Supramolecular Double Helices from Small C₃-Symmetrical Molecules Aggregated in Water

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INTRODUCTION

The structure and function of biological macromolecules and synthetic polymers are intimately connected. Structural studies are therefore key to our understanding of how these macromolecules operate in their (biological) environment. Whereas groundbreaking structural studies on, for example, G-protein coupled receptors, ribosomes, and ion channels were performed using X-ray crystallography, recent technical developments in cryo-electron microscopy (cryo-TEM) are currently accelerating their structural characterization. Single-particle cryo-TEM allows researchers to obtain high-resolution structures through the imaging of many identical copies of biological assemblies. In the past few years, one-dimensional (1D) supramolecular polymers that are formed from proteins involved in neurodegenerative diseases were characterized in great detail as well. In patient-derived material, the polymers were observed to display different 3D architectures depending on the disease, illustrating the importance of detailed analyses.

For synthetic 1D supramolecular polymers, such structure-to-function relationships also exist. For example, the self-assembled architectures formed from building blocks as simple as tripeptides can be used to control the optical and electrochemical properties of pigments. In addition, the morphology of supramolecular nanostructures formed from tetrapeptides has been shown to affect their therapeutic potential. Photocatalytic hydrogen production was observed with supramolecular polymers that feature a crystalline packing of chromophores. Because of the high order in the packing of the molecules, such polymers can be structurally characterized in great detail. However, usually, synthetic supramolecular polymers lack any form of high order or periodicity at the nanometer level. Drawing molecular models that fit the nanoscopic to microscopic dimensions of the synthetic polymers is therefore common practice. To experimentally determine the molecular packing of noncrystalline architectures, one cannot rely on many atomically identical copies, as

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compared to the biological macromolecules. Moreover, recent studies show that these one-dimensional assemblies often exhibit a diversity in molecular order and hence molecular dynamics. For these reasons, the number of synthetic supramolecular polymers resolved at molecular resolution pales in comparison to the biological macromolecules and is a major hurdle to overcome in this part of nanotechnology. Cryo-TEM is also of crucial importance for the direct structural characterization of solution-born nanostructures. When multiple views from different directions of a self-assembled aggregate are combined, cryo-electron tomography (cryo-ET) can yield three-dimensional (3D) information that cannot be obtained using any other experimental technique. Recent developments allow researchers to better visualize organic structures in their native solution environment, including the introduction of graphene oxide (GOx) supports to realize thin but mechanically stable samples. In addition, the use of a Volta phase plate during image acquisition allows one to enhance the contrast without sacrificing resolution. Attractive candidates for these three-dimensional structural characterizations are synthetic supramolecular polymers in water, structures that are key components for the development of biomaterials in nanomedicine. We selected polymers that are formed from amphiphiles with a benzene-1,3,5-tricarboxamide core (BTAs, Figure 1a). Super-resolution microscopy showed micrometer-long fibers, but the resolution was not high enough to determine the exact diameter of several nanometers. Cryo-TEM imaging showed fibers with some periodicity; however, it was impossible to decide on the structure at the molecular level. Hence, most publications where these polymers were studied used cartoons of stacks of single molecules. These polymers were recently observed to be less dynamic than expected. The full deuteration of polymers that were diluted from H2O into excess D2O for hydrogen/deuterium exchange experiments required multiple days, and the polymers are remarkably resistent to disassembly by a good solvent. When mixed with more water-soluble analogues, the obtained copolymers became even more stable in time. Similar results have been recently obtained with BTAs that have carbohydrate groups at the periphery. In the past, we were never able to elucidate the detailed structure of such fibers. For that, advanced cryo-TEM and analyses are required to characterize these polymers at a length scale that is close to the resolution and noise limit.

