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Solvent Composition Dictates Emergence in Dynamic Molecular Networks Containing Competing Replicators

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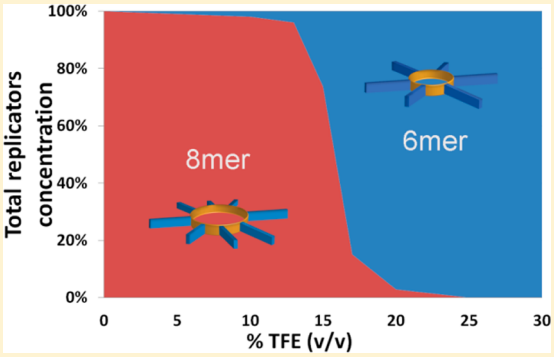
Supporting Information

ABSTRACT: In Darwinian evolution, species that are better adapted to their environment win the competition for common resources from less well-adapted competitors. Thus, in such scenarios the nature of the environment may dictate the outcome of the competition. We investigated to what degree these biological principles acting at the level of species extend to the molecular level into systems based on fully synthetic self-replicating molecules. We now report two systems in which two replicators compete for a common building block and where the environment dictates which of the two replicators wins. We observed that subtle changes in the environment can lead to dramatic differences in the outcome of the competition.



INTRODUCTION

The interplay between biological species and their environment has an important role in Darwinian evolution. Natural selection favors species that are best adapted to their environment. Experimental[1] and theoretical[2,3] work has shown that changes in the environment can enhance the rate of evolution. On the basis of these observations it is likely that the environment has also played an important role in the origin of life: the transition from inanimate to animate matter.[4] Similarly, the influence of the environment should also be considered in attempts to synthesize life denovo.

Self-replicating molecules[5−7] are likely to have played an important role in the origin of life[8] and are a promising starting point for synthesizing life denovo. Simple chemical systems able to self-replicate without any biological assistance were designed for the first time by von Kiedrowski in 1986, based on a palindromic DNA hexanucleotide.[9,10] Other remarkable examples are provided by von Kiedrowski,[11,12] Rebek,[13,14] Joyce,[15,16] Lehman,[17] Philp,[18−20] Ghadiri[21,22] and Ashkenasy.[23−26] In these examples, self-replicating molecules are invariably obtained as dimeric species produced from the reaction between two precursor molecules with one reactive group each (Figure 1a). We recently developed a new family of self-replicating molecules for which the architecture of the replicator is not predetermined.[27,28] Our replicators are formed from molecules with two reactive ends, allowing for the formation of macrocyclic molecules of different ring sizes (Figure 1b). We reasoned that the variety of potential products should translate into an enhanced plasticity and adaptability of the system to changes in the environment. We now report that the environment can indeed determine which self-replicator emerges. In systems in which two replicators compete for a common building block a subtle change in solvent composition was found to have a dramatic effect on the outcome of the competition.

Self-Replication by a Fiber Elongation-Breakage Mechanism. We recently reported the emergence of a single self-replicating molecule from separate small dynamic combinatorial libraries[29,30] made from peptide-functionalized dithiols **1a−c** (Figure 2).[28] Allowing a stirred solution made from any of the three building blocks in water to oxidize resulted in a mixture containing differently sized macrocyclic products (Figure 2) that continuously interconvert as a result of the disulfide exchange reaction.[31] After an initial phase where the smaller trimer and tetramer macrocycles dominate, self-replication of a specific larger macrocycle occurred, that rapidly grew to dominate the mixture. Self-replication in these systems is a result of self-assembly by the replicator into stacks, driven by β-sheet interactions between the peptides. This self-assembly process stabilizes the macrocycle that assembles, draining the equilibrium of this compound.[32] Self-replication is critically dependent on agitation, which breaks growing fibers, increasing the number of ends from which the fibers grow (Figure 1b). The assembly and replication process bears similarities to amyloid formation.[33−37] For the phenylalanine peptide **1a**, a hexameric replicator emerged, while for the more hydrophilic alanine or serine-containing peptide (**1b** or **1c**) the main product was the octameric macrocycle.[28] This difference in replicator size was rationalized as follows: for macrocycles to self-assemble the interactions between them need to exceed a critical energy. Small macrocycles carry too few peptide chains to allow self-assembly, but at a certain macrocycle size the compounds exhibit sufficient multivalency to enable stacking.

