric scFv construct. All measurements done using surface plasmon resonance. (C) Assembly diagram and transmission electron micrographs of “headless” C1q protein constructed from the replacement of the C1q C-terminal globular regions with the NC2 domains of type IX collagen. Adapted with permission from [150] and [155].

when fused to the correct termini of the V-domain [142], and the tunability of the triple helix melting temperature using multiple inputs (e.g., pH [143], V-domain selection [142], collagen-like domain selection/length/patterning [141,144–146]) makes bacterial CLPs interesting for use in different biomaterials applications. Furthermore, many examples show the ability to express bacterial CLPs at high yields [147] and recover the CLPs using simple, large-scale purification techniques [148], demonstrating that these polypeptides could be applicable to the construction of biomaterials. Furthermore, the collagen-like domains of bacterial polypeptides do not elicit immune responses based on studies in mice [149], showing their potential for application in drug delivery.

6.1. Recombinant CLPs in drug delivery applications

The triple helical structure of collagen can be leveraged as a scaffold for the trimeric presentation of different protein domains based on the amenability of the CLP chains to terminal genetic fusions. Many examples exist in nature, with various subclasses of collagen containing terminal globular domains [125], and bacterial CLPs presenting terminal globular V-domains [13]. This trimeric presentation can be utilized to increase the affinity of a binding domain to an antigen target by increasing the avidity in binding, which is especially pertinent for low affinity binding domains. This affinity effect was shown in the development of a “collabody,” in which an scFv to the human epidermal growth factor receptor’s extracellular domain was expressed in a genetic fusion to (GPP)₁₀, linked by a short flexible linker. (Fig. 3A) Using mouse myeloma NS0 cells for expression, the authors demonstrated complete hydroxylation of all the Y-position proline residues; moreover, trimerization improved the dissociation constant by 1000-fold as compared to the monomeric scFv construct (Fig. 3B) [150]. The

trimerization of recombinant antibody fragments was important beyond its capacity to increase binding avidity. Multimerization increased the effective size of the overall structure, thus greatly diminishing renal clearance following intravenous delivery [151]. The collabody structure can be further modified to increase avidity or specificity via functionalization of the free termini of the (GPP)₁₀ triple helix with the same scFv or another binding domain. Additionally, different collagen-like sequences could be utilized to create heterotrimeric collabodies with more complex binding specificity. For example, many platforms have been designed for creating heterotrimeric collagen-like triple helices with precise control of chain composition including ABC heterotrimers composed of a 1:1:1 composition of (PKG)₁₀, (DKG)₁₀, and (EPG)₁₀ collagen-like peptides [152]; AAB heterotrimers composed of a 2:1 composition of (PKGEOG)₅ and (POGDOG)₅ collagen-like peptides [153]; and ABC heterotrimers formed from the 1:1:1 mixing of genetic fusions to the AAB heterotrimeric NC2 domain of type IX collagen [154]. The heteromerization of collabodies might pose an elegant solution to control the targeting specificity of a trimeric scaffold, as many disease states cause cells to upregulate multiple receptors at different relative densities. Manipulating the valency of multiple binding domains within one structure could function to greatly improve targeting specificity. Additionally, similar to the functions of the collabody, a platform based on complement component C1q that was made up of six individual collagenous domain ABC heterotrimers was recently developed by replacing the C1q C-terminal globular regions with the NC2 domains of type IX collagen (Fig. 3C) [155]. This platform could be further amended for the presentation of different recombinant antibody fragments or binding domains, with greater possibilities for control of the number and relative densities of presented domains compared to the collabody platform.

7. Recombinant intrinsically disordered pseudo-repeating polypeptides: XTEN and PAS

Recombinant polypeptides with intrinsic conformational disorder and high solubility have been designed with the intention of creating biodegradable and less immunogenic alternatives to polyethylene glycol (PEG) that will improve the biophysical and pharmacokinetic properties of protein and peptide drugs [156]. XTEN [14] and PAS [15] recombinant pseudo-repeating polypeptides both function with a near-identical mechanism to PEG: both greatly increase the hydrated volume of the tagged molecule, which minimizes renal clearance, while simultaneously providing a shielding effect to reduce phagocytic clearance and proteolysis. Similar to PEG, XTEN and PAS fusions to targeting domains slightly increase the target dissociation constant through a reduction in the on-rate, yet this decrease in apparent affinity can be more than compensated for in vivo through the increase in the fusion's circulation half-life [15,157,158]. Therefore, both XTEN and PAS can be utilized to greatly improve the delivery of antibody fragments, nanobodies and other binding domains, especially those with a sub- μ M dissociation constant.