Here, we show that these BTA molecules can assemble into different architectures in water. To our surprise, most of the species present are double helices, and the pitch can be tuned by the composition of the fibers. Two of the BTA-based monomers contain three linear C12-aliphatic tails that are covalently attached to a linear tetra(ethylene glycol) or a dendritic oligoglycerol-based side chain, C12-nBTA and C12-dBTA, respectively. One BTA molecule, C12-MeBTA, is similar to C12-nBTA but with a stereogenic center in the

Figure 1. Molecular structures and cryo-TEM images of C12-nBTA. (a) Chemical structures of C12-nBTA, C12-dBTA, C12-MeBTA, and C16-dBTA. Cryo-TEM images of supramolecular polymers formed from C12-nBTA at a concentration of 0.75 mg/mL in H2O (b) at a point resolution of 5 nm, as obtained from the contrast transfer function (CTF) at a defocus value of −10 μm (scale bar = 100 nm), and (c) at a point resolution of 2.4 nm as obtained from the CTF at a defocus value of −3 μm (scale bar = 50 nm).
C12-aliphatic tail. In another structure, C16-dBTA, the dendritic oligoglycerol-based BTA has a C16-aliphatic tail (Figure 1a). The ethylene glycol and glycerol units enable water solubility of the supramolecular polymers, whereas the aliphatic tails are required to shield the hydrogen bonds between the amides when the monomers stack on top of one another from water.41 Given the influence of temperatures encountered during the assembly process,42 the supramolecular polymers and copolymers were all prepared using the same heating–cooling protocol.43 First, a detailed analysis of the polymers formed from the C12-nBTA will be given, followed by the other three molecules in Figure 1a. Finally, we present the structures of the copolymers that are formed by mixing C12-nBTA with both C12-dBTA and C16-dBTA.

**RESULTS AND DISCUSSION**

**Double Helix of C12-nBTA by Electron Microscopy.** Conventional cryo-TEM imaging (0° projections) results in enough contrast to observe the high-aspect ratio supramolecular polymers formed by C12-nBTA (Figure 1b). The very long 1D polymers have a diameter below 10 nm and polymer ends cannot be observed. Interestingly, when we were fortunate to have a thin vitrified film and used zero loss energy filtering, the contrast is enhanced such that also a fingerprint-like pattern becomes visible. The image in Figure 1c has been recorded at a nominal magnification of 61000× and is dominated by a fingerprint-like pattern that consists of uniformly spaced dark lines. Fast Fourier transforming of the images shows that the observed spacing is about 4.7 nm (Figure S1). The width of the hydrophobic part of C12-nBTA is between 2 and 2.5 nm, which is roughly half the width of the repeat pattern of the fingerprint. Hence, we conclude that the dark lines originate from the more densely packed part of the C12-nBTA, which is the benzene core and the aliphatic tails. The bright interspaces are assigned to hydrated ethylene glycol, known to have an electron density comparable to vitrified water and hence to create poor contrast. It is hypothesized that the fingerprint originates from C12-nBTA molecules that are assembled at the air–water interface into long polymers (vide infra).43

Next to the fingerprint pattern, fibers can be clearly observed. When the long axis of the polymers is followed, there is a clear undulating variation in the diameter of the polymers (Figure 1c, Figure S2). 3D data is required to study the structure of the polymers in detail. However, the difference in contrast between the polymers and the surrounding vitrified water is small. To improve the signal-to-noise ratio in the images, we performed cryo-ET with vitrified films using GOx as a support layer to prepare thinner films (see Supporting Information),42 combined with zero-loss energy filtering. The sample was tilted from −64° to +64°, and low-dose electron diffraction spots at 2 Å, recorded after the tomographic tilt series, confirm the presence of a GOx support layer (Figure S3). Alignment and reconstruction of the tilt series was
Figure 3. Structural analysis of the polymers formed by C_{12}-nBTA, C_{16}-dBTA, and C_{12}-MeBTA. (a) Cryo-TEM image of C_{12}-nBTA polymers that was used for image analysis. (b) Class sum image of the 100 best aligned individual extracts, revealing the pattern of a double helix with a defined half pitch of hp = 19.9 ± 0.4 nm. (c) Surface representation of the volume reconstruction from the sum image (b) according to a previously reported helix reconstruction scheme.52 (d) Cryo-TEM image of C_{16}-dBTA polymers. (e) Class sum image of aligned image extracts with a diameter of 6.3 ± 0.4 nm for the pair of two stacks, with a random pitch. (f) Schematic showing the orientation of the two stacks relative to each other upon twisting. Arrows correspond to the two different orientations as indicated in (d). The diameter is approximately halved 2.9 ± 0.4 nm at a side view orientation. (g) CryoTEM image of C_{12}-MeBTA polymers. (h) Class sum image of aligned image extracts reveals a motif consisting of a single stack with a diameter of 3.7 ± 0.4 nm. Scale bars are 50 nm (a,d,g), 6 nm (e), and 3.5 nm (h).

