Chapter 2

The Influence of Building Block Design on the Outcome of Type and Size of Supramolecular Block Copolymers
2. The Influence of Building Block Design on the Outcome of Type and Size of Supramolecular Block Copolymers

2.1 Introduction

Supramolecular polymers find applications in bioactive materials\(^1\) and material science.\(^2\) Apart from their chemical structure, controlling the size and dimensions of the supramolecular polymers is important to produce well-defined materials.\(^3\) In conventional polymer synthesis, there are many examples where only covalent linkages between blocks yield assembling materials with controlled lengths. In 2007, Winnik and co-workers reported one of the earliest examples of such systems.\(^4\) They found out that block copolymers made from polyferrocenyldimethylsilane (PFS) forms cylindrical micelles under thermodynamic control with low polydispersity from which rigid block co-micelles can grow also with controlled lengths. As the monomer:seed ratio varies, crystallization driven self-assembly (CDSA) also allowed to synthesize monodisperse cylinders with controllable lengths in micro-meter scale.\(^5\) Additionally, this feature could also be used to obtain fiber-like block co-micelles from poly(3-hexylthiophene) (P3HT) seeds that were generated by the CDSA process and polystyrene (PS) units.\(^6\) The group later extended the scope of their crystallization driven block copolymers with controllable lengths including polycarbonate derivatives, polyselenophenes and perylenediimide amphiphiles.\(^7\)–\(^9\) However, controlling the size and shape of supramolecular polymers in aqueous solutions is a challenging task. Meijer and co-workers showed that handedness of benzene-1,3,5-tricarboxamide (BTA) based helical architectures in water can be defined with certain Coulombic interactions.\(^10\) Moreover, as in conventional polymer synthesis, living supramolecular polymerization has recently enabled access to self-synthesizing materials with very well-defined properties like uniformity and low polydispersity.\(^11\) In a more recent example, Takeuchi and co-workers reported that porphyrin-based supramolecular assemblies, with a polydispersity index of 1.1, form through a process resembling living polymerization featuring a nucleation-elongation mechanism.\(^12\)

Our group previously developed a system\(^13\) in which living polymerization was achieved through a nucleation-elongation mechanism with a peptide-based building block (Figure 2.1). As discussed in detail in Chapter 1 of this thesis, in an agitated solution, primary nuclei (short hexamer fibers) are formed and fragmented to produce more fiber ends (secondary nuclei) from which the highly polydisperse fibers grow. The key to enable control over fiber length is controllable primary and secondary nucleation. This is achieved by using pre-formed small seeds with uniform size so that fibers can grow with equal rate. The uniform seeds can be produced by either applying high shear stress or chemical degradation. More detailed discussion can be found in the following sub-chapters. We took steps to further elaborate the system by means of tuning the composition of the supramolecular block copolymers made out of two structurally closely related self-replicators. We investigated the morphology
of the system at different stages of self-assembly and the origins of the compositional preference of the system. The results show that small changes in structure design impact strongly on the nature of the self-assembling material.

Figure 2.1: Cartoon representation of self-synthesizing fibers growing through a nucleation-elongation mechanism. The living nature of the fibers was demonstrated with sequential addition of ‘food’ consisting mostly of smaller macrocycles. Steps i, ii and iii show the cycles in which half of the solution containing the fibers was replaced with food mixture. Average fiber length distribution (bottom-left) proves that almost perfect control of dispersity has been made possible.

In the second part of the chapter, we describe studies of a set of DCLs made from building blocks with halogen modifications on the amino acid side chain to expand the scope of our supramolecular block copolymers. Furthermore, we aimed to provide a direct proof of supramolecular block copolymer formation by directly visualizing the polymers without the need of a stain or a fluorescent dye.
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2.2 Results and Discussion

2.2.1 Building blocks utilized in mixed block co-fiber formation

For the supramolecular block copolymer experiments, we selected two building blocks both of which can give self-replicating 6-ring macrocycles (Figure 2.2). Building block 1 contains a phenylalanine unit in the peptide backbone and has previously been used to demonstrate the living supramolecular nature of our peptide replicators. Building block 2 differs only by one amino acid in the side chain; cyclo-hexylalanine instead of phenylalanine. By such selection, we aimed to minimize the differences in the self-assembly propensities of the two peptide building blocks.

![Figure 2.2](image)

**Figure 2.2:** Cartoon representation of building blocks utilized in mixed block co-fiber systems.

2.2.2 Experimental proof for the formation of A-B-A type supramolecular block copolymers

Formation of mixed block co-fibers was first confirmed with an initial control experiment. We prepared a DCL from building block 2 and let the system evolve until the DCL contains mostly the replicator 2_6_. We used 2_6_ as seed and mixed it with a pre-oxidized solution containing mostly 1_3_/1_4_ with a seed:food ratio of 1:3. Since the fibers of the two building blocks are indistinguishable by electron microscopy, we monitored the composition of the fibers by reducing them from their ends. Addition of consecutive batches of reducing agent showed a step-wise decrease in the amount of 1_6_. In contrast, the amount of 2_6_ remained almost unchanged until 25 % DTT was added (Figure 2.3b). These data suggest the formation of an A-B-A type supramolecular block copolymer in our system.
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Figure 2.3: a) Cartoon representation of the preparation of A-B-A type supramolecular copolymer made from building block 1 (3.8 mM in borate buffer, pH 7.8) which was pre-oxidized at least 80% with respect to monomer with 80 mM sodium perborate and a solution containing 2 as seed (3.4 mM in 50 mM borate buffer, pH 7.8) with a seed:food volume ratio of 3:1, b) Change in hexamer peak area in supramolecular block co-fibers upon DTT (1.9 mM) mediated partial reduction from the fiber ends. Sample was monitored by UPLC over a period of two days after adding the seed. Figure adapted from reference.13

We then performed a control experiment in which pre-formed hexamers 1 and 2 were mixed in a ratio of 3:1. This time, even a very small amount of DTT addition resulted in reduction of both of the hexamers (Figure 2.4b).