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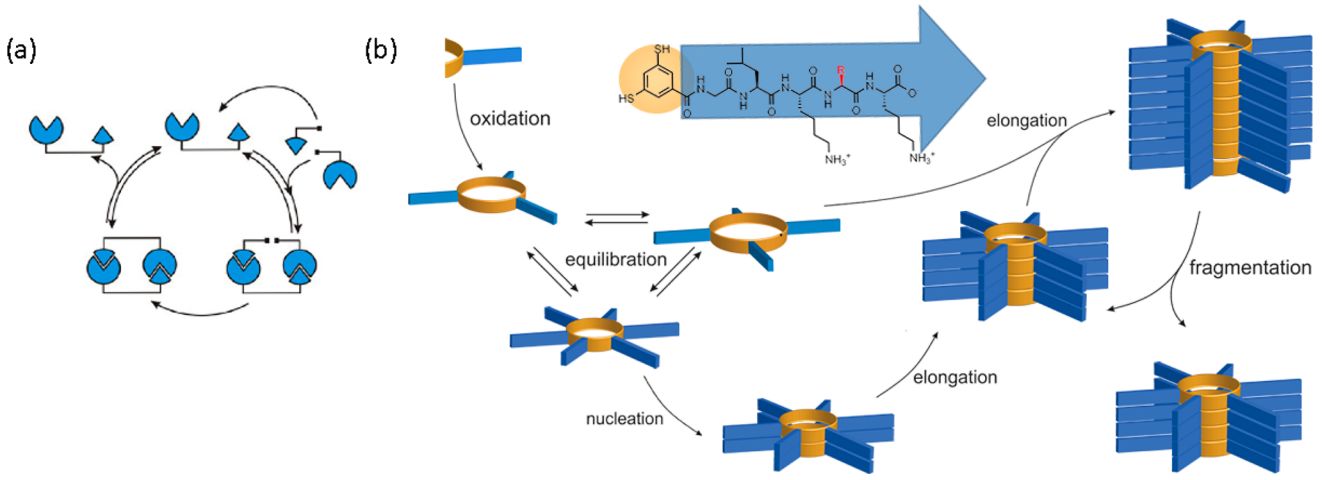

Figure 1. (a) General scheme for self-replication starting from molecules with only a single reactive end. (b) Mechanism of replication in a system of bifunctional building blocks which initially form an exchanging mixture of macrocycles of different sizes via oxidation of thiols to disulfide bonds and subsequent disulfide exchange. Macrocycles of a specific size (in this case the hexamers) self-assemble into fibers as the peptide chains (arrows) form beta sheets through a nucleation-elongation mechanism. The fibers grow from their ends and break upon mechanical agitation, doubling the number of fiber ends that further promote the formation of self-replicating hexamer.

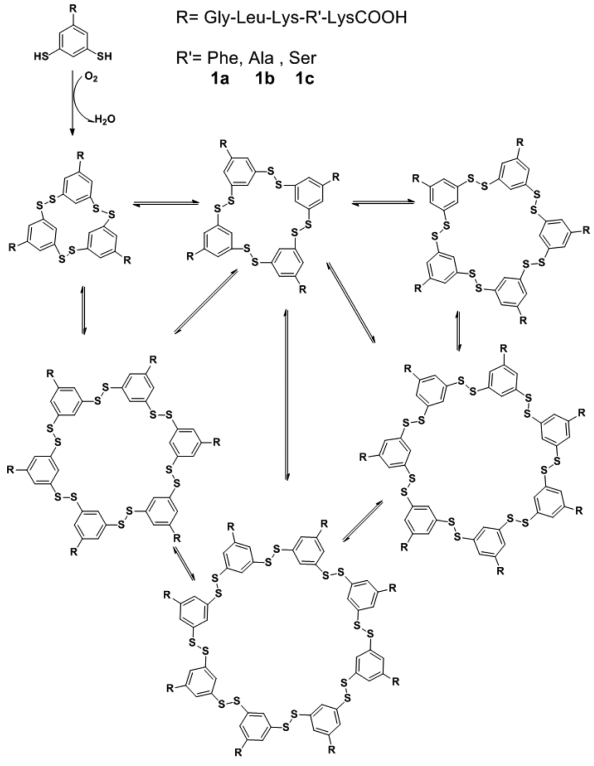

Figure 2. Oxidation of peptide-functionalized dithiol building blocks **1a**–**c** results in the formation of an equilibrium mixture of differently sized macrocyclic disulfides.

