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Uncovering the Selection Criteria for the Emergence of Multi-Building-Block Replicators from Dynamic Combinatorial Libraries

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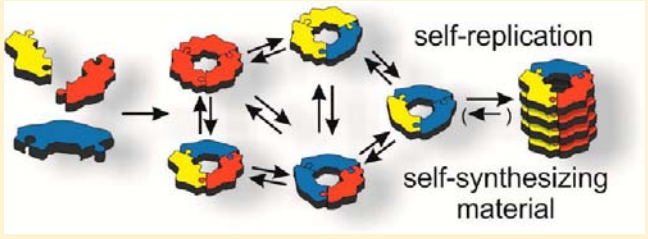
Supporting Information

ABSTRACT: A family of self-replicating macrocycles was developed using dynamic combinatorial chemistry. Replication is driven by self-assembly of the replicators into fibrils and relies critically on mechanically induced fibril fragmentation. Analysis of separate dynamic combinatorial libraries made from one of six peptide-functionalized building blocks of different hydrophobicity revealed two selection criteria that govern the emergence of replicators from these systems. First, the replicators need to have a critical macrocycle size that endows them with sufficient multivalency to enable their self-assembly into fibrils. Second, efficient replication occurs only for library members that are of low abundance in the absence of a replication pathway. This work has led to spontaneous emergence of replicators with unrivalled structural complexity, being built from up to eight identical subunits and reaching a MW of up to 5.6 kDa. The insights obtained in this work provide valuable guidance that should facilitate future discovery of new complex self-replicating molecules. They may also assist in the development of new self-synthesizing materials, where self-assembly drives the synthesis of the very molecules that self-assemble. To illustrate the potential of this concept, the present system enables access to self-assembling materials made from self-synthesizing macrocycles with tunable ring size ranging from trimers to octamers.



INTRODUCTION

Self-replicating molecules[1−8] play a central role in studies of the origin of life[9−13] and are key components in attempts to create de novo life. Until recently, the strategy toward the development of self-replicating molecules has been based on design of replicators following the general scheme shown in Figure 1a, in which a replicator contains two recognition sites through which it binds two precursor molecules and accelerates their ligation. This approach has been implemented successfully for replicators based on nucleic acids,[14−27] peptides,[28−43] hybrids thereof,[44−46] and fully synthetic structures.[47−62] Recently, an alternative to the design approach was introduced in which self-selection from dynamic combinatorial libraries (DCLs; Figure 1b)[63] leads to the emergence of a self-duplicator,[64] self-replicators,[65−68] and autopoietic self-assembled systems.[69,70] DCLs are equilibrium mixtures that are made by linking building blocks together through reversible covalent (or noncovalent) bonds.[71−76] The resulting molecular network is, in most cases, under thermodynamic control; that is, the concentration of each of the molecular constituents is determined by its stability relative to the other network members. This characteristic makes these networks responsive to external and internal influences that alter the relative stabilities of the molecules in the network. Any library member that is capable of recognizing and binding to copies of itself will shift the library composition toward its own formation. This can be considered as a form of self-replication, provided that the formation of the self-recognizing molecule is autocatalytic. Self-recognition may lead to replicator dimerization[64−66] or to assembly into larger self-assembled structures,[67−70] which we have termed "self-synthesizing materials"[67] since the self-assembly process drives the synthesis (i.e., replication) of the very molecule that self-assembles.

Nearly all replicators (dynamic combinatorial or otherwise) reported to date have featured limited structural diversity; replicators are formed through the ligation of two precursor molecules each having a single reactive group. However, we recently developed a system where replicators construct themselves from six or seven components.[67] Starting from a single peptide-functionalized building block with two reactive groups, a small DCL was obtained of differently sized macrocycles from which six- or seven-membered replicators emerged spontaneously, provided the mixtures were agitated. We could not have predicted these outcomes, which raises two more general questions: what are the selection criteria that determine (i) whether replication occurs in a dynamic combinatorial library and (ii) which potential replicator will arise? Answers to these questions are important to facilitate future discoveries of replicators.

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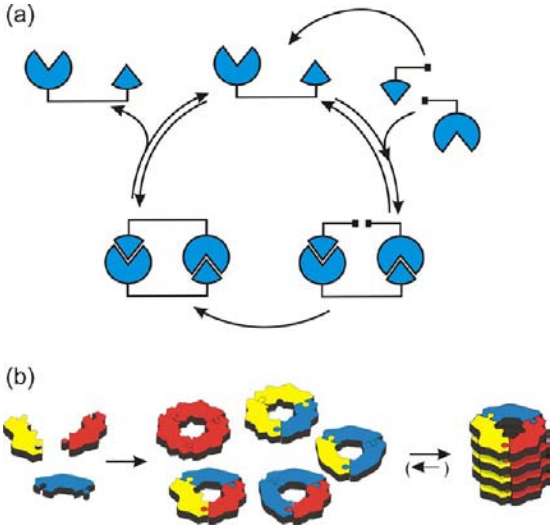


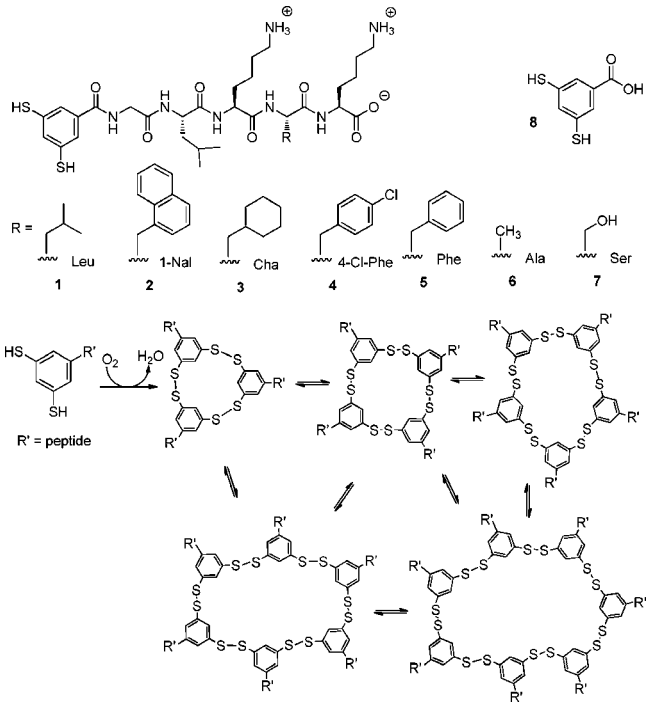
Figure 1. Schematic representation showing how (a) a replicator accelerates ligation of its two precursor molecules; (b) a molecule that is able to bind to copies of itself is able to promote its own formation by shifting the equilibrium in a dynamic molecular network.

