Folding and replication in complex dynamic molecular networks

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Chapter 5  Self-replication Promotes the Formation of Complex Folded Structures

The replication of genetic information and its expression into folded functional structures are key biochemical processes in life. However, it is very challenging to construct synthetic chemical systems capturing these complex behaviors. Herein, we show that both processes can emerge from dynamic libraries made from simple building blocks. Specifically, we show that folded structures are promoted by the formation of a replicator. The composition of the resulting libraries is dominated by compounds that form non-covalent interactions either intramolecularly (giving rise to highly specific complex folded structures, composed of 15 identical subunits), or intermolecularly (resulting in self-replication driven by self-assembly). Whether foldamers or replicators emerge is dictated by the ratio of the building blocks. Moreover, transient formation of a self-replicator was also observed, again, depending on the ratio of the two building blocks.
Chapter 5

5.1 Introduction

Supramolecular assembly in complex systems has attracted increasing attention over the past two decades.\(^1,2\) It has been used to design functional systems that capture important processes in living organisms.\(^3,4\) Self-replication\(^5-7\) is one of the most important ingredients in the origin of life, and self-replicating molecules are a promising possible starting point for the de novo synthesis of life.\(^8,9\) Since von Kiedrowski demonstrated the first self-replicating system,\(^10\) many such systems have been developed based on synthetic molecules\(^11-17\) or biomolecules such as DNA\(^18,19\), RNA\(^20-26\) and peptides.\(^27-30\) However, it remains a huge challenge to develop new self-replicating systems that adaptively respond to a changing environment. In addition, the construction of dissipative self-replicating systems, like those found in living systems, is even more challenging.\(^31,32\) Recently, dynamic combinatorial chemistry\(^33-37\) (DCC) has been shown to be a promising approach for the fabrication of self-replicating systems from complex mixtures of interconverting molecules by self-selection.\(^38\)

More importantly, since the structure of the building block does not necessarily predetermine the nature of the self-replicator, the emergence of replicator from DCLs can be susceptible to environmental changes. The emergence of self-replicators from DCLs can be affected by templates,\(^39\) mechanical agitation,\(^38\) solvent environments\(^40\) and pre-existing replicators.\(^41,42\) Moreover, dynamic self-replication systems can exhibit parasitic phenomena similar to biological systems.\(^43\)

Folding is one of the most important processes in which biomolecules adopt specific three-dimensional shapes in order to achieve their special biological properties and functions.\(^44-47\) Scientists have made great efforts to achieve the synthesis of folded structures, foldamers, with different functions and properties.\(^48-58\) However, designing simple motifs that can be folded into a specific shape, such as a peptide or nucleic acid, remains challenging. Most of these synthetic folds only feature secondary structures, such as helices and sheets, rather than more complex tertiary or quaternary arrangements.\(^59\) Our recent work shows that a dynamic combinatorial approach can allow access to self-synthesizing foldamers with complex tertiary structures with remarkable yields and minimal synthetic effort. The dynamic combinatorial approach may also provide us the possibility to self-sort different assemblies which require different building blocks in complex chemical networks. Self-sorting of different assemblies in complex systems may reveal behavior that goes beyond that accessible in individual subsystems.\(^60\)
Key processes of Darwinian evolution are the replication of genetic information and its expression into functional molecules such as folded proteins. The RNA world hypothesis suggests replication and the expression of genetic information depend on folded RNA enzymes. Replication and folding processes play very important roles in biological systems. Yet, there is no report on the relationship between these two processes within chemical systems. Most synthetic systems only capture a single process, and no examples exist of chemical systems that facture both processes simultaneously. The limiting design rules for developing self-replicating molecules and folded assemblies in synthetic systems makes it challenging to access systems that integrate folding and replication. A primary sequence can, in principle, either replicate or fold, where a delicate balance between inter and intramolecular interactions might favor one over the other.