This macrocycle size is dependent on the strength of the interactions between the peptides. With the more hydrophobic peptide (phenylalanine **1a**) self-assembly into fibers is feasible for a smaller macrocycle size (hexamer) than for the less hydrophobic alanine and serine containing peptides (**1b** and **1c**), which, in water, only assemble as octameric macrocycles. We reasoned that changing the nature of the solvent may change the interaction energy between the peptides and thereby change the size of the emerging self-replicator, which would allow us to identify systems in which differently sized replicators would compete for the same building block. We focused on mixtures of water and 2,2,2-trifluoroethanol (TFE), as the latter cosolvent is known to enhance secondary structure formation in proteins and peptides.[38]

RESULTS AND DISCUSSION

Experiments were set up by allowing building blocks **1a**–**c** to oxidize in the presence of oxygen from the air in stirred aqueous solutions containing different percentages of TFE. We monitored the composition of the mixtures over time using UPLC-MS[39] and observed that, for phenylalanine peptide **1a**, mostly trimer and tetramer macrocycles were formed (Figure 3a and b). However, for the less hydrophobic peptides **1b** and **1c**, depending on the percentage of TFE, dramatically different results were obtained. Molecular networks made from alanine

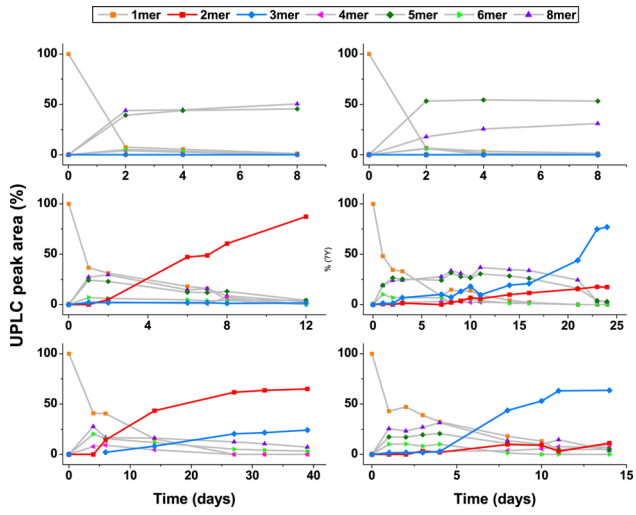

Figure 3. Change in product distributions of DCLs (3.8 mM dithiol building block in 50 mM borate buffer pH 8.2) made from (a) phenylalanine-containing peptide **1a** at 10% v/v TFE, (b) **1a** at 30% v/v TFE, (c) alanine-containing peptide **1b** at 10% v/v TFE, (d) **1b** at 13% TFE, (e) serine-containing peptide **1c** at 15% TFE, and (f) **1c** at 17% TFE.[39]

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peptide **1b** gave rise to the predominant emergence of octamer self-replicator in the presence of 10% v/v of cosolvent, similar to the behavior in pure water (Figure 3c). However, upon increasing the percentage of TFE from 10 to 13% (Figure 3d) hexameric macrocycles were the dominant species. Similar behavior was observed for systems made from the least hydrophobic building block **1c**, but at a somewhat larger percentage of TFE. Until 15% v/v of this cosolvent the octamer emerged as the predominant replicator (Figure 3e), like in pure water, while at 17% TFE the hexamer dominated (Figure 3f).

