Synthetic Replicators

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Self-Replicating Amphiphilic β-Sheet Peptides**

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Several different non-enzymatic molecular replication systems have been prepared and analyzed, including nucleic acids, fatty acids, peptides, and organic molecules.[1–11] This research was evidently motivated by the chemists' enthusiasm to shine light on plausible scenarios that may have lead to the origin of life on earth and early molecular evolution,[12–19] and it also provided new opportunities for understanding fundamental principles, such as molecular recognition and autocatalysis. The study of non-enzymatic replication has been expanded recently beyond autocatalysis, to small molecular networks, in which the replication is also a product of template-assisted cross-catalysis.[10]

The design of replicating peptides has centered mainly on helical coiled-coil structures,[20–26] in which monomeric or dimeric peptides, twenty-five to forty amino acids in length, serve as templates for substrate binding and thus for enhanced condensation and replication. However, it has been postulated that shorter peptides with simpler sequences may also serve as templates for self replication, provided that they are able to arrange themselves into unique and well-defined structures. We show herein that rather simple peptides, close analogues of the synthetic amphiphilic Glu-(Phe-Glu)$_n$ peptides,[27] can form soluble, one-dimensional β-sheet aggregates in water, which serve to significantly accelerate peptide ligation and self replication.

It has been postulated[28–30] and demonstrated that native amyloid fibrils can replicate, albeit with moderate efficiency.[31] Specific design of synthetic peptides can be used to make soluble aggregates with defined structures and higher replication efficiencies. Towards this aim, it has been shown that peptides comprising of repetitive dyads of hydrophilic and hydrophobic amino acid residues tend to adopt β-pleated sheet arrangements. Recently, it was further revealed that the terminal proline residue, which is rigid, does not have a backbone N–H group available for hydrogen bonding, and often acts as a β-sheet breaker, can be used to enhance the formation of ordered β-sheet assemblies.[27,32–35] Based on this evidence, we have synthesized the sequence Aba-Glu-(Phe-Glu)$_5$-Pro of peptide **2** in which the N-terminus proline of the β-sheet forming peptide P$_{FE}$-5[34] has been replaced by the capping aromatic 4-acetamidobenzoate (ABA; Table 1). The

Table 1: Peptide sequences.

Peptide	Sequence[a]	MW[b]
1	ABA-Glu-Phe-Glu-Phe-Ala-Cys-Glu-Phe-Glu-Phe-Glu-Pro-CONH$_2$	1683
E$_1$	ABA-Glu-Phe-Glu-Phe-Ala-COSR[c]	890
N$_1$	Cys-Glu-Phe-Glu-Phe-Glu-Pro-CONH$_2$	899
2	ABA-Glu-Phe-Glu-Phe-Glu-Phe-Glu-Phe-Glu-Phe-Glu-Pro-CONH$_2$	1786
P$_{FE}$-5[34]	Pro-Glu-Phe-Glu-Phe-Glu-Phe-Glu-Phe-Glu-Phe-Glu-Pro	
1^{gg}	ABA-Glu-Phe-Gly-Phe-Ala-Cys-Glu-Phe-Gly-Phe-Glu-Pro-CONH$_2$	1539
E^g	ABA-Glu-Phe-Gly-Phe-Ala-COSR[c]	818
N^g	Cys-Glu-Phe-Gly-Phe-Glu-Pro-CONH$_2$	827
S$_c$	ABA-Lys-Phe-Val-Ala-Lys-Ala-Lys-Lys-Leu-Val-Gly-Phe-CONH$_2$	1496

[a] All peptides were synthesized on solid phase, purified by preparative HPLC, and their purity and molecular weights were characterized by HPLC and LCMS. [b] MW = molecular weight. [c] R = $CH_2CH_2CONH_2$.

structure of peptide **2** was then modified to include an Ala-Cys dyad at the middle of the sequence, resulting in the sequence of peptide **1** (Figure 1) that can be formed by condensation of electrophilic and nucleophilic fragments (E$_1$ and N$_1$ in Table 1) through native chemical ligation.[36]

