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Atomic-level Engineering and Imaging of Polypeptoid Crystal Lattices

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Abstract

Rational design of supramolecular nanomaterials fundamentally depends upon an atomic-level understanding of their structure and how it responds to chemical modifications. Here we studied a series of crystalline diblock copolypeptoids by a combination of sequence-controlled synthesis, cryogenic transmission electron microscopy and molecular dynamics simulation. This family of amphiphilic polypeptoids formed free floating 2D monolayer nanosheets, in which individual polymer chains and their relative orientations could be directly observed. Furthermore, bromine atom side chain substituents in nanosheets were directly visualized by cryo-TEM. This is the first time electron microscopy has been used to localize distinct atoms in polymer crystals in position space, revealing atomic details inaccessible by conventional scattering techniques. While the polypeptoid backbone conformation was conserved across the set of molecules, the nanosheets exhibited different lattice packing geometries dependent on the aromatic side chain *para*-substitutions. Peptoids are inherently achiral, yet we showed that sequences containing an asymmetric aromatic substitution pattern pack with alternating rows adopting opposite backbone chiralities. These atomic-

level insights into peptoid nanosheet crystal structure provide guidance for the future design of bioinspired nanomaterials with more precisely controlled structures and properties.

Keywords: peptoid polymers, supramolecular assembly, peptoid 2D nanosheet, polymer amphiphiles, cryo-TEM, peptoid nanostructures

Significance

A fundamental challenge in material science is to understand the atomic-level structures of nanoarchitectures assembled from synthetic polymers. Here, we reported a family of sequence-defined polypeptoids that form free floating crystalline 2D nanosheets, in which not only individual polymer chains and their relative orientations but also atoms in nanosheets were directly observed by cryo-TEM. These atomic details are inaccessible by conventional scattering techniques. Using the feedback between sequence-controlled synthesis and atomic imaging, we observed how the nanosheet structure responses to chemical modifications at the atomic-length scale. These atomic-level insights open the door to the design of bioinspired nanomaterials with more precisely controlled structures and properties.

Introduction

Synthetic polymer-based 2D nanomaterials have large surface-area-to-volume ratios and can be produced with highly tunable chemical diversity; which gives them promise as platforms for filtration, catalysis, sensing, as well as, components in semiconductors, circuits and photovoltaics (1, 2). However, despite ongoing advances in design, synthesis and characterization, it is still a significant challenge to generate high aspect ratio 2D structures with atomic precision across micron (or greater) length scales.

Poly(*N*-substituted glycines) (a.k.a. polypeptoids), are a family of non-natural sequence-defined polymers that have received growing attention as

a platform to create biomimetic nanomaterials (3). Compared to polypeptides, polypeptoids (in which the side chain is appended to the amide nitrogen rather than the alpha carbon), lack hydrogen-bond donors and chiral centers along their backbones. This leads to excellent thermal processability, good solubility in common solvents, as well as enhanced resistance toward enzymatic and hydrolytic degradation (4, 5). The reduced structural complexity means that the peptoid properties and structure are dominated by the side chain identity and monomer sequence, which simplifies their design and engineering. In addition, the efficient, iterative submonomer solid-phase synthesis method, using primary amines as synthons, allows the incorporation of enormous side chain diversity and precise control over monomer sequence and chain length (4, 6). Polypeptoids can form ordered 2D materials with crystal-like packing of monomers into a hierarchical assembly (3, 7, 8). All these attributes, combined with their similarity to polypeptides in biocompatibility and potent biological activities, make polypeptoids an attractive material for self-assembly of 2D materials with biomedical and materials science relevance. Polypeptoid nanosheets have already shown promise as antibody-mimetic scaffolds for molecular recognition (9, 10), self-repairing membrane mimetics (7, 11), and as templates for biomineralization (12).

Polypeptoid 2D nanosheet crystals can be made from sequences that contain a small set of amphiphilic monomers, arranged either in an alternating (3, 13) or diblock sequence pattern (7). Amphiphilic diblock copolypeptoids can form nanosheets in high yields by simply dissolving in organic solvent/water mixture followed by subsequent evaporation of the organic solvent to induce the crystallization of the hydrophobic block (7, 8, 11). Amphiphilic diblock copolypeptoids of poly(*N*-decylglycine)-*b*-poly(*N*-2-(2-(2-methoxyethoxy)ethoxy)ethylglycine) (Ac-Ndc-Nte) (8) and poly(*N*-4-chlorophenylethylglycine)-*b*-poly(*N*-carboxyethylglycine) (N4Clpe-Nce) (7, 11) have been shown to form highly crystalline 2D nanosheet using this

