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Self-assembling nanocarriers from engineered proteins: Design, functionalization, and application for drug delivery

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ABSTRACT

Self-assembling proteins are valuable building blocks for constructing drug nanocarriers due to their self-assembly behavior, monodispersity, biocompatibility, and biodegradability. Genetic and chemical modifications allow for modular design of protein nanocarriers with effective drug encapsulation, targetability, stimuli responsiveness, and *in vivo* half-life. Protein nanocarriers have been developed to deliver various therapeutic molecules including small molecules, proteins, and nucleic acids with proven *in vitro* and *in vivo* efficacy. This article reviews recent advances in protein nanocarriers that are not derived from natural protein nanostructures, such as protein cages or virus like particles. The protein nanocarriers described here are self-assembled from rationally or *de novo* designed recombinant proteins, as well as recombinant proteins complexed with other biomolecules, presenting properties that are unique from those of natural protein carriers. Design, functionalization, and therapeutic application of protein nanocarriers will be discussed.

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1. Introduction

Significant progress in medicine has expanded the therapeutic landscape, resulting in a wide variety of drugs to treat various diseases, including not only small molecule drugs, but also biomacromolecules such as nucleic acids, peptides, and proteins. However, the efficacy of biologic and small molecule drugs is constrained by poor pharmacokinetics, limited bioavailability, absence of specificity, and inherent toxicity. In response, drug delivery systems (DDS) made from many different types of materials have evolved to address these issues [1–3]. DDS are designed to optimize pharmacokinetics and biodistribution for enhanced therapeutic efficacy and reduced systematic exposure to decrease side effects or toxicity. Among all types of DDS, nanocarriers with a size range of 10–200 nm have emerged as platforms with significant potential for efficient drug delivery via a wide variety of administration routes [4–6]. To develop nanocarriers for drug delivery, the following functionalities should be achieved. (1) Nanocarriers encapsulate drugs with high loading efficiency and maintain stability *in vivo* to protect drugs from degradation. (2) Prolonged *in vivo* circulation in blood or residence time in other tissues help improve the fraction of drug-loaded nanocarriers that reach the target site or cells. One common approach is to coat nanocarriers with hydrophilic polymers, such as poly(ethylene glycol) (PEG), which delays opsonization and clearance by phagocytes [7]. (3) Drug-loaded nanocarriers reach their target sites via passive or active targeting. In passive targeting, the size of nanocarriers, for example, can affect biodistribution, allowing them to preferably accumulate at targeted tissue [8]. Meanwhile, active targeting can be achieved by attachment of targeting ligands, such as targeting peptides and proteins or antibodies [9]. Targeted nanocarriers bind cells through receptor-ligand interactions, often followed by receptor-mediated endocytosis [10]. Additionally, non-specific moieties such as cell-penetrating peptides (CPP) can be employed for enhanced cellular uptake [11]. (4) After reaching the target cell nucleus or cytoplasm, drug release is triggered by diffusion, nanocarrier degradation or stimuli-sensitive mechanisms. Stimuli responsiveness enables spatial and/or temporal control of drug release for improved efficacy. Natural stimuli include redox potential, enzyme activity, and pH, and external stimuli includes temperature and electrical, magnetic, and ultrasound fields [12].

Lipid and synthetic polymer nanocarriers have been extensively investigated for drug delivery with some clinical success [13–16]. For instance, Doxil® was the first US Food and Drug Administration (FDA)-approved liposomal nanocarrier [17]. Two FDA-approved SARS-CoV-2 mRNA vaccines, Pfizer-BioNTech (BNT162b2) [18] and Moderna (mRNA-1273) [19], use lipid nanoparticles to deliver antigen mRNA to prevent viral infection. Poly(lactic-*co*-glycolic acid), poly(glycolic acid), and poly(lactic acid) have been approved by the FDA for therapeutic applications [20]. However, application of synthetic polymers is hindered by polymer heterogeneity, acidic degradation products, and biocompatibility issues in some cases [21,22]. Lipid-based nanocarriers can be limited by low drug loading capacity, limited shelf life, and drug leakage during storage [23]. Both lipid and polymer nanocarriers face the challenges of complex functionalization methods and the use of organic solvents during fabrication [23].

In contrast, self-assembling proteins have garnered much attention for constructing nanocarriers due to their self-assembly behavior in aqueous buffers, monodispersity, ease of functionalization, biocompatibility, and biodegradability [22,24,25]. Proteins are biodegradable into amino acids without acidic or toxic degradation products. Further, in some cases the degradation rate can be controlled to match the application [20,26]. Protein self-assembly is the spontaneous organization of protein building blocks into ordered structures through non-covalent interactions including hydrophobic, electrostatic, hydrogen bonding, and van der Waals interactions. Self-assembly is a highly attractive method for fabricating nanocarriers as it is simple and versatile. Furthermore, it allows protein nanocarriers to form at room temperature in mild aqueous environments, while lipid and polymer nanocarriers are often prepared in harsher conditions [27,28]. Nature has evolved numerous self-assembled protein nanostructures with diverse biological functions, where proteins serve both structural and functional purposes. Viral capsids and bacterial microcompartments are perfect examples of natural protein assemblies [29]. Taking inspiration from nature's toolkit, scientists have investigated sequence-structure–function relationships in natural proteins. Consequently, numerous rational engineered proteins and *de novo* designed proteins have been developed as building blocks for "bottom-up" construction of protein nanoassemblies [30–32]. The use of well-defined protein nanoassemblies is of great interest for designing nanocarriers for drug delivery.

Advances in recombinant DNA technology enable the design of proteins at the DNA level with precise control over amino acid sequence and protein size and structure. Recombinant proteins are synthesized in living cells, such as *Escherichia coli* (*E. coli*), yeast, and mammalian cells, or cell-free systems, resulting in monodisperse protein production. Molecular monodispersity, which is impossible for synthetic polymers but standard for recombinant proteins, is an important feature for constructing nanocarriers with reproducible structures and functions [25,33]. Genetic engineering also permits the incorporation of functional therapeutic or targeting proteins or peptides, or reactive amino acids in a site-specific manner to combine multiple functionalities into a single building block. In some cases, building blocks are not tolerant to genetic fusion or mutation. To address this, affinity proteins, such as protein A or the SpyTag/SpyCatcher system, can be used to incorporate functional ligands or drugs into protein nanocarriers after self-assembly. Further, incorporation of unnatural amino acids (UAA) has opened up new routes to endow physicochemical properties and biological functions not seen in natural proteins, as well as enable orthogonal chemistries for crosslinking or attachment of functional molecules [32,34–36]. Chemical modifications are common methods but can be less desirable due to lack of control of orientation and site specificity. Functionalization strategies to provide stability, encapsulation capability, long *in vivo* half-life, targetability, and stimuli responsiveness to protein nanocarriers are summarized in Table 1.

Given the ability to manipulate proteins and assemble them into nanoscale structures, protein nanocarriers are of great interest in drug delivery, since they can be fabricated with accurately tuned functionalities, chemical and physical properties, and self-assembly behavior. Broadly speaking, protein nanocarriers can be divided into three groups: natural carriers, process fabricated carriers, and self-assembled carriers. Natural protein nanocarriers include a wide variety of virus-like particles (VLP) and protein cages, including ferritin cages and protein vaults, and have been extensively reviewed in [69,70]. Two VLP-based human papillomavirus (HPV) vaccines, GARDASIL® [71] and Cervarix™ [72], have been approved in many countries to prevent HPV-16 and -18 cervical cancer and precancerous lesions. Process fabricated carriers refers to protein nanoparticles made by engineered processes such as desolvation or spray drying. [24,73] Nanoparticles produced by these methods can have excellent delivery properties for some drugs. However, the use of solvents, temperature, or phase changes, creates an environment for the protein building blocks that is often harsher than for self-assembly. Self-assembled protein nanocarriers are typically made from rationally designed recombinant proteins and, while they may use or are inspired by natural

Table 1
Methods of functionalization of protein nanocarriers. The first reference in each row is for the example given and subsequent references are for other literature covered in this review that utilize the same sort of functionalization method.

Modification	Method	Functionality	Example	Ref
Genetic modification	Introduce reactive natural amino acids	Encapsulation	Introduce arginine to the lumen surface of protein cages, creating positively charged lumen for siRNA encapsulation.	[37–40]
		Stimuli responsiveness	pH responsive 6x histidine peptides in protein cages and coiled coil nanocarriers promote endosomal escape followed by intracellular drug release.	[37,41–44]
	Introduce reactive UAA	Stability	Incorporate *para*-azido phenylalanine into proteins for photo-crosslinking of nanocarriers.	[45,46]
		Encapsulation	Incorporate p-acetylphenylalanine into proteins for site specific drug attachment.	[47]
	Incorporate targeting peptides or proteins	Targetability	Genetically fuse a tumor targeting protein or peptide to protein building blocks for tumor targeting.	[47–56]
	Incorporate peptides to inhibit phagocytosis	Long *in vivo* half-life	Genetically fuse a "self-peptide" to coiled coils to reduce macrophage-mediated clearance.	[57]
	Incorporate modular affinity proteins	Encapsulation	Incorporate Spycatcher and protein A to encapsulate antigen and antibody, respectively.	[57–61]
	Post-translational modification for hybrid proteins	Encapsulation	Use post-translational modification to synthesize lipid-protein hybrids, resulting in lipid cores to encapsulate hydrophobic drugs.	[62–64]
	Incorporate enzyme-cleavable peptide	Stimuli responsiveness	Introduce thrombin- or matrix metalloproteinase-cleavable peptides to induce nanocarrier disassembly or drug release.	[55–57]
Chemical modification	Conjugation of drugs	Encapsulation	Conjugate paclitaxel with cysteine residues via a pH-sensitive hydrazone bond.	[65,66]
	Conjugation of functional peptides or proteins	Cellular internalization	Conjugate a cell penetrating peptide to protein cage via cysteine and lysine residues on the surface.	[42,43,67,68]
	Conjugation of stealth polymer	Long *in vivo* half-life	Conjugate PEG to protein nanoparticles for longer *in vivo* circulation.	[57]

proteins, the structures are unique from those of natural carriers. In this review, we will discuss the design, functionalization, and application of this class of recombinant self-assembled protein nanocarriers for drug delivery.

2. Self-assembling protein nanocarriers

2.1. Artificial nanocages

Protein cages are hollow, symmetric nano-sized structures self-assembled from protein building blocks. Numerous protein cages are found in nature as functional protein assemblies to store and protect inner components that are toxic or unstable. Examples of natural protein cages include viruses, ferritins, and small heat shock proteins [29,74,75]. Protein cages are of great interest as drug delivery carriers due to their intrinsic self-assembling properties and inner cavity for drug encapsulation. Cage structures can be further functionalized through genetic and chemical modifications. Naturally derived protein cages, including virus-like particles (VLPs), ferritin protein cages, and vaults, have been developed to encapsulate and deliver small molecule drugs, nucleic acids, and therapeutic proteins [58,69,76–78]. Inspired by naturally occurring cages, artificial nanocages have emerged as alternatives, presenting geometrical and chemical features not seen in natural cages. Artificial nanocages are composed of proteins that do not naturally form cages, so the interfacing surfaces between protein building blocks must be modified to induce self-assembly [79,80]. However, artificial nanocages share some constraints with natural cages. First, the large size of protein nanocages causes challenges in protein folding and expression. Second, the sequence of each building block is explicitly designed so it is generally not tolerant to genetic fusion to a large protein cargo or significant genetic modification. Third, these cage systems show limited loading efficiency with macromolecule cargos, such as nucleic acids and proteins. Advances in computational techniques and protein engineering have led to the design and production of many artificial nanocages that overcome these challenges [81–84], including pioneering work using a symmetry-based method to design symmetric nanoassemblies [85].

