Inducible Fibril Formation of Silk–Elastin Diblocks

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ABSTRACT: Silk–elastin block copolymers have such physical and biological properties that make them attractive biomaterials for applications ranging from tissue regeneration to drug delivery. Silk–elastin block copolymers that only assemble into fibrils at high concentrations can be used for a template-induced fibril assembly. This can be achieved by additionally including template-binding blocks that promote high local concentrations of polymers on the template, leading to a template-induced fibril assembly. We hypothesize that template-inducible silk-fibril formation, and hence high critical concentrations for fibril formation, requires careful tuning of the block lengths, to be close to a critical set of block lengths that separates fibril forming from nonfibril forming polymer architectures. Therefore, we explore herein the impact of tuning block lengths for silk–elastin diblock polypeptides on fibril formation. For silk–elastin diblocks E$_n$S$_m$−S$_n$E$_m$, in which the elastin pentamer repeat is E$^5$ = GSGVP and the crystallizable silk octamer repeat is S$^8$ = GAGAGAGQ, we find that no fibril formation occurs for $n = 6$ but that the $n = 10$ and 14 diblocks do show concentration-dependent fibril formation. For $n = 14$ diblocks, no effect is observed of the length $m$ (with $m = 40, 60, 80$) of the amorphous block on the lengths of the fibrils. In contrast, for the $n = 10$ diblocks that are closest to the critical boundary for fibril formation, we find that long amorphous blocks ($m = 80$) oppose the growth of fibrils at low concentrations, making them suitable for engineering template-inducible fibril formation.

INTRODUCTION

Silk is a naturally occurring biomaterial with a unique combination of elasticity and strength approaching the mechanical properties of materials like Kevlar.1,2 For millennia, humans have repurposed naturally occurring silk in textiles, and the unique properties of this material have led to its widespread use as a biomaterial.3 Silk films have been used as wound dressings to protect against infections,4 silks hydrogels and sponges and porous microtubes have been used as scaffolds for the delivery of cells and/or cytokines5–7 and for tissue regeneration,8,9 and silk capsules have been proven useful for drug delivery.5,10

Applications of silk proteins and engineered silklike polypeptides are to a large extent determined by their unique self-assembly behavior, which is controlled by an alternation of crystallizable and amorphous sequence motifs. In early studies on the structure–property relationship of silklike polymers, Tirrell and co-workers synthesized and studied polypeptides with the general sequence [(AG)$_x$EG]$_n$ inspired by the crystallizable blocks of Bombyx mori silks. Next, by mimicking the alternation of crystallizable and amorphous blocks of natural silks, various groups have developed block copolymers with both crystallizable and amorphous blocks. In particular, silk–elastin-like polypeptides (ELPs)11,12 have been developed that are composed of multiple crystallizable silklike blocks, such as (GAGAG)$_n$ and amorphous elastin-like (GXGVP)$_m$ blocks, where X is the so-called guest residue in the consensus motif for elastin-like polypeptides (ELPs). Another type of amorphous block that has been explored extensively for use in silk-block copolymers13–18 is a collagen-like polypeptide (GXaaYa)$_m$ with the Xaa and Yaa residues chosen to be mostly uncharged and hydrophilic such that these blocks adopt random coils rather than triple helical configurations.19

Silklike polymers often assemble into fibrils and for many applications, such as the encapsulation of therapeutic nucleic acids, it would be advantageous if silk-fibril formation could be engineered to be induced by specific templates. Ideally, the template-induced fibrils can be easily separated from self-assembled fibrils that do not harbor the template. One approach is to use a low concentration of silklike polymers that only self-assemble into fibrils at high concentrations and to locally concentrate them on the template. This approach, of driving fibril formation by a change in concentration, has been realized for the specific case of the viruslike encapsulation of single-DNA molecules, using a silklike polymer endowed with a K$_{12}$ oligolysine DNA binding domain.20 For the trilobe...
copolymer (GXaYaa)_{12}−(GAGAGAGQ)_{10}−K_{12}, it was shown that in the absence of a DNA template it only forms fibrils at high concentrations (above 80 μM). However, already at much lower concentrations, when mixed with DNA, the triblock spontaneously forms rod-shaped viruslike particles, each encapsulating a single DNA molecule, with a core formed by a silk fibril.