Figure 2.4: a) Cartoon representation of a control experiment in which pre-formed cyclic hexamer fibers consist of 1 (3.8 mM in 50 mM borate buffer, pH 7.8) and 2 (3.4 mM in 50 mM borate buffer, pH 7.8) were mixed and b) DTT (1.9 mM) mediated partial reduction of the mixture of fibers showing the decrease in the amount of both macrocycles right after adding 5% batches of DTT. Sample was monitored by UPLC over a period of two days after mixing the two solutions. Figure adapted from reference.13
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2.2.3 Control of fiber length and composition of the supramolecular block copolymers

In previous works from our group,\textsuperscript{15,16} it was shown that replication kinetics are greatly influenced by the mode of agitation. As the number of catalytically active fiber ends increase with mechanical agitation, the rate of replication is also increased. However, one common feature in these studies is the polydispersity of the fibers. Irrespective of the mode of agitation (stirring with varying rpm, shaking, or standing), fiber lengths were found to be far from uniform with relatively high polydispersity indices.\textsuperscript{17} In order to control the length, to obtain monodisperse fibers, we used a specially designed Couette cell which can apply high shear stress to a fiber solution as this is placed between two cylindrical surfaces one of which rotates at high speed (Figure 2.5).\textsuperscript{18}

![Figure 2.5](image)

**Figure 2.5:** Cartoon representation (left) of the Couette cell with a cylindrical inner part (1) that applies a high shear stress by rotation and picture of the cell used in the study (right).

We performed the experiments in three phases: The first involved obtaining the monodisperse seeds of fibers formed from the first building block using the Couette cell. In the second phase, we added food solution of the second building block and let the system assemble for several days allowing newly formed macrocycles to assemble on the ends of the short seed. We followed the growth by monitoring the solution by UPLC (at 254 nm). Lastly, once the growth was completed, we confirmed the actual block copolymer composition by TCEP-mediated step-wise reduction followed by monitoring the species distribution by UPLC. The reason for changing the reducing agent from DTT to TCEP is that TCEP is less toxic and more stable at room temperature than DTT. The entire experiment is shown schematically in Figure 2.6.
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Figure 2.6: Cartoon representation for the experimental design for the synthesis of block co-fibers and confirming their composition by means of partial reduction with TCEP from fiber ends. Unless otherwise stated, seed and food solutions were prepared from 1.9 mM building block 1 or 2 in 50 mM borate buffer (pH 8.2). Block co-fiber solutions were left unagitated and monitored by UPLC for at least one week before reducing small aliquots with a 1.9 mM aqueous solution of TCEP.

In the course of synthesizing different copolymers, we followed a systematic approach and set up the experiments with two different replicators (1 or 2) as seed with seed:food ratios of 1:2 and 1:4 from libraries that were 1.9 mM in building block. Before reducing the fiber solutions, we analysed the fiber lengths by electron microscopy (Figure 2.7). As Figure 2.7b shows, in the solution with seed:food ratio of 1:2, resulting fiber lengths are approximately three times of the length of 2 seeds with a PDI=1.1. When compared with the seed dispersity (PDI=1.05) we can clearly say that growth is still under control in such composition. We, then took a step further and increased the amount of food and prepared a solution with a seed:food ratio of 1:4. This time, the resulting fibers were considerably more polydisperse with PDI=1.20. One potential drawback of increasing the amount of food could be the self-assembly of 1 (instead of block co-fibers). However, when compared with previous studies, the time required for the formation of the block co-fibers is much less than the time required for the spontaneous nucleation of assemblies made from the ‘food’ without agitation. Based on this, we could eliminate the possibility of spontaneous assembly of cyclic hexamers of building block 1. These results show an important aspect of supramolecular block copolymers in which the relative amount of food (1mer/3mer/4mer solution) plays a decisive role in our ability to control the dispersity of the fibers.
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Figure 2.7: Histograms for fiber length distribution and corresponding polydispersity indices of a) $2_6$ fibers (1.9 mM building block concentration in 50 mM borate buffer, pH 8.2) after shearing at 67405 s$^{-1}$ for 30 minutes in a Couette cell, b) fibers from a pre-oxidized (80% with respect to monomer concentration with 40 mM sodium perborate) solution containing a mixture of $1_1$, $1_3$ and $1_4$ as ‘food’ and $2_6$ as ‘seed’ with seed:food volume ratio of 1:2 and c) fibers from a solution containing the same ‘food’ and ‘seed’ as in (b) with seed:food volume ratio of 1:4. Samples for fiber length analyses were blotted on TEM grids at least 10 days after cross-seeding.

We followed the species distribution by UPLC. Once essentially all food had been converted to hexamers, we reduced small aliquots from the solution with TCEP and followed the decrease in percent area of the hexamers and increase of the subsequent monomer formation. The results are shown in Figure 2.8.

Figure 2.9a and 2.9b shows that, in the first system (1:2 seed:food ratio), at least until 15% reduction, the amount of seed ($2_6$) remains almost constant and the percentage of $2_1$ is almost negligible compared to $1_1$. This finding indicates the formation of A-B-A type block fibers. Even with increased amount of food, the change in percent seed ($2_6$) is almost constant until at least 30% reduction (Figure 2.9c and 2.9d).
Figure 2.8: UPLC chromatograms (monitored at 254 nm) 12 days after preparation showing species the distribution after 5 µL aliquots from a DCL made from building blocks 1 and 2 as described above (with seed:food ratio of 1:4) were chemically reduced with different percentages of a 1.9 mM aqueous solution of TCEP: a) 0 %, b) 30 %, c) 50 % and d) 100 %.
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Figure 2.9: UPLC peak area over amount of reducing agent for the two cyclic hexamers (1\textsubscript{6} and 2\textsubscript{6}) in the fibers and monomers (1\textsubscript{1} and 2\textsubscript{1}) in the solution as a result of reducing with 1.9 mM aqueous TCEP solution: a) change in hexamer peak area b) change in monomer peak area when the seed:food volume ratio is 1:2; c) change in hexamer peak area and d) change in monomer peak area when the seed:food volume ratio is 1:4 (1.9 mM each in 50 mM borate buffer, pH 8.2).