2.1.1. Artificial protein nanocages

The Baker group has developed Rosetta software to computationally design protein–protein interfaces to guide cage design [86], enabling engineering of artificial nanocages to deliver various types of cargos with genetic or chemical modifications [87,88]. King et al. utilized the computational approach to develop *de novo* designed protein nanocages [86]. Through symmetrical docking of protein building blocks and designing protein–protein interfaces with low energy, trimeric protein building blocks were used to create a non-viral protein cage with octahedral symmetry. This octahedral protein cage, O3-33, comprised 24 monomers with an external diameter of ~13 nm, a spherical internal cavity of ~8 nm, and six pores of ~3.5 nm. The stable O3-33 cage was obtained by overexpression in *E. coli*. Edwardson et al., further engineered the O3-33 cage by introducing positively charged arginine to the inner surface for siRNA loading (Fig. 1a) [37]. 6x histidine tags in siRNA-loaded cages promoted endosomal escape and the high concentration of tRNA in the cytoplasm displaced siRNA in the cage lumen, leading to the liberation of siRNA for *in vitro* gene silencing in HeLa cells (Fig. 1b). More recently, the positively charged inner face was loaded with negatively charged surfactants, creating a micellar hydrophobic core (Fig. 1c). The resulting cage/surfactant hybrid system efficiently encapsulated nonpolar small molecules and improved the cellular uptake of a small-molecule fluorophore. Furthermore, the release kinetics for different cargos were tuned by altering the lipid composition [89].

Another icosahedral protein cage, I53-50, composed of 120 subunits via computational design was reported by Bale and coworkers [38]. The I53-50 cage was assembled from two distinct oligomeric protein components. Both protein components were expressed in *E. coli* and purified separately and assembled efficiently *in vitro* into icosahedral protein cages with diameters rang-

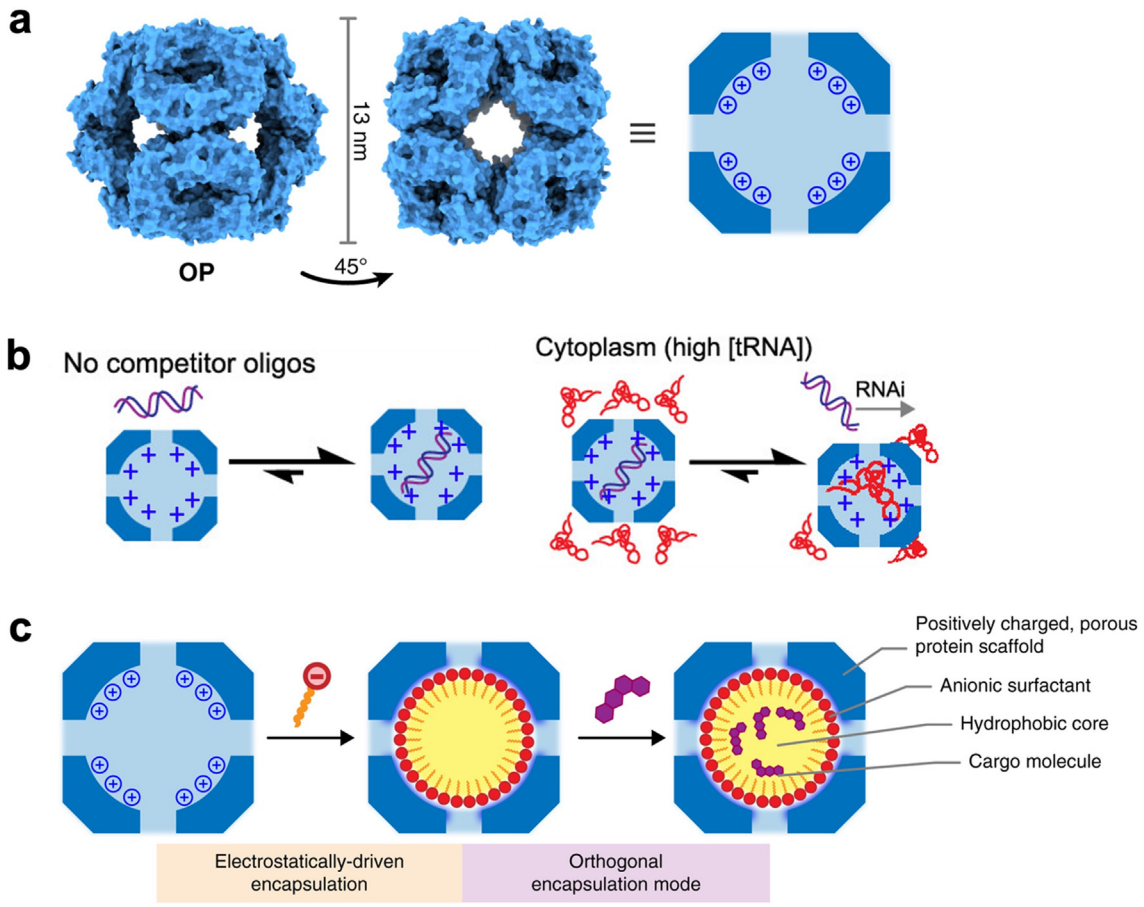

Fig. 1. (a) Surface structure of the OP cage with positively charged lumen. (b) Encapsulation of siRNA into the lumen and release of siRNA due to displacement by tRNA in cytoplasm. (c) Encapsulation of anionic surfactants through electrostatic interactions followed by encapsulation of nonpolar small molecules via hydrophobic effect into micellar hydrophobic core within the OP cage. Adapted with permission from [37]; Copyright 2017 American Chemical Society.

ing from 24 to 40 nm. An I53-50 variant with a positively charged interior surface successfully encapsulated a negatively charged green fluorescent protein (GFP) into the lumen through *in vitro* packaging of the trimeric and pentameric components and GFP. Furthermore, the charged I53-50 cage encapsulated its own mRNA genome *in vivo* in a similar manner as viral nucleocapsids [40]. More recently, this platform has been engineered to display F glycoprotein trimer antigen [90] and native-like HIV-1 envelope trimer antigen [91] for designing vaccines against respiratory syncytial virus and human immunodeficiency virus, respectively. The two-component I53-50 cage allowed the incorporation and stabilization of trimeric antigens with tunable antigen density, resulting in highly ordered, monodisperse immunogens. Potent neutralizing antibody responses induced by immunogens demonstrated the efficacy of the I53-50 cage for vaccine delivery.

Hsia et al. computationally designed an icosahedral protein cage, I3-01, with a diameter of 25 nm [92]. The I3-01 cage was self-assembled from trimeric protein building blocks and robust to genetic fusion to GFP. Bruun et al. rationally engineered a mutated I3-01 (mi3) cage for improved cage uniformity and stability [59]. The mutated mi3 building block was genetically fused to SpyCatcher and the SpyCatcher-mi3 cage was expressed with high soluble yield in *E. coli*. Consequently, antigen protein fused with SpyTag can be displayed on the cage surface through spontaneous isopeptide bond formation between the SpyCatcher and SpyTag. This modular "Plug-and-Display" platform efficiently conjugated *E. coli* maltose-binding protein and a range of antigens bearing SpyTags onto the cage surface. High avidity antibody response generated with SpyCatcher-mi3 conjugated with a protective antigen against *Plasmodium falciparum* demonstrated the potential of SpyCatcher-mi3 for antigen protein delivery. Moreover, SpyCatcher-mi3 allowed the display of antigens with diverse sizes and multimeric structures. SpyCatcher-mi3 not only displayed monomeric antigen (receptor-binding domain (RBD) from SARS-CoV-2) but also dimeric (outer surface protein C), trimeric (influenza hemagglutinin) and tetrameric (influenza neuraminidase) antigens [93]. A recent study reported that RBD conjugated SpyCatcher-mi3 elicited a strong neutralizing antibody response in mice and pigs [94]. Beyond vaccines, the SpyCatcher-mi3 cage holds promise for displaying targeting or therapeutic proteins on the surface for drug delivery.

Taking advantage of the abundance of endogenous retroelements in the human genome, Segel et al. utilized computational tools to identify cage-forming capsid proteins, PEG10, that are common retroelements in human and mouse genomes. PEG10 proteins formed VLPs that bound and secreted their own mRNA, which could be useful for mRNA delivery. To reprogram PEG10 to bind mRNA cargos of interest, mRNA cargos were flanked with untranslated regions of PEG10 gene, which provided a packing signal for PEG10 VLPs. Furthermore, VLPs were pseudotyped by adding fusogenic envelope proteins for cell entry. The resulting pseudotyped VLP system consisting of PEG10, fusogen and mRNA cargo is a modular platform for mRNA delivery to cells, named selective endogenous encapsidation (SEND). SEND was shown to deliver Cas9 mRNA into Neuro-2a mouse neuroblasts expressing a single guide RNA against KRAS, resulting in 60 % insertions and deletions

in cells. Due to its human origin, the SEND system may mitigate the immunogenicity concerns of current viral vectors for mRNA and CRISPR/Cas9 delivery [95].

While computational design offers distinct advantages, rational design can also be used to create new protein cages. Yanofsky et al. described a synthetic protein cage composed of a 11-mer ring-shaped protein subunit, trp RNA-binding attenuation protein (TRAP), from *Geobacillus stearothermophilus* [96]. The TRAP cage was self-assembled from 24 TRAP ring subunits, forming a 22 nm-diameter hollow sphere with a 16 nm-diameter lumen. The ring subunits were connected by disulfide bonds that formed between cysteine residues on TRAP and gold nanoparticles. Thus, the resulting cage could be triggered to break apart in the presence of cellular reducing agents [97,98]. Recently, Naskalska et al. reported that the TRAP cage encapsulated a negatively supercharged variant of GFP into the lumen, though with low loading efficiency. Further, they decorated the cage with a cell-penetrating peptide for enhanced cellular uptake in MCF7 breast cancer cells. GFP was successfully delivered and released intracellularly by TRAP cages [42].

While cages are usually made from proteins whose function is to form the cage, they can also be made from proteins that have biological functions. Recently, Divine et al. reported antibody nanocages with controlled valency and symmetry [60]. Building blocks were composed of protein A, cyclic oligomers, and helical repeat connectors. Protein A is a Fc binding protein that binds to the Fc region in an antibody, which incorporates antibodies into the building blocks. Antibody nanocages could be formed with 2, 6, 12, or 30 antibodies simply by mixing antibodies with the corresponding building blocks, resulting in 8 nanocages with different symmetries and diameters ranging from 15 to 40 nm. Functional Fc-angiopoietin-1 fusions and death receptor 5, CD40, CD3, and CD28 antibodies were assembled in antibody nanocages. In *in vitro* studies, cell surface receptor-targeting antibody nanocages enhanced cell signaling for angiogenesis, apoptosis, CD40 activation, and T cell proliferation compared with free antibodies. Further, SARS-CoV-2 antibody nanocages showed increased *in vitro* viral neutralization against SARS-CoV-2 pseudovirus.

