Enabling Darwinian evolution in chemical replicators
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Life can only be attained in far-from-equilibrium conditions. Dissipative systems belong to a field that, due to the inherent complexities thereof, is still not yet fully explored or understood. In this chapter an experimental setup is described that enables replicators to simultaneously replicate and undergo destruction in far-from-equilibrium conditions, upon providing energy in the form of redox agents. Computer modelling is used to aid the understanding of the active processes in the system and to predict the optimal conditions for replication and destruction to proceed simultaneously and efficiently. Deterministic and stochastic models developed for the purpose are described in detail.

A manuscript containing parts of this chapter is in preparation.
Far-from-equilibrium conditions are crucial for evolution, as they allow attainment of dynamic kinetic stability (DKS), i.e., stability in conditions of simultaneous replication and destruction. Chapter 1 presented an overview on DKS. Inert, not-evolvable species are obtained at equilibrium: the most stable aggregate forms. By contrast, a state of dynamic kinetic stability, or DKS state, leads to the constant evolvability of the replicating system and to the survival of the fittest replicator based on kinetic effects.

Living systems have developed a way to produce diversity towards functional needs in far-from-equilibrium conditions, by means of the inherent capability of DKS systems to yield multiple diverging products, due to their autocatalytic and nonlinear nature, as depicted in Figure 1.2.6. The formose reaction, while constituting an example of chemical diversification, does not lead to evolution (even within its limited chemical space), as it lacks dynamic conditions, i.e., recycling mechanisms that would act as a selection force for the destruction of some of the products.1–7

Evolution can only be achieved by means of continuous far-from-equilibrium production and destruction of replicating species (the example in Figure 3.1 refers to the experimental system that was studied in this thesis), leading to multiple selection cycles.

In this Chapter 3, we describe the realization of an experimental platform for far-from-equilibrium replication, involving destruction of the replicator, and examine extensive deterministic and stochastic computational models developed to aid the understanding of the complexity of these systems and aimed at the identification of appropriate kinetic conditions for enabling chemical evolution. The work in this chapter is based on the same experimental framework presented in Section 2.1 of Chapter 2.
Chapter 3. Far-from-equilibrium replication

3.1. AN EXPERIMENTAL PLATFORM FOR FAR-FROM-EQUILIBRIUM REPLICATION

Above and in Section 1.2 of Chapter 1 the prominent features of replication were amply illustrated. It was described how recycling far-from-equilibrium conditions are required for evolution. In this Section 3.1, an experimental platform for far-from-equilibrium replication is introduced. In the following Sections 3.2 and 3.3, computational models are described that offer insights into the dynamics of these far-from-equilibrium replicating systems.

3.1.1 Replication and reversible redox chemistry

In Section 1.2.1 and in Figure 1.2.2 a few replicators described in the literature were introduced. A common characteristic of most of them is the irreversible nature of the replication reaction: the formation of a highly stable bond, such as in an ester, an amide, or a stable Diels-Alder product, generally results in a very stable product, i.e., an inert replicator that would not undergo any further spontaneous transformations in the course of a few years.

In order to attain dynamic kinetic stability, or DKS, the system should have a certain degree of reversibility. There are replicating systems where reversibility in the reagent pool allows for diversity in the product mixture, however in many of these cases the replication reaction can be considered irreversible for all practical purposes. For these systems it is therefore relatively difficult to reach DKS conditions. While other replicating systems show sufficient reversibility to be brought into different equilibrium or kinetically trapped states based on external stimuli in different conditions, it is important that there be independent irreversible pathways by which the replicator can be formed and destroyed. Such systems have not yet been described.

In our system, the formation of disulfide bridges in the hexamers guarantees reversibility by means of irreversible reactions: as shown in Figures 3.1.1.1 and 3.1.1.2, mild redox reagents such as DTT and perborate can alter the oxidation state of the replicating species by forming thiols from disulfides and vice versa.

![Figure 3.1.1.1 | DTT-mediated reduction of disulfides. Dithiothreitol (DTT) reduces disulfides to thiols under mild conditions, producing an inert cyclic disulfide as a byproduct.](image)
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3.1.1.2 The individual effects of reduction and oxidation

As observed, individual reduction and oxidation processes are able to decrease the concentration of the replicating hexamers. Figures 3.1.2.1 and 3.1.2.2 illustrate the outcomes of such reduction and oxidation experiments, respectively. The replicators can therefore be destroyed back to building blocks and reoxidized to smaller macrocycles such as trimers and tetramers.

3.1.2 The individual effects of reduction and oxidation

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Figure 3.1.1.2 | Perborate-mediated oxidation of thiols. Perborate oxidizes thiols to disulfides under mild conditions, producing borate as a byproduct, i.e., the same species used as the pH buffer.

Since the only species capable of forming supramolecular fibres and therefore capable of replication in our system are hexamers, destruction of the disulfide bond is expected to yield control over the aggregation state of the replicators and therefore over the replication process itself.

As the figures show, reduction and oxidation affect all species, including trimers and tetramers, i.e., non-replicating macrocycles, and hexamers, i.e., the replicators, albeit to different extents.

Figure 3.1.2.1 | Chromatograms showing the effects of batch stepwise DTT-mediated reduction of replicators: trimers, tetramers and replicating hexamers are all reduced efficiently, albeit the latter with relatively lower selectivity. The various plots correspond to different stages in the reduction process. Building block concentration: 3.8 mM. Concentration of reducing agent: 38 mM. The red curve shows the initial library composition. Blue to green curves show the library composition after stepwise reductions of 25% of the disulfide bonds per step.
Figure 3.1.2.2 | Chromatograms showing the effects of batch stepwise perborate-mediated oxidation of building blocks in the presence of replicating hexamers: the oxidation yields almost exclusively trimers and tetramers. The chromatograms correspond to different stages in the oxidation process. Building block concentration: 3.8 mM. Concentration of oxidizing agent: 38 mM. The red curve shows the initial library composition. Blue to green curves show the library composition after stepwise oxidations of 25% of the thiol functionalities per step.

In order for a replicating system to act as a far-from-equilibrium recycling system, replicators should be continuously and irreversibly destroyed back to their constituent building blocks and re-formed from these. Therefore, it is desirable for redox processes to have as a net outcome the irreversible conversion of replicators into food molecules. Note that conversion of food molecules back to replicators (i.e., replication) can occur without changing the overall oxidation state of the thiols.

3.1.3 Replicator destruction in simultaneous batch processes

By combining reduction and oxidation reactions in a simultaneous batch process where the reducing and oxidizing agents were simultaneously and rapidly added to a sample containing macrocycles of various sizes, it is experimentally observed that hexamers can be turned over into trimers and tetramers. This outcome is shown in Figure 3.1.3.1.

It is important to notice that, given that the rate of reduction is orders of magnitude higher than the rate of oxidation (see Table 3.2.1.1 in Section 3.2 for the experimentally determined rate constants), the two processes are never perfectly simultaneous. An initial fast reduction is expected to be followed by an oxidation phase.

The replicators have this way been shown to be destroyed back to their building blocks, by means of batch experiments.

Living systems display DKS. For such kind of stability to be attained with our replicators, it is necessary to bring the system into steady flow conditions.
Figure 3.1.3.1 | Chromatograms showing the effects of batch simultaneous reduction/oxidation of a library containing reduced species (monomers, dimers), non-replicating oxidized species (trimers, tetramers) and oxidized replicators (hexamers): a relative decrease in hexamers concentration and increase in trimers and tetramers concentration is observed. Building block concentration: 3.8 mM. Concentration of oxidizing and reducing agents: 38 mM. Equivalents of oxidizing and reducing agents used: 0.9 (turnover of 90% of the library). Black chromatogram: before batch reduction/oxidation processes. Blue chromatogram: afterwards. As the zoomed figure below shows, simultaneous reduction/oxidation yields net partial destruction of hexamers to trimers and tetramers, alongside other processes.

3.1.4 Replicator destruction in a flow setup

As discussed in Chapter 1, Sections 1.2.3 and 1.2.4, living systems exist in stable steady states that display a high DKS. In order to achieve DKS in an experimental system, it is therefore necessary to carry out replication in far-from-equilibrium conditions. The latter can be achieved in a flow setup such as the one illustrated in Figure 3.1.4.1, where reducing and oxidizing agents are continuously fed into the system to provide chemical energy to turn back replicators into building blocks, as illustrated in Section 3.1.3, while replication takes place.
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Figure 3.1.4.1 | Flow setup for far-from-equilibrium replication/destruction. A reducing agent such as DTT and an oxidizing agent such as perborate are flowed into the system containing food (building blocks) and replicators to reach dynamic replication/destruction far-from-equilibrium states.

Considering zeroth-order destruction mechanisms, a non-trivial stable steady state is created where replication and destruction of the replicator take place at the same time, as shown in the kinetic rates scheme in Figure 3.1.4.2 and in the steady state curves in Figure 3.1.4.3. The flat line corresponding to the destruction process represents a zeroth-order pathway which rate is independent of the fraction of replicator in the system. The bell-shaped replication rate curve is typical of an autocatalytic reaction that is first order in the amount of replicator. As the reaction rate will also depend upon the concentration of the smaller macrocycles (no replication can take place without food molecules), the reaction rate decreases to zero if the system is dominated by replicators, i.e., for high fractions of replicator. Most destruction mechanisms are however first order, hence a single steady state is expected, as shown in the kinetic rates scheme in Figure 3.1.4.4, where replicators are partially destroyed back into building blocks and a steady state containing both smaller macrocycles and replicating hexamers is expected.

Achieving such steady states in the replicator concentrations experimentally has however proven itself to be challenging. The difficulty is not in the observation of a steady ratio between replicators and smaller macrocycles itself, but in the achievement of a constant oxidation level in the library. In all experiments, the oxidation state drifted towards either full oxidation or full reduction of the library within times short enough to hinder the experimental studies aimed at the observation of a steady state.

