Enabling Darwinian evolution in chemical replicators
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Self-replicating molecules containing labile reversible covalent bonds are becoming increasingly topical. Yet their robustness to component exchange and the threat of loss of information has received very little attention. We show here, by means of UPLC-MS studies of structures patterned with $^{15}$N isotopic labels, that supramolecular interactions can efficiently protect self-replicating molecules from otherwise rapid component exchange. Thanks to supramolecular interactions, fibres of macrocyclic peptide derivatives can be grown and deconstructed in a highly selective fashion at their extremities, while macrocycles within the fibre cores remain accessible for component exchange only as a result of exposure due to fibre breakage. Our results unveil several molecular details on the pathways of fibre formation, destruction, breakage and exchange, and provide insights into the role of information transfer during molecular replication.

Parts of this chapter have been published:
Self-replication is of key importance to the question of the origin of life and in the quest for the first de novo synthesis of life, as discussed in Chapter 1. In the process of replication molecular information is copied and transferred to the next generation of replicating molecules. Adequate propagation of such information requires not only sufficiently accurate copying, but also sufficiently long persistence of this information. The latter issue is particularly relevant for the rapidly increasing number of self-replicating systems that feature relatively labile reversible covalent bonds, but received comparatively little attention.

We now report a detailed mechanistic investigation into the pathways that lead to information loss through building-block exchange in systems of stacked self-assembled self-replicating macrocycles. Understanding the mechanisms by which kinetically frozen assemblies can be obtained and can be further transformed leads to an enhanced awareness of their design properties and a higher potential for their targeted exploitation. We conclude that exchange is effectively limited to the stack ends, while the replicator compositions inside the stacks remain stable for months, even though exchange kinetics of nonassembled macrocycles occurs on a timescale of minutes.

Supramolecular chemistry was initially focused on self-assembly at thermodynamic equilibrium. Nowadays, structures frozen in kinetically trapped states are increasingly becoming of interest, together with far-from-equilibrium supramolecular systems chemistry, which is gradually attracting attention. Control of kinetic pathways is key to understand and engineer far-from-equilibrium systems, which in turn are the result of the interplay of many kinetically controlled processes. However, this knowledge is often elusive.

Studies on the origins of life benefit enormously from gaining a better understanding on the pathway complexity of formation, approach to equilibrium and destruction of replicating systems such as the kinetically trapped supramolecular fibres that we have described in the previous chapters: these insights represent a major step towards being able to control the evolutionary traits of simple, molecular replicators.

Ultimately, the way information is maintained and propagated during a replication process will determine the dynamic kinetic stability of the corresponding far-from-equilibrium system. When the peptide replicator system described in the previous chapters is allowed to feature building blocks with more than one peptide sequence, information can be found on many coordinates: the variety of macrocycles that can be formed from the individual building blocks, the composition in building blocks of the solution, and the distribution of the various macrocycles along the length of the fibres, i.e., the degree of mixing of the building blocks in the fibres. Information is not borne in the peptide sequence of an individual building block as, due to its stability, the probability of the sequence to change in the observation time of an experiment is close to zero. Nor is it found in a kinetically trapped system where the possibilities of chemical change are zero or close to zero.
If reaction pathways become available for more than one configuration to be possible, then such a system bears a higher information content than a kinetically locked state. A specific configuration then brings about different evolutionary possibilities compared to any other state. In order to account for such possibilities, it is important to take into account how information can evolve. Configurations that are highly unlikely will not get the chance to compete in the evolutionary race and will not be able to propagate information to subsequent generations of replicators. Other configurations that are accessible to the system, on the other hand, might feature specific proneness to further evolution, e.g., by displaying cooperation. In general, if the information content of a system is expanded to involve more potential configurations, then an extended set of chemical evolutionary pathways becomes available.

