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Elastin-like polypeptide-based micelles as a promising platform in nanomedicine

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ABSTRACT

New and improved nanomaterials are constantly being developed for biomedical purposes. Nanomaterials based on elastin-like polypeptides (ELPs) have increasingly shown potential over the past two decades. These polymers are artificial proteins of which the design is based on human tropoelastin. Due to this similarity, ELP-based nanomaterials are biodegradable and therefore well suited to drug delivery. The assembly of ELP molecules into nanoparticles spontaneously occurs at temperatures above a transition temperature (T_t). The ELP sequence influences both the T_t and the physicochemical properties of the assembled nanomaterial. Nanoparticles with desired properties can hence be designed by choosing the appropriate sequence. A promising class of ELP nanoparticles are micelles assembled from amphiphilic ELP diblock copolymers. Such micelles are generally uniform and well defined. Furthermore, site-specific attachment of cargo to the hydrophobic block results in micelles with the cargo shielded inside their core, while conjugation to the hydrophilic block causes the cargo to reside in the corona where it is available for interactions. Such control over particle design is one of the main contributing factors for the potential of ELP-based micelles as a drug delivery system. Additionally, the micelles are easily loaded with protein or peptide-based cargo by expressing it as a fusion protein. Small molecule drugs and other cargo types can be either covalently conjugated to ELP domains or physically entrapped inside the micelle core. This review aims to give an overview of ELP-based micelles and their applications in nanomedicine.

1. Introduction

Nanomedicine—the development and application of nanoscale drug delivery systems—has a revolutionary impact on the treatment of many diseases, since it offers a large toolbox for site-specific and/or time-controlled delivery of therapeutic agents [1,2]. Nanoparticle-based drug delivery systems can be rationally designed with different compositions, sizes, shapes, and surface properties, in order to be effective and safe [3,4]. Hydrophilic and hydrophobic drugs can either be incorporated into the particle core or attached to the particle surface. In addition, nanoparticles protect cargos from degradation or clearance, leading to prolonged blood circulation time. Due to their small size (10–100 nm), nanoparticles can penetrate through biological barriers, and conjugation with site-specific ligands allows nanoparticles to target tissues or cells where cargo can be released in a controlled manner, while minimizing drug-related side effects [5–7].

Different materials such as polymers, lipids, polysaccharides, and proteins are used for the preparation of nanoparticle drug delivery systems. In particular, elastin-like polypeptides (ELPs), a new class of protein-based biomaterials, have recently gained considerable attention over other variants of materials owing to their unique ability to reversibly self-assemble into a variety of architectures, including micelles, in response to temperature changes [8–10]. Furthermore, ELPs have been proven to be non-immunogenic, biocompatible and biodegradable, and they can be designed to carry and deliver biologically active molecules; as a consequence, they have been successfully employed in a wide range of biomedical applications, including drug and vaccine delivery [11,12].

The potential use of ELPs in nanomedicine has been extensively reviewed by others [8,10–12]; however, none of these reviews specifically cover ELP micelles. The present article reviews the recent developments in engineering ELP micelles for drug delivery applications, because of their well-defined character and control over cargo loading. First, the ELP micelles display monodispersed size distributions and

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particle diameters ranging from 30 to 60 nm, matching the expected size for micelles assembled from polypeptides [13,14]. Second, the positioning of both termini is restricted to the core or corona, enabling cargo to be either properly shielded or completely accessible to interact with its biological environment. Third, the cargo density is easily controlled by changing the ratio of different (functionalized) ELPs. Fourth, the physicochemical properties of ELP can be designed as well as the temperature at which assembly occurs [14]. Fifth, ELPs can be functionalized with various drug molecules, targeting moieties, antigens and molecular adjuvants. Finally, like all ELP-based materials ELP micelles are biocompatible, non-immunogenic and easily produced [9,10].

2. Elastin-like polypeptides (ELPs)

Elastin is an intrinsically disordered protein present in the extracellular matrix as insoluble fibers. Its main function is to confer the mechanical properties of elasticity and resilience to biological connective tissues [15,16]. Elastin fibers are formed through self-assembly and cross-linking of tropoelastin, a water-soluble monomeric protein that contains an alternating arrangement of hydrophobic and hydrophilic domains [17]. The hydrophobic domains are characterized by repetitive valine-proline-glycine-valine-glycine motifs that enable tropoelastin to self-assemble into highly ordered structures called "coacervates" in response to temperature increase; while the hydrophilic domains rich in lysine and alanine stabilize the mature elastin structure by forming covalent cross-links [15,17,18].

The temperature-dependent self-assembling property of tropoelastin's hydrophobic domains has attracted a lot of research interest, and several elastin-based materials known as ELPs have been constructed in order to obtain a new class of thermo-responsive protein polymers, which are being used in various biomedical applications. ELPs are generally composed of a pentapeptide repeat unit of valine-proline-glycine-X-glycine (VPGXG)n where X—the "guest" residue—can be replaced by any amino acid with the exception of proline, and n is the number of pentapeptide repeat units [19–21]. Like tropoelastin, ELPs in an aqueous environment undergo a reversible transparent-to-cloudy phase transition when temperature becomes higher than a characteristic temperature usually called the transition temperature (T_t) [22].

2.1. Temperature-triggered self-assembly of ELPs

From the structural point of view, ELPs are water-soluble at temperatures below their T_t and prefer a disordered random coil state [23]. Hydration of the hydrophobic ELP molecules induces an arrangement of water molecules in a specific ordered structure formed by hydrogen-bound networks [24,25]. In contrast, at temperatures above their T_t, ELPs fold into type II β-turn spirals because of dehydration of the hydrophobic ELP molecules that occurs due to molecular agitation caused by an increase in temperature, which in turn leads to hydrogen-bound disruption and breakdown of ordered water. As a consequence, ELPs aggregate into coacervates driven by hydrophobic interactions (Fig. 1) [25–27]. This coacervation process is reversible and lowering the temperature below the T_t results in complete disassembly into fully soluble unimers. On the other hand, the reversible phase transition of ELPs takes place in a narrow temperature range of 2–3 °C, and it is greatly influenced by the composition, molecular weight, and concentration of the ELP (intrinsic parameters) and medium concentration, ionic strength, pH (extrinsic parameters); therefore, the T_t of ELPs can be precisely tuned by controlling these parameters [28,29].

In addition to experimental studies, computational methods such as molecular dynamic (MD) simulations have been used to gain insights into the molecular basis of the thermo-responsive behavior of ELPs [30,31]. MD simulations is also a powerful tool for rational or knowledge-based design of *de novo* ELPs as it can predict the molecular structure-thermal behavior of ELPs with accuracy similar to experiments, allowing to know which ELP sequences might exhibit the desired properties, leading to a significant reduction of the number of trials. Furthermore, advanced sampling methods such as replica exchange molecular dynamics (REMD) can be used to speed up the sampling of any type of simulation, which in turn can accelerate the screening of ELPs [32]. Moreover, the combination of molecular modeling with *in silico* methods such as quantitative structure-activity and structure-property relationships (QSAR and QSPR) is an important strategy for making accurate predictions of every change made to proteins [33]. As a proof of concept, López Barreiro et al., developed and validated a computational framework to predict the thermo-responsive behavior of a library of eight *de novo* ELPs, which contained 48 repeats of the VPGVG sequence with varying numbers and locations of hydrophobic/hydrophilic and physical/chemical-crosslinking blocks [34]. As a result, computational approaches open the possibility to design and fabricate new ELP-based materials with desired properties, as a larger number of ELP sequences can be explored before their synthesis.

