In natural systems, temperature-induced assembly of biomolecules can lead to the formation of distinct assembly states, created out of the same set of starting compounds, based on the heating trajectory followed. Until now it has been difficult to achieve similar behavior in synthetic polymer mixtures. Here, a novel pathway-dependent assembly based on stimulus-responsive polymers is shown. When a mixture of mono- and diblock copolymers, based on elastin-like polypeptides, is heated with a critical heating rate co-assembled particles are created that are monodisperse, stable, and have tunable hydrodynamic radii between 20 and 120 nm. Below this critical heating rate, the constituents separately form polymer assemblies. This process is kinetically driven and reversible in thermodynamically closed systems. Using the co-assembly pathway, fluorescent proteins and bioluminescent enzymes are encapsulated with high efficiency. Encapsulated cargo shows unperturbed function even after delivery into cells. The pathway-dependent co-assembly of elastin-like polypeptides is not only of fundamental interest from a materials science perspective, allowing the formation of multiple distinct assemblies from the same starting compounds, which can be interconverted by going back to the molecularly dissolved states. It also enables a versatile way for constructing highly effective vehicles for the cellular delivery of biomolecular cargo.

1. Introduction

Self-assembly is an intriguing phenomenon found in nature that chemists are increasingly able to employ in order to create supramolecular smart materials. Information-rich natural building blocks based on DNA,[1,2] peptides/proteins,[3-6] and synthetic polymers[7] have been used to create supramolecular assemblies with different morphologies and functions by utilizing non-covalent interactions and stimuli such as pH,[8] electrolyte concentration,[9,10] solvent switching,[11-13] chemical, and enzymatic reactions,[14-16] shear forces,[17] seeding,[18,19] light,[20] and temperature.[21] Especially the latter stimulus has been applied extensively both in natural and synthetic systems. For instance, plants adjust gene expression patterns depending on thermal changes by altering binding equilibria of chromatin-modulating proteins.[23] In designed systems, DNA origami architectures are assembled by self-sorting during cooling.[1]

In the latter case, structures are formed in thermodynamically closed systems; within thermodynamically closed systems, no matter is added or removed, and energy is only exchanged via heat.

Both synthetic and natural polymers often undergo structural rearrangements based on thermal changes that affect their solubility and assembly features, and which are characterized by their lower and upper critical solution temperatures. In contrast to “natural” polymers such as nucleic acids, which can be kinetically trapped by rapid cooling, the respective assembly state of synthetic polymers is usually reached independently of the thermal pathway taken.[11,21] Here we show pathway-dependent assembly in a thermodynamically closed system of a mixture of nature-inspired synthetic polymers based on elastin-like polypeptides (ELPs). Upon rapid heating stable supramolecular co-assemblies are formed, whereas a slow heating process leads to the separate assembly of the individual ELP polymers. The assembly process is completely reversible, and by cooling and (slow or fast) heating the two assembly states can be interchanged. This co-assembly process is furthermore demonstrated to be a highly versatile method for the construction of multifunctional nanoparticles.

2. Co-Assembly is Pathway-Dependent and Reversible via an Intermediate State

ELPs are intrinsically disordered, temperature-responsive polymers that reversibly change conformation above their transition
temperature ($T_0$), leading to a transition from a molecularly dissolved state to a coacervate phase.\textsuperscript{[24-29]} Their transition temperature is dependent on the guest residue $X$ in their monomer sequence GxGVxP, polymer length, polymer, and electrolyte concentration. In contrast to other temperature-sensitive polymers such as poly(N-isopropylacrylamide), which show a blended transition temperature,\textsuperscript{[30]} ELPs retain their respective $T_x$ in the presence of ELP species with different lengths.\textsuperscript{[31]} guest residues or modifications such as genetic fusions to other proteins.\textsuperscript{[32,33]} This has allowed purification of fusion proteins by supplementation\textsuperscript{[34]} and intracellular “sorting” of distinct ELP populations\textsuperscript{[35]} to control, for example, receptor clustering,\textsuperscript{[36]} but at the same time prohibits the directed co-assembly of ELPs with different transition temperatures to create more complex assemblies. We found that by kinetically overwriting distinct assembly pathways, controlled co-assembly of ELPs can be achieved. Different heating pathways lead to different assemblies, which are interconverted in a thermodynamically closed system (Figure 1).