2.1.2. Artificial peptide nanocages

In addition to protein-based artificial cages, peptides with secondary structure, such as α-helices, are building blocks for constructing artificial cages since they contain critical elements of oligomeric protein interfaces [99]. Due to extensive studies on sequence-structure relationships of α-helices, natural and *de novo* designed coiled coils have been designed for self-assembled nanostructures [100,101]. Here, we focus on synthetic nanocages self-assembled from coiled coils. Self-assembled peptide cages (SAGEs) made from *de novo* coiled coil peptides were reported by Fletcher et al. [102]. In the SAGE system, homotrimeric coiled coils and heterodimeric coiled coils were covalently linked together via disulfide bonds to form two complementary hubs. Hubs mixed in aqueous solution co-assembled into hexagonal networks to form 100 nm hollow spherical cages (Fig. 2a) [103]. Exposure of the N terminus of peptides on the outer surfaces of the SAGEs allowed the incorporation of GFP, mCherry, maltose binding protein (MBP), and luciferase onto SAGEs by genetically fusing proteins with building blocks (Fig. 2b). SAGEs were also modified with short, charged peptides to alter cellular uptake [104]. Further, modular SAGEs functionalized with antigenic peptides tetanus toxoid$_{632\text{-}651}$,ovalbumin$_{323\text{-}339}$, and hemagglutinin$_{518\text{-}526}$, induced antigen-specific T cell and B cell responses in mouse models [105]. Given their robustness to genetic fusion of proteins and peptides, SAGEs hold promise in displaying targeting proteins or peptides on the surface for drug delivery application.

Inspired by DNA origami design [106], Gradišar et al. presented *de novo* designed coiled coil protein origami (CCPO) cages by creating a long single-chain polypeptide that self-assembled into a polyhedral structure [107]. The single-chain polypeptide was composed of 12 concatenated coiled-coil-forming building blocks connected by short, flexible peptide linkers. The self-assembly process was driven by orthogonal pairwise interaction between the coiled-coil building blocks, resulting in polyhedral structures comprised of six different rod-like edges of coiled-coil pairs enclosing an empty core. The CCPO cage was further engineered with enhanced solubility that enabled self-assembly in bacteria, mammalian cells, and mice without signs of inflammation [107]. To further expand the versatility of CCPO cages, nanobodies were generated that recognize different coiled-coil modules, providing opportunities for further functionalization of the coiled-coil modules [108].

Another coiled coil-based self-assembling protein nanoparticle (SAPN) platform was reported by Raman and coworkers [109]. The building block consisted of a pentameric coiled coil domain fused to a *de novo* designed trimeric coiled coil via a glycine linker, and disulfide bonds between the two forms of coiled coils provided stabilization. Folding of the building block resulted in the self-assembly of 60 monomers into polyhedral nanoparticles with a diameter of 16 nm. The multivalent SAPN platform was utilized for repetitive antigen display. *In vivo* immunization efficacy of modular SAPN against malaria [110], HIV [111] and influenza [112] in mouse models was demonstrated.

Combining chemical and *de novo* design, Noble et al. designed a dimeric coiled-coil that self-assembled into dendrimeric anionic virus-like nanoshells with a diameter of 20 nm. Each helix had one hydrophobic interface and two polar facets that allowed it to interact with three neighboring helices. Interactions between helices led to a branching and curved network which closed into a shell. Though the net charge of the shell was negative, the free *N*-terminus of the peptide subunit was positively charged, which allowed shells to bind negatively charged nucleic acids. These shells were able to encapsulate siRNA and plasmid DNA (pDNA) and deliver them into HeLa cells for gene knockdown and expression [113].

2.2. Bioinspired polypeptide nanocarriers

Natural proteins exhibit unique properties that are useful for designing nanomaterials for therapeutic applications. Inspired by natural proteins, scientists have utilized recombinant DNA technology to produce protein polymers consisting of amino acid sequences derived from nature [30,31,114]. Recombinant polypeptides are composed of repetitive natural or engineered amino acid sequences, mimicking the structure or function of natural proteins. They have emerged as valuable building blocks for constructing nanostructures. [22,25,114]. Among the existing recombinant polypeptides, elastin-like polypeptides (ELP) and silk-like proteins (SLP) have been extensively investigated for drug delivery applications because of their self-assembling nature, biocompatibility, and modularity with genetic and chemical modification [35,115].

2.2.1. Elastin-like polypeptide nanocarriers

Elastin-like polypeptides (ELPs) are biopolymers derived from human elastin [116]. ELPs are composed of the pentapeptide repeat valine-proline-glycine-X-glycine (VPGXG), where X can be any amino acid except proline. ELPs are thermally responsive biopolymers, exhibiting lower critical solution temperature (LCST) behavior. Below the critical transition temperature (Tt), they are soluble, while above the Tt, they undergo a hydrophobic phase transition from soluble proteins to insoluble coacervates. The phase transition is reversible such that ELP coacervates can be res-

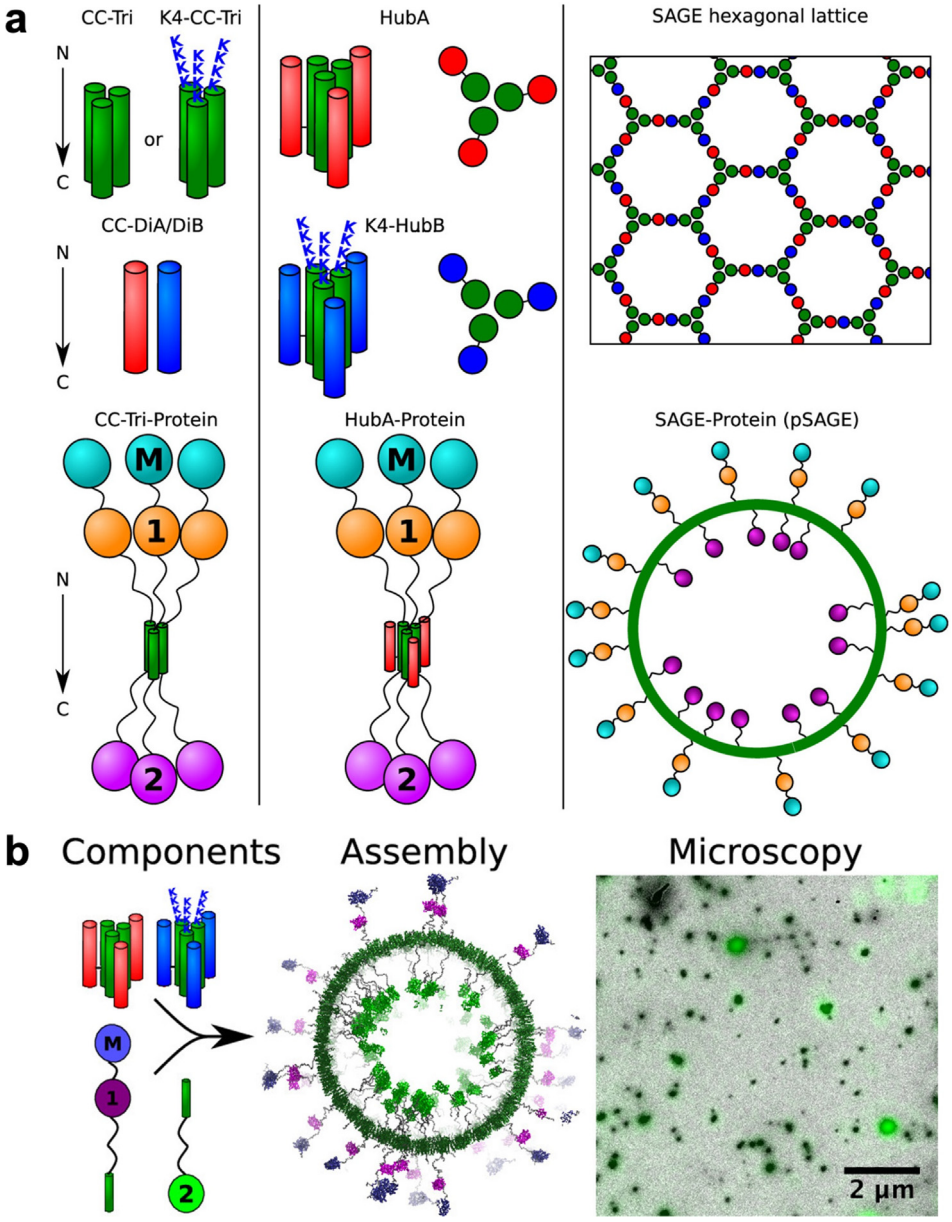

Fig. 2. (a) Coiled coils (green) were linked via disulfide bonds to heterodimeric acidic (red) and basic (blue) coiled coils to form hub A and hub B, respectively. Mixing of hub A and hub B resulted in formation of a hexagonal network. (b) Coiled coils were fused to GFP, mCherry, MBP, and luciferase at N terminus and/or C terminus and assembled into protein-decorated SAGEs. Adapted with permission from [103]; Copyright 2017 American Chemical Society.

olubilized by decreasing the temperature below the Tt [117,118]. ELPs present several advantages as biopolymers for constructing drug nanocarriers. First, the Tt can be modified by changing the ionic strength [119], the number of pentapeptide repeats [120], or guest residue X [121], allowing ELP coacervation at physiological conditions. Second, pH responsiveness can be imbued by incorporating histidine as the guest residue [120], which enables pH-dependent nanoparticle disassembly [122] and can be employed for pH-dependent drug release [123] or endosomal escape [124]. Third, the biocompatibility of ELP supports its development for biomedical applications [125]. ELP-based biomaterials have been extensively reviewed [115,126]. Here, we will focus on recent progress specifically on self-assembled ELP nanocarriers for delivering small molecule drugs, siRNA, and peptides.

ELPs have been exploited to increase the solubility of hydrophobic drugs. Banskota et al. rationally designed a zwitterionic ELP (ZIPP) for delivering a hydrophobic drug, paclitaxel (PTX) [65]. ZIPP contains a cationic and anionic amino acid pair within one pentapeptide motif, displaying stealth properties for extended *in vivo* circulation [127]. ZIPP containing a combination of lysine and glutamic acid showed *in vivo* elimination half-life of up to 12 h via intravenous administration and 15.6 h via subcutaneous administration. A hydrophobic ELP domain was fused to the C-terminus of ZIPP, which permitted the conjugation of PTX to the cysteine residues in the drug attachment domain via a pH-sensitive hydrazone bond [65]. PTX conjugation resulted in the self-assembly of ZIPP-PTX into stable 58 nm micelles, displaying stealth ZIPP on the corona and enclosing PTX in the core. In a mouse model, ZIPP-PTX showed better *in vivo* antitumor efficacy than Abraxane, an FDA-approved PTX protein-bound particles formulation, due to increased drug delivery to tumors.