evaporation method. Despite the efforts on investigating the structures of the diblock copolypeptoid nanosheets by a variety of techniques (e.g., X-ray scattering, atomic force microscopy (AFM), transmission electron microscopy (TEM) and molecular dynamics (MD) simulation), a comprehensive understanding of the nanosheet structures on the atomic length scale is lacking. In the reported studies of diblock copolypeptoid nanosheets, it was proposed that polypeptoid chains are packed in rectangular lattices anti-parallel along the *c* direction and parallel in the *a* direction (Fig. 1f) (7, 8, 11). X-ray scattering revealed that the nanosheet lattice has a universal *a* spacing between adjacent backbones (~ 4.5 Å) along the *a* direction, while the *c* spacing between adjacent backbones in the *c* direction varied depending on the chemistry of the side chains. However, scattering techniques, by their very nature, provide information across many unit cells in the material, and the data is obtained in reciprocal space, not in position space. Therefore, X-ray scattering alone cannot reveal atomic details of heterogeneity within a polymer crystal lattice. So far there has been no method capable of revealing how the side chain chemistry (e.g, size and electron distribution etc.) can affect the atomic-level packing interactions in polypeptoid assemblies. In order to rationally design the nanosheets with more precise control over their properties and structures, it is crucial to fully understand their crystal structures in atomic detail and investigate the impact of side chain chemistry and sequence on their atomic-level structures.

To elucidate the atomic-level structures of nanosheets, direct visualization of the individual chains within the nanosheet crystals is needed. Recent advances in low-dose cryogenic transmission electron microscopy (cryo-TEM) allow high-resolution imaging of radiation-sensitive biological macromolecules such as proteins with minimum electron beam damage (14-16); however, it remains a significant challenge to image synthetic soft materials, such as polymer crystals, at atomic resolution due to their

inherent structural heterogeneity. In our recent study, high-resolution images of crystalline diblock copolypeptoid nanosheets were obtained with direct visualization of crystalline grains and grain boundaries on atomic length scales, using a combination of crystallographic and single particle methods developed for cryo-TEM of biological macromolecules (8). Briefly, the electron micrographs were divided into small boxes comprising unit cells, which were then classified and averaged to reveal the shape and positioning of individual chains, and to map out the distribution of structural heterogeneity in the crystalline nanosheet. However, due to the substantial heterogeneity of those samples, the atomic model matching the major crystal motifs observed by cryo-TEM has not been established in our previous study (8). To obtain more detailed atomic-scale structural information, polypeptoid nanosheet crystals with high atomic-level homogeneity are desired. Additionally, we aimed to investigate the effect of side chain chemistry on the structure of crystalline nanosheets by engineering the electron-dense heavy atoms (e.g., Br or I) to particular locations in the crystal lattice. The increased contrast in low-dose EM micrographs enables the direct imaging of heavy atoms after image processing.

In this contribution, we designed and synthesized a series of amphiphilic diblock copolypeptoids with the same hydrophilic poly(*N*-2-(2-(2-methoxyethoxy)ethoxy)ethylglycine) block (Nte) and a series of *N*-2-phenylethylglycine-based hydrophobic blocks bearing a systematic series of aromatic ring substituents, varying in size and electron withdrawing or donating character. We synthesized analogs with hydrogen, fluorine, chlorine, bromine, iodine, nitro, methyl and methoxy substituents at the *para* position of the ring, and bromine substituent at the *meta* position of the ring, and examined the impact of these substituents on the crystal packing in the lattice. We previously demonstrated that *ortho* substituents are not tolerated in *N*-(2-phenylethyl)glycine containing peptoid nanosheets (13). All these diblock copolypeptoids (except the one with the nitro substituent) formed 2D

nanosheet crystals in water. The atomic-scale structures of the crystalline nanosheets and the effect of aromatic side chain substituents on their crystal packing were studied by low-dose cryo-TEM and X-ray scattering. The combination of sequence control offered by submonomer peptoid synthesis, atomic-level imaging and molecular dynamics (MD) simulations, provides a powerful platform to structurally engineer synthetic materials with the same precision as found in protein engineering. The atomic-level insights revealed in the polypeptoid crystals in this study will open more fundamental questions on structure-property relationships, and provides the foundation upon which to direct the rational design of polypeptoid 2D materials with atomic precision.