Up to now, we focused on a system in which hexamers of 1 were grown on fibers constituted of hexamers of 2. As a next step, we inverted the system so that core of the anticipated supramolecular polymers contained the less hydrophobic phenylalanine instead of cyclohexylalanine. By doing so, we aimed to investigate how the nature of building blocks affect the block copolymer formation.

Different to the first system, this time we observed slightly shorter and more polydisperse 1\textsubscript{6} seeds (Figure 2.10a). In addition, regardless of the fiber composition, the length distribution showed better control than the first system. However, especially with a seed:food ratio of 1:4, many of longer fibers could not be counted due to bending of the fibers on the TEM grid. Therefore, the histograms and the PDI values are representative of only the stiffer fibers (Figure 2.10b and Figure 2.10c).
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Figure 2.10: Histograms as a result of the fiber length analyses and the corresponding polydispersity indices of a) seed \(1_6\) fibers in a DCL made from 1.9 mM building block 1 (in 50 mM borate buffer, pH 8.2) after shearing at 67405 s\(^{-1}\) for 30 minutes in a Couette cell, b) fibers in a 1.9 mM DCL made from 1.9 mM building block 2 (in 50 mM borate buffer, pH 8.2) and cross-seeded with short \(1_6\) fibers with the seed:food volume ratio of 1:2 and c) fibers with the seed:food volume ratio of 1:4.

We again followed the change in UPLC peak area for both \(1_6\) and \(2_6\) and the subsequent monomer formation. Partial reduction results showed that, despite the improved PDI values of fibers, both of the hexamers were reduced (Figure 2.11). This unexpected behaviour suggested unidirectional growth rather than a A-B-A type triblock formation.
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Figure 2.11: UPLC peak area over amount of reducing agent for the hexamers (1_6 and 2_6) in the fibers and monomers (1_1 and 2_1) in the solution as a result of reducing: a) change in hexamer peak area b) change in monomer peak area when the seed:food volume ratio is 1:2; c) change in hexamer peak area and d) change in monomer peak area when the seed:food volume ratio is 1:4 (1.9 mM each in 50 mM borate buffer, pH 8.2).

We lastly designed a control experiment which could shed light on the assembly mechanism. We added small portions of food mixture stepwise. In each step, we changed the composition and increased the fraction of 2_3/2_4 to form a gradient between the blocks. By doing so, we were still able to introduce small amount of 1_3/1_4 to 1_6 solution and were able to test if there is a recognition mechanism to induce triblock copolymer formation. Although the resulting fibers have a low PDI (1.05), concurrent reduction of the two hexamers was observed indicative of unidirectional growth of diblock co-fibers (Figure 2.12).
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Figure 2.12: a) Cartoon representation for the control experiment where 1.9 mM food solution containing different volume fractions is added stepwise to a solution of shortened 1\(_6\) seeds over days. Composition of the food is shown as volume percentage as the concentration is the same for both food solutions (1.9 mM in 50 mM borate buffer pH 8.2). Solution was kept two days without agitation between each step. b) Histogram showing the fiber length distribution after seeded-growth. Change in UPLC peak area of c) the hexamers (1\(_6\) and 2\(_6\)) and d) the monomers (1\(_1\) and 2\(_1\)) upon step-wise reduction with TCEP.

In conclusion, we showed that good control over fiber polydispersity could be achieved in seeded growth of fibers made from different building blocks. We observed an unexpected trend upon partially reducing the fibers from their ends: A-B-A type triblock co-fibers in which hexamers of 1 were grown on fibers constituted of hexamers of 2 and possible unidirectional growth when hexamers of 1 were used as seed instead of 2. In order to get more insight on self-assembly process and to shed light on any potential competing processes, we focused on the morphology of the two systems in the following sub-chapters.

2.2.4 Morphology and recombination of the fibers at different stages of supramolecular polymerization

At this stage, we focused on the morphology of the assembling fibers both before and after shortening them in the Couette cell. Since we observed controlled growth in either case, we reasoned that the morphology of the fibers might play a decisive role
in determining the resulting composition. For imaging, we first used atomic force microscopy (AFM).

While the air oxidized DCLs made from building block 1 gave rise to cyclic 6-mer fibers assembling as pairs with a twist with a period of approximately 40 nm, cyclic hexamer fibers from building block 2 showed long single fibers without any twisting (Figure 2.13).

![AFM images](image)

Figure 2.13: AFM images for the air-oxidized DCLs made from 1.9 mM building block 1 and 2 (in 50 mM borate buffer, pH 8.2) under mechanical agitation for; a) pairs of fibers of 1₆ and b) single fibers of 2₆.

After shortening the fibers using a Couette cell, the twisting in the 1₆ fibers could no longer be observed. Instead, there were more single fibers and almost no association with surrounding fibers was observed (Figure 2.14a). On the contrary, higher level of lateral association in shorhtened 2₆ fibers was observed with varying number of single fibers in each assembling unit (Figure 2.14b). AFM images also revealed that strongly sheared fibers were much thicker (up to 12.6 nm) than the fibers before applying high shear stress.
Subsequently the short $2_6$ seeds were mixed with $2_3/2_4$ containing ‘food’. In contrast to fibers in air-oxidized DCL made from building block 2, seeded growth showed a considerable difference in thickness of different segments of the same fiber (Figure 2.15). This result suggests that $2_6$ containing fibers are shortened and irreversibly transformed to a different morphology when high shear stress is applied. Yet they retain their ability to act as seeds and it appears that fibers can grow off these seeds from two sides.

