Elastin-Like Polypeptide Based Nanoparticles: Design Rationale Toward Nanomedicine

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Elastin-like polypeptides (ELPs) are characterized by a high sequence control, temperature responsiveness and biocompatibility, which make them highly interesting as smart materials for application in nanomedicine. In particular the construction of ELP-based nanoparticles has recently become a focal point of attention in materials research. This review will give an overview of the ELP-based nanoparticles that have been developed until now and their underlying design principles. First a short introduction on ELPs and their stimulus-responsive behavior will be given. This characteristic has been applied for the development of ELP-based block copolymers that can self-assemble into nanoparticles. Both the fully ELP-based as well as several ELP hybrid materials that have been reported to form nanoparticles will be discussed, which is followed by a concise description of the promising biomedical applications reported for this class of materials.

1. Introduction

Elastin and its derivatives have increasingly attracted interest in several disciplines of research ranging from tissue engineering and drug delivery to water purification. This interest has been motivated by the remarkable properties of elastin-based materials. Natural elastin is an extracellular matrix protein that provides elasticity to a wide variety of tissues such as arteries, lung, and skin.[1] Pioneering work in the field of elastin-like polypeptides (ELPs) was performed by Urry and coworkers,[2] who showed that a repeating sequence found in natural elastin, poly(Val-Pro-Gly-Val-Gly), can coacervate and form a variety of self-assembled structures in a manner similar to natural elastin. This review will first describe the characteristics of natural elastin and its water-soluble precursor tropoelastin. Subsequently, the tunable coacervative behavior of ELPs will be discussed, followed by an overview of the possibilities to control this behavior for the assembly into nanoparticles. Both fully ELP-based nanoparticles will be discussed as well as ELP hybrids. The focus will be on the design of the ELP-based materials and their ability to form nanoparticles mostly upon increasing the temperature, but also upon changes in pH and ionic strength. Next to this, different crosslinking methods that increase the stability of these nanoparticles will be presented. ELP-based hydrogels will not be discussed in this review, for an overview of ELP-based hydrogels and their applications we refer to other articles.[3–7] Finally applications of the ELP-based nanoparticles will be discussed briefly, with an emphasis on nanomedicine. Importantly, the work presented here discusses ELPs based on the VPGVG motif, and will not be concerned with other derivatives of tropoelastin, like those based on alternating hydrophobic and crosslinking domains of human tropoelastin[8] or elastin-based side-chain polymers.[9]
2. Natural Elastin and Tropoelastin

Elastin is a major extracellular matrix protein found in many tissues, where it undergoes thousands to billions of cycles of extension and recoil. Tropoelastin is its precursor molecule which is chemically crosslinked to form networks of elastin, which are insoluble in water and highly durable with a half-life of 70 years. The structure of tropoelastin is characterized by alternating hydrophobic and hydrophilic regions with each region generally encoded by a different exon. The hydrophobic regions contain repeats of glycine (Gly), valine (Val), alanine (Ala), and proline (Pro) residues in several motifs, and the hydrophilic regions mainly contain lysine (Lys) residues interspersed by Ala repeats. Tropoelastin is soluble in aqueous solution below its phase transition temperature (Tt), but undergoes a sharp phase transition if the temperature is raised above Tt, which leads to desolvation and aggregation of the polypeptide. During this coacervation process formation of fibrillar structures was observed, which led to the hypothesis that the alignment of tropoelastin molecules was an important factor in the assembly of elastin networks.

Keeley et al. showed that small recombinantly expressed polypeptides, based on human elastin, indeed have the ability to align themselves and form fibrils. They proposed that the interactions of the hydrophobic domains are fundamental for positioning the hydrophilic regions containing Lys residues for intermolecular crosslinking (Figure 1).

The coacervation of tropoelastin upon heating through the Tt is governed by the thermodynamically driven increase in both intra- and intermolecular ordering within the protein. The thermodynamic preference for this order at higher temperatures can be explained by the large contribution of the entropy of hydrating water molecules to the system. At temperatures below Tt, the non-polar residues are hydrated by layers of water molecules of decreased entropy compared to bulk water. Upon an increase in temperature, the non-polar side chains make intra- and intermolecular contacts, thereby liberating the bound water molecules, whose increase in entropy compensates for the decreased entropy of the protein.

Importantly, this process is completely reversible upon cooling below Tt. Since the solubility of tropoelastin decreases upon raising temperature, this process is also referred to as lower critical solution temperature (LCST) behavior, a terminology regularly used in polymer science.

3. LCST Behavior of Elastin-Like Polypeptides

Urry and coworkers have extensively studied tropoelastin and the repeats found in its sequence. The Val-Pro-Gly-Val-Gly (VPGVG) repeat sequence was the first synthetic polypeptide found to coacervate in a manner similar to tropoelastin. This VPGVG repeat provided a simple model for the coacervation of tropoelastin, and research into the structural changes induced upon heating above its Tt has led to the model outlined in Figure 2. This model indicates that the polypeptide changes conformation from a random coil below Tt to a β-spiral structure above Tt, while simultaneously releasing hydrating water molecules.

The β-spiral consists of consecutive type II β-turns that are formed by the VPGVG repeats. The Pro and Gly residues are pivotal for maintaining the β-turn behavior, a terminology regularly used in polymer science.
conformation, whereas the other two positions in the pentapeptide repeat can be substituted by virtually every natural and some non-natural amino acids.\cite{14,19,20} Although the \( \beta \)-spiral model is most frequently used to describe the conformation of these repeats, they have also been reported to adopt a (distorted) \( \beta \)-strand conformation.\cite{19} Other studies have also shown ELPs to adopt a \( \beta \)-spiral conformation below \( T_t \), suggesting mechanisms other than the before mentioned conformational change of random coil to structured \( \beta \)-spiral might play a role for the LCST behavior.\cite{21}

A recent study using molecular dynamics simulations indicated the presence of \( \beta \)-turns at all temperatures, but showed an increase in ordering of the secondary structure at high temperatures.\cite{22} This indicates that there still is some controversy on the mechanism underlying the LCST behavior of ELPs.

