Structure-Function Relationships in Dynamic Combinatorial Libraries

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DOI:
10.33612/diss.90038152

Chapter 5

Towards Replicator Diversification Under Far-from-Equilibrium Conditions

5.1 Introduction

Defining life is probably one of the most intriguing and difficult challenges in the scientific world.[1] Another great challenge is the creation of simple living systems in the laboratory.[2–4] A subject, among many others, which has strong connections with life's definition, is the formation of new species from existing ones. Different speciation mechanisms in evolutionary biology have one common characteristic: isolation of a group of individuals that adapt to their new environment and gradually evolve into distinct species. There has been remarkably little effort on achieving speciation in systems of self-replicators. Peripherally related theoretical work are perhaps the hypercycles of cross-catalytic replicators proposed by Eigen.[5,6] In a 'hypercycle', a set of self-replicating macromolecules are linked in a cross-catalytic manner, with or without enzymatic translation (Figure 5.1).[7] Each macromolecule catalyzes the replication of a co-existing one until the last one catalyzes the replication of the very first molecule.

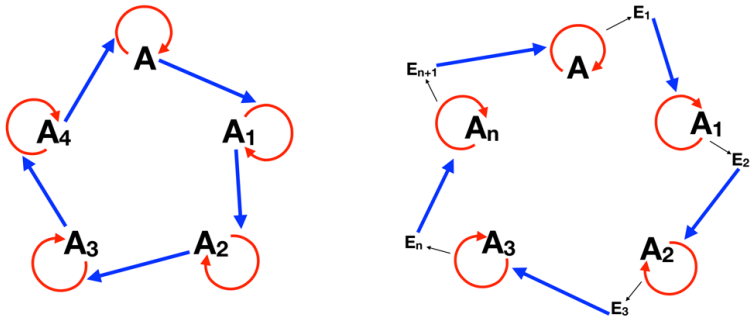

Figure 5.1: Typical hypercycles without translation (left) and with translation (right) functionality induced by enzymes.

There are benefits to such organizations like increased local concentration of the molecules for increased synthesis rate and diversified genetic information in the same local environment. Most importantly hypercycles were proposed as a solution to the Eigen paradox, which states that for a genome to evolve longer than the information threshold, it would need a more complex replication machinery and that would only be encoded in a longer genome.[8] However, after the hypercycle was proposed potential problems have also been identified. These problems can be summarized as: cross-catalysis is an altruistic behavior since it is a competing process with self-reproduction. Thus, hypercycles are vulnerable to invasion by parasites when there

is no compartment to protect them and to stochastic events that could kill one of the species.[9]

In experimental work, Sadownik *et al.* showed that self-replicating molecules could be diversified into distinct sets, where a second was the descendant of a first one.[10] This example shows that fundamental evolutionary processes can occur in abiotic laboratory environments. A shortcoming of this work is that it was performed in a system that was approaching equilibrium, while evolution and many of the biochemical processes act in a dissipative manner and which and rely on continuous inflow of energy.

Aim of the work described in this chapter was to design a system of cross-catalytic replicators acting in a far-from-equilibrium manner, in which the composition of nutrients was gradually changed. Unlike the hypercycle model of self-replication, we aimed to produce a cross-catalytic pathway in which, after a certain number of steps, newly formed species cannot cross-catalyze formation of their predecessors.

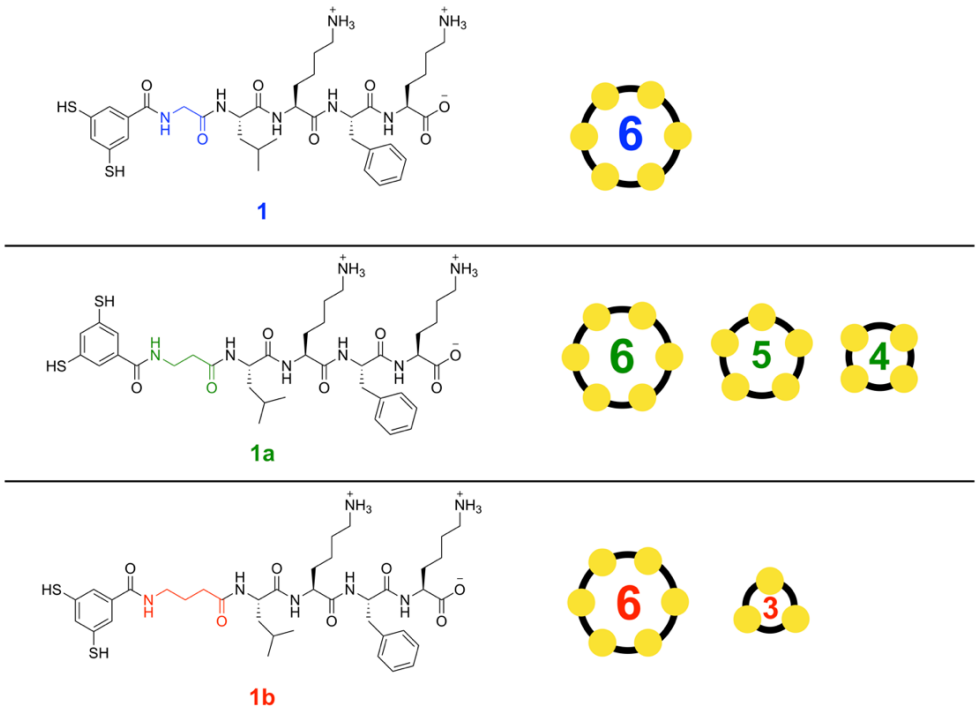

Figure 5.2: Chemical structures of the selected building blocks (left) and the sizes of accessible self-assembling macrocycles (right) in DCLs made from individual building blocks.

5.2 Results and Discussion

Several factors were considered while designing a suitable system. First of all, the overall hydrophobicity of the building blocks is important to be able to monitor the distribution of the species using UPLC. We aimed to maintain good chromatographic separation while having relatively minimal differences in the building block structures. Therefore, we used building block **1** which bears a phenyl alanine in the short peptide sequence and building blocks **1a** and **1b** as its 'mutants'. As was discussed in Chapter 3, differently sized macrocycles were accessible depending on the environment. To achieve best chromatographic separation, we first utilized building blocks **1** and **1b** and **1a** at a later stage of the experiments.

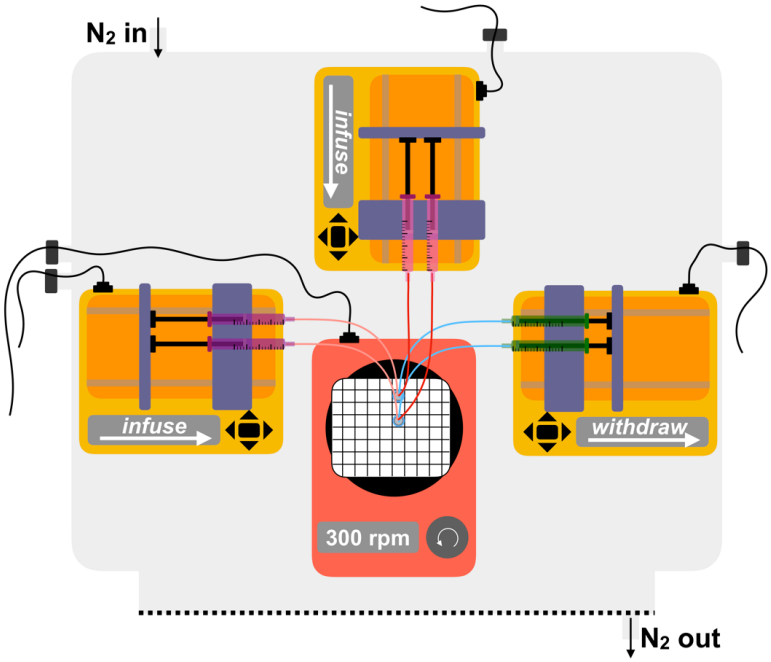

Figure 5.3: Cartoon representation of the experimental flow set-up viewed from the top with two infusion and one withdrawal syringe pumps.

5.2.1 Flow set-up

The evolvability of self-replicating entities is very much dependent on how they adapt to changes in their environment. We designed flow set-ups in a way that the composition of the nutrients required for replication was gradually modified. In order

to achieve that, we used three separate syringe pumps: two pumps for infusion and one for withdrawal (Figure 5.3). While one of the infusion pumps starts with a flow rate of 9.0 µL/h and goes down to 1.0 µL/h over a certain period of time, the second infusion pump starts at 1.0 µL/h and increases to 9.0 µL/h in the same time period. In order to keep the total building block content constant, the withdrawal pump removes material at a constant flow rate (10 µL/h). Each experiment was performed with two vials: one containing the replicator and the other with only buffer solution. Therefore, we were able to see if the replicators are capable of emerging under flow conditions in the absence of any replicator at the beginning. The infusion syringes were prepared in the glove box with previously oxidized solutions that were prepared from different building blocks in borate buffer and oxidized with sodium perborate in an oxygen free environment. Overall, the whole set up was always kept oxygen free in a bench-top glove bag under constant nitrogen flow as it is important to prevent undesired oxidation and uncontrolled emergence of replicators in the infusion syringes. Lastly, in order to allow the system to replicate continuously, we agitated the solutions on a stirring plate at 300 rpm.