Several recent examples have shown that ELPs can be genetically fused to biofunctional peptides or proteins without hampering their self-assembling activities. A hydrophobic and

cytoprotective peptide, humanin (HN), was genetically fused to ELPs by Li et al. for designing an ocular delivery system [128]. HN-ELP fusions assembled into stable ~ 40 nm nanostructures or greater than 1000 nm coacervates at physiological temperature. Both structures bound and protected human retinal pigment epithelium cells from oxidative stress *in vitro*, demonstrating their potential for ophthalmological applications. Costa et al. generated ELP fused to an engineered targeting protein, EgA1 nanobody, at the C terminus, which recognized human epidermal growth factor receptor (EGFR) [47]. Additionally, an unnatural amino acid, p-acetylphenylalanine (pAcF), was incorporated at the N terminus of ELP for site specific attachment of small molecule drugs without cross-reactivity with other protein domains. DOX was conjugated to the ketone group of pAcF via a pH-sensitive linker. The resulting 45 nm micelles displayed nanobodies on the corona and encapsulated DOX in the core. After receptor-mediated cellular uptake, the pH sensitive linker was cleaved in the acidic lysosomal compartments of cells, allowing for the release of DOX intracellularly in EGFR positive A431 and SKOV-3 cancer cells. Lee et al. presented antibody nanoworms self-assembled from ELPs fused to single chain antibodies (scFv) that recognize cell surface receptors on B cells and T cells [49]. All ELP fusions assembled into worm-like nanostructures that were 56–89 nm in length and 5–9 nm in width. Nanoworms displaying more than 100 scFvs per particle clustered bound cell surface receptors multivalently and activated corresponding intracellular signaling for apoptosis, cell cycle arrest, or cell activation. Peddi et al. studied the nanotoxicology of an ELP-based nanocarrier for delivering a hydrophobic small molecule drug, rapamycin (Rapa) [51]. A cognate receptor for Rapa, FKBP12, and an integrin-binding peptide, RGD, were genetically fused to ELPs separately, generating two functional ELP fusions. The two ELP fusions co-assembled into stable bifunctional nanoparticles with FKBP12 solubilizing Rapa through non-covalent binding and RGD mediating cellular uptake through RGD/integrin binding. Rapa-loaded ELP effectively suppressed the mTOR signaling pathway in a mouse model of breast cancer without hepatotoxicity, suggesting the safety and efficacy of this ELP-based nanocarrier for Rapa delivery.

In addition to the direct genetic fusion of functional proteins to ELPs, coiled coil domains have been added to ELPs to mediate protein oligomerization and incorporate functional proteins. Our group has previously reported hollow protein vesicles self-assembled from recombinant protein amphiphiles composed of globular proteins, coiled-coil leucine zippers, and ELPs. Hydrophilic globular model protein, mCherry, was fused to glutamic acid-rich leucine zipper motif (Z_E) and hydrophobic ELPs were fused to arginine-rich leucine zipper motif (Z_R). The heterodimeric Z_E/Z_R interactions led to formation of mCherry-Z_E/Z_R-ELP protein amphiphiles, which self-assembled into hollow protein vesicles with temperature- and salt-triggered ELP phase transition [129]. Our group has demonstrated the versatility of vesicle platform by fusing Z_E to charged variants of superfolder GFP and enzymes, incorporating them into vesicles via self-assembly [130]. Moreover, we incorporated a photocrosslinkable unnatural amino acid, *para*-azido phenylalanine (pAzF), into the ELP domain for enhanced stability in the physiological environment. pAzF-containing Z_R-ELP enabled site-specific crosslinking of protein vesicles under UV irradiation. Vesicle size was reduced to ~ 100 nm by changing ELP hydrophobicity and ionic strength. Protein vesicles successfully encapsulated and delivered DOX and mCherry *in vitro* in HeLa cells (Fig. 3), showing the potential for small molecule drug/protein combination therapies [45]. Additionally, the vesicles have been made pH responsive with incorporation of histidine into the ELP sequence, resulting in vesicle disassembly at acidic pH [219]. Another coiled coil-ELP system was described by Hill et al. for theragnostic application. The fluorinated thermoresponsive protein polymer consisted of a coiled-coil pentamer incorporating fluorine-19 (19F) and an ELP domain, which self-assembled into micelles with a diameter of 30 nm. The resulting micelles could be traced by 19F magnetic resonance imaging (MRI) and showed the ability to encapsulate DOX and release DOX in MCF7 breast cancer cells [131].

2.2.2. Silk-like protein nanocarriers

Silks are natural fibrous proteins produced by silkworms and spiders. Due to the high β-sheet content in silk proteins, they are semi-crystalline biopolymers with high mechanical strength. Silk proteins have been extensively investigated for developing biomaterials because of their self-assembling capability, mechanical properties, biocompatibility, and biodegradability [132]. Silk fibroin (SF) from *Bombyx mori* is the most common silk protein, consisting of a 25 kDa light chain, a 325 kDa heavy chain, and a 30 kDa glycoprotein. SFs are frequently extracted from natural sources and have been widely used as biomaterials for drug delivery [133]. Recombinant production of SLPs overcomes the limited availability and potential immunogenicity of natural silk proteins that are caused by the trace sericin left with silk fibroins during processing [134]. Further, recombinant technology allows for precise control over sequence and incorporation of functionality through genetic modification. Most recombinant SLPs are derived from natural spider silk protein ADF4 from *Araneus diadematus* and MaSp1 and MaSp2 from *Nephila clavipes*. Peptide repeats in SLPs contain a hydrophobic poly-alanine domain and a hydrophilic glycine-rich domain. Recombinant SLPs present similar features as natural silk proteins and have also been implemented for drug delivery. [25,35,135].

Recombinant SLP-based nanocarriers have been genetically functionalized with cell-targeting peptides, cell-penetrating peptides, and polycation domains for enhanced cellular internalization or cargo encapsulation. Small molecule drugs, nucleic acids, and proteins can be loaded into SLP-based nanocarriers through hydrophobic or electrostatic interactions. Florczak et al. prepared ~ 400 nm tumor-targeting SLP-nanospheres by fusing SLPs with a breast cancer cell-targeting peptide [52]. DOX-loaded nanospheres showed pH-dependent drug release *in vitro* due to charge inversion of SLPs at acidic pH. Nanospheres were internalized into tumor cells in a cell type-specific manner. Though negatively charged SLPs cannot complex with nucleic acids, Numata et al. genetically fused SLPs to poly(L-lysine) domains to condense pDNA [53]. RGD was incorporated in the SLP sequence for enhanced cell binding and transfection efficiency. SLP and pDNA were mixed and incubated at 20 °C overnight. The resulting pDNA/SLP complexes with a diameter of ~ 186 nm were delivered into HeLa and human embryonic kidney (HEK) cells without cytotoxicity, though they did exhibit lower transfection efficiency than the lipofectamine control. In a follow-up study, SLP-poly(L-lysine) fusions were functionalized with tumor-targeting peptides for tumor-specific gene delivery [54]. pDNA/SLP complexes were delivered to MDA-MB-435 and MDA-MB-231 breast cancer cells *in vitro*, and mice models bearing MDA-MB-231 breast tumors. *In vitro* and *in vivo* gene transfection resulted in luciferase expression without side effects associated with toxicity. Kozlowska et al. reported nanospheres made from SLP-poly(L-lysine) fusions for RNA-based therapeutics [48]. CpG-siRNA-loaded nanospheres were obtained by salting out SLP-poly(L-lysine) and CpG-siRNA together. *In vitro* work showed delivery of CpG-siRNA into TLR9-positive macrophages via nanospheres, resulting in immune cell activation and prolonged gene silencing (Fig. 4).

One unique feature of SLPs is their mechanical robustness and slow degradation rate, which make them desirable for sustained drug release [136]. Lammel et al. prepared SLP nanoparticles with ~ 330 nm diameter and screened small molecule drugs that

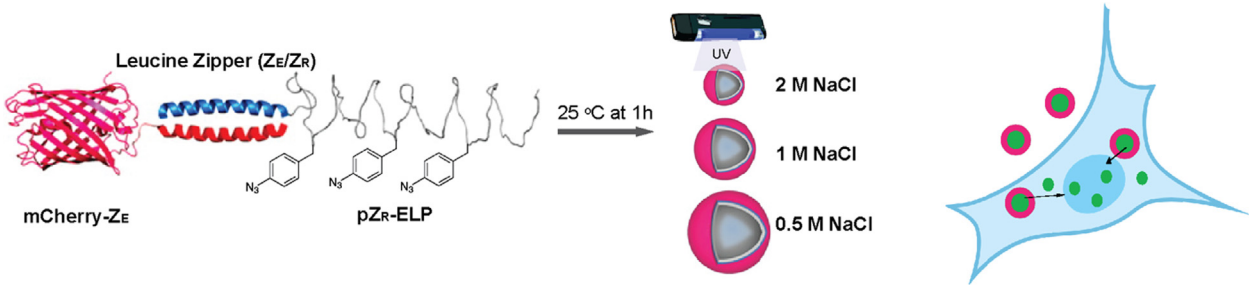

Fig. 3. Photocrosslinkable protein vesicles were self-assembled from thermally responsive Z_R-ELP and mCherry-Z_E. The size of protein vesicles was tuned by altering protein hydrophobicity and ionic strength. The resulting vesicles achieved dual delivery of doxorubicin and fluorescent protein into HeLa cells. Reproduced with permission from [45]. Copyright 2021 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

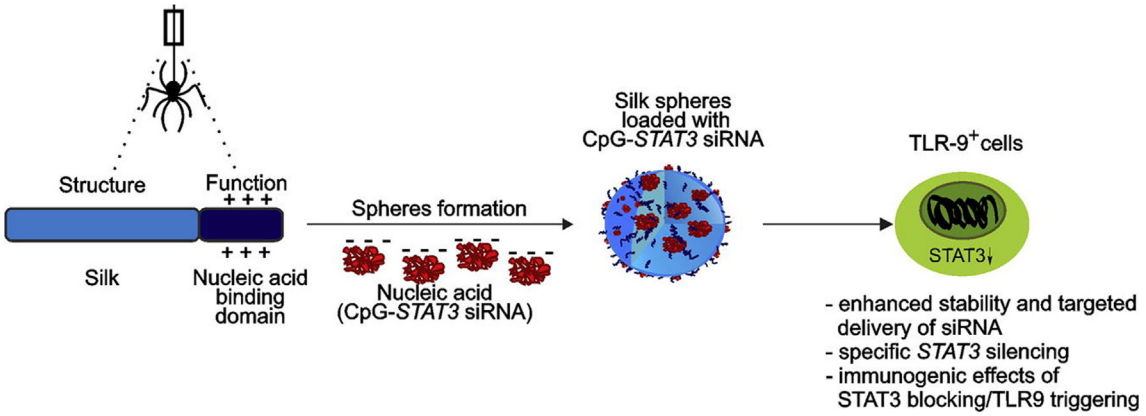

Fig. 4. Fabrication of SLP nanospheres loaded with CpG-siRNA and delivery of CpG-siRNA to target macrophages. Reproduced from [48], Copyright 2017, with permission from Elsevier.

were compatible for encapsulation [137]. *In vitro* release profiles showed constant release rates for two weeks at physiological conditions, though faster release was observed in acidic environments. Moreover, *in vitro* enzymatic degradation studies showed that particles degraded and their sizes decreased slowly. In another work by Hofer et al., SLP nanoparticles were employed for sustained protein delivery [138]. Model protein cargo, lysozyme, was loaded into nanoparticles with almost 100 % loading efficiency through electrostatic interactions. The *in vitro* protein release was dependent on pH and ionic strength, where sustained release of FITC-labeled lysozyme was observed over 28 days at pH 7.4 and 100 mM ionic strength. However, further cell and animal studies are needed to confirm the efficacy of SLP nanocarriers for sustained drug release.

2.2.3. Silk-elastin-like polypeptide (SELP) mixed nanocarriers

SELPs consist of repetitive sequences from both SLP and ELP where the hydrophobic silk domains (GAGAGS) self-assemble into insoluble tightly packed structures and the thermoresponsive elastin domain (GXGVP) undergoes thermally triggered phase transition. Various SELP-based structures have been reported, such as nanoparticles, nanofibers, and hydrogels with a range of tunable properties. Given the benefits of structural stability provided by the silk domain and stimuli responsiveness provided by the elastin domain, SELPs serve as attractive protein polymers for designing advanced drug nanocarriers [35,139]. Xia et al. reported a self-assembling SELP micelle consisting of a silk core and an ELP corona [140]. The diameter of the micelles increased from ~ 40 nm to ~ 69 nm by increasing the silk to elastin ratio from 1:8 to 1:2. Further, the same group demonstrated that the encapsulation of hydrophobic DOX triggered the self-assembly of SELPs into micelles. DOX-loaded SLP micelles were delivered into HeLa cells, showing cytotoxicity due to the release of DOX [141]. Moreover, Parker et al. implemented mucoadhesive properties into SELPs by changing guest residues in the elastin domain [142]. Charged and thiol-containing amino acids were incorporated into the elastin domain for enhanced interaction with mucus. SELP with thiol-containing cysteine displayed the strongest affinity for mucus in both an artificial mucus model and *in vitro* cell study. The results indicated the potential of SELPs for mucosal drug delivery.