An outstanding question that remains, and one that we attempt to answer in this study for silk–elastin diblocks, is what the requirements are on the lengths of both the silk and elastin block to arrive at silk polymers for which assembly can be induced by an external template. More specifically, we aim at silk polymers that only assemble into long fibrils at rather high concentrations, which is a situation that could be created locally by the accumulation of the silk polymers onto the template via a template-binding domain. With this in mind, we study the concentration-dependent fibril formation of silk–elastin-like diblock polypeptides (GSGVP)_m−(GAGAGAGQ)_n for a range of values of the block lengths n and m.

### RESULTS

**Polypeptide Production.** To study the effect of block lengths on the self-assembly behavior of silk–elastin diblocks, genes for a set of diblock polypeptides (GSGVP)_m−(GAGAGAGQ)_n were prepared using the PRe-RDL strategy, as described previously. Herein, we use the nomenclature E^\beta = GSGVP for the elastin pentamer motif and S^Q = GAGAGAGQ for the silk motif; diblock sequences are abbreviated as E^{\beta}_n−S^{Q}_m. Genes were prepared for diblocks with m = 40, 60, or 80 E^\beta pentamer repeats and n = 6, 10, or 14 S^Q silk repeats. The final PRe-RDL step of combining genes for silk and elastin blocks to form genes for diblocks is illustrated in Figure 1, together with a depiction of polypeptide architecture. We depict the silk block in Figure 1 as a \( β \)-solenoid configuration since this is the configuration that S^{Q}_{10} silk blocks were shown to adopt in fibrils in recent atomistic molecular dynamics simulations. Fibrils are formed by \( β \)-solenoids stacking on top of each other. Stacking is thought to be driven by the formation of multiple hydrogen bonds.

It has been described before that the elastin pentamers within silk–elastin multiblock copolymers may increase the rate of fibril formation when used at temperatures above the transition temperature (T_t) of the elastin blocks. Note, however, that we here use a very hydrophilic E^\beta elastin pentamer motif, resulting in silk–elastin polypeptides with very high T_t, such that not thermal transitions but only accumulation to high concentrations on the template will drive fibril formation.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for the E^{\beta}_n−S^{Q}_m diblock after purification is shown in Figure 2a and is representative for the other silk–elastin diblock copolymers used herein. All polypeptides showed high purity, as confirmed by spectrophotometry via the absence of any absorption at 280 nm but a high absorption at 230 nm. Note that for SDS-PAGE, silk–elastin diblocks stain rather poorly by the coomassie-based PageBlue staining. Similarly, poor results in terms of staining were obtained using copper chloride. Also, as observed for many nonglobular protein-based polymers with poor SDS binding, the diblocks have anomalous electrophoretic mobility as compared with typically used molecular weight markers such that we cannot directly estimate molar masses on the basis of the SDS-PAGE by comparing with molecular weight markers. Precise molar masses were obtained from matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). An example spectrum for the selected diblocks is shown in Figure 2b, and experimental molar masses (as well as yields) for all diblocks are given in Table 1. Assuming full removal of N-terminal methionines, the molar masses were obtained from matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). An example spectrum for the selected diblocks is shown in Figure 2b, and experimental molar masses (as well as yields) for all diblocks are given in Table 1. Assuming full removal of N-terminal methionines, the molar masses were obtained from matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). An example spectrum for the selected diblocks is shown in Figure 2b, and experimental molar masses (as well as yields) for all diblocks are given in Table 1. 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implementally determined masses agree with the expected ones within the experimental error.

Table 1. Expected and Experimentally Determined Molar Masses (MALDI-TOF) and Production Yield of of $E_m^S-S_n^Q$ Diblocks (Milligrams of Polypeptide per 2 L of Culture Medium)

|          | expected MW (Da) | experimental MW (Da) | yield (mg/2 L) |
|----------|------------------|----------------------|---------------|
| $E_{40}^S-S_{14}^Q$ | 19 389.77        | 19 386.4            | 14.5          |
| $E_{50}^S-S_{10}^Q$ | 27 338.39        | 27 335.8            | 19.1          |
| $E_{60}^S-S_{6}^Q$  | 35 287.02        | 35 290.9            | 32.5          |
| $E_{70}^S-S_{10}^Q$ | 21 668.07        | 21660.4             | 14.1          |
| $E_{80}^S-S_{4}^Q$  | 29 616.69        | 29 620.0            | 16.8          |
| $E_{90}^S-S_{10}^Q$ | 37 565.32        | 37 567.9            | 20.7          |
| $E_{10}^S-S_{14}^Q$ | 23 946.36        | 23 944.6            | 6.7           |
| $E_{11}^S-S_{10}^Q$ | 31 894.99        | 31 900.7            | 10.6          |
| $E_{12}^S-S_{10}^Q$ | 39 843.62        | 39 850.3            | 12.2          |