Kinetically trapped supramolecular assemblies allow access to a variety of functional materials, the properties of which depend upon the preparation method, hence on the history of the system, and not on the thermodynamically most stable structure, as numerous recent examples show. Among kinetically trapped assemblies, nanofibres occupy a niche role as they are of interest for biological systems (e.g., membrane nanotubes), for the formation of functional gels and materials, and for their role as replicators in studies on the origins of life. While it is generally observed that different preparation methods yield different structures, the mechanistic details by which supramolecular fibres are produced and how they undergo further processes are often elusive. Nonetheless, in kinetically trapped systems, control of the molecular organization of the functional building blocks dictates the expression of the functionalities of a given system. Hence, mastery of these pathways would lead to enhanced awareness of the fundamental properties of the fibres, which would in turn grant capability to design new similar assemblies and to exploit these structures in a targeted way. Recent focus has been dedicated to understanding and controlling kinetic pathways of formation of kinetically controlled self-assemblies, as these indirectly allow to gain control over the properties of the assemblies. This has been achieved through studying the complexity of the pathways of formation of the interesting assemblies, as well as of the pathways of approach to equilibrium once the kinetically trapped assemblies have been formed.

In Chapter 2, experimental and computational insights on the breakage mechanism were described. These insights allow conclusions to be drawn regarding the replication order and the overall fibre length distributions. In this Chapter 4 we illustrate the results of experiments probing building block exchange, i.e., how molecular and supramolecular information can be propagated and lost via equilibration of macrocycles and fibre breakage. Specifically, here we provide mechanistic insights in the steps of (1) kinetically controlled fibre formation; (2) breakage-mediated approach to equilibrium and (3) chemically-mediated deconstruction. Our supramolecular structures are derived from a dynamic combinatorial library. Insights obtained through this work reveal
that block copolymers can be obtained as kinetically stable products by using dynamic combinatorial chemistry (DCC) and can be further processed by using the mechanistic awareness of the underlying pathways.

4.1. PATHWAY COMPLEXITY OF REPLICATION/EXCHANGE

We based our study on a previously reported system, introduced in Section 1.3.2 of Chapter 1, described in Section 2.1 of Chapter 2 and shown in Figure 4.1.1, which plays a role in the studies on the origins of life.14 A thiol-functionalized peptide building block \( \mathbf{1} \) forms a dynamic combinatorial library of disulfide macrocycles, among which hexamers \( \mathbf{1}_6 \) nucleate and elongate into supramolecular nanofibres that can subsequently undergo fragmentation by mechanical forces in solution, leading to sigmoidal growth of hexamer concentration, which is typical of autocatalytic processes. Notably, autocatalysis in our system enables exponential replication, as shown in Chapter 2.20

The mechanisms of the fibre assembly, equilibration and deconstruction processes were analyzed via UPLC-MS techniques, whereby fibres are disassembled and hexamers are eluted individually. We used building blocks \( \mathbf{1} \) and its isotopically labelled analogue \( \mathbf{1}^* \) (Figure 4.1.1). Macrocycles of the same size formed by any combination of \( \mathbf{1} \) and \( \mathbf{1}^* \) co-elute during chromatography, whereby ratios between the two building blocks are easily assessed by MS analysis of the corresponding chromatographic peak (e.g., hexamer).

The use of \( \mathbf{1} \) and \( \mathbf{1}^* \) in the same mixture results in the potential formation of many combinations of macrocycles with different isotopic labelling patterns. These macrocycles are in constant exchange in solution (Figure 4.1.2).

In order to study exchange pathways, we prepared kinetically trapped supramolecular block copolymers by growing labelled \( \mathbf{1}^*_6 \) on both fibre ends of previously prepared unlabelled \( \mathbf{1}_6 \) fibres, in 1:1 ratio (Figure 4.1.3a). This methodology pioneered in this thesis has since been improved within our group in order to produce fibres with low polydispersity and tuneable average length.21

These methods make use of Couette cells as the source of the shear stress. Furthermore, they introduce the formation of block copolymers by means of incubation of polymers made up of different macrocycles with no source of mechanical agitation. The studies in this chapter consider such block copolymer fibres and focus on the effects of mechanically-induced fibre breakage and macrocycle exchange with building blocks in the solution as causes of information propagation and loss.