Two different ELPs were used: a homo ELP block consisting of 60 GXGVP repeats with isoleucine as guest residue $X$, abbreviated as [I-60] (Figures S1–S3, Supporting Information), and a diblock copolymer [A$_3$G$_2$-60]-[I-60], containing an additional, more hydrophilic block with the guest residues alanine and glycine at a ratio of 3 to 2, respectively. Only the diblock copolymer was capable of forming micelles on its own (Figures S1–S3, Supporting Information). A 1 to 1 molar mixture of monoblock and diblock copolymer, heated slowly with a gradient of 0.2 °C min$^{-1}$, showed a sharp increase in optical density at around 16 °C, corresponding to the transition temperature of [I-60]; the solution became visibly turbid as expected with the presence of large coacervates (Figure 1b). When the mixture was instead heated rapidly to 37 °C, above the transition temperature of [I-60] and the more hydrophobic block of [A$_3$G$_2$-60]-[I-60], the increase in optical density was attenuated, indicating the presence of smaller particles instead, which was confirmed by dynamic light scattering (Figure 1c). The same pathway-dependent co-assembly could be observed by the addition of electrolytes (Figure S4a, Supporting Information).

This co-assembly is kinetically driven; when a state was reached by slow heating at which [I-60] started to coacervate but [A$_3$G$_2$-60]-[I-60] remained dissolved, further heating or the addition of electrolytes did not lead to interactions between monoblock and diblock copolymer; micelles formed by [A$_3$G$_2$-60]-[I-60] co-existed as a separate state from the monoblock aggregate (Figure S4b, Supporting Information). Exceeding the transition temperature of both species at a sufficient heating rate however resulted in the co-assembly of hydrophobic domains during the process of coacervation. These were stabilized by the hydrophilic part of the diblock copolymer, which remained dissolved in the conditions used for co-assembly. Gratifyingly, this process was completely reversible (Figure 1c). Repeated cooling and heating resulted in distinct supramolecular assemblies based only on the pathway taken; rapid heating created well-defined nanoparticles, while slow heating resulted in less-defined, large coacervate structures. Slow cooling of co-assembled ELPs revealed that the disassembly occurred via an intermediate state, in which [A$_3$G$_2$-60]-[I-60] started to dissolve, leading to the unhindered coacervation of [I-60] into large aggregates and subsequent increase in turbidity (Figure 1d).

Further cooling dissolved these at the respective transition temperature of [I-60]. Therefore, while co-assembly could be kinetically induced, each ELP species retained its respective transition temperature independent of the presence of other ELPs and irrespective of being part of the same coacervate.

Since the assembly process of ELPs happens rapidly within the order of seconds, the heating rate must be sufficiently large to allow stabilization of the monoblock coacervate by the diblock copolymer. A series of experiments with different heating rates was therefore performed to determine if a critical rate could be identified. For the determination of the difference in transition temperatures, the theoretical transition temperature of the respective hydrophobic blocks was calculated according to:\textsuperscript{[37]}

$$T_x = \alpha + \beta \log_{10}(x)$$

In which $T_x$ is the transition temperature, $x$ is the concentration in μM, $\alpha$ is the transition temperature for log(x) = 0 and $\beta$ is the slope. As shown in Figure 1e,f and Table S1, Supporting Information, a critical heating rate required for co-assembly was detected, which correlated with the difference in transition temperature between monoblock and diblock copolymer.

3. Size and Stability of Co-Assembled Particles

We next investigated the size of co-assembled nanoparticles after formation by heat or electrolyte shock depending on the molar percentage of [I-60] present (Figure 2). While retaining low dispersity, particle size could be controlled between 21 to 120 nm hydrodynamic radius (Table S2, Supporting Information), a size range regarded optimal for biomedical applications.\textsuperscript{[38,39]} Particle size and mass were also probed with stochastic optical reconstruction microscopy and size-exclusion chromatography coupled to multi-angle light scattering (Table 1 and Figure 2b; Figures S5 and S6, Supporting Information). Integration of particle and monomer peak shows an encapsulation efficiency of at least 95%. The ratio of radius of gyration to hydrodynamic radius indicated micellar structures with an increasingly homogenous core. Co-assembled particles were stable for prolonged periods of time without significant changes in size (Figure 2d). In order to investigate whether the interacting ELP blocks are interchangeable, we have created a different amphiphilic ELP block, [A$_3$G$_2$-60]-[V$_4$F$_2$-50] (Figure S7, Supporting Information), for co-assembly with [I-60] (Table S2, Supporting Information). Heat-induced co-assembly formed well-defined particles that increased in size with the proportion of [I-60] used in the formulation as expected, showing that different ELP sequences can be used interchangeably.

