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# Out-of-Equilibrium Self-Replication Allows Selection for Dynamic Kinetic Stability in a System of Competing Replicators

Bin Liu, Juntian Wu, Marc Geerts, Omer Markovitch, Charalampos G. Pappas, Kai Liu, and Sijbren Otto\*

**Abstract:** Among the key characteristics of living systems are their ability to self-replicate and the fact that they exist in an open system away from equilibrium. Herein, we show how the outcome of the competition between two self-replicators, differing in size and building block composition, is different depending on whether the experiments are conducted in a closed vial or in an open and out-of-equilibrium replication–destruction regime. In the closed system, the slower replicator eventually prevails over the faster competitor. In a replication–destruction regime, implemented through a flow system, the outcome of the competition is reversed and the faster replicator dominates. The interpretation of the experimental observations is supported by a mass-action-kinetics model. These results represent one of the few experimental manifestations of selection among competing self-replicators based on dynamic kinetic stability and pave the way towards Darwinian evolution of abiotic systems.

**H**ow life can emerge from inanimate matter remains one of the biggest open questions in science. Self-replication, the ability to produce copies of oneself, such that information contained in the molecules that constitute the system is transferred to the next generation, is a key characteristic of life<sup>[1]</sup> and is likely to play an important role in its emergence.<sup>[2]</sup> Another key feature of life is the ability to

undergo Darwinian evolution, a process in which self-replicators undergo mutation<sup>[3]</sup> and subsequent selection<sup>[4]</sup> under out-of-equilibrium conditions,<sup>[5]</sup> thus relying on an input of energy. Such input of energy (or matter) also allows living systems to maintain their structure and function.<sup>[1a,6]</sup> Structural or compositional differences could provide self-replicators with evolutionary advantages such as enhanced stability, replication rate, ability to adapt to changing conditions, and selectivity with respect to competing reactions.<sup>[4]</sup> In order for such advantages to be selected for, systems of competing replicators need to be subjected to a regime where both self-replication and replicator destruction are enabled.<sup>[4]</sup> This regime is most easily accessible in a stirred open system where replicators are continuously supplied with precursors and where part of the reaction volume is flown out. In such continuously stirred tank reactor (CSTR) setup, destruction is implemented by out-flow, and replicators only persist as long as their replication can keep up with the outflow rate.<sup>[1a]</sup> The steady state in such a system is determined by the balance between the rates of replication and destruction. The replicator composition in this state is not reflecting the thermodynamic stability of the system but rather the dynamic kinetic stability (DKS)<sup>[2b,4,7]</sup> of the replicators. Implementing selection of self-replicators based on their DKS is crucial in order to advance the synthesis of de novo life and improve our understanding of life's origin.

In the past few decades, autocatalytic<sup>[8]</sup> and self-replicating molecular systems have been developed, using various building blocks, including nucleic acids,<sup>[9]</sup> peptides,<sup>[10]</sup> and fully synthetic molecules.<sup>[11]</sup> Traditionally, most reported self-replicators form through energetically down-hill processes and yield either thermodynamically stable or kinetically trapped states.<sup>[12]</sup> More recently, several self-replicating systems have been reported that incorporate selection and competition<sup>[13]</sup> as well as examples of out-of-equilibrium self-replication<sup>[13b,14]</sup> or autopoiesis.<sup>[15]</sup> In several of these examples, destruction of self-replicators was implemented through serial transfer experiments.<sup>[1a]</sup> Examples of systems where self-replicators are selected for based on their DKS are scarce.<sup>[14a]</sup>

We previously reported how self-replicators can be generated from 3,5-dimercaptobenzoyl-containing building blocks. Upon oxidation these dithiols form dynamic combinatorial libraries (DCLs)<sup>[13b,16]</sup> of macrocycles composed of multiple building blocks linked through disulfide bonds. These macrocycles continuously exchange building blocks through reversible thiol-disulfide chemistry. Macrocycles