We performed similar experiments for a range of different TFE fractions. The final ratios between octamer and hexamer macrocycles of **1b** and **1c** as a function of the percentage of TFE in the reaction medium are shown in Figure 4,

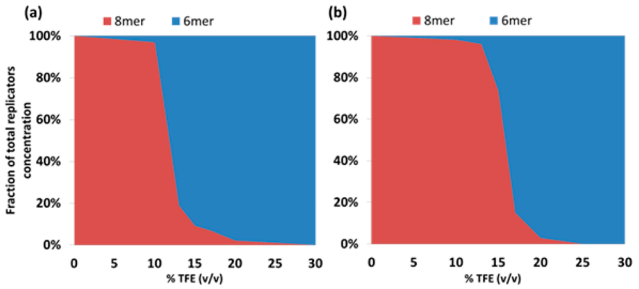

Figure 4. Summary of the experiments at different percentages of TFE (up to 30%) for (a) building block **1b** and (b) building block **1c**. The area in red indicates the relative peak area of the cyclic octamer, while the blue area shows the relative peak area of the cyclic hexamer. The octamer is the predominant replicator (a) until 10% TFE or (b) until 15% TFE.

demonstrating that the switch from octamer to hexamer is a strongly nonlinear function of the solvent composition. Such pronounced nonlinear behavior is explained by the fact that the replicators are capable of exponential growth.[40] Simulation of a simplified model of two exponential replicators competing for a common building block shows a similar sudden transition from one replicator to the other as the relative rate of replication changes. See Supporting Information Figure S28.

As a control, libraries were set up in different water/ethanol mixtures of different compositions (see Supporting Information Figures S25 and S26). In all cases the octamer self-replicator emerged, but at a somewhat reduced rate compared with libraries set up in borate buffer only. No traces of hexamer macrocycles could be detected.

We then verified whether the hexamers and octamers of **1b** and **1c** are self-replicators under the different conditions of the experiments. Samples were made by oxidizing **1b** or **1c** to produce solutions that were dominated by trimer and tetramer, without the presence of any suspected replicators. Solutions were made in 10% TFE (for the octamers) and 30% TFE (for the hexamers) and to these solutions 10% of octamer or hexamer seeds were added, respectively. We then monitored the rate of growth of octamer or hexamer and compared it with the corresponding rate in the absence of seed. The results are shown in Figure 5 and demonstrate that the addition of the suspected replicators indeed enhanced the rate of their own formation, confirming that both octamers and hexamers are self-replicators; i.e. capable of catalyzing their own formation.

Replication is accompanied by fiber formation, as evident from transmission electron microscopy (TEM) micrographs (see Supporting Information Figures S23 and S24). The

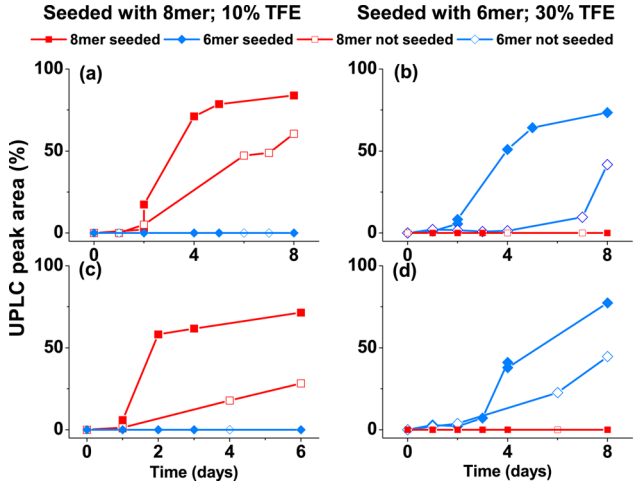

Figure 5. Seeding-induced growth of suspected self-replicating macrocycles under conditions that favor their formation. In all cases the libraries were seeded with 10 mol% of suspected replicator. (a) Octamer seeded at day 2 in a 10% TFE solution containing **1b**. (b) Hexamer seeded at day 2 in a 30% TFE solution containing **1b**. (c) Octamer seeded at day 1 in a 10% TFE solution containing **1c**. (d) Hexamer seeded at day 3 in a 30% TFE solution containing **1c**. The data are compared with libraries that were not seeded (open symbols).[39]

hexamer fibers formed from **1b** and **1c** in the presence of 30% TFE showed substantial lateral association, as was observed previously for the octamers of these building blocks.[28] The fibers of the hexamers of **1b** and **1c** were further characterized by circular dichroism and thioflavin T fluorescence experiments (see Supporting Information Figures S20 and S21). Both techniques confirmed the presence of β-sheets, similar to what we observed previously for the octamers.[28]