In this paper, these questions are addressed by investigating a series of small DCLs made from building blocks featuring peptide sequences that differ in their ability to engage in hydrophobic interactions. Decreasing the hydrophobicity of the building blocks resulted in the preferred self-assembly driven formation of macrocycles of increasing ring size. Thus, trimers and tetramers dominated for the most hydrophobic peptides and macrocycles up to octamers were obtained for the least hydrophobic building blocks. The latter includes a replicator of MW 5.6 kDa, constructing itself from as many as eight identical building blocks, which is probably the most complex replication process yet described for a fully synthetic replicator. These results suggest an important role for multivalency:[77,78] the emergence of a replicator requires a critical macrocycle size that is able to form sufficiently strong interactions with fellow replicators to allow the assembly into fibrils. However, we also observed that the self-assembly of the macrocycles is not sufficient for replication, as the formation of trimers, tetramers, and even pentamers was not autocatalytic, while the formation of hexameric or larger rings was autocatalytic. Thus, autocatalysis appears to be associated with structures that are unlikely to be produced spontaneously, that is, that do not already have an efficient uncatalyzed pathway leading to their formation.

RESULTS AND DISCUSSION

We previously reported the emergence of two self-replicators from a small DCL made from building block **1**.[67,68] This building block features two thiol groups for reversible disulfide chemistry[79] and a peptide chain that has alternating hydrophobic and hydrophilic amino acid residues. Such sequences are predisposed to form β-sheets,[80] although they are too short to do so in isolation (vide infra). Stirring a solution of dithiol **1** in borate buffer (50 mM; pH 8.2) led to the oxidation of the thiols, resulting in the formation of a DCL of macrocyclic disulfides of different ring sizes (Scheme 1). Disulfide exchange takes place through reaction of residual thiolate anion with the disulfides.[79] Without agitation, the DCL was dominated by the cyclic trimer and tetramer that did not appear to self-assemble

Scheme 1. Oxidation of Peptide-Functionalized Dithiol Building Blocks 1−7 or Control Compound 8 Results in a Dynamic Combinatorial Library of Different Macrocyclic Disulfides



or self-replicate. Trimer and tetramer are the smallest unstrained macrocycles that can be formed, and these are expected to dominate the equilibrium mixture in the absence of any molecular recognition since the formation of many small macrocycles is entropically favored over forming a smaller number of larger macrocycles. Thus, under relatively dilute conditions (<10 mM), large macrocycles containing more than four building blocks do not form in significant concentrations in the absence of special influences that would enhance their relative stability. This behavior is exemplified by the DCL of control compound **8** that lacks the peptide chain, which forms predominantly trimer and tetramer macrocycles.[67] However, upon agitating the peptide DCLs made from **1**, large macrocyclic replicators emerged: stirring resulted in the emergence of cyclic heptamer, while shaking gave cyclic hexamer. The hexamer and heptamer (but not the trimer or tetramer) were found to self-associate to form long fibrils held together by β-sheets. We proposed a model for self-assembly and self-replication that features two processes: (i) elongation of the fibrils by sequestration of more of the corresponding macrocycle from the DCL[81,82] and (ii) mechanically induced breakage of the growing fibrils, increasing the number of growing fibril ends (Figure 2). Growth from fibril ends gives rise to a linear increase in fibril-forming macrocycles with time (at constant building block concentration), while fibril breakage may potentially give rise to exponential growth. Note that the fibrils are sufficiently small that they do not precipitate from solution, which remains transparent but shows a small increase in viscosity.

While these results demonstrated that replicators may self-select and emerge spontaneously from DCLs, it remained unclear why specific macrocycle sizes emerged as replicators while others did not. In order to uncover the selection criteria

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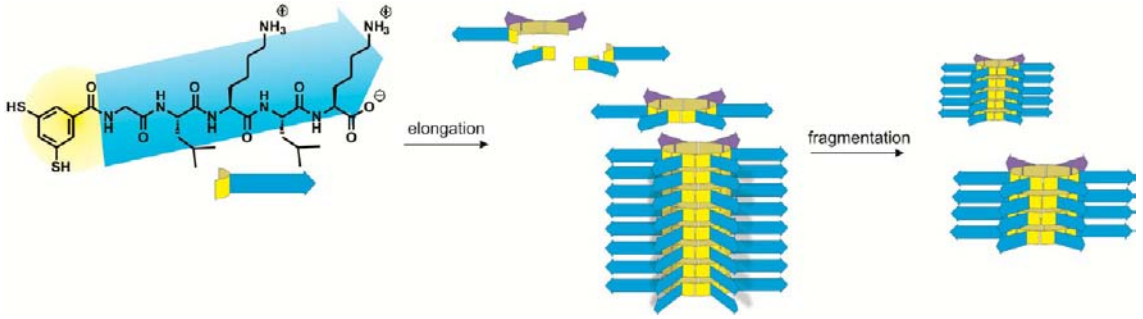

Figure 2. β-Sheet formation between the peptide chains of $\mathbf{1}_6$ drives its assembly into fibrils that grow from their ends. Mechanically induced fragmentation accelerates fibril growth by increasing the number of growing ends.

that govern the emergence of replicators from such mixtures, we have studied the behavior of DCLs made from a family of building blocks of which the structure was varied systematically. Our expectation was that multivalency[77,78] plays an important role in the assembly process. We did not obtain any evidence for self-assembly by the individual dithiol building blocks, suggesting that the interactions that these building blocks could potentially form between themselves are too weak to overcome the entropic penalty of bringing the molecules together. However, our results with peptide **1** show that, when six or seven building blocks are brought together in a large macrocycle, the interactions between the macrocycles are now sufficiently strong to enable assembly into fibrils. The large macrocycle can now form interactions with other same-sized macrocycles using six or seven appended peptide chains. If our hypothesis regarding the role of multivalency as a selection criterion for the emergence of replicators is correct, then building blocks with more hydrophobic peptide sequences should give rise to more stable β-sheets, so that fibril formation (and concomitant self-replication) becomes feasible for a reduced macrocycle size.

Conversely, a less hydrophobic peptide should only give fibrils assembled from larger macrocycles. Thus, building blocks carrying new peptide sequences **2**–**7** (Scheme 1) were designed to be either less or more hydrophobic than peptide **1**, by substituting the C-terminal leucine (Leu) of peptide **1** with more hydrophobic amino acids such as 1-naphthylalanine (1-Nal in **2**), cyclohexylalanine (Cha in **3**), *para*-chlorophenylalanine (*p*-Cl-Phe in **4**), and phenylalanine (Phe in **5**) or less hydrophobic amino acids, including alanine (Ala in **6**) and serine (Ser in **7**).

Since peptide hydrophobicity does not always follow the same order as amino acid hydrophobicity,[83] we first estimated the relative hydrophobicity of the seven building blocks based on their retention times on a reversed-phase HPLC column (Table 1). In this case, the peptide hydrophobicity turned out to correlate well with the hydrophobicity of the amino acid residue that was varied.

We will now first discuss how the product distributions of the DCLs change with time under different agitation conditions. We will then present the results of seeding experiments to establish which of the emerging macrocycles are self-replicators. Finally, we will show the characterization of the self-assembled structures by electron microscopy, circular dichroism, and fluorescence microscopy, confirming that replication is accompanied by amyloid-like peptide self-assembly.

