Self-assembly or coassembly of multiresponsive histidine-containing elastin-like polypeptide block copolymers

Citation for published version (APA):
Abdelghani, M., Shao, J., Le, D. H. T., Wu, H., & van Hest, J. C. M. (2021). Self-assembly or coassembly of multiresponsive histidine-containing elastin-like polypeptide block copolymers. Macromolecular Bioscience, 21(6), Article 2100081. https://doi.org/10.1002/mabi.202100081

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DOI:
10.1002/mabi.202100081

Document status and date:
Published: 01/06/2021

Document Version:
Publisher’s PDF, also known as Version of Record (includes final page, issue and volume numbers)

Please check the document version of this publication:
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Download date: 17. Oct. 2024
In this study a histidine containing elastin-like polypeptide (ELP) diblock copolymer is described with multiresponsive assembly behavior. Self-assembly into micelles is examined by two methods. First, the self-assembly is triggered by the addition of divalent metal ions, with Zn\(^{2+}\) being the most suitable one. Increasing the Zn\(^{2+}\) concentration stabilizes the nanoparticles over a large temperature window (4–45 °C). This diblock exhibits furthermore pH-responsiveness, and particles disassemble under mildly acidic conditions. Second, the coassembly of this ELP with a diblock ELP is examined, which is not responsive to pH and metal ions. Coassembly is triggered by heating the ELPs quickly above the transition temperature of the less hydrophobic block, which results in stable nanoparticles without the need to add metal ions. This novel ELP system offers a versatile modular nanocarrier platform that can respond to different stimuli and can be tuned effectively.

1. Introduction

Assemblies of elastin-like polypeptides (ELPs) are an interesting class of nanoparticles (NPs) for biological applications, as they provide many benefits such as biocompatibility and biodegradability.\(^{[1]}\) Moreover, ELP-based nanoparticles are reported to show improved systemic circulation, and biodistribution.\(^{[2]}\) ELPs are derived from human tropoelastin, the precursor of elastin, an essential protein that provides the required elasticity to connective tissues. Tropoelastin consists of well-conserved sequences, including a hydrophobic domain of Val-Pro-Gly-Val-Gly (VPGVP). ELPs are composed of multimeric repeats of these domains and were first developed by Urry and coworkers as a model system to study elastin-related diseases.\(^{[3]}\) Since then, a large variety of ELP sequences have been designed based on repeats of the pentapeptide VPGXG where X named the guest residue, can be any amino acid except proline. ELPs are typically noted as \[A_XB_YC_Z-n\], where A, B, and C denote the guest residues, X:Y:Z their ratio within the ELP block, and n the number of pentapeptide repeats. ELPs are considered as intrinsically disordered proteins (IDPs). Such kinds of proteins have repetitive low complexity sequences rich in proline and glycine with flexibility, enabling them to undergo phase transitions when exposed to different stimuli such as temperature.\(^{[4,5]}\)

ELPs show lower critical solution temperature (LCST) behavior, which means that the polypeptides reversibly switch between a hydrophilic state at lower and an aggregated state at elevated temperatures. Changing the fourth residue alters the transition temperature (\(T_t\)), giving flexibility in the polypeptide design.\(^{[6,7]}\)

When two ELP sequences are fused, a polypeptide structure is created with two transition temperatures. Upon heating, the block with the lowest \(T_t\) will undergo a conformational change and become hydrophobic, thereby turning the block copolypeptide amphiphilic. This will result in self-assembly, for example into spherical micelles with a dehydrated hydrophobic core and soluble hydrophilic corona. In order to have stable micellar nanoparticles for biomedical applications, there should be a temperature difference between the hydrophobic block transition and the bulk transition of the hydrophilic block of around 10 °C.\(^{[8,9]}\) In addition, charged amino acids such as histidine, lysine, and glutamic acid can be used to install pH-sensitive behavior in the nanoparticles. This is particularly relevant for cancer therapy. The tumor microenvironment exhibits a lower pH of 6.2–6.9 compared to healthy tissue (pH 7.4–6.4).\(^{[10,11]}\) This allows the design of pH-responsive ELP-based nanoparticles which can undergo programmed disassembly within a tumor when, due to protonation of ELP residues, the increase in polarity leads to a decrease in transition temperature. This would lead to a more enhanced accumulation of the particles and the bioactive ingredients they transport in the tumor cells, accompanied with greater
For example, the [V₁H₄]-based block copolymer adopts a hydrophobic, aggregated state at physiological pH, which is switched to a hydrophilic state in acidic environments due to the protonation of the histidine residues. Another interesting feature of [V₁H₄]-based ELPs is the metal chelating features of the histidine residues. Imidazoles are known to interact with many divalent metal ions. This interaction can also be used to tailor the responsiveness and stability of ELP based micelles. Indeed, it was shown that [V₁H₄]-based ELPs can form nanoparticles at 37 °C, which decreased in size and became more stable when Zn²⁺ ions were added. Although histidine-rich ELP sequences have shown interesting results in vitro and in vivo, a systematic investigation of their stimulus responsive features remains underexplored.