Herein, we show how self-replication promotes the formation of a foldamer through self-sorting in a two building blocks DCL: the emergence of self-replicators is resulting from intermolecular non-covalent self-assembly and the formation of foldamers driven by intramolecular interactions. The foldamer is formed following the emergence of replicators, and the distribution of replicators and foldamers in the DCL is determined by the proportion of the two building blocks. Moreover, the process of simultaneous formation and destruction of self-replicating molecules was also observed from the same DCL. Such processes may help us to further understand life-like behavior.

5.2 Results and discussion

5.2.1 Self-sorting of a self-replicator and a foldamer

We have previously reported the emergence of self-replicating molecules and folded complex structures from DCLs made from dithiol-functionalized building blocks. Self-replicating molecules emerge in DCLs made from building blocks functionalized with short peptide chains composed of alternating hydrophobic and hydrophilic amino acid residues to facilitate the generation of β-sheets and an aromatic dithiol core for thiol-disulfide exchange. Building block 1 (Scheme 5.1, left) has a structure similar to those giving rise to the previously reported self-replicating molecules, except that the amino acid attached to the aromatic core is modified from glycine to β-alanine. Stirring building block 1 (2.0 mM) in borate buffer (50 mM, pH = 8.0) containing 1.0 M NaCl in the presence of air results in a small DCL containing different macrocyclic disulfides. Initially, cyclic tetramer (1₄) emerged and became the dominant product (Figure 5.1b). Seeding experiments were performed to
verify that 1₄ is a self-replicator by adding 10 mol% of preformed 1₄ into a fresh DCL of building block 1. The results show that the addition of preformed 1₄ significantly accelerates the formation of itself, suggesting autocatalytic behavior (Figure 5.5.1). The fact that a replicator with a relatively small ring size (comparing with the previously reported cyclic hexamer and octamer replicators) emerged from this DCL is because building block 1 is relatively hydrophobic, which facilitates the formation of small macrocyclic replicators. Building block 2 (Scheme 5.1, right) features an amino acid (aspartic acid) and a nucleobase (adenine) attached to the same aromatic dithiol core as building block 1. However, a different self-assembled structure is formed in the library. Foldamer 2₁₅ was selectively formed by stirring an aqueous solution of building block 2 (2.0 mM) in borate buffer (50 mM, pH = 8.0) containing 1.0 M NaCl with the presence of air (Figure 5.1a). X-ray crystallography shows that 2₁₅ is a cyclic product formed by folding of 15 subunits through intramolecular hydrogen bonds, hydrophobic interactions and π-π stacking.

Scheme 5.1. Dynamic combinatorial libraries made from building blocks 1 and 2: the left part shows the general replication mechanism for the short peptide functionalized building block (1₄ in this case); the right part shows the folding for the nucleobase equipped building block (2₁₅ in this case); in the mixed library of building blocks 1 and 2, replicator 1₄ is formed first, but then gives way to replicator 1₂₂₁₅, and foldamer 2₁₅ is generated at the end.
Self-replication Promotes the Formation of Complex Folded Structures

The different nature of the noncovalent interaction associated with self-replication and folding make it possible to investigate the self-sorting of replicators and foldamers in a single chemical network. Moreover, both building blocks 1 and 2 have the same aromatic dithiol core which ensures that replicators and folded structures are in principle, able to exchange building blocks with each other by thiol-disulfide exchange.

In order to prove this conjecture, a small DCL of disulfides was generated by stirring building block 1 and 2 ([1] = [2] = 1.0 mM) in aqueous borate buffer (50 mM, pH = 8.0) in the presence of 1.0 M NaCl. The library reached equilibrium after 7 days and was mainly composed of cyclic trimer 121 and foldamer 215, accompanied by a small amount of another cyclic trimer 122 (Figure 5.1c). Instead of narcissistic self-sorting (which should result in a mixture of replicator 14 and foldamer 215), social self-sorting occurred, giving rise to the selective formation of mixed cyclic trimer 121 and foldamer 215.