The tendency of the amphiphilic peptides **1** and **2** to form β-sheet assemblies at the water–air interface, as well as in aqueous solutions, was studied using a battery of analytical techniques. Langmuir surface–pressure versus molecular area (π-A) isotherms of **1** and **2** on deionized water indicated that both peptides form stable monolayers. The peptide chains in these monolayers are oriented with their long molecular axes parallel to the water surface, as suggested by the limiting area per molecule (see π-A isotherms in the Supporting Information, Figure S1), which was found to be 250 Å^2 (**1**) and 240 Å^2 (**2**). Grazing-incidence X-ray diffraction (GIXD) experiments on films of **1** or **2** on deionized water provided evidence for assembly of the peptides into two-dimensional crystalline β-sheet monolayers. Diffraction patterns obtained in the expanded state of the monolayer showed Bragg peaks at $q_{xy} = 0.108$ Å^{-1}, $d = 58.1$ Å and at $q_{xy} = 1.315$ Å^{-1}, $d = 4.8$ Å for **1**.

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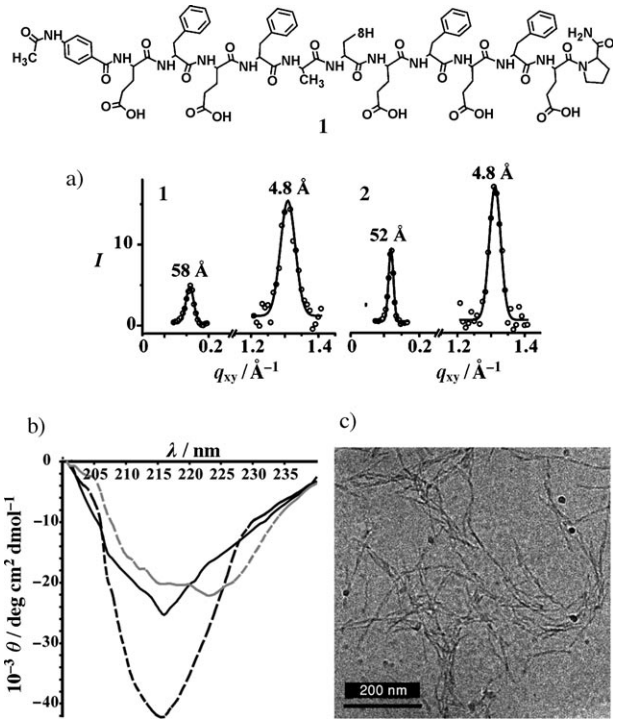
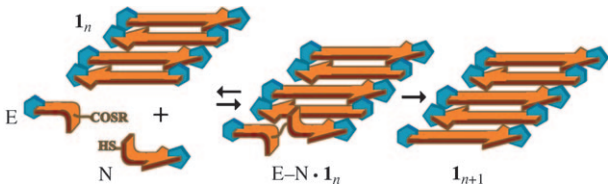


Figure 1. Chemical structure of the self-replicating peptide **1** and structural characterization of the β sheet assemblies formed by peptides **1**, **2**, and $\mathbf{1}^{gg}$. a) GIXD patterns with Bragg peaks corresponding to hydrogen bond spacings and peptide lengths indicated. b) CD spectrum of 50 μM solutions of **1** (solid black line), **2** (dashed black), and $\mathbf{1}^{gg}$ (dashed gray) in ammonium carbonate buffer at pH 7. c) Cryo-TEM images of frozen one-dimensional aggregates formed from 100 μM solution of **1**.

The GIXD pattern of **2** was similar, with Bragg peaks at $q_{xy} = 0.121$ Å^{-1}, $d = 51.9$ Å and at $q_{xy} = 1.310$ Å^{-1}, $d = 4.8$ Å (Figure 1a). The 4.8 Å spacing is characteristic of inter-strand hydrogen bonds along β-sheet ribbons. The 58 Å and 52 Å spacings for **1** and **2**, respectively, are close to the estimated projected length of the β sheet on the water interface, according to a molecular model (data not shown), with the first being slightly longer, probably because of minor alterations in the peptide conformation in which Ala-Cys residues replace Glu-Phe.

The circular dichroism (CD) spectra of both peptides **1** and **2** provided evidence for formation of β-sheet aggregates in buffered (pH 7) and non-buffered (pH of circa 5.5) water solution. At pH 7 (Figure 1b), the spectra of both peptides showed a clear minimum at 216 nm, which is similar to previously described anti-parallel β-sheet structures.[37,38] The spectrum of **1** included additional "knees", of which the minima at about 206 and 225 nm can correlate with some α-helix contribution. The formation of β-sheet structures in water could also be ascertained from a CD minimum at 216 nm, and a maximum at about 195 nm (Supporting Information, Figure S2).[37,38] Another minimum was found in these conditions at around 201 nm and correlated with some random coil contribution, and the spectrum of **1** showed again a broad minimum at 227 nm, which accounts for a small fraction of α helix. The propensity to form soluble β sheets both in water and buffer is an important characteristics of the new system, because as described below, all the replication experiment are performed by first equilibrating the starting materials and templates in water, and then initiating ligation (and replication) by adding buffer.