The work of Urry and coworkers launched a new class of polypeptides based on this VPGVG repeat in elastin, termed ELP. ELPs include all polypeptides that contain Val-Pro-Gly-Xaa-Gly (VPGXG) repeats, where the fourth residue can be any naturally occurring amino acid except L-proline. They are usually described using the notation ELP-[XiYjZk-n], where the capital letters between the brackets indicate the single letter amino acid code for the Xaa-replacing residue in the pentapeptide repeat, and the subscripts represent the ratio of these guest residues. The total number of pentapeptide repeats is denoted by \( n \). However, not only the fourth residue can be varied without disturbing the LCST behavior of the polypeptide, the valine on the first position of the pentamer is often replaced by an isoleucine and the glycine on the third position by an alanine residue. This last substitution yields the polymer \((VPAVG)_n\), which exhibits properties very different from other ELPs as it shows hysteresis upon aggregation, making it more plastic than elastomeric.\cite{23,24} The substitution of the last glycine, resulting in the pentapeptide \((VPGVA)_n\), results in a material that shows no reversible elasticity,\cite{15,25} which is also the case when the first valine is substituted by an alanine, giving \((APGVG)_n\). On the other hand, replacement of this residue by an isoleucine does not change the reversibility of the LCST behavior.\cite{26}

Changing the fourth residue of the ELP pentapeptide \((VPGXG)_n\), often referred to as the guest residue, tunes the LCST behavior of the ELP. Increasing the hydrophobicity of this residue decreases the \( T_t \) (Figure 3A).\cite{14} Because of its profound influence on and good predictability of \( T_t \), substitution of the guest residue is a widely used method to modify the LCST behavior of an ELP during the design process. In addition to these variations in hydrophobicity, the incorporation of charged guest residues in the ELP sequence allows for pH-dependent control over their coacervation behavior. MacKay et al.\cite{27} have developed a quantitative model describing the influence of pH on the \( T_t \) of both basic and acidic ELPs. Basic guest residues are neutral above their pK\textsubscript{a}, which increases the

Figure 1. Hydrophobic effects between the Gly-, Val-, Ala-, and Pro-rich regions in tropoelastin align the protein for intermolecular chemical crosslinking upon coacervation. The hydrophobic regions are represented by square structures with large hydrophobic side chains protruding from them. The interactions between these side chains bring the lysine-containing crosslinking regions, depicted as waves with protruding amine groups, in close proximity. The lysine residues are oxidatively deaminated by lysyl oxidase and subsequently condensed to form desmosine or isodesmosine crosslinks between two pairs of lysine residues of different tropoelastin molecules. Figure is adapted from Keeley et al.\cite{13}

Figure 2. Poly(VPGVG) adopts a \( \beta \)-spiral structure at temperatures above \( T_t \). The VPGVG repeats form \( \beta \)-turns, stabilized by intramolecular hydrogen bonds between the backbones of the first and fourth residues of the pentapeptide. These \( \beta \)-turns arrange into helical \( \beta \)-spirals, which are represented with and without displaying the structure of the \( \beta \)-turns in the turns of the helix. Reprinted with permission from Urry D. W., Physical chemistry of biological free energy transduction as demonstrated by elastic protein-based polymers. J. Phys. Chem. B 1997, 101, 11007–11028. © (1997) American Chemical Society.\cite{24}
hydrophobicity of the polypeptide, leading to a lower $T_t$. On the other hand, deprotonation of acidic guest residues above their $pK_a$ leads to increased charge distribution along the polypeptide chain, increasing the $T_t$. Of course, the opposite effect is observed below the respective $pK_a$’s. Besides the amino acid composition, the total chain length of the ELP also has a profound influence on $T_t$. Even though this effect is strongest for low molecular weight ELPs, it can be used to design ELPs with LCST behavior in a specific temperature range (Figure 3B). [28,29] The design of the ELP, including the distribution of guest residues and total polypeptide length, does not completely fix its $T_t$ at a specific temperature. An important factor that can be used to modify the LCST behavior of a certain ELP is the salt concentration of the ELP solution. [30,31] Cho et al. showed that for a broad range of chaotropic and kosmotropic anions in the Hofmeister series, the $T_t$ decreases with increasing salt concentrations (Figure 3C). For kosmotropes, this decrease is linear to the salt concentration, whereas for chaotropes a non-linear binding isotherm is needed to fit the experimental results. [32,33] In addition, changing the concentration of ELP is another variable that can be used to alter $T_t$ after the design of the ELP has been established (Figure 3D). [28] However, due to the logarithmic dependence of $T_t$ on concentration and the limited range of variation that is possible for the sample concentration (for instance regarding in vivo applications), the effect of the concentration of ELP on $T_t$ is usually restricted in practice, and focus is primarily on the design of the ELP sequence.

The emergence of recombinant DNA technologies has greatly enhanced the possibilities to create polypeptides with a precisely defined sequence and molecular weight to a far larger extent than conventional polymer chemistry can achieve. A common way to produce ELPs uses *Escherichia coli* transformed by a plasmid encoding the ELP sequence, which is commonly generated by recursive
directional ligation, although several other techniques are also available. The excellent control over sequence these techniques provide, has enabled the design of ELPs with specific thermal properties and self-assembling behavior. Moreover, increasingly complex polypeptides, like ELP block copolymers can be easily created using recombinant protein engineering. The individual blocks of the ELP block copolymer can be designed to have very different conformational, chemical, mechanical, and biological properties, resulting in complex LCST behavior of the ELP block copolymers. Not only the mean polarity and total length of the polypeptide are important in predicting the \( T_t \) value of ELP block copolymers constructed, but also the distribution of the polar and apolar regions along the polypeptide chain should be taken into account when designing an ELP block copolymer with a specific \( T_t \).

As these considerations above indicate, the design of an ELP with a specific \( T_t \) and additional stimuli-responsive behavior ("smart" ELPs) is a multivariable process for which a comprehensive, quantitative model is lacking at this moment. The models established by Chilkoti and coworkers can be used to predict \( T_t \) values for a range of concentrations and lengths of a given ELP sequence and of ELPs composed of combinations of these ELP blocks. It however still remains difficult to accurately predict the \( T_t \) values of novel ELP sequences. In practice, a range of ELPs which are thought to possess the desired properties are synthesized, and subsequently the best-performing ELP is selected for further research.

4. ELP Nanoparticles

ELPs have been shown to form nanoparticles in a stimulus-responsive fashion, usually by an increase in temperature. This behavior is tuned by the design of the ELPs; diblock copolymers have been developed that rely on at least one ELP block to facilitate their thermostresponsive behavior. The other block can be another ELP sequence or a chain of different molecular entity. Figure 4 demonstrates the general principle underlying the assembly of ELP-based diblock copolymers upon transition through their \( T_t \). The two ELP blocks are designed to have a significantly different \( T_t \) for instance by using guest residues of different polarity. Because the \( T_t \) of the hydrophobic block is below that of the hydrophilic block, a range of temperatures exist under which the hydrophobic block is in its ordered, condensed state and the hydrophilic block is in its random coil conformation. Within this temperature window, amphiphilicity is thus induced and the ELP block copolymers self-assemble into particles with a narrow size distribution, whose morphology is commonly classified as micelle-like. Subsequent heating above the \( T_t \) of the hydrophilic block induces collapse of the total structure, which is generally undesirable since it prevents control over morphology and size.