2.2. Synthesis of ELPs

ELPs are synthesized in two major approaches including chemical methods and recombinant DNA techniques. The chemical approach *via* solid-phase peptide synthesis provides the possibility to obtain synthetic ELPs by employing either natural or unnatural amino acids as monomers [19,25,35,36]. This approach also allows facile introduction of synthetic molecules such as drugs, dyes, and polymers in the ELPs in order to produce multifunctional hybrid materials [37,38]. However, chain

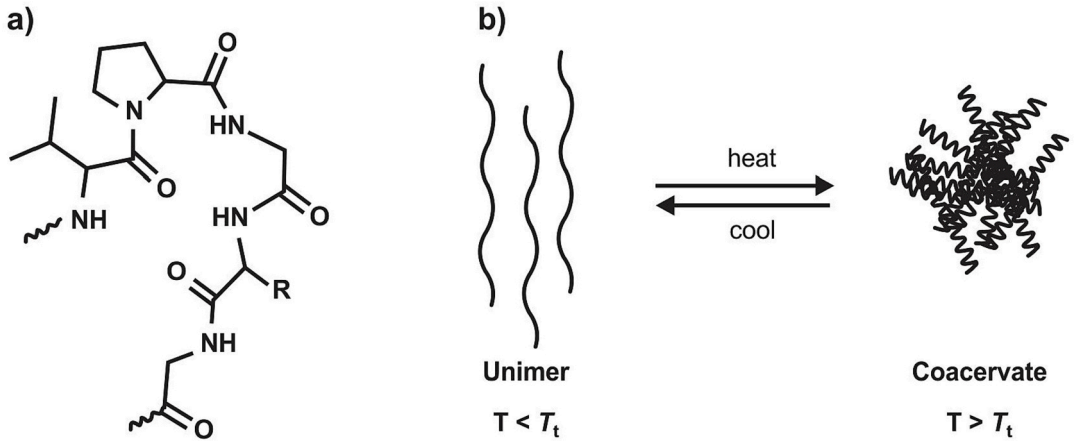

Fig. 1. Inverse temperature transition of ELPs. a) ELPs assemble by forming a type II β turn in the repeating motifs of the backbone. The side group of the guest residue is denoted "R". b) The ELPs are soluble at temperatures below Tt and aggregate into coacervates at temperatures above Tt.

length and stereochemistry are difficult to control [39]. Genetic engineering through recombinant DNA technology appears to be the most convenient method for the synthesis of larger ELP polymers with specified molecular weights, avoiding stereochemical problems [39,40]. The production of genetically encoded ELPs typically comprises three main stages: 1) synthesis of a DNA fragment encoding the desired ELP sequence. 2) insertion of this DNA fragment into a plasmid in order to create a recombinant DNA molecule; and 3) introduction of the recombinant DNA into a host cell (*e.g.*, *E. coli*), where the ELP is produced. Concatemerization and recursive directional ligation methods are commonly used to construct genes that encode ELPs [8,40]. Since repetitive genes are difficult to replicate with PCR, these ELP genes are carefully designed with different combinations of codons encoding for the same repeating pentapeptide [41]. This 'codon scrambling' technique makes the DNA sequence much less repetitive than the resulting polypeptide sequence. By genetic engineering, it is possible to create ELPs with a variety of architectures such as mono-, di-, tri-block copolymers that can be further conjugated with functional peptide/protein motifs [11].

The chemical diversity of ELPs synthesized by recombinant DNA techniques is often limited to the use of canonical amino acid sequences. Noncanonical amino acids are particularly attractive for ELP modification as they can provide distinct bioorthogonal reactive groups [42]. In this sense, expansion of the genetic code of cells represents a versatile strategy to introduce noncanonical amino acids into ELPs. For example, expression of recombinant polypeptides containing noncanonical amino acids can be achieved by using an auxotrophic host strain, where a noncanonical amino acid replaces the corresponding canonical amino acid at all cognate codons [43]. However, the site-specific incorporation of the noncanonical amino acids is not possible using this approach. Suppression of translation stop codons—ochre (UAA), opal (UGA) or amber (UAG)—with an orthogonal aminoacetyl tRNA synthetase/suppressor tRNA pair enables the translational incorporation of noncanonical amino acids in a site-specific manner [43–45]. In this way, Wu et al. were able to incorporate noncanonical amino acids into recombinant ELPs by using the *E.coli* MRA30, an auxotrophic host strain that allows the suppression of amber codons and an orthogonal aminoacyl-tRNA synthetase that is able to charge noncanonical amino acids to the suppressor tRNA [46,47]. This allowed the incorporation of multiple noncanonical amino acids at specific positions within the ELP sequence [46]. On the other hand, Amiram et al. developed novel aminoacyl-tRNA synthetases with tuneable specificities for a variety of noncanonical amino acids [48]. These authors demonstrated the incorporation of 30 noncanonical amino acids per ELP sequence with high incorporation accuracy and high yield production of the corresponding recombinant protein.

Finally, post-translational modifications—enzymatic modifications of proteins after their biosynthesis—are another orthogonal strategy to diversify the ELP design [49,50]. Chilkoti et al. reported for first time the production of lipid-ELP hybrid conjugates using a combination of recombinant expression and post-translational modifications, which allows to functionalize ELPs with either cholesterol [50] or myristic acid [51].

2.3. Purification of ELPs

ELPs are commonly purified by using an inverse transition cycling (ITC) technique, since it takes advantage of the thermal transition behavior of ELPs [9,52,53]. As depicted in Fig. 2, ITC involves consecutive cycles consisting of a so-called "hot spin" and a "cold spin". The hot spin involves adding salt to lower the LCST and incubation at room temperature. This causes the ELPs to form aggregates, which are collected by centrifugation. The cold spin entails resuspending these aggregates in cold, low-salt buffer. Under these conditions the ELPs redissolve, while other proteins remain aggregated and are removed by centrifugation. Depending on the sequence, three to five cycles of ITC are usually sufficient to obtain purified ELPs [9,53]. Additional purification using chromatography procedures could be required to ensure complete homogeneity of the purified ELPs and remove trace amounts of endotoxins [9]. ELP fusion proteins can also be purified by ITC, provided that the ELP domain is relatively large, ensuring the formation of coacervates [54]. ELPs have therefore been used as cleavable purification tags for proteins and have been shown to prevent these proteins from forming inclusion bodies [55]. Generally, fusion of a protein of interest with ELPs increases the yield and simplifies the purification of recombinant proteins [56].

3. ELP-based micelles

Whereas the temperature-controlled formation of large ELP coacervates is useful for purification purposes, drug delivery strategies often require smaller and more well-defined particles for enhanced biodistribution, targeting and tissue penetration [8,10]. Depending on the exact composition and molecular weight, ELPs can be designed to assemble into various nanostructures [57], including spherical micelles [58], worm-like micelles [59], vesicles [60], and nanogels [61]. Spherical micelles are typically assembled from ELPs composed of a hydrophobic block connected to a more hydrophilic block, while worm-like micelles can be assembled from amphiphilic triblock copolymers with a hydrophilic middle block capped by two hydrophobic blocks

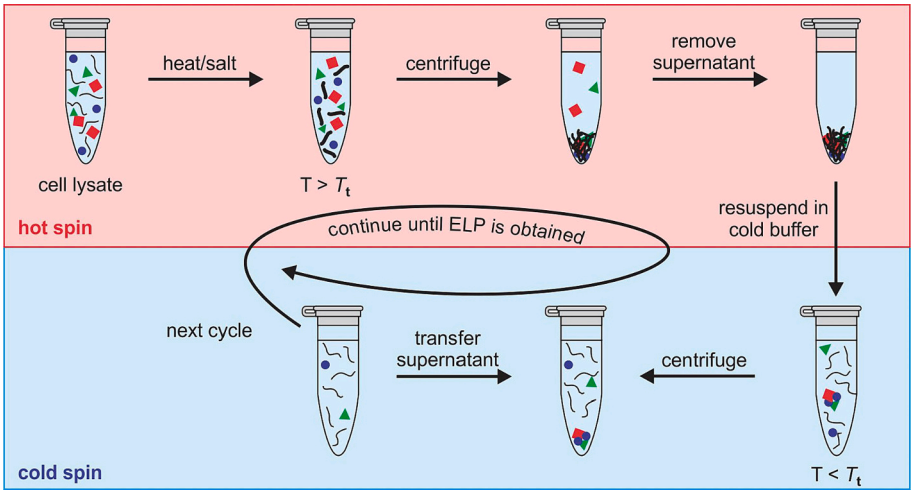

Fig. 2. Purification of ELPs (depicted in black) from other compounds (colored symbols) by inverse transition cycling.