![Figure 5.1. UPLC analysis of DCLs made from (a) 2.0 mM building block 1, (b) 2.0 mM building block 2, (c) 1.0 mM 1 and 1.0 mM 2 in borate buffer (50 mM, pH = 8.0, 1.0 M NaCl) after stirring for 10 days.](image)

In order to obtain more insights into the self-sorting process in the two building blocks DCL, the time evolution of the library was monitored (Figure 5.2a). Initially, the library was dominated by small macrocyclic trimers and tetramers, containing one or two building blocks. After 2 days, the concentration of the mixed cyclic trimer 121 rapidly increased and this macrocycle became the dominant product after 4 days stirring. Foldamer 215 started to
Chapter 5

form after all of building block 1 was consumed and the library ended up consisting mainly of cyclic trimer $1_22_1$ and foldamer $2_{15}$. A seeding experiment focused on cyclic trimer $1_22_1$ was performed in order to assess whether it is a self-replicator (Figure 5.2b). The concentration of $1_22_1$ increased rapidly upon adding 6 mol% preformed $1_22_1$ into a fresh DCL containing building blocks 1 (1.0 mM) and 2 (0.50 mM) in the ratio biased towards the formation of $1_22_1$, which proves that the small cyclic trimer $1_22_1$ is a replicator. Note that the formation of the folded complex structure in the above library is delayed compared to its formation in a separate library which only contains building block 2 under otherwise identical conditions. The preformed foldamer $2_{15}$ was disassembled upon adding building block 1 through thiol-disulfide exchange (Figure S5.5). Note also that, no foldamer $2_{15}$ was observed before all of building block 1 was consumed. Afterwards it rapidly formed and all remaining building block 2 was converted to foldamer after the process of self-replication had reached completion. These results suggest that the formation of foldamer in this DCL requires the emergence of self-replicator to consume those building blocks which cannot form the folded structure. Thus, the emergence of self-replicator promotes the formation of folded complex structures in this two component dynamic chemical network.

![Figure 5.2](image)

**Figure 5.2.** Time evolution of DCLs made from (a) 1.0 mM 1 and 1.0 mM 2, (b) 1.0 mM 1 and 0.5 mM 2 with 6 mol% $1_22_1$ in borate buffer (50 mM, pH = 8.0, 1.0 M NaCl).

**5.2.2 Characterization of new formed self-replicators**

In order to understand whether the mechanism of self-replication is the same as that observed previously for other peptides-based replication, the newly formed replicators were further characterized by circular dichroism (CD), thioflavin T fluorescence assays and transmission electron microscopy (TEM). The CD spectra of replicator $1_4$ showed positive
Self-replication Promotes the Formation of Complex Folded Structures

Helicity at 206 nm and negative helicity around 220 nm, indicative of the presence of β-sheet structure (Figure 5.3a). Similar helicities were observed for replicator 1₂₂₁, but the intensities were relatively weak. Thioflavin T fluorescence measurements also confirmed the presence of β-sheet structures for replicators 1₄ and 1₂₂₁ (Figure 5.3b). Negative staining TEM micrographs of the libraries dominated by replicators 1₄ and 1₂₂₁ showed bundled fibrous nanostructures (Figures 3c and 3d). These results demonstrate that the newly formed self-replicators were driven by the formation of β-sheet, which is consistent with the behavior of previous replicators.⁶²

**Figure 5.3.** (a) CD spectra (recorded at identical concentrations), (b) thioflavin T fluorescence emission spectra, (c) and (d) TEM micrographs of libraries made from building blocks 1 and 2 (borate buffer, 50 mM, pH = 8.0, 1.0 M NaCl) that were dominated by 1₄ and 1₂₂₁, respectively.