Dependence of Self-Assembly of Silk–Elastin Diblocks on Block Lengths. We used atomic force microscopy (AFM) and cryogenic transmission electron microscopy (cryo-TEM) imaging to explore at which block lengths the self-assembly of silk–elastin diblocks could be driven by accumulation onto a template. Solutions of $E_m^S-S_n^Q$ diblocks with $m = 40$ or 80 and $n = 6$, 10, or 14 were prepared from purified polypeptide powders and incubated at room temperature for 24 h (for AFM) or 16 h (for cryo-TEM) to allow for their self-assembly into fibrils. Both these assembly times are long enough for fibrils to reach their terminal lengths, on the basis of the assembly times for very similar silk-block-containing polymers that we have studied before.20

In our previous studies on silk fibril templating on DNA by the (GXaaYaa)$_{132}$−(GAGAGAGQ)$_{10}$–K$_{12}$ triblock copolymer, we found that the templated fibril assembly already occurred at a low concentration of 1.8 μM. In the absence of a template, however, fibrils were only found at concentrations above 80 μM.20 Here, we study the assembly of the silk–elastin diblocks at these same two concentrations, with the goal of identifying optimal block lengths for a templated self-assembly: those for which the polypeptides form no or only very short fibrils at the low concentration but do form fibrils at the high concentration. This would mimic the situation of a dilute polypeptide solution in which the polypeptides only form fibrils upon accumulation onto a template. Results for AFM and cryo-TEM imaging are shown in Figure 3.

We first examined the dependence of the self-assembly on the length of the silk block with the elastin block fixed at $m = 80$. We find that a silk-block length of $n = 6$ is not enough to allow for fibril formation at either the low or high concentration that we have used in our experiments (see Figure 3a). For silk-block lengths of $n = 10$ and 14 (Figure 3b,c), we find concentration-dependent fibril formation: at a polypeptide concentration of 1.8 μM, both diblocks self-assemble into relatively short fibrils (left column), whereas at a high concentration of 100 μM, they self-assemble into very long fibrils (middle column).

Next, we examined the influence of the length $m$ of the amorphous elastin blocks on the fibril formation by the $E_m^S-S_n^Q$ silk–elastin diblocks. As none of the polypeptides with $n = 6$ were able to form fibrils at high concentration, which is a requirement for template-induced fibril formation, this case is not further discussed here. We compare the $n = 10$ or 14 diblocks for lengths $m$ of the amorphous block of $m = 40$ and 80 ($E_{40}^S-S_{10}^Q$ vs $E_{80}^S-S_{10}^Q$ and $E_{40}^S-S_{14}^Q$ vs $E_{80}^S-S_{14}^Q$; Figure 3b,d and e,f). At high concentrations, all fibrils for the diblocks with $n = 10$, 14 are very long irrespective of the length $m$ of the amorphous block (Figure 3, middle column). This is an important feature that will allow assembly into fibrils upon accumulation onto a template. At low concentration, it appears that the fibrils become shorter as the length of the amorphous block increases from $m = 40$ to 80; ascertaining this requires a more quantitative analysis that we perform below. To exclude the possibility that the AFM sample preparation procedure somehow affects our observations on fibril growth, we also used cryo-TEM to visualize fibrils in solution, at a polypeptide concentration of 100 μM. Representative micrographs for a silk-block length of $n = 10$ and amorphous block lengths of $m = 40$, 80 are shown in Figure 3d,b, respectively (right column). As observed using AFM for the same conditions, long fibrils are formed for both cases (indicated by black arrowheads).

Quantitative Analysis of Fibril Lengths. For a template-induced assembly, it is desirable that the fibrils formed at low polypeptide concentration are as short as possible so that self-assembled fibrils are easily distinguished and or separated from template-induced fibrils. We, therefore, quantitatively characterize the subtle differences in the assembly of the diblocks into fibrils at the low concentrations of 1.8 μM for the diblocks with $n = 10$ or 14 and varying lengths of $m$. Fibril lengths for these diblocks were determined from AFM images, and the histograms are shown in Figure 4. The distributions are very broad, with many short fibrils and a smaller number of longer fibrils. Despite their lower number, the longer fibrils represent a significant fraction of the total polypeptide mass. Therefore, the length distributions are given as the fraction of total polypeptide mass (weight fraction) versus fibril length. In view of the broad distributions, we give both the number-averaged fibril lengths ($L_n$) and the weight-averaged fibril lengths ($\langle L \rangle_w$) for the silk–elastin diblocks in Table 2. Finally, Figure 5 shows the number-averaged fibril lengths ($L_n$) for all diblocks with silk-block lengths $n = 10$ and 14.