[62]. Alternatively, ELP vesicles can be prepared from a hydrophobic block capped by two hydrophilic blocks [60]. Several examples of ELP-based nanoparticles are based on amphiphiles that contain an ELP conjugated to a non-ELP moiety, resulting in variously shaped micelles [50,63–66], double-core micelles [67], vesicles [64,68], dendrimers [69], and DNA-containing spheres [70]. Such non-ELP domains can be fully protein-based [63,65,66,68,70], or can comprise a synthetic polymer [64,67,69] or low-molecular weight hydrophobic molecules like cholesterol [50]. Increasing the size of the hydrophobic domain relative to the hydrophilic ELP block controlled the shape and size change of nanoparticles from spherical micelles, to worm-like micelles and vesicles [64,65].

Among the more extensively researched variations of ELP-based nanoparticles are spherical micelles with typical hydrodynamic diameters in the range of 40-100 nm. Generally, ELP diblock copolymers are used consisting of two blocks with a different amino acid at position X in the pentapeptide (VPGXG) repeating units. The resulting amphiphilic structure has a different T_t for each block and the associated temperature-dependent transitions are schematically shown in Fig. 3. At the lower T_t only the more hydrophobic block undergoes hydrophobic collapse, while the more hydrophilic block remains fully solvated. This induces multiple ELPs to self-assemble into spherical micelles. Therefore, the lower T_t is also called the critical micelle temperature (CMT). Above the higher T_t the more hydrophilic block also collapses and coacervates are formed. Thus, the CMT and higher T_t are controlled by the amino acid sequence and the molecular weight of the hydrophobic and the hydrophilic building block, respectively. In summary, by selecting the appropriate amino acid sequence, micelles can be designed with specific properties with respect to size, surface charge and temperature-dependent behavior [14,71–73]. For example, ELP block copolymers were designed with a CMT between 37 °C and 42 °C, inducing micelle assembly inside externally heated tissue [74]. Another interesting design strategy are pH-switchable ELPs sensitive to mild acidic environments common for lysosomes and tumors. ELPs with histidine at position X in the hydrophobic block formed pH-sensitive micelles that are designed to disassemble at low pH. This localized dissociation of the ELPs is beneficial for the penetration and distribution of the micelle cargo in tumors [75]. A similar strategy was applied in another study, in which the incorporation of histidine residues in the hydrophobic block of ELP diblock copolymers resulted in micelles that were responsive to pH as well as Zn^{2+} ions [76]. This amphiphilic polypeptide reversibly assembled into ~40 nm micelles in the presence of 60 μM $ZnCl_2$ over a wide temperature range (4 °C < T < 45 °C). The micelles disassembled upon lowering the pH to 5.5, mimicking the conditions of early endosomes.

Besides ELP diblock copolymers, single segment ELPs can also assemble into micelles if attached to a hydrophobic moiety, such as doxorubicin [77], cholesterol [50], myristic acid [51,78] or a Zn(II)-phthalocyanine photosensitizer [79]. These molecules are more hydrophobic than any amino acid, resulting in more stable micelles as compared to fully ELP-based micelles. The higher stability enables the conjugation of increasingly charged cargo to the corona and facilitates the encapsulation of hydrophobic molecules in the core [50,78] Doxorubicin and phthalocyanine can have a dual purpose: facilitating micelle formation by making the ELP amphiphilic and simultaneously adding a functionality to these micelles [77,79]. The downside of these hybrid amphiphiles is the requirement of an extra synthesis step, as opposed to the expression of a single fusion protein.

4. Functionalization of ELP micelles

Functionalization of nanocarriers with either natural or synthetic entities is a useful strategy to confer them desired properties such as targeting ability and can be achieved by both genetic and chemical methods, either alone or in combination. In order to introduce further functionality, a wide variety of molecules such as drugs [80], polyelectrolytes [81,82], fluorescence dyes [83], targeting ligands [74,84] and peptides/proteins [85–87] have been attached to ELP micelles by either covalent or non-covalent conjugation. In general, hydrophobic molecules usually reside in the micelle core, whereas the hydrophilic ones are typically conjugated to the micelle corona. However, even hydrophilic and charged entities like Gemcitabine [88] and metallodrugs [89] can be incorporated into hydrophobic ELP micelle cores. Generally, cytotoxic payloads can be shielded in the micelle core by attaching it to the hydrophobic block, while targeting ligands are displayed on the corona by conjugation to the hydrophilic segment [90].

4.1. Covalent functionalization of ELP diblock copolymers

Functional ELP-based constructs comprising of an ELP domain and a peptide or protein of interest can be designed and synthesized by genetic engineering [56]. The resulting ELP-based construct combines the thermo-responsiveness of ELPs with a biological function of the peptide/protein motif. For example, a multifunctional carrier was recombinantly synthesized for hyperthermia-enhanced targeted delivery (Fig. 4) [84]. This system consists of an ELP block copolymer [85] with a cell penetrating peptide (CPP) motif appended to C-terminus of the hydrophilic block of the copolymer and a proapoptotic peptide drug bound to the N-terminus of the hydrophobic block. Upon self-assembly, CPP and peptide drug would be located at the micelle corona and the micelle core,

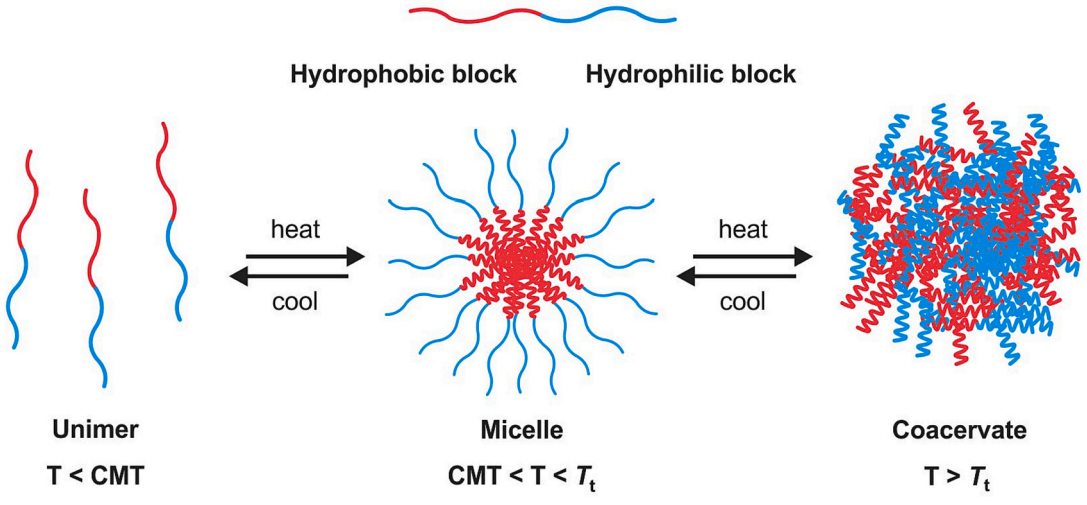

Fig. 3. Phase transition behavior of ELP diblock copolymers.

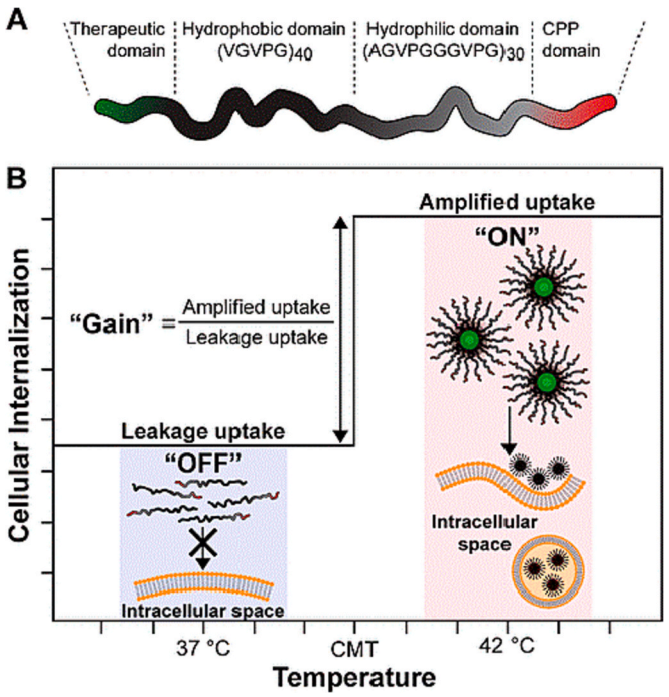

Fig. 4. Design of a multifunctional ELP-based nanocarrier produced recombinantly. (A) This carrier comprises a therapeutic cargo appended to the hydrophobic block of an ELP diblock copolymer and a CPP fused to the hydrophilic block. (B) Self-assembly of this ELP constructs into micelles occurred when temperature increased from 37 °C to 42 °C, and CPP contained in the micelle corona mediated cellular uptake. Reprinted with permission from [84]. Copyright 2014 American Chemical Society.