Furthermore, overoxidation of library members takes place in such a flow setup: [M+2O+H]^+ and [M+4O+H]^+ peaks are observed in UPLC-MS chromatograms of species that dominate the product distribution after a twofold nominal turnover of the library. Overoxidation pathways make it necessary to replenish monomers into the system while performing these studies.
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Figure 3.1.4.2 | Rates diagram in a replication/destruction scenario, zeroth order destruction.

Reaction rates are plotted in arbitrary units against the fraction of replicator. Considering zeroth-order destruction processes, three steady states arise in the concentration of replicator: a repeller and two attractors. The blue areas indicate states for which destruction will prevail, thereby decreasing replicator concentration. The grey area indicates states for which replication will prevail, thereby increasing replicator concentration.

Figure 3.1.4.3 | Replicator concentration as a function of flow rates, or distance from equilibrium.

In equilibration conditions the irreversible replication reaction yields the highest possible replicator concentration. Distance from equilibrium (i.e., higher flow rates in a flow far-from-equilibrium setup) results in a dynamic system with three steady states, among which one stable, another one unstable, and a third one trivial, i.e., containing no replicator. In the two non-trivial steady states, both replication and destruction take place continuously. The blue areas indicate states for which destruction will prevail, thereby decreasing replicator concentration. The grey area indicates states for which replication will prevail, thereby increasing replicator concentration.
Finally, it is always important to stabilize the oxidizing agent perborate, in order to prevent its chemical decomposition, by decreasing its pH. Given the experimental constraints, more insights on this system were discovered by means of computational studies. These are explained and illustrated in Sections 3.2 and 3.3 of this chapter.

3.1.5 Feedback systems

Given the complexities of the chemical kinetics involved, as discussed in Section 3.1.4, a feedback system can be envisioned to maintain a constant oxidation level in the library.

As shown in Figure 3.1.5.1, UV absorbance or an electrochemical potential could be used as a measure of the redox state of the library and fed back into a computer system that could regulate the inflow of reducing and oxidizing agents in order to keep the chemical library at a constant redox state.

Such systems would require the use of an external feedback mechanism to maintain steady state in the oxidation level. Eventually, such feedback mechanisms would have to be integrated in the chemical system for DKS to be displayed and for a system to be classified as autonomous and life-like.
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Figure 3.1.5.1 | Far-from-equilibrium replication system with feedback mechanism. An analytic signal such as UV absorbance or an electrochemical potential can be used to measure the degree of oxidation in the library, which in turn can be used to dynamically adjust flow rates of reducing and oxidizing agents in order to maintain the library partially oxidized while in a far-from-equilibrium state, thereby avoiding oxidation state drifts.

3.1.6 The effects of concentration

In order to gain insights into the complex kinetics of this system, concentration experiments were performed. Libraries were prepared at different monomer concentrations and the emergence of replicators was observed. As shown in Figure 3.1.6.1, higher concentration libraries counterintuitively showed later emergence of hexamers.

The observation can be justified by considering that the rate at which oxygen is supplied to the system is limited by the surface area of the part of the solution exposed to air and by the fact that the vials in which experiments are carried out are capped.

This evidence is compared with computational results in Section 3.2.3.

3.1.7 Conclusions – Experimental far-from-equilibrium replication

An experimental far-from-equilibrium replication platform has been developed based on our known replicator system. We showed how it is possible to revert a replication reaction by only using chemical energy, i.e., without the need for inflow of building blocks and by only using external redox reagents. Maintaining the system in a steady state has proven challenging, due to drifting oxidations states towards either complete reduction or complete oxidation.
Figure 3.1.6.1 | \( t_{50} \), defined as the time (days) for replicator concentration to reach 50% of the total concentration, as a function of total library concentration (mM). Experiments carried out for concentrations of 11.4 and 38 mM showed that the replicator concentration remained under 10% after 30 days. The corresponding \( t_{50} \) values were not measured and are therefore not included in this figure.

In order to further address the complexity of this experimental system, computational models were built and the results thereof studied in detail. Specifically, these models were aimed at the identification of the experimental conditions in which coupling of the reduction and oxidation reactions in the experimental systems would be the most efficient. These models are described and discussed in Sections 3.2 and 3.3 of this chapter.

3.1.8 Experimental methods

Library preparation and monitoring. Dynamic combinatorial libraries were prepared by dissolving building block 1, obtained from Cambridge Peptides, in a 50 mM pH 8.1 potassium borate buffer to a final concentration of 3.8 mM. The pH of the resulting solution was adjusted to 8.1-8.2 by addition of small amounts of a 2.0 M KOH solution. All libraries were contained in HPLC vials (12 × 32 mm) tightly closed with Teflon-lined snap caps. The libraries were stirred using a Teflon coated magnetic stirrer bar (5 × 2 mm, obtained from VWR), on an IKA RCT basic stirrer hotplate at 1200 rpm unless otherwise specified. Library compositions were monitored by quenching 2.0 µL samples of the library in 98 µL of a solution of doubly distilled H₂O containing 0.6% TFA, in a glass UPLC vial, and injecting 5.0 µL of this sample on the UPLC. For samples that were monitored over time it was confirmed that the total peak area in the UPLC chromatograms remained constant.

UPLC-MS analysis. UPLC analyses were performed on a Waters Acquity UPLC I-class system equipped with a PDA detector. All analyses were performed using a reversed-phase UPLC column (Aeris Widepore 3.6 µm XB-C18 150 × 2.10 mm, purchased from Phenomenex). UV absorbance was monitored at 254 nm. Column
temperature was kept at 35 °C. UPLC-MS was performed using a Waters Acquity UPLC H-class system coupled to a Waters Xevo-G2 TOF. The mass spectrometer was operated in the positive electrospray ionization mode. Injection volume was 5 µL of a 3.8 mM library subjected to a 1:50 dilution in a solution of 0.6 v% of trifluoroacetic acid in doubly distilled water. Eluent flow was 0.3 mL/min; eluent A: UPLC grade water (0.1 v% trifluoroacetic acid); eluent B: UPLC grade acetonitrile (0.1 v% trifluoroacetic acid).

<table>
<thead>
<tr>
<th>time (min)</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>90.0</td>
<td>10.0</td>
</tr>
<tr>
<td>1.0</td>
<td>90.0</td>
<td>10.0</td>
</tr>
<tr>
<td>1.3</td>
<td>75.0</td>
<td>25.0</td>
</tr>
<tr>
<td>3.0</td>
<td>72.0</td>
<td>28.0</td>
</tr>
<tr>
<td>11.0</td>
<td>89.0</td>
<td>11.0</td>
</tr>
<tr>
<td>11.5</td>
<td>5.0</td>
<td>95.0</td>
</tr>
<tr>
<td>12.0</td>
<td>5.0</td>
<td>95.0</td>
</tr>
<tr>
<td>12.5</td>
<td>90.0</td>
<td>10.0</td>
</tr>
<tr>
<td>15.0</td>
<td>90.0</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Table 3.1.8.1 | UPLC method. Eluent gradient used for UPLC analysis of libraries formed from building block 1 where eluent A: UPLC grade water (0.1 v% trifluoroacetic acid); eluent B: UPLC grade acetonitrile (0.1 v% trifluoroacetic acid).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
<th>m/z calculated</th>
<th>m/z observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)_2</td>
<td>6.2</td>
<td>1517.7 [M+H]+, 759.4 [M+2H]+, 506.6 [M+3H]+, 380.2 [M+5H]+</td>
<td>1517.5 [M+H]+, 759.6 [M+2H]+, 506.8 [M+3H]+, 380.5 [M+5H]+</td>
</tr>
<tr>
<td>(1)_3</td>
<td>8.7</td>
<td>1137.50 [(M+1)+2H]+, 759.01 [(M+2)+3H]+, 569.25 [(M+1)+4H]+, 455.61 [(M+1)+5H]+</td>
<td>1137.48 [(M+1)+2H]+, 758.98 [(M+2)+3H]+, 569.23 [(M+1)+4H]+, 455.58 [(M+1)+5H]+</td>
</tr>
<tr>
<td>(1)_4</td>
<td>6.8</td>
<td>1516.67 [(M+2)+2H]+, 1011.45 [(M+2)+3H]+, 758.59 [(M+1)+4H]+</td>
<td>1516.64 [(M+2)+2H]+, 1011.42 [(M+2)+3H]+, 758.56 [(M+1)+4H]+</td>
</tr>
<tr>
<td>(1)_6</td>
<td>8.2</td>
<td>1516.67 [(M+3)+3H]+, 1137.75 [(M+3)+4H]+, 910.80 [(M+5)+5H]+, 759.00 [(M+4)+6H]</td>
<td>1516.62 [(M+3)+3H]+, 1137.72 [(M+3)+4H]+, 910.77 [(M+5)+5H]+, 758.99 [(M+4)+6H]</td>
</tr>
</tbody>
</table>

Table 3.1.8.2 | UPLC-MS compound identification.
Flow experiments. A library was prepared by dissolving 3.8 mM $\mathbf{1}$ in 50 mM borate buffer at pH 8.2. The library was then oxidized up to 50% using a freshly prepared solution of sodium perborate (38 mM, pH 8.0). This solution was mixed in a 1:1 ratio with a pre-formed library rich in $\mathbf{1}$ hexamers (building block concentration 3.8 mM in 50 mM borate buffer at pH 8.2, continuously stirred at 1200 rpm). The composition of the mixture was at this point: 23% monomer $\mathbf{1}$, 3% linear dimer ($\mathbf{1}$)$_2$, 12% cyclic trimer ($\mathbf{1}$)$_3$, 12% cyclic tetramer ($\mathbf{1}$)$_4$, 50% cyclic hexamer ($\mathbf{1}$)$_6$. The resulting solution was split into samples of 500 µL and reducing and oxidizing agents DTT (38 mM) and perborate (38 mM) were flowed into the sample on a continuous basis while the library was stirred at 1200 rpm. Samples of the library were taken every 15 minutes and analyzed by UPLC.

3.2. DETERMINISTIC MODELLING – EFFICIENT REPLICATOR RECYCLING CONDITIONS

As an aid to experimental studies, computational models were built. The number of processes involved in such experimental systems is large enough that the qualitative behaviour of the entire process becomes complex and difficult to predict.