The original XTEN recombinant polypeptide developed by Amunix Pharmaceuticals, which was recently acquired by Sanofi in 2021, consists of an 864-residue polypeptide chain containing a proportional yet randomized sequence of alanine, glutamic acid, glycine, proline, serine, and threonine built from iterative expression level screening and extension from randomized 36 amino acid length segments [14]. The finalized XTEN sequence was shown to completely lack secondary structure, present an extremely large hydrodynamic volume with respect to its molecular weight, and exhibit no observed propensity to aggregate in response to either elevated concentration or elevated temperature [14,159]. Starting from this original 864 residue sequence, multiple fractional truncations of XTEN as well as an XTEN tandem dimer have been characterized to function similarly [159]. Most studies show that longer XTEN sequences give greater improvements to circulation half-life (Fig. 4A), yet conversely, longer lengths could have adverse effects on expression yields, interfere with folding at internal insertion sites, or dampen the rate of diffusion into a tumor; accordingly, many applications benefit from selecting small XTEN sequences [158,160].

The PAS recombinant polypeptide (e.g., Pro/Ala-rich Sequence) was created using a more restrictive design principle as the XTEN polypeptide. The original PAS sequence of 20 amino acids was built by randomly arranging proline, alanine, and serine residues while minimizing the presence of two-residue repeats and prohibiting any longer length repeats to prevent the potential formation of secondary structures at localized areas of single-residue homopolymers [15]. This randomly designed 20 amino acid sequence (ASPAAPAPASPAAPASAPA) serves as the traditional template for creating higher molecular PAS tandem multimers, often going all the way up to 30-repeat PAS protein polymers [161]. However, PAS polymers are not restricted to the same arrangement of proline, alanine, and serine amino acids. Similarly acting polymers can be created using the same design technique without including serine, and also, while altering the proportion of proline to alanine residues within a broad range [162]. When compared to PEG polymers of identical molecular weight, PAS biopolymers show larger hydrodynamic volumes and lower concentration-dependent viscosities, outperforming PEG in aspects influential to the administration of polymer-drug conjugates via injection [162]. When compared to XTEN, PAS shows similar trends of increased biopolymer lengths yielding prolonged circulation of fusions (Fig. 4A) [158,163] while the upper range of biopolymer lengths can decrease tumor uptake due to a combination of increasing the apparent target dissociation constant and/or dampening the rate of diffusion into the tumor [160,163]. (Fig. 4B) This has manifested into many examples of the use of PAS fusions of different lengths, ranging from as small as 20 residues to as large as 600 amino acids [161].

Both XTEN and PAS polypeptides can serve as excellent biodegradable alternatives for prolonging circulation in therapeutic protein delivery. Utilizing recombinant polypeptides in a genetic fusion to a protein cargo will always generate a homogeneous product as long as the chosen purification techniques can remove any truncation products. By contrast, many examples have shown that utilizing PEG can yield a difficult to separate heterogeneous mixture of products due to non-specific conjugation and the inherent polydispersity of PEG [164]. Additionally, using genetic fusions allows for the incorporation of two XTEN or PAS biopolymers of the desired lengths by conjugation to both the termini of the protein [165], whereas implementing such a scheme with