4. Encapsulation of Macromolecular Cargo and Cellular Delivery of Encapsulated Macromolecular Cargo

To demonstrate the concept of protein encapsulation, we created genetic fusions of the Förster resonance energy transfer (FRET) protein pair mTurquoise2\textsuperscript{[40]} (donor) and mNemGreen\textsuperscript{[41]}
Figure 1. Heating rate specifies two different ELP assembly pathways. a) Below their respective transition temperatures, both biopolymers are fully hydrated and show a disordered structure. Slow heating triggers the transition of the hydrophobic monoblock selectively. Upon further heating, the diblock ELP copolymer adopts a micellar morphology next to the monoblock coacervates. This process is fully reversible upon cooling. If the system is heated from the dissolved state rapidly above the transition temperatures of both the hydrophobic monoblock and the hydrophobic block in the diblock copolymer, a stable co-assembled state is reached, in which the hydrophilic part of the diblock stabilizes the coacervates formed. This state is reversible upon cooling via an intermediate pathway. The same behavior can be induced by the addition of electrolytes, which lower the transition temperatures of ELPS. b) Optical density measurements of a 1 to 1 molar mixture of [I-60] and [A3G2-60]-[I-60], which was heated gradually (black line) or rapidly from 10 to 37 °C (red line). c) Formation of particles is independent of previous assembly pathways as observed by spectrometry (left axis) and dynamic light scattering (right axis). A 1 to 1 molar mixture of [I-60] and [A3G2-60]-[I-60] was repeatedly heated, either rapidly (red line) or gradually (black line). Optical density and hydrodynamic radii (green triangles) were measured after each transition step. Hydrodynamic radii are displayed as mean ± standard deviation of three independent measurements. d) Cooling of co-assembled particles measured by spectrometry. Horizontal, colored lines indicate the gap between determined transition temperatures of the monoblock and the hydrophobic block in the diblock copolymer, annotated by flanking vertical lines, respectively. Optical density values are displayed as mean ± standard deviation from three independent measurements. e) Mixtures of ELPS with different diblock to monoblock ratios were heated at different rates. Full circles indicate successful co-assembly, empty circles represent a segregated state. db/mb ratio: molar ratio of [A3G2-60]-[I-60] to [I-60]. Total ELP: sum of [A3G2-60]-[I-60] and [I-60] concentration present in the sample. f) Theoretical transition temperature difference plotted against the critical heating rate, at which co-assembly was first observed.
[acceptor] with [I-60] (see Figures S8–S12, Supporting Information). Successful co-assembly together with [I-60] and [A3G2-60]-[I-60] was demonstrated by the high energy transfer with a FRET efficiency of 82% in the case of 10 mol% mNeonGreen/mTurquoise2 (Figure 3), and by protection of the encapsulated proteins from proteolysis (Figure S15, Supporting Information). FRET efficiency scaled with the concentration of encapsulated acceptor in presence of the donor in the coacervate core (Figure S8, Supporting Information). Co-encapsulation of a bioluminescent luciferase NanoLuc-[I-60] conjugate (Figures S13 and S14, Supporting Information) with mNeonGreen-[I-60] showed successful bioluminescence energy transfer (BRET) (Figure 3b).

We subsequently proceeded to add a single-domain antibody fragment-ELP conjugate, 7D12-[A3G2-60]-[I-60], to the co-assembly formulation to stimulate uptake of particles containing both fluorescent proteins by cells expressing the epidermal growth factor receptor.

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**Table 1.** Properties of nanoparticles as a function of mol% [I-60] measured by size-exclusion chromatography coupled to multi-angle light scattering.

| Molar percentage [I-60] [mol%] | \( R_h \) [nm] | \( R_g \) [nm] | \( MW \) [MDa] | \( P \) | \( C_{app} \) [mg mL\(^{-1}\)] |
|-------------------------------|----------------|-------------|----------------|-----|------------------|
| 0                             | 25.6 ± 0.9     | 14.6 ± 0.5  | 12.1 ± 0.1     | 0.570 ± 0.029 | 286  |
| 17.5                          | 28.5 ± 1.2     | 21.2 ± 0.4  | 17.3 ± 0.1     | 0.583 ± 0.029 | 297  |
| 35                            | 33.0 ± 1.2     | 20.8 ± 0.4  | 27.5 ± 0.1     | 0.629 ± 0.026 | 302  |
| 52.5                          | 35.8 ± 1.2     | 22.9 ± 0.5  | 44.4 ± 0.1     | 0.639 ± 0.027 | 383  |
| 70                            | 41.6 ± 1.4     | 27.1 ± 0.3  | 89.0 ± 0.1     | 0.651 ± 0.022 | 490  |

\( R_h \) Hydrodynamic radius; \( R_g \) radius of gyration; \( MW \) molecular weight; \( P \) is defined as \( R_g / R_h \); \( C_{app} \) apparent protein concentration within the particle, assuming a perfect sphere of homogenous density with a radius of \( R_h \) and molecular weight \( MW \). The determined \( dn/dc \) of the material was 0.1586 ± 0.0003 mL g\(^{-1}\).