We monitored the recombination of short seeds on TEM by measuring their poly-
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dispersity over time. By doing so, we hoped to obtain more information on the growth mechanism of fibers from single or stacks of sheared seeds. Figure 2.16a and Figure 2.16b show the change in average length and PDI for $1_6$ fibers with time. The length of the fibers shows a sharp increase in the first day followed by a more gradual increase up to an average close to 120 nm. Although this increase was previously shown to have no effect on seeded-growth of same type of $1_6$ fibers, it may dictate a unidirectional growth upon cross-seeding. In contrast, sheared $2_6$ fibers showed almost no change in terms of length and polydispersity. This observation suggests that $2_6$ fibers were shortened irreversibly to a new morphology which is not prone to recombination. We conclude that fibers can grow from both ends of the stacks of fibers which do not recombine.

Figure 2.16: Change in fiber length dispersities and consequent PDI values as a result of seed recombination in 1.9 mM DCLs made from building blocks 1 and 2 (equimolar in 50 mM borate buffer, pH 8.2). The DCLs were sheared at 67405 s$^{-1}$ for 30 minutes in a Couette cell and incubated without agitation for two weeks: a) length distribution and b) corresponding PDI values for $1_6$ fibers; c) length distribution and d) corresponding PDI values of $2_6$ fibers.
2.2.5 Steps towards directly visualizing the fibers

Due to the very similar dimensions of building blocks 1 and 2 and almost no difference in their contrast in electron microscopy we expanded the toolbox of building blocks by modifying phenylalanine residue in the peptide with heavy atom(s) attached to aromatic unit (Figure 2.17). We hoped that the introduction of heavy atoms would enable visualising block co-fibers directly by TEM using element mapping.

![Chemical structures of halogenated building blocks which were employed in direct visualization experiments.](image)

**Figure 2.17**: Chemical structures of halogenated building blocks which were employed in direct visualization experiments.

Halogenated peptides were synthesized via conventional solid phase methods except building block 3 which was prepared previously and shown to form a cyclic hexamer (3₆) replicator.¹⁴ DCLs made from peptides 4, 5 and 6 were set up in aqueous solution with 10 % (v/v) DMF as co-solvent to obtain homogeneous solution (Figure 2.18). Only the building block 7 could not be utilized in the following experiments due to difficulties in purification and dissolution in aqueous conditions.
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Figure 2.18: Relative UPLC peak area (%) for species in agitated 1.0 mM DCLs (in 50 mM borate buffer and 10% (v/v) DMF, pH 8.2) made from building blocks a) 4, b) 5, and c) 6. Side products arising from disulphide exchange with deletion products are grouped as ‘other’ for simplicity. UPLC chromatograms (monitored at 254 nm) of the same DCLs made from d) 4, e) 5, and f) 6 after 13 days under constant mechanical agitation (1200 rpm). Insets show the halogen functionality in each building block.
Among the four building blocks (3 to 6) to be used in serial cross-seeding experiments, we started with building block 3 which is already known to form a replicator of the same size as our seed 16.\textsuperscript{14} We first tested the fibers only with building block 3. Using electron microscopy, we wanted to see if we can take advantage of elemental analysis with such small molecular entities. In order to do that, we first changed our staining solution from uranyl acetate (UA) to phosphotungstic acid (PTA) which is another popular negative staining agent especially for biological specimens. Since characteristic X-ray signal for tungsten (1.775 keV from the M shell) is further away from the region of interest than that of uranium (3.164 keV from the M shell) we hoped to obtain more clear signals from sulphur and chlorine which are two specific elements contained in our self-assembled fibers.

\textbf{Figure 2.19:} Elemental analysis (EDX) of 3\textsubscript{6} fibers formed in a DCL made from building block 3 (1.9 mM in borate buffer, pH 8.2) from a point selection on the fiber: barely detectable S and Cl signals were obtained. The background spectrum is shown in red.

Unfortunately, as Figure 2.19 shows, we obtained very low signal intensities for the two distinctive elements in our system which were barely visible when compared to the background signal (shown in red). Thus, we changed our strategy to X-ray mapping of fibers instead of elemental analysis based on a single point selection. This part of the experiments was performed on a FEI Talos F200X instrument that has an improved detector geometry compared to the FEI T20 electron microscope used for the experiments shown in Figure 2.19. Elemental mapping was performed based on
an area selection from high-angle annular dark-field (HAADF) images (Figure 2.20).

![Figure 2.20: HAAFD image of bundled 3₅ fibers in a 1 mM DCL made from building block 3 (in 50 mM borate buffer and 10% (v/v) DMF, pH 8.2). Data collection: 1000 seconds.](image)

Elemental mapping was performed with three elements: tungsten (W) for the staining agent, sulphur (S) and chlorine (Cl) for the fibers. Although this technique is generally more useful for mapping larger assemblies, the resolution of the microscope allowed us to distinguish the fibers based on tungsten and sulphur. However, mapping only based on chlorine showed high interference with the background (Figure 2.21). This observation can be explained by the fact that one \(3₅\) macrocycle contains twelve sulphur atoms that are localized at the core. However, the smaller number of chlorine atoms are on the side chain of each phenylalanine unit. When compared with mapping based on sulphur, the local concentration of chlorines appears to be not high enough to map the fibers. However, when combined with length analyses from TEM images, this technique could still be qualitatively informative and help to visualize the diblock- or triblock-fibers.
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Figure 2.21: Electron mapping of fibers of 3_6 in a 1 mM DCL made from building block 3 (in 50 mM borate buffer and 10% (v/v) DMF) based on three elements: a) tungsten (W), b) sulphur (S) and c) chlorine (Cl).