First, we examined the effect of the silk-block length $n$ on fibril lengths by comparing the fibril length distributions for diblocks with a fixed length of the amorphous block ($m = 40$ or 80), for silk-block lengths of $n = 10$ and 14 (Figure 4a vs b and c vs d). For $n = 14$, the tails of the distributions stretch to much larger lengths as compared with the distribution for $n = 10$, leading to higher weight-averaged fibril lengths ($\langle L \rangle_w$) of 185 ± 44.8 nm for $E_{40}^S-S_{14}^Q$, as opposed to $\langle L \rangle_w = 67 ± 9.0$ nm for $E_{40}^S-S_{10}^Q$, and $\langle L \rangle_w = 168 ± 41.6$ nm for $E_{40}^S-S_{14}^Q$ vs $E_{40}^S-S_{10}^Q$; see Table 2 and Figure 4). This shows that at the low concentration of 1.8 μM, the self-assembly is sensitive to the precise length of the silk block, with the shorter silk-block length $n = 10$ resulting in shorter fibrils.

Next, we examined the effect of the length $m$ of the amorphous elastin block by comparing the fibril length distributions for diblocks with a fixed silk-block length of $n = 10$ or 14 and a varying length $m$ of the elastin-like blocks (Figure 4a vs c and b vs d). For diblocks with silk-block length $n = 10$, the histograms of Figure 4 (Figure 4a vs c) corroborate the qualitative observation that fibrils become shorter as the length of the amorphous block increases from $m = 40$ to 80: fibrils formed by $m = 80$ diblocks are significantly shorter than...
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brils formed by the $m = 40$ diblocks at the same concentration $(\langle L \rangle_w = 67 \pm 9$ nm vs $(\langle L \rangle_w = 129 \pm 16$ nm, respectively). This shows that for $n = 10$, the elastin blocks are able to modulate assembly of the silk blocks in such a way that longer elastin blocks lead to shorter fi

brils at low polypeptide concentrations. In contrast, the histograms and weight-averaged fi

bril lengths $(\langle L \rangle_w)$ for diblocks with a silk-block length of $n = 14$ (Figure 4b vs d) are rather similar to each other and are less affected by the elastin block length $m$.

The influence of the length $m$ of the amorphous block for a silk-block length of $n = 10$ on the fi

bril lengths at low concentrations is even more evident from Figure 5, which shows plots of the weight-averaged fi

bril lengths $(\langle L \rangle_w)$ for $n = 10$ and 14 as a function of $m$ (Figure 5). To better evaluate the trend in fi

bril lengths with varying lengths $m$ of the amorphous block, we here also provide the average fi

bril lengths for diblocks with $m = 60$ in addition to those with $m = 40$ or 80. For the $n = 10$ diblock fi

bril lengths clearly decrease when the length $m$ of the amorphous block increases, whereas for $n = 14$, there is no clear trend.

**DISCUSSION**

Block lengths and block length ratios are one of the key parameters of protein-based block copolymers governing their self-assembly, coassembly with a template, and other functionalities.\textsuperscript{11,20,28–30} The synergy between ordered and amorphous polypeptide domains has similarly been shown to heavily influence the resulting material properties of protein-
based materials.\textsuperscript{31} In the case of silkworm protein-based polymers, this means that the amorphous blocks influence the molecular interactions of the crystallizable blocks and vice versa. By precisely tuning the lengths of the amorphous and crystallizable blocks, it will be possible to develop silkworm-like polymers for which assembly into fibrils is induced by an external template. Such a template-induced assembly may be advantageous for various applications, for example, the encapsulation of a therapeutic nucleic acid template for gene therapy purposes. The diblocks used here are an excellent model system to study the mutual influence of amorphous and crystallizable blocks in these polymers and were therefore used here to explore how the block lengths can be optimized for a template-induced assembly.