Results and Discussion

Design and synthesis of amphiphilic diblock copolypeptoids. Polypeptoids lack chiral centers and -NH···O=C- hydrogen bonding along the backbone, which results in self-assembly governed predominantly by interactions between side chains. Our goal is to elaborate the atomic structure of the polypeptoid nanosheet crystal lattice, without disrupting their packing motif, by making subtle variations to the side chain structure and monomer sequence. We chose to examine diblocks based on the *N*-(2-phenylethyl)glycine (Npe) monomer with a 4:6 block composition of four Nte monomer and six Npe monomer as this sequence motif was established as one of the shortest known crystalline nanosheet-forming sequences (7). We then explored their packing rules by varying the *para*-substituents of the aromatic side chains. Thus, a series of sequence-defined diblock copolypeptoid decamers containing a hydrophilic block of poly(*N*-2-(2-(2-methoxyethoxy)ethoxy)ethylglycine) (Nte) and a hydrophobic aromatic block bearing different *para*-substituted 2-phenylethyl side chains, were synthesized by solid-phase submonomer synthesis (Fig. 1a). A series of *para*-substituents, ranging in size and containing both electron-withdrawing groups (F, Cl, Br, I, NO_2) and electron-donating groups (CH_3, OMe) were

chosen. The compound with bromine at the *meta* position of the phenyl side chains was also synthesized to investigate the effect of substitution position on the formation of nanosheets and the crystal lattice packing. All the polypeptoids were purified by reverse-phase HPLC to obtain ≥95% molecular purity. Our ability to directly image peptoid nanosheet lattices with atomic resolution by cryo-TEM (8), coupled with our ability to produce these samples from high-purity chains, provides a unique opportunity to understand the atomic-level structures and detect atomic structural differences in crystal packing resulting from the different sequences. A non-charged hydrophilic Nte block was chosen to eliminate interference of charged molecules with the electron beam.

Self-assembly of diblock copolypeptoids into crystalline nanosheets. Diblock copolypeptoid nanosheets were formed in aqueous/THF solution by an evaporation method to drive the crystallization of the aromatic hydrophobic blocks. Interestingly, all the polypeptoids except Nte_4-$N4NO_2pe_6$ (**6**) formed high aspect ratio nanosheets with nanometer scale thickness (≤ 4 nm, Tables 1 and 2), as evidenced by TEM and AFM analysis (Figs. 1b-e and SI Appendix, Figs. S21-S26). The most electron-withdrawing nitro group (Hammett parameter $\sigma_p = 0.75$, σ_p is a measure of inductive electron-withdrawal or donation by the substituent at the *para* position of benzene (17)) in compound **6** somehow disfavors its packing into ordered nanosheets. Sharp diffraction peaks from X-ray scattering (see WAXS section below) and endothermic thermal transitions from DSC analysis (see calorimetry section below) demonstrate that all the polypeptoid nanosheets are highly crystalline. As expected, the Nte_4-$N4NO_2pe_6$ (**6**), without forming ordered nanosheet structures, showed broad X-ray scattering peaks and no melting transition.

Cryo-TEM imaging of nanosheets. All the nanosheets adopt the same rectangular crystal lattice packing with polypeptoid chains packed in an anti-parallel orientation along the *c* direction and parallel along the *a*

direction (Fig.1f), as evidenced by the universal *a* spacing (4.5 Å) and varied *c* spacing dependent on the atomic size of the aromatic substituents obtained from X-ray scattering measurement of the nanosheets. The *c* spacing gradually increased from 16.4 Å to 19.0 Å as the *para* substitution was changed from fluorine to iodine (Table 2). In this study, four nanosheets (**1**, **4**, **7** and **9**) were chosen to further investigate their packing structures at atomic level. Nte_4-Npe_6 (**1**) with hydrogen as a *para*-substituent is the parent compound. The Nte_4-$N4Brpe_6$ (**4**) with electron-dense bromine atoms was chosen to provide increased contrast in low-dose cryo-TEM micrographs for atom localization. The electron-withdrawing bromine atoms at the *para* positions (σ_p = 0.27) (17), also change the electron distribution of the phenylethyl side chains. Nte_4-$N4mpe_6$ (**7**) is similarly sized to the bromine-substituted compound but contains slightly electron-donating methyl groups at the *para* position (σ_p = -0.19) (17) and was included as a counterpart to **4**. Polypeptoid crystals have been previously shown to form rectangular lattice (Fig. 1f) (7, 18). We therefore designed Nte_4-$(N4BrpeNpe)_3$ (**9**) to have an asymmetric side chain substitution pattern, where one face of the molecule contains phenylethyl and the other face has 4-bromophenylethyl side chains, in order to disrupt the substitution symmetry of the polypeptoid chains and see its impact on the crystal packing.