2.3. Coiled coil nanocarriers

Coiled coils are one of the most abundant protein motifs in nature, found in fibrous proteins and transcription factors in various oligomerization states [143,144]. A coiled coil motif consists of two or more α-helices that self-assemble into a left-handed helix. In heptad repeats (abcdefg)n of coiled coils, amino acids a and d form the hydrophobic core of the coiled coil oligomer, and amino acids e and g are charged or polar amino acids that form stabilizing salt bridges and hydrogen bonds between coils (Fig. 5a) [145]. Due to well-studied sequence-to-structure relationships, coiled coils have been engineered with programmable association and affinity dictated by tailored amino acid sequences. The library of coiled coils provides valuable tools for designing functional protein assemblies in various structures, such as hydrogels, fibers, and nanostructures [146,147]. Natural and *de novo* designed coiled coils have been exploited in nanocarrier design for drug delivery [148–150]. The use of coiled coils can be expanded by adding functional proteins or peptides. The oligomerization of coiled coils also introduces multivalency on nanocarriers, which can enhance binding affinity. Furthermore, pH- [151] and temperature- [152] sensitive coiled coils enable the design of stimuli-responsive nanocarriers.

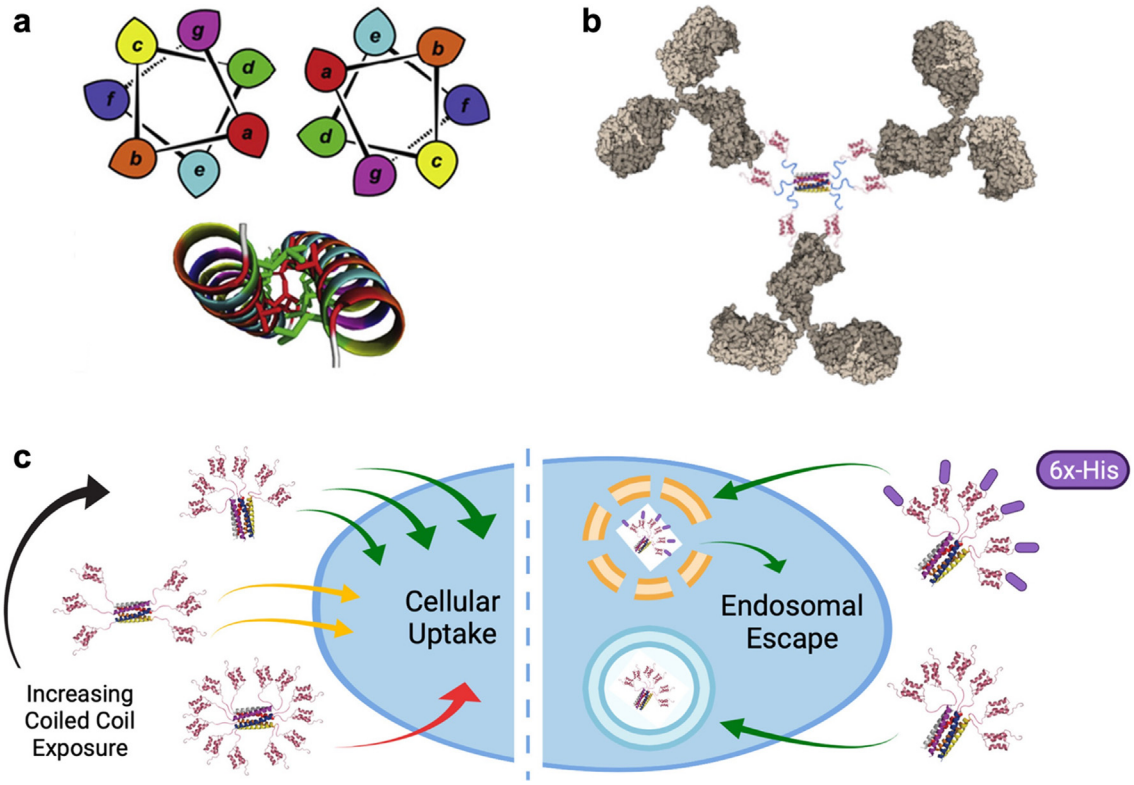

Fig. 5. (a) Helical wheel diagram of a coiled coil indicating the relative positions of amino acids. (b) Schematic of Hex-antibody complex with one Hex bound to three antibodies. (c) Coiled coil exposure enhanced cellular uptake and 6x-His tag promoted endosomal escape. 5a is adapted with permission from [145]. Copyright 2012 American Chemical Society. 5b and 5c are adapted from [41], Copyright 2021, with permission from Elsevier.

Coiled coils have been developed as self-assembled nanocarriers for the delivery of therapeutic proteins. Delivery of antibodies to intracellular targets is limited by their inability to efficiently penetrate cells and reach the cytosol [153]. To address this challenge, our group employed a *de novo* designed hexameric coiled coil (Hex) reported by Zaccai et al. for cytosolic antibody delivery [154]. Hex was genetically fused to Protein A domain B (SPAB), which binds the Fc domain of antibodies. The resulting Hex-SPAB fusions self-assembled into multivalent antibody carriers of 25–35 nm (Fig. 5b) [155]. The stoichiometry of assembled Hex–antibody complexes were characterized with different incubation temperature and time. A stable Hex-antibody complex was observed when Hex bound 3 antibodies under physiological conditions [156]. Detailed *in vitro* studies showed the cytosolic delivery of functional antibodies into HeLa cells via endocytosis [155,157]. Furthermore, it was proven that increased coiled coil exposure to cells increased internalization and 6x-histidine tags increased endosomal escape (Fig. 5c) [41]. The Hex nanocarriers were demonstrated to deliver protein cargos of different sizes, either directly fused to Hex or affinity bound.

Yu et al. designed activatable protein nanoparticles (APNPs) for targeted delivery of therapeutic peptides [57]. APNPs were made from three polypeptides where each polypeptide consisted of one or more therapeutic peptides flanked by coiled coils. Therapeutic peptides and coiled coils were spaced by a thrombin- or matrix metalloproteinase-cleavable peptide for controlled release. Highly specific pairwise coiled coil dimerization drove the self-assembly of polypeptides into nanoparticles with a diameter of 5–7 nm. Furthermore, the APNPs were modified with a "self" peptide derived from human CD47 to inhibit phagocytic clearance, a cell targeting peptide, and PEG for longer *in vivo* blood circulation. *In vitro* cell studies confirmed the release of peptides in response to enzymes in the microenvironment and subsequent cellular internalization of a neuroprotective peptide NR2B9c by U87MG brain epithelial cells and a cytolytic melittin peptide by MDA-MB-231 breast cancer cells. *In vivo* studies demonstrated the efficacy of APNPs for delivering NR2B9c and melittin peptides in rats for treating stroke and cancer, respectively.

2.4. Nanocarriers made from other self-assembling protein polymers

A wealth of natural proteins provides scientists a toolkit to diversify protein sequences and design functional protein nanomaterials. Guerette et al. developed bioinspired suckerin proteins derived from squid, which displayed a block copolymer-like structure and self-assembled into nanoconfined β-sheet networks [158]. Suckerin-19 proteins self-assembled into 100–200 nm nanoparticles stabilized by β-sheets. The suckerin-19 nanoparticles encapsulated DOX through hydrophobic interactions and released DOX in a pH-dependent manner *in vitro* in HeLa cells [44]. The anti-cancer efficacy of the DOX-loaded formulation was confirmed in tumor-bearing mice with reduced tumor size. The suckerin-19 nanoparticles could also complex pDNA through electrostatic interactions. Histidine residues in suckerin-19 triggered endosomal escape, thereby resulting in luciferase expression in both HeLa cells and mouse models. The self-assembly behavior of recombinant suckerin-12 was responsive to protein concentration, salt, and pH [46]. Suckerin-12 was functionalized with pAzF and formed ~ 100 nm nanoassemblies by a salting-out method but has not yet been tested for drug encapsulation or delivery.

Natural surfactant protein, oleosin, from sunflower proteins was engineered by Vargo et al. for constructing protein suprastructures [159]. Tunable self-assembly of recombinant oleosin was observed by altering solution ionic strength and protein hydrophilic fraction, resulting in nanoscale fibers, sheets, and vesicles. Recombinant oleosin was genetically functionalized with RGD pep-

tides protected by a thrombin-cleavable domain [56]. Functionalized oleosin self-assembled into protein micelles and exposed RGD on the surface upon thrombin cleavage, leading to increased cellular internalization by MDA-MB-231 breast cancer cells. The oleosin micelles were further functionalized with TAT and RGD, which encapsulated and delivered hydrophobic PTX into MDA-MB-231 cells with enhanced cell killing compared with free PTX [55].

Matsuura et al. reported a β-annulus peptide that self-assembled into artificial peptide nanocapsules with diameters ranging from 30 to 50 nm [160,161]. The β-annulus cage was engineered to encapsulate DNA, anionic GFP, and quantum dots in its cationic lumen [162–164]. The C-terminal of β-annulus peptides could be conjugated with nanoparticles, coiled coils, DNA, and albumin to enable surface display of cargos without interfering with the self-assembly [165,166]. Furthermore, β-annulus peptides were conjugated with a 20-mer oligothymine (dT20) at the *N*-terminus, which enabled encapsulation of mCherry mRNA bearing a poly(A) tail via hybridization of dT20 and poly(A) during co-assembly. By modifying the exterior with a cell-penetrating peptide, β-annulus delivered mRNA, and induced expression of mCherry in human hepatoma HepG2 cells, though with low efficiency [68].

2.5. Hybrid protein nanocarriers

While protein nanocarriers display incredible diversity in terms of properties and function, the restriction of proteins to twenty natural amino acids plus additional non-natural amino acids does present a limit to the types of structures and physicochemical properties that can be achieved. In the natural world, proteins are frequently modified after translation with molecules such as glycans and lipids [167]. Additionally, some proteins, such as transcription factors, have strong, specific interactions with nucleic acids [168]. In the synthetic realm, soluble therapeutic proteins have been conjugated to polymers such as polyethylene glycol to prolong their half-life *in vivo* [169]. Any natural or synthetic protein modification or conjugation can alter protein properties, contribute new types of attractive or repulsive interactions, and ultimately affect assembly into nanostructures that can be used for drug delivery.