An important development in the field of peptide-based nanoparticle design is to include multiple functionalities and physicochemical characteristics via the coassembly of different building blocks. Coassembly has also recently been applied to ELP building blocks. For example, when two ELP diblocks with the same hydrophobic sequence and a different hydrophilic corona were mixed, coassembled micelles were formed at the Tₘ of the hydrophobic block. Upon further heating, the less hydrophilic block of the ELPs constituting the corona collapsed, exposing more effectively the most hydrophilic ELP sequence. We have recently demonstrated that ELPs with two different hydrophobic domains can also be coassembled in the same particle. Upon fast heating, a difference in transition temperature between hydrophobic blocks was overruled, leading to kinetically trapped stable particles composed of both building blocks. This method was also used to form coassembly coacervates of ELP monoblocks and diblocks with the potential for encapsulation of bioactive macromolecular cargo.

Until now, this method of coassembly has been performed with hydrophobic blocks that were not susceptible to any other trigger than temperature. The aim of this study is to investigate whether the presence of additional stimulus-responsive units in the hydrophobic part of mixtures of two ELP diblock copolymers could affect their assembly behavior (Scheme 1). For this purpose, first, the His-containing diblock ELP [I₁H₄-60][A₃G₂-60] was produced and its stimulus-responsive character and assembly behavior were investigated. Second, the coassembly of [I₁H₄-60][A₃G₂-60] with [A₃G₂-60][I-60] was studied in presence and absence of zinc ions and as a function of pH. The great variety in function installed in the particles by the combination of stimulus-responsiveness and coassembly makes this class of polypeptide nanoparticles of great interest for application as smart delivery devices.

2. Results and Discussion

2.1. Design of a Stimulus Responsive ELP Diblock

Our ELP block copolymer design was based on developing a hydrophilic polymer with stimulus-responsive features other than temperature that could be used to induce amphiphilic properties. This responsive character would allow the formation of stable nanoparticles upon interacting with either metal ions, changes in pH or coassembly with other ELP diblocks. The block copolypeptide was designed to be hydrophilic enough to prevent undesired aggregation, without prohibiting purification protocols that rely on the aggregation of micelles by addition of salts. By exploiting other stimuli for assembly than mere temperature, particle stability could be achieved over larger temperature windows, which should be beneficial for characterization and formulation. Previous studies reported the use of His-rich ELP with valine as additional guest residue (V₁H₄). These ELP diblocks formed micelles at 37 °C which responded upon interacting with metal ions by shrinking in size. In our design, isoleucine was chosen as aliphatic amino acid, next to the stimulus-responsive histidine as guest residues for the hydrophobic core block in a ratio of 1 to 4. We selected a ratio of alanine and glycine of 3 to 2 as guest residues for the hydrophilic block, forming the corona of the micellar structure. Both blocks had equal lengths of 60 pentapeptides. We set the hydrophilic:hydrophobic block length ratio in the range of 1:1 as previous studies showed that this block ratio led to formation of micelles with hydrodynamic radii around 20–40 nm.

2.2. Expression and Purification of ELP [I₁H₄-60][A₃G₂-60]

We utilized the recursive directional ligation method (RDL) to create recombinant plasmids harboring the ELP-encoding gene. The plasmids were digested with either type II restriction enzymes AcuI or BseRI, which generate the same overhang but cut
on different sites of the gene, and BglI. The generated fragments were then ligated to obtain a fusion of both reading frames. The ligation product was transformed into *Escherichia coli* XL1-Blue cells. After the successful construction of the plasmids, the clones were transformed into *E. coli* BLR-DE3 cells. Protein expression was performed in auto induction medium (AIM), which induced production of target protein automatically, usually near saturation at high cell density.²²

After protein expression, we optimized the protein purification methods according to the expected properties of the assigned ELP. For the His-rich polypeptide, the pellet was resuspended in ethylene diamine tetraacetate- (EDTA)-free buffer to facilitate the protein aggregation induced by salt. EDTA is a well-known chelating agent that forms stable complexes with metal ions.²⁶ The interactions between the His-containing ELPs and the metal ions present in the buffer help salt-induced aggregation of ELPs, which could be interfered by EDTA.

