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Coiled coil-based therapeutics and drug delivery systems

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ABSTRACT

Coiled coils are characterized by an arrangement of two or more α-helices into a superhelix and one of few protein motifs where the sequence-to-structure relationship to a large extent have been decoded and understood. The abundance of both natural and *de novo* designed coil coils provides a rich molecular toolbox for self-assembly of elaborate bespoke molecular architectures, nanostructures, and materials. Leveraging on the numerous possibilities to tune both affinities and preferences for polypeptide oligomerization, coiled coils offer unique possibilities to design modular and dynamic assemblies that can respond in a predictable manner to biomolecular interactions and subtle physicochemical cues. In this review, strategies to use coiled coils in design of novel therapeutics and advanced drug delivery systems are discussed. The applications of coiled coils for generating drug carriers and vaccines, and various aspects of using coiled coils for controlling and triggering drug release, and for improving drug targeting and drug uptake are described. The plethora of innovative coiled coil-based molecular systems provide new knowledge and techniques for improving efficacy of existing drugs and can facilitate development of novel therapeutic strategies.

Contents

Abbreviations: Ala, Alanine; APNP, Activatable protein nanoparticle; Asn, Asparagine; bFGF, Basic fibroblast growth factor; CBD, Collagen-binding-domain; CC, Cytochrome C; CD, Circular dichroism; CCPO, Coiled coil protein origami; CHO, Chinese Hamster Ovary; COMP, Cartilage oligomerization matrix protein; CPP, Cell penetrating peptide; Cys, Cysteine; DFMT, Drug-free macromolecular therapeutics; DOX, Doxorubicin; ECM, Extracellular matrix; EGF, Epidermal growth factor; ELP, Elastin like protein; GB, Glioblastoma; Glu, Glutamic acid; HA, Hyaluronan; HIV, Human immunodeficiency virus; HPMA, N-(2-Hydroxypropyl)methacrylamide; HUVEC, Human umbilical vein endothelial cell; HSA, Human serum albumin; Ile, Isoleucine; Leu, Leucine; LMWH, Low-molecular-heparin; LTSL, Lysolipid-containing temperature-sensitive liposomes; Lys, Lysine; K_d, Dissociation constant; KIH, Knobs-into-holes; mAb, Monoclonal antibody; MMP, Matrix metalloproteinase; MRI, Magnetic resonance imaging; MRS, Magnetic resonance spectroscopy; MSN, Mesoporous silica nanoparticles; PEG, Polyethylene glycol; QD, Quantum dot; RHCC, Right-handed coiled coil domain; SAGE, Self-assembled peptide cage; SAP, Saporin; SAPN, Self-assembling protein nanoparticles; scFv, Single-chain fragment; Ser, Serine; SVLP, Synthetic virus-like particle; TAT, Transactivating transcriptional activator; T_m, Melting temperature; Trp, Tryptophan; Tyr, Tyrosine; UV, Ultraviolet; Val, Valine; VEGF, Vascular endothelial growth factor; VLP, Virus-like particle.

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1. Introduction

Coiled coils are abundant structural motifs found in many fibrous proteins and transcription factors and are often involved in assembly of higher order protein structures [1]. Comprised of two or more α-helices that are arranged into a superhelix, coiled coils form a very distinct and unique structure [1]. Because of their biological importance and striking appearance, the sequence to structure relationship of coiled coils have been extensively studied, and to a large extent also decoded [2–5]. The knowledge generated from systematic investigations of the roles of individual amino acids on folding and oligomerization in both natural proteins and synthetic peptides have facilitated *de novo* design of numerous different polypeptides that form well-defined coiled coils under various conditions [6–8]. A large number of coiled coils with various preferences and affinities for oligomerization have been developed and a wealth of natural coiled coil sequences have been explored. This has in turn enabled engineering of an amazing repertoire of higher-order coiled coil supramolecular assemblies, such as fibers [9–11], hydrogels [12–18], and structurally defined discrete nanostructures [19,20]. These intricate molecular architectures form as a result of specific supramolecular interactions that can be tailored with great precision, dictated by the amino acid sequence of the peptides. In many instances, also external factors, such as presence of certain ions and ionic strength, temperature, pH, and interactions with surfaces or biomolecules influence these interactions and thus, the structure and stability of the coiled coils [18,21–23]. The fine balance between attractive and repulsive interactions that can regulate folding and assembly of coiled coils make these molecules highly interesting and versatile components in design of stimuli responsive materials and nanostructures for fabrication of innovative drug delivery systems and development of novel therapeutic strategies.

The possibilities to use coiled coils as key molecular components in drug delivery systems, biomaterials, vaccines and therapeutics can be further expanded by engineering of fusion proteins or by combining them with synthetic polymers, proteins, carbohydrates, lipids and inorganic nanoparticles, using physical interactions or chemical conjugation, to create coiled coil hybrids and nanocomposites [24–26]. Due to the large number of combinations, these hybrids comprise a very diverse group of molecules and nanostructures, but with the common denominator that they leverage on the ability of the peptides to fold and oligomerize through specific intermolecular interactions into coiled coils.

The function of the coiled coils in these systems can broadly be divided into six main areas where the coiled coils either function primarily as structural components for generating or functionalizing (1) hydrogels and (2) discrete nanoscale drug carriers, or (3) particle-like architectures for antigen presentation in vaccine development platforms, or (4) for improving drug targeting, (5) triggering the release of drugs from drug delivery vehicles, or (6) for tethering drugs to polymers, particles or hydrogel scaffolds (Fig. 1). In this review, we cover all these areas and highlight and discuss recent work on applications of coiled coils in design of novel therapeutic strategies and advanced drug delivery applications.

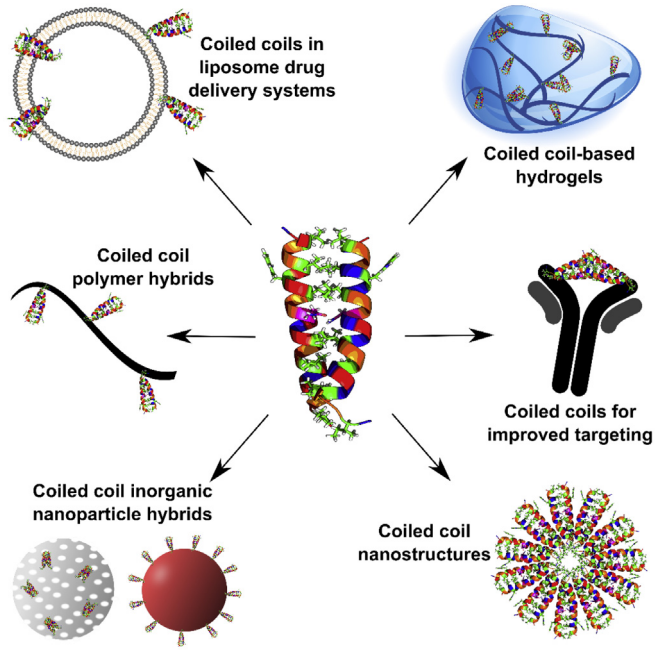


Fig. 1. Coiled coils and coiled coil-hybrids for drug delivery and therapeutics.

2. The coiled coil motif

The coiled coil motif was one of the first more complex protein structures that was successfully determined. This work was initiated already in the late 1920s in Leeds, in the heart of British textile industry, where William Astbury was studying the X-ray diffraction patterns of a large number of different protein fibers. Astbury discovered three main spectral patterns that he referred to as α, β and γ forms. Proteins that gave rise to the structural pattern of the α-form appeared to be common and he referred to them as 'k-m-e-f', for keratin, myosin, epidermin, and fibrinogen. These proteins all gave rise to diffraction pattern with a strong meridional arc at 5.15 Å [27]. In 1950, Linus Pauling proposed a model structure for the α-form, where he suggested two hydrogen bounded helical structures, one of which was an α-helix [28]. This structure could, however, not explain the diffraction pattern recorded by Astbury for the 'k-m-e-f' proteins. Two years later, Francis Crick offered a full parametrized model for a structure of α-helices that were twisted around each other. He called this bundle of supercoiled helices, coiled coil. Crick proposed that two or more α-helices can pack together to form a left-handed supercoil structure with a knobs-into-holes (KIH) arrangement of the amino acids [29].

The structural and functional diversity of coiled coils can be appreciated by studying their role in proteins, spanning from controlling the dimerization of many transcription factors, and components in longer segments in dimeric and trimeric fibrous structural proteins, to complex structures involved in virus-host membrane fusion (Fig. 2A) [30]. In 2001, Walshaw and Woolfson introduced a software (SOCKET) that identified KIH interactions in proteins [31]. By applying this software on data in the Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB) they identified and studied no less than 766 coiled coil containing protein structures [30]. This data was then used to develop a periodic table for coiled coils based on the number of helices in the motifs and the relationships between them (Fig. 2B). A classical coiled coil structures was defined as having only one hydrophobic core. There are classical coiled coils that consist of more than two helices and that assemble through a contiguous ring of KIH interactions between adjacent helices, but they still share a single consolidated central hydrophobic core. Structures comprised of two or more interacting, or connected, classical coiled coils were referred to as complex coiled coil structures [30].

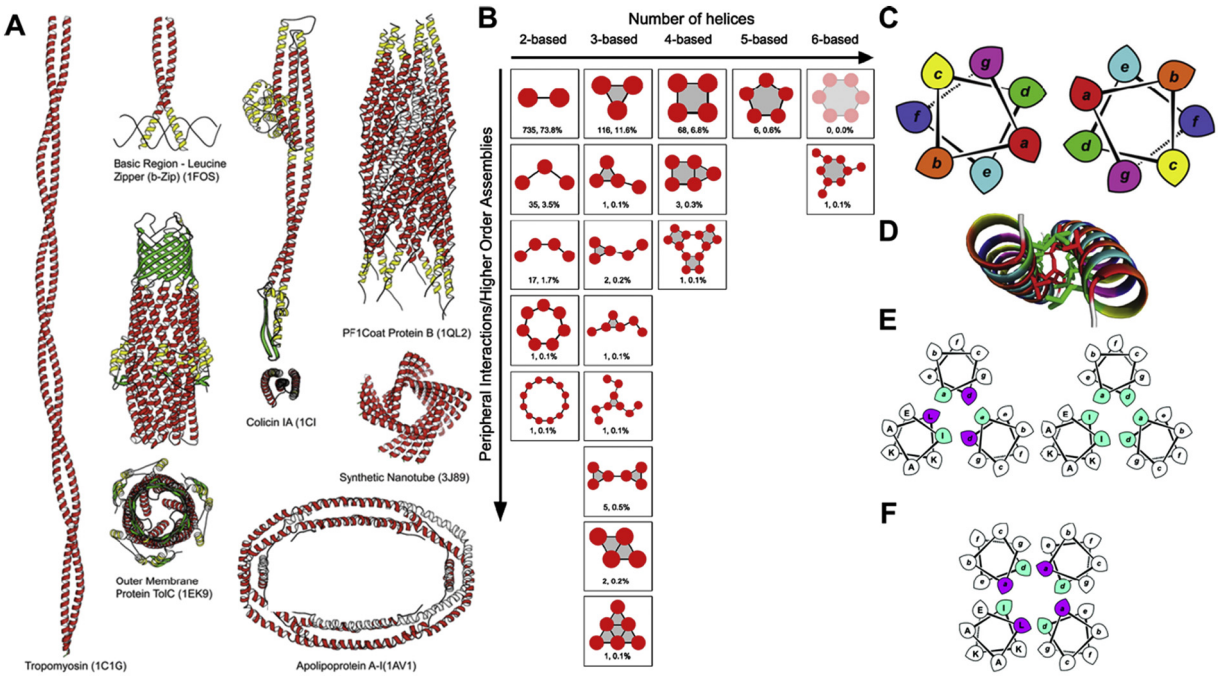


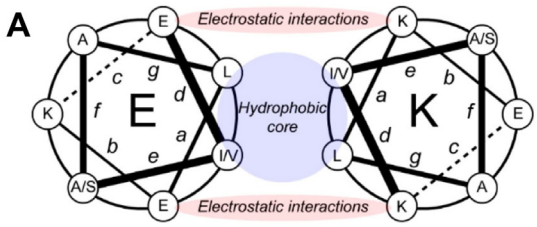
Fig. 2. The structure of coiled coils. A) Examples of natural and synthetic coiled coil-based proteins and structures. Reproduced with permission from [32], copyright Springer International Publishing AG 2017. B) The coiled coil periodic table. Reproduced with permission from [30], copyright Elsevier Ltd. 2008. C) Helical wheel diagram of a coiled coil indicating the relative positions of amino acids. D) A typical parallel dimeric coiled coil colored based on the heptad positions: *absdefg*, *a* = red; *b* = orange; *c* = yellow; *d* = green; *e* = cyan; *f* = blue; *g* = magenta. E) Helical-wheel diagram for trimeric, and F) tetrameric coiled coils. Adopted with permission from [33], copyright 2012 American Chemical Society.