5.2.2 Flow experiments utilizing two building blocks

With the aim of creating new replicator species, we followed a step-wise approach and started our experiments by using two building blocks as nutrients of which were infused into a solution containing a single type of replicator. We tested the system by starting the experiment with a solution containing a mixture of 6-ring species (Table 5.1).

Table 5.1: Summary of the flow experiments with conditions tested in each set-up including building blocks **1** and **1b**. Only in set-up V, **1a** was used as the third building block.

Set-up	[C]	**Total flow rate**	**Replicator composition at t=0 h**	**Sample volume**	**Δt/ step**	**Total flow time**	**Number of turn overs**
I	0.5 mM	10 µL/h	$\mathbf{1}_6$	750 µL	10 h	200 h	2
II, III	0.5 mM	10 µL/h	$\mathbf{1}_6$	750 µL	20 h	180 h	2
IV	0.5 mM	10 µL/h	$\mathbf{1}_n\mathbf{1b}_{6\text{-}n}$	750 µL	20 h	100 h	2
V	0.5 mM	10 µL/h	$\mathbf{1}_n\mathbf{1b}_{6\text{-}n}$	750 µL	20 h	280 h	3

Due to the ease of their chromatographic separation on a UPLC system, we first used oxidized solutions made from building blocks **1** and **1b** in the infusion syringes as nutrients (Figure 5.4). We prepared DCLs made from building blocks **1** and **1b** (0.5 mM each) in borate buffer (50 mM, pH 8.2) and oxidized with 80 mM sodium

perborate solution. While building block **1** was oxidized ⩾90%, the oxidation level of **1b** was ∼70% with respect to the monomer. In Chapter 3 of this thesis, we observed that oxidation of building block **1b** takes place much quicker than that of building block **1**. Therefore, when preparing the infusion material, due to possible risks of uncontrolled oxidation by air in the infusion syringes, we oxidized the solution of building block **1b** less than the solution made from building block **1**.

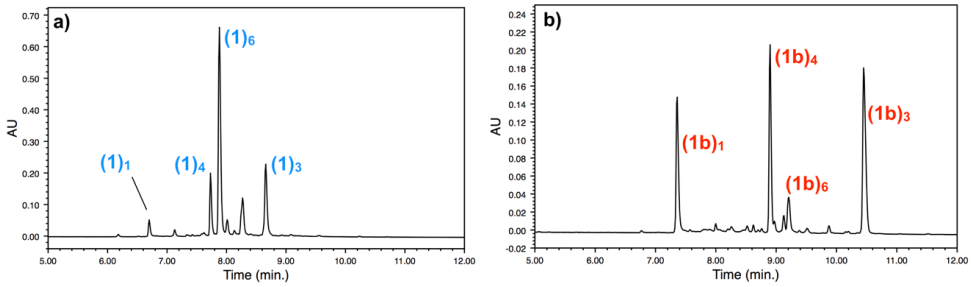

Figure 5.4: UPLC chromatograms (monitored at 254 nm) for DCLs (0.5 mM each) made from a) building block **1** and b) for **1b** in borate buffer (50 mM, pH 8.2). The stock solutions were left in the glove box without further agitation during the experiment.

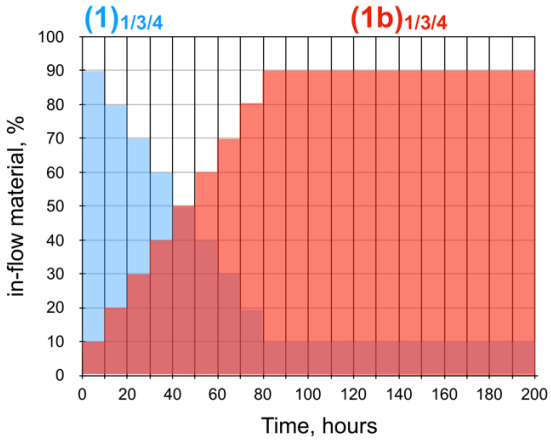

Figure 5.5: Change in % (v/v) of infusion material over time for set-up I. Blue part represents the oxidized solution containing building block **1** and red part represents the corresponding solution made from building block **1b**.

In our first set-up, we gradually changed the composition of nutrients by 10%

(v/v) every 20 hours, by changing flow rates of each infusion pump over a total amount of 90 hours. Since the solution initially contained only $\mathbf{1}_6$, we started infusion at rates of 9.0 µL/h for nutrients of **1** and 1.0 µL/h for nutrients of **1b** (Figure 5.5). After 90 hours, we continued infusion of nutrients with the same composition for another period of 90 hours to test the ability of any replicator present to sustain itself.

After 90 hours, which is slightly more than two turnovers, we observed that the solution contained mostly the cyclic hexamer $\mathbf{1}_6$ and smaller amounts of other 6-ring species (Figure 5.6a). Moreover, even after 200 hours, we observed mostly $\mathbf{1}_6$ and $\mathbf{1b}_3$ which indicates that hardly any exchange occurred between different macrocycles (Figure 5.6b). These observations raise two issues related to experimental design: first, the high oxidation level of building block **1** might have caused the undesirable emergence of $\mathbf{1}_6$ already in the infusion syringe. Moreover, since replication may be slowed down with decreased stirring rate (from 1200 rpm to 300 rpm), the infusion rate may have been too fast to allow the system to adapt to the new source of nutrients provided.

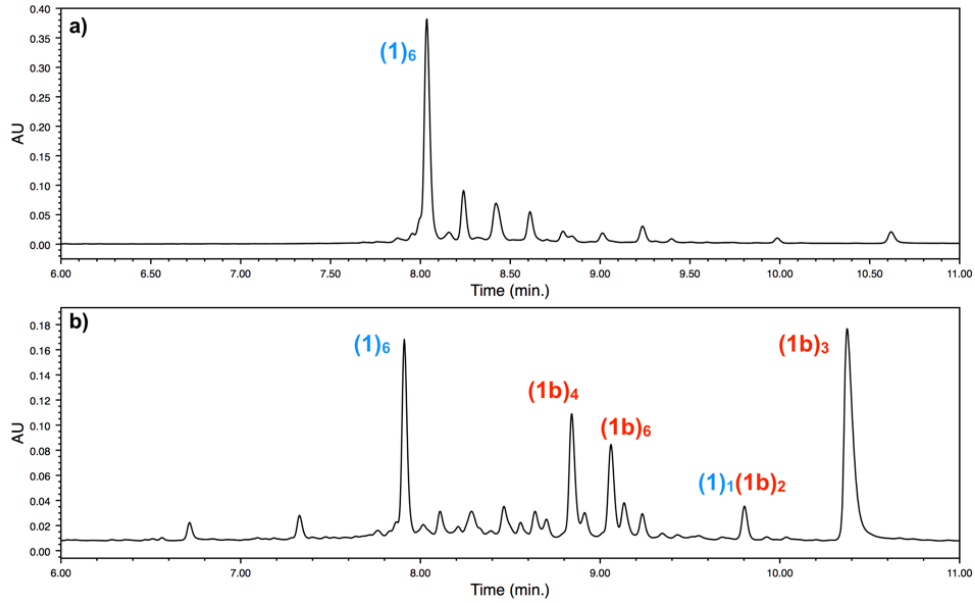

Figure 5.6: UPLC chromatograms (monitored at 254 nm) showing the species distribution in set-up I after: a) 90 hours, b) 200 hours.

In order to allow the replicator(s) more time to adapt in the presence of differ-

ent nutrients, we modified our experimental design in a way that the compositional change in the nutrients took place in time intervals of 20 hours in each step (Figure 5.7). In addition, in order to avoid problems with sample evaporation and oxidation in the infusion syringes, we stopped the flow after 180 hours, which corresponds to more than 2 turnovers.

We performed the experiment on two different solutions in parallel: one using 0.5 mM replicator $\mathbf{1}_6$ and the other using only borate buffer solution. The control sample gave us the chance to test if replicators can emerge spontaneously under flow conditions. We monitored the distribution of the species over time using a UPLC system. As Figure 5.8 shows, in the solution containing $\mathbf{1}_6$ at t=0, all other 6-ring species could be detected in addition to a mixture of 3-ring species after 180 hours. In contrast, in the control sample, we could detect only $\mathbf{1}_6$ and a mixture of cyclic trimers and tetramers. This observation shows that emergence of mixed-building-block 6-ring species depends on the sample history and requires a pre-existing replicator. It also shows that nucleation of $\mathbf{1}_6$ in the infusion syringe does not induce the formation of other 6-ring species in the solution.

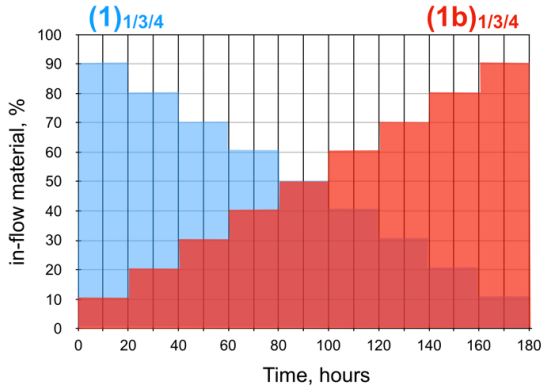

Figure 5.7: Change in % (v/v) of infusion material over time for set-up II. Blue part represents the oxidized solution containing building block **1** and red part represents the corresponding solution made from building block **1b**.