ELP purification was performed using salt-induced precipitation at relatively higher temperature. In accordance with the Hofmeister series, it was found that a saturated solution of the more kosmotropic ammonium sulfate was very effective in protein purification. We precipitated the ELPs using 20–40 vol% of a saturated ammonium sulfate solution at 25 °C. We adopted different rounds of inverse transition cycling (ITC) purification. The first round employed pH-dependent precipitation in which we precipitated the polypeptide using basic pH conditions (8.0–9.0) during the hot ITC cycle. At basic pH the histidines are neutralized and subsequently the T of the hydrophobic block will be decreased. At the cold ITC spin the ELP-containing pellet was resuspended in low pH (6.0) phosphate buffered saline (PBS) to allow ELP disassembly and removal of *E. coli* contaminants after centrifugation. The second purification step employed the usage of EDTA-free buffer that prevented the chelation of complex metal ions allowing histidine residues to form stable complexes with the metal ion content present in the medium.²² After 3 ITCs the polypeptides were finally purified using size-exclusion chromatography (SEC) to remove minor residual impurities. The purification procedure was visualized using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 1). Compared to the protein ladder, the ELPs migrated further than the expected *M*<sub>W</sub> because of the high content of histidine residues; similar results were previously observed by McPherson et al. and Meyer and Chilkoti.²⁸,²⁹ To further verify the *M*<sub>W</sub> of the ELPs, purity and exact mass of the protein were determined using qTOF mass spectrometry (Table 1).

### 2.3. Physicochemical Characterization of the Responsiveness of ELP Nanoparticles to Metal Ions and pH

To investigate the LCST behavior of the ELPs, we measured the transition temperature at different protein concentrations (2.5–40 × 10<sup>−6</sup> m) using UV–vis spectroscopy. As expected, it was not feasible to measure the phase transition within 10–60 °C without the presence of divalent ions. Due to the presence of 48 histidine residues distributed along the hydrophobic core, metal ions were required to complex with the imidazole nitrogen of the histidines to stabilize the assembly. Based on literature reports,³⁰ addition of 20 × 10<sup>−6</sup> m of Zn<sup>2+</sup> as chloride salt to the ELP solution was used to induce the LCST behavior of the ELPs. The absorbance of [I<sub>1</sub>H<sub>4</sub>-60][A<sub>3</sub>G<sub>2</sub>-60] at 350 nm was measured during a temperature ramp experiment between 10 and 60 °C (0.2 °C min<sup>−1</sup>).

**Figure 2** shows the absorbance at concentrations of 40, 20, 10, and 2.5 × 10<sup>−6</sup> m ELP in PBS, pH 7.4 supplemented with 20 × 10<sup>−6</sup> m Zn<sup>2+</sup>. The absorbance was slightly increased when the nanoparticles were formed. When the temperature increased, the block copolymer underwent an inverse temperature transition resulting in a collapse of the hydrophobic core forming micellar nanoparticles, surrounded by a solvated hydrophilic corona. This formation of the micellar nanoparticles caused a linear increase in the absorbance between 25 and 45 °C. Upon raising the temperature above 47 °C the transition from nanoparticles to the aggregated state occurred. In addition, we observed that at lower concentrations of the ELPs the transition temperature was increased.²⁸ For the lowest concentration of ELP assembly could only be observed upon addition of 60 × 10<sup>−6</sup> m Zn<sup>2+</sup>.