Emergence of Replicators from Dynamic Combinatorial Libraries. DCLs were set up starting from the new building blocks **2**–**7**. Three solutions (0.50 mL; 3.8 mM in peptide building block, contained in a 2 mL HPLC vial) were prepared for each peptide in borate buffer (50 mM, pH 8.2): one was stirred using a magnetic stir bar, one was shaken using an orbital shaker, and one was not agitated. Under the conditions used for stirring and shaking, the stirred samples experienced a higher maximum shear stress than the shaken samples.[84] The product distributions of the DCLs were analyzed using LC-MS, which allows the separation and identification of the individual macrocycles. Note that any self-assembled structures that are formed from the library members fall apart into their constituent macrocycles under the conditions of the HPLC analysis. A representative analysis of the DCL prepared from building block **4** is shown in Figure 3.

Table 1. Peptide Sequences and Their Retention Time in Reversed-Phase HPLC[a]

peptide	sequence[b]	retention time (min)
2	X-Gly-Leu-Lys-*1-Nal*-Lys-COOH	12.4
3	X-Gly-Leu-Lys-*Cha*-Lys-COOH	12.3
4	X-Gly-Leu-Lys-*p-Cl-Phe*-Lys-COOH	12.2
5	X-Gly-Leu-Lys-*Phe*-Lys-COOH	11.1
1	X-Gly-Leu-Lys-*Leu*-Lys-COOH	10.7
6	X-Gly-Leu-Lys-*Ala*-Lys-COOH	9.5
7	X-Gly-Leu-Lys-*Ser*-Lys-COOH	9.4

[a]Gradient from 5 to 95% CH_3CN in water in 30 min using a Phenomenex phenylhexyl C18 column 4.6 × 75 mm, 3 μm. [b]X is 3,5-dimercaptobenzoic acid.

The evolution of the product distributions of the small DCLs was monitored over the course of 14–30 days. The results obtained for DCLs prepared from peptides **2**–**7** are shown in Figure 4a–f and will be discussed in order of decreasing peptide hydrophobicity.

The stirred, shaken, and nonagitated DCLs prepared from the most hydrophobic peptide (**2**, containing a naphthylalanine residue) all gave comparable results (Figure 4a). In all three samples, cyclic trimer $\mathbf{2}_3$ and tetramer $\mathbf{2}_4$ were the main cyclic species after 23 days. The same behavior was observed previously for control compound **8** without the peptide sequence. The oxidation of building block **2** was slow, most likely due to the fact that the experiments for this peptide had to be performed at a relatively low pH (4–5) because of solubility problems at higher pH. No significant quantities of macrocycles larger than the tetramer were observed for this peptide. However, unlike for control compound **8**, fibrous aggregates were observed in the small DCL (vide infra).

DCLs made from the less hydrophobic cyclohexylalanine peptide **3** also gave trimer and tetramer as the main species

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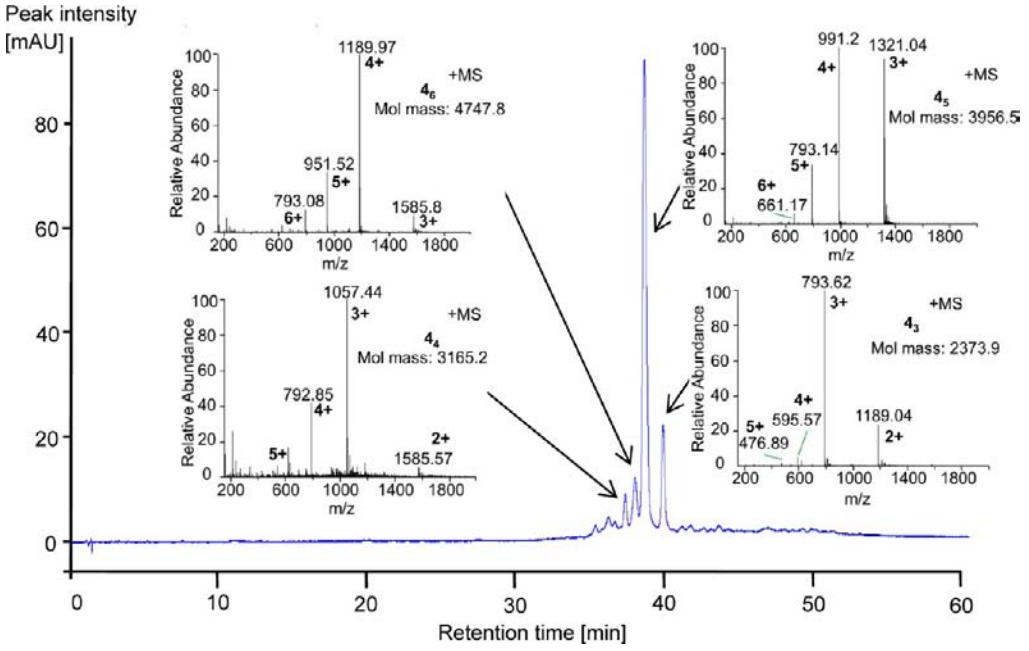

Figure 3. HPLC analysis (monitored at 254 nm) of a nonagitated DCL prepared from building block **4** after 9 days. The insets show the positive-ion mass spectra of the peaks due to (in order of elution) cyclic tetramer, hexamer, pentamer, and trimer.

after 7 days (Figure 4b). While this composition remained unchanged for the nonagitated sample, a relatively sudden growth of hexamer took place in the agitated samples. The onset of this event occurred after 7 days in the stirred sample and after 12 days in the shaken sample. The longer lag phase in the shaken sample is consistent with the fact that stirring subjects the samples to higher shear stress than shaking so that fibril fragmentation is more efficient upon stirring, leading to more efficient replication.

DCLs made from *p*-chlorophenylalanine containing peptide **4** demonstrated unusual behavior (Figure 4c). Oxidation is rapid, and after 1 day, the trimer and tetramer dominate the mixture, irrespective of the mode of agitation. During the following 5 days, the cyclic pentamer grows rapidly in all samples. In the nonagitated sample, it continues growing to become the dominant species. However, in the stirred and shaken samples, the hexamer emerges after about 7 days and grows at the expense of the pentamer and the other macrocycles in the system. Hexamer and pentamer compete for a common resource, and the experimental conditions apparently determine which of the two macrocycles wins the competition.

The nonagitated and shaken DCLs made from phenylalanine containing peptide **5** were initially dominated by cyclic trimer $\mathbf{5}_3$ and cyclic tetramer $\mathbf{5}_4$ (Figure 4d). However, after approximately 11 days, the hexamer emerged in the shaken sample. The same species also emerged in the stirred sample, but more rapidly. In sharp contrast, the library that was not agitated showed only a slow increase in the amount of $\mathbf{5}_6$ while $\mathbf{5}_3$ and $\mathbf{5}_4$ continued to dominate the mixture over the time course of the experiment.

Similar to the observations made for peptide **5**, DCLs made from alanine containing peptide **6** showed the emergence of a replicator only in the agitated samples (Figure 4e). However, for this peptide, the cyclic octamer emerged. Note that upon milder agitation by shaking the samples prepared from **6** gave results that were similar to those obtained by stronger agitation through stirring.

Finally, agitated DCLs made from the least hydrophobic peptide in the series (serine containing **7**) also resulted in the emergence of cyclic octamer (Figure 4f). In the stirred sample, octamer growth could be detected already after 4 days, whereas in the shaken sample, the octamer starts emerging only after 8 days. The subsequent rate of octamer growth is significantly smaller when the sample is shaken compared to when it is stirred, in line with less efficient fibril breakage (and, hence, lower replication rate) when agitation is milder.