In the following, hexameric macrocycles are represented as colored rectangles as shown in Figure 4.1.3a (blue for \( \mathbf{1}_6 \); red for \( \mathbf{1}^*_6 \) and different shades of purple for the mixed macrocycles) The use of labelled and unlabelled peptide building blocks \( \mathbf{1} \) and \( \mathbf{1}^* \) introduces sufficient diversity to be able to study the pathways of formation,
approach to equilibrium and deconstruction, unencumbered by enthalpic factors which would be present upon mixing building blocks with different peptide sequences or using building blocks that can form macrocycles of different sizes.

Figure 4.1.1. Thiol functionalized peptide building blocks I and isotopically labelled I* form a mixture of interconverting macrocycles upon formation of disulfide bridges (the structure of the cyclic trimer is shown as an example). Among the macrocycles, hexamers selectively self-assemble by nucleating and elongating into supramolecular nanofibres, which undergo mechanically-induced fragmentation.

Figure 4.1.3 shows schematically how mixing can occur over time in a system prepared as a 1:1 block copolymer of unlabelled 1_6 and labelled 1*_6 macrocycles, resulting in two parallel trends, both driven by the entropy of mixing of labelled and unlabelled material: (1) At the level of the fibre, the distribution of labelled material over the length of an average fibre becomes uniform; (2) At the level of
individual macrocycles: From initially pure \( 1_6 \) and \( 1^*_6 \) macrocycles, mixed hexamers \( 11^*_5, 11^*_4, 11^*_3, 11^*_2, 11^*_1 \) (in various isomeric forms) will form in statistical (binomially distributed) amounts.

![Figure 4.1.2](image)

**Figure 4.1.2.** Exchange between building blocks in solution leading to formation of mixed macrocycles (of which four are shown).

In **Figure 4.1.3** and in the following ones, average fibres are taken into consideration. Purple rectangles indicate both similar probabilities of finding a labelled or an unlabelled macrocycle and also an increased probability to find a mixed macrocycle, rather than a homogeneous one, in a particular position of the average fibre.

![Figure 4.1.3](image)

**Figure 4.1.3.** Supramolecular block copolymers (a) of \( 1_6 \) (blue rectangles) and \( 1^*_6 \) (red rectangles) undergo mixing over time, along the equilibration pathway. Colors show the distribution of the isotope label (blue = unlabelled; red = fully labelled; purple = partially labelled) along the fibre length of an average hexamer in an average fibre during the mixing process. An intermediate gradient state (b) is reached, eventually yielding fibres where hexamers have a uniform probability of containing \( 1 \) and \( 1^* \) (c).

The mechanism by which the approach to equilibrium proceeds may involve three different mixing pathways A, B and C, as shown in **Figure 4.1.4**. Mechanism A only involves exchange at the fibre ends: macrocycles transfer from fibre ends, where exchange can potentially happen (**Figure 4.1.2**), to the solution. Mechanism B
involves fibre ends exchange as in Mechanism A, but also features fibre breakage, exposing fibre cores to the exchanging solution.

Figure 4.1.4. Mixing pathways. a) Mechanism A: fibre end exchange; slow mixing expected. b) Mechanism B: breakage combined with fibre end exchange; mixing sped up by breakage. c) Mechanism C: diffusion limited exchange with building blocks in the fibre cores; fibre breakage irrelevant for mixing rates.
Mechanism C implies exchange between macrocycles in solution and macrocycles in the fibre cores. Note that exchange at sites other than the fibre ends does not occur to a significant degree in our system (vide infra), as confirmed by the observation that non-agitated samples show hardly any mixing, which excludes Mechanism C from being viable.