Next, the stability of micelle formation of the [I<sub>1</sub>H<sub>4</sub>-60][A<sub>3</sub>G<sub>2</sub>-60] diblock as a function of Zn<sup>2+</sup> concentration was further analyzed. We started with addition of 20 × 10<sup>−6</sup> m of Zn<sup>2+</sup> as chloride salt to a PBS solution of the ELP. The mixture was incubated at room temperature (RT) for 15 min. The samples were then

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**Table 1.** Yield and measured masses of [I<sub>1</sub>H<sub>4</sub>-60][A<sub>3</sub>G<sub>2</sub>-60] ELP. The expected mass was calculated with ProtParam, ExPASyonline software. The actual mass was measured with qTOF.

| Protein                  | Yield [mg L<sup>−1</sup>] | Theoretical *M*<sub>W</sub> [gmol<sup>−1</sup>] | Measured *M*<sub>W</sub> [gmol<sup>−1</sup>] |
|--------------------------|--------------------------|-----------------------------------------------|-----------------------------------------------|
| [I<sub>1</sub>H<sub>4</sub>-60][A<sub>3</sub>G<sub>2</sub>-60] | 10–20                    | 49 349.38                                     | 49 349.60                                     |
Figure 2. Phase transition behavior of [I,H₄-60][A₃G₂-60] copolymer. A) Above 25 °C, hydrophobic interactions between the segments of the hydrophobic [I,H₄-60] resulted in micelle formation and a small increase in the absorbance. A sharp increase in the absorbance was observed when the [A₃G₂-60] block collapsed, resulting in a transition from nanoparticles to aggregates. B) Absorbance of the [I,H₄-60][A₃G₂-60] assemblies at different protein concentrations. In case of the 2.5 × 10⁻⁶ M concentration, 60 × 10⁻⁶ M Zn²⁺ was used to ensure particle formation.

Figure 3. Example of DLS analysis of zinc-induced assembly. A) ELPs in PBS pH 7.4 at 37 °C. With no zinc ions present, the polypeptide remained soluble (red). The addition of 60–80 × 10⁻⁶ M Zn²⁺ induced the formation of well-defined nanoparticles of around 40 nm in diameter with PDI ≈ 0.1 (black). Protein concentration was 10 × 10⁻⁶ M. B) Cryo-TEM image of ELP micelles. Scale bar = 50 nm.

We also observed that the amount of zinc ions to induce and stabilize the assemblies differed depending on the ELP concentration and the buffers or media in which the ELPS were prepared. We prepared different solutions of the nanoparticles in PBS, HEPES, and OptiMEM in final concentrations of 10 × 10⁻⁶ M with the addition of 60–80 × 10⁻⁶ M Zn²⁺. The DLS measurements confirmed the presence of monodisperse nanoparticles of the same size among all the ELP solutions (Figure 4). The addition of >100 × 10⁻⁶ M Zn²⁺ could even stabilize the nanoparticles at 4 °C (Table 2). The micellar assembly process was reversed by chelation of Zn²⁺ with EDTA. Table S2 (Supporting Information) shows an overview of the amount of the zinc ions needed to...
stabilize the ELP nanoparticles in different media. Generally, the lower the ELP concentration the higher the zinc amount needed. To study in more detail the effect of the type of metal ions on the self-assembly of the nanoparticles, we tested other ions such as Mg²⁺, Ca²⁺, and Ni²⁺. All the ions were prepared as dichloride salts and added to different dispersions of the nanoparticles in PBS, HEPES, and OptiMEM. The ELP solutions were prepared in concentrations of 10⁻⁶ M. The mixtures were incubated at RT for 15 min to induce metal-histidine complexation. We observed using DLS measurements that Ni²⁺ ions had an assembly-inducing effect on ELPs prepared in PBS buffer and OptiMEM, but not in HEPES, whereas only Zn²⁺ induced the assembly in all solutions (Table 3).

Also, the pH responsiveness was evaluated by DLS. We prepared 10⁻⁶ M ELP solutions in PBS at different pH values; 7.4, 6.5, 5.5, and 4.5 with the addition of 60 x 10⁻⁶ M ZnCl₂. The ELP nanoparticles remained stable at pH 7.4 and 6.5 and disassembled as the pH was decreased to 5.5 (Figure 5). At pH values below 6.0, the imidazole side chains are protonated, become hydrophilic, and lose their affinity for Zn²⁺. These results are consistent with the V₇H₄₄-based nanoparticles developed by Huang et al.,[14] but in contrast to the behavior of the ELP [V₄G₇A₈-80][V₄H₄-100] nanoparticles developed by Callahan et al.[13] Their particles remained assembled even after lowering the pH, which could be a result of the longer hydrophobic block.