Additional characterization of peptide **1** assemblies in 100 μM aqueous solutions was obtained by cryoelectron microscopy (Figure 1c). Long and entangled fibrils were observed, which are as small as about 5 nm in width, and correspond well with the molecular length of **1** along the β-strand long axis. All the structural measurements thus suggested the formation of β-sheet aggregates of **1** and **2** at the water–air interface and in solution, depending on the working conditions. Scheme 1 illustrates the anti-parallel β sheet formed by self assembly of peptide **1**, demonstrating the availability of the β sheet edges for interactions with incoming reactants during the replication processes.



Scheme 1. The autocatalytic process of **1**. The antiparallel β sheet $\mathbf{1}_n$ serves as template for registered association of E and N, which react through native chemical ligation to form a new molecule of **1**. The larger aggregate $\mathbf{1}_{n+1}$ is then available for the next catalytic reaction.

A clear signature of template-assisted autocatalytic production of **1** was observed in the ligation reaction between equimolar amounts of the electrophile thioester E_1 and the nucleophile N_1. Figure 2a shows that, after a lag phase of about three hours, during which **1** has formed slowly, the rate of formation increased significantly; therefore, the assembly of peptide **1** becomes more pronounced only after reaching a certain critical concentration, thus accelerating the autocatalysis. In a control reaction, we followed the rate of formation of a mutated peptide, $\mathbf{1}^{gg}$, from its precursors E^g and N^g. Owing to introduction of two glycine residues into its sequence, $\mathbf{1}^{gg}$ hardly forms stable β sheets in water, and its production proceeded linearly over the entire nine hours of inspection time. The CD spectra of $\mathbf{1}^{gg}$ indicated weak and rather spread absorptions, which could indicate the formation of mixed sheets, helices, and random structures (Figure 1b and Supporting Information, Figure S2; gray dashed lines). In addition, this peptide did not form a monolayer at the air–water interface according to Langmuir π-*A* measurements in which no signal was detected.

To further study the autocatalytic properties of **1**, the rate of ligation of E_1 and N_1 in reactions that were initially seeded with different amounts of **1** was estimated (Figure 2b). Although less than 1 μM of **1** was formed after one hour of the template-free reaction, its production was enhanced autocatalytically in proportion to the higher seeding concentrations. The fastest reaction, seeded with 116 μM template,

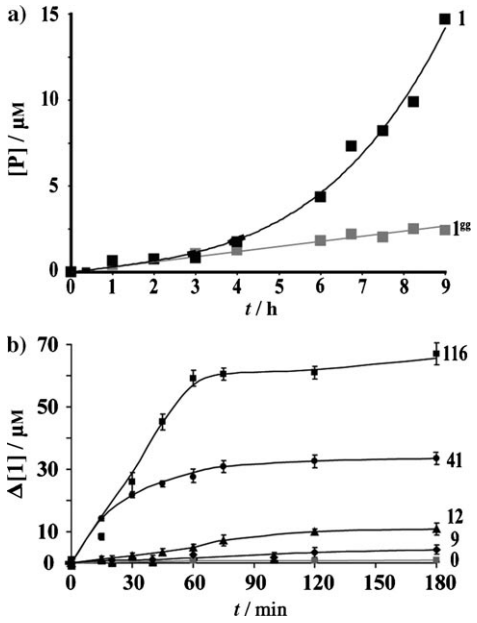


Figure 2. Kinetic analysis of the self-replication process of peptide **1**. a) Production of **1** over time in reactions between E_1 and N_1. The linear production over time of a control peptide $\mathbf{1}^{gg}$, which cannot form β sheets, is shown for comparison. b) Production of **1** over time in reactions between E_1 and N_1 that were initially seeded with 9–116 μM amounts of **1** as a template (the seeded amounts are marked next to the curves). Production of **1** in the template-free reaction is shown in gray for comparison. All the reactions were performed with 250 μM precursors (E_1 and N_1 (**1**), or E^g and N^g ($\mathbf{1}^{gg}$)), at (22 ± 1) °C in aqueous 3-(4-morpholinyl)-1-propanesulfonic acid (MOPS) buffer solutions at pH 7, and with tris(2-carboxyethyl)phosphine (TCEP; 5 mM) as a reducing agent (see the Experimental Section in the Supporting Information). The amounts of formed product were determined by HPLC using a foreign ABA-labeled peptide as standard (Supporting Information, Figure S3). Curves were generated to guide the eye.