5. ELP Block Copolymers

The first example of an ELP diblock copolymer which underwent temperature responsive self-assembly into nanoparticles was presented by Conticello and coworkers. For this purpose a block copolymer was developed based on two different ELP sequences, [VPGEG(IPGAG)]\textsubscript{4}\textsubscript{14} as the hydrophilic and [VPGFG(IPGVG)]\textsubscript{4}\textsubscript{16} as the hydrophobic domain; the (guest) residues were chosen to achieve a large polarity difference and trigger phase segregation at ambient temperature. In addition, the glutamic acid residues were hypothesized to facilitate micelle formation under physiological conditions by forming stabilizing charge–dipole interactions between

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**Figure 4.** General design principle illustrating the formation of ELP nanoparticles. In this example, an ELP block copolymer containing a hydrophobic and a hydrophilic block self-assembles into micelles upon heating above the \( T_t \) of the hydrophobic block. Similar structures can be formed by other diblock copolymers consisting of only one ELP sequence and a domain of different molecular entity. Further heating above the \( T_t \) of the hydrophilic block induces aggregation of the complete polymer, abolishing the well-defined morphology and size of the nanoparticles. Subsequent cooling leads to reversion of the process. Reprinted with permission from adapted from Hassouneh W., Fischer K., MacEwan S. R., Branscheid R., Fu C. L., Liu R., Schmidt M. and Chilkoti A., Unexpected multivalent display of proteins by temperature triggered self-assembly of elastin-like polypeptide block copolymers. Biomacromolecules 2012, 13, 1598–1605. © (2012) American Chemical Society.
ELASTIN-LIKE POLYPEPTIDE BASED nanoparticles: DESIGN RATIONALE TOWARD NANOMEDICINE

Figure 5. ELP diblock copolymers can form stable nanoparticles in aqueous solution. (A) At 5 °C (below the Tt of both blocks), the copolymers existed as unimers with a diameter of 7.2 ± 2.4 nm. At 40 °C (> Tt of the hydrophobic block), a population of nanoparticles with a small size distribution of 87 ± 15 nm was observed by DLS. (B) In neutral aqueous solution at 25 °C, TEM indicated the presence of spherical nanoparticles which corresponded well with the particles detected by DLS. Additionally, cylindrical aggregates were observed, which were thought to result from fusion of the smaller spherical nanoparticles. Reprinted with permission from Thermo-mimetic polypeptides. Lee T. A. T., Cooper A., Apkarian R. P. and Conticello V. P., Advanced Materials, 12. © (2000) (WILEY-VCH Verlag GmbH, Weinheim, Fed. Rep. of Germany). [40]

The finding is well as a tuneable system for the construction of ELP-based nanoparticles. This study was the first to demonstrate nanoparticle formation from ELP block copolymers. Moreover, it showed that the dynamics of nanoparticle formation using these ELP block copolymers can be adjusted by changing the temperature and pH, allowing a high degree of control over the morphology of the particles, which can find useful applications in materials science and drug delivery. In addition, the precise control over the ELP composition gives additional control over the assembly characteristics of these systems. Much work has been done since on ELP block copolymers for the assembly of nanoparticles, often directed to nanomedicine applications.

The lab of Chilkoti has extensively studied ELP nanoparticles. They developed block copolymers based on two different ELP blocks that can self-assemble into well-defined spherical nanoparticles,[34,41] By changing the ELP guest residue, a more hydrophilic ELP-[VPGVG-(VPGG)7(VPGAG)8]n, block and a more hydrophobic ELP-[VPGVG]m, block were designed and fused to create a block copolymer. Fusions of blocks with different molecular weight and different hydrophilic-to-hydrophobic ratios resulted in the formation of a series of 10 different block copolymers, with different Tt’s for both blocks. Upon heating through the lower Tt, the hydrophobic block collapsed, leading to subsequent formation of monodisperse, spherical nanoparticles which are stable over an 8–10 °C temperature range. This unimer-to-micelle Tt, scaled with the Tt of the more hydrophobic monoblock ELP-[VPGVG]m, it was slightly increased with respect to the monoblock, supposedly due to the influence of the hydrophilic block. A subsequent rise in temperature induced collapse of the hydrophilic block, leading to polydisperse, micrometer-sized aggregates (Figure 4). Varying the relative and absolute lengths of the two blocks allowed control over the critical micelle temperature (CMT), the temperature at which the block copolymers assembled into nanoparticles. Only ELP block copolymers with a hydrophilic-to-hydrophobic block length ratio between 1:2 and 2:1 showed assembly into nanoparticles. It was reasoned that the difference in solubility at higher ratios is insufficient to promote self-assembly; these block copolymers behave like unimeric ELP. The influence of the length of the hydrophobic block was also investigated, demonstrating that a hydrophobic block length of 60 monomers yields the most stable nanoparticles. Increasing this length led to a less defined micelle-to-aggregate Tt, as the nanoparticles gradually grew bigger in size until they collapsed into large aggregates. Logically, the size of the micelles was dependent on the length of the ELP block copolymer and the hydrophilic-to-hydrophobic block ratio. This study demonstrated the influence of varying the composition of the ELP block copolymer and the subsequent influence of these changes to the characteristics of the assembly process.

Similarly, another study showed that a minimum molecular weight of the ELP was needed to form stable nanoparticles. It was furthermore found that the CMT is mostly determined by the most hydrophobic ELP-block,
whereas the hydrophilic ELP-block determines the micelle-to-aggregate $T_m$. In order to circumvent this micelle-to-aggregate transition it has been reported that addition of poly(ethylene glycol) (PEG) to the solution destabilizes the coacervates of an ELP20-24$^4$ (composed of exons found in human elastin) diblock copolymer, subsequently leading to increased stability of the smaller non-aggregated nanoparticles.$^{[39]}$

To investigate if addition of another protein to the ELP diblock copolymer affects nanoparticle formation, two ≈10 kDa proteins thioredoxin (Trx; hydrophilic) and a fibronectin type III domain (Fn3; hydrophobic) were fused to the block copolymer via a protein engineering approach. These proteins were connected to the hydrophilic end to study their effect on the CMT.$^{[39]}$ Fusion of the proteins to the ELP diblock copolymers did not change the CMT as compared to the corresponding free ELP diblock copolymer, suggesting that the $T_m$ of the hydrophobic block had not changed. However, extension of the hydrophilic ELP-block did decrease micelle-to-aggregate $T_m$ for Trx, as expected for an elongation of the hydrophilic ELP block. Surprisingly, for Fn3 the effect was the opposite. This observation was explained by the hypothesis that different proteins will have different interactions with both ELP blocks, which might lead to unexpected differences in transition temperatures. These triblock polypeptides were designed with the purpose to create self-assembled nanoparticles with multivalent display of the functional proteins on the surface. Indeed, upon assembly an increase in avidity for their receptors was observed, a behavior which had been shown before for peptides.$^{[41,44,45]}$ The multivalent targeting of these well-defined nanoparticles was found to be more efficient than for large polydisperse micrometer sized aggregates.$^{[44]}$

In a recent study this multivalency was triggered, leading to an amplified uptake of a therapeutic domain into targeted cells. For this purpose an ELP diblock copolymer was designed with a cell penetrating peptide (CPP) domain at the hydrophilic end and a therapeutic domain at the hydrophobic end. Upon an extrinsic thermal trigger, these block copolymers formed micellar structures that showed multivalent display of the CPP, inducing internalization of the micelles, together with the therapeutic domain.$^{[45,46]}$ (Figure 6) These examples demonstrate some of the possibilities designed ELP nanoparticles can offer for application in drug targeting.