### 5.2.3 Ratio effects

We then investigated how the ratio of the two building blocks affects the outcome of the library. The results described above already indicated that the library behavior depends strongly on the ratio of the two building blocks, because replicator 1₂₂₁ contains both building blocks, while the formation of folded macrocycles 2₁₅ requires the self-replicating
molecules to consume all of the other building blocks that are incapable of giving rise to foldamers. A series of libraries were set up at in a total concentration of 2.0 mM with different ratios \([\text{[1]}/\text{[2]} = 90/10, 80/20, 70/30, 60/40, 50/50, 40/60, 30/70, 20/80, 10/90]\) under the same conditions as the described above. The libraries were stirred for 10 days, when they had reached equilibrium as observed by UPLC/MS. As shown in Figure 5.4, different product distributions were observed from the libraries with different ratios of building blocks 1 and 2. The libraries were dominated by replicators \(1_4\) and \(1_22_1\) when the mole fraction of building block 2 is below than 30%. In this range, the concentrations of replicator \(1_22_1\) increased with the increase of building block 2 in the libraries and reached a maximum at 0.6 mM of building block 2. Replicator tetramer \(1_4\) disappeared from the libraries when the concentration of building block 2 was more than 0.8 mM and the main products of the libraries were replicator \(1_22_1\) and foldamer \(2_{15}\). As the concentration of building block 2 in the libraries was raised further, more foldamer \(2_{15}\) was generated and the concentration of replicator \(1_22_1\) decreased. Note that in all cases, building block 1 was incorporated into a replicator (either \(1_4\) or \(1_22_1\)), and building block 2 formed foldamer \(2_{15}\) after all of building block 1 had been integrated into replicators.

![Figure 5.4. Summary of the experiments at different ratios of building blocks 1 and 2. Total concentration of the two building blocks is 2.0 mM and all of the libraries were set up in borate buffer (50 mM, pH = 8.0, 1.0 M NaCl).](image)
5.2.4 Transient self-replication

Interestingly, transient self-replication of 1₄ was observed when the concentration of building block 2 is below 30 mol% in the libraries. In those libraries (Figure 5.5a, b and c), the emergence of replicators 1₄ was observed before the formation of replicator 1₂2₁, whereas, once replicator 1₂2₁ emerged, replicator 1₄ was transformed into replicator 1₂2₁ until all of building block 2 was consumed.

To investigate whether transient self-replication of 1₄ can affect self-sorting between replicator 1₂2₁ and foldamer 2₁₅, cross-seeding experiments were performed (Figure 5.5). As shown in Figure 5.5a, the concentration of replicator 1₄ rapidly increased at first when 10 mol% preformed replicators 1₄ was seeded into the DCL made from 1.0 mM building block 1 and 1.0 mM building block 2. Then replicator 1₂2₁ emerged after 70 hours stirring, and rapidly became the dominant species. Meanwhile, the concentration of self-replicator 1₄ decreased and essentially disappeared from the library. Finally, foldamer 2₁₅ emerged from the library after all of building block 1 was transferred to replicator 1₂2₁. In contrast, there was no emergence of replicator 1₄ when 10 mol% preformed replicator 1₂2₁ was seeded into the same library (Figure 5.5b). Although the pathways to form the final products are different in these two experiments, both gave the same product distribution dominated by replicator 1₂2₁ and foldamer 2₁₅. These results indicate that seeding-induced transient self-replication can change the pathway for the formation of the compounds in the library, but not the final product distribution. In addition, cross catalysis did not change the requirement for the formation of folded macrocycles: self-replicating molecules must sequester all of the building blocks incapable of giving rise to foldamers.

Figure 5.5. Time evolution of DCLs made from 1.0 mM 1 and 1.0 mM 2 with (a) 10 mol% 1₄ and (b) 10 mol% 1₂2₁ in borate buffer (50 mM, pH = 8.0, 1.0 M NaCl).
Chapter 5