We also performed cross seeding experiments to verify whether the hexamer and octamer macrocycles are replicators in environments in which they do not grow to significant concentrations in the absence of seeding. Thus, seeding mixtures of trimer and tetramer macrocycles of **1b** or **1c** with hexamer seed in 10% TFE and octamer seed in 30% TFE in all cases showed that the seeded species was capable of self-replication, albeit to a very limited extent (Figure 6, but more clearly visible in Figure 7). However, the growth of the species that was added as a seed was in all cases much slower than the competing replicator, despite that no seed of the latter had been added (Figure 6).

Thus, even when competing octamer replicator was added as a seed, the hexamer of **1b** and **1c** emerged as the predominant replicator in 30% TFE libraries (Figure 6b, d). Conversely, the octamer became the dominant replicator in 10% TFE experiments (Figure 6a, c) even when these were seeded with competing hexamer replicator. Thus, these systems do not behave as instructable replicator networks in the way recently reported by Philp, where the seed that was added dictated which of the competing replicators became dominant.[20]

Most surprisingly, the growth of the replicator `nativè to a specific environment appears to benefit from seeding by its competing `non-nativè replicator, suggesting that cross-catalysis between replicators of different macrocycle size is taking place.

The final compositions of the solutions of all eight seeding experiments on **1b** and **1c** are summarized in Figure 7 and

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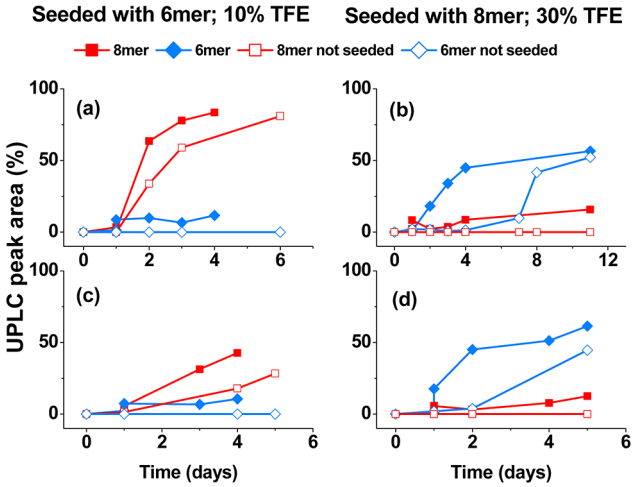

Figure 6. Seeding-induced growth of suspected self-replicating macrocycles under conditions that *do not* favor their formation. (a) Peptide **1b** at 10% TFE; (b) peptide **1b** at 30%TFE; (c) peptide **1c** at 10% TFE; (d) peptide **1c** at 30% TFE. The libraries were seeded on day 1 with 10 mol % of (a, c) hexamer and (b, d) octamer. The data are compared with libraries that were not seeded (open symbols).[39]

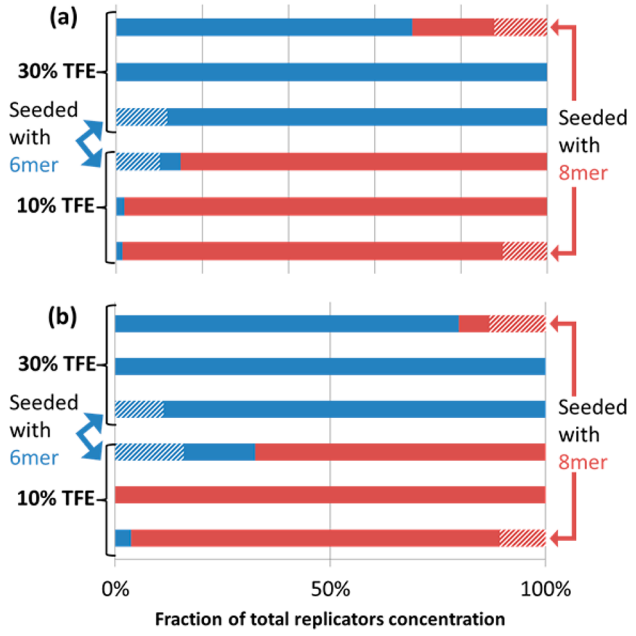

Figure 7. Summary of the seeding and cross-seeding experiments for (a) building block **1b** and (b) building block **1c**, compared with nonseeded experiments. The hexamer is shown in blue and the octamer in red. The striped areas correspond to the amount of added seed.

compared with the compositions of the corresponding samples that were not seeded.