Targeting by receptor–ligand interactions is one way to achieve delivery of the ELP nanoparticles to a specific site in vivo; another approach, which would be particularly interesting for tumor targeting, is pH-sensitive (dis) assembly. Chilkoti and coworkers$^{[47]}$ designed an ELP block copolymer with histidine guest residues in the hydrophobic ELP block, which were thought to induce pH-sensitive behavior. As the $pK_a$ of histidine is close to physiological pH, it was envisioned that using histidines, pH-dependent self-assembly, would occur in the clinically relevant pH-range. The pH-sensitive ELP-[VPGVG(VPGGG)7(VPGAG)8]5-[VPGVG(VPGHG)4]20 was developed; upon heating from ambient to physiological temperature, these copolymers self-assembled into nanoparticles. A further increase in temperature led to a micelle-to-aggregate transition, as was also observed for pH-insensitive nanoparticles. However, because of the introduction of histidines within the ELP sequence, the $T_m$ of the hydrophobic block could be controlled by the pH. At a pH of 6.4 and lower, only a monomer-to-aggregate transition was observed. At this low pH the histidines become increasingly protonated, leading to a higher $T_m$ of the hydrophobic block that approaches the $T_m$ of the hydrophilic ELP-[VPGVG(VPGGG)4(VPGAG)10] block, resulting in a block copolymer that behaves like an ELP unimer. On the other hand, increasing the pH led to a larger difference in $T_m$ for the two
blocks, giving rise to a larger range of temperatures at which the nanoparticles were stable. Addition of Zn$^{2+}$-ions also increased the stability of the nanoparticles, making them not only temperature responsive, but also pH- and [Zn$^{2+}$]-sensitive (Figure 7). Their ability to disassemble at low pH makes these pH-responsive ELP nanoparticles interesting candidates for drug nanocarriers, for instance in targeting the acidic microenvironment of tumors or release of cargo upon internalization into the endosomes of cells.

The stability of ELP nanoparticles has been an area of concern for the implementation of ELP-based nanoparticles in vivo. Because self-assembled structures are formed by non-covalent interactions only, their stability is of particular importance for various applications in which the ELP nanoparticles come into contact with other amphiphiles, like plasma proteins and lipopeptides. One way to increase the stability of the nanoparticles after formation would be to introduce crosslinking sites. This can be done by using cysteine residues in the copolymer, which can form stabilizing disulfide bonds. Importantly, this design does not compromise the reversible assembly of the nanoparticles, because the disulfide bonds can be broken in a reducing environment. An ELP-based system, ELP-[(VPGVG)$_3$(VPGEG)$_2$]$_{10}$-[(C$_4$G$_3$)-([IPGVG]$_4$(VPGYG))$_{12/15}$ incorporating four cysteine residues at the region between the two ELP blocks was reported. The two ELP blocks were designed to have a large difference in hydrophobicity to enable micelle formation, as discussed before. However, these nanoparticles were shown to be capable of crosslinking by formation of covalent disulfide bonds upon assembly. This assembly was not reversed upon cooling below $T_t$ ($\approx$10 °C), but reduction of the disulfide bonds by the reducing agent tris(2-carboxyethyl)phosphine (TCEP) destabilized the nanoparticles. This indicates the important role the disulfide bonds play in particle stabilization.[48] To test the feasibility of these nanoparticles to be used in drug delivery applications, the uptake and release kinetics of the hydrophobic anti-inflammatory drug dipyridamole (DIP) were studied. DIP was shown to be solubilized and encapsulated in the hydrophobic core of these micelles; subsequently, the solubility of the drug in water increased 60- to 70-fold.[49] In vitro drug release studies showed a rapid initial release of DIP in the first 3 h, followed by extended release over a 40 h period. Addition of a reducing agent that disrupts the disulfide crosslinking bonds at the core–shell interface of the micelles showed an increase in the rate of release; on the other hand, increasing the hydrophobic block length slowed down the DIP release rate, probably because the solubility of DIP was higher in the more densely packed core. Although these results show that these nanoparticles can be used to optimize the release kinetics of a hydrophobic drug, the kinetic profile is not optimal since only a small fraction of the drug is gradually released with the majority either released within the first 3 h or remaining firmly associated with the particles.

A different example of crosslinking ELP diblock copolymeric nanoparticles was described by Smits et al.[50] In this approach lysine residues were incorporated at the N-terminus of the ELP block copolymer using genetic engineering. Upon self-assembly and subsequent crosslinking of the ε-amine of the lysines and the N-terminus using the naturally occurring amine-reactive reagent genipin stable nanoparticles were formed that did not disassemble upon lowering the temperature below the lowest $T_t$. Upon changing the ε-amines into azides, using a diazotransfer reaction,[51] a bioorthogonal and faster crosslinking reaction could be employed, using the strain-promoted azide-alkyne cycloaddition (SPAAC). For this purpose the azides were crosslinked using a linear molecule with a bicyclononyne (BCN) moiety at both ends, which also resulted in stable nanoparticle formation, be it much faster (Figure 8).

An approach to induce different morphological changes using temperature was recently described by Soon and co-workers. They created double temperature responsive mixed micelles, consisting of two different ELP block copolymers, ELP[(VPGAG)$_4$(VPGVG)]$_{16}$-[(VPGIG)$_4$(VPGVG)]$_{12}$ and ELP[(VPGPG)$_4$(VPGVG)]$_{16}$-[(VPGIG)$_4$(VPGVG)]$_{12}$. When the temperature was above the $T_t$ of the more hydrophobic 160 ELP block, mixed micelles were formed. One of the blocks,
perform. Even further heating through the full switch in avidity was not seen for some of the assays onto the nanoparticle. It should be noted though that this in release of fibrinogen, as the binding peptide was collapsed through the micelle, as mentioned before. Subsequent transition multivalency of the binding peptide presented on the were shown to bind fibrinogen, probably induced by fibrinogen-binding peptide. Upon micelle formation these micelles were found to bind fibrinogen upon collapse of the ELP [(VPGAG)4(VPGVG)]16 blocks. Going through the lowest Tt of the hydrophobic block. Further investigation showed that heating above this Tt decreased the hydrodynamic radius of the nanoparticle and dehydrated its hydrophobic micelle core. These observations indicate that the micelles condense when heated above the Tt of the hydrophobic block. This process is both rapid and reversible, and is accompanied by a helix-to-sheet transition, which was confirmed by circular dichroism (CD) and infrared (IR) spectroscopy. Together, these results indicate that a temperature-triggered switch of secondary structure can induce morphological changes (i.e., compactness and size) in these particles. Furthermore, this block design allows formation of nanoparticles at temperatures below the Tt of the hydrophobic block. This combination of properties can find application in stimuli-responsive nanomaterials; in particular it increases the range of possibilities for showed multi-temperature induced changes in morphology leading to differences in fibrinogen binding. Not only ELP diblock copolymers can self-assemble into defined structures. A triblock copolymer, BAB (where A is a hydrophilic and B is a hydrophobic ELP block), was designed to self-assemble into micelle-like particles at a temperature around 20 °C. To this end, the hydrophobic sequence [(IPAVG)4VPAVG]16 formed the ends of the block copolymer, separated by a [VPVG12]2 central hydrophilic block. Fluorescence measurements with pyrene demonstrated the presence of micellar structures above and below the Tt of the hydrophobic block. Further investigation showed that heating above this Tt decreased the hydrodynamic radius of the nanoparticle and dehydrated its hydrophobic micelle core. These observations indicate that the micelles condense when heated above the Tt of the hydrophobic block. This process is both rapid and reversible, and is accompanied by a helix-to-sheet transition, which was confirmed by circular dichroism (CD) and infrared (IR) spectroscopy. Together, these results indicate that a temperature-triggered switch of secondary structure can induce morphological changes (i.e., compactness and size) in these particles. Furthermore, this block design allows formation of nanoparticles at temperatures below the Tt of the hydrophobic block. This combination of properties can find application in stimuli-responsive nanomaterials; in particular it increases the range of possibilities for showed multi-temperature induced changes in morphology leading to differences in fibrinogen binding.