4.2. MECHANISTIC STUDIES BY MEANS OF REDUCTION EXPERIMENTS

Figure 4.2.1 shows a comparison between the MS spectra of hexamer macrocycles immediately after fibre growth (Figure 4.2.1a) and after 45 days of stirring at 200 rpm (Figure 4.2.1b). Note that a period of stirring of 45 days approximately corresponds to twice the length of an experiment that leads to full fibre growth from an initial library of unoxidized building blocks. A merged envelope of isotope peaks in the final MS spectrum, rather than the bimodal distribution of the two original envelopes of the peaks, shows that mixed macrocycles are formed (Figure 4.1.2). This indicates that the stability of the initial fibres is indeed only transient and confirms the kinetic nature of the fibres.

To confirm the block copolymer nature of the supramolecular aggregates, progressive reduction of the fibres was performed by using dithiothreitol (DTT) as a reducing agent. As shown in Figure 4.2.2, DTT irreversibly destroys the disulfide bonds of the hexamers predominantly at the fibre ends, reducing the latter back to building blocks (1 and 1*). This experiment showed high selectivity in the first stages of the destruction process (Figure 4.2.2c-g): the isotopically labelled macrocycles 1* are reduced first, followed by the unlabelled macrocycles 1. In the first half of the initial reduction experiment, the selectivity towards 1* hexamers is 80-90%, depending on the time interval between preparation and execution of the first experiment, indicating that the outer blocks are almost exclusively targeted. The small amount (10-20%) unlabelled 1 that is also reduced may be a result of fibre breakage. These data confirm that growth and deconstruction pathways predominantly involve reactions at the fibre ends.

To gather further insights into the equilibration pathways, we performed the experiments of Figure 4.2.2 at different times during equilibration. A freshly prepared block copolymer loses all of the labelled macrocycles in the initial stages of reduction (Figure 4.2.3a), leaving only unlabelled macrocycles to be detected in the second half of the reduction process. In contrast, the composition of a perfectly mixed fibre does not vary as the fibres is progressively reduced (Figure 4.2.3c), as the constituent macrocycles are fully mixed. Intermediate gradient distributions (Figure 4.2.3b) yield a more gradual change in the isotopic distribution during the reduction experiment.
Figure 4.2.1. MS spectra of quadruply charged hexamer ions from \(1+1^*\) fibres containing isotopically labelled macrocycles grown on previously prepared isotopically unlabelled macrocycle fibres: a) immediately after preparation; b) after 45 days stirring at 200 rpm. \([1]+[1^*] = 3.8 \text{ mM}\).
Figure 4.2.2. MS spectra of quadruply charged hexamer ions from a) fibres grown from unlabelled macrocycles; b) \(1^*_{16-16} \), block-co-fibres and the same block-co-fibres after progressive reduction with c) 15%; d) 30%; e) 45%; f) 60% and g) 75% DTT. [I]+[I\(^*\)] = 3.8 mM. Sample prepared at 1200 rpm.
Figure 4.2.3. Reduction experiments are performed on a sample by gradually deconstructing macrocycles from the fibre ends by reduction. The remaining mixture is allowed time to mix, after which another sample is subjected to gradual reduction. The same experiment performed at various stages of the equilibration pathway provides insights on the isotopic distribution within the fibres.