respectively. At mild hyperthermia conditions (42 °C), this ELP-based construct self-assembles into spherical micelles exhibiting increased cellular uptake and cytotoxicity compared to the non-functionalized one. ELP micelles can also be functionalized for *in vivo* tumor targeting purposes. For this, the NGR peptide, a targeting moiety that has shown to target CD-13 positive tumor cells, was genetically appended to an ELP diblock copolymer [86]. These NGR-functionalized ELP micelles showed to have the ability to selectively target the angiogenic tumor vasculature in a mouse tumor model, leading to a greater accumulation of the micelles in tumor tissue in comparison with healthy tissue. Proteins can also be conjugated to ELP micelles through genetic engineering. In this respect, an ELP diblock copolymer was genetically fused to the knob domain of adenovirus type 5 fiber protein, which is known to promote cellular virus entry by binding to the coxsackie and adenovirus receptor (CAR)—a cell receptor commonly found in several mammalian tissues such as brain, heart, liver, *etc.* [91,92]. As expected, the Knob-ELP micelles were more internalized—*via* CAR-dependent endocytosis—by hepatocytes than non-modified ELP micelles.

Optimal peptide display at the ELP micelle corona is dependent on the linker connecting this peptide to the hydrophilic block [93]. Positively charged linkers can counteract the negative surface charge originating from free hydrophilic C-termini, reducing electrostatic repulsions between ELP micelles and cell membranes to a minimum. Increasing the linker length can prevent hydrophobic peptide ligands from interacting with the hydrophobic core and thereby reducing the availability to interact with the intended target. The density of displayed peptide ligands is also of great importance for effective interaction with target cells [94].

Genetic engineering is a valuable tool to fuse peptides or proteins to ELPs; however, synthetic molecules cannot be incorporated to the ELP motifs using this approach [82]; therefore, chemical modification represents a powerful alternative. Conjugation of synthetic molecules usually involves covalent chemical modification of cysteines [77,88,95–97], lysines [98], unnatural amino acids [80], or the N-terminus [99] of ELPs. For example, N-hydroxysuccinimide (NHS) chemistry was used to covalently attach fluorescence dyes to the surface of ELP micelles *via* N-terminal conjugation [83]. The N-terminus of the ELP diblock copolymer is located in the hydrophilic block thus upon temperature-triggered micelle assembly, it was available on the ELP micelle corona and modified with NHS-ester fluorescence dyes, NHS-Texas red and NHS-Fluorescein. The resulting fluorescently-labeled ELP micelles showed great potential for targeted *in vivo* imaging.

4.2. Non-covalent functionalization of ELP diblock copolymers

Non-covalent conjugation of functional moieties to nanocarriers is driven by a large number of weak interactions including electrostatic, van der Waals and hydrophobic interactions, hydrogen bonding, π–π stacking, *etc.* [100,101]. Hydrophobic molecules such as rapamycin [102,103], doxorubicin [104], paclitaxel [105], and dipyridamole [106], can be physically entrapped within the ELP micelle core through hydrophobic interactions. An alternative supramolecular modification approach involves conjugation of ELPs to a binding motif specific for the drug. For example, an efficient drug conjugation strategy for ELP micelles was developed based on a fusion protein composed of ELP and the receptor FKBP that specifically binds the ligand rapamycin [102,103,107]. Other tools for supramolecular binding of cargo to nanocarriers could entail any set of interacting moieties that exhibits a strong and specific binding affinity. For example, functional molecules can be conjugated to ELP-based nanoparticles using a coiled-coil motif. For instance, Assal and coworkers conjugated a single-chain vascular endothelial growth factor ($scVEGF_{121}$) to a genetically engineered ELPs fused with polyaspartic acid tails *via* a heterodimeric coiled coil (Fig. 5) [108]. This ELP construct assembled into 30-35 nm micelles at 50 °C and the cytotoxic drug paclitaxel was physically entrapped in the micelle core. Non-covalent conjugation of $scVEGF_{121}$ *via* coiled coil formation enabled the targeting of the endothelial growth factor of HeLa cells, resulting in induced apoptosis. Control experiments confirmed that the cytotoxicity was exclusively caused by paclitaxel and was dependent on VEGF targeting.

Whereas the release of covalently linked drugs is controlled by degradation processes, the release rate of non-covalently linked drugs can be tailored by appropriate selection of interactions between the nanocarrier and the drug, resulting in an optimized binding affinity although non-covalent coupling can be easily influenced by different factors, such as pH and ionic strength [101,109,110].

5. ELP micelles in medicine

ELPs and ELP micelles have recently attracted increasing attention in drug delivery because of their thermo-responsive self-assembly behavior combined with their inherent biodegradability, low toxicity and apparent non-immunogenicity [8–10,24,111–114]. In particular, various ELP micelles carrying either small drugs or biomacromolecules are being explored for vaccination as well as for the treatment of cancer, eye-related diseases and for wound healing (Table 1) [74–76,80,87,91,95,97,102,107,115–122].

5.1. ELP micelles as vaccine carriers

A tuberculosis vaccine was developed based on ELP micelles [115]. A fusion protein comprising a *Mycobacterium tuberculosis* antigen conjugated to an amphiphilic ELP diblock copolymer self-assembled into spherical micelles. Upon subcutaneous administration in mice, these antigen-ELP micelles induced higher levels of antigen-specific IgG antibodies as compared to free antigen, suggesting that these antigen-ELP construct elicits a humoral immune response.

ELP micelles carrying the ovalbumin derived SIINFEKL epitope

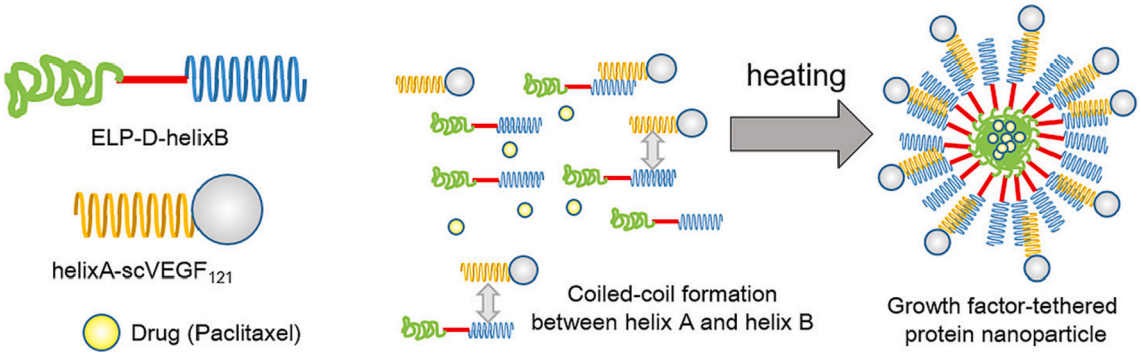

Fig. 5. Non-covalent conjugation of a protein to ELP micelles *via* coiled-coil formation, which was achieved by fusing helix A and helix B to scVEGF121 and ELPs, respectively. Reprinted with permission from [108]. Copyright 2015 American Chemical Society.