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the diblock copolymers formed both micelle and vesicle-like structures in aqueous solution based on indications from TEM and atomic force microscopy (AFM), which was supported by measurements of the density distribution in these particles, expressed as \( \rho \). For these diblock copolymers \( \rho = 0.91 \), which is close to the reported value of 1.0 for vesicles, and farther from the 0.77 for spherical micelles. For the diblock copolymer \( \rho = 0.83 \), which is closer to 0.77, and suggests a mixture of micelles and vesicles. Additional encapsulation of water-soluble cargo would be a nice follow-up study for these systems to show their application towards nanomedicine.

6. Hybrid Systems

The systems described previously dealt with copolymers consisting of two or three different ELP blocks. In addition, many examples have been described in literature in which an ELP can transfer its LCST properties onto molecules fused to it.\(^{[30,46]}\) These include, amongst others, the fusion of poly(aspartic acid), small hydrophobic drugs, protein receptors, single stranded DNA, virus coat proteins, silk-based polypeptides and synthetic polymers. Some of these examples will be described here.

Hybrid block copolymers consisting of ELP-[VPGVG]\(_{10}\)-KI repeats fused to a poly(aspartic acid) chain were synthesized, yielding a library of amphiphilic block copolymers capable of self-assembling into particles upon heating above the \( T_1 \). The aim was to investigate the possibility of these block copolymers to form micelles with a size close to 100 nm at a temperature around 37 °C. Particle diameter and \( T_1 \) were controlled by the ratio and the lengths of the ELP and poly(aspartic acid) blocks, with a high ELP molecular weight resulting in larger nanoparticles formed at lower temperatures. The hybrid with the largest ELP to poly(aspartic acid) ratio, ELP-[VPGVG]\(_{30}\)-KI\(_{16}\)-D\(_{32}\), gave exceptionally large nanoparticles with a large distribution as measured with DLS, which indicates the formation of adhered nanoparticles. Assembly of nanoparticles with a diameter of less than 100 nm was observed for ELP-to-poly(aspartic acid) ratios below 4.9. The capability of these nanoparticles to retain the hydrophobic dye 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS) showed these nanoparticles to be promising nanocarrier candidates.\(^{[56]}\)

When designing self-assembling block copolymers, the orientation of the blocks can be an important factor to take into account. This was illustrated by a study in which four different proteins were fused to the N- or C-terminal end of an ELP protein. The four proteins used were blue fluorescent protein (BFP), chloramphenicol acetyltransferase (CAT), thioredoxin (Trx), and interleukin-1 receptor agonist (IL1Ra). All four fusion proteins showed higher expression levels when located at the N-terminus of the ELP block. In addition, three out of four (not BFP) showed improved activity when located N-terminally. This indicates that the fusion protein should preferably be located at the N-terminus in ELP-fusions.\(^{[57]}\)

An important class of hybrid protein-based biomaterials comprising ELP is formed by the silk-elastin-like protein polymers (SELPs). These polypeptides have found widespread application in the fabrication of biomaterials mainly directed at drug delivery and tissue engineering, and have also been employed for the preparation of nanoparticles.\(^{[58–60]}\) Most of the designs usually utilize the precipitation behavior of the silk domain only, neglecting the LCST behavior of the ELP block. In a system that does combine the self-assembly of both domains, it was found that the assembly behavior of such a SELP system is a two-step process. First, the silk block formed intermolecular hydrogen bonds, leading to a micellar-like structure composed of a core of silk protein and a shell of free ELP chains. Upon heating above the \( T_1 \) of the ELP, the micellar-like structures assembled into ordered aggregates, leading to a variety of structures, including nanoparticles, hydrogels, and nanofibers. The morphology and size of the resulting aggregates could be influenced by the ratio of silk to ELP blocks, although the degree of control is restricted.\(^{[61]}\)

In another investigation, also combining the responsiveness of both materials, two different SELP systems were studied, (GAGAGAB)\(_{24}\)-ELP-[VPGVG]\(_{5}\)(VPGAG)\(_{3}\)(VPGGG)\(_{2}\)\(_{16}\), referred to as S\(_{24}\)ELP\(_{40}\), and (GAGAGAB)\(_{12}\)-spacer-ELP-[VPGVG]\(_{4}\)(VPGAG)\(_{3}\)(VPGGG)\(_{16}\) referred to as S\(_{12}\)-spacer-ELP\(_{40}\). S\(_{24}\)ELP\(_{40}\) is a diblock copolymer composed of 24 silk-like and 40 ELP repeats, S\(_{12}\)-spacer-ELP\(_{40}\) is a diblock copolymer of 12 silk-like repeats and 40 ELP repeats that are connected by a hydrophilic random coil sequence (spacer). Both these block copolymers showed pH-induced self-assembly into fibrils, which upon a change in temperature yielded “sticky” fibrils that interacted with each other, due to the hydrophobic interactions of the ELP blocks.\(^{[62]}\)