For every measurement, we calculated the fraction of isotopically labelled building blocks in the fibres \( F \), defined below, and plotted it against the equivalents of reducing agent used.

\[
F = \frac{[1^*]_{fibre}}{[1]_{fibre} + [1^*]_{fibre}} = \frac{m/z(1_{6}^{4+} + 1^*_{6}^{4+}) - m/z(1_{6}^{4+})}{m/z(1_{6}^{4+})} \times \frac{2}{3}
\]

where \([1^*]_{fibre}\) is the concentration of \(1^*\) as supramolecular fibres in the system and \([1]_{fibre}\) is the concentration of \(1\) as supramolecular fibres in the system, \(m/z(1_{6}^{4+} + 1^*_{6}^{4+})\) is the average mass to charge ratio of the isotopic peak distribution of the mixture of \(1\) and \(1^*\) in the supramolecular fibres (calculated from integration on the MS spectra), \(m/z(1_{6}^{4+})\) is the average mass to charge ratio of the isotopic peak distribution of pure \(1\) in the supramolecular fibres (calculated from integration on the MS spectra), \(2/3\) is a correction factor that accounts for the observation by MS of hexamers of \(1\) with a quadruple charge.

Starting with a 1:1 mixture of \(1\) and \(1^*\), when fibres have not been reduced yet, \(F = 0.5\). As we progressively add more reducing agent, \(F\) decreases (Figure 4.2.4), indicating that the remaining macrocycles in the fibre cores are richer in unlabelled macrocycles. As shown in Figure 4.2.4, the initial slope (which we refer to as \(M\)) of the plot of \(F\) against the amount of added reducing agent is less negative as the \(1^*\)-\(1\) block-co-fibres samples are given more time to equilibrate. The non-linear shape of the \(F\) plot reflects the initial block-copolymer nature of the fibres. The initial slope \(M\) of the \(F\) plot serves as a measure of the degree of mixing of the labelled building blocks from the cores to the periphery of the fibres: the higher the mixing, the higher (more positive) the slope \(M\).

As mixing occurs, the distribution in Figure 4.1.3a (block copolymer), shifts towards the one in Figure 4.1.3b (gradient distribution). This observation confirms that equilibration is proceeding at the fibre level, but is not yet complete (i.e., \(M \neq 0\)), even after 45 days.

Figure 4.2.5 shows the corresponding plot of \(F\) versus degree of reduction for a system where we mixed unlabelled and labelled building blocks \(1\) and \(1^*\) giving rise
to a completely random homogeneous distribution of labels (as shown in Figure 4.1.3c). As expected, Figure 4.2.5 shows a substantially flat line with a value of $F$ close to 0.5, indicating uniform mixing.

Figure 4.2.4. Fraction of labelled building block in the fibres ($F$) plotted against the equivalents of reducing agent DTT (with respect to $[1]$+$[1^*]$) supplied to a system of supramolecular fibres prepared by growing $1^*$ hexamers on previously prepared $1$ hexamer fibres ($[1]$+$[1^*]$=3.8 mM) and stirred at 200 rpm. Blue squares: 0 days; light blue circles: 4 days; light green upwards pointing triangles: 12 days; green downwards pointing triangles: 45 days.

Figure 4.2.5. Fraction of labelled building block in the fibres ($F$) plotted against the equivalents of reducing agent DTT (with respect to $[1]$+$[1^*]$) for uniformly mixed fibres formed from a 1:1 mixture of building blocks 1 and $1^*$. 
From the evidence in Figure 4.2.4 and Figure 4.2.5, the slope of the first part of the plot of $F$ versus degree of reduction provides a measure of the approach to equilibrium. An increase in the slope (towards less negative values) indicates that mixing of the building blocks has occurred. A slope of 0 indicates complete statistically uniform mixing. Thus, we have evidence of equilibration at the level of the macrocycles from the data in Figure 4.2.1 and evidence of equilibration at the level of the fibre from the data in Figure 4.2.2 and Figure 4.2.4.