As a result of numerous thorough studies on the structure of classical coiled coils in proteins, such as the leucine zippers [6,34,35], and significant efforts in coiled coil *de novo* design [3,4,7,8,36], the sequence to structure relationship of coiled coils is now well understood [6]. This knowledge has facilitated efforts in *de novo* design of coiled coils with a variety of oligomeric states, with parallel [37] or antiparallel helices [38], and different homo- and heteromer combinations [33,39–41]. The primary structure of a canonical coiled coil shows a repeating seven-residue pattern of hydrophobic (*h*) and polar (*p*) residues, *hpphppp* [42,43]. The positions of the amino acids in these heptad repeats are designated *a*, *b*, *c*, *d*, *e*, *f* and *g* and can be schematically represented in a helical wheel diagram (Fig. 2C, D). The size of the coiled coil domains can vary significantly, from just 1–2 heptads in the smallest designed coiled coils, to sequences with more than 3000 amino acids in the protein giantin [42]. Hydrophobic amino acids are conserved at position *a* and *d*, forming a hydrophobic patch running along the length of each helix in the coiled coils, and that is flanked by polar and charged residues. Since seven residues is not enough to form two full turns in a regular a-helix, the hydrophobic patch twists around the helix axis. As a consequence, interacting helices in a coiled coil are arranged into a supercoil in order to align their hydrophobic patches to form a stable hydrophobic core. Isoleucine (Ile) and leucine (Leu) commonly form the hydrophobic core of coiled coils at position *a* and *d*. Ile at position *a* and Leu at position *d* typically result in parallel dimers, whereas peptides with Ile at position *a* and *d* induces assembly of trimers (Fig. 2E), while tetrameric assemblies are formed with Leu at *a* position and Ile at *d* position (Fig. 2F) [6,44]. The presence of charged amino acids, in particular glutamic acid (Glu) and lysine (Lys), in position *e* and *g* stabilizes the coiled coil structure, defines heterospecificity and the orientation of the structure [39,45,46]. Position *b* and *c* are often occupied by alanine (Ala) as a small helix favoring residue and *f* position by glutamine (Glu) and lysine (Lys) to increase the solubility of the structure. Tryptophan (Trp) and tyrosine (Tyr) that absorb strongly in the ultraviolet (UV) wavelength range are also often included at *f* position to facilitate spectroscopic monitoring of the peptides [6,45].

The interface formed by the residues at *a* and *d* positions must not necessarily by occupied by hydrophobic residues. Polar residues in these positions, and that are conserved through evolution, have been found in various structurally defined coiled coils [47]. The by far most abundant of these polar residues is asparagine (Asn) [47]. The presence of Asn at *a* position destabilizes dimeric coiled coils but can increase the preference of parallel dimers over antiparallel dimers or parallel trimers [47].

To have better control over the self-assembly and stability of coiled coils, several different strategies have been developed to tune the oligomerization process [7,21,33,41,48,49]. Woolfsons and co-workers designed a set of *de novo* designed coiled coils peptides in which the dissociation constants varied from the micromolar to the subnanomolar range [45]. Aronsson et al. investigated a positive design approach that exploited the difference in packing efficiencies of Val and Ile in the hydrophobic core and the difference in α-helical propensities of serine (Ser) and Ala, to produce parallel heterodimeric coiled coils with dissociation constants spanning from the low micromolar to the picomolar range [41], (Fig. 3A). The large differences in affinities for heterodimerization resulted in social self-sorting of the peptides when all monomers were combined. The number of heptads also has a drastic effect on the stability of coiled coils and can be exploited to tune the stability of heterodimers. The stability of coiled coils increases by increasing the number of heptads. Thomas et al. showed that by increasing the number of heptads in a heterodimeric coiled coil from 3 to 4, the dissociation constant (K_d) of the dimer decreased by four orders of magnitude [45], (Fig. 3B).

Assembly of higher order coiled coil structures can be promoted by extending the hydrophobic helical interfaces past the *a* and *d* residues and involving residues at *e* and *g* position in KIH interactions. By changing the canonical heptad repeat from *hpphppp* (Type N) to *hpphpph* or *hpphhpp* (Type 1); *hpphhph* (Type 2); and *hphhphp* or *hhphphp*



	KV	KI
EV	$(1.4 \pm 1.5) \times 10^{-6}$	$(8.5 \pm 7.1) \times 10^{-8}$
EI	$(7.2 \pm 4.7) \times 10^{-8}$	$< 1.0 \times 10^{-10}$ $((0.43 \pm 4.9) \times 10^{-16})^{*}$

*Calculated value

B

	$CC\text{-}A_N^3$	$CC\text{-}A_N^{3,5}$	$CC\text{-}A_{N4}$
$CC\text{-}B_N^3$	$(3.10 \pm 0.58) \times 10^{-6}$	$(2.79 \pm 1.42) \times 10^{-7}$	$(7.47 \pm 3.30) \times 10^{-8}$
$CC\text{-}B_N^{3,5}$	$(6.40 \pm 2.77) \times 10^{-7}$	$(5.15 \pm 2.05) \times 10^{-9}$	$(2.08 \pm 0.73) \times 10^{-10}$
$CC\text{-}B_N^4$	$(3.87 \pm 1.45) \times 10^{-7}$	$(4.74 \pm 4.49) \times 10^{-10}$	$<1.0 \times 10^{-10}$

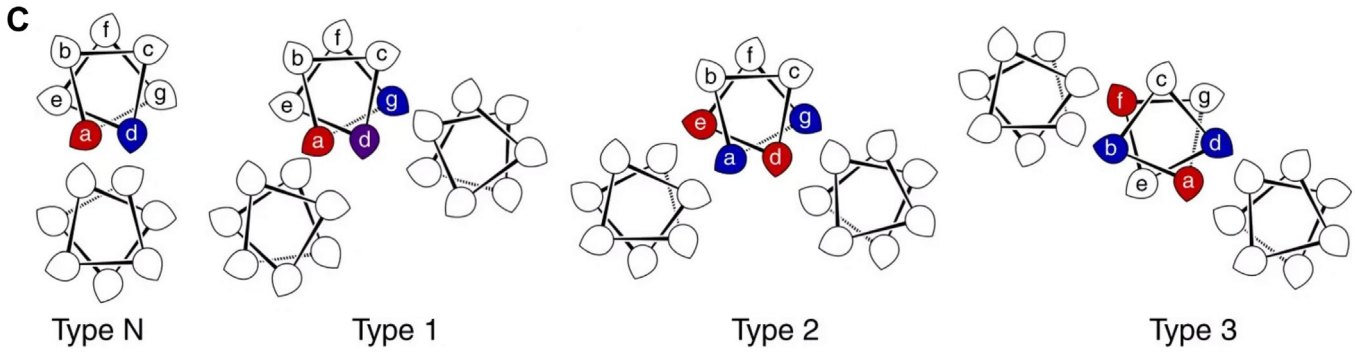


Fig. 3. Design of coiled coils with different affinities and preferences for oligomerization. A) Helical-wheel representation of the EI/EV/KI/KV peptides, *de novo* designed to form parallel coiled coil heterodimers (reproduced with permission from [15], copyright 2016 American Chemical Society), and the corresponding dissociation constants for the different combinations of heterodimers (in molar) at 20 °C. Adopted with permission from [41], copyright Springer Nature 2015. B) Helical-wheel representation of the *de novo* designed coiled coils CC-A_N and CC-B_N and the corresponding dissociation constants (in molar) at 20 °C. Reproduced with permission from [45], copyright 2013 American Chemical Society. C) Helical-wheel diagrams for different types of coiled coil interfaces. Classical Type N and Type 1–3 coiled coils labelled using the canonical a-g nomenclature. The teardrop shapes show the direction of Cα-Cβ bonds. Red, blue and purple colored positions highlights knobs in knob-into-hole interactions as parallel, perpendicular and X-layer respectively, where core-packing angels describe the orientation of a knob side-chain with respect to the hole into which it fit as introduced by Harbury et al. [4]. Reproduced with permission from [53], copyright 2016 Springer Nature.

(Type 3), three different types of coiled coil assemblies can be obtained (Fig. 3C). However, residues at positions *e* and *g* do not have to be hydrophobic. Type 2 sequences (*hpphhph*) can form α-helical barrels with 5–7 helices, where each α-helix contains two seams allowing it to interact with two neighboring helices [50]. For instance, Liu et al. showed that by replacing all *e* and *g* residues with Ala, they can form a

Table 1
Examples of coiled coil-based therapeutics and drug delivery systems.

Peptide system	Properties	Application	Reference
E_x/K_x	*De novo* designed, heterodimeric, $(EIAALEK)_x/(KIAALKE)_x$	Vesicle fusion, Polymer-drug linker	[22,36,110]
GCN4 pLI	Heterodimeric, naturally derived but engineered	pH and temperature responsive liposomal drug release	[68,70]
Leucine zipper peptide	Heterodimerization	Temperature responsive liposomal drug release, Activation of cell penetrating peptides	[71,151,152,154]
JR2KC/JR2E	Four-helix-bundle, heterodimeric	Enzymatically triggered liposomal drug release	[72,165]
KV/KI/EV/EI	Heterodimeric, *de novo* designed	Triggered liposomal drug release	[74,75]
SVLP	Lipopeptide	Vaccine platform	[79]
SAGE	Combination of heterodimeric and homotrimeric coiled coils	Vaccine platform	[88]
SAPN	Fusion of a modified COMP domain and a *de novo* designed trimeric coiled coil	Vaccine platform	[84]
CCE/CCK	Heterodimeric, antiparallel	Drug-free therapeutics	[13,16]
Heparin-binding coiled coil peptide	Heterodimeric	Encapsulation and release of FGF	[128]
Collagen-binding-domain	Heterodimeric	Tethering growth factor to artificial ECM	[134]
COMP	Pentameric bundle	Temperature responsive hydrogel for release of curcumin, Carrier for vitamin A, D curcumin and for theranostics	[98–100]
RHCC	Tetrameric bundle	Carrier for cisplatin and other Pt-based drugs	[101,102]
Helix A and Helix B	Heterodimers fusion to ELP	Vesicles for VGEF delivery	[106]

stable heptameric coiled coil structure [51]. Involvement of amino acids in *e* and *g* positions in KIH interactions have been seen in natural extreme proteins, such as GCN4 variants and in pentamers like the cartilage oligomerization matrix protein (COMP), as well as in phospholamban and the rotavirus enterotoxin CorA, which all follow the *hhpphhp* heptad repeat pattern [52].