The fact that we do not observe any consumption of the non-native replicators that were added as seed, also not during later stages of replication, suggests that the macrocycles that are incorporated into fibers do not readily undergo disulfide exchange and appear to be kinetically trapped within these fibers.

We previously reported that in water solution peptide hydrophobicity determines the macrocycle size of the self-replicator that emerges. When the hydrophobicity decreases the size of the macrocycle increases.[28] The above data show that it is also possible to affect the macrocycle size of the emerging replicator by changing the solvent. With the use of TFE cosolvent, peptide−peptide interactions are strengthened. MD simulations by Mark et al.[41] suggest that preferential solvation of peptides by TFE enhances intrapeptide hydrogen bonding by reducing competition by water and providing a low dielectric environment. TFE shows only limited association with hydrophobic residues of the peptides, so hydrophobic interactions between the peptides are not significantly affected. With TFE mediated strengthening of the interactions between peptide building blocks, stacking becomes feasible for smaller macrocycles. Thus, in the presence of TFE oxidation of building block **1a** (which formed hexamers in water) resulted in the fibers made from trimer and/or tetramer (see Supporting Information Figure S22).[42] For **1b** and **1c**, the addition of TFE causes stacking to become feasible for hexameric macrocycles, while octamers were required in pure water. The concentration of TFE required for the changeover was higher for the less hydrophobic and therefore less strongly interacting serine-containing building block **1c** as compared to alanine-containing **1b**. These trends are summarized in Figure 8, which shows

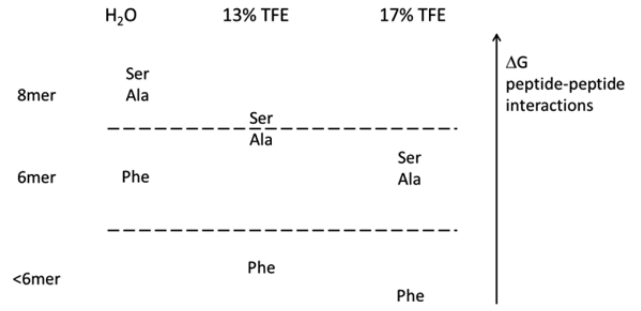

Figure 8. Qualitative comparison of the strength of the peptide−peptide interactions for the different peptides in water/TFE mixtures. Introduction of TFE strengthens the peptide−peptide interactions, making stacking feasible for smaller macrocycles.

qualitatively the strength of the peptide-peptide interactions for **1a**−**c** in different water/TFE mixtures. The horizontal lines separate the areas in which specific macrocycles dominate.

CONCLUSIONS

Our results capture for the first time at a molecular level the role of the environment in the competition between two replicating molecules for a common building block. Perhaps not surprisingly the environment plays a decisive role in the emergence of self-replicators from complex mixtures. The proportion of two competing replicators was found to be a strongly nonlinear function of the solvent composition. This finding opens new possibilities in systems chemistry,[8,43,44] specifically for investigating Darwinian evolution at the molecular level, since we are now able to rationally affect fitness (i.e., the rate of replication) of competing replicators by changing the environment. The results suggest that it should be possible to define niches for specific replicators by creating specific environments.

EXPERIMENTAL SECTION

Materials. Water was doubly distilled prior to use. Boric acid and potassium hydroxide utilized for the preparation of buffers and pH adjustment were obtained from Acros Organics and Merck Chemicals,

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respectively. 2,2,2-Trifluoroethanol was purchased from Sigma-Aldrich. Ethanol was purchased from J. T. Baker. Acetonitrile (UPLC grade), water (UPLC grade), and trifluoroacetic acid were purchased from Biosolve BV.