The octamer of **7** is the largest macrocycle yet that is amplified in a DCL. It has a molecular weight of 5.6 kDa, similar to that of a small protein, and is formed in more than 90% yield in a single step, simply by stirring an aqueous solution of the corresponding building block.

Taken together, these findings support our hypothesis for multivalency as a selection criterion for the emergence of replicators. The size of the self-assembling macrocycles that emerge from the DCL increases with decreasing hydrophobicity of the building block and, hence, with decreasing strength of the peptide−peptide interactions. *This leads us to propose the first selection criterion: a critical macrocycle size is needed in order to afford sufficiently strong interactions between the macrocycles to drive self-assembly.*

Seeding Experiments. In order to establish whether the larger macrocycles that emerge in the DCLs are self-replicating and, hence, capable of enhancing the rate of their own formation, we have performed a series of seeding experiments. In these experiments, a small amount of assembled macrocycle was added to a DCL at a stage where little or none of this macrocycle was present. A seeding-induced increase in the rate of formation of the specific macrocycle is a clear indication of autocatalysis. We have carried out such seeding experiments for all peptide DCLs except for those made from **2**, as this system did not show the emergence of macrocycles larger than trimer and tetramer (which are also the products formed in a DCL of

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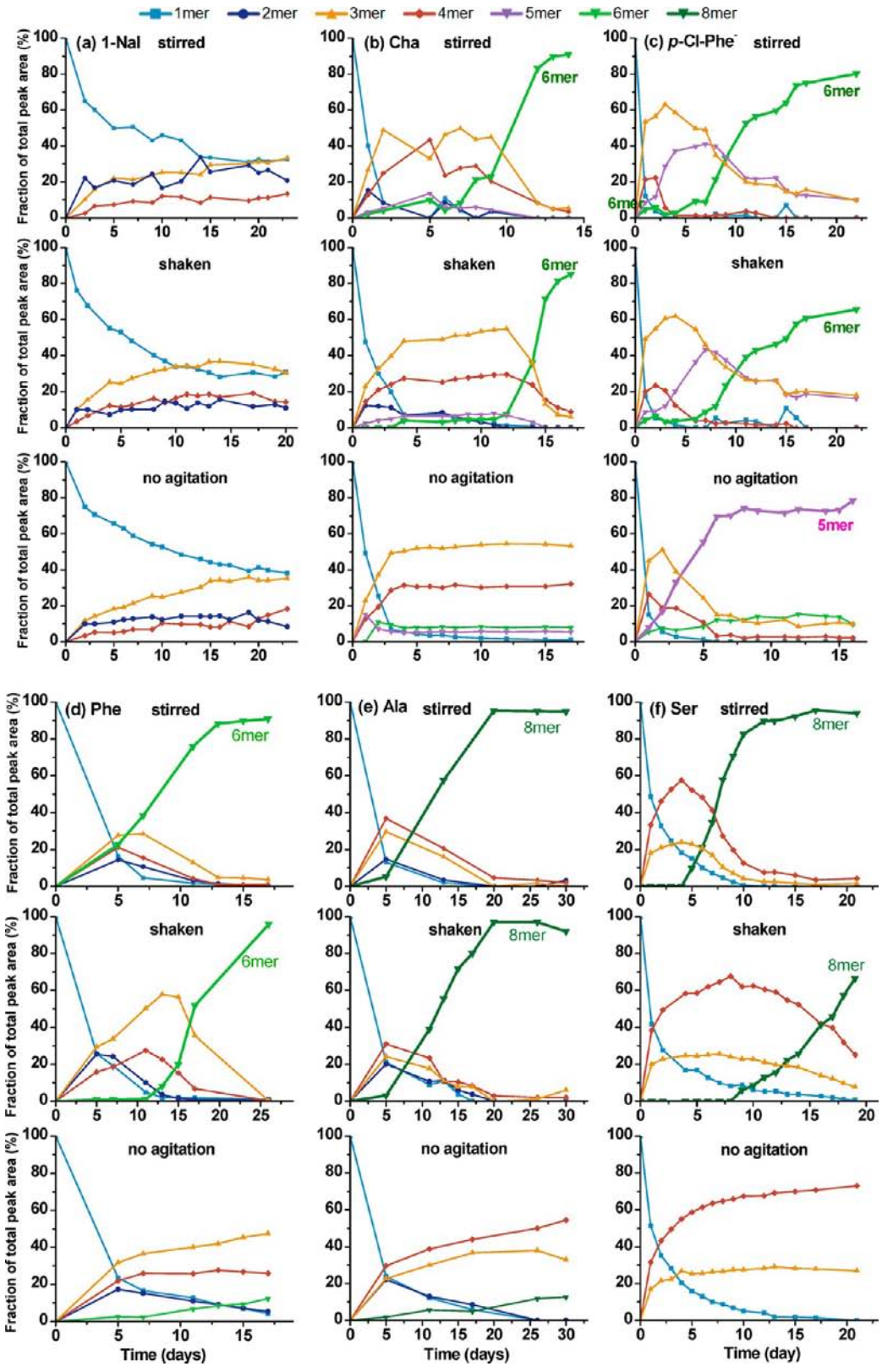

Figure 4. Evolution of the product distribution of small DCLs (3.8 mM dithiol building block in 50 mM borate buffer pH 8.2) made from (a) 1-naphthylalanine containing peptide **2**; (b) cyclohexylalanine containing peptide **3**; (c) *p*-chlorophenylalanine containing peptide **4**; (d) phenylalanine containing peptide **5**; (e) alanine containing peptide **6**; and (f) serine containing peptide **7** while stirred at 1200 rpm; shaken at 1200 rpm or not agitated. To maintain disulfide exchange in the samples made from **3**, 5−30 mol % of monomer **3** was added to the stirred sample at t = 2, 5, and 9 days and 15 mol % of monomer **3** was added to the shaken sample at t = 6 days. Similarly, to the DCLs made from **4** was added 10−20 mol % of monomer **4** to the stirred and shaken samples at t = 7, 9, and 14 days. Key: (blue square) 1mer; (purple circle) linear 2mer; (yellow triangle) cyclic 3mer; (red diamond) cyclic 4mer; (purple triangle) cyclic 5mer; (light green triangle) cyclic 6mer; (dark green triangle) cyclic 8mer.