3. Coiled coils and coiled coil-hybrids for drug delivery and therapeutics

The abundance of natural coiled coil motifs and the increasing knowledge in *de novo* design have facilitated development of a wide range of intricate molecular structures and bioresponsive assemblies for drug delivery and therapeutic applications (Table 1). To further leverage on their rich chemical and structural diversity, coiled coils are often combined with other molecules and nanostructures, such as synthetic polymers, biopolymers, lipids and inorganic nanoparticles, to generate hybrid and composite materials. The role of the coiled coils in these materials range from pure structural components to being more actively involved in drug encapsulation and release or in mediating a therapeutic effect.

3.1. Coiled coils in liposome drug delivery systems

Liposome-based drug delivery systems have been thoroughly studied because of their biocompatibility and biodegradability [54]. Drug molecules can be efficiently encapsulated in liposomes and significant improvements in drug pharmacokinetics and biodistribution can be achieved when combined with techniques for obtaining stable liposomes with long circulation times by *e.g.* incorporating polyethylene glycol (PEG) [55]. Further improvements can be achieved by using bioresponsive elements for targeted delivery [56] or triggered drug release [57]. Currently there are about ten liposome-based drug formulations in clinical use for treatment of for example breast cancer, ovarian cancer, and leukemia, and a large number of liposome-based drug formulations are in various stages of clinical trials [58].

Coiled coils offer numerous possibilities to enhance the performance of liposomal drug delivery systems by facilitating cellular uptake or tailoring the release rate by either exogenous factors or endogenous disease biomarkers. The group of Alexander Kros has explored multiple strategies for both coiled coil mediated drug delivery and for enhancing cellular uptake of liposomes, using a heterodimeric coiled coil system consisting of the E (EIAALEK) and K (KIAALKE) [36] peptides with either 3 [59] or 4 [26,60] heptad repeats. These coiled coils were designed and optimized to mimic features of the SNARE protein complex, involved in vesicle fusion processes [61], when tethered to lipid membranes. The two oppositely charged peptides were separately tethered to two different sets of liposomes using a hydrophobic lipid anchor, conjugated to the peptides *via* a short PEG linker (Fig. 4A). When combining the two sets of liposomes decorated with either the E or K lipopeptides, a fusion process was initiated as a result of peptide folding and heterodimerization, resulting in both lipid and liposome content mixing (Fig. 4B–D) [62,63]. Daudey et al. studied the individual roles of the three-heptad peptides E3 and K3 in this process [64]. The positively charged peptide K3 was found to be driving the fusion by immersing into the neighboring liposome after dimerizing with the liposome-conjugated complementary E peptide, which thus acted as a handle to initiate liposomal docking. Zope et al. developed this concept further by demonstrating that it was possible to functionalize Chinese Hamster Ovary (CHO) cells and Zebrafish embryos with these peptides while retaining their ability to form coiled coil heterodimers with complementary peptides anchored in liposomes (Fig. 4E) [65]. This process resulted in accumulation of liposomes at the cell surface, liposomal docking and uptake. Yang et al. continued this work by demonstrating the possibility to target genetically engineered HeLa cells that constantly expressed the four-heptad K4 peptide on their cell surface (HeLa-K) [26]. These cells were injected into Zebrafish embryos followed by administration of E4-liposomes encapsulating the cytotoxic drug doxorubicin (DOX). The proliferation of the xenografted HeLa-K cells was significantly reduced when treated with DOX-containing E4-liposomes compared to non-peptide-decorated DOX-liposomes and free DOX [26]. This effect was further demonstrated to be a consequence of membrane fusion between the liposomes and the cells and not a result of endosomal uptake [66]. Although this system show that the coiled coils can dimerize and fold with high specificity also in complex biological environments, the strategy apparently suffers from the

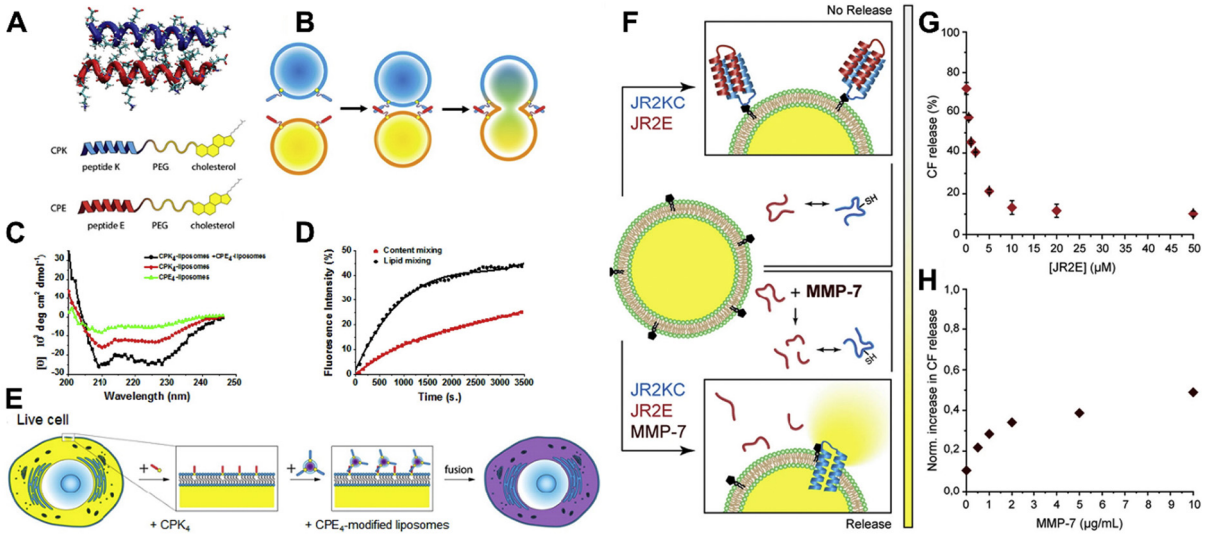


Fig. 4. Coiled coil heterodimerization and folding mediated modulation of liposome fusion and permeability. A) The E/K peptides heterodimerize and fold into a coiled coil and can be tethered to liposomes when modified with a terminal cholesterol moiety. B) Dimerization and folding of the *E*- and K-peptides tethered to separate liposomes result in liposome fusion, content and lipid mixing. C) CD spectra of liposomes decorated with E or K peptides alone and when combined. D) Lipid and content mixing as indicated by fluorescence spectroscopy. E) Illustration of targeted liposome-cell fusion. Reproduced with permission from [66], copyright 2016 American Chemical Society. F) Illustration of JR2KC-triggered liposomal release. G) Release was inhibited upon folding and heterodimerization with the complementary peptide JR2E. H) The JR2KC-triggered release was recovered after cleavage of JR2E by MMP-7. Reproduced with permission from [72], copyright 2016 Springer Nature.

requirement to label cells with one of the two peptides to enhance drug uptake, which reduces applicability for drug delivery [26,66]. Despite this drawback, there are several other interesting *ex vivo*/*in vitro* applications, for example to improve specific non-viral gene delivery as proposed by Blenke et al. [59].

Coiled coils can also be utilized to increase the membrane permeability of liposomes to facilitate release of encapsulated compounds. Reja et al. explored an engineered variant of GCN4 pLI coiled coil [67] for this purpose, where the Ile at the second *d* position in the four-heptad repeat was substituted for fluorescent environmentally sensitive amino acids [68]. These substitutions resulted in unfolding of the coiled coil (P3P5) at pH values deviating significantly from pH 7.4. In contrast, the non-substituted coiled coil (P1P3) remained stable over a wide pH range. Incorporation of P3P5 into liposome bilayers resulted in enhanced drug release at pH 5.5 compared to P1P3 liposomes as a result of the unfolding of P3P5 and subsequent destabilization of the lipid bilayer. *In vitro* cell experiments with encapsulated proflavine, demonstrated that this concept could reduce viability of glioblastoma cells from 95% to 20% when using P3P5 liposomes. Confocal laser scanning microscopy revealed the acidic lysosomes as the main drug localization, further indicating that the pH sensitivity of the coiled coil was contributing to the drug release [68]. The pH responsive release could potentially also facilitate treatment of solid tumors as the tumor microenvironment typically is slightly acidic [69], which could promote a localized drug release.

Temperature responsive coiled coils incorporated in liposome bilayers for triggered drug delivery have also been reported based on the same engineered variant of GCN4 pLI [70]. To adjust the temperature sensitivity to a range that was suitable for *in vivo* applications, the α-Ile at the third *d* position in the four-heptad repeat was substituted for a γ-Ile in both peptides of the heterodimeric coiled coil. As a consequence, a drastic reduction in the melting temperature (T_m) from 67 °C for the non-substituted coiled coil (P1/P2) to 40 °C was observed for the substituted coiled coil (P3/P4). Incorporation of P3/P4 into liposome bilayers resulted in a slow but significant release of encapsulated carboxyfluorescein for up to 26 h at 40 °C, which was not seen when the more thermally stable P1/P2 coiled coil was used [70]. The potential of using thermally sensitive coiled coils for triggering release has also been investigated by Kostarelos and co-workers using a leucine zipper tailored for modulating lipid membrane integrity [71]. The peptides self-assemble into a coiled coil comprising of two or more α-helices when anchored within the lipid membrane. The T_m of the amphiphilic peptides were around 40 °C when incorporated in liposomes. Temperature responsive liposomes combined with this peptide could as a result of mild hyperthermia deliver and release encapsulated DOX while showing better stability in serum than the liposome alone and lysolipid-containing temperature-sensitive liposomes (LTSL). Their accumulation in B16F10 melanoma tumors in C57BL/6 tumor-bearing mice were comparable to LTSL after 1 h but showed a three-fold higher DOX accumulation 24 h after local hyperthermia.

The possibility to use endogenous stimuli, such as enzymes overexpressed in tumor microenvironments, is also an attractive strategy for triggering liposomal release of cancer drugs. Lim et al. explored enzyme-mediated liposomal release using a set of peptides designed to fold into a helix-loop-helix motif and heterodimerize into four-helix bundles [72]. The lysine (Lys) rich peptide (JR2KC) was covalently conjugated to maleimide head group functionalized lipids incorporated in the lipid bilayer of liposomes using the thiol moiety of cysteine (Cys) in the loop region. The peptide was not membrane active prior conjugation, but rapidly triggered release of encapsulated fluorophores when bound to the liposomes. Allowing JR2KC to heterodimerize with the complementary glutamic acid (Glu) rich peptide (JR2E) before addition to the liposomes, reduced the triggered release significantly as a result of the competition between heterodimerization and lipid membrane partitioning (Fig. 4F–G). JR2E has two cleavage sites for matrix metalloproteinase 7 (MMP-7), an enzyme often overexpressed in tumors [73]. After exposing JR2E to MMP-7 for about two hours, the membrane-activity of JR2KC was recovered (Fig. 4H) [72]. Skyttner et al. showed that the possibility to modulate membrane integrity by coiled coil dimerization also could be implemented using classic coiled coils using the *de novo* designed peptides KVC and KIC [41,74]. Similar to JR2KC, these peptides also lacked membrane activity unless conjugated to liposomes. In the presence of the complementary peptides (EI and EV), dimerization and folding into coiled coils significantly reduced the release [74]. By increasing the amphipathic character of the KVC peptide by substituting one Asn and one Trp for a Val and Glu, respectively, the release after conjugation was significantly improved [75]. With stable enough coiled coils, the possibility to inhibit the lipid membrane permeation could allow the liposomes to reach and accumulate in the target tissue prior dissociation of the heterodimers, resulting in enhanced and localized drug release.