inside the core were studied as CTL vaccine carriers [117]. For this, ELPs comprising non-typical amino acid sequences (VPG-Xy-G)n and (VLPG-Xy-G)n, were designed to be non-immunogenic, since immune responses against a vaccine carrier can impair the immune response against the antigen. However, the consensus is that ELPs generally have minimal immunogenicity [8–10,111–113,115,123]. The rationale for the sequence of these immune-tolerant elastin-like polypeptides (iTEPs) was the higher similarity to murine and human elastin, compared to canonical ELPs. Additional factors that were included in the design in order to decrease the immunogenicity were a higher glycine content at the X positions and a longer repeating motif than typical B-cell receptor epitopes. Various iTEPs were injected twice in mice and the resulting iTEP-specific antibody levels in the sera were negligible, as determined with an enzyme-linked immunosorbent assay (ELISA). These results confirmed that iTEPs are poorly immunogenic. In the same study, SIINFEKL was recombinantly conjugated to the hydrophobic segment of an amphiphilic iTEP, resulting in SIINFEKL buried in the core of micelles. DCs presented significantly more SIINFEKL epitope after exposure to these nanoparticles compared to native ovalbumin, and to the soluble fusion protein of SIINFEKL conjugated to the hydrophilic block of the iTEP amphiphiles (Fig. 6). Next, a coculture of ELP-treated DCs with $CD8^+$ T-cells resulted in increased antigen presentation and T-cell activation. Mice were immunized with the various formulations together with incomplete Freund's adjuvant (IFA). Unfortunately, the resulting formulation was not characterized, but it seems unlikely that the ELP micelles remain intact in the presence of the IFA emulsion. Nevertheless, injections with the mixture of SIINFEKL-iTEP micelles and IFA induced the highest SIINFEKL-specific CTL response. Unfortunately, this study did not include a group of SIINFEKL-iTEP micelles without IFA, so it remains unclear whether these ELP micelles can function as an adjuvant. Nonetheless, these results illustrate that this ELP-based delivery system is a promising carrier for CTL peptide vaccines.

ELP micelles were recently shown to be a suitable platform for allergen immunotherapy (AIT) [116]. An amphiphilic ELP conjugated to birch pollen allergen Bet v 1 was coassembled with unconjugated ELP amphiphiles into micelles displaying Bet v 1 at the periphery. Compared to the current standard for birch pollen immunotherapy of birch pollen extract mixed with alum, these ELP-Bet v 1 micelles induced a stronger and earlier Bet v 1-specific IgG1 and IgG2a response in naïve mice (Fig. 6). Moreover, IL-4, IL-5 and IL-13 levels were lower in the ELP-Bet v 1 micelle group compared to the alum-adsorbed birch pollen extract group, which is indicative of a weak $CD4^+$ T-cell response, potentially resulting in less adverse effects.

5.2. ELP micelles for cancer treatment

Diblock copolymer iTEPs have also been studied as a potential delivery system for the treatment of programmed cell death 1 (PD-1) positive tumors. For this, iTEP micelles were prepared displaying a single chain variable antibody fragment directed to the PD-1 protein. The resulting nanoparticles were shown to be effective as immune checkpoint inhibitors, both *in vitro* and *in vivo* [119].

ELP block copolymers with a CMT of ~40 °C were designed to form micelles in externally heated tissues. These micelles contained covalently attached cargo in their core and corona [85]. For example, various amphiphilic ELPs were equipped with an RGD domain [95] or a fibronectin type III (fn3) domain [120] on the hydrophilic terminus to target αVβ3 integrins, which are overexpressed in tumor blood vessels. Most ELPs of this series were designed to form micelles in externally heated tissues, whereas some had a CMT below 37 °C for comparison. One of the ELPs in the latter category was also functionalized with the tripeptide ligand NGR to target the CD13 receptor in tumor vasculature [86]. The hydrophilic blocks of the amphiphiles used in these studies were combined with pH-sensitive hydrophobic ELP blocks containing histidines. These diblock copolymers assembled at pH $\geq$ 6.8 and were designed to disassemble in mildly acidic environments that are common for the extracellular regions of tumors. The pH-sensitive micelles were injected in mice bearing tumor xenografts derived from a human colon cancer cell line. Compared to similar micelles that were pH-insensitive, the histidine-rich ELPs penetrated the entire tumor tissue much more evenly. This may improve the therapeutic efficacy by enabling cargo to reach relatively inaccessible parts of tumors [75].

In another study interferon alpha (IFNα) was genetically fused to an ELP diblock copolymer and the resulting micelles displayed IFNα at the corona above room temperature [118]. While the antiproliferative activity to Daudi cells of these micelles was 2.8-fold lower than free IFNα, it was 5.7-fold higher than pegylated IFNα. To study the proteolytic stability of the micelles, various formulations were treated with protease K, resulting in decreased levels of antiproliferative activity: only 1.09-fold for IFNα micelles as opposed to complete activity loss for free IFNα. The ELP-based micelles were superior to both pegylated IFNα and free IFNα with respect to the circulation half-life in mice: 54.7 h, compared to 39.0 h and 0.44 h, respectively. Finally, mice bearing ovarian tumors were intravenously injected with these samples, resulting in the highest tumor retention and anti-tumor efficacy for the IFNα-displaying micelles.

The MacKay group conjugated the cytostatic and immunomodulatory drug rapamycin to micelles by expressing a fusion protein of an ELP diblock copolymer and FK506 binding protein 12 (FKBP), which is the receptor for rapamycin. These micelles were used to treat mice with human breast cancer xenografts. A 26-fold increase of the half-life of rapamycin using these micelles upon administration was reported. Furthermore, the ELP-rapamycin micelles also had a lower toxicity and higher anti-tumor efficacy compared to free rapamycin [103]. Authors combined this rapamycin-FKBP-diblock ELP in another study with a similar ELP diblock copolymer extended with RGD at the hydrophilic terminus, directing the micelles to αVβ3 integrin receptors which are overexpressed in tumor-associated endothelial cells [107]. Mixed

Table 1
Characteristics of ELP micelles used for the delivery of therapeutic agents.