Therefore, we set up DCLs in parallel with different seed:food ratios by using cyclic hexamer 1_6 as seed and 3_3/3_4 as food to investigate the block copolymer propensity of the system. We started with sheared 1_6 fibers of approximately 50 nm length (Figure 2.22) and performed cross-seedings with seed:food ratios of 1:1, 1:3 and 1:5 (Figure 2.23).
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Figure 2.22: TEM image for 16 seeds (1.9 mM in 50 mM borate buffer, pH 8.2) (left) that were sheared at 67405 s⁻¹ for 30 minutes in a Couette cell and the corresponding dispersity (right). The seeds were used for cross-seeding the 1 mM DCLs made from building block 3 (50 mM borate buffer and 10% (v/v) DMF, pH 8.2).

Figure 2.23: UPLC chromatograms (left) and the corresponding length analyses after seeding (right) of the libraries made from building block 31 (1.9 mM in 50 mM borate buffer and 10% (v/v) DMF, pH 8.2) and seeded with short 16 fibers (1.9 mM in 50 mM borate buffer, pH 8.2) with seed:food volume ratios of a) 1:1, b) 1:3 and c) 1:5.
As the amount of food was increased, the polydispersity of the fibers changed from 1.03 to 1.11. When the seed:food ratio was 1:1 or 1:3 we observed a larger proportion of mixed species which may hamper directly visualizing the blocks within the fibers. Therefore, for elemental mapping, we picked the fibers made from the library with seed:food ratio of 1:5.

This time, we performed elemental mapping on an area containing bundled fibers with a scale of 70 nm and collected data again for 1000 seconds (Figure 2.24 and Figure 2.25).

\[\text{Figure 2.24: HAADF image of bundled fibers from a DCL made from building block 3 (1.9 mM in 50 mM borate buffer containing 10% (v/v) DMF, pH 8.2) and seeded with short 16 fibers (1.9 mM in 50 mM borate buffer, pH 8.2) with seed:food volume ratio of 1:5. Data collection: 1000 seconds.}\]
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Figure 2.25: Electron mapping of fibers from a DCL made from building block 3 (1.9 mM in 50 mM borate buffer containing 10% (v/v) DMF, pH 8.2) and seeded with short 1₆ fibers (1.9 mM in 50 mM borate buffer, pH 8.2) with seed:food volume ratio of 1:5 based on three elements: a) tungsten (W), b) sulphur (S) and c) chlorine (Cl).

Unfortunately, as evident from Figure 2.25 utilizing building block 3, which bears a single chlorine on the para-position of the phenylalanine side chain, was not sufficient to provide a full electron mapping for self-assembling fibers. Therefore, we switched to other halogenated building blocks (4-6 in Figure 2.17) which formed cyclic 3mer in the DCLs made from each building block individually. However, upon cross-seeding with 1₆, we could not observe emergence of a specific species in the DCLs (Figure 2.26). Even though the cross-seeding with 1₆ resulted in formation of cyclic hexamers of the second building block, other species such as cyclic hexamers containing both of the building blocks and small amount of deletion products were found to be co-eluting.
2.3 Conclusions

We have shown that supramolecular polymerization is an effective tool to access A-B-A type block copolymers, but also that this behaviour is very dependent on the nature of the building block. A factor greatly affecting the formation of different types of supramolecular polymers appears to be the morphology of the sheared seeds which could potentially dictate whether triblock- or diblock-fibers are formed. Regarding the growth mechanism from single or stacks of nuclei and organization within these fibers, we can speculatively conclude that single fibers grow preferentially from one...
2. The Influence of Building Block Design on the Outcome of Type and Size of Supramolecular Block Copolymers of their two ends. Furthermore, the fact that we observe growth from both ends with stacks of short seeds suggests that the fibers within these seeds are (at least partially) oriented in an antiparallel fashion. For direct visualization of the type and size of these polymers, further improvement in building block design is needed which would require more localized heavy atoms together with complete dissolution of the building blocks in aqueous solution.

2.4 Acknowledgements

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2.5 Experimental Section

2.5.1 Materials

Doubly distilled water was used in all experiments. For preparing buffer solution, boric anhydride was purchased from Sigma Aldrich. Building Blocks 1, 2 and 3 were purchased from Cambridge Peptides Ltd. (Birmingham, UK) and had ≥95% purity. For the peptide synthesis N,N’-diisopropylcarbodiimide, oxyma, 1,2-ethanedithiol were purchased from Sigma Aldrich. Wang resin (0.57 mmol/g substitution) and Fmoc-protected amino acids were purchased from Merck Chemicals. Amino acid coupling reagents N,N-diisopropylcarbodiimide (DIC) and oxyma were purchased from Merck Chemicals. During 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.

2.5.2 General Methods

Peptide Synthesis and Purification

Building blocks 4 to 7 were synthesized on pre-loaded Wang resin by conventional SPPS methodology on 0.1 mmol scale. Amino acid couplings were made by using DIC/Oxyma methodology and cleaved from the polymeric resin with using a concentrated TFA (95 v/v%) solution. After cleavage from the polymeric resin, the crude peptides were purified on a Schimadzu prep-HPLC system by using a Phenomenex, Jupiter (10 µm, C5, 300 Å, 250 × 21.2 mm) prep-column.

Library and Sample Preparation

Building blocks 1 and 2 were dissolved to a concentration of 1.9 mM in borate buffer (50 mM, pH 8.2). More hydrophobic building blocks 4 to 7 were dissolved to a concentration of 1.0 mM in borate buffer (50 mM, pH 8.2) and 10% (v/v) DMF as co-solvent to achieve complete dissolution. DCLs were equilibrated in HPLC vials (12 × 32 mm) with Teflon caps and were left agitated on an IKA RCT hot plate stirrer at 1200 rpm. Prior to UPLC and LC-MS analyses, 5 µL from each (1.9 mM) sample was diluted 10 times with 5 µL DMF and 40 µL doubly distilled water. The same sampling protocol was followed for all UPLC and LC-MS analyses including block copolymer solutions.
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UPLC and UPLC-MS Analyses

UPLC analyses were performed on a Waters Acquity H- and/or I-class equipped with a diode array UV/Vis detector. UPLC-MS analyses were performed on a Water Xevo G2 UPLC/TOF with ESI ionization. All analyses were performed using a reversed-phase UPLC column (Phenomenex Aeris Peptide, 2.1 × 150 mm; 1.7µm). UV absorbance was monitored at 254 nm. Injection volumes were 10 µL (UPLC) and 5 µL (UPLC-MS) of freshly aliquoted sample with 0.3 mL/min flow rate.