Figure 3.2.1 shows schematically the model that was built to understand the behaviour of our replicators in far-from-equilibrium conditions. This model was verified under thermodynamic equilibration conditions and studied extensively under far-from-equilibrium conditions. The source code for the model is provided in Appendix A, Section A.2.

An important process in our model is destruction, which is achieved by means of simultaneously supplying a reducing agent and an oxidizing agent to the system, in a flow setup. These conditions are equivalent to the experimental setup, under the approximation of highly concentrated redox agents, i.e., when dilution effects from supplying redox agents are ignored.

3.2.1 Thermodynamic equilibration conditions

The model was calibrated based on experimentally determined reaction orders and constants. The experimental work was performed in collaboration with Shuo Yang (S.Y.), Gaël Schaeffer (G.S.) and Andreas Hussain (A.H.). S.Y. and G.S. designed the experiments to measure oxidation, reduction and exchange rate constants and relative orders. S.Y. synthesized the compounds used for the experiments and performed the measurements. S.Y. and G.S. analyzed the kinetic data. A.H. measured the replication rate constant and relative orders in trimers and tetramers. G.S. estimated the order of the quenching reaction.
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Figure 3.2.1 | Far-from-equilibrium replication model, as a template for numerical simulations.
The role of destruction processes, i.e., reducing replicating hexamer fibres to monomers and dimers and reoxidizing the latter to non-assembled trimers and tetramers, is shown as a large backwards arrow and is at the core of the studies in Section 3.2.

An overview of the orders and constants used for the purpose is provided in Table 3.2.1.1.
Flows of DTT and perborate of $8 \cdot 10^{-6}$ M min$^{-1}$ correspond to a nominal turnover of 3.8 mM in 8 hours. Experimentally, flows can be varied with relative ease and the studies in the following sections are based on altering this parameter in a range between $1 \cdot 10^{-10}$ M min$^{-1}$ (nominal turnover 73 years) and $5 \cdot 10^{-5}$ M min$^{-1}$ (nominal turnover 1.3 hours) to determine the behaviour of the library in various regimes.
Air oxidation was not studied in depth. For the purposes of modelling, this process was considered to take place under a steady state of oxygen concentration from air and regarded as a zeroth-order process in molecular oxygen, with an oxidation constant of $1 \cdot 10^{-3}$ min$^{-1}$ that fits the experimentally observed timescales of oxidation.
The order of replication in the replicator was determined experimentally to be 1 (Chapter 2). The experimentally determined replication constant could not be used directly in the model, as explicit nucleation, elongation and breakage processes are modelled, instead. An indirectly derived replication constant based on first order kinetics in the replicator and second order kinetics in trimers and tetramers could be determined numerically. The indirectly obtained replication constant of $110$ M$^{-2}$ min$^{-1}$ is in the same order of magnitude of the experimentally measured one ($35.6$ M$^{-2}$ min$^{-1}$) and therefore in good agreement with the experiments.
The kinetic constants used to model the replication process are in Table 3.2.1.2, which lists all kinetic constants used in the model, including their notation, used in the rest of the chapter and in the code. These symbols are also used in the parameters panels in Appendix A, Section A.2.5, to which the figure captions refer.
Furthermore, a breakage probability profile was used, with zero probability for short fibres and linearly increasing probability for fibres above a length threshold, up to a maximum value (kBm in Table 3.2.1.2). Longer fibres are therefore more likely to break than shorter ones.11

<table>
<thead>
<tr>
<th>Process</th>
<th>Relative orders</th>
<th>Rate constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduction by DTT</td>
<td>1 (DTT) + 1 (disulfide)</td>
<td>1.9 ( \times ) 10^5 M^{-1} s^{-1}</td>
</tr>
<tr>
<td>Oxidation by perborate</td>
<td>1 (perborate) + 1 (thiol)</td>
<td>1.15 M^{-1} s^{-1}</td>
</tr>
<tr>
<td>DTT-perborate quenching</td>
<td>1 (DTT) + 1 (perborate)</td>
<td>15 M^{-1} s^{-1}</td>
</tr>
<tr>
<td>Replication</td>
<td>1 (6mers) + 2 (3mers,4mers)</td>
<td>0.593 M^{-2} s^{-1}</td>
</tr>
<tr>
<td>Exchange (thiol-disulfide)</td>
<td>1 + 1</td>
<td>7.95 ( \times ) 10^3 M^{-1} s^{-1}</td>
</tr>
<tr>
<td>Flow of DTT and perborate</td>
<td>0</td>
<td>8 ( \times ) 10^6 M min^{-1}</td>
</tr>
</tbody>
</table>

Table 3.2.1.1 | Experimentally determined rate constants for the far-from-equilibrium kinetic model. Values per second are converted in values per minute in order to fit the units in the model.

<table>
<thead>
<tr>
<th>Process</th>
<th>Notation</th>
<th>Relative orders</th>
<th>Rate constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidation by oxygen</td>
<td>kO2</td>
<td>0 (O_2) + 1 (thiol)</td>
<td>1.0 ( \times ) 10^{-3} min^{-1}</td>
</tr>
<tr>
<td>Reduction by DTT</td>
<td>kred</td>
<td>1 (DTT) + 1 (disulfide)</td>
<td>1.1 ( \times ) 10^{7} M^{-1} min^{-1}</td>
</tr>
<tr>
<td>Oxidation by perborate</td>
<td>kox</td>
<td>1 (perborate) + 1 (thiol)</td>
<td>69 M^{-1} min^{-1}</td>
</tr>
<tr>
<td>DTT-perborate quenching</td>
<td>kquench</td>
<td>1 (DTT) + 1 (perborate)</td>
<td>900 M^{-1} min^{-1}</td>
</tr>
<tr>
<td>Nucleation</td>
<td>kN</td>
<td>2 (3mers,4mers)</td>
<td>1 ( \times ) 10^{-7} M^{-1} min^{-1}</td>
</tr>
<tr>
<td>Catalyzed elongation</td>
<td>kCE</td>
<td>2 (3mers,4mers) + 1 (fibre ends)</td>
<td>4 ( \times ) 10^{3} M^{-2} min^{-1}</td>
</tr>
<tr>
<td>Breakage</td>
<td>kBm</td>
<td>1 (6mers)</td>
<td>1 ( \times ) 10^{2} min^{-1}</td>
</tr>
<tr>
<td>Exchange (thiol-disulfide)</td>
<td>kX</td>
<td>1 + 1</td>
<td>4.77 ( \times ) 10^{5} M^{-1} min^{-1}</td>
</tr>
<tr>
<td>Flow of DTT and perborate</td>
<td>kflow</td>
<td>0</td>
<td>8 ( \times ) 10^{6} M min^{-1}</td>
</tr>
</tbody>
</table>

Table 3.2.1.2 | Rate constants used in the model, including their notation. Nucleation, elongation and breakage pathways are explicitly modelled.

An initial equilibration phase lasting ca. two weeks (2 \( \times \) 10^4 minutes) shows similar results as observed experimentally, as illustrated in Figure 3.2.1.1. The latter figure and the following ones contain two panels: on the left, library species are plotted...
against time (reduced species in red, trimers and tetramers in blue and hexamers in black), while on the right, reducing and oxidizing agent concentrations (red and dark blue, respectively, in order to represent the colours of their corresponding main products in the panel on the left) are plotted against time. All concentrations in both panels are relative to total building block concentration. In the captions, the configuration panels used to produce the corresponding dynamics are referred to. The configuration panels are provided in Appendix A, Section A.2.5.

Figure 3.2.1.2 shows the outcome of a longer equilibration phase, prolonged to 6 weeks, without including any far-from-equilibrium effects: full replication is observed.

Figure 3.2.1.1 | Replication kinetics simulation in thermodynamic conditions. At a building block concentration of 3.8 mM, hexamers dominate the product mixture. All concentrations are relative to total monomer concentration. Monomers, dimers: red. Trimers, tetramers: blue. Replicating hexamers: black. Appendix A.2.5, Parameters panel E1.

Figure 3.2.1.2 | Replication kinetics simulation in thermodynamic conditions. After 6 weeks, the model shows full replication of the starting material. Building block concentration: 3.8 mM. All concentrations are relative to total monomer concentration. Monomers, dimers: red. Trimers, tetramers: blue. Replicating hexamers: black. Appendix A.2.5, Parameters panel E2.
3.2.2 Far-from-equilibrium replicator recycling conditions

In order to study replication in far-from-equilibrium conditions by means of the computational model, it is important to bring the system in glove box conditions: after two weeks, when redox flow is introduced (indicated in the plots by a vertical dotted line), oxidation by air oxygen is also prevented from taking place. This decouples the destruction redox pathways from oxygen mediated oxidation and allows for a better understanding of the underlying processes. Destruction conditions are achieved by providing equimolar flows of a reducing agent such as dithiothreitol and an oxidizing agent such as perborate. The studies in this section assume that reduction is selective towards the destruction of hexamers and oxidation is selective towards the production of trimers and tetramers. Section 3.2.3 covers the general case and presents an extensive analysis of the effects of selectivity on the system.

Flows of $1 \cdot 10^{-7}$ M min$^{-1}$ guarantee dynamic destruction and replication, as shown in Figure 3.2.2.1. Relative replicator concentration at the steady state is about 85%. Monomers and dimers are present at the steady state, alongside low amounts of trimers and tetramers. The concentration of the oxidizing agent at steady state is higher than that of the reducing agent, due to oxidation being slower (lower rate constant) than reduction. The reducing agent is already active at lower concentrations.

![Figure 3.2.2.1](image)

Experimentally and in the models, it is possible to add a batch of oxidizing agent at the start of the flow experiment. Equal flows of reducing and oxidizing agents...
can be applied subsequently. As shown in Figure 3.2.2.2, if the final concentration of the oxidizing agent from the previous experiment is taken as the initial batch concentration of oxidizing agent at the start of the flow, then the steady state replicator concentration at this flow is slightly higher, i.e., ca. 86%. The concentration of the oxidizing agent increases towards the steady state, to a higher steady state value compared to the previous experiment. Note that while the change in steady state replicator concentration might be minimal, using an initial batch concentration of oxidizing agent can be helpful in the experiments in order to avoid drifting of the system to a completely reduced state.