In order to investigate the contributions of mechanisms A and B (Figure 4.1.4) to the approach of equilibrium, we monitored supramolecular block copolymer fibres at different stirring rates, i.e., 0 rpm (quiescent sample), 200 rpm, 800 rpm, and 1200 rpm, and performed reduction experiments at different points in time on the samples at each stirring rate. The stirring rates were chosen in order to reflect absence (quiescent sample) or presence (stirred samples) of mechanical forces in solution and to cover different average fibre lengths and rates of fibre breakage. The 1200 rpm, 800 rpm and 200 rpm samples contain fibres of increasing average length, while the quiescent sample contains the longest fibres. Samples were prepared starting with unlabelled hexamers $1_6$ formed at 1200 rpm and brought to the aforementioned stirring rates. Labelled building blocks $1^*$ were subsequently added. Over the course of 2 days, labelled hexamers $1^*_6$ would grow on the previously existing fibres in a growth/breakage process as described in Chapter 2.

As a result of this growth dynamics, all $F$ plots in Figure 4.2.4 do not resemble the ideal $F$ plot of perfect block copolymer fibres, which should have a slope $M$ equal to -1 up to complete destruction of the labelled building blocks (0.5 equivalents of DTT) and a slope of 0 up to complete destruction of all hexamers: actual block copolymer fibres that are grown under stirring conditions result in a statistical distribution in the positioning of the unlabelled cores, due to statistical fibre breakage during fibre growth. The resulting supramolecular block copolymer fibres were then monitored over the course of 6 weeks by taking samples and performing reduction experiments to obtain $F$ plots as described previously.

Figure 4.2.6 shows the MS spectra of hexamer macrocycle ions for the various stirring rates, at day 0, i.e., before the experiment (left) and at day 45, i.e., at the end of the experiment (right). These data show that mixing only occurs in the stirred samples. Furthermore, macrocycles mixing at the molecular level is slightly faster with the 200 and 800 rpm samples than for the 1200 rpm sample, while the quiescent sample shows no significant mixing with respect to the original sample. Figure 4.2.7 shows the variations in the slope of the plots of $F$ versus degree of reduction, which provides a measure of the degree of mixing with time, for samples at the different stirring rates. We found that over the course of 45 days the stirred sample show a significantly faster mixing than the quiescent sample. The samples at 200 and 800 rpm also show a slightly faster mixing than the sample at 1200 rpm.
The data in Figure 4.2.6 probing exchange of building blocks among the individual macrocycles are consistent with the data in Figure 4.2.7 probing mixing within the fibres. The samples that show faster building block mixing (Figure 4.2.6) also show faster mixing at the fibre level (Figure 4.2.7).
Figure 4.2.7. Initial slope ($M$) of the plots of $F$ versus the degree of reduction, against exchange time for block copolymers of $1_6$ containing isotopically labelled macrocycles grown on previously prepared isotopically unlabelled macrocycle fibres, comparing quiescent samples with samples agitated at different stirring rates.

The mixing at a molecular level that Figure 4.2.6 shows is confirmed by quantitative analysis by using the standard deviation, computed around the peak at m/z=1138.45 (the trough in the isotopic distribution of mixed labelled/unlabelled hexamers), of the isotopic distributions of the samples at the various stirring rates, as shown in Figure 4.2.8.

Figure 4.2.8. Plot of minus the standard deviation (around the peak at m/z=1138.45) of the isotopic distributions of samples at different stirring rates (before carrying out the reduction experiments) against exchange time, as a measure of building block mixing in the macrocycles.
The observed results (Figure 4.2.6, Figure 4.2.7, and Figure 4.2.8) can only be explained by introducing fibre breakage activating exchange at the fibre ends as the main responsible for macrocycles mixing, i.e., Mechanism B. The stark difference in mixing rates between the quiescent and the stirring samples over the observation period of 6 weeks cannot be explained by simply involving exchange at the fibre ends (Mechanism A), as the difference in fibre length between the quiescent and any stirred sample is not much larger than the differences in average fibre length between any two stirring rates chosen.

While comparing mixing rates for the stirred samples with the non-agitated samples allows us to make the conclusions above regarding Mechanism B being the prevailing exchange pathway, the differences in mixing rates in the samples at the various stirring rates cannot be deemed significant enough to draw any further conclusions from them.