We preformed low-dose cryo-TEM imaging on the vitrified hydrated crystalline nanosheets **1, 4, 7** and **9** and the low-dose micrographs were further analyzed by sorting and averaging the small section of micrographs comprising unit cells using the protocol reported in our previous study (8). The parameters and contrast transfer function (CTF) corrections of low-dose micrographs that were used for processing can be found in SI Appendix, Figs. S27 and S28 and Table S2. The averaged images of nanosheets are shown in Figs. 2c, 3c, 4c and SI Appendix, Fig. S30. The corresponding Fourier transforms (FFT) are shown in SI Appendix, Figs. S29 and S30. The signal-to-noise (SNR) ratio in the averaged images is significantly enhanced compared

to that in original low-dose micrographs. The reflections at 1.5 Å along *a* direction can be clearly observed. In addition, the CTF corrections of low-dose micrographs suggest the presence of detectable Thon rings in FFTs up to 1.4 Å. These results indicate the reliable image phase information in the averaged images (see SI Appendix, Fig. S28 and Table S2). In addition, at least 3 micrographs with different defocus values for each sample were used for CTF compensation during processing (see defocus values in SI Appendix, Table S2). The images shown in Figs. 2c, 3c, 4c and SI Appendix, Fig. S30 are the projection views from the top view of the untilted nanosheets (*b* direction). Cryo-TEM imaging of the sheets revealed that the viewing angle is aligned with polypeptoid chains in the *b* direction, which are oriented perpendicular to the plane of the sheet, as evidenced by the projection of superimposed bromine atoms at the side chains shown in averaged images. The V shape shown is the projection of a single peptoid chain along the *b* direction. The bright dots lining up along the apex of a V-shaped structure and the arms extending out from both sides of the bright lines are the projections of backbones and side chains from the top view of the sheets, respectively. Interestingly, the dihedral angles of V shapes in the four sheets are similar (~104°). Also, all the sheets in this study show a universal *a* spacing (~ 4.5 Å), which is the adjacent backbone distance along the *a* direction and is consistent with our previous study (18). The *c* spacing, the distance between backbones in adjacent rows along the *c* direction (adjacent vertical bright lines in Figs. 2c, 3c, 4c and SI Appendix, Fig. S30), increased with increasing size of the aromatic side chains: (sheet **1**, 16.2 Å) < (sheet **9**, 17.2 Å) < (sheet **4**, 18.2 Å) = (sheet **7**, 18.2 Å).

The chosen crystalline nanosheets (except sheet **7**) exhibit homogeneity at atomic scale in terms of the presence of crystal motif, as shown in the unit cell distribution maps (SI Appendix, Figs. S31 and S32). Fig. 2c shows that the polypeptoid molecules with phenylethyl side chains in sheet **1** arranges into parallel V-shaped rows along *c* direction. In sheet **4**,

the bromine atoms (three atoms superposed in column) at the *para* position of the phenylethyl side chains are clearly observed, showing a tip-to-tip packing from the top view of the sheet (Fig. 3c). This is, to our best knowledge, the first time to localize atoms in polymer crystals in position space with image phase information. Interestingly, the V-shaped structure, which is the projection of individual polypeptoid chain from the top view of the nanosheets, is packed in different orientation (parallel vs. anti-parallel) along the *c* direction depending on the *para*-substitution of the phenylethyl side chains: sheet **1** bearing phenylethyl side chains showed all parallel V-shaped packing while sheet **4** bearing 4-bromophenylethyl side chains exhibited all anti-parallel V-shaped packing (Figs. 2c and 3c).

We asked if the all anti-parallel V-shaped packing in sheet **4** is due to the electron-withdrawing character of *para* Br atoms (σ_p = 0.27) by synthesizing Nte_4-$N4mpe_6$ (sheet **7**), which has similarly sized but slightly electron-donating methyl groups at the *para* position (σ_p = -0.19) (17). Surprisingly, in contrast to sheets **1** and **4**, sheet **7** exhibited about 60% anti-parallel V shapes and 40% parallel V shapes, initially suggesting that the electron-withdrawing character of Br is not the primary contributor to the anti-parallel V packing motif. The heterogeneity in sheet **7** is more prevalent along the *c* direction than the *a* direction, as shown in the distribution map (SI Appendix, Fig. S32), suggesting that polypeptoid chains are more likely to flip in the *c* direction during self-assembly.