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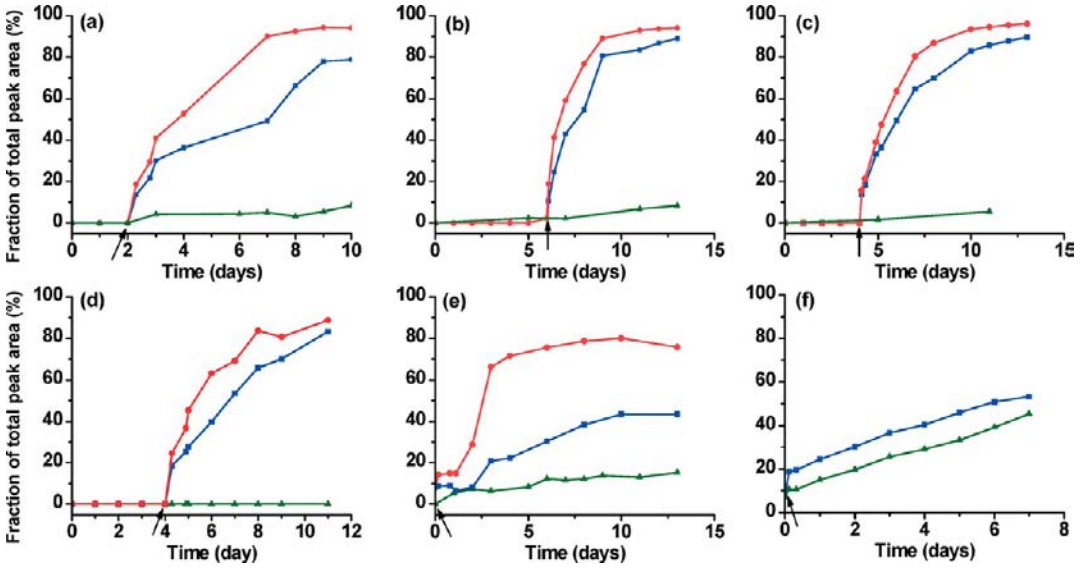

Figure 5. Seeding-induced growth of suspected self-replicating macrocycles when stirred at 1200 rpm (red diamond); in the absence of agitation (blue square) compared to the nonseeded nonagitated sample (green triangle). In all cases, a nonagitated DCL dominated by cyclic trimer and tetramer was seeded with 5 mol % of suspected replicator (a) $\mathbf{3}_6$; (b) $\mathbf{5}_6$; (c) $\mathbf{6}_8$; (d) $\mathbf{7}_8$; (e) $\mathbf{4}_6$; (f) $\mathbf{4}_5$. The time of addition of the seed is indicated with an arrow.

a control compound **8** lacking the peptide chain). For all other peptides, except **4**, the nonagitated DCLs are dominated by cyclic trimer and tetramer and largely devoid of any replicator. Addition of a small amount (5 mol %) of a sample that is rich in the suspected replicator (hexamer for **3** and **5** and octamer for **6** and **7**) should induce a substantial increase in the rate of replicator formation in these samples. The data in Figure 5 show that the rate of formation of the larger macrocycles increases dramatically upon seeding, confirming that $\mathbf{3}_6$, $\mathbf{5}_6$, $\mathbf{6}_8$, and $\mathbf{7}_8$ are indeed self-replicators. For every replicator, we performed two seeding experiments: one where the sample was stirred after being seeded and one where it was left nonagitated. Self-replication is somewhat more efficient in the stirred samples, although the difference is relatively small. The growth of hexamer and octamer in the nonagitated samples indicates the ability of these macrocycles to grow in the absence of agitation, once they have reached a certain concentration, even though under these conditions these compounds do not reach significant concentrations in the absence of seed on the time scale on which we monitored our experiments. This result highlights the importance of mechanically induced fibril breakage in the early stages of the emergence of the replicators. Apparently, the formation of nuclei from which the replicator fibrils can grow is an unlikely event, which is not surprising, given the low concentrations of larger macrocycles in the DCLs in the absence of any self-recognition (as evident from the behavior of control compound **8**).

Seeding experiments for peptide **4** were complicated by the fact that the unstirred sample already contained a large amount of cyclic pentamer. In order to obtain a sample that contained only a small amount of this potential replicator, a solution of peptide **4** was oxidized rapidly using sodium perborate, producing a kinetically controlled mixture dominated by trimer and tetramer. Seeding of this sample with 5 mol % of 6mer and stirring dramatically increased the rate of hexamer formation, compared to the unseeded nonagitated sample (Figure 5e). Thus, also the hexamer of **4** is a replicator. However, performing a similar experiment, but now using a sample rich in the pentamer of **4** as a seed, failed to accelerate the formation of this pentamer, even if we seeded with as much as 15 mol % of pentamer (Figure 5f). Thus, the pentamer of **4** is not a self-replicator under the conditions of these experiments.

It appears to form readily, already in the absence of any autocatalytic pathway. We speculate that this is due to the fact that the pentamer is still a relatively accessible species in the dynamic library that (in the absence of any recognition between macrocycles) would be dominated by trimer and tetramer. Note that, under thermodynamic control and in the absence of ring strain and specific recognition events, DCLs are dominated by the smallest macrocycles since it is entropically favorable to form many small molecules as opposed to fewer larger molecules. Thus, the concentration at equilibrium of larger macrocycles falls off rapidly with increasing macrocycle size. *These considerations suggest a second selection criterion for self-replication: apart from requiring a critical macrocycle size, self-replication also requires the replicator to be a poorly populated species in the absence of an autocatalytic pathway; i.e. its spontaneous formation should not be too fast.*

Fibril Characterization. The extent to which fibrils were present in the various samples was evaluated by (cryo-)TEM and thioflavin T fluorescence. The organization of the peptides within the fibrils was analyzed by circular dichroism (CD) and fluorescence spectroscopy.

(Cryo-)TEM images show that in all DCLs that were agitated fibrils were present. Furthermore, DCLs made from the most hydrophobic naphthylalanine peptide **2**, which contained mainly trimer and tetramer macrocycles, showed long twisted fibrils even in the nonagitated sample (Figure 6a–c). Apparently, the hydrophobic interactions between the peptide chains are strong enough to form fibrils even with the small trimer and tetramer macrocycles. DCLs made from cyclohexylalanine containing peptide **3** formed nontwisted fibrils when agitated. Fibrils were also observed in the nonagitated sample of peptide **3** but in much lower abundance than in the agitated samples (Figure 6d–f). DCLs made from *p*-chlorophenylalanine containing peptide **4** formed twisted