A template-induced assembly requires that the polypeptides do not self-assemble into fibrils at low polypeptide concentrations (i.e., the normal working concentration), whereas at high polypeptide concentrations (which resembles the accumulation of polypeptide onto a template), they should form fibrils. For \( E_{m}^{\beta}-S_{n}^{\alpha} \) silk–elastin diblock polypeptides, we have shown herein that a minimal length of the crystallizable silk block is required to drive fibril formation at these high polypeptide concentrations. We established that for amorphous block lengths \( m \geq 40, n = 6 \) is insufficient whereas \( n \geq 10 \) is sufficient for fibril formation. Next, we have shown that these \( E_{m}^{\beta}-S_{n}^{\alpha} \) silk–elastin diblock polypeptides with \( n \geq 10 \) also form fibrils at low concentrations. Only for some cases we find that the length of these fibrils can be controlled by the length \( m \) of the amorphous blocks. Specifically, it appears that the diblocks with block lengths \( n \) that are closest to the boundary for fibril formation (\( n = 10 \)) respond most strongly

**Table 2. Number- and Weight-Averaged Fibril Lengths for the \( E_{m}^{\beta}-S_{n}^{\alpha} \) Diblocks\textsuperscript{a}

|       | \( N \) | \( \langle L \rangle_{n} \) (nm)\textsuperscript{a} | \( \langle L \rangle_{w} \) (nm)\textsuperscript{b} |
|-------|-------|-----------------|-----------------|
| \( E_{60}^{\beta}-S_{10}^{\alpha} \) | 567   | 70 (±2.7)       | 129 (±16.0)     |
| \( E_{60}^{\beta}-S_{10}^{\alpha} \) | 85    | 54 (±3.7)       | 75 (±13.8)      |
| \( E_{60}^{\beta}-S_{10}^{\alpha} \) | 636   | 43 (±1.3)       | 67 (±9.0)       |
| \( E_{60}^{\beta}-S_{10}^{\alpha} \) | 222   | 81 (±5.6)       | 168 (±41.6)     |
| \( E_{60}^{\beta}-S_{14}^{\alpha} \) | 159   | 57 (±3.9)       | 100 (±30.2)     |
| \( E_{60}^{\beta}-S_{14}^{\alpha} \) | 185   | 91 (±6.8)       | 185 (±44.8)     |

\( N \) is the number of fibrils analyzed for each diblock.\textsuperscript{b} Number- and weight-averaged fibril lengths (±standard error of the mean (SEM)) were calculated using the data obtained from AFM images.

**Figure 4.** Fibril length distributions of \( E_{m}^{\beta}-S_{n}^{\alpha} \) diblocks. Fibril lengths were determined from AFM images of diblocks incubated at 1.8 \( \mu M \) for 24 h. Weight fractions of each binned length category were calculated with the assumption that the polypeptide mass of a given fibril is proportional to the fibril length. (a) \( E_{60}^{\beta}-S_{10}^{\alpha} \); (b) \( E_{60}^{\beta}-S_{14}^{\alpha} \); (c) \( E_{40}^{\beta}-S_{10}^{\alpha} \); and (d) \( E_{40}^{\beta}-S_{14}^{\alpha} \). For each polypeptide sample, the number (\( \langle L \rangle_{n} \))- and weight (\( \langle L \rangle_{w} \))-averaged fibril lengths (±SEM) are also shown in the plots.

**Figure 5.** Influence of the elastin block length \( m \) on fibril lengths of the \( E_{m}^{\beta}-S_{n}^{\alpha} \) diblocks. Number-averaged fibril lengths at low polypeptide concentration (1.8 \( \mu M \)) versus the block length \( m \) of the amorphous elastin block is plotted for (a) \( E_{60}^{\beta}-S_{10}^{\alpha} \) and (b) \( E_{40}^{\beta}-S_{14}^{\alpha} \). The error bars represent the standard error of the mean (SEM). *\( p < 0.01 \), as determined by a Welch’s \( t \)-test for two independent samples with unequal variances.\textsuperscript{*}}
to the length of the amorphous block, with longer amorphous blocks leading to shorter fibrils at low concentrations, suggesting that the amorphous blocks in that case can effectively oppose fibril growth. For longer silk blocks \( n = 14 \), the driving force for fibril growth is apparently already too large to be opposed by the amorphous blocks, since in that case we do not find a significant dependence of fibril growth on the length of the amorphous blocks. Our report of continuous tuning of fibril growth by adjusting the length of the amorphous blocks is new but also consistent with our previous report on “on/off” switching of fibril formation via amorphous blocks: triblocks \((GXXaYa)_{67}-(GAGAGA)_{n}-(GXXaYa)_{67}\) with \( n = 16 \) do not form fibrils in the presence of the amorphous blocks, but when the latter are proteolytically shortened, fibrils are formed. For diblocks such as \( E_{m}S_{10}Q \), which form only very short fibrils at low concentrations but very long fibrils at high concentrations, template-induced assembly may be realized by adding a template-binding block, for example, a positively charged block to bind negatively charged (nucleic acid) templates.\(^{20}\) The binding blocks may of course influence the self-assembly behavior of the silk—elastin part to some extent, but this could then be compensated for by rational tuning of the block lengths in the triblock designs.