Another hybrid system that uses both the LCST behavior of ELP together with the self-assembling properties of the fusion partner, is based on the cowpea chlorotic mottle virus (CCMV) capsid protein (CP) to which a short ELP sequence (ELP-[VPGVG]\(_{4}\)(VPGAG)\(_{4}\)(VPGGG)\(_{4}\)) was introduced N-terminally.\(^{[63]}\) CCMV CPs normally form virus-like particles (VLPs) via a pH-induced self-assembly process.\(^{[64,65]}\) The presence of the ELP moiety now allowed \( T_1 \)-induced assembly to take place. When pH was used to trigger assembly, at pH 5.0 and 0.5M NaCl, the 180 ELP-CP protein subunits self-assembled into 28 nm diameter VLPs (Figure 10), which was similar to the wild type CP. ELP-induced assembly could be achieved at physiological pH 7.5 and increased salt concentration, which triggered ELP coacervation. This yielded a monodisperse population of VLPs with a diameter of 18 nm via assembly of 60 protein subunits. The tighter packing
provide a new method to assemble homo- or hetero-
over the number of hybridized hybrids. These findings
tary DNA sequences capable of forming a four-armed
of 46 bases. By designing four different partly complemen-
aggregates above (cODN). Unhybridized ODN-ELP formed micrometer-sized
their LCST behavior in the presence of complementary ODN
A second interesting feature of these ODN-ELPs involves
defined number of polypeptides through molecular recognition by a
predesigned number of complementary (bio)molecules. For instance in a number of studies DNA has
control over particle size and symmetry.
Besides all-protein based hybrid ELPs, there are also many
examples of systems in which ELPs are combined with other
(bio)molecules. For instance in a number of studies DNA has
been fused to ELP. One example combines the LCST behavior of ELP with the molecular recognition characteristics of single-stranded DNA oligonucleotides (ODN). Genetically expressed ELP-[VPGVG]<sub>n</sub> was ligated to a synthetic ODN of 46 bases. By designing four different partly complementary DNA sequences capable of forming a four-armed junction, ELP-ODN hybrids could be assembled with control over the number of hybridized hybrids. These findings provide a new method to assemble homo- or hetero-<em>n</em>-mers of polypeptides through molecular recognition by a predesigned number of complementary oligonucleotides. A second interesting feature of these ODN-ELPs involves their LCST behavior in the presence of complementary ODN (cODN). Unhybridized ODN-ELP formed micrometer-sized aggregates above <em>T</em><sub>c</sub>, whereas double-stranded ODN-ELP hybridized to cODN yielded monodisperse 39 nm particles, which are thought to resemble micelles. These ODN-ELP hybrids therefore provide a versatile system for controlling particle size, complexity and dynamics by combining the molecular recognition ability of the oligonucleotides with the LCST behavior of ELPs.

Hybrids of DNA and ELP have also been studied for gene delivery. An ELP block fused to an oligolysine block K<sub>8</sub>-ELP[(VPGVG)<sub>5</sub>(VPGLG)<sub>2</sub>(VPGAG)<sub>3</sub>]<sub>13</sub>, referred to as ELP130, ELP[(VPGVG)<sub>5</sub>(VPGLG)<sub>2</sub>(VPGAG)<sub>3</sub>]<sub>9</sub>, referred to as ELP90 and ELP[(VPGVG)<sub>5</sub>(VPGLG)<sub>2</sub>(VPGAG)<sub>3</sub>]<sub>4</sub>, referred to as ELP40, ELP[(VPGVG)<sub>5</sub>(VPGLG)<sub>2</sub>(VPGAG)<sub>3</sub>]<sub>1</sub>, referred to as ELP130, either once or twice with PEG2000 and PEG5000. This resulted in the formation of 12 hybrids, 6 linear, and 6 miktoarm polymers. As ELP in this case was the hydrophobic and PEG the hydrophilic block, these materials self-assembled into well-defined micelles upon inducing the ELP transition by increasing the salt concentration. Again, composition of the hybrid was important for the self-assembly, as the branched hybrids, ELP90-2xPEG5000 and ELP130-PEG2x-PEG5000, showed to assemble in the most well-defined nanoparticles. Another ELP-synthetic polymer hybrid was obtained by the formation of poly(oligo(ethylene glycol) methyl ether methacrylate) (poly(OGEMA)) brushes on an ELP triblock copolymer. Two of the three ELP blocks contained lysine residues, which were modified into initiator sites for the in situ atom transfer radical polymerization (ATRP) toward poly(OGEMA). This resulted in a hybrid material of which the LCST behavior of poly(OGEMA) directed the overall <em>T</em><sub>c</sub> as formation of the poly(OGEMA) brushes seemed to overrule the ELP LCST behavior. These results show that the use of ELP together with synthetic polymers can lead to interesting new materials, that can either self-assemble into well-defined
transfection of MCF-7 (human breast cancer) cells, after which expression of the target gene in these cells was detected. Bacterial production of this ELP hybrid was hampered by the introduction of the cationic block and it furthermore showed considerable cytotoxicity, in the same range as branched poly(ethylene imine) (PEI). In a follow-up study, instead of introducing a cationic block by fusion with an oligolysine, the ELP block was synthetically conjugated to a benzyl-protected poly(aspartic acid) block. This was done by polymerization of the N-carboxyanhydride of β-benzyl-l-aspartate (BLA) using the ELP block as macronitiator in the ring-opening polymerization. Subsequent amidation of the benzyl ester side chains with diethylene-triamine (DET) resulted in the formation of a cationic block. This hybrid showed the ability to form polyplexes that could be used for transfection. Furthermore, the block copolymer was found to be well tolerated by cells, with a cell viability that was significantly higher in comparison with PEI.

Also hybrids containing ELP conjugated to synthetic materials have been described. In one example a series of ELP-PEG hybrids was synthesized by combining three different ELPs that differed only in length, namely ELP[(VPGVG)<sub>5</sub>(VPGLG)<sub>2</sub>(VPGAG)<sub>3</sub>]<sub>1</sub>, referred to as ELP40, ELP[(VPGVG)<sub>5</sub>(VPGLG)<sub>2</sub>(VPGAG)<sub>3</sub>]<sub>9</sub>, referred to as ELP90 and ELP[(VPGVG)<sub>5</sub>(VPGLG)<sub>2</sub>(VPGAG)<sub>3</sub>]<sub>13</sub>, referred to as ELP130, either once or twice with PEG2000 and PEG5000. This resulted in the formation of 12 hybrids, 6 linear, and 6 miktoarm polymers. As ELP in this case was the hydrophobic and PEG the hydrophilic block, these materials self-assembled into well-defined micelles upon inducing the ELP transition by increasing the salt concentration. Again, composition of the hybrid was important for the self-assembly, as the branched hybrids, ELP90-2xPEG5000 and ELP130-PEG2x-PEG5000, showed to assemble in the most well-defined nanoparticles. Another ELP-synthetic polymer hybrid was obtained by the formation of poly(oligo(ethylene glycol) methyl ether methacrylate) (poly(OGEMA)) brushes on an ELP triblock copolymer. Two of the three ELP blocks contained lysine residues, which were modified into initiator sites for the in situ atom transfer radical polymerization (ATRP) toward poly(OGEMA). This resulted in a hybrid material of which the LCST behavior of poly(OGEMA) directed the overall <em>T</em><sub>c</sub> as formation of the poly(OGEMA) brushes seemed to overrule the ELP LCST behavior. These results show that the use of ELP together with synthetic polymers can lead to interesting new materials, that can either self-assemble into well-defined
nanoparticles, or that can influence the characteristic LCST behavior of the ELP.