The nanosheets with phenylethyl and 4-bromophenylethyl side chains exhibited all parallel and anti-parallel V shapes, respectively. This motivated us to design the Nte_4-$(N4BrpeNpe)_3$ (sheet **9**) with an asymmetric pattern of alternating phenylethyl and 4-bromophenylethyl side chains. Surprisingly, sheet **9** adopted all parallel V shapes and the phenylethyl side chains are packed against 4-bromophenylethyl side chains. However, the packing between the shorter phenylethyl side chain and the longer 4-bromophenylethyl side chain is clearly distinguishable in the cryo-TEM image

(Fig. 4c). Moreover, only one *c* spacing of 17.2 Å is observed in sheet **9**, which is intermediate between the *c* spacings of sheet **1** (16.2 Å in Fig. 2c) and sheet **4** (18.2 Å in Fig. 3c). The difference of spacing is caused by different van der Waal's radius of bromine (~ 1.85 Å) and hydrogen atoms (1.2 Å).

The different orientations of V shapes (parallel vs. anti-parallel) is the result of multiple weak interactions between the interfaces along the *c* direction, making it difficult to predict. However, our studies indicated that there is not clearly one dominant factor, steric or electrostatic character of *para*-substituents, for the different orientations of V shapes. Further investigation probing the origin of the packing is underway.

Molecular dynamics simulations. We used the information derived from the atomic-scale resolution averaged images to perform molecular dynamics (MD) simulations to get a better understanding of the nanosheet structures at an atomic level. Molecular dynamics simulations were conducted via NAMD package (19) together with MFTOID force field (20) and the resultant models are shown in Figs. 2-4. Initial coordinates for MD simulations were generated with all backbones in the *cis*-amide conformation as suggested in recent studies (18, 21). The backbone Φ and Ψ angles of the Cα alternated between Z_R (~ 90, ~ 150) and Z_S (~ -90, ~ -150) (22). Peptoid chains were arranged in blocks of 12 stacked parallel in the *a* dimension, with these blocks arranged ether parallel or anti-parallel to one another in the *c* dimension to form a periodic monolayer configuration. The models generated are well matched with the averaged images, as shown in Figs. 2c, 3c and 4c of their overlapped structures. Remarkably, the polypeptoid backbone fold itself is identical in all four sheet lattices (sheets **1, 4, 7** and **9**), as shown by their superimposable structures (SI Appendix, Fig. S34), suggesting the capability of atomic engineering of the sheet structure predictively. Interestingly, the molecular simulation required racemic pairing of backbones with opposite chirality in sheet **9** bearing alternating

phenylethyl and 4-bromophenylethyl side chains (as seen in Fig. 4b). Many studies have shown that racemic crystals of oligoamides (23, 24), peptides (25, 26) and proteins (27, 28) grow much more readily than the enantiomeric pure crystals. There is also a genuine tendency that racemic crystals are more stable and denser than their chiral counterparts (29). Our findings in sheet **9** are consistent with those studies. In addition, the relaxed molecules and their potential projection maps exhibit the features as same as that those observed in the averaged cryo-TEM images (SI Appendix, Fig. S33).

Nanosheet calorimetry. To investigate the effect of *para*-substitution of phenylethyl side chains on the thermal properties of the nanosheets, DSC measurements were conducted on nanosheets both in solution and in the dry state. As the size of the *para* substituents increased from H to F, Cl, Br and I, the melting temperature of the dry nanosheets gradually increased from 93 ^{o}C to 172 ^{o}C due to the enhanced Van der Waals interaction between molecules (Tables 1 and 2). Surprisingly, sheets **4** and **7** with the *para*-positions of phenylethyl groups fully substituted with -Br and -CH_3, respectively, exhibited a much higher melting temperatures (~150 ^{o}C) than those of sheets **1** and **9** (~95 ^{o}C) with either no or partial *para*- substitution (Table 1 and Fig. 5). We attribute this to the different packing geometry of side chains between rows. Since the overall molecular conformation and face-to-face *a* direction stacking is identical among all four molecules (see MD simulations section), one of the primary differences among their lattices becomes the interface between adjacent rows in the *c* direction. The *para*-substituents of adjacent rows essentially point directly at one another. Sheets **4** and **7** with fully *para*-substituted side chains had stronger interaction between rows due to the interdigitated packing between *para*-substituents of the side chains. Sheets **1** and **9** on the other hand, with either no or partial *para*-substituents had a slight offset between rows along the *b* direction, resulting in a thicker sheet and less chain overlap. This effect is evidenced by the AFM and XRD data, showing that sheets **1** (3.2

nm) and **9** (3.0 nm) are thicker than that of sheets **4** (2.6 nm) and **7** (2.7 nm) (Table 1). The thickness of the sheets obtained from AFM is lower than that measured from XRD, which should be due to the different techniques used, the different interaction between the sheets and that between the sheets and the AFM substrate. Analysis of the MD simulations of these four lattices enabled us to compare their calculated total non-bonded internal energies (i.e. the sum of the electrostatic and van der Waals contributions). Sheets **4** and **7** with significantly higher melting temperatures (T_m: ~150 °C) showed much lower non-bonded internal energy ($E_{internal}$ < 3500 Kcal/mol) than those of sheets **1** and **9** (T_m: ~90 °C and $E_{internal}$ > 5300 Kcal/mol) (Table1), suggesting that the structures of sheets **4** and **7** are more stable than that of sheets **1** and **9**.