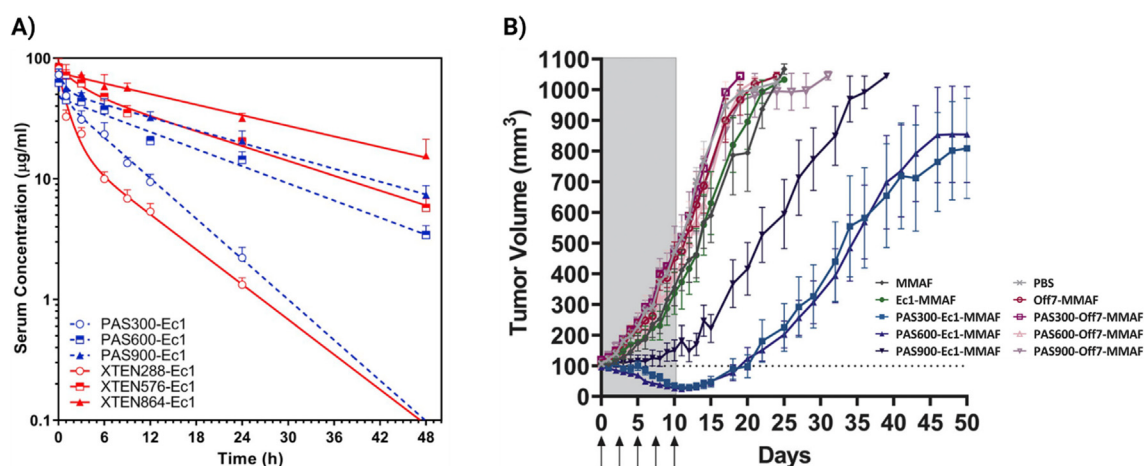


Fig. 4. In vivo pharmacokinetics of PASylated and XTENylated DARPin fusions of different biopolymer lengths. (A) Effect of recombinant PAS and XTEN polypeptide lengths on serum concentration of DARPin fusions. All proteins were injected intravenously into five mice at 4.5 mg/kg for each construct and all serum concentrations were measured with sandwich ELISA. (B) Effect of recombinant PAS lengths on tumor volume of DARPin fusion with monomethyl auristatin F (MMAF) conjugation. All protein-MMAF conjugates were injected intravenously into six mice with EpCAM-positive HT29 tumor xenografts with either 300 nmol/kg of the EC1 DARPin or a negative control Off7 DARPin. Adapted with permission from [158] and [160].

PEG would require use of two orthogonal conjugation groups, further increasing the complexity of synthesis and down-stream processing steps [164]. Furthermore, both the XTEN and PAS polypeptide sequences have additional properties that make them even more useful for protein delivery. Both XTEN and PAS only contain reactive nitrogen functionalities at their *N*-terminus and have been shown to tolerate incorporation of multiple cysteine residues within the polypeptide chain, allowing for creation of site-specific and multivalent chemically conjugated products with *N*-hydroxysuccinimide ester and maleimide functionalized payloads [158,166–168]. PAS polypeptides also contain only one reactive carboxyl functional group, at the C-terminus, enabling additional orthogonal chemical conjugations. The functionalization of a protein cargo with XTEN or PAS polypeptides also eliminates the necessity for incorporation of purification tags, which typically requires further downstream processing for tag cleavage and removal. The tolerance of XTEN fusions to prolonged heating at elevated temperatures has been leveraged to create a purification scheme wherein an initial heating step is used to precipitate a majority of whole cell lysate protein. Subsequently, the low isoelectric point of the XTEN sequence is used to enable an anion exchange chromatography step to further purify XTEN-fusion protein constructs [14,159,169]. A single-step chromatographic purification scheme without harsh conditions has also been recently developed for PAS fusion constructs by using a moderately low-affinity PAS-specific antibody Fab fragment with fast dissociation kinetics and a 1 M L-prolinamide elution step [170]. This Fab was generated using a 40 amino acid PAS polypeptide conjugated to a strong immunogenic carrier protein, with multiple immunizations to several mice generating only a small pool of six monoclonal antibodies [171]. A similar technique was used to create an anti-XTEN monoclonal antibody [159]. The low number of antibodies generated for both XTEN and PAS despite substantial effort, coupled with the lack of observed immune response with either sequence, emphasizes the potential suitability of translating from PEG to either of these polypeptides for prolonging circulation in therapeutic protein delivery.

7.1. Recombinant XTEN and PAS polypeptides in antibody drug delivery applications

The primary application of XTEN and PAS recombinant polypeptides has been to prolong the circulation of different therapeutic protein cargo. This is particularly relevant for proteins below 70 kDa, as this molecular weight represents the general renal threshold limit for globular proteins, which includes a majority of the recombinant antibody fragment domains and all nanobodies and non-immunoglobulin scaffolds [151]. To this end, XTEN and PAS recombinant polypeptides have been shown to prolong the circulation of Fab domains [15,163,172–176], nanobodies [177,178], anticalins [179,180], DARPin [158,160], and affibodies [168]. The fusion of these polypeptides can increase circulation half-life as compared to circulation of the untagged protein by 2-fold [163,171] to 114-fold [158,160]. This change greatly improves the therapeutic output of these molecules by prolonging their circulation in mice from approximately a few minutes to nearly 24 h, in the upper-range of XTEN and PAS tagged molecules. These differences in half-lives in mice are even more substantial after taking into account the allometric scaling of these values to circulation in humans.