To confirm the micellar shape of the ELP [I₁H₄-60][A₃G₂-60] nanoparticles, we examined their topology using hollow fiber-flow field-flow fractionation (HF5). The results showed that the nanoparticles had an average hydrodynamic radius of 24.4 ± 1.0 nm and the ratio between the radius of gyration (Rg) and the hydrodynamic radius (Rh) was 0.6 indicating spherical micellar nanoparticles with a condensed hydrophobic core (Figure 6).

### 2.4. Effect of Coassembly on the Stability of the ELP Nanoparticles

After having established the effect of metal ion concentration and pH on the self-assembly of ELP [I₁H₄-60][A₃G₂-60], we now investigated how these stimulus responsive features could be included in a coassembly approach with an ELP not sensitive to pH and metal ions. Coassembly of two ELPs with different hydrophobic blocks in well-defined nanoparticles is achieved upon fast heating above the transition temperature of the less hydrophobic block. By mixing a responsive with a nonresponsive hydrophobic block, the sensitivity to stimuli could possibly be altered. To investigate the effect of the coassembly approach on the stability of the nanoparticles, [I₁H₄-60][A₃G₂-60] was mixed with [A₃G₂-60][I₁-60] in the presence and absence of zinc ions and as a function of pH. From now on, we refer to [I₁H₄-60][A₃G₂-60] as ELP 1 and [A₃G₂-60][I₁-60] as ELP 2. ELP 2 was previously produced and characterized by Pille et al.[21] ELP2 was shown to form nanoparticles at nanomolar concentrations when heated above the Tc of the hydrophobic block. Table 4 shows a brief comparison between both ELP diblocks.

Taking advantage of their properties, we utilized a simple coassembly between both diblocks with preset heating speed, molar ratios, and salt concentrations. In order to evaluate the coassembly of the ELP diblocks, we prepared protein solutions of 10 x 10⁻⁶ M in PBS and mixed the diblocks at different

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**Table 2.** Effect of Zn²⁺ concentration and temperature on micelle stabilization. The protein concentration was 10 x 10⁻⁶ M. SD = standard deviation.

| Temperature [°C] | ZnCl₂ [x 10⁻⁶ M] | Hydrodynamic radius [nm] | Pdi |
|------------------|------------------|--------------------------|-----|
| 37               | 0                | 2.6 ± 0.9 (no micelles)  | 1.0 |
| 37               | 60               | 18.1 ± 4.8               | 0.06|
| 25               | 60               | 19.7 ± 5.0               | 0.08|
| 4                | 60               | 4.2 ± 1.1 (no micelles)  | 1.0 |
| 4                | >100             | 20 ± 4.7                 | 0.16|

**Table 3.** Overview of the concentrations (x 10⁻⁶ M) of different metal ions required to stabilize the ELP assemblies. Protein concentration was 10 x 10⁻⁶ M.

|        | PBS   | HEPES | OptiMEM |
|--------|-------|-------|---------|
| Zn²⁺   | 60–80 | 60–80 | 80      |
| Ca²⁺   | No response up to 100 x 10⁻⁶ M | No response up to 200 x 10⁻⁶ M | 80 |
| Mg²⁺   | No response up to 200 x 10⁻⁶ M | No response up to 40 x 10⁻⁶ M | 40 |
| Ni²⁺   | 40    | No response up to 40 x 10⁻⁶ M | 40 |

**Figure 5.** pH-responsiveness of His-rich ELP nanoparticles. Below pH 6.5, the nanoparticles disassembled in presence of 60 x 10⁻⁶ M ZnCl₂. Protein concentration was 10 x 10⁻⁶ M.

**Figure 6.** Hollow fiber-flow field-flow fractionation of the ELP nanoparticles. The nanoparticles showed an average Rg of 24 nm and average Rh of 15.2 nm.
Table 4. Overview of the assembly behavior of ELPs. Average values obtained using AF5 and DLS. Protein samples were prepared at 10 \times 10^{-6} \, \text{m} in PBS pH 7.4.

| Protein | Measured $M_w$ [g mol$^{-1}$] | First $T_t$ [°C] | Second $T_t$ [°C] | $R_h$ [nm] | $R_g$ [nm] | $R_h/R_g$ | Pdl | Shape   |
|---------|-------------------------------|-----------------|-----------------|-----------|-----------|-----------|-----|---------|
| ELP1    | 49 149.60                     | 20$^{a)}$       | 47              | 24.0      | 15.2      | 0.63      | 0.06| Micelles|
| ELP2    | 48 198                        | 21              | 57              | 29.3      | 17.1      | 0.706     | 0.03| Micelles|

$^{a)}$ Assembly with addition of 60 \times 10^{-6} \, \text{m} \text{Zn}^{2+}.