Note that for the third Mechanism C to be dominating, the quiescent sample should show similar exchange rates as the stirred ones, as mixing would be limited by diffusion only (Figure 4.1.4c). This is not the case, as shown in Figure 4.2.6 and Figure 4.2.7, which shows that Mechanism C is not viable in our system.

TEM data in Figure 4.2.9 suggest that fibre length is highest for the 200 rpm sample and 800 rpm samples and lowest for the 1200 rpm sample. The fibre length of the quiescent sample is on average higher than for agitated samples and difficult to determine quantitatively due to most fibres spanning a length higher than the diameter of the TEM grids used for the measurements.

The observations clearly confirm that fibre end exchange needs to be activated by fibre breakage in order for mixing at the macrocycle level and at the fibre level to occur.

The fact that for the quiescent sample no significant mixing is observed, neither as a gradient variation throughout the average fibre, nor at the molecular level on the individual MS spectra, proves further that the main mechanism responsible for the mixing of the macrocycles after the fibres are built is fibre breakage, as the higher exchange rates for stirred samples cannot be accounted for by considering shorter fibres only. Fibre breakage therefore allows exchange with macrocycles in solution as the core macrocycles become the new fibre ends, after breakage.

It is interesting to notice, finally, that fibre breakage by itself wouldn’t explain the observed data, either. Macrocycles in the fibres also exchange building blocks, as this is observed from the MS spectra (Figure 4.2.1 and Figure 4.2.6). Hence, fibre exchange at the fibre ends, together with fibre breakage which activates it, must both be considered as important pathways to account for approach to equilibrium in our system.
4.3. CONCLUSIONS

In conclusion, we have explored the complexity of the kinetic pathways involved in kinetically controlled supramolecular self-assembly, approach to equilibrium and destruction by kinetically controlled processes.

Taken together, the data suggest that building block exchange during both fibre formation and destruction take place predominantly at the fibre ends. In quiescent samples exchange of building blocks between fibres is prohibitively slow. Macrocycles in the fibre cores will only exchange if they become exposed at a fibre end through a stochastic fibre disassembly-assembly process. But even with agitation, exchange is still not complete after 45 days, even though nonassembled macrocycles exchange building blocks within minutes. Thus, supramolecular interactions are remarkably efficient in conserving the structural make-up of, and information contained within, self-assembled self-replicating molecules.

Approach to equilibrium, which involves mixing of building blocks within the macrocycles and mixing of macrocycles within fibres, happens via fibre end exchange of macrocycles with the solution. Given the length of the replicator fibres (several hundred nanometers), such exposure through random dynamic variation in fibre length is highly unlikely. Hence, only agitation-mediated fibre breakage will allow macrocycles that were initially hidden within the fibre cores to become exposed at the fibre ends, where they can then undergo building-block exchange. Fibres not exposed to mechanical forces do not equilibrate efficiently, providing evidence on the nature of the exchange mechanisms at the fibre ends and activated by fibre breakage.

Our results imply that information contained in peptide-based replicators can be relatively robust, even where all reactions and interactions through which this
information is generated are reversible and can therefore also induce loss of information. An important requirement is that the time required to propagate the information (i.e., the time needed for replication) be shorter than the timescale with which information is lost. Thus, limited stability of information carriers is perhaps less problematic than often believed, as long as systems are sufficiently dynamic; that is, replication dynamics are sufficiently fast.

We expect the new insights to be useful in order to exert control on the supramolecular and macroscopic properties of nanofibrous materials. As growth and destruction are important to synthesize block copolymers, different blocks could have different roles, e.g., the core could have a structural role and the fibre ends be endowed with catalytic activity. Evidence that macrocycles can only undergo mixing by fibre breakage could be employed to have building blocks keep their structure or vary it depending on mechanical forces. Using the previous example, if fibre cores are strongly held together, then fibre ends will not mix with the cores, keeping functionalities well separated. Once the fibre ends are grown on the fibre cores, the latter will be shielded from exchange, unless breakage is introduced. Without this detailed knowledge, such control could not be planned.