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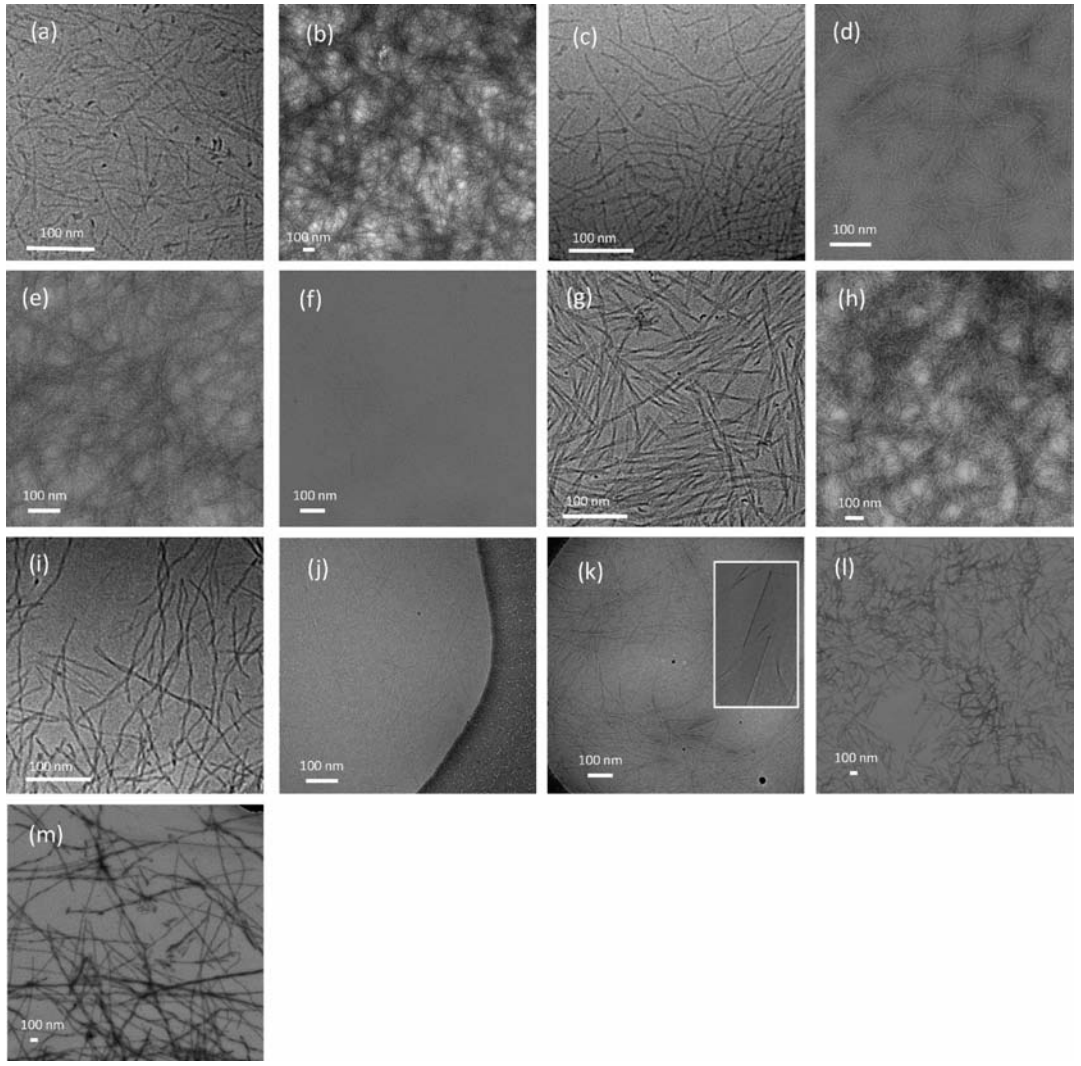

Figure 6. (Cryo-)TEM images of DCLs made from peptide **2** (a) stirred, (b) shaken, (c) nonagitated; peptide **3** (d) stirred, (e) shaken, (f) nonagitated; peptide **4** (g) stirred, (h) shaken, (i) nonagitated; peptide **5** (j) stirred; peptide **6** (k) stirred and peptide **7** (l) stirred, (m) shaken. Images a, c, f, g, i, j, and k were obtained using cryo-TEM. Images b, d, e, h, l, and m were obtained using negative staining.

fibrils in the nonagitated (pentamer-rich) and agitated (hexamer-rich) samples (Figure 6g–i). In DCLs made from the more hydrophilic peptides **5**, **6**, and **7** fibrils could only be detected by TEM in the agitated samples (Figure 6j–m). In the case of peptide **6**, the fibrils showed a tendency to associate laterally into twisted sheets (inset in Figure 6k). The nonagitated samples of peptides **5**–**7** contained mostly trimer and tetramer (vide supra), and no aggregates were detectable in the TEM analysis of these samples.

Of all the peptides studied, DCLs made from the serine containing peptide **7** showed the largest difference in replication kinetics between shaken and stirred samples (Figure 4f). Comparison of the TEM images of both samples reveals that fibrils formed in the stirred sample are substantially shorter than those from the shaken sample (155 ± 77 nm versus 1.49 ± 0.59 μm respectively, Figure 6l,m), indicating that these fibrils are more readily fragmented when stirred compared to shaken.

To obtain more insight into the structure of the fibrils at the molecular level, we studied the samples using circular dichroism making use of the distinct spectral features in the far-UV (below 250 nm) of random-coil, β-sheet, and α-helix motifs.[85] With the exception of the stirred sample of peptide **2**, the CD spectra of peptides in the agitated DCLs made from building blocks **2**–**4** (Figure 7a–c) show the typical CD signature for β-sheets (maximum at 195–200 nm, minimum at 216–218 nm). Most of the agitated samples made from dithiols **2** and **4** show an additional minimum at around 238 nm. This unusual feature may be attributed to interactions between the aromatic amino acid side groups.[86,87] All of these samples contained fibrils (as evident from cryo-TEM). The same applies to the agitated DCLs made from peptides **5**–**7**, which showed CD spectra similar to those observed for the agitated samples made from **2**–**4**, but shifted to shorter wavelengths (Figure 7d–f). As such, these CD spectra are not readily assigned to any particular secondary structure, while they certainly do not correspond to random-coil peptide. In order to shed more light on the structure of the assemblies in the DCLs made from these peptides, we have performed fluorescence experiments (vide infra).

The nonagitated samples of DCLs made from peptides **5**–**7** (from which no replicators emerged) all show CD signatures characteristic of random-coil peptides (Figure 7d–f). This is in agreement with the fact that these samples showed no evidence

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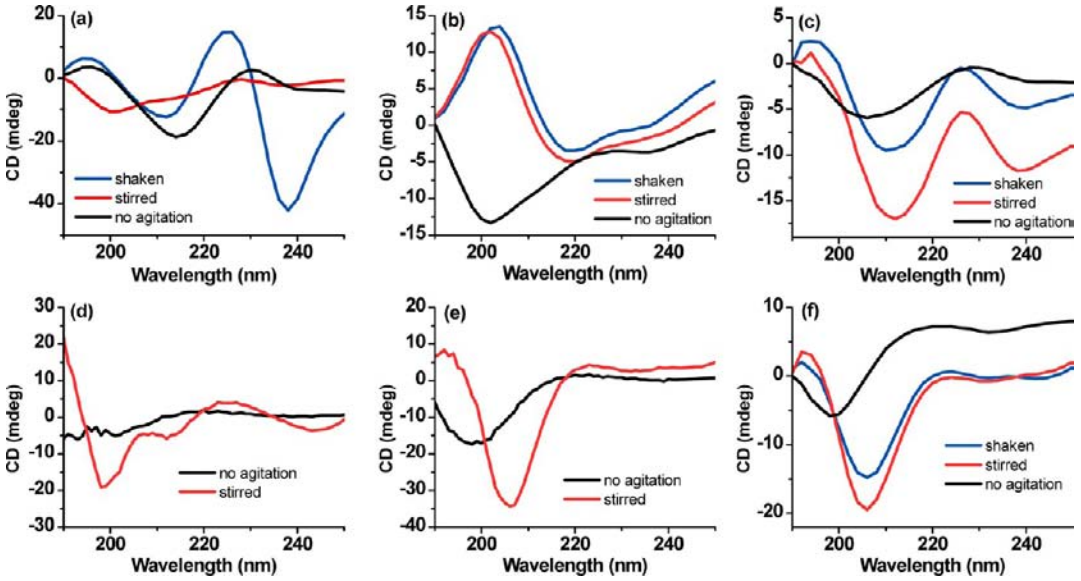

Figure 7. CD spectra of samples of DCLs made from peptides (a) **2**; (b) **3**; (c) **4**; (d) **5**; (e) **6**; and (f) **7** under different agitation conditions: shaken (blue), stirred (red), or in the absence of agitation (black).