We then explored the competition between replicators 1<sub>4</sub> and 1<sub>2</sub>2<sub>1</sub> when these are provided with different building blocks as “food”. We prepared a DCL containing building blocks 1 (1.7 mM) and 2 (0.3 mM) under at the same conditions as described above. As expected, initially only replicators 1<sub>4</sub> emerged from the library which was subsequently converted to replicator 1<sub>2</sub>2<sub>1</sub>. Since the amount of building block 2 in this library was insufficient to convert all of building block 1 into replicator 1<sub>2</sub>2<sub>1</sub>, the final library contained both replicator 1<sub>4</sub> and replicator 1<sub>2</sub>2<sub>1</sub>. After the library reached equilibrium, 85 mol% (relative to the total concentration of building blocks 1 and 2) of building block 1 was added. Self-replicator 1<sub>4</sub> rapidly grew while part of replicator 1<sub>2</sub>2<sub>1</sub> was disassembled and the library was dominated by self-replicator 1<sub>4</sub>. Then 15 mol% building block 2 was added and most of replicator 1<sub>4</sub> was rapidly destroyed and converted into replicator 1<sub>2</sub>2<sub>1</sub>, reaching a product distribution similar to that of the library formed by mixing the corresponding amounts of building blocks 1 and 2 at the start of the experiment. A second round of building block addition was also performed and similar results were obtained (Figure 5.6a).

![Graph](image)

**Figure 5.6.** Alternating additions of portions of building blocks 1 and 2 to a mixture made from 1.7 mM 1 and 0.3 mM 2 in borate buffer (50 mM, pH = 8.0, 1 M NaCl), results in switching between states dominated by replicators 1<sub>4</sub> and 1<sub>2</sub>2<sub>1</sub>. (a) Addition of 1 and 2, separately, (b) addition of 1 and 2 simultaneously.

These results indicate this system is able to simultaneously create and disintegrate self-replicators in response to the addition of building blocks. We also conducted experiments in which building blocks 1 and 2 were added at the same time. The results are shown in **Figure 5.5b**. Again, simultaneous creation and destruction of self-replicators was observed. These results, combined with the previous observations, suggest that replicator 1<sub>4</sub> in these two building blocks DCLs is thermodynamically less stable, even if it is the thermodynamic
Self-replication Promotes the Formation of Complex Folded Structures

product in the library which only contains building block 1. In the presence of a sufficient amount of building block 2, all of the species made from building block 1, including replicator 14, will convert to replicator 12. Yet replicator 14 was able to grow transiently, even in the presence of competition replicator 12. Thus, replicator 14 appears to be a more proficient replicator.

5.3 Conclusion

In conclusion, we were able to witness two of the most important processes of living systems, self-replication and folding in a single fully synthetic chemical system. The most critical aspect of this system is the self-sorting between self-replicators and foldamers. Self-replication is the result of intermolecular self-assembly while the formation of foldamer is driven by intramolecular non-covalent interactions. The emergence of self-replicators consumes all of the non-foldable building blocks (whose existence will destroy any foldamer) and result in a stable fibrous structure. The remaining foldable building blocks then react to give rise to a complex folded structure. The competition between self-replicators and foldamer is controllable and the emergence of self-replicators or foldamer can be directed by adjusting the ratio of the building blocks. We also observed the transient formation of a self-replicator, the formation of which can be induced by adding the respective starting building block to the library. The metastable self-replicator is eventually consumed and transformed into a stable self-replicator. The proportion of metastable self-replicator and stable self-replicator in the library can be controlled by the ratio of building blocks.

Our results provide a way for non-templated self-sorting of different structures in covalent disulfide based dynamic combinatorial libraries, which paves the way for designing complex dynamic systems with multiple coupled functions in the future, such as catalysis for self-replicators and binding for foldamers.
Chapter 5

5.4. Experimental section

5.4.1 General methods

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich and used as received. Acetonitrile (ULC-MS grade), water (ULC-MS grade) and trifluoroacetic acid (HPLC grade) were purchased from Biosolve BV. Peptide building block 1 was purchased from Cambridge Peptides Ltd. (Birmingham, UK) with 95% purity. The synthesis of building block 2 was described in Chapter 2.

Buffer preparation

Borate buffer (50 mM, pH = 8.0) was prepared by dissolving sodium tetraborate (3.84 g, Na$_2$B$_4$O$_7$.10H$_2$O) in 200 mL doubly distilled water. Then the pH was adjusted to 8.0 by addition of concentrated HCl.