At very low flows of $1 \cdot 10^{-9}$ M min$^{-1}$, the oxidizing agent increases in concentration, while a steady state is not yet reached (Figure 3.2.2.3). A quench vs. recycling relative flux analysis shows that at low flows, quenching of the reducing agent with the oxidizing agent is 6-7 orders of magnitude slower than redox processes with library members. The redox agents flowed in the system are too diluted to quench each other and mostly react with the library members.

Increasing flows to $5 \cdot 10^{-7}$ M min$^{-1}$ induces a steady state with lower hexamer content (ca. 60% in relative terms), as shown in Figure 3.2.2.4. Redox fluxes are 4 orders of magnitude higher than the quenching flux, indicating that the destroying agents are still coupled to the library.

![Figure 3.2.2.2 | Replication kinetics simulation in far-from-equilibrium conditions. Flow of $1 \cdot 10^{-9}$ M min$^{-1}$. Added initial oxidizing agent at the start of the flow. The dashed line represents the start of the flow of redox agents. Building block concentration: 3.8 mM. All concentrations are relative to total monomer concentration. Monomers, dimers: red. Trimers, tetramers: blue. Replicating hexamers: black. In the panel on the right, red represents reducing agent and blue oxidizing agent. Appendix A.2.5, Parameters panel F2.](image)

As shown in Figure 3.2.2.2, the final concentration of the oxidizing agent from the previous experiment can be taken as the initial batch oxidizing agent concentration. The steady state hexamer concentration will therefore be higher.
Figure 3.2.2.3 | Replication kinetics simulation in far-from-equilibrium conditions. Flow of $1 \cdot 10^{-9}$ M min$^{-1}$. Negligible destruction is shown at the steady state: the replicator dominates the system. The dashed line represents the start of the flow of redox agents. Building block concentration: 3.8 mM. All concentrations are relative to total monomer concentration. Monomers, dimers: red. Trimers, tetramers: blue. Replicating hexamers: black. In the panel on the right, red represents reducing agent and blue oxidizing agent. Appendix A.2.5, Parameters panel F3.

Figure 3.2.2.4 | Replication kinetics simulation in far-from-equilibrium conditions. Flow of $5 \cdot 10^{-7}$ M min$^{-1}$. At the steady state, a higher portion of the library is composed by trimers and tetramers, compared to the results in Figure 3.2.2.1. The dashed line represents the start of the flow of redox agents. Building block concentration: 3.8 mM. All concentrations are relative to total monomer concentration. Monomers, dimers: red. Trimers, tetramers: blue. Replicating hexamers: black. In the panel on the right, red represents reducing agent and blue oxidizing agent. Appendix A.2.5, Parameters panel F4.
Figure 3.2.2.5 | Replication kinetics simulation in far-from-equilibrium conditions, recursive alteration of the initial concentration of the oxidizing agent. Flow of $5 \cdot 10^{-7}$ M min$^{-1}$. Steady state values from the previous simulation are recursively used for the next iteration. The initial oxidizing agent concentrations for the two experiments above are (in the relative units of initial monomer concentration): 0.0250 (simulation above, corresponding to the steady state value of the simulation in Figure 3.2.2.4), 0.0405 (simulation below, corresponding to the steady state value in the simulation above), corresponding to absolute concentrations of 95.2 µM and 154 µM, respectively. The dashed line represents the start of the flow of redox agents. Building block concentration: 3.8 mM. All concentrations are relative to total monomer concentration. Monomers, dimers: red. Trimers, tetramers: blue. Replicating hexamers: black. In the panel on the right, red represents reducing agent and blue oxidizing agent. Appendix A.2.5, Parameters panel F4, adjusted cox parameter.

The initial oxidizing agent concentration is therefore a useful experimental parameter that can be used to tune steady states. A relative concentration of hexamers between 20% and 80% is an ideal steady state condition as it allows for constant recycling of replicators and building blocks. Increasing the initial oxidizing agent concentration recursively based on the previous values, in order to
find a steady state value, can therefore be used as an aid to experiments. **Figures 3.2.2.5 and 3.2.2.6** show an example of such a study, starting from the previously analyzed conditions.

The initial oxidizing agent concentration removes the need and the additional time for the oxidizing agent to first accumulate, in order for oxidation to take place at a steady rate.

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**Figure 3.2.2.6** | Replication kinetics simulation in far-from-equilibrium conditions, recursive alteration of the initial concentration of the oxidizing agent. Flow of $5 \times 10^{-7}$ M min$^{-1}$. Above: an initial relative initial concentration of oxidizing agent of 10 induces a steady state value of 10.0001. Below: an initial relative concentration of 100 induces a steady state value of 100. The dashed line represents the start of the flow of redox agents. Building block concentration: 3.8 mM. All concentrations are relative to total monomer concentration. Monomers, dimers: red. Trimers, tetramers: blue. Replicating hexamers: black. In the panel on the right, red represents reducing agent and blue oxidizing agent. Appendix A.2.5, Parameters panel F4, adjusted cox parameter.

The difference in hexamer concentration at the steady state compared to the start of the flow is due to the reduction capability of the initial reducing agent, up to
when the oxidizing agent reaches a sufficient concentration to start oxidizing efficiently. A low initial concentration of monomers available for oxidation is another underlying cause of the observed lag between reduction and oxidation rates. Overoxidation pathways can play a role in the initial phases of such far-from-equilibrium replication experiments.

Another important parameter that can be used to tune experimental conditions is the time at which flow is started (indicated as a vertical dotted line in the left plot in the figures within this Section 3.2). This can be combined with the initial oxidizing agent concentration. Hexamers are not dominant at the start of the far-from-equilibrium experiment if the flow is started after ca. 6 days (9000 minutes) instead of after 2 week. As shown in Figure 3.2.2.7, replication proceeds (given its high rate at the start of the flow) and hexamers achieve a relative steady state concentration of 85% while replicating and being destroyed simultaneously, comparable to the results in Figure 3.2.2.1. A slightly lower oxidation state is observed.

These experiments show how initial conditions such as time of start of the flow and initial oxidizing agent concentration can be used alongside the flow rate as important parameters to control the steady state of the experimental far-from-equilibrium replicating system.

Upon increasing the flow further to $5 \cdot 10^{-6}$ M min$^{-1}$, destruction prevails and replicators are in the order of $1 \cdot 10^{-6}$ M at the steady state (Figure 3.2.2.8).
Figure 3.2.2.8 | Replication kinetics simulation in far-from-equilibrium conditions. A high redox agents flow of $5 \cdot 10^{-6} \text{ M min}^{-1}$ induces almost complete destruction of the replicator at the steady state. The dashed line represents the start of the flow of redox agents. Building block concentration: 3.8 mM. All concentrations are relative to total monomer concentration. Monomers, dimers: red. Trimers, tetramers: blue. Replicating hexamers: black. In the panel on the right, red represents reducing agent and blue oxidizing agent. Appendix A.2.5, Parameters panel F6.

Figure 3.2.2.9 | Replication kinetics simulation in far-from-equilibrium, overoxidizing conditions, without the effects of overoxidation. The flow of the oxidizing agent ($5.5 \cdot 10^{-7} \text{ M min}^{-1}$) is set to be higher than the flow of the reducing agent ($5.0 \cdot 10^{-7} \text{ M min}^{-1}$). The dashed line represents the start of the flow of redox agents. Building block concentration: 3.8 mM. All concentrations are relative to total monomer concentration. Monomers, dimers: red. Trimers, tetramers: blue. Replicating hexamers: black. In the panel on the right, red represents reducing agent and blue oxidizing agent. Appendix A.2.5, Parameters panel F7.

If the flow of the oxidizing agent is set to be higher compared to the flow of the reducing agent, an initial destruction phase is followed by reoxidation of monomers/dimers to a trimer/tetramer/hexamer system (Figure 3.2.2.9).
Experimentally, however, overoxidation products are also expected in such conditions. These therefore do not qualify as a viable method to attain DKS replication conditions.

Based on the studies in this section, a few considerations can be made, which are relevant for the experimental studies (using the kinetic parameters in Table 3.2.1.2):

- Up to a flow rate (kflow) of $1 \cdot 10^{-9} \text{ M min}^{-1}$, quenching processes are not relevant and redox flow is coupled to the library. Most of the library consists of replicators. Replication does take place at the steady state: its rate also depends upon the concentration of smaller macrocycles.
- Flows between $1 \cdot 10^{-9} \text{ M min}^{-1}$ and $1 \cdot 10^{-7} \text{ M min}^{-1}$ induce optimal redox recycling.
- For higher flows, the library contains almost no hexamers that could catalyze replication: replicator destruction prevails.
- High initial oxidizing agent concentrations (cox) require higher flow rates for redox recycling to yield similar steady state hexamer concentrations, due to the sample not being reduced by initial excess reducing agent.
- Low flow start times (fstart) could expand the range of flow rates where redox recycling takes place, due to locking the library into a partially oxidized dynamic state.
- Disulfide exchange processes are often dominant, but do not necessarily compete with the replication/destruction cycle, as disulfide exchange mostly affects the non-assembled non-replicating macrocycles.

### 3.2.3 Flux analysis and selectivity studies

The studies presented in Section 3.2.2 show that, under conditions where the reducing agent destroys hexamers and the oxidizing agent produces trimers and tetramers, higher redox agents flows induce replicator destruction, with trimers and tetramers as the prevailing species at the steady state. While other factors such as initial oxidizing agent concentration (compare Figures 3.2.2.1 and 3.2.2.2) and flow start time (compare Figures 3.2.2.1 and 3.2.2.7) could be used to tune the evolution to the steady state and, to a minor extent, the steady state composition itself, the flow rate of supplying the redox agents was identified as the major factor that affects steady state composition.