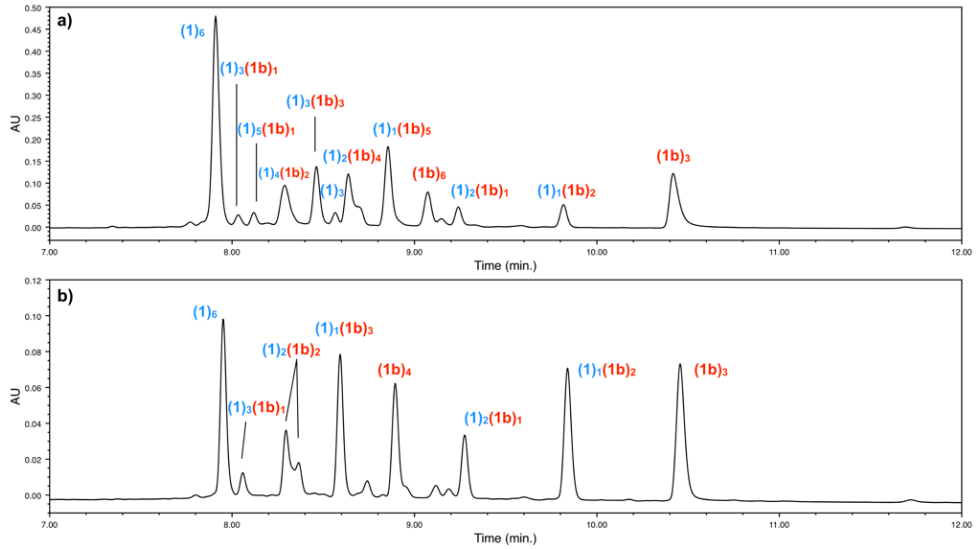

Figure 5.8: UPLC chromatograms (monitored at 254 nm) after 180 h for a) DCL that initially contains only $\mathbf{1}_6$ and b) control sample in which nutrient solutions were infused into a solution initially containing only buffer.

Despite the fact that the original replicator $\mathbf{1}_6$ was still dominating the overall library composition, we were able to perform a kinetic analysis on overall distribution of the species (Figure 5.9). The kinetic profile of the cyclic hexamers shows that $(\mathbf{1b})_6$ appeared only after the first turnover. Figure 5.10 shows how the relative UPLC peak areas (%) of each cyclic hexamer and cyclic trimer changed over time. Intensities of most cyclic hexamers showed a clear increase over time. Only $\mathbf{1}_6$ and $(\mathbf{1})_5(\mathbf{1b})_1$ were unable to sustain themselves, which was most likely a result of the change in composition of nutrients over time. This exploratory experiment showed that formation of different species originating from two building blocks can be manipulated with a change in nutrients composition.

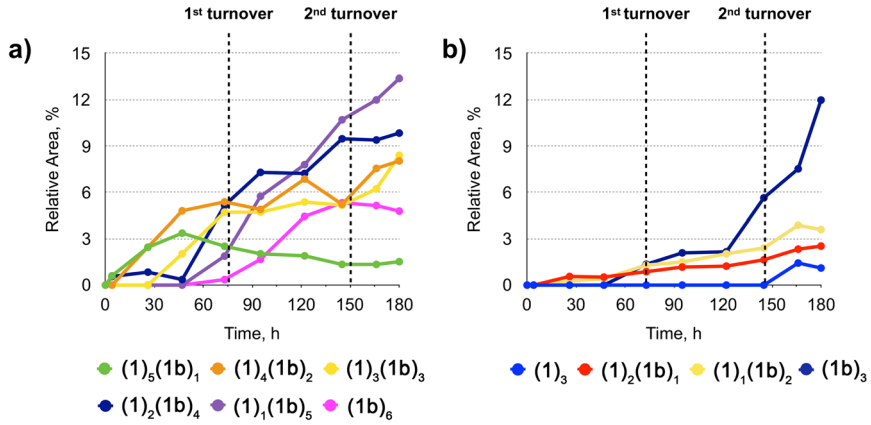

Figure 5.9: Relative UPLC peak area over time showing a), b) overall species distribution separated into two graphs for clarity from the same DCL used in set-up II.

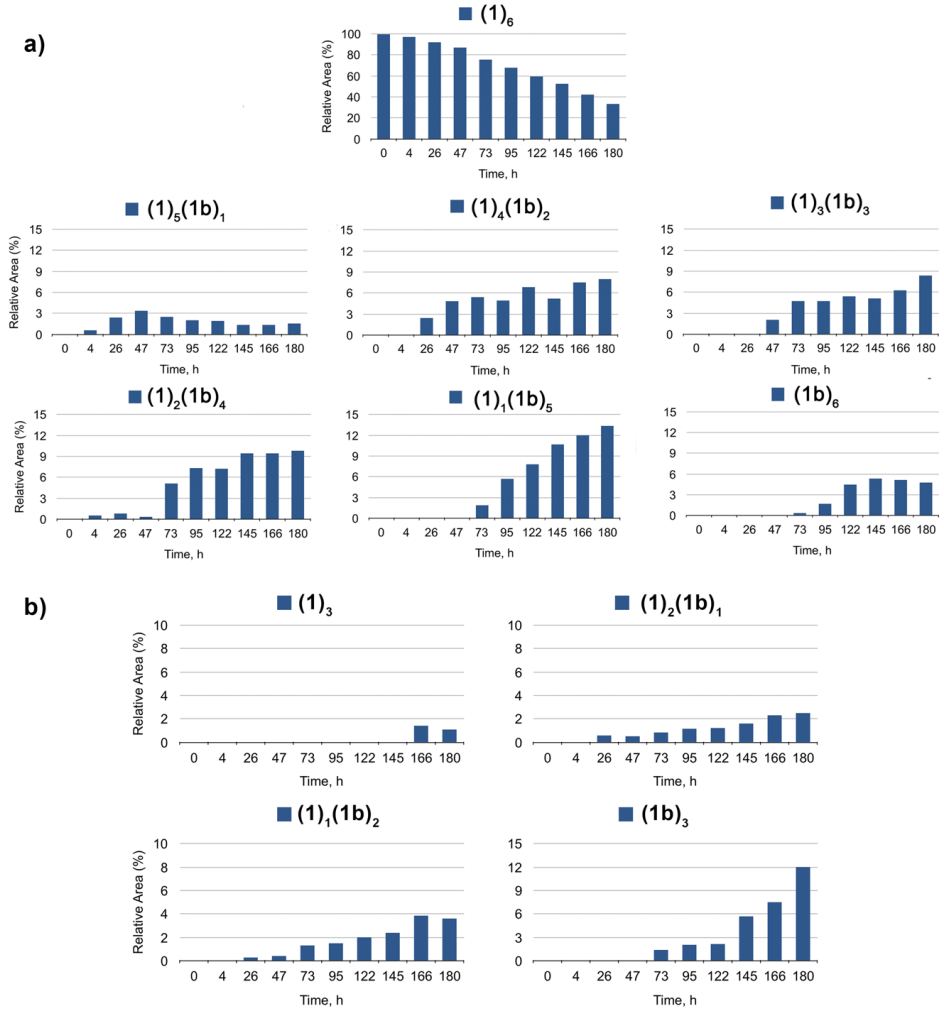

Figure 5.10: a) 6-ring species and b) 3-ring species distribution from the DCL used in set-up II.

As we briefly mentioned earlier in this chapter, there are certain experimental considerations that have to be taken into account. One of them is the oxidation level of the nutrients. In our preliminary experiments, we used nutrients that were prepared by dissolving building block **1** to a concentration of 0.5 mM and then oxidizing it to more than 90% with 80 mM sodium perborate solution. Such a high-level oxidation triggered the self-nucleation of $\mathbf{1}_6$ even before the infusion started which caused $\mathbf{1}_6$ to dominate the library composition. Moreover, although we tried

our best to exclude oxygen, even trace amounts of oxygen may completely oxidize the remaining monomer in the infusion syringe disabling the disulfide exchange in the solution. For these reasons, we monitored the composition of the nutrients made from building block **1** at different stages of the experiment (Figure 5.11).

Depending on the infusion rate, we were able to use the same infusion syringe without re-filling it up to 4 days. However, Figure 5.11c shows that after 4 days, $\mathbf{1}_6$ became the main species in the solution, even if the syringe was kept in a glove bag. These observations suggest that either the oxidation level should be slightly lower at t=0 or the infusion material should be re-filled more frequently from a stock solution that is kept under inert atmosphere during the experiment.