In summary, we examined the interplay between crystallizable and amorphous blocks in \( E_{m}S_{n}Q \) silk—elastin diblock polypeptides. We find that for diblocks with lengths of crystallizable blocks just over the threshold for fibril formation, the amorphous block acts as a break on fibril growth at low concentrations, with longer amorphous blocks leading to shorter fibrils. Since template-inducible silk fibril formation requires that fibril formation only occurs at high concentrations, we conclude that for engineering (template)-inducible silk fibril formation of \( E_{m}S_{n}Q \) silk—elastin diblock polypeptides, suitable block lengths are most likely those with crystallizable block lengths \( n \) close to the threshold for fibril formation as possible. At such critical silk-block lengths \( n \), the amorphous block length \( m \) can be used for fine-tuning the assembly behavior. In combination with suitable template-binding blocks, such diblocks allow for nucleic acid encapsulation in silk fibrils,\(^{20}\) and, for example, may also allow for engineering-induced silk fibril formation on the surfaces of biomaterials, which has also been explored for other silks.\(^{32–34}\)

**EXPERIMENTAL SECTION**

**Materials.** pET24a(+) was previously modified for PRe-RDL (recursive directional ligation by plasmid reconstruction) cloning.\(^{21}\) PRe-RDL cloning vectors containing genes for various numbers of the ELP repeat \((E_{m}S_{n}Q_{m}S_{n}Q\) where \( m = 40, 60, \) or \( 80 \)) were previously developed.\(^{35}\) They were digested for 3 h at 37 °C using BglII and AcuI enzymes. BglII \( \times \) AcuI fragments containing the \( E_{m}S_{n}Q_{m}S_{n}Q\) genes, the so-called A-fragments, were purified using gel electrophoresis by separating the digestion products on a 1% agarose gel. To obtain the B-fragments, vectors containing the genes for a number of repeats of the silklke repeat \((S_{n}Q_{m}S_{n}Q\) where \( n = 6, 10, \) or 14) were digested in 3 h at 37 °C using BglII and BseRI enzymes and the fragments containing the genes for the \( S_{n}Q_{m}S_{n}Q\) sequences were again purified by gel purification. Genes for \( E_{m}S_{n}Q_{m}S_{n}Q\) diblocks were then constructed by ligation of the A- and B-fragments using Quick ligase for 5 min at room temperature in a 1X Quick ligase buffer. The ligation products were transformed into *E. coli* EBSAlpha chemically competent cells, and the cells were plated on TBdry-Agar plates supplemented with 45 μg/mL kanamycin. Colony PCR was performed to select those colonies that contained plasmids with one or more copies of the sequence encoding the silklke repeat \( S_{n}Q_{m}S_{n}Q\) and identity of the plasmids was confirmed by DNA sequencing.

**Gene Construction by PRe-RDL.** To construct genes encoding for a set of \( E_{m}S_{n}Q_{m}S_{n}Q\) diblock polypeptides with various lengths of the ELP and silklke blocks, we used the PRe-RDL approach previously developed.\(^{21}\) The pET24a(+) PRe-RDL cloning vectors containing genes for various numbers of the ELP repeat \((E_{m}S_{n}Q_{m}S_{n}Q\) where \( m = 40, 60, \) or \( 80 \)) were previously developed.\(^{35}\) They were digested for 3 h at 37 °C using BglII and AcuI enzymes. BglII \( \times \) AcuI fragments containing the \( E_{m}S_{n}Q_{m}S_{n}Q\) genes, the so-called A-fragments, were purified using gel electrophoresis by separating the digestion products on a 1% agarose gel. To obtain the B-fragments, vectors containing the genes for a number of repeats of the silklke repeat \((S_{n}Q_{m}S_{n}Q\) where \( n = 6, 10, \) or 14) were digested in 3 h at 37 °C using BglII and BseRI enzymes and the fragments containing the genes for the \( S_{n}Q_{m}S_{n}Q\) sequences were again purified by gel purification. Genes for \( E_{m}S_{n}Q_{m}S_{n}Q\) diblocks were then constructed by ligation of the A- and B-fragments using Quick ligase for 5 min at room temperature in a 1X Quick ligase buffer. The ligation products were transformed into *E. coli* EBSAlha chemically competent cells, and the cells were plated on TBdry-Agar plates supplemented with 45 μg/mL kanamycin. All sequences were confirmed by DNA sequencing.