All of the DCLs were analyzed using the following gradient method: Solvent A: ULC/MS grade water purchased from Biosolve (with 0.1 V/V % TFA as modifier) Solvent B: ULC/MS grade acetonitrile purchased from Biosolve (with 0.1 V/V TFA as modifier)

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<tr>
<th>Time, min.</th>
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<td>20.00</td>
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Transmission Electron Microscopy (TEM)

All images were obtained following negatively staining the sample on the grid. Samples were diluted 20-fold prior to deposition. A small aliquot (5 µL) of diluted sample was deposited on a 400-mesh copper grid covered with a thin carbon film (Agar Scientific). After 30 s of deposition, the droplet was gently blotted on filter paper. The sample was stained twice (4 µL each time) with a 2% uranyl acetate solution and blotted again on filter paper after deposition (30 s each time). The grids were observed on a Philips CM12 transmission electron microscope operating at 120 kV. All images were recorded on a slow scan CCD camera. 2D-chemical mapping experiments were performed at Utrecht University on a FEI Talos F200X (S)TEM operating at 200 kV.
Atomic Force Microscopy

AFM samples were prepared with freshly aliquoted 100 µL sample. Samples were diluted to a concentration of 10 µM and deposited to a mica surface (Grade V1, Van Loenen Instruments). Afterwards, extra solvent was evaporated under air and the surface was washed twice with UPLC grade water followed by blotting on paper. AFM images were recorded at room temperature on a Bruker Multimode 8 in Scan Asyst-Air imaging mode. A Scan-Asyst Air, silicon tip (Bruker) on a nitride cantilever was used with following parameters: 115 µm length, 25 µm width, 70 kHz resonance frequency, 0.4 N/m force constant. All images were analyzed on NanoScope Analysis 1.50 software.

2.6 Appendix

2.6.1 Histograms for seed recombination over days

Figure 2.27: Histograms showing fiber length distribution over two weeks for I₆ fibers in a DCL made from building block 1 (1.9 mM in 50 mM borate buffer, pH 8.2) and sheared at 67405 s⁻¹ for 30 minutes in a Couette cell at day 0.
2. The Influence of Building Block Design on the Outcome of Type and Size of Supramolecular Block Copolymers

Figure 2.28: Histograms showing fiber length distribution over two weeks for \( 2_6 \) fibers in a DCL made from building block 2 (1.9 mM in 50 mM borate buffer, pH 8.2) and sheared at 67405 s\(^{-1} \) for 30 minutes in a Couette cell at day 0.
Figure 2.29: Histograms showing fiber width distribution over two weeks for $2_6$ fibers in a DCL made from building block 2 (1.9 mM in 50 mM borate buffer, pH 8.2) and sheared at 67405 s$^{-1}$ for 30 minutes in a Couette cell at day 0.
2.6.2 AFM images of shortened seeds over days

**Figure 2.30**: AFM images for $\mathbf{1}_6$ fibers (1.9 mM in 50 mM borate buffer, pH 8.2) that were sheared at $67405 \text{ s}^{-1}$ for 30 minutes in a Couette cell at day 0 and monitored over the first four days of recombination.
Figure 2.31: AFM images for $2_6$ fibers (1.9 mM in 50 mM borate buffer, pH 8.2) that were sheared at 67405 s$^{-1}$ for 30 minutes in a Couette cell at day 0 and monitored over the first four days of recombination.
2.6.3 UPLC-MS Analyses

Figure 2.32: Mass spectrum of the cyclic pentamer $3_5$ from the UPLC-MS analysis of an agitated library made from building block 3 and cross-seeded with sheared $1_6$ fibers. Calculated isotopic profiles (species, abundance) for $[M+3H]^{3+}$: 1321.37 (M, 100%), 1321.71 (M+1, 99.95%), 1322.04 (M+2, 79.18%), 1322.37 (M+3, 63.25%); m/z calculated: 1981.26 [M+2H]$^{2+}$, 1321.37 [M+3H]$^{3+}$, 991.38 [M+4H]$^{4+}$; m/z observed: 1981.52 [M+2H]$^{2+}$, 1321.37 [M+3H]$^{3+}$, 991.28 [M+4H]$^{4+}$.

Figure 2.33: Mass spectrum of the cyclic hexamer $3_6$ from the UPLC-MS analysis of an agitated library made from building block 3 and cross-seeded with sheared $1_6$ fibers. Calculated isotopic profiles (species, abundance) for $[M+4H]^{4+}$: 1189.58 (M, 100%), 1189.83 (M+1, 88.93%), 1190.07 (M+2, 78.53%), 1190.33 (M+3, 63.91%), 1190.58 (M+4, 48.91%); m/z calculated: 1585.61 [M+3H]$^{3+}$, 1189.46 [M+4H]$^{4+}$, 951.96 [M+5H]$^{5+}$; m/z observed: 1585.45 [M+3H]$^{3+}$, 1189.58 [M+4H]$^{4+}$, 951.96 [M+5H]$^{5+}$. 
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Figure 2.34: Mass spectrum of the cyclic hexamer 1,3,5 from the UPLC-MS analysis of an agitated library made from building block 3 and cross-seeded with sheared 1,6 fibers. Calculated isotopic profiles (species, abundance) for [M+4H]4+: 1181.09 (M, 100%), 1181.33 (M+1, 87.27%), 1181.58 (M+2, 71.83%), 1181.83 (M+3, 54.61%), 1182.07 (M+4, 38.09%); m/z calculated: 1573.62 [M+3H]3+, 1181.21 [M+4H]4+, 944.77 [M+5H]5+; m/z observed: 1573.80 [M+3H]3+, 1181.09 [M+4H]4+, 944.88 [M+5H]5+.