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**Figure 2.** Size and stability of co-assembled ELP nanoparticles. a) Scheme of particle formation. With increasing amounts of monoblock, bigger particles are created upon co-assembly. b) Hydrodynamic radii of nanoparticles formed by heat or salt shock increased with the presence of [I-60] in the co-assembled system. c) 3D reconstruction of an ELP co-assembled nanoparticle with 92 mol% [I-60] as measured by stochastic optical reconstruction microscopy. Z-axis values are represented as colour gradient. d) Particles remained stable over extended periods of time without significant changes in hydrodynamic radius. Values are presented as mean ± standard deviations from three independent measurements. Amount of [I-60] is given in mol percentages.
Figure 3. Encapsulation and cellular delivery of macromolecular cargo. mTq2 = mTurquoise2; mNG = mNeonGreen. NanoLuc = NanoLuc luciferase.

a) FRET measurements by using mTq2 and mNG as a donor/acceptor pair. Fusion proteins with [I-60] were encapsulated in the coacervate core, conjugates with [A3G2-60] served as non-encapsulated control. A hyphen indicates no donor/acceptor in the nanoparticle formulation, respectively. b) Bioluminescent resonance energy transfer (BRET) using NanoLuc and mNG as a donor/acceptor pair. Spectra are normalized to the emission maximum of NanoLuc luciferase. c) Scheme of particle formation. Fluorescent proteins to be encapsulated were fused to the hydrophobic [I-60] monoblock, the targeting group 7D12 was fused to [A3G2-60]-[I-60]. Interaction of fluorescent proteins after cell uptake was probed by fluorescence imaging. d) Live cell confocal imaging. Cell membrane stain is visualized in red, mTurquoise2 excitation signal is shown in cyan, mNeonGreen signal, raw and corrected FRET signal is shown in green. The FRET signal was corrected for the spectral bleed-through by using particles with only either mTurquoise2 or mNeonGreen (Figure S15, Supporting Information). Scale bars = 50 µm.
receptor (EGFR) (Figure 3c).[44] A431, a cell line known to highly express EGFR, was selected as the model cell line, since 7D12 efficiently targets A431 cells and induces uptake of attached cargo as shown in our previous work.[44] Live cell confocal imaging showed that functionalized particles were taken up after an incubation time of 1 hour as expected. The punctuated fluorescence within cells indicates endosomal or lysosomal localization, as would be expected given the size of co-assembled particles and the targeting moiety. mTurquoise2 and mNeonGreen continued to show fluorescence and energy transfer after correction for their respective spectral bleed-through, indicating that cargo and particle integrity were retained significantly after uptake (Figure 3d and Figure S16, Supporting Information).

This pathway-dependent co-assembly of ELPs offers interesting opportunities. The method is highly modular, as macromolecular cargo modified with the appropriate ELP sequence can become a structural part of the co-assembly itself, leading to high encapsulation efficiencies. This can be employed to a large number of therapeutically interesting peptides and enzymes which are known to tolerate a genetically or chemically fused ELP tag. It also widens the chemical space for modifications of the corona-forming di-block polymer, as these can now be done completely independent of the cargo before co-encapsulation. Different ELP sequences can be combined, opening up great flexibility in particle design. The ELP responsiveness allows building in pathway-dependent (dis)assembly to tune interaction and release.[45–47] The process of encapsulation also greatly concentrates macromolecular cargo, which can be beneficial to study concentration-dependent behavior of macromolecules.[48] Brining, for example, enzymes into sufficient proximity can be critical for understanding and steering sequential catalysis.[49]

The presented pathway-dependent co-assembly of ELPs therefore not only leads to further insight into their thermodynamic properties but also opens up new perspectives for the further development of functional nanoparticles for biomedical applications.

5. Experimental Section
Experimental details can be found in the Supporting Information.

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

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Conflict of Interest
The authors declare no conflict of interest.

Data Availability Statement
Research data are not shared.

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