**Polypeptide Expression.** Between 100 and 150 ng of the plasmids encoding the \( E_{m}S_{n}Q_{m}S_{n}Q\) diblocks were transformed into BL21(DE3) *E. coli* chemically competent cells. These cells were used to inoculate starter cultures of 10 mL of TB medium supplemented with 45 μg/mL kanamycin. Starter cultures were incubated at 37 °C overnight on a shaker at 200 rpm and used to inoculate cultures of 2 L TB medium with 45 μg/mL kanamycin. Cells were grown at 37 °C on a shaker at 200 rpm for a total of 24 h. Polypeptide expression was induced in these cells 8 h after inoculation by supplying isopropyl β-D-1-
thiogalactopyranoside at a final concentration of 1 mM to the medium.

**Polypeptide Purification.** After 24 h, cells were centrifuged at 3000 × g for 10 min at 10 °C and the pellet was resuspended in 25 mL of cold phosphate-buffered saline (PBS). The cells were lysed on ice by 18 sonication cycles of 10 s with 40 s intervals (Sonicator 3000, Misonix). Inclusion bodies were pelleted by centrifugation at 29 000 × g for 12 min at 4 °C, and the supernatant (soluble lysate), containing the polypeptide, was collected. Next, nucleic acids were removed by mixing the soluble lysate with 4 mL of 10% polyethylenimine and centrifugating at 29 000 × g for 12 min at 4 °C. Polypeptides in the remaining supernatant (clearsed lysate) were further purified using inverse transition cycling (ITC). During each round of ITC, polypeptides are subjected to the following four steps: (1) addition of 10−

**S10** M ammonium sulfate or sodium chloride to lower the transition of the ELP-containing polypeptides. (2) Incubation at 37 °C for 15 min to induce phase separation of the ELP-containing polypeptides, and precipitation of the ELP-containing polypeptides by centrifugation at 40 °C for 12 min at 29 000 × g. Eₘ₋Sₘ polypeptides were purified by multiple rounds of ITC, but it was found that this did not lead to the desired level of purity of the polypeptides. To further increase purity, we also used a "bake-out" procedure: samples were incubated at 95 °C for 15 min to induce denaturation of any remaining cellular proteins. Next, the samples were cooled down on ice for 30 min to ensure that the ELP-containing polypeptides dissolved, and finally, the remaining denatured cellular proteins were removed by centrifugation at 4 °C for 12 min at 29 000 × g. A bake-out only was found to be insufficient for polypeptide purification (as determined spectroscopically and with SDS-PAGE) so that this procedure was always combined with several rounds or ITC. For the Eₘ₋Sₘ polypeptides, three rounds of ITC were used, followed by a bake-out. For the Eₘ₋Sₘ polypeptides, three rounds of ITC were used with a bake-out after the 1st and 2nd rounds of ITC. Finally, for the Eₘ₋Sₘ polypeptides, bake-out was integrated in ITC: after centrifugation of aggregated ELP-containing polypeptides (ITC step 2), the pellet was resuspended and solubilized in PBS by incubation for 15 min at 95 °C (step 3). To remove denatured proteins and other insoluble matter, the sample was cooled down on ice for 30 min and centrifuged at 4 °C for 12 min at 29 000 × g (step 4). Two rounds of ITC including bake-out were performed to purify the Eₘ₋Sₘ polypeptides. Finally, all polypeptides were dialyzed against Milli-Q water, lyophilized, and stored at room temperature.

**Polypeptide Characterization.** Polypeptide purity was assessed with SDS-PAGE. SDS-PAGE was carried out using 4−20% Ready Gel Tris−HCl precast gels, 1x Laemmli running buffer, and Precision Plus Protein Kaleidoscope Prestained Protein Standard. Gels were stained with PageBlue. To confirm the molecular weight of the polypeptides, we used matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). MALDI-TOF MS was carried out on an UltraFlexXTreme mass spectrometer (Bruker). Weighed portions of freeze-dried polypeptides were mixed with the matrix to a final concentration of 0.3 mg/mL and dried on the 800 μm spots of an MTP AnchorChip 384 target (Bruker). After mixing, final concentrations of the matrix components were 5 mg/mL 2,5-dihydroxyacetophenone, 1.5 mg/mL di-ammonium hydrogen citrate, 25% (v/v) ethanol, and 1% (v/v) trifluoroacetic acid.