Temperature-dependent WAXS analysis. To further investigate the crystal structures of the sheets, WAXS measurements were conducted at increasing temperatures. Fig. 6 shows the WAXS intensity of dry sheets versus magnitude of the scattering vector, *q*, at room temperature. The *a* (100) and *c* (001) spacings of the sheets obtained from WAXS measurements are consistent with that observed from cryo-TEM imaging, as shown in Table 1. For sheet **4** bearing the 4-bromophenylethyl side chains, the scattering peak of the *c* spacing (18.2 Å) was not observed in WAXS, XRD and solution SAXS measurements (Fig. 6a and SI Appendix, Figs. S35 and S38); however, higher order scattering peaks of the *c* spacing (9.2 Å and 6.1 Å) were clearly seen in Fig. 6a and SI Appendix, Fig. S35. This is probably due to the interference of the strong scattering from the bromine atoms between the side chains. Sheets **1** and **9** containing all parallel V shapes showed different diffraction patterns in the high *q* region (q = 1.1-1.7 $Å^{-1}$) corresponding to the *a* spacing compared to sheets **4** and **7** having anti-parallel V shapes (Fig. 6b). This is consistent with the observation from cryo-TEM imaging showing different crystal lattices: parallel vs. anti-parallel V-shaped packing.

The trend of the order-disorder temperatures of the sheets (T_{ODT}: sheet **7** > sheet **4** > sheet **9** > sheet **1,** SI Appendix, Figs. S39-42) is consistent with the melting temperature trend obtained from DSC measurements. The change of the scattering peaks as a function of increasing temperature for all the sheets in this study differs from that of the aliphatic nanosheet (Ac-Ndc_9-Nte_9) system (30) we studied previously. The broad scattering peak characterizing a loss of order along the *a* direction with no loss of order along the *c* direction with increasing temperature – a distinct signature of the sanidic liquid crystalline phase in the Ac-Ndc_9-Nte_9 crystal – is not observed in the sheets in this study. In this study, the scattering peaks of the sheets from *a* and *c* spacings exhibit no discernible changes before reaching the order-disorder transition temperature, where all the scattering peaks disappear simultaneously (SI Appendix, Figs. S39-42). This suggests that the sheets in the current study are not sanidic liquid crystalline, which is probably due to the more rigid aromatic side chains compared to the aliphatic side chains in the Ac-Ndc_9-Nte_9 crystal. The sanidic liquid crystalline phase has generally been found in polymers with flexible alkyl side chains emanating from an extended and rigid aromatic backbone (31, 32).

Conclusions

We present atomic-level structural tuning of a peptoid nanosheet crystal lattice. The precision of peptoid synthesis, coupled with our advances in cryo-TEM imaging to directly image individual molecules in the lattice, allow us to study the polypeptoid sheet crystals at the atomic level. Remarkably, bromine atoms superposed in column were directly and distinctly observed in the nanosheet crystal bearing 4-bromophenethyl side chains. To the best of our knowledge, this is the first time atoms were directly localized in polymer crystals in position space with image phase. We explored the relationship between the atomic structure and the aromatic side chain substitution of the polypeptoid nanosheet crystals. Interestingly, all the sheets exhibited the same *cis* backbone fold but different lattice

packing geometry (parallel vs. anti-parallel V shapes) depending on the *para*-substitution of the aromatic side chains. This allows us to further engineer the crystal structures by tailoring the aromatic side chains. It is interesting that breaking the symmetry of the polypeptoid, by putting alternating phenylethyl and 4-bromophenylethyl side chains, resulted in packing of phenylethyl side chains against the 4-bromophenylethyl side chains across the interface between rows in the *c* direction. This provides a precise platform to study subtle pairwise interactions between lattice faces within a polymer crystal. Interestingly, the MD simulation of this sheet required adjacent rows to pack with alternating chirality. This is consistent with other studies showing that racemic crystals grow more readily than their enantiomeric counterparts. Nanosheets exhibiting higher melting temperatures (~150 °C) are more stable, as evidenced by their lower thickness and lower non-bonded internal energy. Our study on the investigation of polypeptoid crystal structures at the atomic level is not only a tremendous advance in soft material imaging, but also in enabling the future design of biomimetic nanomaterials with more precisely controlled structures and properties.