ELP sequence	Cargo (cargo location: core/corona)	Conjugation method	Application	D_h (nm)	Tested *in vitro*/*in vivo* (preclinical)	Ref
$(V_2EV_2)_{10}I_{60}$	Antigen from membrane of *M. tuberculosis* (corona)	Recombinantly fused	Vaccination against tuberculosis	63	*In vivo*	[115]
$I_{48}S_{48}$	Bet v 1 (corona)	Recombinantly fused	Birch pollen immunotherapy	45	*In vitro* and *in vivo*	[116]
[Gly-Ala-Gly-Val-Pro-Gly]$_{70}$-Gly-[Gly-Val-Leu-Pro-Gly-Val-Gly]$_{28}$	SIINFEKL (core)	Recombinantly fused	Vaccination model (OVA)	72-81	*In vitro* and *in vivo*	[117]
[Gly-Ala-Gly-Val-Pro-Gly]$_{70}$-Gly-[Gly-Val-Leu-Pro-Gly-Val-Gly]$_{28}$	Antibody fragment specific for PD-1 (corona)	Recombinantly fused	Immune checkpoint inhibition for cancer treatment	44	*In vitro* and *in vivo*	[119]
$(A_3G_2)_{12}I_{60}$	1. Heavy chain antibody fragment (corona) 2. IRDye700DX (core)	1. Recombinantly fused 2. NHS ester on N-terminus	1. Cancer targeting (EGFR) 2. Cytotoxic drug delivery for cancer treatment	68	*In vitro*	[87]
$V_{80}S_{80}$	Nanobody (corona)	Recombinantly fused	Cancer targeting (EGFR)	89	*In vitro*	[97]
$V_{80}S_{80}$	Doxorubicin (core)	Modification of *p*AcF residue, incorporated *via* amber codon suppression	Cytotoxic drug delivery for cancer treatment	47	*In vitro*	[97]
$(VAGAGAGAGAGAGAGA)_4V_{60}$	RGD (corona)	Recombinantly fused	Cancer targeting ($\alpha_v\beta_3$ integrin)	49	*In vitro*	[95]
$(VAGAGAGAGAGAGAGA)_6V_{60}$	RGD (corona)	Recombinantly fused	Cancer targeting ($\alpha_v\beta_3$ integrin)	50	*In vitro*	[95]
$(VAGAGAGAGAGAGAGA)_6V_{60}$	Fn3 domain (corona)	Recombinantly fused	Cancer targeting ($\alpha_v\beta_3$ integrin)	65	Not applicable	[120]
$(VAGAGAGAGAGAGAGA)_8V_{60}$	Fn3 domain (corona)	Recombinantly fused	Cancer targeting ($\alpha_v\beta_3$ integrin)	62	Not applicable	[120]
$(VAGAGAGAGAGAGAGA)_6V_{90}$	Fn3 domain (corona)	Recombinantly fused	Cancer targeting ($\alpha_v\beta_3$ integrin)	70	*In vitro*	[120]
$(VAGAGAGAGAGAGAGA)_4V_{120}$	Fn3 domain (corona)	Recombinantly fused	Cancer targeting ($\alpha_v\beta_3$ integrin)	73	Not applicable	[120]
$(VAGAGAGAGAGAGAGA)_4V_{90}$	RGD (corona)	Recombinantly fused	Cancer targeting ($\alpha_v\beta_3$ integrin)	62	*In vitro*	[95]
$(VAGAGAGAGAGAGAGA)_4V_{90}$	Fn3 domain (corona)	Recombinantly fused	Cancer targeting ($\alpha_v\beta_3$ integrin)	62	Not applicable	[120]
$(VAGAGAGAGAGAGAGA)_4V_{90}$	NGR (corona)	Recombinantly fused	Cancer targeting (CD13)	~60	*In vivo*	[86]
$(VAGAGAGAGAGAGAGA)_5$-$(VH_4)_{20}$	Not applicable	Not applicable	Tumor penetration for cancer treatment	~60	*In vivo*	[75]
$(IH_4)_{60}$-$(A_3G_2)_{60}$	Not applicable	Not applicable	Cargo release in tumor environment	~40	Not applicable	[76]
[Val-Pro-Lys-Glu-Gly]$_{120}$-$(CV)_8$	Paclitaxel (core)	Cysteine modification	Cytotoxic drug delivery for cancer treatment	116	*In vitro* and *in vivo*	[97]
$(AG)_{80}(CV)_8$	Paclitaxel (core)	Cysteine modification	Cytotoxic drug delivery for cancer treatment	94	*In vitro* and *in vivo*	[97]
$S_{48}I_{48}$	FKBP bound to rapamycin (corona)	Recombinantly fused	Cytotoxic drug delivery for cancer treatment	48	*In vitro* and *in vivo*	[103]
$S_{48}I_{48}$	FKBP bound to rapamycin (corona)	Recombinantly fused	Treatment of Sjögren's syndrome	48	*In vivo*	[102]
$I_{48}S_{48}$	RGD (corona)	Recombinantly fused	Cancer targeting ($\alpha_v\beta_3$ integrin)	48	*In vitro* and *in vivo*	[107]
$S_{48}I_{48}$	Knob domain of adenovirus fiber protein (corona)	Recombinantly fused	Endocytosis in cells expressing CAR	43	*In vitro*	[91]
$S_{48}I_{48}$	Knob domain of adenovirus fiber protein (corona)	Recombinantly fused	Transcytosis across the lacrimal gland for treatment of ocular diseases	43	*In vitro* and *in vivo*	[121]
$A_{48}I_{48}$	IFNα (corona)	Recombinantly fused	Cancer treatment	51	*In vitro* and *in vivo*	[118]
$V_{40}(AG)_{30}$	Arg_5 (corona)	Recombinantly fused	Cell penetration for cancer treatment	42	*In vitro*	[74]
$V_{60}(AG)_{30}$	keratinocyte growth factor (corona)	Recombinantly fused	Epidermal and dermal regeneration in wound healing	68	*In vitro* and *in vivo*	[122]
$V_{60}(AG)_{30}$	stromal cell-derived growth factor 1	Recombinantly fused	Epidermal and dermal regeneration in wound healing	63	*In vitro* and *in vivo*	[122]
$V_{60}(AG)_{30}$	Cathelicidin	Recombinantly fused	Anti-microbial protection in wound healing	57	*In vitro* and *in vivo*	[122]

micelles composed of these two fusion proteins combined with rapamycin resulted in suppressed tumor growth in a breast cancer mouse model at a 3-fold lower dose compared to untargeted rapamycin carrying micelles. Accumulation inside the tumor of these particles was higher compared to these untargeted particles. Mice treated with FKBP- and RGD-functionalized ELP-based particles all survived for the full duration of the study, whereas the mice in the control groups treated with either PBS, free rapamycin or the ELPs without RGD all died before the study was completed (Fig. 7).

Pille et al. designed ELP-based nanoparticles targeting epidermal growth factor receptor (EGFR) positive tumors. For this, an ELP diblock copolymer was conjugated to an EGFR specific heavy chain antibody fragment (VHH) [87]. Mixing this fusion protein with the same block copolymer fused to the cytotoxic photosensitizer IRDye700DX resulted in micelles carrying both the VHH and IRDye700DX at the corona. The micelles were shown to have a strong EGFR-specific cytotoxic effect in a

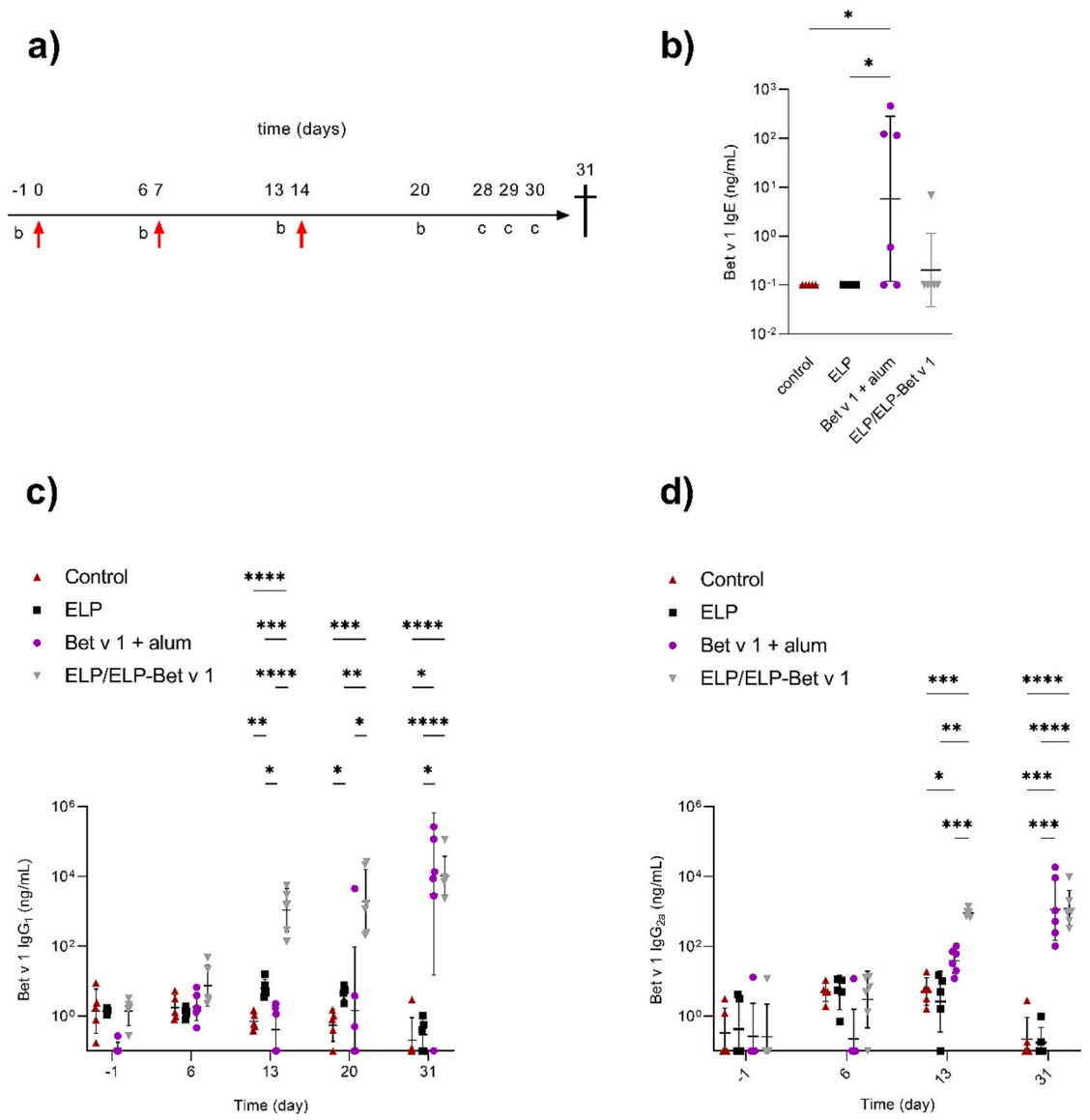

Fig. 6. *In vivo* immunogenicity in naïve mice. A) Mice were immunized on day 0, 7 and 14 by subcutaneous injection (red arrows). Blood samples (b) were taken on days -1, 6, 13 and 20. The mice received intranasal challenges with birch pollen extract (c) to boost immunoglobulin production on days 28, 29 and 30. The mice were sacrificed on day 31. B—D) Serum Bet v 1-specific IgE (B), IgG1 (C) and IgG2a (D) levels over the course of the study or for IgE at day 31. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$. Reprinted with permission from [116]. Copyright 2022 Frontiers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

human epidermoid carcinoma cell line.