In addition to SDS-PAGE, polypeptide purity was monitored during purification by spectrophotometry using a Thermo Scientific NanoDrop spectrophotometer. After each round of ITC and/or bake-out, 2 μL of the sample was applied onto the NanoDrop to record the 220−350 nm spectra. For pure samples, no absorbance at 280 nm is observed as the silk−elastin diblocks do not contain any amino acid that absorbs at this wavelength, viz., tryptophan, tyrosine, and cysteine. The presence of the silk−elastin diblocks in these pure samples is confirmed by high absorbance at 230nm, which is caused by the peptide bonds. Nonpure samples are characterized by absorbance at 230 and 280 nm, due to the presence of the above-mentioned amino acids in natural (bacterial) proteins.

**Polypeptide Self-Assembly.** For atomic force microscopy experiments, polypeptide stock solutions of 100 μM were prepared by vortexing weighed portions of freeze-dried polypeptides in 10 mM phosphate buffer (pH 7.4) at 95 °C for 10 min. Solutions at lower concentrations were prepared by immediately diluting the 100 μM polypeptide solutions in 10 mM phosphate buffer (pH 7.4). Next, samples were incubated at room temperature for 24 h to allow self-assembly of the silk−elastin fibrils. For cryo-TEM, weighed portions of freeze-dried Eₘ₋Sₘ and Eₘ₋Sₘ polypeptides were solubilized in PBS to a concentration of 100 μM by a combination of vortexing and heating to 65 °C for 15 min. Solubilized polypeptides were incubated for 16 h at room temperature to allow for the assembly of the silk−elastin fibrils.

**Atomic Force Microscopy (AFM).** Samples for AFM imaging were prepared by depositing 5 μL of 24 h incubated polypeptide solutions onto clean silicon surfaces. Salts and nonabsorbed particles were removed after 2 min by rinsing the surfaces with 1 mL of Milli-Q water. Surfaces were slowly dried under a N₂ stream. Samples were analyzed in air in the ScanAsyst (PeakForce Tapping) imaging mode on a NanoScope MultiMode 8 system (Bruker) using ScanAsyst-Air cantilevers (Bruker). The scan area was set to 5 × 5 μm², and images were recorded at 512 × 512 pixels and a line rate of 0.977 Hz. A second-order flattening was performed on all images using NanoScope Analysis 1.40 software. Lengths of the self-assembled silk−elastin fibrils were measured using FiberApp software with settings: α = 0, μ = 500, γ = 20, κ₁ = 20, κ₂ = 10, step = 1 pixel, and iterations = 100, "Use A* pathfinding algorithm". Lengths obtained with FiberApp software were binned to obtain length distributions and to calculate the number and weight averages of lengths of the self-assembled silk−elastin fibrils (assuming fibril mass is proportional to fibril length). The standard error of the mean for the weight averages was estimated using the method of block averages: fibril length observations were split into 5 blocks, and the standard error of the mean was taken to be the standard deviation of the weight-averaged fibril lengths for the 5 blocks.

**Cryogenic Transmission Electron Microscopy (cryo-TEM).** Solutions containing 100 μM Eₘ₋Sₘ or Eₘ₋Sₘ and incubated for 16 h at room temperature were prepared for cryo-TEM. The cryo-TEM measurements were performed at Duke University’s Shared Materials Instrumentation Facility. First, lacey holey carbon grids (Ted Pella) were cleaned in a PELCO EastGlow cleaning system (Ted Pella). For each sample, 3 μL was deposited onto a grid. Samples were then placed on a臺cc-Omesa.9b1025

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vitrified using a FEI Vitrobot Mark III by blotting the sample for 3 s with an offset of −3 mm and vitrifying it in liquid ethane. To prevent sample evaporation prior to vitrification, the sample chamber was kept at 22 °C and 100% humidity. Finally, the prepared grids were placed into a Gatan 626 cryoholder and imaged on a FEI Tecnai G2 Twin.

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L.W. designed, biosynthesized, and characterized the polypeptides and performed cryo-TEM and AFM experiments. S.R. and I.W. contributed to biosynthesis of the polypeptides and cryo-TEM experiments. A.C. advised on polypeptide design; E.M. and J.V.D.O. contributed to the design of the project. R.D.V. designed the project. L.W. and R.D.V. wrote the manuscript; all authors have edited the manuscript and have given approval to the final version of the manuscript.

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The authors declare no competing financial interest.

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