Finally, an interesting group of hybrids are the systems in which small hydrophobic molecules are covalently conjugated to ELP, and that may find application in the area of drug delivery. In one example the C-terminus of a monoblock ELP was extended by a cysteine-rich, -{GGC}₈, scaffold domain that allowed the conjugation of maleimide-containing small molecules. At concentrations above 3 µM under physiological conditions, these conjugates assembled into nanoparticles. A hydrazone linker between the small molecules and the ELP carrier introduced the possibility of disassembly and release of the small molecules at low pH, as in endosomes and lysosomes. In this design, switchable amphiphilicity was created by functionalizing a hydrophilic ELP scaffold domain with hydrophobic moieties. This design can be applied for several neutral or anionic ELPs and a range of hydrophobic small molecules. More generally it has also been shown that small molecules with a logD > 1.5 are capable of introducing sufficient amphiphility to induce self-assembly into nanoparticles.¹⁷

Next to small molecules, also fusion of a monoblock ELP to a small peptide has been investigated. In this study L4F, an alpha-helical peptide, that shows potential for the reduction of inflammatory response in cardiovascular disease and liver fibrosis, was fused N-terminally to a large ELP of 192 pentapeptide repeats. These hybrids formed nanoparticles below Tₛ with a size around 50 nm. Interestingly, according to cryo-TEM and TEM data, they seemed to form unilamellar vesicles. In these vesicles, the L4F peptide is supposed to be located in the membrane, but retained the ability to inhibit the activation of hepatic stellate cells (HSC).²³

The abovementioned examples all demonstrate that ELP-based nanoparticles provide a highly versatile stimulus-responsive platform for drug loading and release, which can easily be extended to the accommodation of other functionalities, like imaging agents. As the design parameters of the ELP systems are well understood, one can create a range of tailor-made structures with the desired functionalities. An overview of the most important systems discussed in this review is listed in Table 1.

7. Applications

The specific properties displayed by ELPs, as described in the previous sections, make them ideal materials for biomedical applications. A very attractive feature is the fact that ELPs consist of amino acids, being thus completely made from natural building blocks, and therefore, are considered to be more biodegradable and biocompatible than polymers that include non-natural building blocks. Indeed, the susceptibility of a small library of ELPs to degradation by elastases and collagenases was reported recently.²⁴ Furthermore, the biocompatibility of ELPs was already shown by Urry and coworkers for their model system poly(VPGVG) and its crosslinked matrix. They performed a series of standard biological tests for the medical use of materials, which all indicated that ELPs are non-toxic, non-mutagenic, and non-immunogenic.²⁵ Most recently, the absence of cytotoxic or systemic inflammatory effects was demonstrated after subcutaneous injection of poly(VPGVG) microparticles in rats. However, after intraocular injection induced fibroblastic activity was detected.²⁶

For the nanoparticle-based systems described in this review, their most promising application is in the field of nanomedicine, in particular as systems for drug loading, targeting, and delivery. In order to efficiently use ELP nanoparticles in vivo as drug delivery vehicles,²⁶,²⁷ control over morphology, size, and polydispersity are of great importance, regarding, for instance, renal clearance, tissue penetration, and their capacity to encapsulate small molecules. The ability of ELP nanoparticles to encapsulate hydrophobic drug-like molecules has been reported in numerous publications;²⁸,²⁹ combined with the stimuli-responsive behavior of ELPs, this property provides a platform for the delivery of drugs to their desired targets. Several possible strategies have been reported; importantly, the passive release of drugs by circulating ELP nanoparticles,²¹ tumor localization by the enhanced permeability and retention (EPR) effect,⁰⁰ hyperthermia-induced assembly of ELP nanoparticles for tumor targeting,⁴⁵,⁸¹ and ligand-based targeting of ELP nanoparticles.⁸⁹ Although hyperthermia-driven targeting has been the primary focus in this field until now, other stimuli, like pH, can also be employed to target ELP nanoparticles to certain tissues (for instance tumors) or to release their contents in a specific environment (e.g., upon exposure to low pH in the endosomes). Next to this, the use of an additional linker, like a hydrazone,²¹ can also be used to induce disassembly upon endosomal entrapment. Moreover, by either fusing or covalently coupling a targeting moiety (for instance a ligand for a specific receptor) to the ELP block copolymer, ELP nanoparticles can be targeted to specific regions in vivo.⁴⁹ Reports on a complete pharmacokinetic model⁸² and tumor therapies⁸³ involving ELP nanoparticles demonstrate that these applications are under rapid development.

An interesting example of an ELP-based nanoparticle used for targeted drug delivery in vivo is based on an ELP block copolymer functionalized with an NTR tripeptide ligand, which was produced by protein engineering.⁸⁵ This NTR tripeptide is a ligand for the CD13 receptor, which is known to be upregulated in tumor vasculature. In this study it was shown that the multivalent display of the ligand on the ELP nanoparticles led to selective targeting of
<table>
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<tr>
<th>ELP sequence[a]</th>
<th>$R_h$ (nm)</th>
<th>Morphology</th>
<th>Stimuli</th>
<th>Threshold</th>
<th>Functionalization</th>
<th>Comments</th>
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<tr>
<td>ELP-{VPGEG}$<em>4${VPFG}$</em>{14}$, VPGEG-{VPFG}$<em>6${VPGV}$</em>{14}$, VPGFG</td>
<td>87 ± 15 (diameter @40°C)</td>
<td>Spherical, Cylindrical</td>
<td>Temperature, pH</td>
<td>11.9°C @ high pH, 9.5°C @ low pH</td>
<td>–</td>
<td>–</td>
<td>Lee et al.[40]</td>
</tr>
<tr>
<td>ELP-{[VPGVG]}$<em>{40}$-{[VPGG]}$</em>{40}$, ELP-{[VPGVG]}$_{60}$</td>
<td>32.0</td>
<td>Spherical</td>
<td>Temperature</td>
<td>36°C</td>
<td>Targeting peptides</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ELP-{[VPGVG]}$<em>{40}$-{[VPGG]}$</em>{40}$, ELP-{[VPGVG]}$_{60}$</td>
<td>30</td>
<td>Spherical</td>
<td>Temperature, pH</td>
<td>See Figure 7</td>
<td>Conjugated imaging agent</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ELP-{[VPGVG]}$<em>{40}$-{[VPGG]}$</em>{40}$, ELP-{[VPGVG]}$_{60}$</td>
<td>25</td>
<td>Spherical</td>
<td>Temperature, limited pH effect</td>
<td>10°C</td>
<td>Stabilization by disulfide bonds, hydrophobic drug reservoir</td>
<td>Reducing agent needed for reversion of assembly</td>
<td>–</td>
</tr>
<tr>
<td>ELP-{[VPAVG]}$<em>{40}$-{[IPAVG]}$</em>{40}$-{[VPGVG]}</td>
<td>Variable (depending on temperature)</td>
<td>Spherical</td>
<td>Temperature</td>
<td>25°C</td>
<td>$T_c$ governs particle density</td>
<td>Little control over particle dissociation</td>
<td>–</td>
</tr>
<tr>
<td>ELP-{[VPGVG]}$<em>{40}$-{[VPGG]}$</em>{40}$-[VPGVG]$<em>{40}$-[VPGVG]$</em>{40}$</td>
<td>96 ± 2</td>
<td>Spherical core-shell or vesicle</td>
<td>Temperature</td>
<td>~50°C</td>
<td>Hollow vesicle-like particles</td>
<td>Strong hysterisis behavior</td>
<td>–</td>
</tr>
<tr>
<td>(VPGVG)$_n$, D$_m$</td>
<td>Variable</td>
<td>Spherical</td>
<td>Temperature</td>
<td>Variable</td>
<td>–</td>
<td>–</td>
<td>Fujita et al.[56]</td>
</tr>
<tr>
<td>ELP-{[VPGVG]}$<em>{40}$-{[VPGG]}$</em>{40}$-[GCC-drug]$_{40}$</td>
<td>21.1 ± 1.5</td>
<td>Spherical</td>
<td>Drug conjugation</td>
<td>–</td>
<td>pH-sensitive drug release</td>
<td>Hydrophobic drugs only</td>
<td>–</td>
</tr>
<tr>
<td>ODN- ELP-{[VPGVG]-m}</td>
<td>39</td>
<td>Tetrameric or compound micelle[b]</td>
<td>Temperature, ODN hybridization</td>
<td>65°C</td>
<td>ODN recognition</td>
<td>Large batch-to-batch variation</td>
<td>–</td>
</tr>
<tr>
<td>ELP-{[VPGVG]}$<em>{40}$-[VPGG]$</em>{40}$-[GCC-drug]$_{40}$-[CMV]CP</td>
<td>diameter: 28 or 18 nm (depending on stimulus)</td>
<td>Spherical</td>
<td>Salt concentration, pH</td>
<td>pH 5 @ low [salt], pH 7.5 @ high [salt]</td>
<td>Virus capsid, possibly hollow architecture</td>
<td>–</td>
<td>Van Eldijk et al.[63]</td>
</tr>
<tr>
<td>ELP-{[VPGVG]}$<em>{40}$-[VPGG]$</em>{40}$-[VPGG]$_{40}$-[CMV]CP</td>
<td>~50</td>
<td>Spherical</td>
<td>Salt concentration</td>
<td>–</td>
<td>Well-defined micelles</td>
<td>Miktoarm nanoparticles most well-defined</td>
<td>–</td>
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This table summarizes and compares the properties of the most relevant nanoparticles described in this paper: their hydrodynamic radius ($R_h$), morphology, the stimuli that trigger their formation and the corresponding threshold values and the functionalization that was demonstrated for these designs. For reasons of clarity, for each literature reference only the most important design is described. In addition, it should be noted that threshold values also depend on factors like ELP and salt concentration. [a]Hydrophobic domains are bold; [b]Depending on the complementary ODN.
tumor vasculature by targeting the CD13 receptor, resulting in a higher localization of the functionalized nanoparticles to tumors than non-functionalized nanoparticles.