subsequently performed in the software IMOD using the simultaneous iterative reconstruction technique.44,45 While varying the z-height of numerical cross sections through the reconstructed volume, we observed parts of the regular fingerprint pattern described above. This structure can be visualized entirely when tilting the cross section to become parallel to the air–water interface, indicating that the structure is present only on the air–water interface of the vitrified film (Figure S4 and Movie S1). Hence, this result confirms our hypothesis that the C_{12}-nBTA polymers form a self-assembled monolayer (SAM) at the air–water interface, which consists of orderly positioned molecules. Because of the small width of the hydrophobic part of C_{12}-nBTA (∼2–2.5 nm) that gives the contrast, and the fact that the fingerprint pattern can be observed only within a few nanometers in z-height at the interface, it is most likely that the molecules are organized in an edge-on orientation at the air–water interface (Figure S5). In addition to the SAM, we have observed small aggregates that are less than 3 nm wide (Figure S6).

To enhance the visibility of the C_{12}-nBTA supramolecular polymers, the signal-to-noise ratio of the reconstructed volume was improved by denoising using either a 3D median filter or a nonlinear anisotropic diffusion (NAD) filter.46 Subsequently, a numerical cross section was obtained in which the polymers are visible but not the SAM to allow an unbiased analysis of the polymers. Again, variations in the diameter of the polymers can be observed (Figure S7). The sizes of the smallest and largest diameters in the not-denoised volume are the same as those detected in Figure 1c (Figures S2 and S8). Remarkably, also the contrast along the length of the polymers appears to be not constant when all contrast from the polymer is included in the intensity averaged cross section (white box in Figure S7). We have reported similar observations also in the first paper on this molecule, but based on conventional cryo-TEM imaging.55 The 3D structures that could give rise to these variations in the contrast and the diameter of the polymers are a double helix and a twisted ribbon.47 Now, the improved contrast by using GOx and zero-loss energy filtering, and cryo-ET, allow for an in-depth analysis of the polymers in 3D.

To further investigate the polymers, we segmented two of them manually, which yielded 3D models of the polymers (see Supporting Information). Using a computer-aided visualization through the long axis of these polymers, we observe that the cross sections perpendicular to the long axis of the polymers are twisting in one direction (Movie S2). One of the polymers was carefully analyzed using its 3D model as a guide. At several locations along the polymer, which are indicated by the red stars in Figure 2a, we extracted vertical numerical cross sections (x−z planes) (Figure 2b). These provide a clearer view after denoising with NAD, and two separate dark spots are observed on opposite sides of the red stars in the top, middle, and bottom images (Figure 2c). In the other two images, which correspond to the high-contrast locations in Figure 2a, these separate dark spots merge into dark ellipses because of missing wedge artifacts.48 Since the high-contrast areas are not connected in the parts of the polymer in which this artifact does not play a role, there must be two separate stacks oriented side by side. Hence, this observation excludes the interpretation of a twisted ribbon structure. Instead, and intriguingly, this analysis on a length scale of several nanometers indicates that two separate stacks of the achiral C_{12}-nBTA exist, and that these wrap around one another to form a double helix. The width of the dark spots (∼2 nm, Figure S9) matches the estimated diameter of the hydrophobic part of the molecule. In addition, the diameter of the polymers as observed in both 2D and 3D (∼6 nm, Figures S2, S8, and S9) corresponds to the 6 nm diameter that was previously found through small-angle X-ray scattering.49