Figure 7. A) DLS measurements of coassembly-induced stability of [I$_1$H$_4$-60]-based nanoparticles. Zinc-stabilized nanoparticles (ELP1) exhibit the same size as the ELP1:ELP2 mixed micelles without zinc. ELP1 was mixed with ELP2 followed by heating at 37 °C for 3 min. Protein concentration was 10 \times 10^{-6} \, \text{m}. B) Cryo-TEM image of coassembled ELP1/ELP2 nanoparticles. Scale bar = 50 nm.

percentages. The mixed polypeptides were allowed to form nanoparticles at 37 °C for 3–5 min. Then, the nanoparticles were characterized using DLS. During the measurements, it was important to determine size distributions by number intensity to detect the presence of monomers in solution. In case of the formation of stable coassemblies, size distribution values by intensity, number, and volume should be consistent.

DLS data showed that only 5–10% of ELP2 was enough to achieve stable coassembled particles without the addition of Zn$^{2+}$ ions. Increasing the ratio of ELP2 did not affect the size of the coassemblies (Figure 7A). Similarly, the coassembled nanoparticle is also stable for at least 24 h, confirmed by DLS (data not shown). The ELP2 diblock determined the properties of the coassembled micelles; the self-assembly was controlled by the transition temperature ($T_t$) of the ELP2 hydrophobic block.$^{[31]}$ Furthermore, the morphology of coassembled ELP nanoparticles was confirmed using cryo-TEM (Figure 7B). Zinc-stabilized ELP1 nanoparticles normally dissociate when either the pH decreases below 6.0 or upon the addition of chelating agents such as EDTA. Interestingly, ELP1 nanoparticles lost their pH-responsiveness when coassembled with ELP2. The coassembled particles stayed intact and only slightly increased in size when the pH reached 4.5. At pH values between 8.0 and 4.5, it is not feasible to form ELP1 nanoparticles regardless of the ionic strength of the buffer in absence of Zn$^{2+}$. The presence of ELP2 particles and the absence of free ELP in this pH window confirms that the ELP1 monomers coassembled with ELP2. Moreover, the mixed micelles disassembled when the temperature decreased below 25 °C. This contrasts with the Zn$^{2+}$-stabilized nanoparticles that are stable at 4 °C (Figure 8). Moreover, we investigated the effect of the addition of zinc to stabilize the ELP1-ELP2 mixture in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) (DMEM-10% FBS). ELP2 does not form stable particles in DMEM-10% FBS while ELP-1 requires 120 \times 10^{-6} \, \text{m} \text{ZnCl}_2 for stabilization. When mixing ELP1 and ELP2, the amount of Zn$^{2+}$ necessary to attain stable nanoparticles decreased to 40 \times 10^{-6} \, \text{m}. This proves the synergistic interaction between the hydrophobic blocks. Addition of zinc can be beneficial to stabilize the mixed micelles for storage after formulation of the nanoparticles and encapsulation of sensitive cargos such as siRNA or fast degrading drugs. If the removal of zinc is needed, EDTA can be added to chelate the ions.