3.2. Lipidated coiled coils for assembly of virus-like particles

Coiled coils combined with lipids are not only utilized for facilitating liposomal drug delivery but have also been studied as a platform for generating supramolecular assemblies for antigen display in vaccine development. The prevention of millions of deaths yearly makes immunization one of the greatest success stories in global health. Although vaccines have been able to control and prevent infectious diseases [7] early vaccines contained dead, attenuated or inactivated viruses which by accident could cause real viral outbreaks [76]. Since then, the safer subunit vaccine approach has emerged where only key microbial components are administered in order to trigger an immune response [77]. However, many of these subunit vaccines do not show a very distinct immunostimulatory response, probably due to the limited antigen display. This can be improved by including adjuvants together with the subunit virus or by using virus-like particles (VLPs). VLPs are designed to resemble the virus in shape and size and to display repetitive antigens but lack the viral genetic material, which make them non-infectious [78]. Boato et al. utilized coiled coils for creating synthetic virus-like particles (SVLP) using a lipopeptide with a lipid tail conjugated to the N-terminal of a coiled coil forming peptide and an antigen attached to the peptide C-terminal [79]. The SVLP self-assembled as a result of clustering of the lipid tails and folding of the peptides into parallel coiled coils, resulting in formation of supramolecular nanoparticles with a diameter of about 20–30 nm with antigens exposed on their surface. The repetitive antigen display on the nanoparticle surface could elicit humoral immune responses in immunized rabbits and mice for several different antigens [79–81]. Additional improvement to this vaccine platform could involve generation of antigen clusters, which is often observed in viruses, in order to enhance the binding selectivity. Along this line, Ang et al. created stable micelles of self-assembling coiled coil peptide-PEG-lipid hybrids which retained their oligomeric state when 3-helix and 4-helix conjugates were mixed. These mixed micelles became patchy, making them a promising SVLP candidate with well-defined ligand clusters by exploiting the possibility to generated coiled coils with different oligomeric states [82].

3.3. Coiled coil nanoparticles

The formation of a hydrophobic core that runs and twist along the axis of the helices is central for the folding and association of peptides into the characteristic rod-like coiled coil motif. In order to generate more complex coiled coil-based peptide structures additional interactions or topological constraints that can promote further assembly of the coiled coils into more intricate geometries must be introduced [83]. This can for example be achieved by connecting coiled coils side-to-side or end-to-end using disulfide bonds or various other linkers [19,84], or by combining coiled coils with different helix orientation and that are capable of inter- or intramolecular dimerization [20]. For peptides connected *via* linkers, the structure of the resulting

supramolecular assemblies have been found to be highly dependent on the linker length [85]. By connecting parallel heterodimeric coiled coils using short linkers, steric constraints have been observed to favor formation of large linear assemblies, whereas longer and more flexible linkers resulted in assembly of different discrete nanostructures involving just a few peptides. Moreover, Woolfson and co-workers showed that it was possible to create self-assembled peptide cages (SAGE) by combining heterodimeric and homotrimeric coiled coils in a non-linear arrangement [19]. The heterodimeric coiled coil peptides were separately conjugated to one peptide designed to form homotrimers using a Cys at *f* position that was included in all sequences (Fig. 5A). The folding of the homotrimers resulted in formation of discrete and soluble assemblies with the heterodimer-forming peptide orphaned on the outside. Further mixing of the two sets of trimeric hubs resulted in self-assembly of hollow spherical particles with a diameter of about 100 nm, suitable for drug encapsulation and controlled release (Fig. 5B).

The SAGEs can be modified with other peptides and proteins to promote cell-specific uptake [86–88]. The homotrimeric coiled coil could for example be extended with a short and charged peptide without effecting the stability of the SAGE structure, which enabled modulation of the endocytic uptake of the particles by HeLa cells. The efficiency of uptake of the particles by cells could be tuned by changing the surface net-charge of the particles. In general, a positively charged surface increased endocytic uptake whereas a negatively charged surface impeded this process. In addition to alter the number of charged residues, changing the stoichiometry of the tetrapeptide extensions allowed for further tunability of particle uptake [86].

Woolfson and co-workers have also investigated the possibilities to use SAGEs as modular scaffolds for antigen presentation [88], similar to how the SVLPs are utilized. These nanoparticles were found to be non-toxic in *in vivo* mice assays, even at very high doses. Antigens could be attached to both the N- and/or C-terminal of the SAGE peptides. When including antigen-presenting peptides, a T-cell response was observed *in vitro*, confirming internalization of SAGE particles in antigen presenting cells. In *in vivo* experiments, antigen functionalized SAGEs gave a larger response than the corresponding free antigen. Interestingly, antigens included at the N-terminal stimulated a stronger $CD4^+$ T-cell response whereas the same antigens included at the C-terminal instead increased antibody production, showing that antigen position on the SAGEs can influence immune response. Another

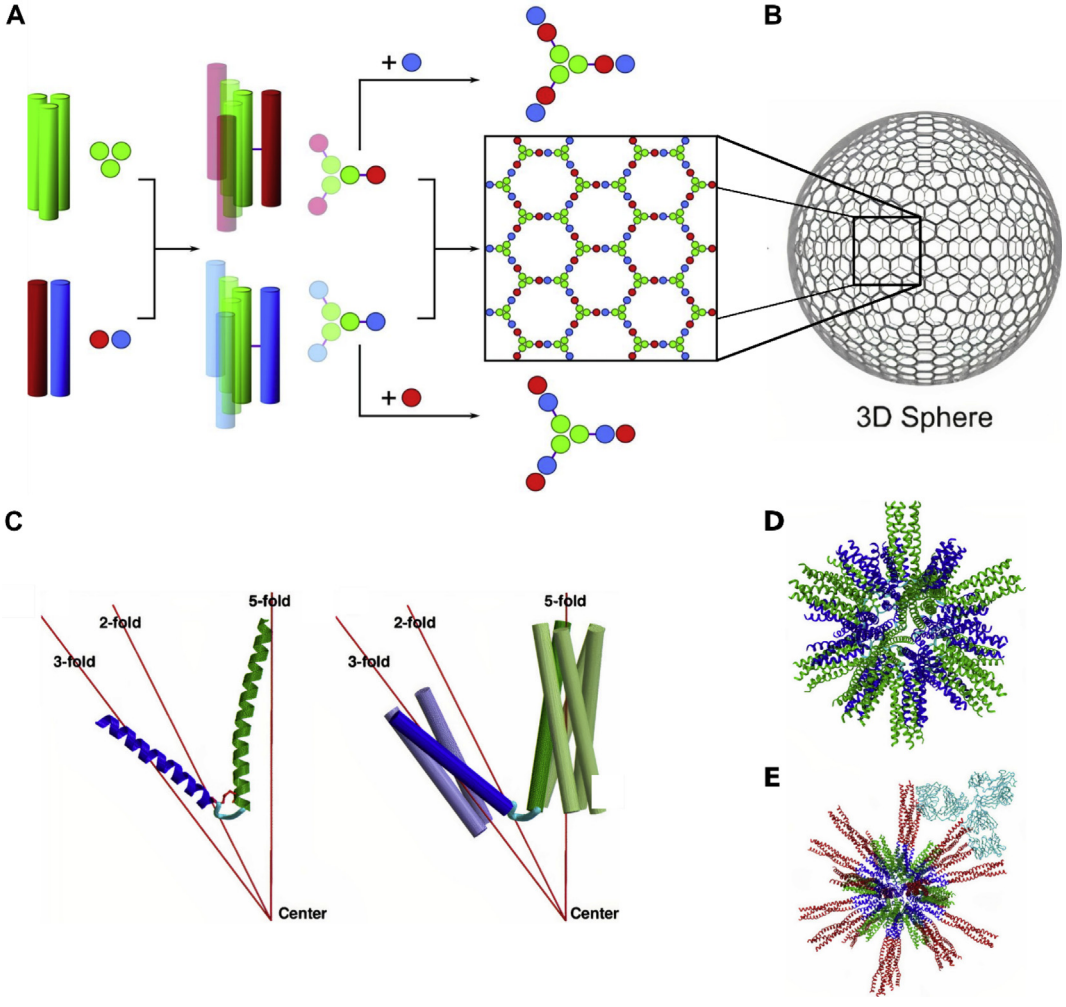


Fig. 5. Self-assembly of coiled coil nanoparticles. A) Homodimeric coiled coils (green) were linked *via* disulfide bonds to heterodimeric acidic (red) and basic (blue) coiled coils to form hub A and hub B, respectively. Mixing of hub A and hub B resulted in formation of a hexagonal network (B) that assembled into hollow spheres. Adopted with permission from [19], copyright 2013 the American Association for the Advancement of Science, and from [86], copyright 2018 American Chemical Society. C) A homotrimeric coiled coil domain (blue) connected *via* a linker segment (cyan), and a homopentameric coiled coil domain (green) 3D model is displayed. The interhelical disulfide bond is highlighted in red. D) A computer model of the self-assembled peptide nanoparticles composed of a pentameric coiled coil domain of cartilage oligomerization matrix protein (COMP) (green) and a *de novo* trimeric coiled coil domain (blue). E) A further extension to the trimeric coiled coil domain of the human immunodeficiency virus (HIV) surface protein gp41 coiled coil motif (red). The particle is comprised of many copies of the trimeric coiled coil domains containing the extension that can be recognized by an antibody (cyan), which induces a strong immune response. Adopted with permission from [84], copyright 2006 Elsevier inc.

interesting advantage with this approach is the possibility to display multiple antigens on the same nanoparticle by mixing different ratios and kinds of antigen presenting peptides in order to increase the immunogenicity [88]. More research is needed before the SAGE vaccine candidate can find clinical applications, but the results are encouraging.

A similar approach for antigen display has been investigated by the group of Peter Burkhard, using a set of novel self-assembling protein nanoparticles (SAPNs) formed by combining coiled coil oligomerization domains [84]. This structure consists of a pentameric coiled coil domain based on COMP, which was slightly modified and combined with a *de novo* designed minimal trimeric coiled coil domain *via* two glycine residues. The two helices were also stabilized by a disulphide bonds between two Cys residues at position *f* in the heptad repeat, one turn away from the end of each helix (Fig. 5C). The folding of the peptides resulted in self-assembly of 16 nm nanoparticle consisting of 60 monomeric building blocks with a regular polyhedral symmetry [84,89], (Fig. 5D). Similar to the SVLPs, the SAPNs also resembles viruses with respect to shape and size and can also be functionalized with antigens, resulting in beneficial repetitive antigen display [78] (Fig. 5E). SAPNs have shown immunization efficacy in animal studies for a variety of diseases, including malaria [90], HIV [91] and influenza [92].