Materials and Methods

Detailed experimental procedures and characterization compounds can be found in SI Appendix.

Synthesis of diblock copolypeptoids. All diblock copolypeptoids were synthesized using automated solid-phase submonomer synthesis on a Symphony X peptide synthesizer at a scale of 200 mg Rink amide resin (0.64 mmol/g) by adapting reported procedures (18). 40-50 mg of final peptoid with > 90% molecular purity was obtained.

Self-assembly of nanosheets. The purified diblock copolypeptoid was dissolved in THF/H_2O (50/50, v/v) at a concentration of 2 mg/mL to form a clear solution, followed by slow evaporation of the THF in the refrigerator

at 4 ºC. Turbid solutions containing a large amount of crystalline nanosheets were obtained after several days.

Cryo-TEM data collection. The atomic-scale low-dose cryo-TEM results reported in this paper are based on vitrified, hydrated nanosheets prepared using Vitrobot (FEI Inc.). The specimens were imaged with a Titan Krios (FEI Inc.) operated at 300 kV with a K2 Summit direct detection camera and post-column energy filter (slit width at 25 eV) (Gatan Inc.). Low magnification images of nanosheets were obtained from dry specimens. Micrographs were collected on a Philips CM200 at 200 kV using a Gatan US1000 CCD camera at liquid nitrogen temperature in low-dose mode to minimize radiation damage.

Image processing. Images of 2D crystals are generally not perfect due to dislocations, distortions from stress and image distortion within the microscope, which cause high resolution diffraction spots to be smeared out. In order to recover the high spatial frequency signal, a crystal unbending process was conducted on all micrographs.

NanoDSC measurement of nanosheet solutions. NanoDSC measurement of aqueous nanosheet solutions were performed on a CSC Model 6100 Nano Differential Scanning of Calorimeter. The nanosheet solutions were degassed and heated from 5 ºC to 100 ºC at 2 ºC/min under a pressure of 3 atm.

DSC measurement of dry nanosheets. DSC measurement of dry nanosheets were performed on a TA Q200 Differential Scanning Calorimeter. The nanosheet solutions were added to a pre-weighed aluminum T Zero pan and dried under vacuum. Repeated this process several times until about 2 mg of dry nanosheets was collected. The DSC pan was sealed with aluminum T Zero lid and heated from 0 ºC to 180 ºC at 10 ºC/min.

AFM imaging of nanosheets. Diluted nanosheet solutions were dropped onto freshly cleaved mica and dried under vacuum for AFM imaging. *Ex* situ (in air) AFM imaging of the nanosheets was performed on an Asylum MFP-3D Atomic Force Microscope using tapping mode imaging with TAP 150 AL-G tips. The resonant frequency and force constant of the tips are 150 kHz and 5 N/m, respectively.

X-ray scattering of crystalline nanosheets. The nanosheet solutions were placed on a Kapton window (25 μm thickness) which is beneath a rubber gasket spacer (0.75 cm thickness) with a hole in the center (d = 0.25 inch) and dried under vacuum. Repeated this process several times until enough nanosheets were placed on the Kapton window. Then another Kapton window (25 μm thickness) was placed on top of the rubber spacer before placing it in the WAXS holder. Wide angle X-ray scattering (WAXS) measurements on the nanosheets were performed at Advanced Light Source (ALS) beamline 7.3.3 located at Lawrence Berkeley National Labratory.

Powder X-ray diffraction. The nanosheet solutions were pipetted onto a MiTeGen micromesh and dried under vacuum. Repeated this process several times until enough nanosheets were placed on the micromesh. Powder X-ray diffraction data of the nanosheets were collected at ALS beamline 8.3.1., a multiple-wavelength anomalous diffraction and monochromatic macromolecular crystallography. Beamline has a superbend source with an energy range of 5-17 keV. Data were collected with the detector 350 mm from the sample.

Small Angle X-ray Scattering (HT-SAXS). The nanosheet solutions were diluted with miliQ water to prepare samples for three different concentrations. SAXS data were collected at the SIBYLS beamline (12.3.1) at the Advanced Light Source in Lawrence Berkeley National Lab. The sample was collected at 11 keV (1.27 Å) X-ray beam. The SAXS data were analyzed by ScÅtter.