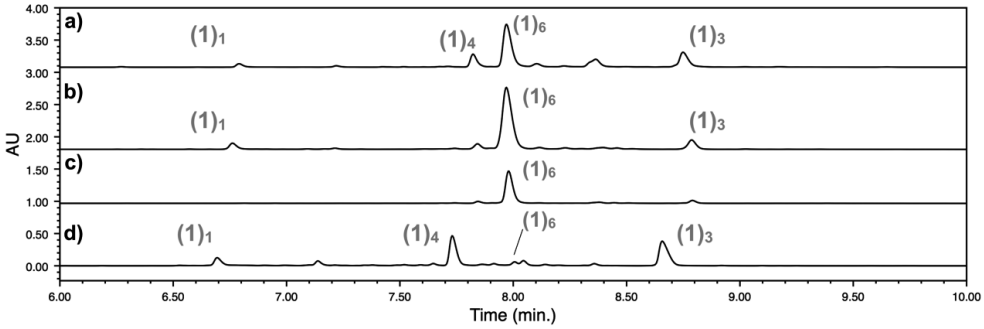

Figure 5.11: UPLC chromatograms (monitored at 254 nm) showing the distribution of species in the DCL used as in-flow material, made from building block **1**; a) at day 1, b) in glove box at day 57, c) at day 4 in the injection syringe under constant N_2 flow and d) freshly prepared with decreased oxidation level and only trace amount of replicator ($\mathbf{1}_6$) in solution.

Therefore, we repeated the experiment with freshly prepared in-flow material of which the oxidation level was still ∼90% with respect to the monomer but which contained only trace amount of replicator (set-up III). When compared with the previous system, we observed certain differences most importantly in the distribution of the species (Figure 5.12). First of all, while in set-up II, the relative percentage of $\mathbf{1}_6$ was almost 40% after 180 hours, this time it was less than 20 % at the end of the experiment (Figure 5.11a). This observation proved that the amount of replicator in the infusion material strongly affects the final distribution of the species. Moreover, the distribution of the other species was correlated with the composition of the infusion material: first **1**-rich hexamers emerged and as more **1b**-rich material was infused, the distribution shifted towards more **1b**-rich hexamers including $(\mathbf{1})_3(\mathbf{1b})_3$.

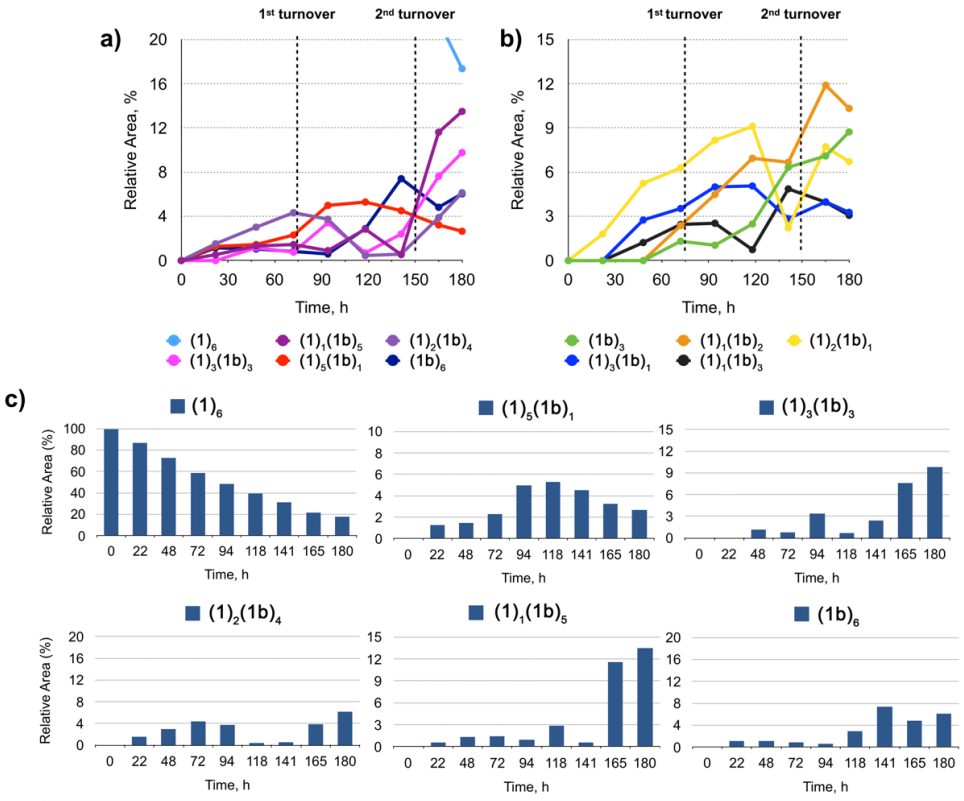

Figure 5.12: Relative UPLC peak area over time showing the distribution of a) 6-ring species, b) 3-ring and 4-ring species and c) 6-ring species in separate bar graphs from the DCLs used in set-up III.

In addition to diversification of cyclic hexamers in the presence of a replicator, we also performed a control experiment infusing nutrients into buffer to check the history dependence of the evolution of these species. As Figure 5.13 shows, we did not observe any cyclic hexamer different from $\mathbf{1}_6$ which had emerged in the infusion syringe. Instead, only mixtures of cyclic trimers and tetramers formed over time, leaving only trace amount of $\mathbf{1}_6$ in the solution. This control experiment proved that diversification of cyclic hexamers requires a relatively high concentration of pre-existing replicator in the solution to assist the formation of further replicators.

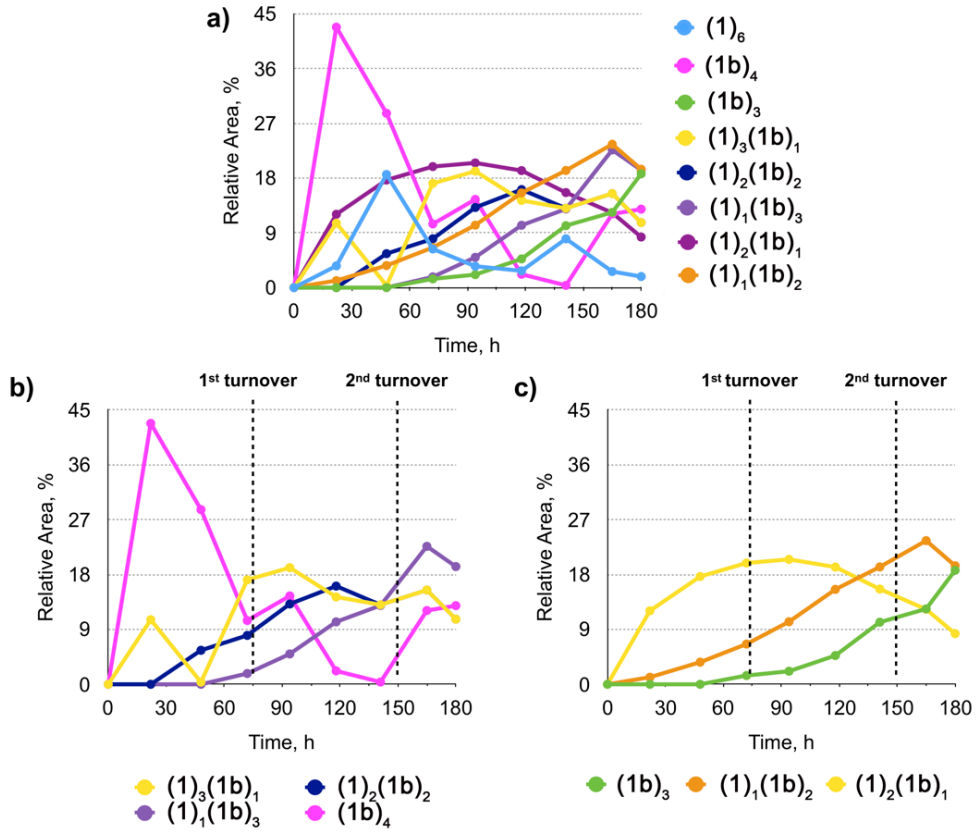

Figure 5.13: Relative UPLC peak area over time for the control sample of set-up III that contained only borate buffer at t=0 a) overall distribution of species detected with UPLC-MS, b) 4-ring species and c) 3-ring species.

As a next step, we modified the species composition in the solution at t=0. Therefore, we prepared a DCL made from an equimolar mixture of building blocks **1** and

1b ([**1**]=[**1b**]=0.25 mM) which was stirred at 300 rpm. We then started infusion in a way that the initial composition of the in-flow material contained equal amounts by volume of each equimolar building block solution at t=0 (Figure 5.14). By doing so, we wanted to probe whether mixed replicators could be sustained in an environment where the composition of the infusion material was changed. Moreover, having thus skipped the infusion of **1**-rich nutrients may provide more time for the system to adjust to the changing nutrient composition.