Since both the specificity and affinity for dimerization and the preference for relative helix orientation into either parallel or anti-parallel orientation can be tuned, coiled coil interactions can be used for programming the self-assembly process of modular nanoscale constructs with highly defined structural properties, similar to DNA origami. The group of Roman Jerala pioneered the work of coiled coil protein origami (CCPO) by designing and producing a long single-chain polypeptide that self-assembled into a polyhedra [20], (Fig. 6). The polypeptides were expressed in *E. coli* and was comprised of no less than 12 concatenated orthogonal hetero- and homomeric coiled coil forming segments. The CCPO structures assembled through multiple cooperative and long-range interactions between complementary modules located far apart in the primary structure, leading to dimerization between complementary sequences. The resulting tetrahedron structure was comprised of six different rod-like edges of paired coiled coils and had a cavity in the core [20]. The approach was later further refined in order to overcome the poor solubility of the peptides and difficult refolding procedure of the structures seen in the initial work [93]. To increase the solubility of the peptides and the CCPO structures, the number of charged residues at non-interacting positions (*b*, *c* and *f*), was increased. The approach enabled self-assembly of more complex structures that could form in bacteria and mammalian cells as well as in live mice without any observed adverse host reactions, indicating a potential for applications in drug delivery and vaccine development [93], (Fig. 6G, H).

The single-chain design used in these CCPOs is powerful but relies entirely on intramolecular interactions, which can limit the number of possible nanostructures that can be fabricated. Intermolecular and modular self-assembly of well-defined origami-like structures using multiple polypeptides is possible but has so far only been demonstrated using 3–4 coiled coil heterodimers, resulting in comparatively simple geometries, such as squares and triangles [85,94]. Additional strategies to generate discrete coiled coil nanoparticles and nanostructures are discussed in a recent review by Park [83].

3.4. Coiled coil nanocarriers

The N-terminal heptad repeat region of COMP has inspired designing of several coiled coil structures of interest for drug delivery [95,96]. Guo et al. observed that the coiled coil domain of COMP could bind to vitamin A and vitamin D3 and a number of signaling molecules and suggested that this molecule could be used in delivery applications [95]. Later other groups tried to use this protein as a carrier for saturated and unsaturated fatty acids [97] and the polyphenolic compound curcumin [11], and to deliver an inverse agonist for osteoarthritis [98]. Montclare and *co*-workers have explored different possible applications of COMP and have for example generated protein block *co*-polymers by combining two domains of COMP and an elastin like protein (ELP) for self-assembly of temperature responsive systems that retained the small molecules binding capacity of COMP [99]. This protein block co-polymer was further used for assembly of fluorinated micelles for encapsulating the chemotherapeutic DOX [100]. The drug was released at elevated temperature. In addition, the micelles could be traced using fluorine-19 (^{19}F) magnetic resonance imaging (MRI) and ^{19}F magnetic resonance spectroscopy (MRS), demonstrating a strategy for development of F-MRI/MRS-traceable theranostics.

In addition to COMP, the right-handed coiled coil domain (RHCC) is another natural derived coiled coil structure that has been explored as a drug carrier. RHCC forms a tetrameric coiled coil structure with four large internal cavities that can bind metals, including gold and platinum [101]. Eriksson et al. used this nano-sized structure as a carrier to deliver platinum containing chemotherapeutic drug cisplatin to cancer cells. They observed that RHCC was able to bind to and enter cells *in vitro*. However, the uptake was not fast enough to be applicable for *in vivo* drug delivery. They also showed that RHCC was not severely toxic or immunogenic in mice [101]. Unfortunately, the short half-life of cisplatin loaded RHCC (CP-RHCC) and difficulties with targeting the intended glioblastoma (GB) cells, made the therapeutic application of CP-RHCC difficult to realize [102]. To overcome this challenge, Thanasupawat et al. investigated the possibility to instead use the Pt(IV) prodrug, with encouraging results. Tests *in vitro* and *in vivo* showed that Pt (IV)-RCHH could induce apoptosis of GB cells at a five-fold lower Pt (IV) concentration that the pro-drug alone while showing no effects on astrocytes [102].

In another attempt to combine coiled coils with ELPs, Park and Champion demonstrated the possibility to assemble vesicle-like structures using recombinant arginine-rich (Z_R) and glutamic acid-rich (Z_E) leucine zipper motifs fused with an ELP motif or a globular protein, respectively (Fig. 7). Z_R forms homodimeric coiled coils with relatively weak affinity ($K_d \approx 10^{-7}$ M) at room temperature and the fusion protein form a temperature-responsive "rod-coil" protein amphiphile when allowed to dimerize. In addition, Z_R can heterodimerize and fold into coiled coils with the complementary Z_E polypeptide with an affinity in the picomolar range. Using fusion proteins of Z_E and the globular fluorescent proteins mCherry and EGFP [103], vesicles were formed that could be used for encapsulation of small molecules, proteins and nanoparticles. Moreover, by tuning the protein concentration and temperature, vesicles with different size and single or bi-layer vesicles could be produced [104]. The effects of net-charge and size of the globular proteins fused to the Z_E peptide were systematically investigated and revealed critical parameters that influenced vesicle size and morphology, while also indicating the potential of these structures in biocatalysis and biosensing [105].

Coiled coil-ELP fusion proteins have also been functionalized with growth factors for self-assembly of nanoparticles targeting growth factor receptors [106]. ELP with a poly(aspartic acid) tail (ELP-D) and the growth factor were genetically fused with the ACID-p1 (helix A) and BASE-p1 (helix B) polypeptides, respectively. Helix A and helix B were originally designed by O'Shea et al. to fold into parallel coiled coil heterodimers [46,107]. In order to create a drug delivery system targeting the vascular endothelial growth factor (VEGF) receptor of cancer cells, helix B was fused with the single-chain VEGF (scVEGF121). When combined with helix A fused to ELP-D nanoparticles with a core diameter of about 30 nm were assembled, that enabled encapsulation of the chemotherapeutic drug paclitaxel by heat treatment. Paclitaxel-loaded protein nanoparticles induced cell death in HeLa cells *in vitro*.

3.5. Coiled coil polymer-hybrids

Coiled coils conjugated to hydrophilic synthetic polymers comprise versatile hybrid systems for drug delivery and have been investigated

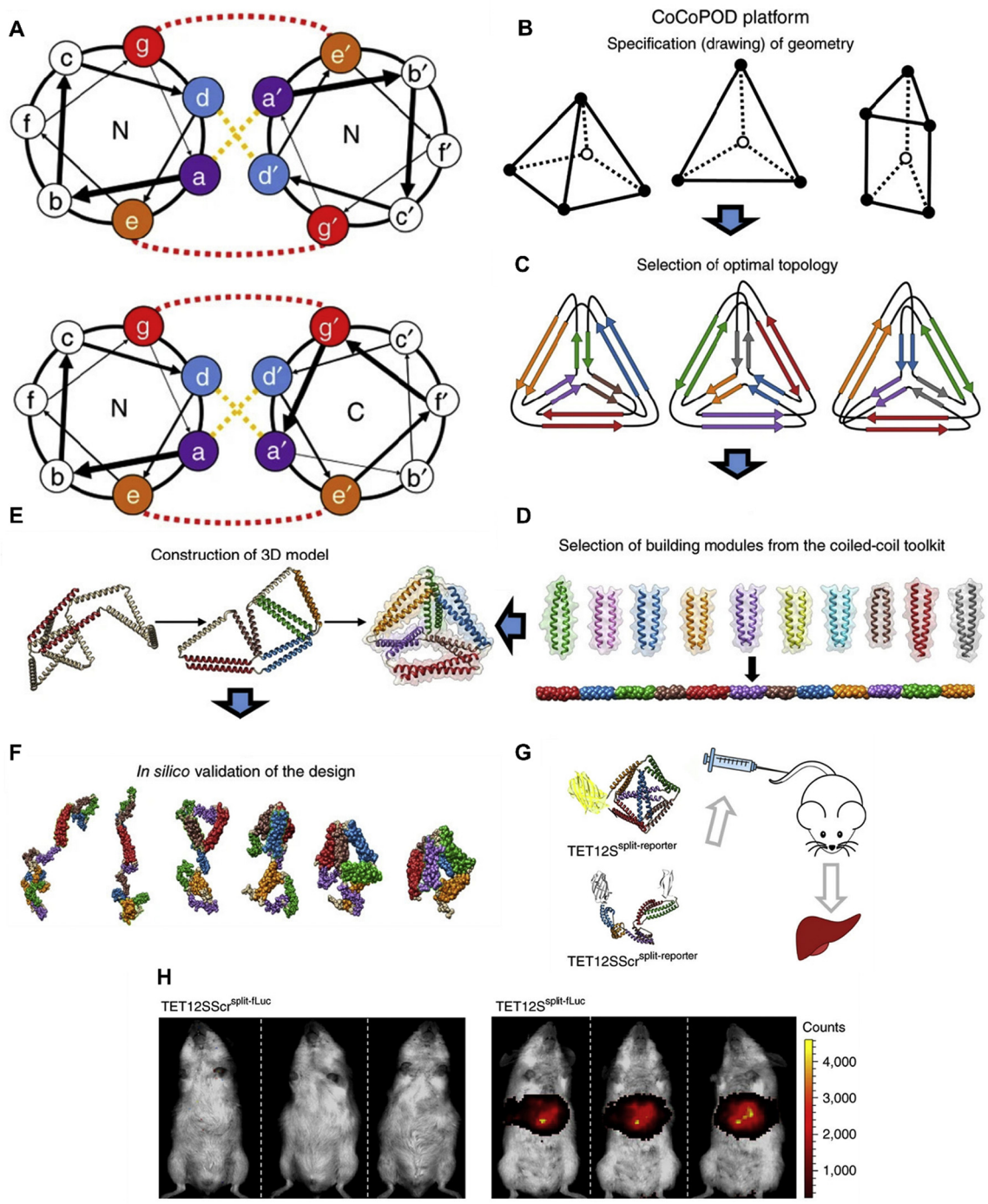


Fig. 6. Coiled coil peptide origami. Multiple *de novo* designed parallel and antiparallel hetero- and homodimeric coiled coils were combined into a single polypeptide sequence for assembly of a peptide origami tetrahedron. A) Helical wheel diagram of a coiled coil with parallel orientation (top) and antiparallel orientation (bottom) used in the design of the origami structures. B) The initial step in the design of the origami structure involves decision of the desired geometry. C) Possible topologies resulting in the desired geometry were computed and all of the circular permutations possible for a topology were examined to identify the one with lowest topological contact order. D) Suitable building modules were determined based on the developed coiled coil tool kit. These coiled coil modules were mapped to the abstract topology identified by graph theory. E) Different all-atom 3D models were used for calculating volume, contact order, and solvent accessible surface. F) All designed coiled coils were further evaluated by coarse-grained structure-based folding simulations. G) To investigate folding of tetrahedrons *in vivo*, the plasmid encoding TET12S$^{split\text{-}fLuc}$ containing subdomains of a split-fluorescent protein that were fused to N- and C-terminus of TET12S, was delivered to the livers of mice by hydrodynamic delivery. H) The level of bioluminescence in mice that produced TET12S$^{split\text{-}fLuc}$ (right) was compared to mice expressing the scrambled version of the protein (left). Adopted with permission from [93], copyright 2017, Springer Nature.

in a wide range of configurations to enhance drug uptake or to promote controlled release. Conjugation of both conventional drugs and biotherapeutics to synthetic polymer carriers can improve the efficacy and half-life of the therapeutic compounds [108] and increase tumor accumulation [109]. Klok and co-workers used the *de novo* designed parallel heterodimeric coiled coil E3/K3 [36] as a non-covalent linker for conjugating drugs to a polymer carrier in order to create a universal strategy for polymer-drug conjugation [110]. The E3/K3 heterodimer is stable at physiological pH but start to unfold at pH 5 as a result of formation of E3 homotrimers [22], opening up for potential release of the drug