2.5.1. Protein-DNA nanocarriers

A variety of different DNA origami nanostructures have been engineered using the high fidelity base pairing of nucleotides, where the sequence of the DNA segments controls the dimensions of structures formed. While pure DNA structures have not found wide use for drug delivery, due to challenges such as degradation by nucleases or immunogenicity [170], there are some promising examples [171]. Additionally, the combination of protein interactions and DNA base pairing can capitalize on the advantages of both types of molecules. Ryu et al. reported a hybrid nanostructure with DNA as a scaffold to attach targeting and therapeutic proteins for intracellular delivery [50]. They used DNA hybridization to create Y-shaped structures made from 3 single strands of DNA. Each end of the Y contained different sequences recognizable by zinc finger proteins, which are sequence specific DNA binding proteins. Fusion proteins were created from the three zinc finger proteins and various protein cargoes. Upon mixing, ~20 nm Y shaped nanostructures were formed with unique proteins attached to each end of the Y (Fig. 6). Targeting of several types of cancer cells expressing high levels of EGFR was achieved using EGFR specific repebody proteins fused to zinc finger proteins. The targeted hybrid structures were demonstrated to deliver both doxorubicin, which intercalates in the DNA, and phosphatase and tension homolog (PTEN) tumor suppressor protein, which was fused to a zinc finger protein, *in vitro*. This combination resulted in loss of cell viability for multiple EGFR positive cancer cell lines. Though repebody proteins were used in this example, this approach could be utilized with a variety of different monovalent binding scaffolds such as DARPins, affibodies, or nanobodies [172,173].

While the use of protein-DNA origami structures for drug delivery is in early stages, there are more DNA nanomaterials in the literature that could be adapted for delivery applications including cages, bundles and ribbons [174–176] Additionally, protein-DNA materials are not limited to origami base pairing, and unstructured DNA can be used as a hybrid building block. For example, biotinylated unstructured DNA was mixed with recombinant enzymes fused to streptavidin [61]. The interaction between biotin and streptavidin led to the formation of nanogels with dimensions on the order of 80–200 nm depending on assembly conditions. The enzymes maintained their activity and the use of therapeutically relevant enzyme beta-galactosidase suggests that in future work these nanogels could be taken up by cells for intracellular enzyme delivery, such as for lysosomal storage diseases.

In addition to serving a structural role, nucleic acids can also be incorporated into protein nanomaterials for use as specific binding domains, for example to achieve targeting. Aptamers are DNA or RNA molecules with 3D folded structures that can bind specific targets, including proteins, with high affinity in the low- to sub-nanomolar regime. Aptamers can be created using directed evolution approaches analogous to methods used for engineered binding proteins [177]. Humenik et al. created silk-aptamer nanohydrogels by conjugating cyclooctyne functionalized aptamers to recombinant SLP that was chemically modified with azide esters to create silk nanohydrogels [66]. The hydrogels, made up of nanofibrils, were 140–150 nm and self-assembled in solution or were spin coated on surfaces. Thrombin, a model therapeutic enzyme that causes clotting, was incorporated into the hydrogels via cooperative binding of thrombin-specific aptamers. The protein could be released on demand by introduction of specific, competitive DNA sequences that bound the aptamers and opened their structures, displacing enzymatically active thrombin into solution.

Thelu et al. combined both DNA origami and aptamers with proteins to create targeted drug delivery nanogels [178]. Biotinylated DNA origami 4-arm crosses were mixed with tetrameric streptavidin and monovalent biotinylated aptamers to form nanogels in the range of 100–300 nm, depending on the ratios of components. DOX was encapsulated in the nanogels by simple mixing with the other components prior to assembly. Nanogels without aptamers were internalized by MCF7, HeLa, and A549 cancer cells cancer cells and the toxic effects of DOX were observed. Nanogels with aptamers targeting human protein tyrosine kinase-7 (PTK7) demonstrated cell specific uptake and DOX toxicity, with PTK7 positive leukemia and HeLa cells exhibiting higher uptake and lower cell viability than PTK7 negative B lymphocytes. Targeting aptamers used in this example and protein drug binding aptamers used above could be varied to achieve targeting or loading of many different proteins for delivery to different cell types in future work.

2.5.2. Protein-Lipid nanocarriers

Lipid based nanocarriers have been extensively developed for drug delivery and vaccine applications, including FDA approved products [179,180]. However, their structural instability is a limitation that requires careful storage and can impact function. Lipid modification of proteins via bioconjugation method enables manipulation of protein properties. For example, Antos et al. utilized sortase-catalyzed transpeptidation to attach lipids to GFP in a site-specific manner. The resulting lipid-protein conjugates displayed enhanced interactions with the plasma membrane and localization to early endosomal compartments [181]. Based on previous research in construction of protein-lipid conjugates, incorpo-

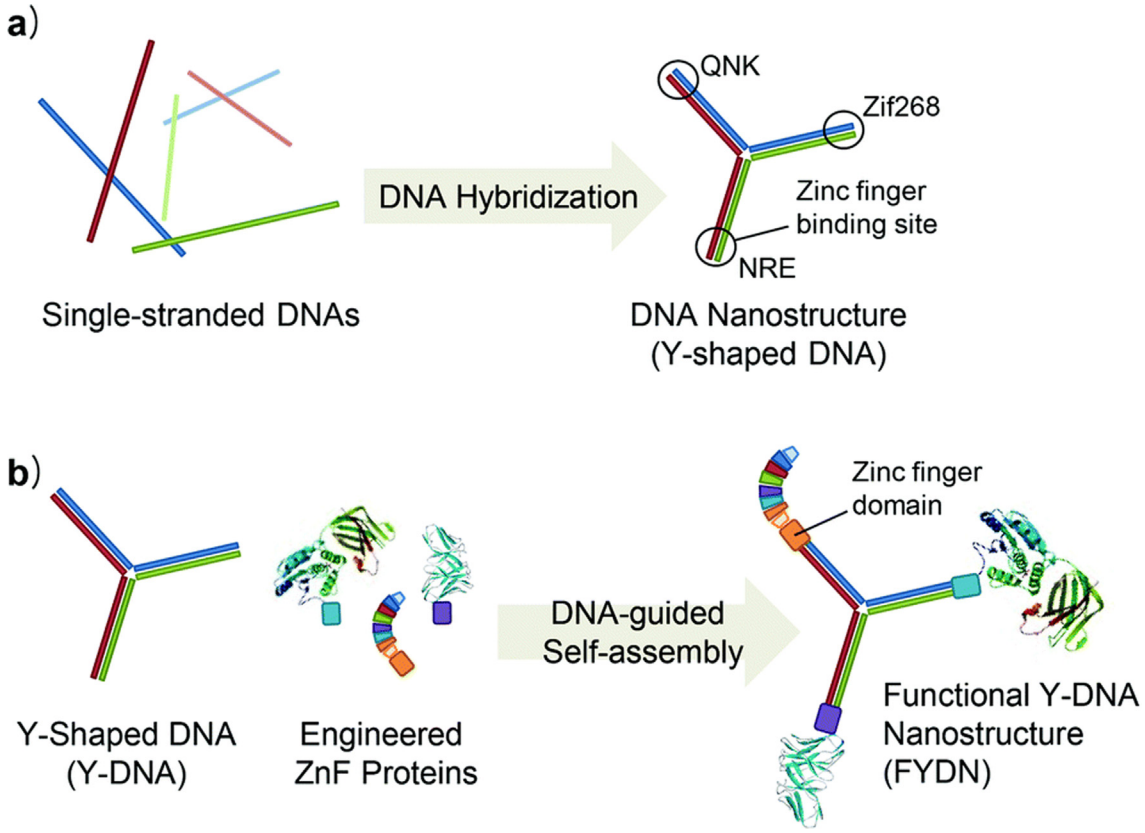

Fig. 6. (a) Single stranded DNA is hybridized to form Y-shaped origami structures. (b) Zinc finger (ZnF) proteins (teal, orange and purple rectangles) fused to functional targeting or therapeutic proteins are mixed with Y-DNA to form protein-DNA hybrid nanoassemblies capable of targeted intracellular protein delivery. Reproduced with permission from [50] from the Royal Society of Chemistry.

ration of proteins into existing lipid nanomaterials or adding lipid modifications to protein assemblies are two approaches that have been explored to improve drug delivery. In one of the early studies that used protein-lipid hybrids for drug delivery, Kyunga et al. conjugated thermoresponsive ELP to the surface of liposomes for hyperthermia treatment [182]. Functionalization by ELP resulted in higher encapsulation efficiency of DOX. *In vitro* study with HeLa cells showed that the thermoresponsive phase transition of ELP on the liposomal surface efficiently enhanced cellular uptake. In the simplest case, large cationic unilamellar vesicles were simply mixed with ovalbumin (OVA) or epidermal growth factor proteins, which induced folding and engulfing processes that generated multilamellar protein lipid hybrid vesicles [183,184]. The multilamellar vesicles had diameters in the range of 150–200 nm and could be further decorated with anionic lipid adjuvant and hyaluronic acid (HA) for administration as a vaccine delivery system. The hybrid vesicles induced OVA specific antibodies and cellular responses in mice.

In a rationally designed example, a pentameric coiled coil protein was engineered to be super positively charged, enabling it to complex with nucleic acids. In the initial application for pDNA delivery presented by Liu et al., the protein-DNA complexes were simply mixed with cationic lipid formulation FuGENE HD to encapsulate the complexes [185]. The ternary assemblies had diameters in the range of 315–350 nm depending on the ratio of components. Murine preosteoblast cells exhibited the highest transfection efficiency with ternary assemblies containing an 8:1 ratio of protein to DNA and controls containing no protein or containing TAT cell penetrating peptide performed significantly worse. In the second application, the supercharged coiled coil protein was complexed with siRNA and DOX before mixing with Lipofectamine 2000 to form ~ 200 nm assemblies [186]. DOX was proposed to interact with the hydrophobic channel of the pentameric coiled coil. Formulations containing the coiled coil exhibited greater delivery of both siRNA and DOX to MCF-7 breast cancer cells, though the presence of the protein did not result in better knockdown than Lipofectamine 2000 alone.

Oude Blenke et al. reported a different design of coiled coil protein liposome, where heterodimeric coiled coil partners, K3 and E3, were used to functionalize liposomes and cells to induce docking and delivery [67]. The coiled coil peptides contained a terminal cysteine that was used to attach a maleimide functionalized PEG-lipid conjugate. Liposomes containing antisense oligonucleotides or siRNA were mixed with the modified peptides, which induced insertion of the lipid tail on the peptides into the liposome membrane at a density of 1 % peptide-lipid out of total lipids. Cells were then modified by incubation with cholesterol-PEG-K3 or -E3 conjugates, which inserted in the membrane, presenting the peptide on the cell surface. K3 functionalized liposomes were capable of delivering functional antisense oligonucleotides or siRNA to E3 functionalized HeLa cells, though not in the reverse scenario or to unmodified cells. The binding of the coiled coil appeared to induce endocytosis. Functionalization with K3-E3 is a novel method for *ex vivo* manipulation of cells and truly orthogonal targeted delivery. This hybrid liposome could be potentially extended to other cell targeting peptides fused to E3 for cell specific disease applications.