Recently, recombinant XTEN polypeptides were applied as a scaffold for small-molecule drugs conjugated to a cysteine-engineered antibody, creating a homogenous antibody-drug conjugate (ADC) with a very high drug-to-antibody ratio [167]. While the implementation of these recombinant polypeptides did not prolong circulation of ADCs because of their relatively large size

and FcRn-mediated recycling, it emphasized the benefits of using recombinant polypeptides as a scaffold for site-specific conjugation of cargo and creation of a homogenous product. This example allowed for drug-to-antibody ratios of 18, where most clinically-approved ADCs only have a ratio of at most 4 [181].

An additional interesting application of XTEN polypeptides utilizes its secondary masking mechanism to create an MMP-inducible protein delivery system. This was originally implemented using a 288 residue XTEN polypeptide linked by an MMP-2 and MMP-9 cleavable linker to a cytostatic and cytotoxic protein, Killin, and 6x-arginine cell-penetrating peptide fusion [169]. The cleavable XTEN mask gated the cytotoxicity of Killin, shown by the approximately 96 % decrease in *in vitro* cell viability of MMP-2 and MMP-9 upregulated human fibrosarcoma HT-1080 cells after delivery of 10 μ M of the conjugate for 24 h, without affecting the growth of BRL3A liver derived non-tumor cells [169]. Amunix Pharmaceuticals has since started implementing this XTEN masking system for the delivery of XTENylated Protease-Activated T-Cell Engagers, or XPATs. The XPAT platform, termed AMX-818, uses a bispecific T-cell engager (BiTE) format constructed from the fusion of an anti-HER2 scFv and an anti-CD3 scFv with N- and C-terminal XTEN masks of different lengths linked by a proprietary sequence of three adjacent protease cleavable domains. The XTEN masks on AMX-818 increase the EC₅₀ for *in vitro* target-directed T cell cytotoxicity of huPBMCs against BT-474 tumor cells by four-orders of magnitude after a 48-hour incubation. Additionally, this masking allows for a large increase in maximum tolerated exposure, with a C_{max} around the single μ M range, an approximately 400-fold improvement to the unmasked variant. This improvement allows for safe dosing of AMX-818 at relatively high doses of around 50 mg/kg in non-human primates [182]. This AMX-818 platform has also been shown to be translatable to other scFv tumor targeting domains [183].

8. Coiled-coil based nanostructures

Coiled-coil peptides are a widely used motif for protein assembly because of their ability to provide highly predictable and tunable interactions [16]. Coiled-coil domains often exhibit 4 or more heptad repeats of hydrophobic (H) and polar (P) residues, HPPHPPP [184], and can be easily combined to generate a wide range of complex assemblies [185]. Many of these sophisticated assemblies have been made possible by sequentially combining different coiled-coil domains into defined morphologies [186–189]. A multiple domain protein containing 12 coiled-coil-forming segments was used to create a tetrahedral nanostructure whose morphology was precisely controlled by the orientation and sequential pairwise interaction of each coiled-coil segment [188]. More recently, dimerizing coiled-coils based on the arginine-rich leucine zipper motif (Z_R) and glutamic acid-rich leucine zipper motif (Z_E) were used to create protein amphiphiles for thermally triggered self-assembly of 5 μ m protein vesicles displaying functional GFP or mCherry [190]. Coiled-coil interactions can also be repurposed to introduce multivalency in nanocarriers for enhanced binding [191]. These examples highlight the structural flexibility achieved simply by creating the appropriate pairwise coiled-coil interactions.

Recently, a *de novo* designed hexameric coiled-coil peptide (Hex) [192] was exploited for antibody delivery. Hex was fused to the domain B of Protein A to enable binding to the Fc region of antibodies [193]. The resulting self-assembled nanoparticles were shown to display up to three antibodies, and these nanoparticles were readily uptaken into HeLa cells via endocytosis. Increased exposure of the Hex coiled-coil on the carriers enhanced cellular internalization, highlighting a role of the coiled-coil in pro-

moting endocytosis [194]. Improved endosomal escape was observed when His-tags were presented on the carrier, suggesting a possible endosomal buffering effect.