An EGFR specific nanobody was attached to the hydrophilic block of a different ELP diblock copolymer. Here, the hydrophobic segment contained the unnatural amino acid *p*-acetylphenylalanine (p-AcF), incorporated by amber codon suppression. Doxorubicin was conjugated to the ketone group of the p-AcF residues. The resulting amphiphiles assembled into micelles encapsulating doxorubicin and displaying the EGFR nanobody at the corona. This resulted in an increased cytotoxicity in various EGFR positive cancer cell lines when compared to the EGFR-negative cells [120].

Banskota et al. developed a zwitterionic ELP (denoted ZIPP) with a non-canonical VPKEG motif to create nanocarriers with an increased plasma half-life [97]. This hydrophilic ELP was attached to a hydrophobic regular ELP domain containing cysteines. The cytotoxic drug paclitaxel was conjugated to the cysteine residues *via* an acid-labile n-ε-maleimidocaproic acid hydrazide linker. These ZIPP micelles were compared to ELPs in which the ZIPP-domain was substituted for an uncharged hydrophilic ELP domain. Both micelle types were more toxic than free paclitaxel and albumin-based nanoparticles carrying paclitaxel (Abraxane) to various human cancer cell lines for prostate and colon cancer and less toxic than free paclitaxel to a triple-negative breast cancer cell line. Confocal microscopy confirmed that the uptake of the ZIPP micelles and the non-charged equivalent in the prostate cells were similar. Both formulations had a maximum tolerable dose in mice of at least twice the value of free paclitaxel. The ZIPP micelles had an extended plasma half-life ($t1/2 = 19$ h), compared to only 12 h for the non-charged micelles. This resulted in a more efficient treatment of tumors in mice compared to control groups (*i.e.* uncharged micelles, Abraxane and free paclitaxel).

5.3. ELP micelles for the treatment of ocular diseases

The previously mentioned rapamycin-FKBP-ELP micelles were also used to treat mice for Sjögren's syndrome [102,103]. This autoimmune disease involves infiltration of lymphocytes in the lacrimal gland, causing severe dry eyes and diminished visual acuity. Intravenous

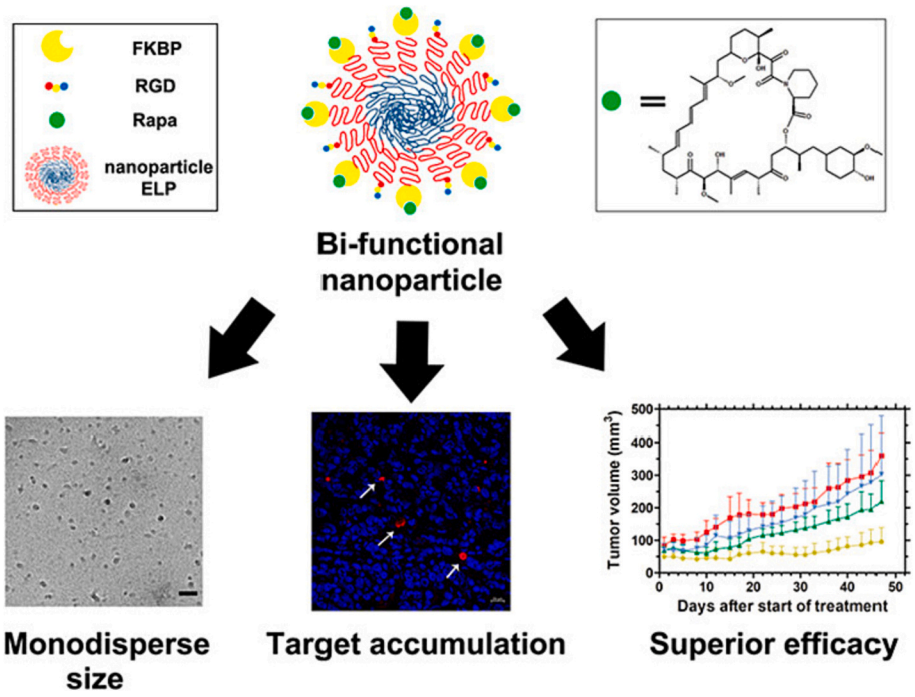

Fig. 7. Graphical overview of rapamycin-containing ELP micelles used for tumor targeting. These micelles had a particle size <100 nm with monodispersed size distribution, showed higher tumor accumulation and superior anti-tumor efficacy compared with controls. Reprinted with permission from [107]. Copyright 2017 American Chemical Society.

injection of rapamycin formulated with ELP micelles significantly suppressed lymphocytic infiltration compared to free rapamycin. Topical treatment of eye-related diseases is challenging because of natural clearance barriers that limit drug absorption [121]. The lacrimal gland expresses CAR, which can be targeted with the adenovirus fiber protein knob domain. The MacKay group therefore developed a fusion protein of this knob domain conjugated to an ELP diblock copolymer. Increased internalization of these micelles in hepatocytes was proven *in vitro*, demonstrating their potential as a drug delivery vehicle that promotes uptake in CAR-expressing cells [91]. Next, *in vivo* studies showed transcytosis of the micelles across the lacrimal gland. Confocal microscopy confirmed the presence of the knob-ELP micelles in the lumen of the lacrimal gland acinar cells [121]. These results present a new opportunity for the treatment of ocular diseases.

Two additional applications of this particular ELP diblock copolymer are related to eye diseases. Fusion of the block copolymer to the protein lacritin [124] or a peptide from αB-crystallin [125] resulted in the formation of large aggregates with hydrodynamic diameters of ~150 nm and ~ 1500 nm respectively, much larger than ELP block copolymer micelles (45 nm). Still, the particles containing ang despanie the αB-crystallin peptide were found to protect human retinal pigment epithelial cells from oxidative stress (Fig. 8). The lacritin-containing particles were a potent remedy for corneal wounds in mice.

5.4. ELP micelles for wound healing

The potential of ELP nanoparticles in wound healing was further demonstrated in a study of ELP diblock copolymer micelles fused to keratinocyte growth factor (KGF), stromal cell-derived growth factor 1 (SDF1) and cathelicidin (LL37) at the hydrophilic terminus (Fig. 9) [122]. Growth factors promote cell proliferation while cathelicidin protects against infection and induces angiogenesis. However, wound treatment with these proteins is hampered by rapid degradation in the protease-rich wound environment. Therefore, each protein was shielded by conjugation to an ELP diblock copolymer and subsequent assembly into micelles at $T >$ 34-36 °C. The KGF displaying micelles induced

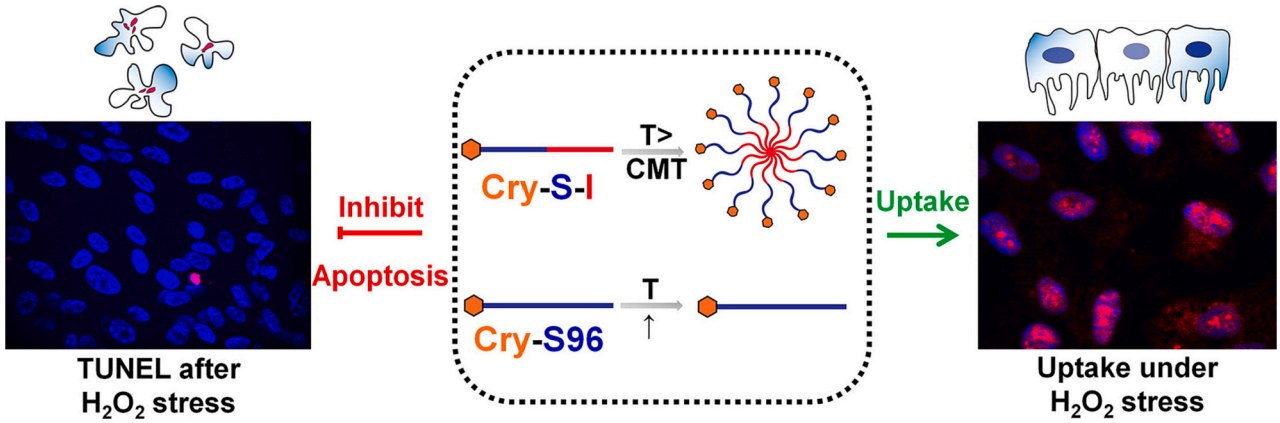

Fig. 8. ELP micelles containing αB-crystallin proved to protect RPE cells from oxidative stress-triggered apoptosis. Reproduced with permission from [125].