The previous lipid-protein hybrid nanostructures were created either by mixing or synthetic conjugation schemes. However, there are several natural post translational modifications that lipidate proteins. Lugenbuhl et al. utilized natural yeast post-translational modification machinery to myristolate ELPs at the *N*-terminus

[62]. This was accomplished by providing *N*-myristoyltransferase and myristic acid and inserting the transferase peptide substrate sequence at the *N*-terminus of ELP. Addition of a single myristic acid resulted in self-assembly of the lipoproteins into spherical 20–30 nm micelles or rod-shaped micelles with lengths of hundreds of nm depending on the exact sequence and length of the ELP. DOX and paclitaxel were encapsulated into the lipid core of the micelles, which were internalized by 4T1 breast cancer cells and resulted in cytotoxicity. Intravenous injection of the myristolated ELP DOX loaded micelles into mice revealed a longer circulation half-time than free DOX. This approach could be applied to other hydrophobic cargo that would benefit from nanoencapsulation. It has also been extended by Mozhdehi et al. to another, less common post-translational lipid modification, cholesterol modification. This was achieved by fusing the cholesterol binding C-terminal domain of hedgehog protein to the C-terminus of ELP [64]. Upon binding cholesterol, hedgehog undergoes a cleavage reaction resulting in covalent transfer of the cholesterol to the C-terminus of ELP and release of hedgehog (Fig. 7). Cholesterol modified ELP also assembles into micelles, 24 nm in diameter. Exendin-4, a peptide used in the treatment of diabetes, was fused to the *N*-terminus of cholesterol modified ELP, which was not possible with *N*-terminal ELP myristolation. The cholesterol peptide micelles exhibited biological activity that was improved compared to exendin-4-ELP micelles without cholesterol. Together, these two methods provide alternatives for lipid modification at either terminus of proteins. While myristolated ELP assemblies have been expanded to include a beta-sheet forming peptide at the *N*-terminus [63], it remains to be seen if nanoassemblies can be formed from other lipid modified proteins, or if it is a property unique to ELP. Recent studies reporting nanoassemblies from farnesylated ELPs [187] and lipidated resilin-inspired polypeptides [188] modified via post-translational modifications expanded the biological toolbox to construct lipidated protein assemblies, suggesting more opportunities for future drug delivery applications.

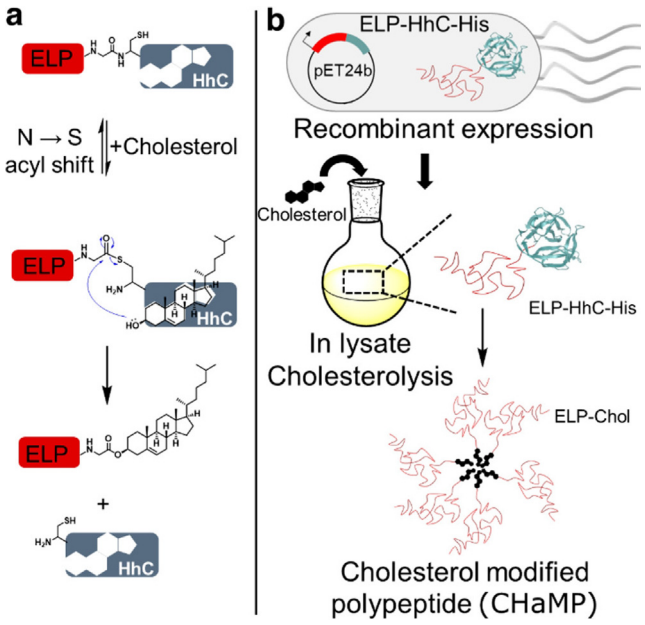

Fig. 7. (a) Chemical reaction of C-terminal domain of hedgehog protein (HhC) with cholesterol that results in cleavage of HhC and replacement by cholesterol. HhC is fused to the C-terminus of ELP. (b) Schematic of fusion protein expression, reaction, and self-assembly processes. Reprinted with permission from [64]. Copyright 2019 American Chemical Society.

2.5.3. Protein-Glycan nanocarriers

Outside of VLPs, there are relatively few hybrid protein-glycan nanostructures and they are limited to covalent modification, as opposed to post-translational gycolsylation. Ding et al. utilized the K3 and E3 coiled coil peptide partners described previously for covalent conjugation to HA chains to create pH sensitive nanogels for intracellular protein delivery [43] (Fig. 8). Maleimide chemistry was used to link thiol terminated peptides to HA at a molar ratio of ~ 7.5 peptides per HA molecule. K3 and E3 functionalized HA plus saporin toxic protein cargo were desolvated, resulting in formation of 176 nm nanogels by stabilized by K3-E3 coiled coils. The nanogels swelled at pH 6.0 or lower and released protein cargo due to the pH sensitivity of the K3-E3 interaction. HA is a natural ligand for CD44 and CD44 positive MCF-7 cells, and nanogels demonstrated cellular uptake, endosomal escape and decreased viability due to delivered saporin. This approach should be extendable to other coiled coils and polysaccharides to create nanogels with different chemical properties, as well as to other cargos, provided their structure is not damaged by desolvant.

Xiao et al. have conjugated HA, dextran, and laminarihexaose to ELP, which self-assembled into micelles with diameters in the range of 220–280 nm [189]. Conjugation was achieved by reaction of the *N*-terminus of ELP with NHS-alkyne ester, followed by click chemistry to azide functionalized polysaccharides. Although cargo encapsulation and delivery have not yet been reported, it is expected that the hydrophobic ELP core of the micelles could carry hydrophobic small molecule drugs similar to the other described ELP nanocarriers. While the few examples here use synthetic conjugation, progress in the area of bacterial glycosylation of recombinant proteins suggests that there may opportunities for new protein-glycan hybrid nanomaterials in the future [190].

2.5.4. Protein-Polymer nanocarriers

While proteins did not evolve to interact with synthetic polymers, the many similarities between protein "polymers" and synthetic polymers have encouraged development of protein-polymer hybrid materials [191]. Compared with other hybrid nanocarriers, protein-polymer hybrids are the most well-studied hybrid structures with diverse synthesis approaches and thorough investigation of pharmacokinetics of protein-polymer hybrids as drug delivery carriers. Protein-polymer hybrids are designed to impart various functionalities provided by polymers, such as amphiphilicity for self-assembly, longer *in vivo* circulation, pH responsiveness, and enhanced stability [191–193]. Additionally, the cost of polymer materials produced in bulk can be less expensive than purified recombinant proteins, providing a way to reduce cost while maintaining a significant component of protein material. As with other hybrid nanocarriers, protein-polymer assemblies can be produced via mixing or covalent conjugation. In many cases of "simple mixing", the protein must either be modified with chemical functional groups or have sufficient naturally occurring surface accessible lysine or cysteine residues. Only one example of covalent conjugation is presented and we direct the reader to a recent review on self-assembly of protein-polymer conjugates for drug delivery for more details on synthetic strategies, assembly of nanocarriers and drug delivery applications [191]. Whether the polymer is directly conjugated to the protein, or the protein is modified to induce interactions with the polymer, it is essential that these chemistries do not disrupt protein structure or inhibit protein activity.

Vanparijs et al. used the "grafting from" approach to create conjugates of model protein, BSA, and thermoresponsive diacrylate polymer containing acid labile dioxolane groups [194]. The BSA was functionalized with approximately 5 chain transfer agents to enable polymer to be grown from the protein using reversible addition-fragmentation chain transfer (RAFT) polymerization.

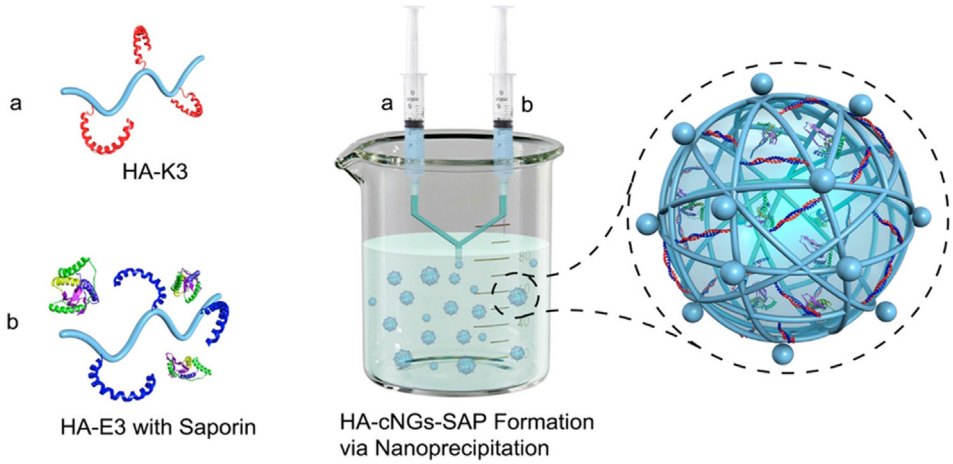

Fig. 8. Hyaluronic acid (HA) covalently functionalized with (a) K3 and (b) E3 peptides are mixed with saporin protein cargo and injected into acetone to induce precipitation of nanogels, which are stabilized by pH sensitive coiled coil formation of K3 and E3. Reprinted with permission from [43]. Copyright 2018 American Chemical Society.

Increasing the temperature above the LCST, which was a function of the degree of polymerization, resulted in self-assembly of hybrid nanoparticles of 50 and 187 nm diameter, for degree of polymerization 100 and 200, respectively. In the presence of acid, the particles disassociated due to hydrolysis of the hydrophobic dioxolane, leaving soluble polymer-protein conjugates even above the LCST. The intact particles were loaded with hydrophobic immune stimulant CL075 via solvent displacement from ethanol to water. Dendritic cells exhibited significantly increased uptake of particles relative to soluble BSA, and CL075 loaded particles induced upregulation of maturation markers indicative of immune stimulation.

While multiple polymer chains grown from a protein may interfere with its function, therapeutic proteins may require less bulky modifications. Zhang et al. produced disulfide connected polymer-protein hybrid micelles called proteosomes [195]. Thermoresponsive diblock copolymer poly[*di*(ethylene glycol) methyl ether methacrylate]-*b*-poly[poly(ethylene glycol) methyl ether methacrylate-*co*-pyridyl disulfide methacrylamide] was synthesized and heated above the LCST to induce micelle formation. Reduced BSA with surface available free thiols reacted with the pyridyl disulfide groups in the polymer, acting as both a cargo and a crosslinker to stabilize the micelles, even when cooled below the LCST. The proteosomes had diameters of ~ 50 nm and released the BSA in reducing conditions while preserving a majority of the protein activity. Fibroblasts and breast cancer cells exhibited high uptake of proteosomes with no negative affect on viability. This approach could be applied to other proteins that have sufficient native surface cysteine residues or that can be recombinantly or chemically modified to add surface thiol groups.

Electrostatic interactions can also be used to form a non-covalent interaction between proteins and polymers. Lee et al. reported polymer-protein nanocomposites formed from positively charged polymer and proteins modified with a negative 20-mer glutamate tag [39]. A series of guanidinium-functionalized poly-oxanorborneneimide homopolymers were produced with varying molecular weight (MW). Simple mixing of tagged protein and polymer resulted in complexes of diameter ~ 100–200 nm, depending on the polymer MW. A wide range of polymer:protein ratios was investigated for HeLa cell delivery using tagged GFP and it was determined that higher MW correlated with better delivery with little dependence on ratio. Mechanistic studies revealed that cytosolic delivery was directly due to polymer-cell membrane interactions and not via endocytosis, which is the route of uptake for most other nanocarriers. Additionally, 10-mer glutamate tagged Cre recombinase was delivered to HEK-293 T cells and over 90 % of cells exhibited recombination. This smaller tag may enable extension of this concept to other recombinant proteins, though optimization of the terminus, linker and length of the tag may be needed to preserve the function of some proteins.

While the previous examples all delivered permanently altered protein cargo (polymer conjugated, reduced, tagged), Dutta et al. developed a self-immolative strategy to enable separation of unmodified protein and polymer following intracellular delivery (Fig. 9) [196]. A copolymer of polyethylene glycol monomethyl ether methacrylate and pyridyl disulfide ethyl methacrylate was produced and covalently reacted with amine groups on the surface of proteins. Assembly of hybrid complexes with diameters of ~ 10–30 nm was induced by addition of bifunctional amine terminated crosslinkers. Exposure to reducing conditions, such as those found in cells, resulted in self-immolative cleavage of the protein-polymer bond and reduction of the polymer crosslinks to release unmodified protein. The assemblies were internalized by HeLa cells much more than unmodified protein and exhibited escape from endo/lysosomes to the cytosol over time. Cytochrome *c* retained its activity following release from the hybrid complexes, as evidenced by induction of apoptosis. Complexes made with lysozyme and ribonuclease A were also fabricated and exhibited similar properties, though the rate of release of the protein from polymer depended on the number of lysines in the protein. Though this approach can be extended to other proteins, there may be a minimum number of surface lysines required for sufficient binding between the protein and polymer as well as a maximum number of lysines that precludes release of the protein from the polymer. It is possible to modify the number of surface accessible lysines either recombinantly or covalently if needed.