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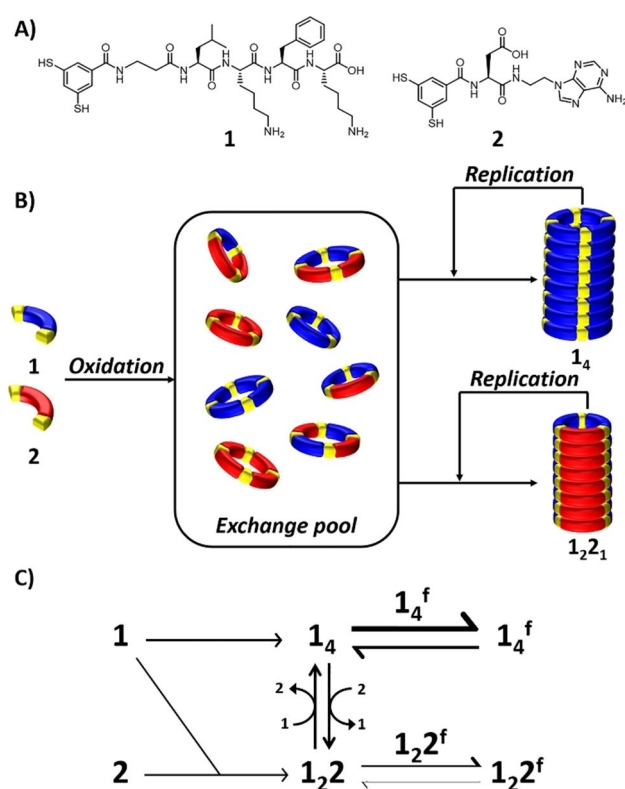
with a sufficiently large ring size can self-assemble into fibrous aggregates and undergo exponential self-replication through fiber elongation and breakage.<sup>[10a,17]</sup> Mixtures of multiple self-replicators differing in ring size and composition can be created by mixing different types of building blocks in a closed system.<sup>[18]</sup> Recent work on an open system showed that, in DCLs made from a single building block, chemical fueling can result in selection for the molecularly more complex replicator.<sup>[14a]</sup>

We now report an open system, maintained in a replication-destruction regime, featuring two building blocks that give two self-replicators of different building block composition. In this regime selection favors the self-replicator that is only metastable otherwise, but has the highest dynamic kinetic stability. This outcome is in stark contrast to the one obtained in a closed vial where reversible disulfide exchange is allowed.

In order to clearly demonstrate selection based on dynamic kinetic stability a system of competing self-replicators is needed for which the product distribution in a closed system is different from that obtained in replication-destruction regime. Given the difficulty in predicting, even qualitatively, the relative thermodynamic and kinetic stabilities of replicators, the a-priori design of such systems is beyond current capabilities. However, from the experimentally observable behavior of replicators in a closed environment such systems may yet be identified: in particular, systems which exhibit a fast but transient emergence of one type of replicator followed by a slower take-over by a second competing replicator are likely candidates. While such behavior is, at least in our experience, rare, we did observe it in the course of investigating the relationship between self-replication (observed in DCLs made solely from building block **1**) and folding (formation of a 15mer of **2** observed in DLCs made solely from building block **2**<sup>[19]</sup>) (Scheme 1).<sup>[20]</sup> Peptide building block **1** features alternating hydrophobic and hydrophilic amino-acid residues to allow for the formation of H-bonded networks such as  $\beta$ -sheets, which allow the macrocycles to assemble into fibers.<sup>[21]</sup> In our previous work we focused mainly on DCLs containing at least 30 mol % of **2**, for which foldamer formation occurred alongside replicator formation. For the present study the focus is on DCLs containing less than 30 mol % of **2**, in which two competing self-replicators form, while no foldamer is produced (most likely due to the low abundance of **2**).