3. Conclusion

Elastin-like polypeptides (ELPs) exhibit many advantages as building blocks for drug carriers. They are biocompatible and biodegradable as well as demonstrate low toxicity. ELPs can self-assemble upon changes in temperature via a process known as lower critical solution temperature (LCST) behavior. When ELP-diblock copolypeptides are used with blocks with different transition temperatures well-defined nanoparticles can be formed. In this study we focused on the development of an interesting biocompatible and pH-responsive diblock, [I$_1$H$_4$-60][A$_3$G$_2$-60] (ELP1). This sequence exhibits pH-responsiveness and disassembles in the mildly acidic conditions that characterize the extracellular regions of tumor cells. The His-rich block copolymer is hydrophilic and fully water soluble at physiological conditions. Self-assembly into micelles could be triggered by the addition of divalent metal ions, with Zn$^{2+}$ being the most suitable one. Zinc is a nontoxic metal ion and has a tolerable plasma concentration of 200 \times 10^{-6} \, \text{m}.$^{[32]}$ Increasing the Zn$^{2+}$ concentration
stabilized the nanoparticles over a large temperature window (4–45 °C). Also, the nanoparticles showed a pH-triggered disassembly when the pH of the incubation conditions was changed from physiological pH 7.4 to pH 5.5 (early endosomal pH) and lower. We further explored the potential of ELP1 in its coassembly with the [A3G2-60][I-60] diblock (ELP2) which is not responsive to pH and metal ions. Combination of ELP1:ELP2 mixtures resulted in stable nanoparticles without the need to add metal ions. Due to ELP1-ELP2 coassembly the pH-sensitivity of the ELP1 diblock was lost and the mixture disassembled at temperatures below 25 °C. Addition of zinc however led to improved stabilization of the coassembled particles. This coassembly approach will allow the creation of modular micelles with multiple functionalities and cargo loading potential. We envision that the multiresponsive coassembled nanoparticles could be used for delivery of both hydrophobic and hydrophilic drug cargos. As an example, negatively charged oligonucleotides such as siRNA can be loaded via interaction with the protonated His residues while hydrophobic drugs such as doxorubicin could be physically encapsulated within the hydrophobic core of ELP1-ELP2 coassemblies. Furthermore, these ELP nanoparticles, once entering the lower pH environment at the targets, could release drugs due to their pH-responsiveness. This can open the door to explore novel systems that are more suitable for targeted drug delivery.

4. Experimental Section

All chemicals were purchased from Sigma-Aldrich and used as received unless specified otherwise. The ELP [A3G2-60][I-60] was kindly provided by Jan Pille and produced according to a literature description.[21] Cloning and Expression of ELPs: The cloning protocol for recursive-directional ligation was adapted from literature.[33] Two separate ELP plasmids were designed. The first plasmid encoded for the [A3G2-60] block and the other one encoded for the [I1H4-60] block. The plasmid containing the N-terminal part of the block was cut with BglI and AcuI while the block containing the C-terminal part was cut with BglII and BseRI. The digested sequences were ligated together using T4 DNA ligase (NEBiolabs). The new plasmid encoding for the [I1H4-60][A3G2-60] diblock was transformed into XL-10 Gold (Agilent) and colonies were cultured in LB medium with 50 µg mL\(^{-1}\) kanamycin. The DNA was extracted and screened for the correct ligation using restriction digestion and DNA sequencing. The sequencing results were analyzed by Benchling software. The ELP plasmids were transformed into E.coli BLR(DE3) cells (Invitrogen) and grown on agar plates containing 30 µg mL\(^{-1}\) kanamycin and grown on agar plates containing 30 µg mL\(^{-1}\) kanamycin overnight at 37 °C. A single colony was selected and grown overnight at 37 °C, 250 rpm in LB medium containing 50 µg mL\(^{-1}\) kanamycin. The overnight culture was diluted to an OD 600 of 0.1 in filter-sterilized AIM TB medium (Formedium) containing 6 g L\(^{-1}\) glycerol, 0.005% Antifoam 204 and 100 µg mL\(^{-1}\) kanamycin. Cells were grown at 300 rpm at 30 °C for 24 h. Cells were collected by centrifugation at 4500 g, 4 °C for 15 min. For cytoplasmic extraction, 1 g of wet cell pellet was resuspended in 10 mL PBS pH 7.4, 1 × 10\(^{-3}\) M PMSF, Complete Protease Inhibitor Cocktail (Roche), 0.5 mg mL\(^{-1}\) lysozyme. Cells were lysed by a Sonics Vibra-Cell VC 750 sonicator (power level 7, 12 cycles of 10 s sonication, 10 s breaks). Cell debris was collected by centrifugation at 13 000 g at 4 °C for 15 min. Residual DNA was precipitated by adding 0.5% w/v poly(ethylene imine) and removed by centrifugation at 13 000 g at 4 °C for 30 min. Once a clear lysate was obtained, the ELPs were precipitated by adding a saturated solution of (NH\(_4\))\(_2\)SO\(_4\) up to 10–25 v/v%. The mixtures were incubated at room temperature to enhance the precipitation. Proteins were collected by centrifugation at 13 000 g at 28–30 °C for 30 min. The pellet was resuspended in PBS and centrifuged to remove insoluble contaminants at 13 000 g at 4 °C for 20 min. These ITC cycles were repeated until sufficient purity was obtained, usually after 2–3 cycles. To obtain higher protein purity, the ELPs were purified on a HiLoad 16/600 Superdex 200 column.