Subsequently, we investigated the periodicity in the twisting nature of the double helix. We fitted the segmented areas in the
x−z planes with an ellipse and calculated the angles of the long axis (yellow lines in Figure 2d) of these ellipses. These yellow lines indicate the directions in which the two stacks lie side by side, resulting in the graph as displayed in Figure 2e. The angles gradually increase from about −60° to +60° and subsequently drop more quickly to −60°, and this pattern has a constant period. Such a sawtooth pattern is expected for a continuous twist in one direction, and angles with absolute values between 60° and 90° are not observed because of missing wedge artifacts. The analysis of the periodicity in 3D cryo-TEM image: 23 (±2) nm (Figure S10). Gratifyingly, this period is in excellent agreement with 3.5 nm, which is only slightly larger than that in the double helix. This structure is reminiscent of the internal packing of protofilaments in amyloid fibrils that are composed of paired β-sheets.51 The class sum images of such protofilaments also show two high-density areas separated by an area of lower density, with a similar axial symmetry along the long axis of the fibrils. The individual stacks of the polymers have a diameter of ~3.5 nm, which is only slightly larger than that in the fingerprint at the air–water interface. The area between the stacks is gray and not black, implying that the individual stacks of BTAs are not completely apart from one another all the time. Transient local interactions between the two stacks of C12-nBTA polymers are expected to occur that may stabilize the double helix. The helical symmetry of the structure allows us to calculate the 3D volume from the sum image.52 The surrounding areas of the double helix repeat in C12-nBTA were masked, and the part of the sum image corresponding to the helix pitch (in agreement with the tomography data) was cyclically shifted in a vertical direction in 360 steps. These are the equivalent of 1° rotation steps of the double helix around the long axis and enable a complete 3D reconstruction of the volume. The resulting 3D reconstruction of one full pitch of the double helix (Figure 3c) and its length corroborate our previous observations (vide supra). It must be noted that the assembled molecules are achiral and no preferred handedness is expected. Therefore, we refrain from discussing the handedness of the double helices, which in principle is present in equal amounts of P and M helices.

Influence of Molecular Changes on the Fiber Structure. Next, we investigated several different supramolecular polymers formed by the other molecules—all based on the BTA core—as displayed in Figure 1a. The water solubility of the C12-nBTA was increased by substituting the tetra(ethylene glycol) unit with a dendritic oligoglycerol-based side chain at the C12-aliphatic tail. As a result, the C12-dBTA aggregates only into spherical micelles (Figure S13).53 Therefore, we re-established the balance between the hydrophobic and hydrophilic parts by increasing the aliphatic tail with 4 methane units in C16-dBTA (see Supporting Information). Gratifyingly, cryo-TEM images of C16-dBTA show that this molecule indeed forms supramolecular polymers (Figure 3d). After the techniques discussed above were applied, the results show that now two stacks are connected, not in a double helix, but as two adjacent stacks that appear as pairs (Figure 3e), as schematically shown in Figure 3f. Upon rotation along the structure, the overall width of the polymer changes by half (Figure 3d,f). This is confirmed by MSA and subsequent classification of a randomly extracted set of fiber environments. In the cryo-TEM images these pairs of two stacks are observed to bundle with other pairs, but also observed as individual pairs. However, no single stacks are observed because of the changes in the width of the polymers following their long axis. These ribbon-like structures differ significantly in their appearance as compared to the double helices because their contrast and thickness varies while they rotate in the volume. By rotation of a pair of two stacks like the one in Figure 3e by 90°, a motif with the diameter of a single stack 2.9 (±0.4) nm is obtained (not shown).

To show how subtle differences in the molecular structure influence the morphology, we show the cryo-TEM analysis of C12-MeBTA (Figure 3g,h). The C12-MeBTA is a chiral analogue with three methyl groups more than C12-nBTA that also forms supramolecular polymers.55 Previous work showed that C12-MeBTA polymers have more order in the arrangement of the BTA cores because of more persistent hydrogen bonds.56 After cryo-TEM imaging and generating a class sum image, the fibers look like a single unstructured polymer stack with a diameter of 3.7 (±0.4) nm (Figure 3h). The distinguished organization into two individual stacks as observed with C12-nBTA and C16-dBTA is obviously lost here. Moreover, MSA and subsequent classification revealed the tendency of the fibers to twist (Figure S14). These results show that careful analyses are a prerequisite for discriminating the differences in the 1D packing of supramolecular polymers that are caused by small differences in the molecular structure of the monomers. In the next section we continue by studying supramolecular copolymers.

Electron Microscopy of Mixtures of C12-dBTA and C16-dBTA with C12-nBTA. We studied mixtures of both dendritic BTAs (C12-dBTA and C16-dBTA) with C12-nBTA in different ratios. For the combination of C12-nBTA and C12-dBTA we have shown before that they copolymerize in ratios of 2:1, 1:1, and 1:2.59 After both molecules were mixed, the same heating–cooling protocol was used to be consistent with the sample preparation of the homopolymers (see Supporting Information). During this protocol the molecules are heated to 80 °C, which is above their cloud point temperature,52 and this is likely causing a temporal dehydration of the ethylene glycol...
and glycerol units. Similar to above, cryo-TEM micrographs of the respective copolymer samples were recorded and these were subsequently analyzed by MSA and classification techniques.