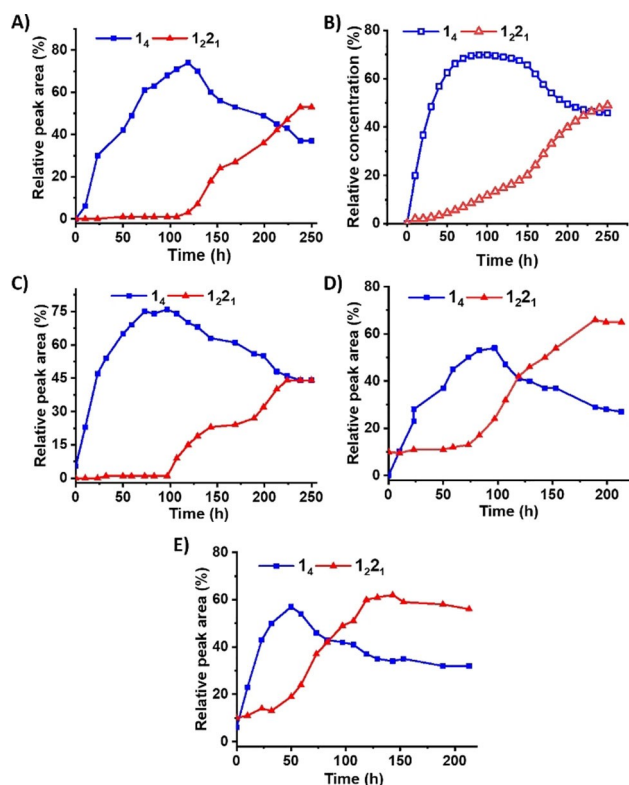
We started by studying a DCL composed of 1.7 mM **1** and 0.30 mM **2** in borate buffer ( $[\text{Na}_2\text{B}_4\text{O}_7] = 50 \text{ mM}$ , pH 8.0, containing 1.0 M NaCl) in a closed vial. The composition of the mixture was monitored over time using UPLC-MS. We chose a wavelength where the molar absorptivity of the building blocks **1** and **2** was comparable (Figure S6), so that UPLC peak areas directly reflect the quantity of library material. The results showed a rapid increase in the amount of **1**<sub>4</sub> followed by a decrease that set in after about 100 hours, which coincided with the emergence of trimer **1**<sub>2</sub>**1** (Figure 1A).

In order to probe whether **1**<sub>4</sub> and **1**<sub>2</sub>**1** are capable of self-replication we performed a number of seeding experiments.

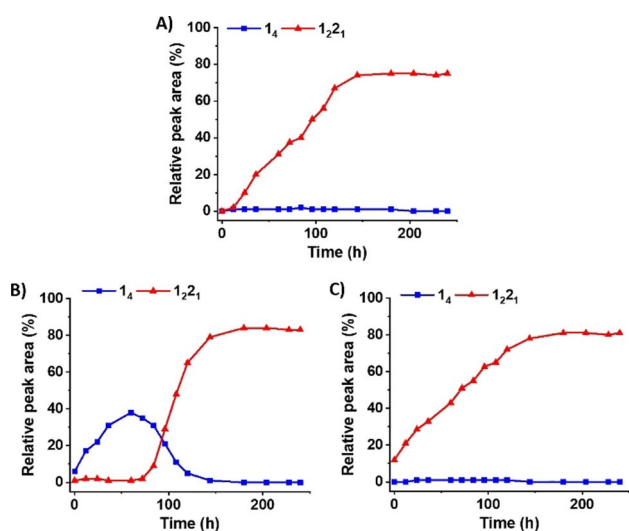


**Scheme 1.** A) Molecular structures of building blocks **1** and **2**. B) A DCL containing a mixture of macrocycles of different sizes and building block compositions is generated upon oxidation of a mixture of building blocks **1** and **2**. From this DCL macrocycles **1**<sub>4</sub> and **1**<sub>2</sub>**1** assemble autocatalytically into fibrous aggregates. C) Simplified mass-action-kinetics model of the system (Supporting Information Section 6). The species **1**<sub>4</sub><sup>f</sup> and **1**<sub>2</sub>**1**<sup>f</sup> represent all the macrocycles that are assembled into fibers. The thickness of arrows reflects the relative values of the rate constants.