In this Section 3.2.3, the study will be extended to include redox agents selectivities, represented by two additional parameters, x and y, defined as the relative selectivities towards hexamers of the reducing and oxidizing agents, respectively. It is important to note that x applies to the reactivity difference between hexamers exposed at the fibre ends (hexamers locked inside fibres are not reduced) and trimers and tetramers in solution. y applies to the relative selectivity in oxidation towards hexamers at the fibre ends (hexamers in solution are assumed to rapidly equilibrate to trimers and tetramers) and trimers and tetramers in solution. The studies in the previous section therefore correspond to x=1, y=0, i.e., under the
assumption that reduction is selective towards the destruction of hexamers and
oxidation is selective towards the production of trimers and tetraters.

In order to aid these studies, detailed flux analyses are described for each one of
the analyzed conditions. At the steady state, the reducing and oxidizing agents
flowed in the system react according to various reaction pathways. The reaction
fluxes are the reaction rates for a specific chemical species on a given reaction
pathway. A sample flux analysis with fictitious data is presented in Figure 3.2.3.1.

Reduction, oxidation and replication fluxes are broken down into various pathways.
In the example in the figure, 30% of both reducing and oxidizing agents react with
each other in the quenching pathway. About 42% of the flowed reducing agent goes
into reducing trimers and tetraters into monomers, while the remaining 28%
reduces hexamers into monomers. On the other hand, about 45% of the oxidizing
agent goes into producing trimers and tetraters, while the remaining 25% yields
hexamers. As a net outcome of reduction and oxidation, hexamers are converted
into trimers and tetraters at 3% of the rate of inflow of the reducing or the oxidizing
agent, calculated as 45%-42% based on trimers and tetraters turnover or as 28%-25%
based on hexamers turnover. At the steady state, this rate is matched by the rate of
replication, which converts trimers and tetraters into hexamers, as shown in the
lowest bar in Figure 3.2.3.1.

On top of gaining an understanding of the underlying pathways in the system at
its steady state, another parameter that can be derived from such analyses is the
real turnover time compared to the nominal turnover time. Based on the example
in Figure 3.2.3.1, given that only 3% of the redox flow actively destroys replicators
back into trimers and tetraters, the real turnover time is 33 times higher than the
nominal turnover time. For a nominal turnover time of 11 hours, the real turnover
time would be of 15 days.

![Figure 3.2.3.1 Reduction, oxidation, and replication flux analysis.](image)

As mentioned above, the simulations presented up to this point assume that the
destruction operated by the redox agents be highly selective: reduction
preferentially depletes hexamers transforming them into monomers, whereas oxidation yields trimers and tetramers from the latter, via dimers. Though useful to understand a possible limit behaviour of this replicating system, the experimental results indicate that the real system is more complex: Figures 3.1.2.1 and 3.1.2.2 indicate that both reduction and oxidation (the latter to a smaller extent) affect both larger replicating macrocycles, i.e., hexamers, and smaller non-replicating ones, i.e., trimers and tetramers, instead. The combined effect, shown in Figure 3.1.3.1, is the turnover of hexamers towards smaller macrocycles, however studying selectivity effects allows us to make conclusions on the kinetics of the whole system and on the final outcome that is to be expected of a far-from-equilibrium regime based upon it. Namely, if reduction is not perfectly selective towards hexamers and oxidation is not perfectly selective towards the formation of trimers and tetramers, full destruction of the replicators at the steady state cannot be expected. Further studies were carried out in order to quantify these selectivity effects.

Let us consider a \((x,y)\) coordinate system where \(x\) and \(y\) are the relative selectivities towards hexamers of the reduction and oxidation reactions, respectively. \(x=0\) indicates that reduction preferentially takes place with trimers and tetramers, while \(x=1\) indicates that reduction is selective towards hexamers. Similarly, \(y=0\) indicates that oxidation yields trimers and tetramers preferentially, while at \(y=1\) oxidation uniquely produces hexamers. Note that \(x\) and \(y\) are relative measures and, as the reaction rates depend upon kinetic constants and the concentrations of the reactants, \(x=0.5\) or \(y=0.5\) do not necessarily indicate that 50\% of the reactants/products are trimers and tetramers and 50\% are hexamers.

Figure 3.2.3.2 | Replication kinetics simulation in far-from-equilibrium conditions. Redox agents flows: \(5 \cdot 10^{-6} \text{ M min}^{-1}\). Hexamer selectivities: \(x=1\), \(y=0\) (reduction only affects hexamers, oxidation only produces trimers and tetramers). Building block concentration: 3.8 mM. All concentrations are relative to total monomer concentration. Monomers, dimers: red. Trimers, tetramers: blue. Replicating hexamers: black. In the panel on the right, red represents reducing agent and blue oxidizing agent. Appendix A.2.5, Parameters panel S1.
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The previous simulations in Section 3.2.2 were carried out by considering a fairly strong destruction pressure for hexamers, i.e., $x=1$ and $y=0$. For reference, Figure 3.2.3.2 shows the typical outcome of such conditions, i.e., full destruction of the replicators at steady state.

In the following, unless mentioned otherwise (Figures 3.2.3.18 and 3.2.3.19), these selectivity studies were carried out with relatively high redox agents flows of $5 \cdot 10^{-6}$ M min$^{-1}$, using a monomer concentration of 3.8 mM at the start of the experiment. Figure 3.2.3.3 shows the flux analysis for the steady state in Figure 3.2.3.2. As the steady state contains a negligible amount of replicator, 99.99999% of the redox flux results in mutual quenching. The remaining 0.00001% of the redox flux induces transformation back into trimers and tetramers of the small amounts of replicators that nucleate and begin to elongate.

**Figure 3.2.3.3** | Flux analysis for the steady state in Figure 3.2.3.2.

**Figure 3.2.3.4** | Replication kinetics simulation in far-from-equilibrium conditions. Redox agents flows: $5 \cdot 10^{-6}$ M min$^{-1}$. Hexamer selectivities: $x=1$, $y=1$ (reduction only affects hexamers, oxidation only produces hexamers). Building block concentration: 3.8 mM. All concentrations are relative to total monomer concentration. Monomers, dimers: red. Trimers, tetramers: blue. Replicating hexamers: black. In the panel on the right, red represents reducing agent and blue oxidizing agent. Appendix A.2.5, Parameters panel S2.
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Figure 3.2.3.5 | Flux analysis for the steady state in Figure 3.2.3.4.

Figure 3.2.3.6 | Replication kinetics simulation in far-from-equilibrium conditions. Redox agents flows: $5 \cdot 10^{-6}$ M min$^{-1}$. Hexamer selectivities: $x=0$, $y=1$ (reduction only affects trimers and tetramers, oxidation only produces hexamers). Building block concentration: 3.8 mM. All concentrations are relative to total monomer concentration. Monomers, dimers: red. Trimers, tetramers: blue. Replicating hexamers: black. In the panel on the right, red represents reducing agent and blue oxidizing agent. Appendix A.2.5, Parameters panel S3.

Figure 3.2.3.7 | Flux analysis for the steady state in Figure 3.2.3.6.
Figure 3.2.3.8 | Replication kinetics simulation in far-from-equilibrium conditions. Redox agents flows: $5 \cdot 10^{-6}$ M min$^{-1}$. Hexamer selectivities: $x=0$, $y=0$ (reduction only affects trimers and tetramers, oxidation only produces trimers and tetramers). Building block concentration: 3.8 mM. All concentrations are relative to total monomer concentration. Monomers, dimers: red. Trimers, tetramers: blue. Replicating hexamers: black. In the panel on the right, red represents reducing agent and blue oxidizing agent. Appendix A.2.5, Parameters panel S4.

The next figures show the outcome of the other extreme situations. If both $x$ and $y$ are 1 (Figures 3.2.3.4 and 3.2.3.5), hexamers are rapidly turned over to monomers and back into hexamers via the rate-limiting dimer formation pathway. Any remaining trimers and tetramers are fully converted into replicators. The steady state contains monomers and dimers as well as hexamers and the entirety of the redox flux acts upon turning over hexamers, with minimal quenching taking place. If $x=0$ and $y=1$ (Figures 3.2.3.6 and 3.2.3.7), the redox processes aid replication. Any remaining building blocks are quickly turned over to replicating hexamers. If both $x$ and $y$ are 0 (Figures 3.2.3.8 and 3.2.3.9), trimers and tetramers are quickly turned over to monomers and back to trimers and tetramers via the slow dimer formation reaction. Production of dimers then becomes rate-limiting, preventing any further
replication, which needs trimers and tetramers as the food molecules. Quenching plays a significant role in the flux analysis.

Useful selectivities were found to be $x=0.05$ and $y=0.35$, as shown in Figure 3.2.3.10. In these conditions, destruction is still prevailing, as shown by the fact that redox conditions decrease the concentration of the hexamers. The steady state still mostly contains replicating hexamers. The flux analysis in Figure 3.2.3.11 indicates a real turnover time of about 20 times the nominal turnover time.

### Figure 3.2.3.10
Replication kinetics simulation in far-from-equilibrium conditions. Redox agents flows: $5 \cdot 10^{-6}$ M min$^{-1}$. Hexamer selectivities: $x=0.05$, $y=0.35$ (reduction very selective towards trimers and tetramers, oxidation selective towards trimers and tetramers). Building block concentration: 3.8 mM. All concentrations are relative to total monomer concentration. Monomers, dimers: red. Trimers, tetramers: blue. Replicating hexamers: black. In the panel on the right, red represents reducing agent and blue oxidizing agent. Appendix A.2.5, Parameters panel S5.

### Figure 3.2.3.11
Flux analysis for the steady state in Figure 3.2.3.10.

### Figures 3.2.3.12 (flux analysis in Figure 3.2.3.13) and 3.2.3.14 (flux analysis in Figure 3.2.3.15) indicate the outcome of higher selectivity towards the reduction of hexamers. These conditions approach the ideal ones, with a fast destruction/replication turnover and a real turnover time of about 6 times the
nominal turnover time. The steady state distribution contains approximately 50% hexamer.