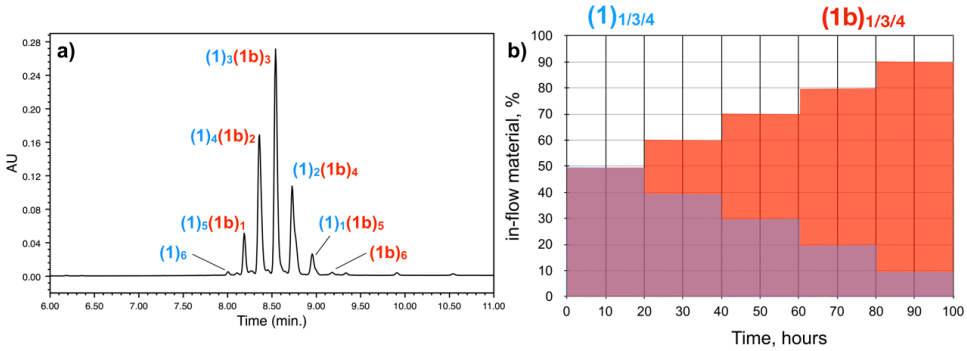

Figure 5.14: a) UPLC chromatogram (monitored at 254 nm) for the DCL made from equimolar amounts of building block **1** and **1b** ([**1**]=[**1b**]=0.25 mM) in borate buffer (50 mM, pH 8.2) and b) composition of infusion material for set-up IV.

After 100 hours, we observed a difference in the distribution of species (Figure 5.15). Instead of replicator growth being dominated by $(\mathbf{1})_1(\mathbf{1b})_5$, this time we observed that the almost statistical cyclic hexamer distribution was conserved (Figure 5.14a). This observation showed that the 6-ring species emerging from the equimolar mixture of building blocks **1** and **1b** were capable of sustaining themselves even when the composition of in-flow material was slowly changed over time. Since more of the **1b**-containing nutrients were infused at later stages of the experiment, a mixture of cyclic trimers was also detected which was enriched in $(\mathbf{1b})_3$. In contrast, in the control sample, we observed only $\mathbf{1}_6$ which was probably a result of self-nucleation, and mixtures of cyclic trimers and tetramers (Figure 5.15b).

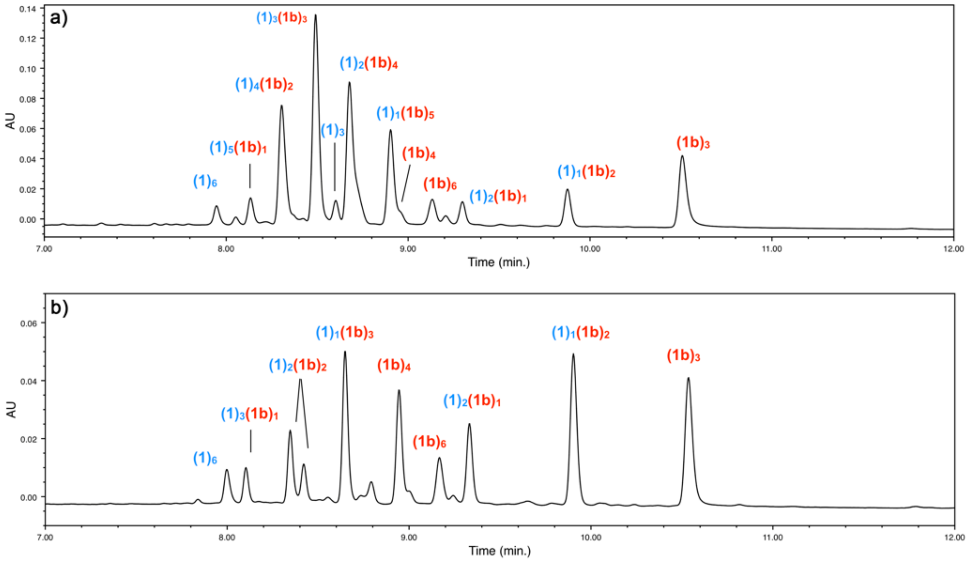

Figure 5.15: Part of UPLC chromatograms (monitored at 254 nm) after 100 hours of infusion using set-up IV a) for the DCL (0.5 mM in 50 mM borate buffer, pH 8.2) containing $(\mathbf{1})_n(\mathbf{1b})_{6\text{-}n}$ at t=0 and b) control sample which initially contained only borate buffer.

The kinetics of the system were again followed by monitoring the relative UPLC peak areas of the species in the solution. The results confirmed that the 6-ring species were able to sustain themselves in the solution, especially during the first 40 hours (Figure 5.16a and Figure 5.16c). After that point, when the infusion material contained $\geqslant$70 % building block **1b**, the percentage of **1b**-rich species clearly increased. Similar to the previous set-ups, 3-ring species started to emerge as more **1b** was infused (Figure 5.16b).

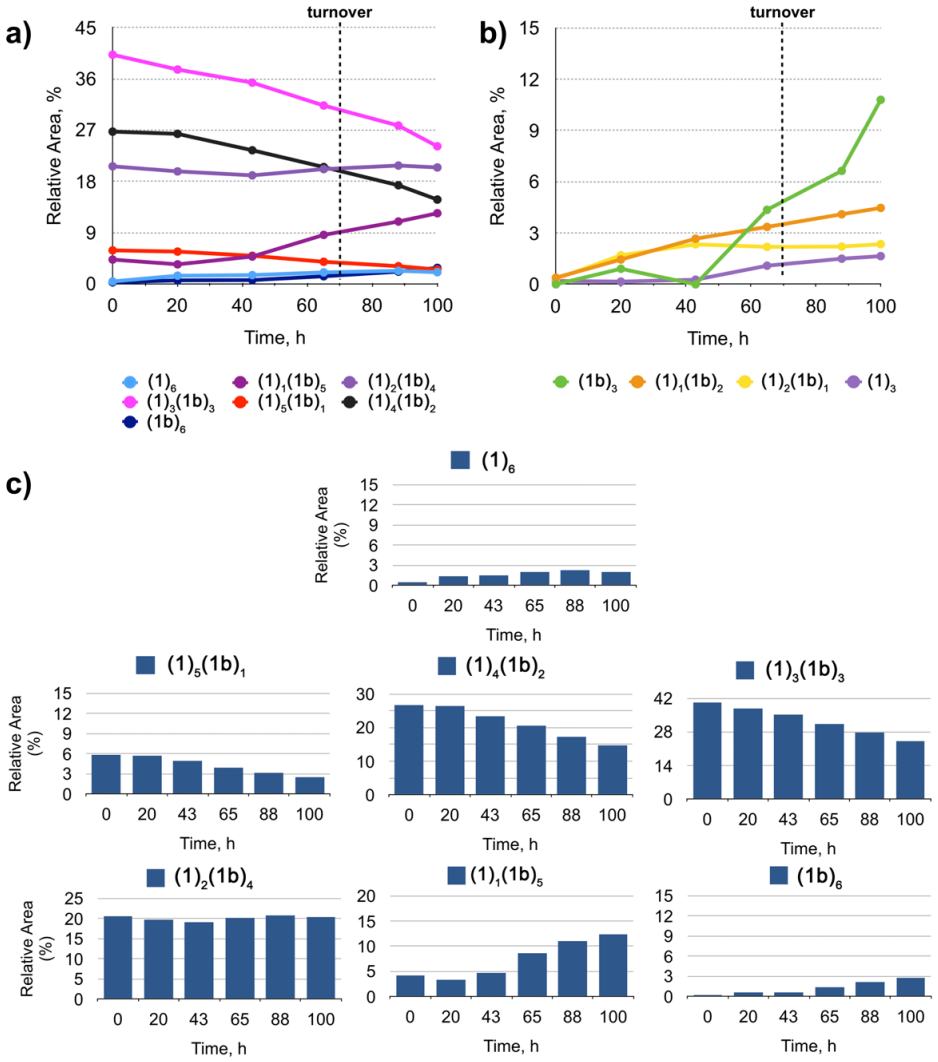

Figure 5.16: Relative UPLC peak area over time for set-up IV, showing the distribution of a) 6-ring species, b) 3-ring species and c) 6-ring species in separate bar graphs.

5.2.3 Infusion of the third building block under flow conditions

Lastly, we attempted to introduce the third building block into the system. It proved challenging to monitor such complex mixtures over a period of 280 hours. In order to keep the system as simple as possible, we started with a mixture of 6-ring species made from DCLs made from building blocks **1** and **1b** ([**1**]=[**1b**]=0.25 mM) in borate buffer (50 mM, pH8.2). In the first 100 hours, we used the same infusion material as in set-up IV. After 100 hours, we changed the composition and started to inject 10% (v/v) building block **1a** and 90% (v/v) building block **1b**. In a period of 180 hours, the composition was changed step by step towards more of the building block 1a-containing nutrients (Figure 5.17).

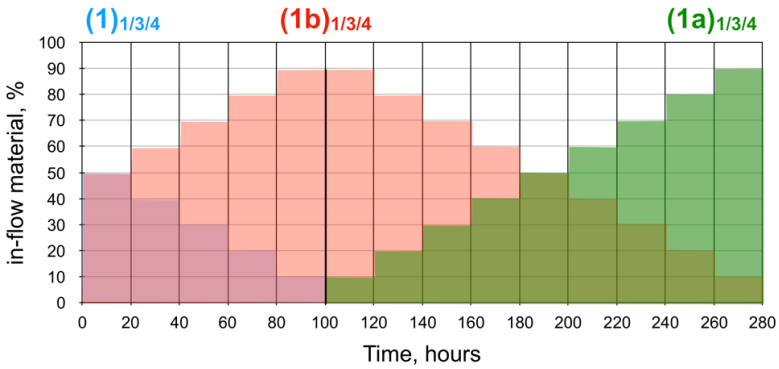

Figure 5.17: Change in % (v/v) of infusion material over time for set-up V which includes nutrients made from building blocks **1**, **1b** and **1a**.