Repeating the experiment shown in Figure 1A but now adding 10 mol % of preformed fibers of **1**<sub>4</sub> at  $t=0$  hours induced a modest increase in the rate of formation of this macrocycle (Figure 1C) hinting at autocatalysis. In order to obtain clearer evidence, we repeated this seeding experiment using a 1:1 ratio of building blocks **1** and **2**, where the spontaneous emergence of **1**<sub>4</sub> is more difficult. While in the absence of seed barely any **1**<sub>4</sub> formed, addition of 10 mol % seed induced a rapid but transient formation of **1**<sub>4</sub> (Figure 2A, B), confirming that **1**<sub>4</sub> is indeed a self-replicator. The addition of **1**<sub>4</sub> did not seem to affect the formation of **1**<sub>2</sub>**1** in the experiment shown in Figure 1A, while it delayed the emergence of **1**<sub>2</sub>**1** in the experiment shown in Figure 2B, suggesting that cross-catalysis by **1**<sub>4</sub> of the formation of **1**<sub>2</sub>**1** is insignificant. When seeding a DCL composed of 1.7 mM **1** and 0.30 mM **2** with preformed fibers of **1**<sub>2</sub>**1** we observed that the lag phase in the growth of **1**<sub>2</sub>**1** shortened compared to that in the unseeded sample (Figure 1D). Furthermore, seeding with **1**<sub>2</sub>**1** did not seem to have an effect on the formation of **1**<sub>4</sub> (compare Figure 1A and D). These results indicate that there is no noticeable cross-catalysis between replicators **1**<sub>4</sub> and **1**<sub>2</sub>**1** which therefore cannot be considered as mutants of each other. Lastly, when seeding an identical



**Figure 1.** Kinetics of formation of replicators  $1_4$  (blue squares) and  $1_2 2_1$  (red triangles) in stirred DCLs made from 1.7 mM **1** and 0.30 mM **2** in borate buffer ( $[\text{Na}_2\text{B}_4\text{O}_7] = 50 \text{ mM}$ , pH 8.0, 1.0 M NaCl). A) Emergence of replicators in the absence of seed. B) Simulation results obtained for a closed system, analogous to the experiment shown in Figure 1A. Kinetics of replicator formation upon seeding with C) 10 mol%  $1_4$ ; D) 10 mol%  $1_2 2_1$ ; or E) 10 mol% of  $1_4$  and  $1_2 2_1$ , each. The kinetics were monitored using ultra performance liquid chromatography (UPLC). Lines are drawn to guide the eye. See Figure S1 for data for the other library members.



**Figure 2.** Kinetic data for DCLs made from 1.0 mM **1** and 1.0 mM **2** in borate buffer ( $[\text{Na}_2\text{B}_4\text{O}_7] = 50 \text{ mM}$ , pH 8.0, 1.0 M NaCl) (A) without seed, and seeded with 10 mol% (B) preformed  $1_4$  or (C) preformed  $1_2 2_1$ . See Figure S2 for data for the other library members.

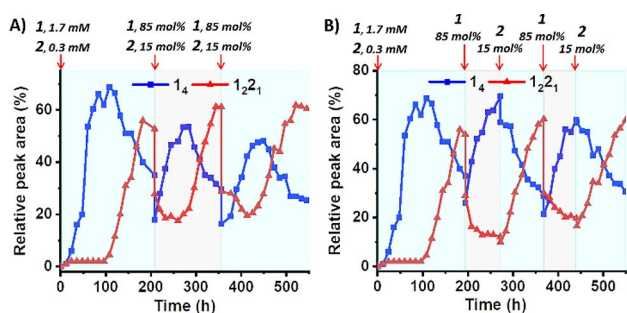
DCL simultaneously with preformed fibers of  $1_4$  and  $1_2 2_1$ , we again observed the transient formation of  $1_4$ , but to a reduced degree and for a shorter period of time compared to the non-seeded sample.

Note that, once  $1_2 2_1$  has emerged, it grows at the expense of  $1_4$  as both replicators require building block **1**, suggesting that the more stable state of the system is one in which both replicators co-exist, with, at a building block ratio of 1.7 mM **1** and 0.30 mM **2**, a slight preference for  $1_2 2_1$ . Note that also in the absence of stirring there is a net conversion of  $1_4$  into  $1_2 2_1$  when starting from a mixture dominated by  $1_4$ , albeit at a much reduced rate (Figure S7). These results indicate that under these conditions  $1_4$  is a faster self-replicator than  $1_2 2_1$  but that the resulting  $1_4$ -rich state is only metastable.