Figure 8. Characterization of coassembly behavior as a function of pH and temperature. All the samples were formulated in PBS at 10 × 10^{-6} m. ELP1 nanoparticles were prepared by the addition of 60 × 10^{-6} m zinc. For the coassembly, ELP1 and ELP2 were mixed and heated at 37 °C for 3 min. A) Formation of mixed micelles with different ratios of ELP1 to ELP2. B) Effect of pH on the self-assembly of ELP1 and coassembly of ELP1 and 2. C) Effect of temperature on micelle formation.
and desalted on a HiPrep 26/10 (GE Healthcare Life Sciences) with an NGC-Chromatography system (Biorad) at 1 mL min⁻¹ PBS and 4 mL min⁻¹ MilliQ, respectively. Residual salt concentration was below 0.01 mg mL⁻¹ as determined by conductivity. The ELP solution was filter-sterilized with 0.22 µm PES syringe filters (Nalgene) and freeze-dried. The yield was determined by weighing.

**SDS-PAGE:** Proteins were run under non-reducing conditions on 4–20% Mini-PROTEAN TGX Precast Gels, Promega. The electrophoresis was carried out at constant voltage (150 V) for 1 h. Gels were stained with Bio-Safe Coomassie Stain (Biorad).

**Quadrupole Time of Flight (Q-toF) Mass Spectrometry (Q-TOF):** Mass was determined using a High-Resolution LC-MS system consisting of a Waters ACQUITY UPLC I-Class system coupled to a Xevo G2 Quadrupole Time of Flight (Q-ToF). Freeze dried samples were resuspended in MilliQ to a concentration of 1 × 10⁻⁶ m. The samples were acidified with 0.1% formic acid upon injection. The protein was separated (0.3 mL min⁻¹) on the column (Polyanis C18A reverse phase column 2.0 × 100 mm, Agilent) using a 15–75% acetonitrile gradient in water supplemented with 0.1% v/v formic acid before analysis in positive mode in the mass spectrometer. Deconvolution of the m/z spectra was done using the MaxENTI algorithm in the Masslynx v4.1 (SCN862).

**UV–vis Spectroscopy:** Spectroscopy experiments were performed with a V-650 UV-Vis spectrophotometer (Jasco). Quartz cuvettes (Hellma) with a path length of 10 mm were used. For ramping experiments, the heating rate was set to 0.2 °C min⁻¹ with data collection each 0.2 °C. DLS: Samples were diluted in PBS pH 7.4. Measurements were performed on a Malvern Zetasizer Nano. The samples were equilibrated for 5 min at 37 °C before data collection. Reported values are averages of three independent measurements. For temperature-induced disassembly, the samples were measured at 4.0 °C.

**Hollow Fiber Flow Field-Flow Fractionation (HF5):** The measurements were performed using the Wyatt Eclipse hollow fiber system connected to a Wyatt DAWN HELEOS II light scattering detector with a Wyatt Optilab REX refractometer detector. The hollow fiber (HF) cartridge was pre-equilibrated with PBS pH 7.4 buffer, followed by calibration using Bovine Serum Albumin. Typically, 40 µL ELP particle solution of 10.0 mg mL⁻¹ (200 × 10⁻⁶ m) was injected while heated up to 37 °C during the experiment. The data were analyzed using Astra 7.1.24 software. 2.5.7. For the detailed description of the protocol, see Table S4 in the Supporting Information.

**Cryogenic Transmission Electron Microscopy (cryo-TEM):** The morphology of the ELP nanoparticles was characterized on the T/J/e CryoTitan (ThermoFisher Scientific) equipped with a field-emission gun operating at 60 kV, a post-column Gatan energy filter and an auto loader station. The grids (R2/2, Cu, Quantifoil Jena grids, Quantifoil Micro Tools GmbH) for cryo-TEM imaging were first treated in a Cressington 208 carbon coater for 40 s. Then, 3 µL of sample solution was pipetted onto the grid and blotted in a Vitrobot MARK IV (ThermoFisher Scientific) at 37 °C with 100% humidity. The grid was blotted for 3.5 s (blotting force -1) and directly plunged and frozen in liquid ethane. Images were acquired using a post-GIF Zk Gatan CCD (charged-coupled device) camera.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

**Acknowledgements**

This work has been financially supported but the European Union’s Horizon 2020 research and innovation program Marie Sklodowska-Curie Innovative Training Networks Nanomed (No. 676137).

**Conflict of Interest**

The authors declare no conflict of interest.
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