The new evidence is also anticipated to play a role within origins of life studies, specifically in the understanding of transmission of information in the prebiotic world. In modern biology, information is processed by machinery that operates by direct access onto compatible genes. Early replicators, however, are more likely to resemble our fibres in their capability to read a building block pool depending on interactions of macrocycles with fibre ends, rather than with the macrocycle sequence in the fibre cores, thereby mimicking a sequential access mechanism. The prebiotic selection rules for replication and evolution could be hidden beneath our current mechanistic insights.

### 4.4. 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 H$_2$O 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.
**Mixed block supramolecular copolymer formation.** Supramolecular block copolymers were obtained starting with the preparation of 500 µL of a 3.8 mM solution of unlabelled monomers 1 up to the formation of hexamers 1₆ at 1200 rpm as described above. These were brought to various stirring rates, i.e., 0 rpm (non-agitated sample), 200 rpm, 800 rpm and 1200 rpm (stirring rate unaltered), and 500 µL of a 3.8 mM solution of labelled building blocks 1* were subsequently added. Over the course of 2 days, labelled hexamers 1*₆ grow on the previously existing 1₆ fibres. The resulting supramolecular block copolymer fibres were then monitored over the course of 6 weeks by taking 2.0 µL samples and performing reduction experiments as described below.

**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 3.6µm 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).

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<td>10,0</td>
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<tr>
<td>15,0</td>
<td>90,0</td>
<td>10,0</td>
</tr>
</tbody>
</table>

Table 4.4.1 | UPLC method. Eluent gradient used for UPLC analysis of libraries formed from building block 1 where 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>(i)₂</td>
<td>6.2</td>
<td>1517.7 [M+H]^+, 759.4 [M+2H]^3+</td>
<td>1517.5 [M+H]^+, 759.6 [M+2H]^3+</td>
</tr>
</tbody>
</table>
Reduction experiments. Over the course of 45 days, aliquots of 200 µL of a 3.8 mM solution of the libraries at the various stirring rates were gradually reduced using a freshly prepared aqueous solution of dithiothreitol (DTT, 38 mM), while the rest of each sample was left stirring. The aliquots were analyzed by UPLC-MS before starting the reduction and after each reduction step with 0.15 equivalents of DTT, which induced the reduction of 15% of the library. Successive reduction steps were applied up to the full reduction of the aliquots. The quadruply charged hexamer ion isotopic distribution at m/z around 1138 was analyzed by means of an Excel application calibrated against two samples rich in hexamers of $i$ and $i^*$, respectively, in order to compute the molar fraction $F$ of labelled hexamers in each sample before the reduction experiment and after each reduction step for every aliquot at the various stirring rates. Based on these data, $F$ plots were produced and initial slopes $M$ of the $F$ plots were calculated and plotted for the samples at the various stirring rates based on the aliquots taken at different points in time over 6 weeks.

Negative staining TEM. An aliquot of a sample taken from a peptide library was diluted 40 times in doubly distilled water. Shortly thereafter, a small drop of the diluted sample was deposited on a 400 mesh copper grid covered with a thin carbon film. After 30 s the droplet was blotted on filter paper. The sample was then stained with a solution of uranyl acetate deposited on the grid and blotted on filter paper after 30 s. The grids were observed in a Philips CM120 electron microscope operating at 120 kV. Images were recorded on a slow scan CCD camera.

Fibre length measurements. TEM micrographs were analysed using ImageJ. A scale was put on each micrograph according to its magnification, and an average length of each sample was determined by measuring fibres from the micrograph, using the measuring tool of ImageJ. The data was then transferred and analysed using MS Excel.
4.5. REFERENCES