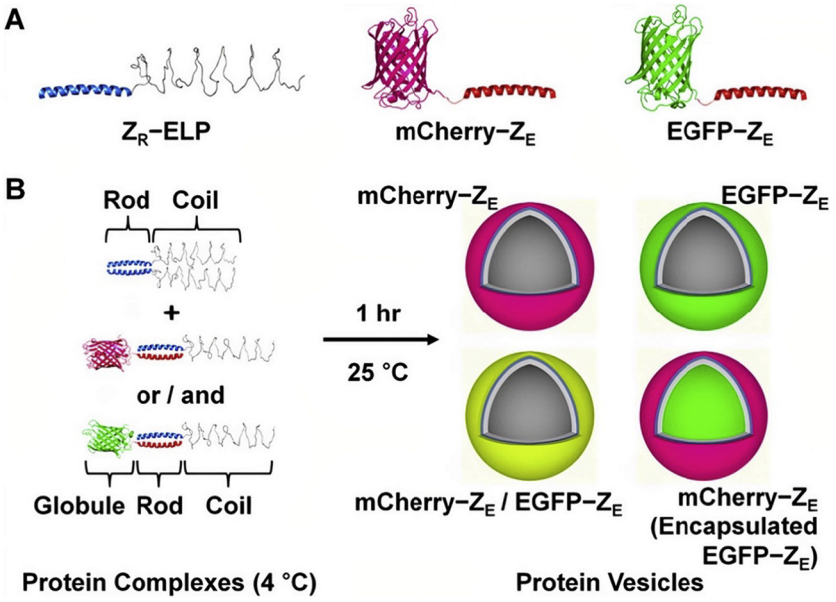


Fig. 7. Self-assembly of ELP-coiled coils hybrid nanoparticles. A) Schematic illustration of the structure of the recombinant diblock copolypeptides used for self-assembly of the nanocarriers (PDB_ID mCherry: 2H5Q and PDB_ID EGFP: 1EMK). B) Solutions of ZR-ELP and globule-rod-coil (mCherry-ZE/ZR-ELP and EGFP-ZE/ZR-ELP) were prepared at 4 °C and upon mixing and incubation at 25°C self-assembled into hollow vesicles. The formation of a coacervate phase of EGFP-ZE encapsulated by mCherry -ZE vesicles is possible in some conditions. Reproduced with permission from [102], copyright 2014 American Chemical Society.

once the complex has been internalized by endocytosis and enters the acidic endosomal and lysosomal compartments. The polymer, N-(2-Hydroxypropyl)methacrylamide (HPMA), was functionalized with either of the two peptides. The polymer-peptide hybrid was subsequently exposed to the drug, tethered to the complementary peptide, resulting in a supramolecular complexation of the drug to the polymer. *In vitro* experiments indicated cellular uptake and that intracellular unfolding, and not enzymatic degradation, of E3/K3 was responsible for drug release [110], and that some of the polymer-drug conjugate was transported to the lysosomes [111].

Gormley et al. used coiled coil-polymer hybrids for fabrication of enzyme responsive microcapsules [112]. The helix-loop-helix polypeptides JR2EC and JR2KC [113], designed to heterodimerize and fold into a four-helix bundle, were conjugated to a N-(2-hydroxypropyl)methacrylamide-*co*-N-(3-aminopropyl)methacrylamide (HPMA-co-APMA) co-polymer backbone. The sequential deposition of the JR2EC and JR2KC functionalized polymers on mesoporous silica particles, driven by peptide heterodimerization and folding, resulted in formation of layer-by-layer (LBL) structures that were retained when the silica core was dissolved. A macromolecular model drug was encapsulated in the microcapsules by first loading it into the mesoporous silica particles prior the assembly of the LBL film. After the LBL deposition process the silica particles were dissolved, resulting in peptide-polymer hybrid capsules encapsulating the drugs. The sequence of JR2EC contains two cleavage sites recognized by matrix metalloproteinase 7 (MMP-7), an enzyme overexpressed in many tumors. Cleavage of JR2EC by MMP-7 disrupt its ability to dimerize with JR2KC, which resulted in destabilization of the capsules and release of the encapsulated cargo.

Coiled coil-based therapies do not necessarily have to involve drug molecules but can instead promote a therapeutic effect by modulating a cellular or physiological response by means of specific biorecognition events. This strategy has been extensively explored by the group of Kopeček for development of novel drug-free macromolecular therapeutics (DFMT), based on coiled coil biorecognition [114–117]. Their work has primarily focused on the therapeutically relevant biomolecular target B-lymphocyte antigen CD20, which is a membrane protein expressed on the surface of differentiating B-cells and often found in non-Hodgkin's lymphomas [118]. CD20 remains unchanged on the cell surface upon antibody binding [119], thus providing a potential platform for targeted therapeutics. The anti-CD20 antibody drug Rituximab has been approved for treatment of some B-cell originated lymphoid malignancies [120]. Binding of Rituximab to the target antigen triggers a cross-linking of CD20 that can initiate apoptosis of the cells [120–122]. Kopeček and co-workers have investigated means to mimic this process by triggering CD20 cross-linking to induce apoptosis in B-cells using coiled coil heterodimerization (Fig. 8). The two complementary pentaheptad peptides CCE and CCK are random coil as monomers but fold into an antiparallel coiled coil upon heterodimerization. CCE was conjugated to the Fab' fragment of the 1F5 antibody (Fab'-CCE), a murine anti-CD20 IgG2a, while CCK was conjugated to a HPMA polymer ((CCK)9-P). Conjugation did not prevent folding into coiled coils as demonstrated by circular dichroism (CD) and dynamic light scattering (DLS). Raji cells were used to validate the *in vitro* function, showing that Fab'-CCE and pre-mixed Fab'-CCE and (CCK)9-P could decorate the cell surface whereas (CCK)9-P alone could not. Interestingly, the cells underwent apoptosis after consecutive treatment with Fab'-CCE followed by (CCK)9-P or a pre-mixed solution of both [114]. Further work by Wu et al. demonstrated the *in vivo* efficacy, where three doses of either consecutive or pre-mixed Fab'-CCE and (CCK)9-P completely removed all traces of malignant B-cells in the bone marrow of a mouse model bearing human B-lymphoma [115]. Zhang et al. used multiple fluorescence imaging techniques to study the cell surface assembly of the system, finding that the CD20 cross-linking initiated lipid raft formation and also discovered that consecutive treatment was more specific and showed less nonspecific toxicity than pre-mixed peptide-hybrids [116]. This DFMT system has also been evaluated using human serum albumin (HSA) instead of a synthetic polymer (Fig. 8) [117], in an effort to facilitate translation into clinical applications [123]. Similar to the peptide-polymer hybrids, peptides conjugated to HSA could induce receptor dimerization resulting in apoptosis of Raji cells *in vitro* [117]. Additionally, substitution of the coiled coil biorecognition element by complementary morpholino oligonucleotides have shown great efficacy as a DFMT system [123].

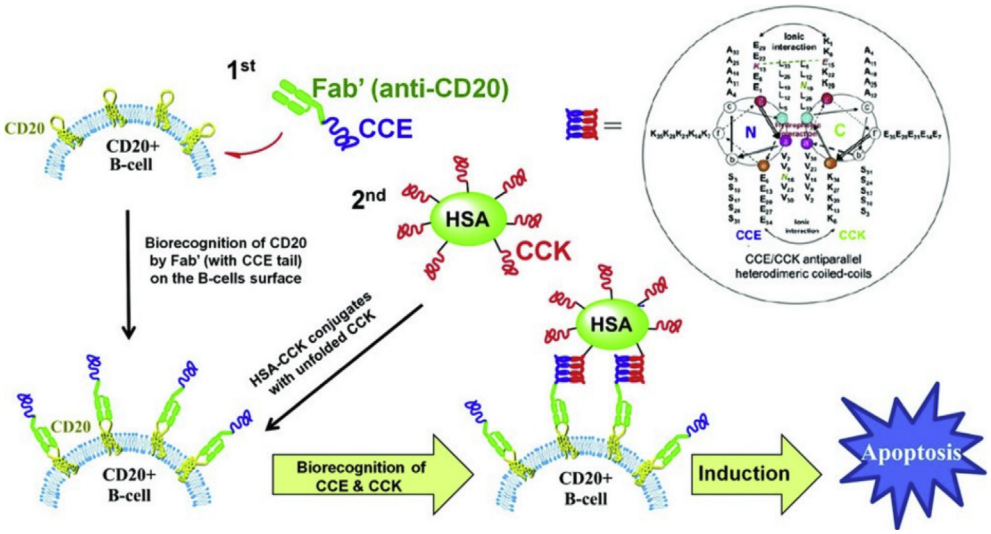


Fig. 8. Drug-free macromolecular therapeutics based on coiled coil heterodimerization. The random coil peptides CCE and CCK were conjugated to anti-CD20 Fab' fragments and human serum albumin (HSA), respectively. Binding of Fab'-CCE to CD20+ Raji cells resulted in accumulation of CCE peptides at the cell surface. Subsequent addition of HSA-CCK resulted in receptor clustering as a result of CCE/CCK heterodimerization and folding into antiparallel coiled coils. The coiled coil induced CD20 cross-linking triggered apoptosis of the cells. Reproduced with permission from [117], copyright 2018 WILEY-VCH Verlag GmbH & Co. KgaA, Weinheim.

3.6. Coiled coil-based hydrogels

Hydrogels are highly hydrated polymer networks that can mimic both physical and biochemical aspects of tissues, which make them very attractive for biomedical applications. A number of hydrogel drug delivery systems are in clinical use or in clinical trials for delivery of for example growth factors, peptides and antimicrobial compounds [124]. Coiled coil-polymer hybrids have been extensively investigated for both functionalization and self-assembly of hydrogels [13,125,126]. Tirell and co-workers pioneered this work by combining two recombinant leucine zipper domains, partly based on the Jun-oncogene product, separated by a flexible and water-soluble random coil polypeptide segment [14]. The association folding of the coiled coils resulted in self-assembly of a hydrated, pH and temperature responsive supramolecular polymer network. A related approach for self-assembly of hydrogels was soon thereafter presented by the group of Kopeček using a *de novo* designed coiled coil and a coiled coil derived from the motor protein kinesin tethered to a synthetic *co*-polymer *via* Ni^{2+} chelating groups [13]. The hydrogels were found to undergo temperature-induced collapse with a mid-point transition temperature of 39 °C as a result of unfolding of the coiled-coils. Several similar strategies using different coiled coils conjugated to various synthetic polymer and biopolymer backbones have later been investigated for fabrication of responsive hydrogels. The possibility to tune the interaction involved in the oligomerization and folding of the coiled coils provides direct molecular scale control over the properties of the resulting hydrogels. Along this line, Aronson et al. used a set of *de novo* designed coiled coils with different affinities for heterodimerization conjugated to 4-arm PEG for self-assembly of programmable hydrogels where the stiffness of the hydrogels was found to be dependent on the affinity for dimerization [15]. The bioorthogonal cross-linking of hydrogels offered by peptide dimerization can facilitate encapsulation and controlled release of protein-based therapeutics and bioactive factors. In addition, the possibility to encapsulate cells and retain high cell viability, make coiled coil-based hydrogels an interesting and dynamic scaffold for 3D cell culture. Because of the supramolecular nature of the interactions the hydrogels tend to be very soft, shear thinning and dissociate over time, which also make these materials attractive for delivery of cells and bioactive factors that are either sensitive to shear forces or benefit from a more protected environment during, or after, administration [127].