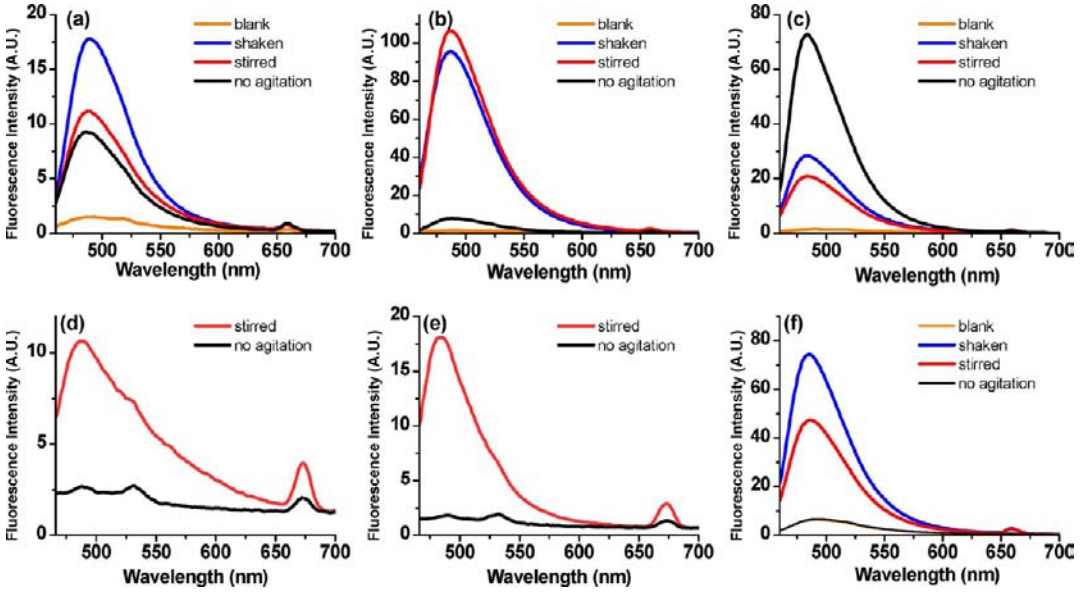

Figure 8. Fluorescence emission spectra of solutions of ThT in the presence of DCLs made from peptides (a) **2**; (b) **3**; (c) **4**; (d) **5**; (e) **6**; and (f) **7** under different agitation conditions: shaken (blue), stirred (red), or in the absence of agitation (black).

for fibrils in the TEM micrographs. Also, the DCL made from peptide **3** in the absence of agitation, for which TEM analysis revealed a small number of fibrils, gave a CD spectrum typical for a random-coil conformation. Unexpectedly, the CD spectrum of the nonagitated DCL made from peptide **4** (Figure 7c; the only DCL to produce cyclic pentamers) resembles that of random-coil peptide, even though an abundance of fibrils was observed in TEM micrographs.

We also studied the structure of the assemblies with fluorescence experiments using thioflavin T (ThT). ThT is a dye that is widely used for staining amyloid-type fibrils that are based on cross-β-sheet interactions.[88] ThT shows enhanced fluorescence following binding to amyloid material. All peptide samples which displayed fibrils in the TEM images showed enhanced ThT fluorescence intensity compared to a blank sample containing only buffer (Figure 8). The fluorescence intensity of the nonagitated DCL made from peptide **3**, which showed some fibrils by TEM, yet no significant replicator formation, is considerably lower compared to the fluorescence intensities of the agitated samples, which contained large concentrations of replicator (Figure 7b). This observation is in line with the lower abundance of fibrils in the nonagitated sample as observed by cryo-TEM (Figure 6f). Taken together with the TEM and CD studies, the ThT fluorescence data suggest that the organization of the peptides in the self-assembled replicators is, for most samples, reminiscent of that in amyloid fibrils.

■ CONCLUSIONS

Using a dynamic combinatorial strategy, we have discovered an entire family of new self-replicating molecules that are formed through a complex and unique mechanism. Exposing aqueous

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solutions of simple peptide-functionalized dithiol building blocks to oxygen from the atmosphere leads to DCLs consisting of a mixture of macrocycles of different ring size. With increasing ring size, the library members display an increasing number of peptide chains, which may potentially drive the self-assembly of the library members into fibrilar aggregates by forming β-sheets. This assembly process drives the formation of the very macrocycle that assembles by shifting the macrocycle equilibrium in its direction,[81] so that the assembling macrocycle may become self-replicating.

This study establishes that peptide hydrophobicity, in combination with multivalency, determines which of several potential self-replicators actually emerges from the individual DCLs. By comparing the behavior of DCLs made from a family of structurally related building blocks, a clear trend emerges: as the hydrophobicity of the building block decreases, the size of the emerging macrocycle increases. Assembly into fibrils appears to require a critical interaction energy between the peptide-functionalized macrocycles. When the peptide is modified to make it more polar, the hydrophobic interactions between the peptides become weaker, so that larger macrocycles carrying more peptides need to be formed before self-assembly becomes feasible. This behavior allows us to define a first selection criterion for replicator emergence: *a critical macrocycle size is needed in order to afford sufficiently strong interactions between the macrocycles to drive self-assembly.* However, this condition is not sufficient to achieve self-replication. It is also necessary that the replicator is predominantly formed through an autocatalytic pathway. Hence, the spontaneous pathway should be less efficient than the autocatalytic pathway. We have found that the cyclic trimers, tetramers, and pentamers from the more hydrophobic peptides already assemble into fibrils unaided. No evidence for autocatalysis could be obtained for these systems. These observations suggest a second selection criterion for self-replication: *self-replication requires the replicator to be a poorly populated species in the DCL in the absence of an autocatalytic pathway; that is, its spontaneous formation should be inefficient relative to its autocatalytic formation.*

These selection criteria should be useful to guide future work aimed at the discovery of new replicators using dynamic combinatorial libraries. They may also assist in further developing the recently emerged concept of self-synthesizing materials. Between this study and our previous report,[67] we now have access to self-assembled materials made from macrocycles covering a continuous range of ring sizes from trimers and tetramers for the most hydrophobic peptide to octamers for the most hydrophilic ones. In most cases, self-assembly drives the synthesis of the macrocycles that assemble.

Our results show that the process by which the fibrils emerge becomes increasingly dependent on agitation as the ring size increases. When the ring size matches the inherent preference of the dithiol building block core (i.e., when trimers and tetramers are formed, just like when using control compound **8**), no agitation is required in order to achieve fibril formation. However, for larger ring sizes (hexamer and beyond), which are unstable relative to the trimer and tetramer in the absence of any self-assembly process, the kinetics of formation of these species becomes prohibitively slow in the absence of agitation-mediated self-replication. This kinetic barrier may be overcome when the process of fibril growth is aided by mechanically induced fibril fragmentation. Fibrils grow from their ends, and the creation of more fibril ends promotes the growth of the replicator. Thus, replication is driven by mechanical energy, and we have observed for several peptide sequences that replication is faster when greater shear stress is exerted by stirring instead of shaking the sample.