Unfortunately, when analysing the library during the course of the experiment, we faced difficulties with identifying the peaks in the UPLC chromatograms due to co-elution of species which have very small differences in hydrophobicity. Therefore, we cannot provide a detailed kinetic analysis for set-up V. Nevertheless, our analysis revealed that, there is an evident shift from a mixture of cyclic hexamers towards cyclic pentamers and tetramers (Figure 5.18).

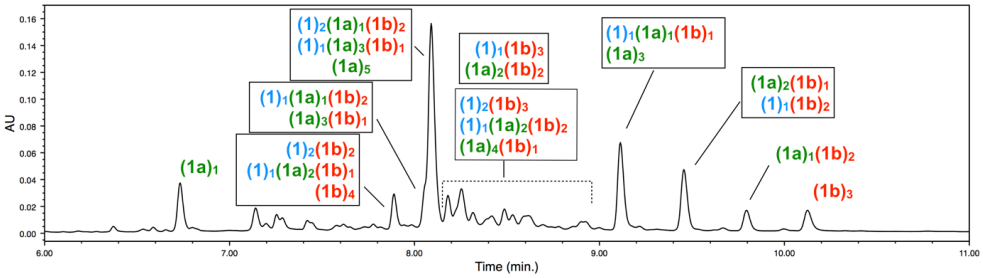

Figure 5.18: Part of the UPLC chromatogram (monitored at 254 nm) after 280 hours of flow for the DCL which initially contained $\mathbf{1}_n\mathbf{1b}_{6\text{-}n}$ (0.5 mM in 50 mM borate buffer, pH 8.2) used in set-up V. Macrocycles that are listed in boxes have the same exact theoretical mass values. Therefore, the exact composition of the peaks cannot be assigned (See Appendix for LC-MS spectra).

5.3 Conclusions

We report our first attempt to build up a flow system where species are diversified by slow changes in the composition of nutrients required for replication. We followed a step-wise approach and first optimized the experimental set-up by utilizing two building blocks. We observed that, using building blocks **1** and **1b**, starting from only $\mathbf{1}_6$ replicator, it was possible to evolve the distribution of the 6-ring species depending on the nutrients' composition. Moreover, although not perfectly, we also observed that the mixture of 6-ring species was able to sustain itself. Upon inclusion of a third block we were only able to make a qualitative analysis and observed a shift from 6-ring species to 4- and 5-ring species. Although the system is promising, it would require optimization of analytical methods to provide a more detailed kinetic analysis of such complex mixtures. Another solution, to provide more simplicity, would be determining conditions where only one size of replicator dominates: 6mer or 3mer for building block **1b** and 4mer, 5mer or 6mer for building block **1a**. Despite the problems in sample analyses, the flow set-ups with two building blocks showed that replicating species can be diversified by cross-catalysis in a dynamic environment. However, the number of steps in the cross-catalytic pathway aimed to produce species that cannot cross-catalyze their initial predecessor appears to be larger than the number realized in this chapter. Once the analytical problems are solved, it would be possible to test how species can be diversified under flow conditions.

5.4 Acknowledgements

Yiğit Altay is gratefully acknowledged for the optimization of flow set-ups and advise on how to keep them oxygen free.

5.5 Experimental Section

5.5.1 Materials

UPLC grade water (Biosolve BV) was used in all experiments where needed. For preparing buffer solution, boric anhydride, disodium phosphate and monosodium phosphate were purchased from Sigma-Aldrich. Sodium hydroxide (Merck Chemicals) and sodium chloride (Merck Chemicals) were used for pH adjustments. Building blocks **1**, **1a** and **1b** were purchased from Cambridge Peptides Ltd. (Birmingham, UK) and had ⩾98 % purity. For UPLC measurements, UPLC grade water, acetonitrile and trifluoroacetic acid (Biosolve BV) were used. In sample dilution, peptide synthesis grade dimethyl formamide (DMF) (Biosolve BV) was used. The Atmosbag (51×58 cm, an inflatable polyethylene glove box alternative) was purchased from Sigma-Aldrich.

5.5.2 Methods

Library preparation and Sampling

Preparation of DCLs:

A stock replicator ($\mathbf{1}_6$) solution was prepared by dissolving building block **1** to a concentration of 1.0 mM in borate buffer (50 mM in $B_4O_7^{2-}$ anions, pH 8.2). Moreover, another stock DCL was prepared by mixing building blocks **1** and **1b** ([**1**]=[**1b**]=0.5 mM) in borate buffer (50 mM, pH 8.2). Stock DCLs were equilibrated in HPLC vials (12 × 32 mm) with Teflon caps and were left agitated on an IKA RCT hot plate at 1200 rpm. Prior to flow experiments, solutions were diluted to a concentration of 0.5 mM (V_{tot}=750 µL) with the same buffer. After the dilution, the DCLs were placed in the glove bag on an IKA RCT stirring plate and agitated at 300 rpm during the experiments.

Preparation of infusion material:

Building blocks **1**, **1a** and **1b** were weighed out in HPLC vials and transferred to a glove box in the powder form. In the glove box, they were dissolved to a concentration of 0.5 mM with borate buffer (50 mM, pH 8.2) which was previously degassed. Prior to flow experiments, the DCLs were left unagitated for approximately a week to allow equilibration.

Sampling:
Prior to UPLC and LC-MS analyses, 10 µL sample was diluted 5 times with 5 µL DMF and 35 µL UPLC grade water in the glove bag. Once the samples were taken out of the glove bag, they were directly analysed using UPLC to minimize exposure to oxygen from the air. The same sampling protocol was followed for all UPLC and LC-MS measurements.

5.5.3 Kinetic profiles

Set-up II

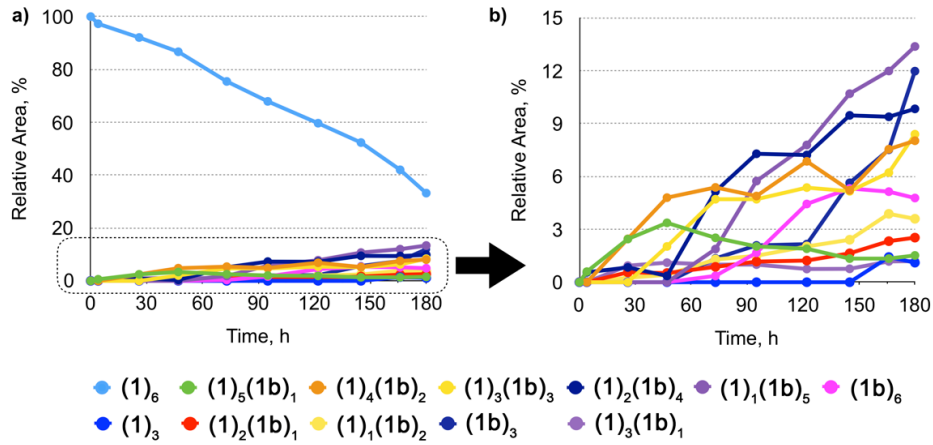

Figure 5.19: Relative UPLC peak area over time for set-up II; a) overall distribution of the species detected with UPLC-MS b) magnified part of the analysis showing detailed kinetics which excludes $\mathbf{1}_6$.

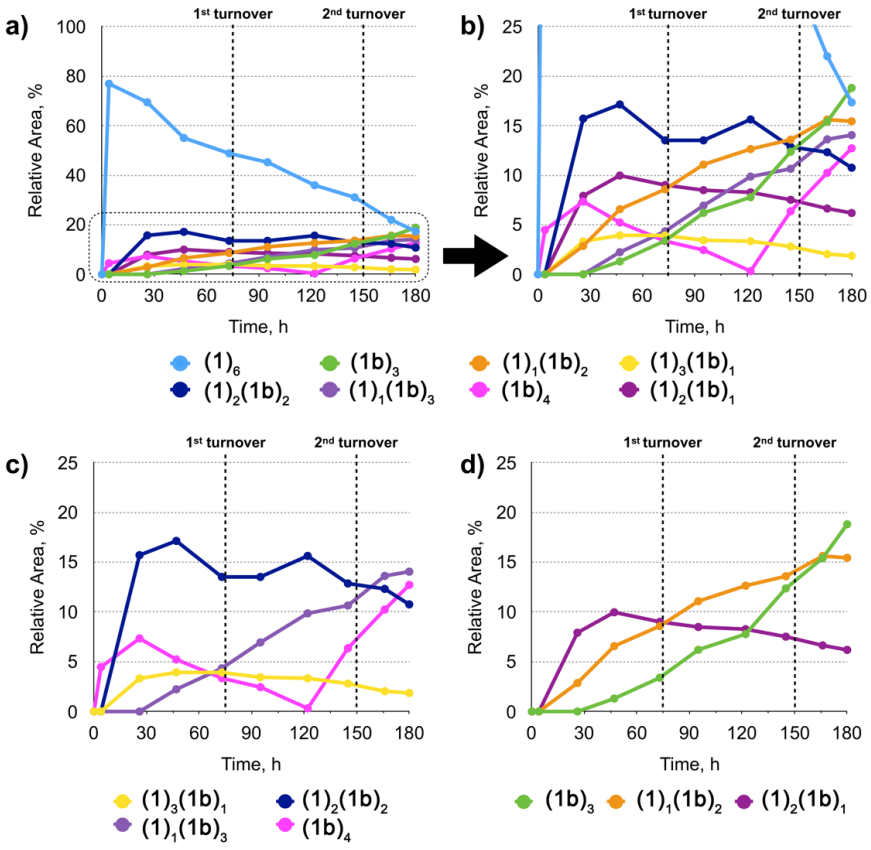

Figure 5.20: Relative UPLC peak area for the control sample a) overall species distribution, b) zoomed part of the analysis showing detailed kinetics of species excluding $\mathbf{1}_6$, c) 4-ring species and d) 3-ring species distribution.