Zhang et al. used a heparin-binding coiled coil peptide, $PF4_{ZIP}$, derived from the heparin-binding domain of human platelet factor 4 for assembly of hydrogels [128]. $PF4_{ZIP}$ and low-molecular-heparin (LMWH) were conjugated to star shape poly(ethylene glycol) (PEG) and hydrogels where formed immediately after mixing the two polymer components. The presence of heparin in the hydrogels allowed for efficient encapsulation of basic fibroblast growth factor (bFGF). A stable complex was formed between heparin and bFGF with retained biological activity. After an initial rapid release of bFGF, the release rate correlated with the erosion of the hydrogel. Roth et al. explored a slightly different approach to encapsulate and release growth factors from hydrogels, using a heterodimeric coiled coil to control the release of growth factor functionalized gold nanoparticles [129]. Epidermal growth factor (EGF) was fused with a Glu-rich peptide (*E*-peptide) and conjugated to gold nanoparticles. The E-peptide was designed to heterodimerize and fold into heterodimeric coiled coils with a complementary Lys-rich peptide (K-peptide) immobilized in an alginate hydrogel. Alginate hydrogels functionalized with the K-peptides could bind and retain the EGF-E-peptide functionalized nanoparticles as a result of peptide dimerization and folding into coiled coils. Interestingly, the released EGF-modified nanoparticles could bind the EGF receptor (EGFR) in A431 cells, resulting in higher EGFR phosphorylation than EGF alone at comparable concentrations. Similar coiled coils were also explored for presentation and release compounds from two dimensional (2D) substrates. Crescenzo and co-workers used a set of heterodimeric coiled coils with the sequences $(EVSALEK)_5$ and $(KVSALKE)_5$, referred to as the E- and K-peptide, respectively [130–132] for this purpose. The E-peptide was used for tethering of E-tagged molecules to different K-decorated surfaces. They also investigated means to tune the stability and affinity of the E/K coiled coils using Leu to Ala mutations in the K peptide and by multivalent presentation of the E peptide in order to control the release rate of tethered biomolecules. By means of this technique they produced heterodimers with dissociation constants ranging from 0.1 pM to 270 nM [133].

Kobatake and co-workers used coiled coils for integrating different growth factors [134,135] and transcription factors [136] in an artificial extracellular matrix (ECM) to enable localized release to direct cell behavior. The artificial ECM was produced recombinantly and was comprised of two repeats of an elastin-derived sequence $(APGVGV)_{12}$, the cell adhesion sequences RGD and IKVAV, and a collagen-binding-domain (CBD). Moreover, the heterodimeric coiled coil forming peptide

helix B was fused to the artificial ECM whereas the complementary peptide, helix A, was fused to three different types of growth factors; bFGF, EGF and scVEGF121. The heterodimerization of helix A and helix B was used for the tethering the growth factors to the artificial ECM. The dissociation of the heterodimers resulted in controlled release of growth factors from the hydrogel, which promoted capillary-like formation and stimulated growth-promoting activity of human umbilical vein endothelial cells (HUVECs) cultured in the gels [134]. A similar strategy was investigated for the controlled release of the transcription factor Olig2, which was evaluated using mouse embryonic carcinoma P19 cells. The release of the tethered transcription factors resulted in internalization by the P19 cells as confirmed by immunostaining [136]. The possibility to introduce and release bioactive factors dynamically to cells cultured in these hydrogel scaffold enabled receptor internalization in a similar manner as in the native ECM [137].

Ding et al. used coiled coil heterodimers for supramolecular cross-linking of pH responsive hyaluronan (HA) based hydrogel nanoparticles formed by nanoprecipitation (Fig. 9) [138]. The resulting nanogels had a diameter of about 180 nm. The peptides, GY(EIAALEK)$_3$GC (E3) and GY(KIAALKE)$_3$GC (K3), were conjugated individually to HA as random coil monomers but folded into coiled coils upon mixing of the two peptide-HA components at pH 7.4. Lowering the pH value of the buffer to pH < 6 resulted in unfolding of the peptides and a substantial swelling of the nanogels. Cytochrome C (CC) could be efficiently loaded into the nanogels. Over 90% of the CC was released at pH 5 after 24 h whereas less than 20% was released at pH 7.4 over the same time period. MCF-7 human breast cancer cells overexpressing the HA-binding CD44 receptor showed a receptor mediated endocytic uptake of the CC loaded nanogels. The pH-mediated disruption and disassembly of the nanogels in the endosomes and subsequent release of free HA-E3 and HA-K3 was hypothesized to promote fusion with endosomal membranes and endosomal escape of the cargo (Fig. 9B). The possibility to deliver drugs was evaluated by loading the highly potent protein toxin saporin (SAP) in the nanogels, resulting in a significant decrease in viability of MCF-7 cells.

The possibility to combine the coiled coil domain of COMP and elastin into block-copolymers have been further investigated by Olsen et al. for self-assembly of thermoresponsive hydrogels and particles capable of binding curcumin [99,139]. By further modifying the elastin-like polypeptide motif domain in a COMP-elastin block-copolymer with the photocrosslinker diazirine, Montclare and co-workers were able to fabricate photopatterned free-standing hydrogels. The patterned hydrogel outperformed the non-photopatterned hydrogel both with respect to stability and adsorption and release of curcumin [140].

3.7. Coiled coil inorganic nanoparticle hybrids

Coiled coils conjugated to various types of inorganic nanoparticles have been widely explored for biomedical applications, primarily for imaging, diagnostics and other bioanalytical applications [141–143]. Yang et al. developed a combined imagining and drug delivery system by conjugating coiled coils to CdSe-ZnS core-shell quantum dots (QDs) [144]. The polypeptide, comprising of a coiled coil domain (P) and a leucine zipper domain (A) separated by a random coil midblock, was conjugated to the QDs trough specific metal-affinity interactions between an N-terminal polyhistidine sequence and the QD surface. The polypeptide both reduced the toxicity of the QDs and incorporated a drug delivery function to the nanoparticle. The resulting QD-polypeptide hybrids retained their QD-imaging properties while showing a pH and temperature responsive release of fluorescent model drugs. Both nonpolar and polar drugs could be loaded into the immobilized polypeptide matrix. *In vitro* experiments showed an enhanced cell uptake and delivery of a model drug when incorporating an RGD sequence at the C-terminal of the polypeptide.

Martelli et al. investigated the possibility to use heterodimeric coiled coils as thermoresponsive valves to control the release of drugs encapsulated in mesoporous silica nanoparticles (MSNs) [145]. The *E*-peptide, C(EIAALEK)$_3$, was conjugated to the MSNs *via* the N-terminal Cys residue. Heterodimerization with the complementary K-peptide, (KIAALKE)$_3$, resulted in formation of coiled coils on the surface of the nanoparticles that blocked the pores and prevented the release of an encapsulated model drug. The drug was released when increasing the temperature of the suspensions above the melting temperature (T_m) of the coiled coils as a result of peptide unfolding. Although the T_m of the particular peptide used here (80 °C) was higher than temperatures relevant for biomedical applications, the results indicate the possibility to tune release through defined coiled coil interactions. For drug delivery applications peptides with lower melting temperatures must be explored.

3.8. Coiled coils combined with cell penetrating peptides

Cell penetrating peptides (CPP) can traverse cell membranes and facilitate delivery of bioactive cargo into cells [146]. CPPs were discovered as result of an unexpected finding that transactivating transcriptional activator (TAT) from human immunodeficiency virus 1 (HIV-1) could traverse cell membranes and localize in the nuclei of the cells [147,148]. It was later found that a short sequence of the full-length protein, rich in Lys and Arg residues, was responsible for the cell penetrating properties of TAT [149]. Conjugation or fusion of TAT-derived

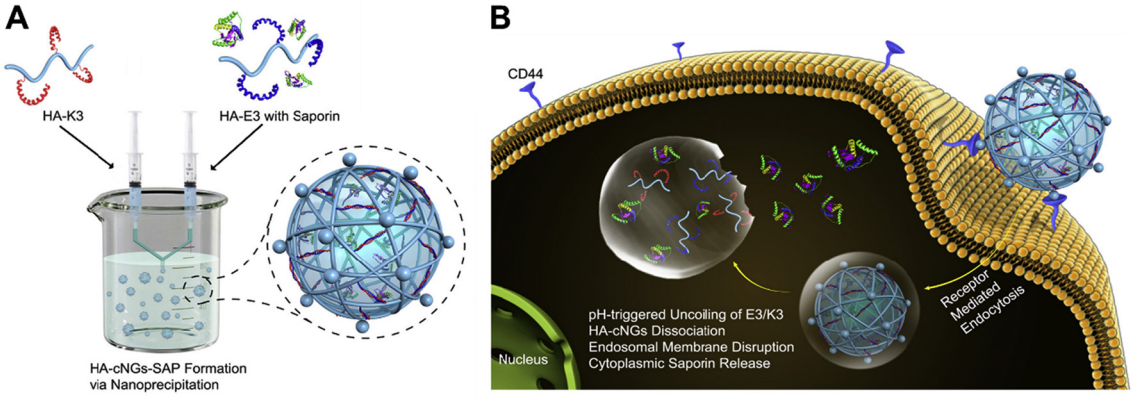


Fig. 9. Coiled coil polymer-hybrid nanogels. A) Strategy for preparing pH-sensitive hyaluronic acid nanogels cross-linked by heterodimeric coiled coil peptides. B) Mechanism for nanogel delivery of drugs into cells by endocytosis mediated internalization. Reproduced with permission from [138], copyright 2018 American Chemical Society.

peptides and many other CPPs, such as Arg-oligopeptides, to drugs and other bioactive factors have been extensively explored for drug delivery [150]. Coiled coils have been used to both improve the bioactivity of the conjugated drug and to control the activity of CPPs. To avoid interference between the positively charged CPP R11, a peptide comprised of eleven Arg residues, and a therapeutic peptide cargo, Kitamatsu and co-workers suggested to link the CPP and the cargo non-covalently using coiled coils [151]. The bioactive peptide was conjugated to the positively charged monomer Lz(K), whereas R11 was conjugated to the complementary Glu rich leucine zipper peptide Lz(E). Using this method, they successfully delivered the autophagy-inducing Beclin 1 peptide into U-251 MG cells without compromising the activity of the cargo. The same group also demonstrated possibilities to use this technology for delivery of larger cargos, such as the homebox protein Nanog, into cells [152]. After internalization of the cargo, Nanog was successfully transported into the nucleus of the cells and remained bioactive. To avoid possible steric hinderance between the CPP and Nanog the position of the CPP was altered from the N-terminus to the C-terminus. In later work, peptide nucleic acids were used instead of leucin zipper motifs to reduce the risk of non-specific interactions between the R11 modified coiled coils and the bioactive peptides [153]. To circumvent such problems, Bode et al. [154] explored alternatives to using a full length CPP. The CPP was divided in two tetra-Arg fragments that were anchored to the N-termini of two separate coiled-coil forming leucine-zipper peptides. Upon dimerization of the peptides in a parallel arrangement, the two CPP fragments were aligned in a geometry mimicking a longer oligo-Arg peptide, leading to activation of the CPP function. The affinities for heterodimerization had a considerable influence on the activation of the CPP. Whereas low affinity dimers needed to be covalently connected to function, coiled coils with a sufficiently high association constant ($K_a > 10^{11}\ M^{-1}$) efficiently penetrated cells.