Library preparation

Building blocks were dissolved in borate buffer (50 mM, pH 8.2) in the presence of 1.0 M NaCl. All libraries were set up in an HPLC vial (12×32 mm) with a Teflon-coated screw cap. All HPLC vials were equipped with a cylindrical stirrer bar (2×5 mm, Teflon coated, purchased from VWR) and were stirred at 1200 rpm using an IKA RCT basic hot plate stirrer. All experiments were performed at ambient conditions.

UPLC and UPLC-MS analysis

UPLC analyses were performed on a Waters Acquity H-class system equipped with a PDA detector, using a detection wavelength of 254 nm. Samples were injected on an Phenomenex Aeris Peptides 1.7 μm (150 × 2.1 mm) column, purchased from Phenomenex, using ULC-MS grade water (eluent A) and ULC-MS grade acetonitrile (eluent B), containing 0.1 V/V % TFA as modifier. A flow rate of 0.3 mL/min and a column temperature of 35 °C were applied.

UPLC-MS analyses were performed using a Waters Acquity UPLC H-class system coupled to a Waters Xevo-G2 TOF. The mass spectrometer was operated in positive electrospray ionization mode with the following ionization parameters: capillary voltage: 3 kV; sampling cone voltage: 20 V; extraction cone voltage: 4 V; source gas temperature: 120 °C; desolvation gas temperature: 450 °C; cone gas flow (nitrogen): 1 L/h; desolvation gas flow (nitrogen): 800 L/h.
Self-replication Promotes the Formation of Complex Folded Structures

Circular Dichroism

Spectra were recorded at room temperature using a JASCO J715 spectrophotometer and HELMA quartz cuvettes with a path length of 1 mm. All spectra were recorded at room temperature from 190 nm to 300 nm, with 2 nm step interval and 3 scans with a speed of 200 nm/min. Solvent spectra were subtracted from all spectra. Samples were diluted to a concentration of 0.15 mM with respect to the concentration of building block.

Thioflavin T (ThT) Fluorescence Assay

Fluorescence measurements were performed on a JASCO FP 6200 fluorimeter using quartz cuvettes with 1 cm path length. For the ThT measurements, a freshly prepared solution of thioflavin T (dissolved to 2.2 mM in 50 mM borate buffer at pH = 8.0 and filtered through a 0.2 µm syringe filter) was diluted to 22 µM with the same buffer. An aliquot of 450 µL of this diluted solution was transferred to the cuvette, followed by the addition of 80 µL of sample (diluted with borate buffer to 80 µM total building block concentration just prior to the measurement). Spectra were recorded after an incubation time of 2 minutes. The excitation wavelength was set at 440 nm and spectra were recorded in the range of 480-700 nm with a slit width of 5 nm, using a cutoff filter at 480 nm. The blank spectrum (i.e. fluorescence of the corresponding dye in solvent only) was subtracted from each spectrum.

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 (supplied by Van Loenen instruments). After 30 seconds, the droplet was blotted on filter paper. The sample was then stained twice with a solution of 2% uranyl acetate (5 µL) deposited on the grid, and blotted on filter paper after 30 seconds. The grids were observed in a Philips CM12 electron microscope operating at 120 kV. Images were recorded on a slow scan CCD camera.

UPLC methods:

Method for the analysis of DCLs made from building blocks 1 and 2:

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201
Chapter 5

5.4.2 Appendix

Kinetic profiles for self-replicator $1_4$ and foldamer $2_{15}$

**Figure S5.1.** Kinetic study of libraries made from (a) building block 1 (2.0 mM) and (b) building block 2 (2.0 mM) in borate buffer (50 mM, pH = 8.0) with 1.0 M NaCl.

Seeding experiment for self-replicator $1_4$

**Figure S5.2.** Growth of $1_4$ with time in a DCL made from 1.0 mM building block 1 in borate buffer (50 mM, pH = 8.0, 1.0 M NaCl) without seeding and upon seeding with 10 mol % (in terms of [1]) preformed $1_4$, demonstrating the autocatalytic nature of the formation of $1_4$. 