Figure 2.35: Mass spectrum of the cyclic hexamer 1,3,4 from the UPLC-MS analysis of an agitated library made from building block 3 and cross-seeded with sheared 1,6 fibers. Calculated isotopic profiles (species, abundance) for [M+4H]4+: 1172.35 (M, 100%), 1172.59 (M+1, 98.08%), 1172.84 (M+2, 80.30%), 1173.09 (M+3, 58.16%), 1173.33 (M+4, 41.84%); m/z calculated: 1562.29 [M+3H]3+, 1172.47 [M+4H]4+, 938.17 [M+5H]5+; m/z observed: 1562.15 [M+3H]3+, 1172.35 [M+4H]4+, 938.29 [M+5H]5+. 
2. The Influence of Building Block Design on the Outcome of Type and Size of Supramolecular Block Copolymers

Figure 2.36: Mass spectrum of the monomer $4_1$ ($[\text{M+H}]^{1+} = 828.19$) from the UPLC-MS analysis of an agitated library made from building block $4$. Calculated isotopic profile for $[\text{M+2H}]^{2+}$ (species, abundance): 414.60 (M, 100%), 415.10 (M+1, 50.36%), 415.60 (M+2, 93.51%); m/z calculated: 414.63 $[\text{M+2H}]^{2+}$; m/z observed: 414.60 $[\text{M+2H}]^{2+}$.

Figure 2.37: Mass spectrum of the cyclic trimer $4_3$ from the UPLC-MS analysis of an agitated library made from building block $4$. Calculated isotopic profile for $[\text{M+3H}]^{3+}$ (species, abundance): 827.42 (M, 100%), 827.76 (M+H, 91.90%), 828.09 (M+2H, 81.61%), 828.42 (M+3H, 61.18%); m/z calculated: 1240.89 $[\text{M+2H}]^{2+}$, 827.26 $[\text{M+3H}]^{3+}$, 620.44 $[\text{M+4H}]^{4+}$; m/z observed: 1240.37 $[\text{M+2H}]^{2+}$, 827.42 $[\text{M+3H}]^{3+}$, 620.92 $[\text{M+4H}]^{4+}$. 
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Figure 2.38: Mass spectrum of the cyclic hexamer $4_6$ from the UPLC-MS analysis of an agitated library made from building block $4$ and cross-seeded with sheared $1_6$ fibers. Calculated isotopic profiles (species, abundance) for $[\text{M+4H}]^{4+}$: 1240.92 (M, 100%), 1241.16 (M+1, 88.17%), 1241.42 (M+2, 75.98%), 1241.66 (M+3, 63.18%), 1241.92 (M+4, 48.04%); m/z calculated: 1653.51 [$\text{M+3H}]^{3+}$, 1240.89 [$\text{M+4H}]^{4+}$, 992.70 [$\text{M+5H}]^{5+}$; m/z observed: 1653.69 [$\text{M+3H}]^{3+}$, 1240.92 [$\text{M+4H}]^{4+}$, 992.85 [$\text{M+5H}]^{5+}$.

Figure 2.39: Mass spectrum of the cyclic hexamer $1_4$ from the UPLC-MS analysis of an agitated library made from building block $4$ and cross-seeded with sheared $1_6$ fibers. Calculated isotopic profiles (species, abundance) for $[\text{M+4H}]^{4+}$: 1223.45 (M, 99.97%), 1223.70 (M+1, 88.40%), 1223.95 (M+2, 66.27%), 1224.22 (M+3, 54.91%), 1224.45 (M+4, 65.72%); m/z calculated: 1631.20 [$\text{M+3H}]^{3+}$, 1223.40 [$\text{M+4H}]^{4+}$, 978.72 [$\text{M+5H}]^{5+}$; m/z observed: 1631.07 [$\text{M+3H}]^{3+}$, 1223.45 [$\text{M+4H}]^{4+}$, 978.67 [$\text{M+5H}]^{5+}$.
2. The Influence of Building Block Design on the Outcome of Type and Size of Supramolecular Block Copolymers

Figure 2.40: Mass spectrum of the monomer 5\textsubscript{1} ([M+H]\textsuperscript{1+} = 840.17) from the UPLC-MS analysis of an agitated library made from building block 5. Calculated isotopic profile for [M+2H]\textsuperscript{2+} (species, abundance): 420.59 (M, 100%), 421.09 (M+1, 55.66%), 421.59 (M+2, 23.27%); m/z calculated: 420.13 [M+2H]\textsuperscript{2+}; m/z observed: 420.59 [M+2H]\textsuperscript{2+}.

Figure 2.41: Mass spectrum of the cyclic trimer 5\textsubscript{3} from the UPLC-MS analysis of an agitated library made from building block 5. Calculated isotopic profile for [M+3H]\textsuperscript{3+} (species, abundance): 837.41 (M, 100%), 837.74 (M+H, 97.35%), 838.08 (M+2H, 87.14%), 838.41 (M+3H, 63.48%); m/z calculated: 1255.88 [M+2H]\textsuperscript{2+}, 837.25 [M+3H]\textsuperscript{3+}, 627.94 [M+4H]\textsuperscript{4+}; m/z observed: 1255.36 [M+2H]\textsuperscript{2+}, 837.41 [M+3H]\textsuperscript{3+}, 628.41 [M+4H]\textsuperscript{4+}. 
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Figure 2.42: Mass spectrum of the cyclic hexamer 5₆ from the UPLC-MS analysis of an agitated library made from building block 5 and cross-seeded with sheared 1₆ fibers. Calculated isotopic profiles (species, abundance) for [M+4H]⁴⁺: 1255.89 (M, 100%), 1256.14 (M+1, 91.37%), 1256.39 (M+2, 77.55%), 1256.64 (M+3, 59.13%), 1256.89 (M+4, 41.56%); m/z calculated: 1674.52 [M+3H]³⁺, 1255.89 [M+4H]⁴⁺, 1004.71 [M+5H]⁵⁺; m/z observed: 1673.99 [M+3H]³⁺, 1255.89 [M+4H]⁴⁺, 1005.02 [M+5H]⁵⁺.