For the three C_{12}-nBTA/C_{12}-dBTA mixtures, several micrometers-long supramolecular polymers are observed as the dominant morphology (Figure S15). In Figure 4a representative class sum images of the copolymers are shown next to the sum image of C_{12}-nBTA. The structures of the 2:1 and 1:1 copolymers are also double helices. Both class sum images are representative, which means that most images show a double helix with only slight variations. The length of the half pitch of the double helices is determined to be 17.9 (±0.4) nm and 17.5 (±0.4) nm for the 2:1 and 1:1 copolymers, respectively. These values are significantly shorter than the 19.9 (±0.4) nm of the pure C_{12}-nBTA. The 1:2 copolymers look slightly different because the double helix seems to vanish (Figure 4a). However, an undulation in the density along the polymer can still be observed, with a half pitch of 14.9 (±0.4) nm, which is even shorter as compared to those of the 2:1 and 1:1 copolymers. Although no distinct separation between two individual stacks can be deduced for the 1:2 copolymer, the similar alternating pattern indicates that either a twisted stack is formed or that more than one stack of molecules interact with one another, as observed for the other compositions. These delicate variations in the length of the half pitch, depending on the composition, indicate that the polymers are likely well mixed at the molecular level and thus probably form copolymers with a regular composition.

The C_{12}-nBTA assembles into a double helix and the C_{16}-dBTA assembles into a pair consisting of two adjacent stacks (vide supra). Micrometers-long supramolecular polymers were also found as the dominant morphology for all mixtures of C_{12}-nBTA and C_{16}-dBTA. Despite the similarities in the cryo-TEM data at first view (Figure S16), also here subtle differences are observed in the images. In the case of the 2:1 and 1:1 copolymer, both twisted and ribbon-like morphologies are observed upon detailed analysis. For the 1:2 copolymer, individual fibers are observed predominantly, although bundles of these fibers are also observed. The variety of aggregates shows the flexibility and adaptivity of supramolecular copolymers. Subtle variations in the parameters of the preparation process can influence the resulting morphologies and induce multiple aggregation types, even within one sample. After qualitatively evaluating similarities and differences in the images, we focus on individual and structurally defined polymers for which structural analysis by MSA and subsequent classification of the cryo-TEM data appeared possible. The resulting class sum images for all copolymers of C_{12}-nBTA and C_{16}-dBTA are shown in Figure 4b. For the 2:1 mixture a double helix can be observed with a half pitch of 21.6 (±0.4) nm, slightly larger as compared to those of the C_{12}-nBTA homopolymers. When the amount of C_{16}-dBTA is increased, resulting in a ratio of 1:1, also a double helix is detected and for this copolymer a half pitch of 32.0 (±0.4) nm is determined. For the 1:2 mixture we observe a different structure, which is comparable to the homopolymers formed by C_{16}-dBTA itself and has two parallel stacks with diameters of 2.2 (±0.4) nm each. The overall width of the structure is determined to be 6.7 (±0.4) nm. According to the measured diameters, these polymers are likely composed of two stacks that each consist of a one-dimensional pile of the molecules. The two parallel stacks can therefore be thought of as a double helix with an infinitely long pitch. Thus, the copolymerization of C_{12}-nBTA with an increasing amount of C_{16}-dBTA leads to the unwinding of the supramolecular double helix. Similar to those of the mixtures with C_{12}-dBTA, the gradual change in the length of the half pitch suggests the formation of random copolymers.