We constructed a simplified mass-action-kinetics model (which disregards fiber stack lengths and breakage) that shows qualitatively that the experimentally observed behavior can indeed result from two competing replicators that can interconvert and that differ in their rate of replication (Scheme 1C and Supporting Information Section 6). Using a model in which the rate constant for replication of  $1_4$  is about 44-fold faster than that of  $1_2 2_1$ , and the disassembly rate constant of  $1_4$  is 2000-fold faster than that of  $1_2 2_1$  (Table S3) produced behavior that is qualitatively similar to that observed in the experiments (Figure 1B). We refrained from a more quantitative approach to modelling, since the simplified nature of the model does not allow direct mapping of the rate constants in the model onto those in the experimental system.

Given that in a closed vial replicator  $1_4$  transiently dominates the DCL composition prior to the emergence of replicator  $1_2 2_1$ , and that  $1_4$  and  $1_2 2_1$  compete for building block **1**, it is intriguing to study how the two replicators compete in a replication-destruction regime. This out-of-equilibrium regime is accessible in an open system through serial transfer or continuous flow.<sup>[1a,22]</sup> We implemented the continuous flow approach by using a DCL solution of constant volume, to which a solution of building blocks **1** and **2** was continuously supplied, while removing part of the sample volume at a flow rate that matches the inflow. The outflow in this system serves to “destroy” DCL members in a non-selective, irreversible manner; i.e. the rate of “destruction” through outflow is the same for all DCL members. Tuning the flow rate thus allows us to tune the rate of destruction, which works in addition to destruction via other reversible pathways (disulfide exchange). We reasoned that, using continuous flow, we may be able to select for self-replicators based on their DKS, such that the replicator with the highest DKS prevails, irrespective of its thermodynamic stability.

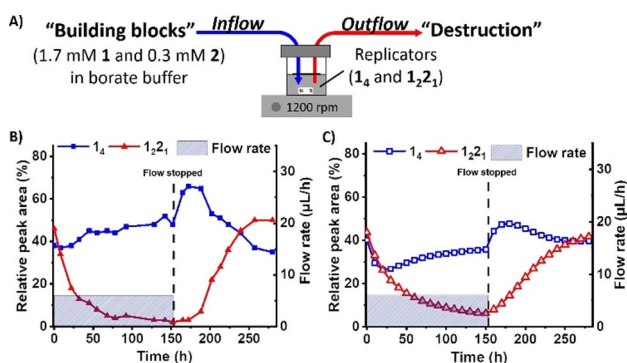
We first tested the possibility for the metastable replicator to prevail in a replication-destruction regime through repetitive addition experiments (Figure 3). We started from a DCL consisting of 1.7 mM **1** and 0.30 mM **2**, prepared under the same conditions as described above. After replicator  $1_2 2_1$  had taken over from  $1_4$  as the dominant replicator, an identical, fresh solution of building blocks **1** and **2** was added. This addition induced a rapid resurgence of self-replicator  $1_4$  while the concentration of replicator  $1_2 2_1$



**Figure 3.** Response of replicator composition to the addition of portions of building blocks 1 and/or 2 to a mixture made from 1.7 mM 1 and 0.30 mM 2 in borate buffer ( $[\text{Na}_2\text{B}_4\text{O}_7] = 50 \text{ mM}$ , pH 8.0, 1 M NaCl). A) Addition of 1 and 2 simultaneously. B) Addition of 1 and 2 alternatingly. See Figure S3 for data for the other library members.

diminished (Figure 3A). However, consistent with the observations in Figure 1, the dominance of replicator  $1_4$  was only temporary and it gave way again to replicator  $1_2,1$ , reaching a product distribution similar to that obtained in Figure 1A. A second addition of building blocks yielded a similar pulse of  $1_4$ . Conducting experiments in which building blocks 1 and 2 were added alternatingly showed even larger swings in replicator composition (Figure 3B). These results echo the observations made in the seeding experiment shown in Figure 1E, showing that  $1_4$  will form rapidly even though  $1_2,1$  is already present in relatively high amounts. These results also indicate that, en route to equilibrium, in this system metastable replicator distributions can be created and destroyed in response to the addition of building blocks.