Next to drug delivery also other applications for ELP-based nanoparticles in nanomedicine have been investigated. One such an example is the use of ELP-based nanoparticles in the development of vaccine carriers. In this study an antigenic peptide from *M. tuberculosis* (Ag) was N-terminally fused to the ELP block copolymer [(VPGVG)2VPGE(VPGVG)2]20(VPGIG)60. This block copolymer self-assembled into micellar structures, where the Ag was located at the exterior of the nanoparticles, as it was fused to the hydrophilic ELP block. Immunization of mice with these Ag-conjugated assemblies led to the induction of an immune response, where IL-5 and IgM and IgG antibody responses were up-regulated. This immune response could not be detected for the non-functionalized nanoparticles. Also the use of ELP-based nanoparticles in gene therapy has gained interest. As mentioned before, ELP nanoparticles can be sensitive to disassembly, especially when used in vivo, where other amphiphilic molecules can disrupt the assembly. This makes it necessary to develop crosslinking methods in order to introduce additional stability in these nanoparticles. Several examples have been described, for example using cysteines, but also using lysine side chains for crosslinking. Though these examples have been described and show a good increase in stability of the nanoparticles, still room for improvement remains, and new crosslinking technologies are needed in order to make ELP nanoparticles stable for use in drug delivery.

Most ELP based nanoparticles have a micellar structure, and can encapsulate hydrophobic drugs. However, it has not yet been reported that these materials could also encapsulate hydrophilic drugs. ELP-based materials that can self-assemble into vesicular structures, of which a few examples have been reported, would hold potential for the encapsulation of hydrophilic molecules. It would be of great value to also have access to such ELP-based vesicles, as this would greatly extend the drug delivery applications for which these materials can be used.

As many different ELP-based nanoparticles have been developed, the focus of the current research is now more and more on drug delivery and in vivo applications. In this regard it would be interesting to investigate the possibilities of drug release within the cell and, concomitantly, endosomal escape routes. For this purpose one could for example optimize the pH-responsiveness of the ELP-based particles in order to induce disassembly and release upon entrapment of the particles in the endosomes and lysosomes. Or one could make use of nanoparticles that are crosslinked by disulfide bonds, which would disassemble in a reducing environment. Several options for drug delivery into the cell have been touched upon, but more research is needed to confirm these methods or develop new

8. Conclusion

This review has described the different designs for ELP-based nanoparticles that have been reported in the literature, and has thereby illustrated the versatility of ELPs for the development of nanoparticles. Progress in the genetic engineering of ELPs has given access to the use of these ELPs by expression in *E. coli*, but also other organisms like yeast and plants. Next to this, it has enabled full control over the exact sequence of the ELP, and thereby simplified the introduction of functional groups at exact locations and the production of ELP-fusion proteins. In addition, control over the ELP sequences provides control over the parameters that modulate the LCST behavior of ELPs (length, guest residues). The temperature responsive behavior of ELPs makes them very interesting for the development of “smart” materials, which can be targeted to specific locations in the body. In combination with the biocompatibility of ELPs, this has made these materials very interesting for in vivo use, for example, in tissue engineering and drug delivery.

Though the design parameters for the temperature responsive behavior of unimeric ELPs are known, and can be controlled with quite good accuracy, it is hard to predict their LCST behavior, when ELPs are used in di-/triblock copolymers or hybrid materials. For example in some reports, fusions of ELP diblock copolymers with proteins with different hydrophilicities did not result in a change of the CMT, whereas fusion of a peptide or protein to unimeric ELPs did show a change of *T*<sub>c</sub>. This demonstrates it is difficult to extrapolate the LCST behavior of unimeric ELPs to their behavior when used to form block copolymers or hybrids. This is an area in which more research is needed in order to develop a general strategy for designing ELP-based block copolymers or hybrids with a specific LCST behavior.

As mentioned before, ELP nanoparticles can be sensitive to disassembly, especially when used in vivo, where other amphiphilic molecules can disrupt the assembly. This makes it necessary to develop crosslinking methods in order to introduce additional stability in these nanoparticles. Several examples have been described, for example using cysteines, but also using lysine side chains for crosslinking. Though these examples have been described and show a good increase in stability of the nanoparticles, still room for improvement remains, and new crosslinking technologies are needed in order to make ELP nanoparticles stable for use in drug delivery.
methods for drug delivery within the cell, escaping or circumventing endosomal uptake.

The systems described in this review are some selected examples to illustrate the potential ELPs, and ELP-based nanoparticles in particular, provide when using the right design. We have given an overview of the parameters that can be used to fine tune the design as well as the possibilities these ELP-based systems have with regard to stimulus responsive assembly, morphology, crosslinking and formation of hybrid particles. With these systems in hand, now is the time to investigate in detail their potential for nanomedicine.

9. Appendix

Author Contributions
The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. These authors contributed equally.

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