Figure 2.43: Mass spectrum of the cyclic hexamer 1₅₆ from the UPLC-MS analysis of an agitated library made from building block 5 and cross-seeded with sheared 1₆ fibers. Calculated isotopic profiles (species, abundance) for [M+4H]⁴⁺: 1236.18 (M, 100.00%), 1236.42 (M+1, 79.01%), 1236.66 (M+2, 47.45%), 1236.93 (M+3, 56.02%), 1237.18 (M+4, 33.29%); m/z calculated: 1646.53 [M+3H]³⁺, 1236.15 [M+4H]⁴⁺, 989.52 [M+5H]⁵⁺; m/z observed: 1646.71 [M+3H]³⁺, 1236.18 [M+4H]⁴⁺, 989.65 [M+5H]⁵⁺.
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Figure 2.44: Mass spectrum of the cyclic hexamer $1_{5}1_{4}$ from the UPLC-MS analysis of an agitated library made from building block 5 and cross-seeded with sheared 16 fibers. Calculated isotopic profiles (species, abundance) for $[M+4H]^{4+}$: 1216.47 (M, 100%), 1216.71 (M+1, 52.13%), 1216.98 (M+2, 40.30%), 1217.22 (M+3, 29.31%), 1217.46 (M+4, 37.76%); m/z calculated: 1621.84 [M+3H]$^{3+}$, 1216.42 [M+4H]$^{4+}$, 974.34 [M+5H]$^{5+}$; m/z observed: 1621.76 [M+3H]$^{3+}$, 1216.47 [M+4H]$^{4+}$, 974.08 [M+5H]$^{5+}$.

Figure 2.45: Mass spectrum of the monomer 61 ($[M+H]^{1+}$ = 850.21) from the UPLC-MS analysis of an agitated library made from building block 6. Calculated isotopic profile for $[M+2H]^{2+}$ (species, abundance): 425.61 (M, 100%), 426.11 (M+1, 53.95%), 426.61 (M+2, 26.26%); m/z calculated: 425.64 [M+2H]$^{2+}$; m/z observed: 425.61 [M+2H]$^{2+}$. 
Figure 2.46: Mass spectrum of the cyclic trimer \(6_3\) from the UPLC-MS analysis of an agitated library made from building block 6. Calculated isotopic profile for \([M+3H]^{3+}\) (species, abundance): 848.45 (M, 100%), 848.78 (M+H, 91.87%), 849.11 (M+2H, 66.66%), 849.45 (M+3H, 39.47%); m/z calculated: 1271.94 [M+2H]^{2+}, 848.28 [M+3H]^{3+}, 636.97 [M+4H]^{4+}; m/z observed: 1271.91 [M+2H]^{2+}, 848.45 [M+3H]^{3+}, 636.69 [M+4H]^{4+}.

Figure 2.47: Mass spectrum of the cyclic hexamer \(6_6\) from the UPLC-MS analysis of an agitated library made from building block 6 and cross-seeded with sheared 16 fibers. Calculated isotopic profiles (species, abundance) for \([M+4H]^{4+}\): 1272.20 (M, 100%), 1272.45 (M+1, 94.89%), 1272.70 (M+2, 80.27%), 1272.95 (M+3, 53.86%), 1273.20 (M+4, 35.32%); m/z calculated: 1695.59 [M+3H]^{3+}, 1272.42 [M+4H]^{4+}, 1017.94 [M+5H]^{5+}; m/z observed: 1695.73 [M+3H]^{3+}, 1272.20 [M+4H]^{4+}, 1018.07 [M+5H]^{5+}.
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Figure 2.48: Mass spectrum of the cyclic hexamer 1₂6₄ from the UPLC-MS analysis of an agitated library made from building block 6 and cross-seeded with sheared 1₆ fibers. Calculated isotopic profiles (species, abundance) for [M+4H]⁴⁺: 1249.98 (M, 100%), 1250.23 (M+1, 78.81%), 1250.47 (M+2, 58.75%), 1250.73 (M+3, 32.87%), 1250.98 (M+4, 22.28%); m/z calculated: 1665.60 [M+3H]³⁺, 1249.95 [M+4H]⁴⁺, 1000.36 [M+5H]⁵⁺; m/z observed: 1665.77 [M+3H]³⁺, 1249.98 [M+4H]⁴⁺, 1000.30 [M+5H]⁵⁺.

Figure 2.49: Mass spectrum of the cyclic hexamer 1₂6₄ from the UPLC-MS analysis of an agitated library made from building block 6 and cross-seeded with sheared 1₆ fibers. Calculated isotopic profiles (species, abundance) for [M+4H]⁴⁺: 1227.51 (M, 100%), 1227.76 (M+1, 82.55%), 1228.01 (M+2, 54.45%), 1228.26 (M+3, 32.23%), 1228.52 (M+4, 21.61%); m/z calculated: 1636.61 [M+3H]³⁺, 1227.46 [M+4H]⁴⁺, 981.96 [M+5H]⁵⁺; m/z observed: 1636.15 [M+3H]³⁺, 1227.51 [M+4H]⁴⁺, 981.92 [M+5H]⁵⁺.
2.7 References


2. The Influence of Building Block Design on the Outcome of Type and Size of Supramolecular Block Copolymers