Set-up III and IV

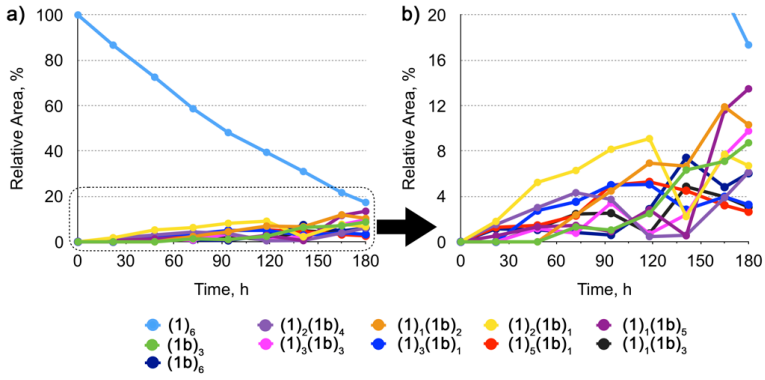

Figure 5.21: Relative UPLC peak area over time for set-up III; a) overall distribution of the species detected with UPLC-MS b) magnified part of the analysis showing detailed kinetics which excludes $\mathbf{1}_6$.

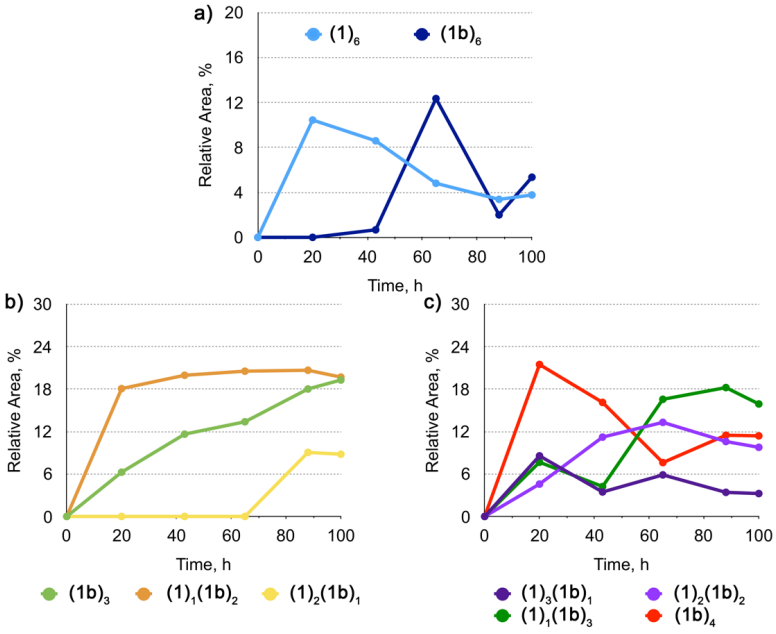

Figure 5.22: Relative UPLC peak area over time for the control sample which contained only borate buffer at t=0 in set-up IV; a) distribution of $\mathbf{1}_6$ and $\mathbf{1b}_6$ b) 3-ring species and c) 4-ring species detected in the solution.

5.5.4 UPLC-MS Analysis of set-up V

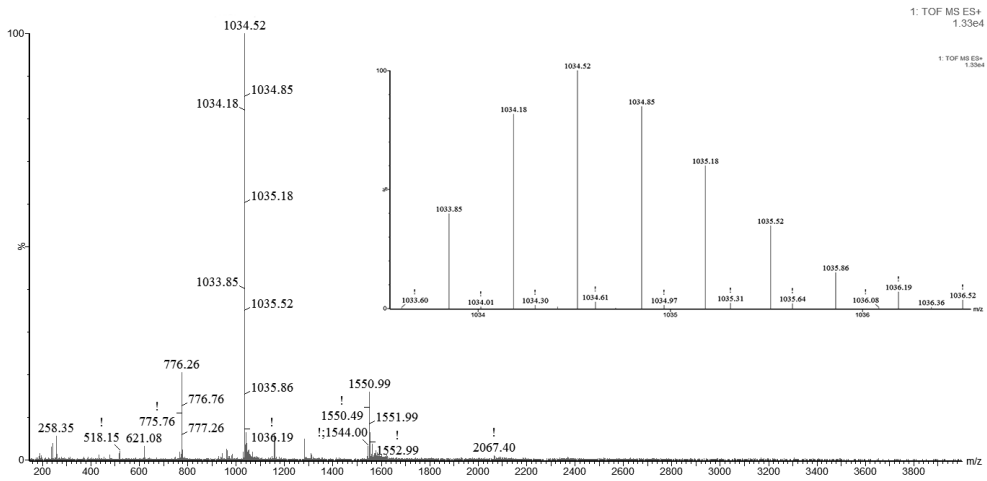

Figure 5.23: Mass spectrum of cyclic tetramers $(\mathbf{1})_1(\mathbf{1a})_1(\mathbf{1b})_2$ and/or $(\mathbf{1a})_3(\mathbf{1b})_1$ from the UPLC-MS analysis of the DCL used in set-up V. Calculated isotopic profile (species abundance) for $[M+3H]^{3+}$: 1034.52 (M, 100 %), 1034.85 (M+1, 84.89 %), 1035.18 (M+2, 60.01 %), 1035.52 (M+3, 34.67 %);m/z observed: 1550.99 $[M+2H]^{2+}$, 1034.52 $[M+3H]^{3+}$, 776.26 $[M+4H]^{4+}$;

$(\mathbf{1})_1(\mathbf{1a})_1(\mathbf{1b})_2$ m/z calculated: 1551.70 $[M+2H]^{2+}$, 1034.47 $[M+3H]^{3+}$, 775.85 $[M+4H]^{4+}$

$(\mathbf{1a})_3(\mathbf{1b})_1$ m/z calculated: 1551.71 $[M+2H]^{2+}$, 1034.47 $[M+3H]^{3+}$, 775.85 $[M+4H]^{4+}$

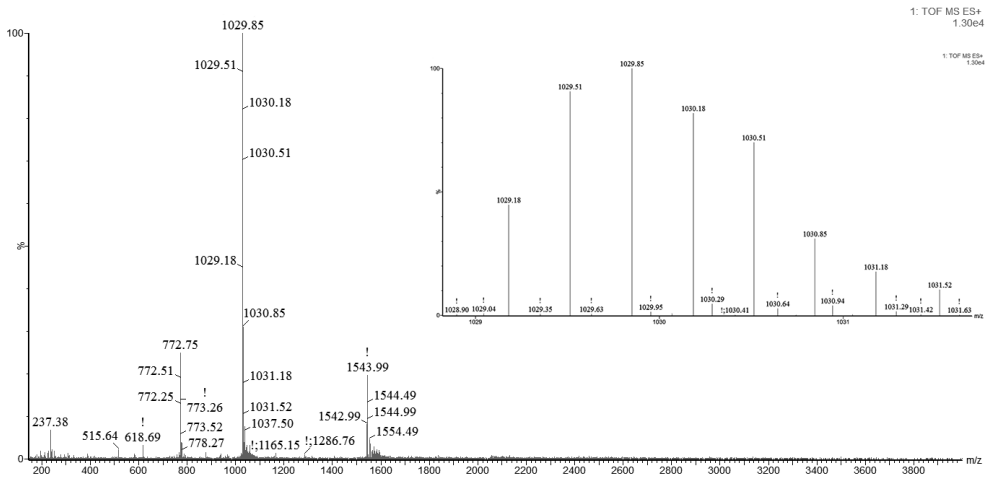

Figure 5.24: Mass spectrum of cyclic tetramers $(\mathbf{1})_1(\mathbf{1a})_2(\mathbf{1b})_1$ and/or $(\mathbf{1})_2(\mathbf{1b})_2$ from the UPLC-MS analysis of the DCL used in set-up V. Calculated isotopic profile (species abundance) for $[M+3H]^{3+}$: 1029.85 (M, 100 %), 1030.18 (M+1, 81.84 %), 1030.51 (M+2, 70.06 %), 1030.85 (M+3, 31.08 %);m/z observed: 1543.99 $[M+2H]^{2+}$, 1029.85$[M+3H]^{3+}$, 772.75 $[M+4H]^{4+}$;