In order to attain ideal conditions, it is important to also tune the model based on the available constraints regarding the expected rate constants. The two following statements are valid approximations to include in the model:

1. The rate constant of reducing a hexamer at a fibre end is 50% (because only one half is accessible) of the rate constant for reducing any of the non-assembled macrocycles. Even though accessing a fibre end is not chemically equivalent to accessing a macrocycle in solution, it is a valid first approximation.

2. The oxidation rate constants match the ratio with which replicating hexamers get produced relative to trimers and tetramers in an experiment where
perborate is added to monomer. While this ratio varies depending on the amount of hexamer already in the system, a valid first approximation based on the experimental observations is that hexamers are produced at 2% of the rate of production of trimers and tetramers.

Figure 3.2.3.14 | Replication kinetics simulation in far-from-equilibrium conditions. Redox agents flows: $5 \times 10^{-6} \text{ M min}^{-1}$. Hexamer selectivities: $x=0.7$, $y=0.1$ (reduction mildly selective towards trimers and tetramers, oxidation very selective towards trimers and tetramers). Building block concentration: 3.8 mM. All concentrations are relative to total monomer concentration. Monomers, dimers: red. Trimers, tetramers: blue. Replicating hexamers: black. In the panel on the right, red represents reducing agent and blue oxidizing agent. Appendix A.2.5, Parameters panel S7.

Figure 3.2.3.15 | Flux analysis for the steady state in Figure 3.2.3.14.

The statements above are taken up in the simulation in Figure 3.2.3.16 (flux analysis in Figure 3.2.3.17). Statement 1 results in an $x$ value of 0.33, while statement 2 results in a $y$ value of 0.02. In these conditions, quenching represents 0.007% of the total redox flow and the nominal and real turnover times are 13 hours and 7 days, respectively. Replicators represent about 65% of the total library at the steady state.
In order to obtain a faster turnover of the replicator, it is possible to increase the flow from \(5 \times 10^{-6} \text{ M min}^{-1}\) to \(5 \times 10^{-5} \text{ M min}^{-1}\). The resulting conditions still include selectivity values that reflect the rate constant constraints and are illustrated in Figure 3.2.3.18 (flux analysis in Figure 3.2.3.19). While replication represents a lower relative fraction of the redox flux, a higher inflow results in a lower real turnover time: in these conditions, the nominal and real turnover times are 1.3 hours and 2.5 days, respectively. Quenching represents 0.01% of the redox inflow and the steady state relative replicator concentration is higher than 20%.

It is important to notice that the conditions above represent realistic conditions, but due to practical issues a stable steady state was not obtained experimentally so far.

Figure 3.2.3.16 | Replication kinetics simulation in far-from-equilibrium conditions. Redox agents flows: \(5 \times 10^{-6} \text{ M min}^{-1}\). Hexamer selectivities: \(x=0.33, y=0.02\) (realistic conditions: reduction selective towards trimers and tetramers, oxidation extremely selective towards trimers and tetramers). Building block concentration: 3.8 mM. All concentrations are relative to total monomer concentration. Monomers, dimers: red. Trimers, tetramers: blue. Replicating hexamers: black. In the panel on the right, red represents reducing agent and blue oxidizing agent. Appendix A.2.5, Parameters panel S8.

Figure 3.2.3.17 | Flux analysis for the steady state in Figure 3.2.3.16.
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Figure 3.2.3.18 | Replication kinetics simulation in far-from-equilibrium conditions. Redox agents flows increased to $5 \cdot 10^{-5} \text{ M min}^{-1}$. Hexamer selectivities: $x=0.33$, $y=0.02$ (realistic conditions: reduction selective towards trimers and tetramers, oxidation extremely selective towards trimers and tetramers). Building block concentration: 3.8 mM. All concentrations are relative to total monomer concentration. Monomers, dimers: red. Trimers, tetramers: blue. Replicating hexamers: black. In the panel on the right, red represents reducing agent and blue oxidizing agent. Appendix A.2.5, Parameters panel S9.

Figure 3.2.3.19 | Flux analysis for the steady state in Figure 3.2.3.18.

Based on the analyses above, redox flow ($k_{\text{flow}}$), reduction selectivity towards hexamers ($x$) and oxidation selectivity towards hexamers ($y$) are three important parameters that have a profound influence on the steady state composition in our far-from-equilibrium replicating system.

It is useful to extend the flow and selectivity studies presented above by analyzing systematically the effects of the parameters $k_{\text{flow}}$, $x$ and $y$, in order to draw general conclusions.

In the following, the **steady state values** of the following parameters were analyzed:

- **Relative hexamer concentration**: compared to total building block concentration.
— **Real turnover times**: the time needed to destroy an equal amount of replicators as the initial monomer concentration, based on actual destruction/replication fluxes.

— **Nominal turnover times**: the time needed to destroy an equal amount of replicators as the initial monomer concentration, based on nominal redox agents flow rates.

Furthermore, the *relative steady state values* of the following fluxes were analyzed:

— **Quenching flux**: the rate of quenching compared to the flow rate.

— **Trimers/tetramers recycling flux**: the rate of reduction and oxidation of trimers and tetramers compared to the flow rate.

— **Hexamers recycling flux**: the rate of reduction and oxidation of hexamers compared to the flow rate.

— **Destruction/replication flux**: the rate of replication compared to the flow rate, also equal, at the steady state, to the rate of destruction of hexamers into trimers and tetramers due to redox processes.

The decomposition in fluxes described above is illustrated in Figure 3.2.3.20, which is based on the fictitious example in Figure 3.2.3.1. The parentheses contain the figure number where the corresponding flux is analyzed against the parameters \( k_{\text{flow}}, x \) and \( y \).

The plots in Figure 3.2.2.21 illustrate the steady state hexamer concentration against the three aforementioned parameters, \( k_{\text{flow}} \) (in logarithmic scale), \( x \), and \( y \). In every subplot in the following figures, isosurfaces are displayed in blue, green, yellow, red, and black to indicate different values of the plotted measures. The thresholds for the isosurfaces vary in the following figures. For Figure 3.2.2.21 they are 10, 20%, 50%, 80%, and 90%, respectively, in terms of relative replicator concentrations. For Figures 3.2.2.22-25 they are 10%, 20%, 50%, 80%, and 90%, respectively, in terms of relative fluxes.

For Figure 3.2.2.21, the semispace delimited by the blue isosurface and not containing any of the other isosurfaces indicates \( k_{\text{flow}}, x \), and \( y \) parameters for
which the steady state relative replicator concentration is lower than 10%, whereas the semispace delimited by the black isosurface and not containing any other isosurface indicates parameters for which a given flux is higher than 90%. All intermediate steady state relative replicator concentrations are contained in the spaces between the various isosurfaces.

The kflow axis is the bottom-left one. In the remaining figures in this section, the same measures are plotted twice for the sake of clarity: on the left plot, the flow rates increase from the bottom to the left, while on the right plot the flow rates increase from the left to the bottom. The axes corresponding to the x and y parameters are not affected.

As shown in Figure 3.2.2.21, hexamer concentration at the steady state is high for flows below $10^{-7}$ M min$^{-1}$ and can decrease for higher flows. Specifically, for selectivity values $x>y$ destruction prevails at the steady state. At $x=1$, $y=0$ and for high flows ($10^{-5}$ M min$^{-1}$), the replicator is almost completely depleted. This is in agreement with the simulations described above.

Figure 3.2.2.22 shows that quenching at the steady state mainly occurs in the two following cases. In both of them, one of the recycling pathways is induced to have negligible fluxes:

1. For $x$ values close to 0, $y$ values above 0.1, and flows above $10^{-8}$ M min$^{-1}$: destruction and redox recycling cannot affect trimers and tetramers but help hexamer formation instead, leading to very high hexamer concentrations (especially if $y>0.1$). Replication then becomes extremely slow due to lack of trimers and tetramers. The redox agents therefore react with each other at the steady state.

---

**Figure 3.2.2.21 | 3D flow/selectivity studies.** Steady state relative hexamer concentration analyzed against parameters $\log(k_{flow})$ (the base 10 logarithm of the flow rate, or flow rate constant), $x$ (the selectivity of the reducing agent towards the reduction of hexamers) and $y$ (the selectivity of the reducing agent towards the reduction of hexamers). Code used to generate the images in Section 3.2.5. Thresholds: 10%, 20%, 50%, 80%, 90% (blue, green, yellow, red, black isosurfaces, respectively).
2. For extreme values of \( x \) and \( y \), close to \( x=1, y=0 \), and high flows (above \( 5 \times 10^{-7} \text{ M min}^{-1} \)), when the redox agents induce complete destruction of the replicating hexamers. As the reduction of hexamers cannot take place, the redox agents flowed in the system also react with each other at the steady state.

**Figure 3.2.2.22 | 3D flow/selectivity studies.** Steady state relative quench flux analyzed against parameters \( \log(k_{\text{flow}}) \) (the base 10 logarithm of the flow rate, or flow rate constant), \( x \) (the selectivity of the reducing agent towards the reduction of hexamers) and \( y \) (the selectivity of the reducing agent towards the reduction of hexamers). Code used to generate the images in Section 3.2.5. Thresholds: 10%, 20%, 50%, 80%, 90% (blue, green, yellow, red, black isosurfaces, respectively).

**Figure 3.2.2.23 | 3D flow/selectivity studies.** Steady state relative trimers/tetramers recycling flux analyzed against parameters \( \log(k_{\text{flow}}) \) (the base 10 logarithm of the flow rate, or flow rate constant), \( x \) (the selectivity of the reducing agent towards the reduction of hexamers) and \( y \) (the selectivity of the reducing agent towards the reduction of hexamers). Code used to generate the images in Section 3.2.5. Thresholds: 10%, 20%, 50%, 80%, 90% (blue, green, yellow, red, black isosurfaces, respectively).
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Figure 3.2.2.24 | 3D flow/selectivity studies. Steady state relative hexamers recycling flux analyzed against parameters log(kflow) (the base 10 logarithm of the flow rate, or flow rate constant), x (the selectivity of the reducing agent towards the reduction of hexamers) and y (the selectivity of the reducing agent towards the reduction of hexamers). Code used to generate the images in Section 3.2.5. Thresholds: 10%, 20%, 50%, 80%, 90% (blue, green, yellow, red, black isosurfaces, respectively).