Parallels exist between this mechanism of mechanically induced assembly and replication and various observations on amyloid fibers (implicated in diseases such as Alzheimer's and Creutzfeldt-Jacob).[89] It is becoming increasingly apparent that amyloid formation may be accelerated by mechanically induced fragmentation of fibrils[90−95] and seeding[96,97] by preformed fibrils. Also, outside the field of peptides and proteins, the interplay between macroscopic fluid dynamics and molecular scale assembly processes, while absent in most traditional analyses of self-assembling systems,[98] may give rise to interesting phenomena, such as chiral organization.[99−101] The new mechanism of mechanically induced self-assembly driven self-replication constitutes an important advance in the field of replicator chemistry. The replicators that emerge are substantially more complex than previous generations of replicators, which invariably contained only two building blocks. In contrast, in the process of replication described herein, up to eight building blocks combine to give rise to a replicator with a molecular weight of up to 5.6 kDa.

The number of building blocks that are incorporated into the replicators is no longer predetermined, but the chemical network is at liberty to select the replicator size that is most efficient under a given set of experimental conditions. Thus, the replicators contain more information than was present in the building blocks. As pointed out recently, in different contexts, by Joyce[102] and by Pross,[103] such spontaneous increase in information content of self-replicators is an important step in closing the gap between inanimate and animate matter.

The newly developed family of replicators opens up exciting opportunities for achieving Darwinian evolution in a fully synthetic system of self-replicators. For example, given the large number of building blocks that are incorporated in a replicator, it is easy to envisage that the system may choose to incorporate other building blocks from the solution during replication if such are provided. Studies are currently underway in our laboratory that focus on the emergence and subsequent evolution of mixed building block replicators in more complex dynamic molecular networks.

■ 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, respectively. Acetonitrile (HPLC-S/LC-MS grade) and trifluoroacetic acid were purchased from Biosolve BV.

Peptide Synthesis. Peptides **2**−**7** were synthesized by Cambridge Peptides Ltd. (Birmingham, UK) from 3,5-bis(tritylthio)benzoic acid, which was prepared via a previously reported procedure.[67] Peptide **4** was also synthesized by Biomatik (Delaware, USA). 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 **2**−**7** were dissolved to a concentration of 3.8 mM in borate buffer (50 mM, pH 8.2). Where necessary, the pH of the solution was adjusted by the addition of 1.0 M KOH solution such that the final pH was 8.0. The volume of each library was 500 μL. Each solution was allowed to equilibrate in an HPLC vial (12 × 32 mm) with a Teflon-lined snap cap. Samples containing a cylindrical microstirrer bar (2 × 5 mm, Teflon-coated, manufactured by Cowie obtained from Fisher) were stirred at 1200 rpm using an IKA RCT basic hot plate stirrer. Shaken samples were placed in an Eppendorf Thermomixer Comfort (orbital

shaker) and shaken at 1200 rpm with an orbital radius of 1.5 mm. All library experiments were performed at ambient temperature. A small aliquot of each sample was removed to another vial prior to each HPLC or LC-MS analysis. Peptides **2** and **4** synthesized by Cambridge Peptides Ltd. were not totally soluble under the experimental conditions. To avoid precipitation, experiments for peptide **2** were performed at pH 5−6.

Seeding Experiments. For peptides **3**, **5**, **6**, and **7**, a solution containing mostly trimer and tetramer was split into two parts, and then to each sample was added a small amount (5.0 mol %) of pre-existing fibrils formed from the corresponding macrocycle. One sample was stirred and another left without any agitation. The samples were monitored by HPLC for the first 8−9 days following the addition of the fibrils. For the seeding experiments with peptide **4**, a solution of this peptide was oxidized by sodium perborate to give a sample containing mostly trimer and tetramer, and then the solution was split in four parts. To the first part 5.0 mol % of pre-existing fibrils formed from the hexamer was added, and the sample was stirred; the second solution was not seeded and stirred. To the third sample was added 15.0 mol % of pre-existing fibrils formed from pentamer, and the last sample was not seeded; both samples were left without agitation. All four samples were monitored by HPLC over a period of 2 weeks following the addition of the fibrils.

HPLC and LC-MS Analyses. HPLC analyses were performed on an Agilent/HP 1050 series equipped with a diode array UV/vis detector. LC-MS analyses were performed on an Accela high-speed LC system (ThermoFisher Scientifics) coupled to a LTQ-Fleet ion trap mass spectrometer, fitted with a Thermo Fisher Ion Max source setup. All analyses were performed at 45 °C using a reversed-phase HPLC column (Prodigy C18, 2 × 150 mm; 5 μm or phenylhexyl C18, 4.6 × 75 mm, 3 μm both obtained from Phenomenex). UV absorbance was monitored at 254 nm. Positive-ion mass spectra were acquired using electrospray ionization. Scan rate = normal; flow to source (after splitter) = 80−100 μL/min; capillary temperature = 275 °C; capillary voltage = 47 V; spray voltage = 4.2 kV; sheath, auxiliary, and sweep gas flow rates were 8, 2, and 2 mL/min, respectively. Injection volume = 2.0 μL of freshly aliquoted sample; column temperature = 45 °C; flow rate = 0.8 mL/min.

Solutions containing peptides **2**, **3**, **4**, and **7** and their oxidation products were analyzed using the phenylhexyl column. Solvent A: double distilled water (0.1 vol % trifluoroacetic acid). Solvent B: acetonitrile (0.1 vol % trifluoroacetic acid). Solutions containing peptides **5** and **6** and their oxidation products were analyzed using the Prodigy column. Solvent A: double distilled water (0.2 vol % heptafluorobutyric acid). Solvent B: acetonitrile (0.2 vol % heptafluorobutyric acid) (see Supporting Information).

Cryo-TEM. A small drop of sample was placed on a Quantifoil 3.5/1 holey carbon-coated grid. Blotting and vitrification in ethane was done in a Vitrobot (FEI, Eindhoven, The Netherlands). The grids were observed in a Philips CM120 cryo-electron microscope operating at 120 kV with a Gatan model 626 cryo-stage. Images were recorded under low-dose conditions with a slow scan CCD camera.

Negative Staining TEM. A small drop of sample was deposited on a 400 mesh copper grid covered with a thin carbon film. After 30 s, the droplet was blotted on filter paper. The sample was then stained with a solution of uranyl acetate deposited on the grid and blotted on filter paper after 30 s. 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 100 μM (with respect to peptides **2**−**7**) with additional borate buffer (50 mM, pH 8.2). The diluted sample (80 μL) was added to a ThT solution (22 μM, 450 μL) in sodium phosphate buffer (50 mM, pH 8.2) and incubated for 2 min. The fluorescence was measured on a JASCO FP6200 spectrophotometer by excitation at 440 nm (5 nm slit width) and recoding the emission from 460 to 700 nm (5 nm slit width, 5 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 = 20 nm/min, continuous scanning) and HELLMA 10 × 2 mm quartz cuvettes. All reported spectra are averages of 5 repeats. Solvent spectra were subtracted from all spectra. All spectra were obtained using samples diluted to 8−18 μM (with respect to building block concentration).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

LC-MS methods and data and extended CD spectra for the DCLs made from peptides **2**−**7**. 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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