proceeded at about a 60-fold higher rate compared to the template-free reaction. The ligation reaction proceeded slowly when seeded with $\mathbf{1}^{gg}$ or with a "scrambled" peptide sequence of similar length, S_c (Supporting Information, Figure S4). The enhanced ligation in mixtures that were seeded with **1** is thus not just due to a "crowding effect", but rather a result of molecular recognition. Furthermore, even seeding with peptide **2**, which forms β sheets, enhanced the ligation only marginally (Supporting Information, Figure S4), demonstrating the specificity of the system.

The exact mechanism by which the β-sheet aggregates of **1** enhance its replication is currently under investigation. In our hypothesis, the two edges of a one-dimensional self-assembled β-sheet ribbon, or areas of imperfect packing along the structure, serve as templates for simultaneous interaction with the precursors E and N, leading to their fast ligation owing to high effective molarity. A simple three-step mechanism that can account for this process is given by Equations (1)–(3). T_n represents a catalyst and growing aggregate of **1**, and T_L represents an aggregate that has reached a critical size and can disassemble into two smaller structures T_S and $T_{S'}$.

$$nT \rightleftharpoons T_n \quad (1)$$

$$E + N + T_n \rightarrow T_{n+1} \quad (2)$$

$$T_L \rightleftharpoons T_S + T_S \quad (3)$$

The suggested mechanism is supported by analysis of the logarithmic plot of the initial rates versus seeded template concentrations derived from the kinetic data given in Figure 2b (Supporting Information, Figure S5), which revealed exponential growth with a catalytic order $p = 1.2$.[39] This value reflects an efficient process, in which after each step of ligation a new catalytic site is formed (in T_{n+1}) with the same catalytic properties as the old one (T_n). The nonlinearity of the catalysis, characterized by $p > 1$, reflects the fact that the kinetic rate is proportional to the number of aggregates and not their length, so as increasingly larger amounts of smaller aggregates are formed in the reaction mixture, the rate increases due to both increase in total concentration of T and the number of aggregates.[40] This phenomenon could be directly ascertained when we compared the rates of replication within the template-seeded mixtures in the original conditions, with the observed rates within such mixtures in which ligation was initiated right after vigorous sonication for five minutes. Within reaction seeded with intermediate (50 μM) or high (130 μM) concentrations of **1**, sonication, namely, disassembly of the aggregates, caused an increase in rate of product formation during the first hour (Supporting Information, Figure S6). Further support to the suggested mechanism was obtained by characterizing aggregates that were formed after equilibration of solutions of different concentrations of **1**, in either buffer at pH 7 or water, by dynamic light scattering measurements. These measurements (Supporting Information, Figure S7) showed that one type of large aggregate is mainly formed in solutions of the lower concentrations, whereas increasingly larger amounts of two kinds of smaller aggregates are obtained as the concentration is elevated.

The study described herein allows expanding the repertoire of families of replicating molecules. We have recently discussed the potential of "high-order" catalysis, in which the replicating template is not a monomer but rather an oligomeric structure, for directing complex processes in systems chemistry.[41,42] Also of significance is the fact that the β-sheet replication is achieved using short peptides that have only alternating aromatic and charged amino acids. This system demonstrates a stepwise mechanism by which short peptides undergo self-assembly to form defined templates, which in turn become efficient replicators. A recent review summarized the catalytic ability of peptide β sheets in water.[43] By pointing out the β-sheet properties, and specifically their efficiency in hydrolyzing oligoribonucleotides and preventing amino acid racemization, it was suggested that such systems may have played a role in the origins of life. This view is also discussed in recent reports that showed how short peptides can be formed under prebiotic conditions.[44,45] In addition, the origin of molecular chirality was also probed in peptide chain-elongation experiments, showing that significant homochiral amplification of peptides is obtained by the formation of β sheets.[46,47] Our results support these hypoth-

eses in providing evidence for the ability of short β-sheet peptides to promote their replication.

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