Figures 3.2.2.23 and 3.2.2.24 illustrate trimers/tetramers recycling flux and hexamers recycling flux at the steady state, respectively. Trimers and tetramers are strongly recycled at higher flows for very low y values, when oxidation produces enough of these two species for the reducing agent to consume. An exception must be made for conditions where quenching prevails. Hexamer recycling prevails for high y values, where oxidation produces enough of them to be reduced. The main exception is for x=0, where the reducing agent is not selective towards hexamers.

Figure 3.2.2.25 illustrates the parameter space for which redox recycling takes place. Low flows always couple the redox agents to the library, inducing high relative turnover compared to the flow, i.e., most of the flow induces destruction and formation of replicators. For higher flows, two trends can be observed:

1. For selectivity values close to x=1, y between 0 and 0.5, most of the flow is employed to destroy the replicators. While an interesting outcome for DKS replication/destruction conditions, the steady state only contains minimal amounts of replicators, which is undesired.

2. For x values above 0.3 and intermediate y values (between the blue and the yellow isosurfaces), redox destruction/replication, while not constituting the majority of the redox flux, is still relatively important. At the same time, steady state replicator concentration is between 40% and 80%, which is ideal in order to observe an evolving population of replicators. Optimizing the experimental conditions should be performed in this parameter region if possible. In the simulations in Figures 3.2.3.16 and 3.2.3.18, x=0.33 and y=0.02 were identified.
as selectivity values in agreement with reaction rate considerations; higher flows (5 \cdot 10^{-5} \text{ M min}^{-1}) were also deemed ideal for experimental DKS studies, with a real turnover time of 2.5 days.

**Figure 3.2.2.25 | 3D flow/selectivity studies.** Steady state relative destruction/replication flux analyzed against parameters \( \log(k_{\text{flow}}) \) (the base 10 logarithm of the flow rate, or flow rate constant), \( x \) (the selectivity of the reducing agent towards the reduction of hexamers) and \( y \) (the selectivity of the reducing agent towards the reduction of hexamers). Code used to generate the images in Section 3.2.5. Thresholds: 10\%, 20\%, 50\%, 80\%, 90\% (blue, green, yellow, red, black isosurfaces, respectively).

**Figures 3.2.2.26 and 3.2.2.27** show a comparison between real and nominal turnover times in the parameter space under study. The yellow and red isosurfaces indicate shorter turnover times (2 and 10 days, respectively), hence more interesting conditions for our studies in the laboratory. The blue and green surfaces correspond to 1000 and 100 days in the nominal turnover time plot, respectively, but to \( 10^{10} \) and \( 10^{20} \) days in the real turnover time plot, respectively, indicating that real turnover times might be orders of magnitude higher than the nominal ones, due to very inefficient redox recycling. The most favourable conditions are obtained at higher flows and within the inner red isosurfaces, located in the same region as the one described for destruction/replication fluxes (**Figure 3.2.2.25**). For these parameters, destruction/replication cycles are relatively efficient and replicators are concentrated enough in the system to allow for recycling to take place.
3.2.4 Conclusions and perspectives – Far-from-equilibrium replication

In the previous section, a few considerations were made regarding the optimal conditions for DKS replication/destruction, based on the flux analyses and on the
3D selectivity/flow studies. These were found to be the following: redox flow of $5 \cdot 10^{-5}$ M min$^{-1}$, $x=0.33$ and $y=0.02$. These conditions yield a real turnover time of about 2.5 days, negligible quenching (0.01% of the total redox flow) and a steady state relative replicator concentration of above 20%.

Considering the effect of selectivity in the redox processes that carry out the destruction of the replicators, further experimental studies can be based upon these results and could focus on additional experimental techniques to show recycling in the system at the steady state. We propose here four possibilities:

— Destruction kinetics could be studied with fibres of different average lengths at the steady state, i.e., prepared at different stirring rates: the destruction reaction could be faster with shorter fibres due to the increased amount of fibre ends, thereby implying a higher selectivity towards the hexamers. At steady state, a lower relative concentration of hexamers is expected, indicating the activity of recycling reactions in the system. The average fibre length can be also tuned in the computational model by varying the elongation and breakage constants. In the simulations carried out for this thesis, typical average fibre lengths are of 15-40 hexamers, depending on the conditions. Experimentally, fibre lengths of about 100 nm (produced at 1200 rpm) correspond to about 200 hexamers (considering preliminary hexamer-hexamer distances of about 0.48 nm, measured from MD simulation results by Pim Frederix). While the qualitative results of the simulations are not expected to vary with different fibre lengths, changing the latter could be a useful handle on the experimental system to verify expected behaviours.

— UPLC-MS studies could be performed to monitor the turnover of replicating macrocycles during the steady state. By using isotopically labelled building blocks, it would be possible to compare the exchange of building blocks with the macrocycles in the fibres, compared to a reaction where stirring, but no redox process, is applied.

— Methods to alter the selectivities could be devised, in order for the experiments to be possibly conducted in various regimes, as needed. Immediate candidates are alternative reducing and oxidizing agents, as well as alternative replicators bearing different peptide sequences, which, by forming macrocycles of different sizes and fibres of different strength, will be differently susceptible of being reduced or of being formed as an oxidation product.

— A vast range of reducing and oxidizing agents and of peptide building blocks could be explored. Characteristics specific to the individual chemicals might allow the observation of interesting steady state behaviour, i.e., efficient recycling and simultaneous coexistence of reduced and oxidized library members and of non-replicating and replicating species.

An important remark is that, while the studies in Section 3.2.3 presented the experimental conditions including the flow rates that should be used based on ideal turnover rates and realistic considerations on kinetic parameters and selectivities,
it was difficult to observe efficient redox recycling experimentally. The cause thereof might be one of the following:

- Selectivities might depend on replicator (or fibre end) concentration, with a nonlinear relationship. As Figure 3.1.2.1 and Figure 3.1.2.2 show, the selectivity of the reduction reaction towards hexamers is higher when trimers and tetramers are lower in concentration, while oxidation seems to produce preferentially trimers and tetramers even when these are relatively high in concentration. The exact details of this relationship might provide further insights in the behaviour of the experimental system. Further studies could focus on determining the exact selectivities at work in the experimental system and their relationship with the concentration of the species (both assembled and in solution). Such studies would be useful to further clarify the actual fluxes involved in any conditions under examination.

- Based on the observations above regarding selectivities, it would be worthwhile to explore, both in the experiments and in the simulations, studies in which reduction and oxidation are not applied simultaneously, but in out-of-phase waves, so that the library oxidation state oscillates steadily within a predefined range. Since reduction of hexamers is more effective once most of the trimers and tetramers have been already reduced, it would be interesting to reduce most of the library to induce a significant destruction of the hexamers, before reoxidizing it to the initial oxidation level, thereby restoring trimers and tetramers at the expense of the replicators. These studies would provide valuable insights into the systems dynamics and on how to induce efficient replication and destruction cycles in order to attain evolution from simple chemical replicators.

- Often, the system was observed to drift in oxidation state towards a fully reduced or fully oxidized system, before a steady state could be observed for long enough. A method to control the flows of the redox agents in order to avoid drifts in the oxidation states would help in the observation of the steady states.

- Overoxidation products were often observed in the experimental studies. A way of reducing the flux on this pathway would greatly help to stabilize the steady states.

3.2.5 Concentration experiments

In order to gain insights in the results of the concentration experiments shown in Figure 3.1.6.1, simulations were performed of the library in thermodynamic equilibration conditions for the first week. The results are not similar to what is observed experimentally: the simulations indicate that higher concentrations induce faster replication, contrary to what was observed in the experiments. The experimental observations can only be explained by oxygen supply from air being the rate limiting step at higher concentrations.
In Figures 3.2.5.1-3, library concentrations of 0.38 mM, 3.8 mM (the concentration used in previous simulations), and 38 mM, respectively, were used for the simulations.

Figure 3.2.5.1 | Replication kinetics simulation in thermodynamic conditions, concentration experiments. Building block concentration: 0.38 mM. All concentrations are relative to total monomer concentration. Monomers, dimers: red. Trimers, tetramers: blue. Replicating hexamers: black. In the panel on the right, red represents reducing agent and blue oxidizing agent. Appendix A.2.5, Parameters panel C1.

Figure 3.2.5.2 | Replication kinetics simulation in thermodynamic conditions, concentration experiments. Building block concentration: 0.38 mM. All concentrations are relative to total monomer concentration. Monomers, dimers: red. Trimers, tetramers: blue. Replicating hexamers: black. In the panel on the right, red represents reducing agent and blue oxidizing agent. Appendix A.2.5, Parameters panel C2.
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Figure 3.2.5.3 | Replication kinetics simulation in thermodynamic conditions, concentration experiments. Building block concentration: 38 mM. All concentrations are relative to total monomer concentration. Monomers, dimers: red. Trimers, tetramers: blue. Replicating hexamers: black. In the panel on the right, red represents reducing agent and blue oxidizing agent. Appendix A.2.5, Parameters panel C3.

The experimental observations in Section 3.1 indicate that higher concentration samples show slower replication. The observation could be justified by considering that the oxidation step is rate-limiting at higher concentration due to the limited surface area of the sample, through which molecular oxygen can enter the system.

Figure 3.2.5.4 | Replication kinetics simulation in thermodynamic conditions, concentration experiments. Building block concentration: 3.8 mM. The oxidation process is rate-limiting due to the slow inflow of oxidizing agent from the air. Appendix A.2.5, Parameters panel C2. Parameter lim_ox set to 10^6 instead of 10^8 in function gfibres (Appendix A.2.2). All concentrations are relative to total monomer concentration. Monomers, dimers: red. Trimers, tetramers: blue. Replicating hexamers: black. In the panel on the right, red represents reducing agent and blue oxidizing agent.
Figures 3.2.5.4 and 3.2.5.5 show the results of such simulations, which confirm that rate-limiting oxidation can be responsible for the observations. It was not possible, however, to obtain faster replication at the lowest concentrations by means of the current model, likely due to oxygen supply already being fast enough for the intermediate concentrations.