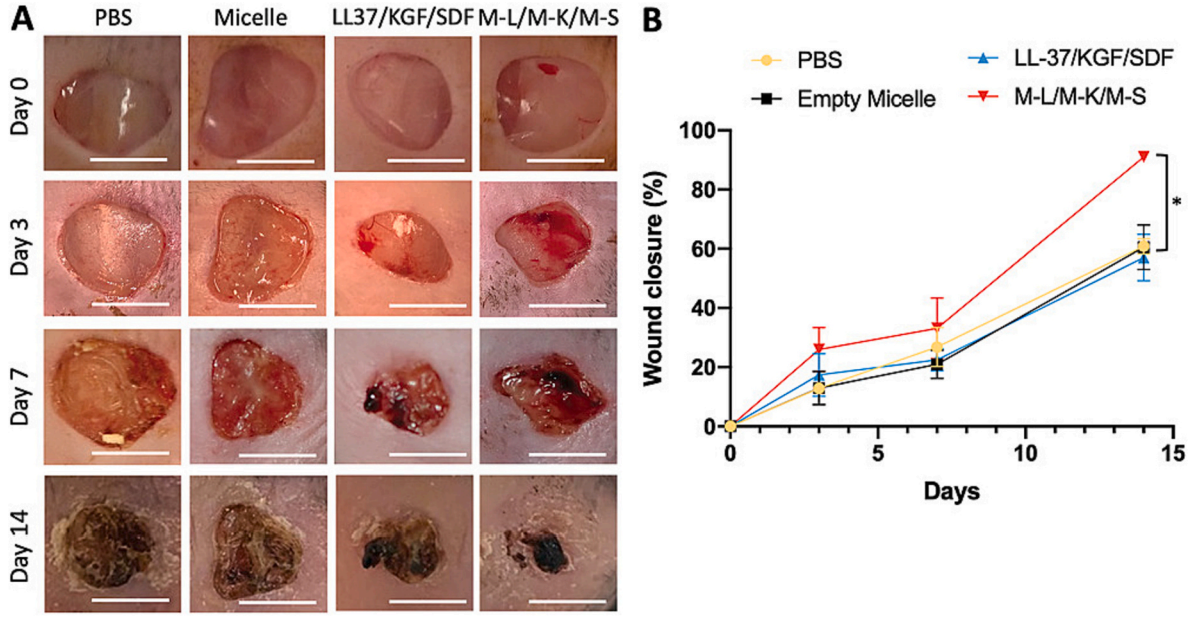

Fig. 9. Wound closure following treatment with drugs delivered by ELP micelles. A mixture of KGF-functionalized micelles, SDF1-functionalized micelles and cathelicidin-functionalized micelles was compared to a mixture of the recombinant proteins, non-functionalized micelles. A) Representative photographs of a healing wound in four stages; B) Percentage of wound closure over time. Reprinted with permission from [122]. Copyright 2022 Wiley-VCH GmbH.

similar proliferation in an endothelial cell line as recombinant KGF, while ELP-KGF aggregates (>600 nm) based on an ELP monoblock induced lower levels of endothelial cell proliferation, showing a reduced availability of KGF in the larger nanoparticles as opposed to the micelles, where KGF is effectively displayed at the periphery. SDF1 also retained its activity when displayed at the corona of ELP micelles, as shown by the similar cell migration towards recombinant SDF1 as towards micelles with conjugated SDF1. Micelles displaying LL37 significantly inhibited bacterial growth, although not as effectively as recombinant cathelicidin. Wounds of diabetic mice were treated with a fibrin hydrogel containing KGF micelles, SDF1 micelles and cathelicidin micelles, resulting in a significantly higher wound closure (91%) than treatment with fibrin gels containing a mixture of recombinant KGF, SDF1 and LL37 (57%), unfunctionalized ELP micelles (61%) or PBS (61%).

5.5. *ELPs in clinical trials*

Although ELP micelles have not been introduced in clinical practice yet, some ELP monoblocks are currently in clinical use. For instance, the company PhaseBio has conducted multiple clinical trials with ELP-based formulations, including a novel treatment for type II diabetes and SARS-CoV-2 [8,126,127]. Moreover, the ELP platform “Pemziviptadil” is used in a phase II clinical trial to treat pulmonary arterial hypertension (PAH) [128]. Pemziviptadil involves fusion proteins of ELPs and vasoactive intestinal peptide (VIP) that form a depot upon subcutaneous injection [129,130]. VIP-ELP proteins in the periphery of the depot are slowly rehydrated and released into circulation over a period of several weeks. Circulating VIP-ELP causes vasodilation, which is beneficial for PAH patients.

6. Conclusions and future perspectives

ELPs form a distinct class of biomaterials that show great promise in the field of nanomedicine, not the least due to their thermosensitive self-assembly properties. Representative examples illustrating the potential benefits of ELPs for therapeutic delivery have been described in this review. The complementary use of genetic engineering techniques and chemical methods allows the development of ELPs with different compositions, architectures, properties, and functionalities. In particular, ELP diblock copolymers that self-assemble into spherical micelles in response to temperature stimuli are very attractive for drug delivery purposes. These ELP micelles are commonly characterized as core-corona structures, where the core serves as a depot for hydrophobic payloads while the hydrophilic corona can be utilized for further conjugation with targeting ligands. In addition to thermo-responsiveness, other properties—such as biocompatibility, biodegradability, non-toxicity and non-immunogenicity—make ELP micelles well suited for biomedical applications, especially in drug delivery.

The future success of nanomedicine relies on the development of more effective delivery systems where ELP micelles represent an emerging platform that offer real opportunities for improving the efficacy of treatments against a large number of diseases. However, some characteristics inherent of micellar drug delivery systems, such as low drug loading capacity and poor physical stability *in vivo*, could be improved. In addition, it has been increasingly recognized that several factors associated with the pathological microenvironment (*e.g.*, low extracellular pH, high intracellular glutathione levels, overexpression of specific enzymes, *etc.*,) affect biodistribution and targeting of nanocarriers [131,132]. Therefore, ELP micelles should be designed to harness different targeting mechanisms for enhancing biodistribution as well as to respond to different biological stimuli (temperature, pH and redox) for controlling drug release in specific locations of the body. However, it is important to consider that any modification of ELPs can change their physicochemical properties and thus their *in vivo* performance, so full characterization and evaluation of physical, chemical and biological properties of these new materials is of the utmost importance. On the other hand, one of the current interest in nanomedicine lies in the development of nucleic acid delivery systems. To date, there are very few examples where ELPs with cationic moieties were synthesized and used for carrying DNA and RNA molecules [71,133], which are expected to expand rapidly in the coming years. It is clear that the use of ELP micelles in drug delivery contexts is mostly limited to preclinical studies; therefore, more investigation in this field is necessary to accelerate their clinical translation. We are convinced that advances in genetic and chemical engineering will provide the opportunity for the rational design and production of novel multi-targeted and multi-stimuli responsive ELP micelles with optimal physicochemical and biological

properties that enable address specific biomedical challenges.

CRediT authorship contribution statement

Jolinde van Strien: Writing – original draft. **Oscar Escalona-Rayo:** Writing – review & editing. **Wim Jiskoot:** Supervision. **Bram Slütter:** Writing – review & editing. **Alexander Kros:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

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

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