202
Effect of the ratio of building blocks on the product distribution of the libraries made from 1 and 2

**Figure S5.3.** UPLC analysis of libraries made from building blocks 1 and 2 in borate buffer (50 mM, pH = 8.0, 1.0 M NaCl) at a total concentration of 2.0 mM at different ratios: (a) 0; (b) 10; (c) 20; (d) 30; (e) 40; (f) 50; (g) 60; (h) 70; (i) 80; (j) 90; (k) 100 mol % of building block 2.
Chapter 5

Kinetic profiles of libraries made from building blocks 1 and 2 with different ratios

Figure S5.4. Kinetic analysis of libraries made from building blocks 1 and 2 in borate buffer (50 mM, pH = 8.0, 1.0 M NaCl) at a total concentration of 2.0 mM at different ratios: (a) 10; (b) 20; (c) 30; (d) 40; (e) 50; (f) 60; (g) 70; (h) 80; (i) 90 mol % of building block 2.
Self-replication Promotes the Formation of Complex Folded Structures

Building block 1 triggered foldamer disassembly

**Figure S5.5.** Kinetic analysis of a library made from foldamer (1.0 mM, in terms of monomer [2]) and building block 1 (1.0 mM) in borate buffer (50 mM, pH = 8.0, 1 M NaCl), showing foldamer 2_{15} disassembling into monomer 2 by building block 1 and transforming into self-replicator 1_{2}1_{2}.

Total peak area of the library made from building blocks 1 and 2

**Figure S5.6.** Total UPLC peak area of libraries made from (a) building block 1 (2.0 mM); (b) building block 2 (2.0 mM); (c) building blocks 1 (1.0 mM) and 2 (1.0 mM), showing that the molar absorptivity of the building blocks are essentially independent of the macrocycles.
Chapter 5

5.4.2 UPLC and UPLC-MS Analyses

Figure S5.7. UPLC analysis of the DCL made from 1 (1.6 mM) and 2 (0.4 mM) in borate buffer (50 mM, pH = 8.0, 1.0 M NaCl) after stirring for 36 hours.

Figure S5.8. Mass spectrum of $2_{15}$ (retention time 3.46 min in Figure S5.7) from the LC-MS analysis of a DCL made from 1 (1.6 mM) and 2 (0.4 mM). Calculated m/z: 2296.40 [M+3H]$^{3+}$, 1722.55 [M+4H]$^{4+}$, 1378.24 [M+5H]$^{5+}$; observed m/z: 2296.84 [M+3H]$^{3+}$, 1722.81 [M+4H]$^{4+}$, 1378.37 [M+5H]$^{5+}$.
**Figure S5.9.** Mass spectrum of 2 (retention time 4.48 min in Figure S5.7) from the LC-MS analysis of a DCL made from 1 (1.6 mM) and 2 (0.4 mM). Calculated m/z: 462.10 [M+1H]^+; observed m/z: 462.23 [M+1H]^+.

**Figure S5.10.** Mass spectrum of 1,2 (retention time 6.20 min in Figure S5.7) from the LC-MS analysis of a DCL made from 1 (1.6 mM) and 2 (0.4 mM). Calculated m/z: 1075.30 [M+2H]^2+, 717.20 [M+3H]^3+; observed m/z: 1075.06 [M+2H]^2+, 717.20 [M+3H]^3+.
Chapter 5

Figure S5.11. Mass spectrum of 1,2 (retention time 7.41 min in Figure S5.7) from the LC-MS analysis of a DCL made from 1 (1.6 mM) and 2 (0.4 mM). Calculated m/z: 1231.43 [M+2H]$^{2+}$, 821.29 [M+3H]$^{3+}$; observed m/z: 1231.58 [M+2H]$^{2+}$, 821.23 [M+3H]$^{3+}$.