![Figure 3.2.5.5](image)

Figure 3.2.5.5 | Replication kinetics simulation in thermodynamic conditions, concentration experiments. Building block concentration: 38 mM. The oxidation process is rate-limiting due to the slow inflow of oxidizing agent from the air. Appendix A.2.5, Parameters panel C3. Parameter \( \text{lim}_{\text{ox}} \) set to \( 10^{-6} \) instead of \( 10^6 \) in function \( \text{gfibres} \) (Appendix A.2.2). All concentrations are relative to total monomer concentration. Monomers, dimers: red. Trimers, tetramers: blue. Replicating hexamers: black. In the panel on the right, red represents reducing agent and blue oxidizing agent.

### 3.3. STOCHASTIC MODELLING – A PLATFORM FOR MULTI-BUILDING-BLOCK SYSTEMS

In addition to deterministic models, we developed a stochastic computational platform to study our peptide replicators in conditions where multiple building blocks are present in the system. Such setups allow the exploration of evolutionary scenarios. The work for this Section 3.3 of Chapter 3 was realized in collaboration with Tenzin Kunsel.

The main reason to use stochastic models for multi-building-block systems is twofold:

1. **Combinatorial explosion of variables and equations.** The number of possible products with multiple building blocks increases dramatically with the number of the latter. This combinatorial explosion is not treatable with deterministic models, as the number of variables and ODEs would be too high and difficult to compute with modern tools. Stochastic models only consider a finite population, so that probabilities of reactions of possible species that are not
part of the current population are not calculated explicitly, making the systems lighter to simulate.

2. **Insights into chemical instability and nonlinear systems.** Deterministic models, due to their intrinsic formulation, do not allow for the observation of phenomena that can only be initiated by statistical fluctuations in the concentration of the species, such as symmetry breaking and phase transitions in general. Such phenomena are precisely what we are looking for in a far-from-equilibrium replicating system, namely transitions from a state with lower DKS to another one with higher DKS, initiated by a random fluctuation in the concentrations that is amplified thereafter towards the new state.

In **Table 3.3.1** a general comparison is performed between deterministic and stochastic models.

<table>
<thead>
<tr>
<th></th>
<th><strong>DETERMINISTIC MODELS</strong></th>
<th><strong>STOCHASTIC MODELS</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Consider concentrations,</strong> e.g., [1], [3], [4], [6soln], [62], [63], [64], ...</td>
<td><strong>Consider populations:</strong> e.g., 3, 4, 1, 6soln, 1, 4, 62, 6soln, 64, 1, 62, 4, 1, 3, 3, 6b, 6soln, ...</td>
<td></td>
</tr>
<tr>
<td><strong>Express the evolution in time of concentrations with kinetic equations</strong> and <strong>kinetic constants:</strong></td>
<td><strong>Express the evolution in time of populations with probabilities of reaction events:</strong></td>
<td></td>
</tr>
<tr>
<td>$- \frac{d[3]}{dt} = -k_{\alpha}[1][2] + ...$</td>
<td>$P_\mu = c_\mu h_\mu$</td>
<td></td>
</tr>
<tr>
<td><strong>Time plots are obtained by numerical integration of the corresponding system of ODEs</strong></td>
<td><strong>Time plots are obtained by generating a series of random reaction collisions and times based on probability distributions</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Useful and easier for systems with one building block, without combinatorial explosions of the number of species and equations</strong></td>
<td><strong>The only feasible method for systems with many building blocks (e.g., XGLKFK + XGLKSK), both for mixed fibres of homogenous hexamers and for mixed fibres of mixed hexamers</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Easy to write, but impractical to simulate (simulation times of years or thousands of years) for systems with many building blocks (e.g., XGLKFK + XGLKSK)</strong></td>
<td><strong>Require advanced statistics and programming for bookkeeping in the more complex scenarios</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Limit or exclude nonlinear effects</strong></td>
<td><strong>Show the interesting phenomena, initiated by fluctuations and correlations in the concentrations:</strong> <strong>chemical instabilities, nonlinear systems, symmetry breaking and evolution become accessible</strong></td>
<td></td>
</tr>
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</table>

**Table 3.3.1** | Comparison between deterministic and stochastic computational modelling of a chemical system.
A deterministic kinetic equation for reaction $\mu$ reads as follows:

$$\frac{d[X]}{dt} = k_{\mu}[X_1]^{x_1}[X_2]^{x_2}$$

Where $k_{\mu}$ is the reaction constant and $x_1$ and $x_2$ are the reaction orders in species $X_1$ and $X_2$, respectively.

In a stochastic model, the probability of reaction is computed as follows:

$$P_{\mu} = c_{\mu}h_{\mu}$$

Where $c_{\mu}$ is the relative probability that a given combination of reactants for reaction $\mu$ will collide and react and $h_{\mu}$ is the number of possible reactant combination found in the population at the current time for reaction $\mu$.

$$c_{\mu} = \frac{k_{\mu}\prod(x_i!)}{\sqrt{\left(\sum x_i\right)^{N_A}}}$$

$$h_{\mu} = \prod\left(\frac{N_A}{x_i}\right)$$

Where $n_{X_1}$ and $n_{X_2}$ are the current populations of species $X_1$ and $X_2$, respectively.

Our algorithm is the direct implementation of Gillespie’s original method, which takes into consideration the formulas above.\textsuperscript{12} In this implementation, two random numbers are generated by means of a uniform random number generator:

1. The first one selects which reaction occurs following the reaction probability distribution $P_{\mu}$ for every reaction $\mu$.
2. The second one is used to calculate the next time when that particular reaction should occur:

$$\tau = \frac{1}{P} \ln \frac{1}{r}$$

Where $r$ is a random number and:

$$P = \sum P_{\mu}$$

The population is subsequently adjusted accordingly, the time is advanced (output is generated when the time runs past given milestones) and the reaction probabilities are recalculated based on the new distribution of species.

3.3.1 Implementation

We implemented a simplified model variant involving just three species: trimers, tetramers and hexamers. The corresponding reaction map is shown below in.
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Figure 3.3.1.1 and involves exchange reactions, irreversible replication reactions and irreversible destruction reactions.

Figure 3.3.1.1 | Kinetic reaction network of the main implemented stochastic model. Double arrowheads indicate exchange reactions. In the model, the same constants are used for forward and backward reactions on a given reaction pathway (e.g., $k_{\text{exchange}}$ is used for both exchange pathways between trimers and hexamers, i.e., from trimers to hexamers and from hexamers to trimers). Exchange, replication, and destruction reaction constants are used to infer reaction probabilities for the stochastic model.

The source code for the implemented model is to be found in Appendix A, Section A.3.

A full stochastic model including fibrous species requires the creation of a hypergraph of species and reactions and advanced bookkeeping, in addition to appropriate algorithms to speed up reaction selection. Such an algorithm is not in the scope of this thesis and is being developed further for the purpose.

3.3.2 Results and discussion

A steady state was obtained as the result of all simulations. Studies were performed in order to verify the behaviour of the model with varying initial population and kinetics constants of exchange, replication and destruction. The results matched qualitatively the behaviour observed in the deterministic models.

Varying the initial population in a system with the same amounts of trimers, tetramers, and hexamers led to qualitatively similar steady state distributions, as shown in Figure 3.3.2.1. Fluctuations are less significant when larger samples are studied in a stochastic model.

Varying the exchange constant $k_{\text{exchange}}$ led to a replication regime for low exchange constant values and a full equilibration of hexamers with trimers and tetramers for high exchange constant values, as shown in Figure 3.3.2.2.

Varying the replication constant led to a replication regime for high replication constants and a full equilibration of hexamers with trimers and tetramers for low replication constants, as shown in Figure 3.3.2.3.
Figure 3.3.2.1 | Distribution of species and attainment of steady state with increasing population. From left to right, initial population increases from 1000, through 10000, to 100000. While the qualitative behaviour is unvaried, higher populations display relatively smaller fluctuations in the individual macrocycle populations.

Figure 3.3.2.2 | Final steady state population by varying the exchange constant. A replication regime is observed for low exchange constants, where trimers and tetramers are preferentially converted into hexamers. For higher exchange constants, full equilibration of hexamers with trimers and tetramers prevails.

Figure 3.3.2.3 | Final steady state population by varying the replication constant. A replication regime is observed for high replication constants, where trimers and tetramers are preferentially converted into hexamers. For lower replication constants, full equilibration of hexamers with trimers and tetramers prevails.
Varying the destruction constant led to a system with three phases, a replication regime with low destruction constants, a replication/destruction regime for intermediate values and a destruction regime for high destruction constants, as shown in Figure 3.3.2.4. The intermediate regime is precisely the most important one for further studies in the area, as a mixture of hexamers and trimers and tetramers is observed at steady state, indicating continuous replication and simultaneous destruction of the replicators.

The behaviours shown in Figure 3.3.2.4 match the results obtained from the deterministic model described in Section 3.2, i.e., higher destruction constants imply a lower steady state concentration of hexamers. The reason for the different populations of trimers and tetramers at high destruction constants is due to the different kinetics of destruction of hexamers towards these two species, compared to their distribution at equilibrium, dominating for very high destruction constants.

3.3.3 Conclusions – Stochastic modelling

Our simple stochastic model matches the qualitative behaviour of the deterministic models.

The stochastic models will play a steadily larger role in understanding the nonlinear behaviour of our replicating systems, especially when far-from-equilibrium conditions and multiple building blocks are taken into consideration. As explained in the introduction, stochastic models are relevant in multiple building blocks scenarios due to the combinatorial explosion of variables and equations with increasing diversity of the library and allow for nonlinear behaviours and chemical instabilities that are expected to have played a major role in the chemical evolution of life.
3.4 REFERENCES