**CONCLUSIONS**

By using detailed cryo-TEM and cryo-ET analyses with a wide variety of approaches, we have shown that supramolecular polymers in water—counterintuitively and against the current understanding—can form double helices. For the different BTA-based supramolecular polymers, we often observe structural diversity, but herein the presence of significant amounts of double helices that are micrometers long is reported. These double helices are observed in combination with single-stacked polymer monolayers at the air—water interface and smaller highly dynamic aggregates that are difficult to characterize. Most remarkable, in most cases the stable structure is a double helix with a regular repeat of which the length (specified as half pitch) varies with the molecular structure of the monomer as well as the composition of monomers in the supramolecular copolymers. In some cases,
even an infinite pitch is observed when two adjacent stacks that do not twist are present. The double helix structure was discovered for the C_{12}-nBTA through direct observation in 3D using cryo-ET. The regular period of \( \sim 23 \text{ nm} \) is remarkable given that the structure originates from an achiral molecule. This allowed us to analyze the polymers with a complementary approach that uses components of the single particle analysis method (alignment, multivariate statistical analysis, and classification), and subsequently perform an image-based 3D helical reconstruction. Another remarkable observation is the lack of helical inversion observed in the double helices over long lengths. Obviously, the number of fibers analyzed is too small to prove the 50:50 ratio between left-handed and right-handed helices. In the vitrified films we did not observe double helices of which the two stacks separate or supramolecular polymers that consist of a stack of single molecules, except the ones at the air–water interface.

Upon mixing of BTA-based amphiphiles in different ratios, the length of the half pitch of the double helix changes in the copolymers. When C_{12}-nBTA, a double helix forming monomer, is mixed with C_{12}-dBTA, a monomer that forms a pair of two stacks, the double helix gets unraveled. In other words, when C_{16}-dBTA is the majority in the mixture, the helix adopts the straight pairing of the majority component. Upon mixing of C_{12}-nBTA with C_{12}-dBTA, a spherical micelle forming monomer, the double helix gets more twisted, resulting in a shorter pitch when more C_{12}-dBTA is added. These data are in-line with a very good mixing of the two molecules and suggests that the number of molecules of each type is more or less constant over each length of the half pitch, although hard evidence for that is lacking. To show that the double helix is not present in all cases, we also show the single-polymer stacks formed by C_{12}-MeBTA, which illustrates how subtle the assembly process is. Although great care was taken in always using the same assembly conditions, small variations in the preparation method of supramolecular polymers can also result in different morphologies. The results presented show the importance of an in-depth analysis of all structures formed, and that, together with data on the dynamic exchange and insights into the mechanism of formation, it is possible to get a better understanding of the properties of these supramolecular assemblies.

Currently, it is experimentally impossible to elucidate which molecular interactions are responsible for the formation of the very regular double helix. Like in lipid bilayers, the hydrophobic shielding in the aggregates is proposed to be the best way to explain the dimer formation promoting double helices. However, this will require further advances in both microscopy and computational approaches. Some indications were already proposed in 2007 by Fejer and Wales. They explored the potential energy landscape for clustering disklike ellipsoidal particles and showed that fibers of one or more stacks can be obtained depending on the potential energy surface.\(^5\)

The double helix revealed in this study may be kinetically very stable and could therefore explain the slow monomer exchange of these polymers that was recently observed to occur during multiple days.\(^5\) However, at present, it is still too difficult to directly correlate the dynamics of supramolecular polymers and their high-resolution structures. We anticipate that the accurate determination of 3D structures in water will be important to establish the structure–function relationships of man-made materials. Moreover, it is expected that many more 1D fibers presently thought to be single stacks turn out to be double helices.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.0c08179.

Details on sample preparation and chemical synthesis, all details on how the TEM measurements were made and analyzed, and additional images (PDF)

- Move 1 (MOV)
- Movie 2 (MOV)

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The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We gratefully acknowledge A.J.H. Spiering for synthetic support. The authors acknowledge financial support from the Dutch Ministry of Education, Culture and Science (Gravity Program 024.001.035) and the ERC Advanced Grant SynMat (788618). H.G., S.H., and C.B. thank SFB76S of the German Science Foundation (DFG) for funding. E.W.M. thanks the Humboldt Foundation for support.

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Title:
Supramolecular Double Helices from Small C3-Symmetrical Molecules Aggregated in Water.

Date:
2020-10-14

Citation:
Lafleur, R. P. M., Herziger, S., Schoenmakers, S. M. C., Keizer, A. D. A., Jahzerah, J., Thota, B. N. S., Su, L., Bomans, P. H. H., Sommerdijk, N. A. J. M., Palmans, A. R. A., Haag, R., Friedrich, H., Böttcher, C. & Meijer, E. W. (2020). Supramolecular Double Helices from Small C3-Symmetrical Molecules Aggregated in Water. Journal of the American Chemical Society, 142 (41), pp. 17644-17652. https://doi.org/10.1021/jacs.0c08179.

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