Figure S5.12. Mass spectrum of 2 (retention time 7.73 min in Figure S5.7) from the LC-MS analysis of a DCL made from 1 (1.6 mM) and 2 (0.4 mM). Calculated m/z: 689.63 [M+2H]$^{2+}$, 460.09 [M+3H]$^{3+}$; observed m/z: 689.64 [M+2H]$^{2+}$, 460.22 [M+3H]$^{3+}$.
Figure S5.13. Mass spectrum of 1, (retention time 8.16 min in Figure S5.7) from the LC-MS analysis of a DCL made from 1 (1.6 mM) and 2 (0.4 mM). Calculated m/z: 774.37 [M+1H]^+, 387.18 [M+2H]^2+; observed m/z: 774.37 [M+1H]^+, 387.87 [M+2H]^2+.

Figure S5.14. Mass spectrum of 1,2 (retention time 8.29 min in Figure S5.7) from the LC-MS analysis of a DCL made from 1 (1.6 mM) and 2 (0.4 mM). Calculated m/z: 845.76 [M+2H]^2+, 564.17 [M+3H]^3+; observed m/z: 845.68 [M+2H]^2+, 564.26 [M+3H]^3+.
Figure S5.15. Mass spectrum of 1,2; (retention time 8.49 min in Figure S5.7) from the LC-MS analysis of a DCL made from 1 (1.6 mM) and 2 (0.4 mM). Calculated m/z: 1182.49 [M+3H]$^3+$, 887.12 [M+4H]$^4+$; observed m/z: 1182.52 [M+3H]$^3+$, 887.27 [M+4H]$^4+$.

Figure S5.16. Mass spectrum of 1a (retention time 8.74 min in Figure S5.7) from the LC-MS analysis of a DCL made from 1 (1.6 mM) and 2 (0.4 mM). Calculated m/z: 1543.70 [M+2H]$^2+$, 1029.47 [M+3H]$^3+$, 772.35 [M+4H]$^4+$; observed m/z: 1543.99 [M+2H]$^2+$, 1029.52 [M+3H]$^3+$, 772.51 [M+4H]$^4+$.
Self-replication Promotes the Formation of Complex Folded Structures

**Figure S5.17.** Mass spectrum of 1<sub>4</sub> (retention time 9.07 min in Figure S5.7) from the LC-MS analysis of a DCL made from 1 (1.6 mM) and 2 (0.4 mM). Calculated m/z: 1929.37 [M+2H]<sup>2+</sup>, 1286.58 [M+3H]<sup>3+</sup>, 965.19 [M+4H]<sup>4+</sup>, 772.35 [M+5H]<sup>5+</sup>; observed m/z: 1929.49 [M+2H]<sup>2+</sup>, 1286.54 [M+3H]<sup>3+</sup>, 965.29 [M+4H]<sup>4+</sup>, 772.53 [M+5H]<sup>5+</sup>.

**Figure S5.18.** Mass spectrum of 1<sub>2</sub> <sub>4</sub> (retention time 9.59 min in Figure S5.7) from the LC-MS analysis of a DCL made from 1 (1.6 mM) and 2 (0.4 mM). Calculated m/z: 2002.78 [M+1H]<sup>+</sup>, 1001.89 [M+2H]<sup>2+</sup>, 668.26 [M+3H]<sup>3+</sup>, 501.45 [M+4H]<sup>4+</sup>; observed m/z: 2002.84 [M+1H]<sup>+</sup>, 1001.71 [M+2H]<sup>2+</sup>, 668.30 [M+3H]<sup>3+</sup>, 501.56 [M+4H]<sup>4+</sup>. 

211
Chapter 5

Figure S5.19. Mass spectrum of 14 (retention time 11.23 min in Figure S5.7) from the LC-MS analysis of a DCL made from 1 (1.6 mM) and 2 (0.4 mM). Calculated m/z: 1158.02 [M+2H]^{2+}, 772.35 [M+3H]^{3+}, 579.51 [M+4H]^{4+}; observed m/z: 1158.25 [M+2H]^{2+}, 772.33 [M+3H]^{3+}, 579.60 [M+4H]^{4+}.

5.5 References

Self-replication Promotes the Formation of Complex Folded Structures

Chapter 5