In another example, Liu et al. used bioorthogonal click chemistry to reversibly link protein and polymer inducing assembly of hybrid complexes that also release unmodified protein [197]. Proteins including RNase, GFP, BSA and β-galactosidase were functionalized at lysine positions with arylboronic acid. PEG-methacrylate copolymer modified with salicylhydroxamate was simply mixed with the protein to enable the fast click reaction between the two functional groups and formation of complexes of ~ 20 nm. Exposure of the complexes to reactive oxygen species (ROS) released the polymer and boric acid from the protein, while the remaining carbamate moiety on the protein self-immolated to restore the protein to its original form. Hybrid protein complexes were internalized significantly more than unmodified or arylboronic acid functionalized protein by HeLa cells. Stimulation of

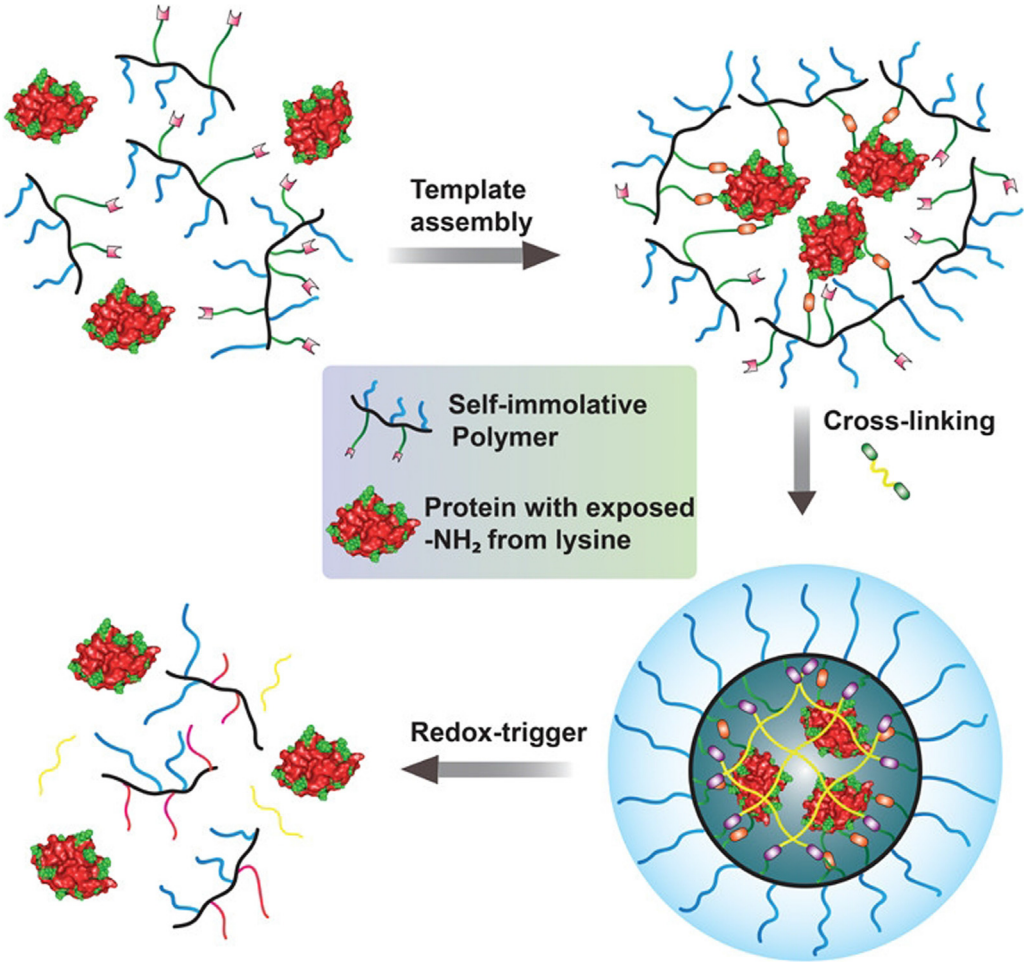

Fig. 9. Copolymer of polyethylene glycol monomethyl ether methacrylate and pyridyl disulfide ethyl methacrylate was assembled with proteins bearing surface exposed lysine residues and crosslinked to stabilize. Reducing conditions disrupt the crosslinks and protein-polymer bonds, releasing unmodified protein. Reprinted with permission from [196]. Copyright 2017 American Chemical Society.

ROS induced functional effects of delivered proteins, such as cell death in the case of RNase. The protein functional linkage was also modified to self-immolate under reducing conditions for use in situations where ROS were not present.

3. Conclusions and outlook

Self-assembling protein nanocarriers have emerged as promising platforms for drug delivery due to their inherent biocompatibility and biodegradability, structural homogeneity, and modularity in design. Thanks to advances in genetic engineering and synthetic biology, functionalization of protein nanocarriers goes beyond chemical modifications, which allows scientists to customize the properties of nanocarriers for specific applications at the genetic level. Self-assembly has been exploited as a simple and effective strategy to synthesize nanocarriers under mild conditions and display functional motifs with control over orientation. Therefore, multifunctional protein nanocarriers have been reported with improved drug encapsulation, targetability, stimuli responsiveness, and *in vivo* half-life. Researchers continue to expand the portfolio of protein materials, from engineered natural proteins to *de novo* designed proteins with diverse properties. Furthermore, hybrid protein materials combining proteins with other biological or synthetic molecules have been developed by chemical methods or post-translational modification. The hybrid protein structures have properties not observed in proteins and show potential to further improve drug delivery.

The selection of protein nanocarriers for drug delivery depends on properties of the nanocarriers, drug cargo, disease physiology, and administration route. However, it is hard to directly compare each system as some systems do not have *in vivo* results and each system delivers different cargos to different types of cells. There is not sufficient data yet to make generalizations about certain particle types or classes being the most ideal for certain cargos or particular applications. Only with the addition of *in vivo* pharmacokinetics and pharmacodynamics data on engineered protein carriers, can recommendations be made for their use. The majority of applications are in the cancer or vaccine space, but this likely reflects the popularity of these areas of research rather than proof that these are the best applications for most protein nanocarriers. Although drug delivery carriers were originally designed for small molecules and many of the protein nanocarriers described here carry traditional hydrophobic drugs, the emergence of more diverse and complex drugs imposes new delivery challenges. Peptides, proteins, and nucleic acids are potent therapeutic molecules with higher selectivity and lower toxicity compared to small molecules. However, their large sizes and complex structures hamper their encapsulation and release efficiency. Biomacromolecules are also more sensitive to stresses present in the environment, such as proteases and changes in pH and temperature. Degradation or aggregation can activate the immune system, leading to rapid

clearance or immunogenicity. Therefore, protein nanocarriers to be used for protein, peptide and nucleic acid cargoes need to adapt to new challenges of encapsulation, release, and protection against degradation [198,199]. Direct genetic fusion allows incorporation of therapeutic peptides and proteins into nanocarriers in a site-specific manner and in some cases presented here, the therapeutic protein serves as both a building block of the nanocarrier and the cargo. Moreover, modular affinity proteins and click chemistry are emerging methods for drug encapsulation. Hydrophobic ion-pairing complexation method has been demonstrated in polymer and lipid nanoparticles for enhanced protein stability and increased encapsulation efficiency [200]. The same approach could be applied to protein nanocarriers with hydrophobic cores. Subsequently, drug release can be triggered by cleavable peptides or programmable disassembly. Additionally, self-immolative linkers have been used to link drug and building block to design prodrug-like properties in polymeric nanocarriers [201,202]. When triggered by specific stimulus, drug conjugates undergo a cascade of head-to-tail disassembling reactions that leads to subsequent release of the drug. It is also possible to combine self-immolative linkers with proteins to enable controlled release from protein nanocarriers.

Recently, mRNA and CRISPR-Cas9 have emerged with high potential for nucleic acid-based therapeutics. However, due to significantly larger sizes of mRNA (~1000–4000 nucleotides) compared with other therapeutic RNAs (siRNA, 21–23 nucleotides), it is more challenging to encapsulate mRNA and deliver it intracellularly [203,204]. PEG 10 VLPs described previously are a promising example of using non-viral origin VLPs to encapsulate and deliver mRNA *in vitro* [95]. Further, natural RNA binding proteins that are rich in cationic amino acids can be used for RNA encapsulation. Simon et al. genetically incorporated a mRNA-binding domain (RBD) into ELP. This RBD was rich in arginine and known to bind RNA through intramolecular interactions. ELP-RBD fusions formed ribonucleoprotein with mRNA and worked as artificial granules that regulate the translation of GFP in a thermally responsive manner [205]. This approach may be combined with many of the ELP nanocarriers presented in this review. Similar to mRNA, safe and efficient delivery of CRISPR/Cas9 remains the biggest challenge for its therapeutic application. Viral vectors are efficient carriers for CRISPR/Cas9 delivery but are limited by cargo sizes and immunogenicity concerns. In contrast, non-viral delivery carriers could be valuable alternatives for CRISPR/Cas9 delivery to address the immunogenicity concerns and reduce off-target editing effect [206,207]. However, few studies using protein nanocarriers for CRISPR/Cas9 delivery have been reported. Natural protein nanocarrier, VLP P22, has been used for the *in vivo* assembly of Cas9 proteins and gRNA by fusing the Cas9 to its building block, but modifications for enhanced cell entry and programmable release are needed [208].

Despite the progress in designing self-assembling protein materials, their toxicology, pharmacokinetics, and biodistribution need to be thoroughly characterized before clinical translation. Investigation of interactions between nanocarriers and biological environments is vital for understanding their *in vivo* fate. Once protein nanocarriers are exposed to biological fluids, nanocarriers are surrounded by proteins and other biomolecules that form the protein corona. The protein corona plays a critical role in immune response and targeting capabilities of all classes of nanocarriers [209]. Remarkable progress has been made in characterizing and understanding the biological relevance of the protein corona [210,211]. However very few studies exist that report on the protein corona of protein nanostructures [212,213]. Controlling and exploiting the protein corona raises new opportunities to better design and predict the *in vivo* fate of protein nanocarriers.

The blood–brain barrier (BBB) represents, arguably, the largest obstacle for drug delivery [214]. So far, none of the self-assembling protein nanocarriers discussed in this review have attempted delivery to brain. Studies are needed on nanocarrier-BBB interactions, brain targeting, and nanocarrier-cerebrospinal fluid interactions to develop protein nanocarriers for brain delivery. Furthermore, protein nanocarriers of nonhuman origin raise immunogenicity concerns. To circumvent these issues, humanized or de-immunized sequence modifications can be introduced in building blocks for reduced immunogenicity [215]. Additionally, proteins produced in bacterial systems suffer from endotoxin contamination, which can result in inflammatory reactions. This challenge can be addressed by exploring alternate expression systems, such as plant [216], mammalian [217], and cell-free expression systems [218]. Altogether, self-assembled protein nanocarriers exhibit promise for delivery of a wide variety of drug classes and their use will expand along with the progress in protein engineering and synthetic biology when combined with materials science and engineering.

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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