Peptide Synthesis. Peptides **1a−c** were synthesized by Cambridge Peptides Ltd. (Birmingham, U.K.) from 3,5-bis(tritylthio)-benzoic acid, which was prepared via a previously reported procedure.[27] All peptides showed purity higher than 85%. Impurities were mostly due to oxidation of thiols to disulfides (i.e., dimer, trimer).

Library Preparation and Sampling. Building blocks **1b−c** were dissolved to a concentration of 3.8 mM in borate buffer (50 mM, pH 8.2) and 2,2,2-trifluoroethanol in 10%, 13%, 15%, 17%, 20%, 25%, and 30% v/v. After the addition of TFE, the pH of the solution was adjusted by the addition of 2.0 M KOH solution such that the final pH was 8.2. The volume of each library was 400 μL. Each solution was allowed to equilibrate in an HPLC vial (12 × 32 mm) with a Teflon-lined snap cap. All the samples contained a cylindrical microstirrer bar (2 × 5 mm, Teflon-coated, purchased from VWR) and were stirred at 1200 rpm using an IKA RCT basic hot plate stirrer. All library experiments were performed at ambient temperature. A small aliquot of each sample was removed to another vial and diluted 20 times with doubly distilled water prior to UPLC or LC-MS analysis.

Seeding Experiments. Two libraries containing building blocks **1b−c** in 10% and 30% TFE were prepared according to the procedure described above. The thiol solutions were oxidized to convert 70% of the thiols to disulfides with sodium perborate (freshly prepared, 38 mM) such that they contained mostly monomer, trimer and tetramer. The libraries were then split in two parts. To one of the samples prepared with 10% TFE a small amount (10 mol %) of pre-existing octamer was added. The same amount of hexamer was added to one of the two libraries prepared with 30% TFE. All four libraries were monitored by UPLC.

Cross Seeding Experiments. For peptides **1b−c**, two solutions containing mostly monomer, trimer, and tetramer in the presence of 10% and 30% TFE were prepared according to the procedure described above. The libraries were then split in two parts. To one of the two libraries prepared with 10% TFE a small amount (10 mol %) of pre-existing hexamer was added. The same amount of octamer was instead added to one of the two libraries prepared with 30% TFE. All four libraries were monitored by UPLC.

Negative Staining Transmission Electron Microscopy. A small drop (5 μL) of sample was deposited on a 400 mesh copper grid covered with a thin carbon film (Agar Scientific). After 30 s, the droplet was blotted on filter paper. The sample was then stained twice (4 μL each time) with a solution of 2% uranyl acetate deposited on the grid and blotted on the filter paper after 30 s each time. The grids were observed in a Philips CM120 cryo-electron microscope operating at 120 kV. Images were recorded on a slow scan CCD camera.

Thioflavine T (ThT) Fluorescence. Sample aliquots were diluted to a concentration of 76 μM with respect to peptides **1b−c** with additional potassium borate buffer (50 mM, pH 8.2). The diluted sample (3.2 μL) was added to a ThT solution (22 μM, 26.8 μL) in potassium borate buffer (50 mM, pH 8.2) and incubated for 5 min. The solution was diluted with additional potassium borate buffer (50 mM, pH 8.2, 100 μL) and transferred into a HELMA 10 × 2 mm quartz cuvette. The fluorescence was measured on a JASCO FP6200 spectrophotometer by excitation at 440 nm (5 nm slit width) and emission from 460 to 700 nm (5 nm slit width, 3 repeats averaged).

Circular Dichroism (CD). Spectra were obtained at 20 °C using a JASCO J715 spectrophotometer (range = 190−400 nm, pitch = 2 nm, bandwidth = 5 nm, response = 2 s, speed = 50 nm/min, continuous scanning) and HELMA 10 × 2 mm quartz cuvettes. All reported spectra are averages of 3 repeats. Solvent spectra were subtracted from all spectra. All spectra were obtained using samples diluted to 8 μM (with respect to building block).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

UPLC and LC-MS methods and data, CD spectra, ThT fluorescence experiments for the DCLs made from peptides **1b−c**, and TEM micrographs for DCLs made from peptides **1a−c**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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