We then continued by studying the same system in a replication-destruction regime using the simple flow setup depicted in Figure 4A. The concentrations of the solutions of 1 and 2, that were added continuously, were such that the



**Figure 4.** Change of the distribution of replicators  $1_4$  and  $1_2,1$  with time in a replication-destruction regime in which a 200  $\mu\text{L}$  mixture of both replicators was provided with a continuous supply ( $6.0 \mu\text{L h}^{-1}$ ; turnover time of 33 hours) of a solution of building blocks 1 and 2 (1.7 mM in 1 and 0.30 mM in 2). A) Schematic depiction of the flow setup. B) Change of the product distribution of replicators  $1_4$  and  $1_2,1$  with time. For two repeats of this experiment, see Figure S4. C) Kinetic simulation (see Scheme 1C) under conditions similar to those in panel B. In and outflow was stopped after about 150 h in experiments and simulations, which is indicated by the vertical, dashed lines.

overall concentrations of the two building blocks in the sample container remain constant throughout the experiments. We started the experiment with a mixture of the two replicators, similar to the end-point of Figure 1A. All other conditions were identical to those described for experiments conducted in closed vials. In the resulting continuous replication/destruction regime self-replicator  $1_4$  was maintained at a high concentration with respect to  $1_2,1$  (Figure 4B). In addition, we observed a rapid decrease in concentration of  $1_2,1$  in the DCL as soon as the flow was started. This observation suggests that  $1_2,1$  is being removed faster than it can replicate, leading to its decrease in concentration. In other words, in the replication-destruction regime,  $1_4$  is better at competing for building block 1 than  $1_2,1$ . The mass that accumulates upon depletion of  $1_2,1$  resides mostly in monomers 1 and 2 (see Figure S4 which shows repeats of the experiment in Figure 4). When the inflow and outflow are stopped, the system returned to the state observed in the closed vials even though  $1_4$  first transiently increased in concentration even further (consuming the building blocks that still remained in the system after the flow was stopped), but later demised and gave way to  $1_2,1$ . The simulations of this experiment using the mass-action-kinetics model described above gave qualitatively similar results (Figure 4C and Supporting Information Section 6). We noticed that changes in flow rate resulted in substantial changes in the outcome of the competition between the replicators. When the flow rate was decreased (to  $4 \mu\text{L h}^{-1}$ ; turn-over time 49.5 hours), the selection pressure is reduced and both replicators are now able to persist (Figure S5A). When the flow rate was increased (to  $12 \mu\text{L h}^{-1}$ ; turn-over time 16.5 hours), building blocks 1 and 2 accumulated at the expense of replicators  $1_4$  and  $1_2,1$  (Figure S5B).

In conclusion, we have shown how two self-replicators that emerge spontaneously from the same DCL compete for a common building block. In closed vials, the faster of the two eventually prevails. In an open system, in a replication-destruction regime, the results of this competition are opposite and the faster, previously metastable replicator unremittingly dominates the mixture. This system is maintained away from equilibrium by continuously supplying building blocks and continuously removing part of the sample volume. Advantage of such CSTR setup is that it is one of the simplest ways of implementing a replication-destruction regime, allowing selection for replicators based on their dynamic kinetic stability. However, the physical implementation of replicator destruction (i.e. by outflow) has the disadvantage of being, in most cases, non-selective; every replicator, and any other DCL member, has the same probability of being removed in a given period of time. Thus, selection for dynamic kinetic stability in such systems equates to selection for the fastest replicator. As became clear in the iconic experiments by Spiegelman<sup>[9b]</sup> selection for speed of replication may lead to a reduction in replicator complexity during evolution. Future experiments of replicators in a replication-destruction regime are therefore best performed using mechanisms through which destruction can

also be selective for certain replicators. These and further studies on competition and selection in a system of multiple self-replicators mark important steps towards creating synthetic self-replicators capable of Darwinian evolution and contribute to improving our understanding of the role of Darwinian evolution in the synthesis and the origin of life. Such studies would complement other recent work towards evolutionary chemical systems.<sup>[23]</sup>

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## Conflict of Interest

The authors declare no conflict of interest.

## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Keywords:** Dynamic Combinatorial Chemistry · Dynamic Kinetic Stability · Self-Assembly · Self-Replication · Out-of-Equilibrium

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