In addition to nanoparticles, de novo designed coiled-coil peptides have been designed to self-assemble into a polyhedral nanocage [188]. A single protein composed of 12 coiled-coil peptides was created to drive the formation of nanostructures based on orthogonal pairwise interactions. The recent discovery of nanobodies that bind specifically to different coiled-coil pairs provided a simple strategy to decorate the nanocage with antibodies for therapeutic applications [195]. Even multimeric coiled-coil interactions have been exploited to create polyhedral nanostructures [196] that are useful for multivalent display of antigens against HIV [197] and malaria [198]. The ability to modulate the displayed protein cargo is an attractive feature that can be extended to antibody or nanobody display.

9. Conclusions and future directions

This review highlights the diverse array of functionalities imparted to antibodies, antibody fragments, nanobodies, and other non-immunoglobulin scaffolds by direct genetic fusion or conjugation to recombinant protein polymers. Each biopolymer instills features such as greater avidity, enhanced circulation half-life, or alternative purification methods that can assist in the translation of these protein therapeutics to the clinic. While no clinical studies to date have applied these biopolymers in combination with antibodies, antibody fragments, nanobodies, or non-immunoglobulin scaffolds, several recombinant protein polymer conjugates to other classes of protein therapeutics have shown promising results in clinical trials [199,200]. Moreover, the continual expansion of the number and diversity of protein biologics on the market will increasingly require cutting-edge drug delivery approaches. For example, the fraction of combination products such as antibody-drug conjugates (ADCs) will continue to increase, supported by advances in antibody design and new linking chemistries, and these ADCs will require more sophisticated control over the drug delivery process to maximize drug potency, obviate off-target toxicities, and improve therapeutic index [201]. Meanwhile, antibodies and antibody-like molecules offer new prospects for reaching intracellular 'undruggable' targets [202], and the advent of new endosomal escape techniques enables new capacity for successful antibody delivery into the cellular cytoplasm [203,204]. Protein polymers offer a natural solution to modify and fine-tune ADCs, intracellular antibodies, and other novel antibody products for increased clinical potential, and the existence of new regulatory frameworks will accelerate the translation and commercialization processes [205]. For these reasons we ascertain that in the near future, recombinant protein polymers will see much more clinical relevance in the delivery of antibody and antibody-like binders.

New frontiers for antibody-protein polymer structures may exist in the use of protein polymers to construct and modify hierarchical biomaterial structures, resulting in novel capacity to organize antibody or antibody-like domains into three-dimensional nano- and microstructures relevant to drug delivery. A large fraction of the application space for elastin-like [206], silk-like [87], resilin-like [115], and collagen-like [207,208] polypeptides and their hybrids [87,209,210] involves the construction of hydrogel structures, and some of these hydrogels have been directly applied for the sustained local delivery of antibodies [211]. Furthermore, the inherent properties of protein polymers such as synthetic collagen-like polypeptides enable unique modes of hydrogel modification with growth factors, nanoparticles, and other drug delivery moieties via utilization of the strand invasion capacity of CLPs for stable and tunable binding to denatured collagen

[132,212]. The self-assembly properties of CLPs and ELPs have been combined to produce vesicular nanoparticles from CLP-ELP fusions [213,214], with covalent linkage of the CLP and ELP permitting temperature-responsive control over nanoparticle assembly state and drug delivery. Moreover, benign bioconjugation techniques such as sortase A ligation [215], SpyCatcher/SpyTag chemistries [216], and unnatural amino click chemistries [217] can be used to allow controlled decorated hydrogels and nanostructures with antibodies or nanobodies without disruption of the assembly process [70,218,219]. The ability to exploit the diverse features of protein polymers in conjunction with antibodies will greatly expand the array of applications of antibody-protein polymer fusions by enabling fine-tuning of antibody presentation, organization, and activity within the context of a wide array of advanced molecules, nanostructures, and biomaterials.

Additional recombinant technologies such as Fc domain fusion and HSA-targeting have been shown to provide some of same advantages as recombinant protein polymers [200]. These fusion partner will likely impact the dissociation constant, solubility, aggregation behavior, and the immunogenicity of each specific antibody [220]. Additional preclinical studies that fully encapsulate these issues must be made on a case-by-case basis.

Data availability

No data was used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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