3.9. Coiled coils for improved targeting

Therapeutic monoclonal antibodies (mAbs) can display high target specificity and affinity [155] and long circulation half-lives [156]. Therapeutic mAbs have improved the treatment of a wide range of diseases including cancer, autoimmune diseases and prevention of transplant rejections [157]. Despite the many benefits of mAbs, adverse side effects associated with the target antigen being expressed on non-target cells can result in for example cardiotoxicity and dermatitis [156,158]. One potential approach to reduce these problems is by masking the antibody through blocking of its antigen binding site with a peptide attached *via* a cleavable linker that can be cleaved by enzymes overexpressed in the target tissue, which will restore antibody activity [159,160]. Trang et al. generalized this strategy in order to be able to use the same mask for several different antibodies [161]. The mask consisted of a coiled coil domain genetically fused with the antibody N-termini through a MMP cleavable sequence, enabling activation in *e.g.* a tumor microenvironment. The coiled coil masking domain was first evaluated by testing different peptides with low- and high inter-coil affinity forming heterodimers with different relative orientations, and peptides covalently linked by disulphide bridges as well as helix-turn-helix homodimers (Fig. 10A). A coiled coil referred to as CC2B, forming parallel heterodimers with high affinity for dimerization, was found suitable for further testing. All four N-termini of the antibody were fused with CC2B peptides to ensure maximum inhibition of antigen binding. The concept was evaluated on different therapeutic mAbs including hBU12 rituximab, trastuzumab, h15H3, and 145-2C11. When masked using the CC2B domain, a decrease in binding affinity to the target antigen of about 1–3 orders of magnitude was observed for all mAbs using flow cytometry of antigen-positive cells. Antibody activity could be restored relatively close to the original affinities for all antibodies after being exposed to MMP-2 (Fig. 10B). *In vivo* experiments in a human CD19+ Ramos non-Hodgkin lymphoma xenograft tumor model showed that unmasked and CC2B masked hBU12 antibodies had similar activities while a masked antibody containing a non-cleavable MMP linker was less active, confirming the roles of both MMP and the cleavable linker in regulating the mAb antigen binding.

Conjugation of antibody fragments to drug carriers offers other ways to improve drug targeting. Pola et al. used a recombinant single-chain fragment (scFv) to improve the localization of a HPMA polymer carrying cytostatic drugs [162]. To prevent both loss of biological activity and the formation of non-well-defined products after scFv conjugation to the polymer, a supramolecular linker in the form of a heterodimeric coiled

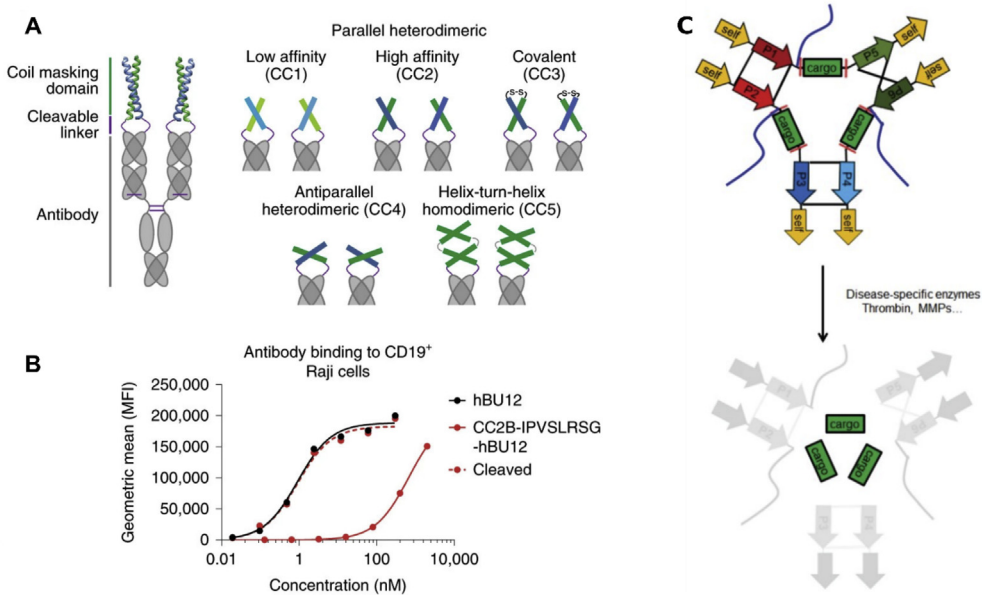


Fig. 10. Improved targeting by mAb masking and activatable protein nanoparticles. A) Schematic illustration of masking of mAb antigen binding sites using different classes of coiled coils fused to the mAb *via* cleavable linkers. B) Binding of mAbs to CD19+ Raji cells with the unmasked antibody hBU12, masked antibody CC2B-IPVSLRSG-hBU12 and pre-cleaved CC2B-IPVSLRSG-hBU12 by MMP-2. Adopted with permission from [161], copyright 2019, Springer Nature. C) Schematic illustration of self-assembling activatable protein nanoparticles and mechanism for triggering of cargo release by disease-specific enzymes. Adopted with permission from [167], copyright 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

coil was used. One of the peptides was conjugated to the scFv while the other peptide was conjugated to the polymer backbone [163]. In initial work, the peptides $(VAALEKE)_4$ and $(VAALKEK)_4$ were used [162,163] but since the two peptides can heterodimerize in both a parallel and an antiparallel orientation they were later exchanged for a heterodimeric coiled coil designed to specifically adopt an antiparallel orientation. This improvement enhanced cell binding efficiency of the polymer therapeutic, likely because of minimized steric hindrance [25,164]. The binding of the scFv to this peptide-polymer hybrid therapeutic resulted in about 100 times higher toxicity to cancer cells compared to the non-targeting polymer conjugates [162]. A similar effect was also observed *in vivo*, where an increased efficacy was obtained for the scFv modified polymers compared to the non-targeting polymer conjugate [164].

Therapeutic mAbs are primarily developed for binding extracellular targets. To improve the versatility of mAbs, Lim et al. developed a coiled coil-based strategy to deliver sufficient amounts of functional mAbs inside cells for targeting intracellular epitopes [165]. A *de novo* designed coiled coil peptide that self-assembled into a hexamer bundle [166] was used and fused with domain B of Protein A *via* a glycine-serine linker. The Protein A domain can bind to the Fc region of IgG with nanomolar affinities and each hexamer could hence be loaded with up to 6 antibodies. *In vitro* experiments using HeLA cells showed that functional antibodies could be delivered to cytosolic targets.

Coiled coil peptide structures can both improve targeting and reduce adverse effects caused by highly potent therapeutics. Yu et al. embedded therapeutic peptides that on their own demonstrated severe side effects in a rather sophisticated construct referred to as designed activatable protein nanoparticles (APNPs) [167]. The APNS consist of three polypeptide sequences where each polypeptide consists of at least one therapeutic peptide flanked by two coiled coil forming motifs. The polypeptides were further modified with a "self" peptide, i.e. a sequence designed to minimize phagocytic uptake of the nanoparticles in order to enhance delivery of the APNSs [168]. A Cys residue was included in each sequence for conjugation of PEG to improve stability and circulation time. Targeting peptides and enzyme-cleavable linkers were also inserted on both sides of the therapeutic peptide to promote drug release by endogenous proteases in the target tissue (Fig. 10C). The *in vivo* efficacy of the APNP system was demonstrated using two different therapeutic peptides, NR2B9c fused with a CPP and melittin for treatment of stroke and cancer, respectively.

4. Conclusions and future perspectives

The current transformation of the pharma industry towards development of precision medicines and biopharmaceuticals, including mAbs and gene- and cell-therapies, highlights the need of flexible and modular drug delivery platforms and improved targeting strategies as well as new materials that can facilitate efficient use of these new therapeutics. As outlined in this review, the structural and functional diversity of coiled coils have enabled development of a plethora of innovative and versatile drug delivery systems and novel therapeutic strategies. The use and function of coiled coils range from purely structural components, such as particles or hydrogels, to providing defined therapeutic actions by facilitating specific interactions with drug targets. All these systems have in common that they leverage on the modularity and programmability of the coiled coils for generating functional entities through the defined interactions involved in coiled coil oligomerization and folding.

The possibilities to use coiled coils that self-assemble into highly defined nanoparticles are attractive for generating bioresponsive and biodegradable drug carriers. The potential to use these particles for antigen presentation also comprise an interesting and highly modular vaccine development platform. The modularity and means to tune oligomerization state can be utilized for controlling both the number and multivalency of relevant antigens in order to tailor the immune responses. As highlighted by the SARS-CoV-19 pandemic, possibilities to facilitate rapid development of new vaccines are key for handling and preventing viral outbreaks.

Exploiting the modularity of coiled coils is also a reoccurring theme in development of strategies to improve drug targeting. Development of generic drug targeting strategies can be facilitated by attaching the active compound, *e.g.* a small molecular drug or a biologics, and the targeting moieties to separate peptides that can be combined by coiled coil formation. Coiled coils also offer means to combine multiple identical or complementary targeting moieties as larger supramolecular entities to achieve avidity effects that can improve drug accumulation and target specificity in comparison to the corresponding single individual target recognition motifs. Coiled coil oligomerization has also been explored as means to bring non-therapeutically active components into close contact, resulting in assembly of functional and bioactive complexes. By combining such strategies with targeting, unwanted side effects could potentially be drastically reduced.

The strategies developed to use coiled coils for triggered release leverages on the numerous ways to tailor the response of the peptides by differing relevant stimuli, such as pH, temperature and enzymatic cleavage. The response of the peptides when subject to stimuli must then be linked to a response in the delivery system used, for example a liposome or a hydrogel, which clearly can be challenging from a design perspective. Coiled coil formation is also often used as a means to retain the drug in a dynamic delivery system for prolonged time. Disruption of the oligomer and subsequent unfolding have been used to increase lipid membrane permeability, to dissociate a hydrogel or to release drugs that are conjugated to one of the peptides. Tethering of drugs to macromolecules or particle carriers using coiled coils provides flexibility and prevent steric hindrance that can occur between the delivery vehicle and the drug target when using covalent linkers. In addition, the drug release rate will be directly related to the affinities for dimerization which can be tailored over a quite large range. However, the activity of the drug can be influenced by the conjugated peptide and the need to modify the drug can also be complicated from a regulatory perspective.

Despite much promising research, there is currently to our knowledge only one designed coiled coil based drug/vaccine candidate undergoing clinical trials [169], highlighting the need to also increase efforts and work towards clinical translation. In addition, large scale peptide synthesis and recombinant protein and peptide production can be expensive and complex. However, the increasing interest in biopharmaceuticals, including peptide-based drugs, generates know-how and spurs development of infrastructure and facilities for large scale GMP-peptide production as well as increasing awareness of regulatory aspects. In comparison to other techniques for generating defined and programmable molecular materials and nanostructures, such as DNA origami, methods for industrial polypeptide production are mature and relatively cost-effective.

Considering the biological importance of coiled coil motifs, the modularity of the systems that can be developed, and our rapidly increasing understanding of coiled coil design rules and improvements in techniques for synthesis and bioproduction of advanced coiled coil-based structures, we expect that the field will continue to thrive and contribute to development of better treatments in the future.

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