$(\mathbf{1})_1(\mathbf{1a})_2(\mathbf{1b})_1$ m/z calculated: 1544.70 $[M+2H]^{2+}$, 1029.80 $[M+3H]^{3+}$, 772.35 $[M+4H]^{4+}$

$(\mathbf{1})_2(\mathbf{1b})_2$ m/z calculated: 1544.70 $[M+2H]^{2+}$, 1029.80 $[M+3H]^{3+}$, 772.35 $[M+4H]^{4+}$

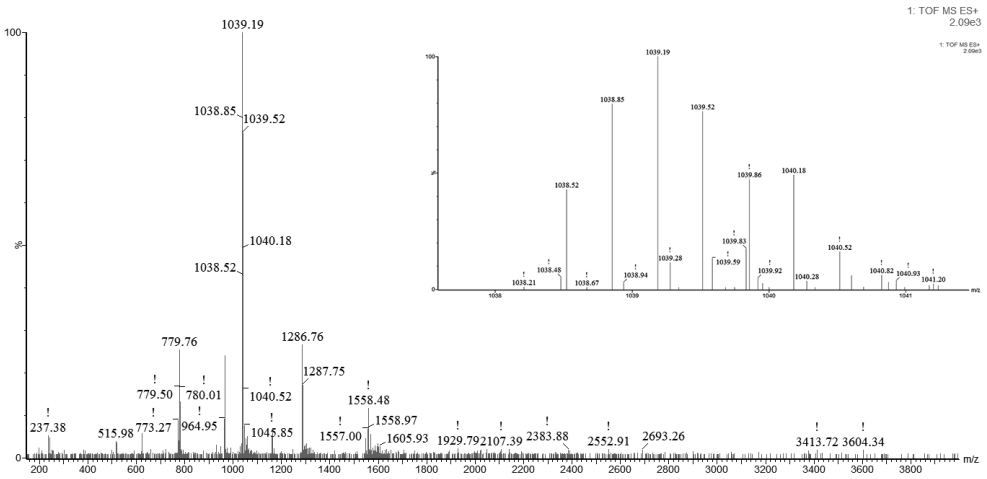

Figure 5.25: Mass spectrum of cyclic tetramers $(\mathbf{1a})_2(\mathbf{1b})_2$ and/or $(\mathbf{1})_1(\mathbf{1b})_3$ from the UPLC-MS analysis of the DCL used in set-up V. Calculated isotopic profile (species abundance) for $[M+3H]^{3+}$: 1039.19 (M, 100 %), 1039.52 (M+1, 76.38 %), 1039.86 (M+2, 47.20 %), 1040.18 (M+3, 49.15 %);m/z observed: 1558.48 $[M+2H]^{2+}$, 1039.19 $[M+3H]^{3+}$, 779.76 $[M+4H]^{4+}$;

$(\mathbf{1a})_2(\mathbf{1b})_2$ m/z calculated: 1558.71 $[M+2H]^{2+}$, 1039.14 $[M+3H]^{3+}$, 779.36 $[M+4H]^{4+}$

$(\mathbf{1})_1(\mathbf{1b})_3$ m/z calculated: 1558.71 $[M+2H]^{2+}$, 1039.14 $[M+3H]^{3+}$, 779.36 $[M+4H]^{4+}$

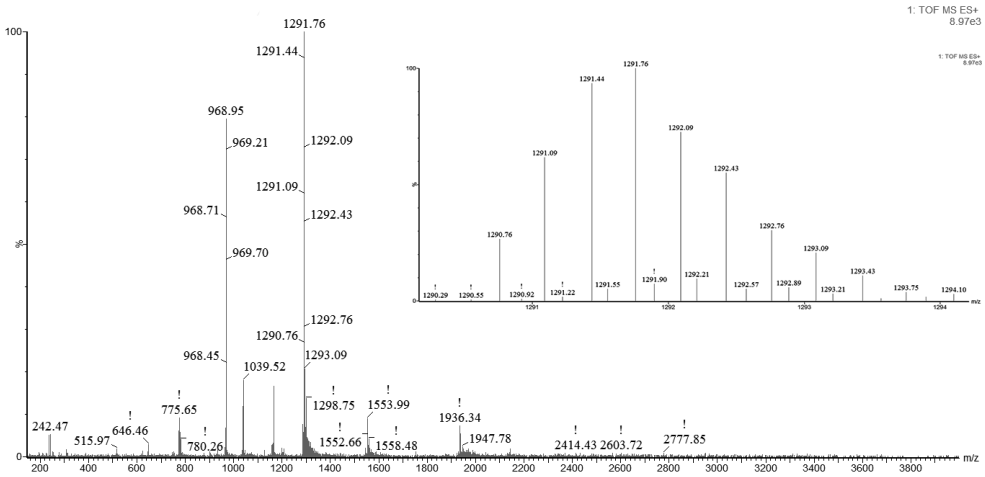

Figure 5.26: Mass spectrum of cyclic pentamers $(\mathbf{1})_2(\mathbf{1b})_3$, $(\mathbf{1})_1(\mathbf{1a})_2(\mathbf{1b})_2$ and/or $(\mathbf{1a})_4(\mathbf{1b})_1$ from the UPLC-MS analysis of the DCL used in set-up V. Calculated isotopic profile (species abundance) for $[M+3H]^{3+}$: 1291.76 (M, 100 %), 1292.09 (M+1, 72.50 %), 1292.43 (M+2, 55.13 %), 1292.76 (M+3, 30.34 %);m/z observed: 1936.34 $[M+2H]^{2+}$, 1291.76 $[M+3H]^{3+}$, 968.95 $[M+4H]^{4+}$;

$(\mathbf{1})_2(\mathbf{1b})_3$ m/z calculated: 1936.38 $[M+2H]^{2+}$, 1291.92 $[M+3H]^{3+}$, 968.94 $[M+4H]^{4+}$

$(\mathbf{1})_1(\mathbf{1a})_2(\mathbf{1b})_2$ m/z calculated: 1936.38 $[M+2H]^{2+}$, 1291.92 $[M+3H]^{3+}$, 968.94 $[M+4H]^{4+}$

$(\mathbf{1a})_4(\mathbf{1b})_1$ m/z calculated: 1935.89 $[M+2H]^{2+}$, 1291.92 $[M+3H]^{3+}$, 968.94 $[M+4H]^{4+}$

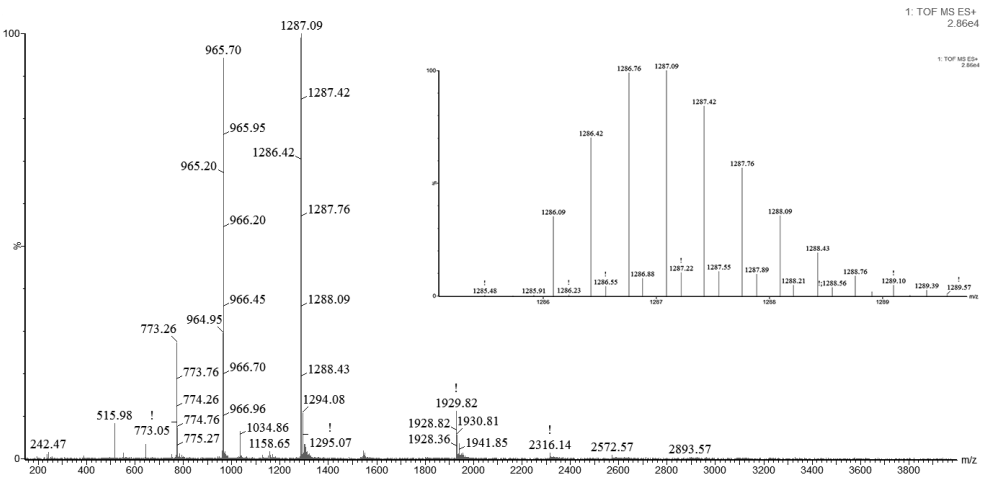

Figure 5.27: Mass spectrum of cyclic pentamers $(\mathbf{1})_2(\mathbf{1a})_1(\mathbf{1b})_2$ and/or $(\mathbf{1})_1(\mathbf{1a})_3(\mathbf{1b})_1$ from the UPLC-MS analysis of the DCL used in set-up V. Calculated isotopic profile (species abundance) for $[M+3H]^{3+}$: 1287.09 (M, 100 %), 1287.42 (M+1, 84.26 %), 1287.76 (M+2, 56.83 %), 1288.09 (M+3, 35.63 %);m/z observed: 1929.82 $[M+2H]^{2+}$, 1287.09 $[M+3H]^{3+}$, 965.70 $[M+4H]^{4+}$;

$(\mathbf{1})_2(\mathbf{1a})_1(\mathbf{1b})_2$ m/z calculated: 1929.37 $[M+2H]^{2+}$, 1287.25 $[M+3H]^{3+}$, 965.43 $[M+4H]^{4+}$

$(\mathbf{1})_1(\mathbf{1a})_3(\mathbf{1b})_1$ m/z calculated: 1929.38 $[M+2H]^{2+}$, 1287.25 $[M+3H]^{3+}$, 965.44 $[M+4H]^{4+}$

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