Molecular dynamics simulations. Initial coordinates for molecular dynamic simulations were generated with all backbones in the cis-amide conformation and with Φ and Ψ angles of the Cα alternated between Z_R (~ 90, ~ 150) and Z_S (~ -90, ~ -150). Monomer chains were arranged in blocks of 12 stacked parallel in the *a* dimension with these blocks arranged ether parallel or anti-parallel to one another in the *c* dimension to form a periodic monolayer configuration. Topology information and psf generation were performed using autopsf in VMD (33). Water molecules were added using the autosol plugin in VMD. Simulations were run in NAMD (19) with CHARMM force-field parameters similar to MFTOID (20). Simulations were run at 300K for at least 50 ns in NPT conditions. The non-bonded internal energy of polypeptoid sheets was calculated using the molecular mechanics energy function in NAMD 2.12. (19) The statistical analysis was carried out on internal energy data for the last 20 ns MD trajectory.

ASSOCIATED CONTENT

Supporting Information

Synthesis of diblock copolypeptoids, Cryo-TEM data collection and image processing, Table of characterization data of polypeptoids **1**-**10**, LC-MS traces of diblock copolypeptoids **1**-**10**, ^{1}H and ^{13}C NMR spectra of diblock copolypeptoids **1**-**10**, TEM of dry sheets and AFM images of dry sheets **2**-**3**, **5**, **7**-**10** , Low-lose cryo-TEM micrographs of sheets **1**, **4**, **7** and **9**, CTF estimation of low-dose cryo-TEM micrographs of sheets **1**, **4**, **7** and **9**, FFTs of averaged images of sheets **1**, **4** and **9**, Averaged images and the corresponding FFTs of sheet Nte_4-$N4mpe_6$ (**7**), Distribution map of motifs in sheets **1**, **4** and **9**, Crystal motifs and distribution map in sheet Nte_4-$N4mpe_6$ (**7**), Comparisons between simulations and cryo-TEM images, Table of summary of properties of micrographs used in the analysis, Overlapped peptoid chains taken from the MD sheet models, DSC measurements of dry sheets **2**-**3**, **5**, **8**, and **10**, NanoDSC measurements of sheets **1** and **9** in solution, XRD measurements of dry sheets **1**-**5**, **7**-**10**, SAXS measurements of

sheets **1**, **4**, **7** and **9** in aqueous solutions, WAXS measurements of dry sheets **1**, **4**, **7** and **9** as a function of increasing temperature.

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Competing interests

The authors declare no competing financial interest.

Author contributions

S.X. and R.N.Z. designed all the polypeptoids. S.X. synthesized all the polypeptoids, including their assembly and characterization. S.X. performed the AFM, DSC and X-ray scattering measurements. X.J. performed the cryo-TEM experiments and data analysis. R.K.S. and N.K.L. performed the MD simulations. D. P. supervised the MD simulations. N.P.B and R.N.Z. were responsible for the overall project and provided project supervision.

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Fig. 1. Assembly of amphiphilic diblock copolypeptoids into nanosheets via evaporation of a THF/water solution. (a) Chemical structures of diblock copolypeptoids **1**-**10.** The Nte block of compound **5** was increased to 6 monomers to increase the water solubility of this peptoid. (b,d) Representative TEM images of nanosheets. (c,e) Representative AFM images of nanosheets. The inset graphs are thickness profiles of nanosheets. (f) Proposed nanosheet structures of amphiphilic diblock copolypeptoids: polypeptoid chains are packed anti-parallel along the *c* direction and parallel along the *a* direction. The hydrophobic block (yellow color) is crystalline and the hydrophilic block (blue color) is amorphous.

Fig. 2. Nanosheet assembled from Nte_4-Npe_6 (**1**). (a) Chemical structure of Nte_4-Npe_6 (**1**). (b) Molecular model of sheet **1**. The molecules are packed anti-parallel along *c* direction and parallel along *a* direction. (c) Cryo-TEM image of sheet **1** from *b* direction (top view) showing parallel V shapes along *c* direction. (d) Top view of the hydrophobic domain in (b) from *b* direction showing parallel V shapes along *c* direction. The structure is overlapped with cryo-TEM image shown in (c).

Fig. 3. Nanosheet assembled from Nte_4-N4$Brpe_6$ (**4**). (a) Chemical structure of Nte_4-N4$Brpe_6$ (**4**). (b) Molecular model of sheet **4**. The molecules are packed anti-parallel along *c* direction and parallel along *a* direction. (c) Cryo-TEM image of sheet **4** from *b* direction (top view) showing anti-parallel V shapes along the *c* direction. The Br atoms show a tip-to-tip packing (red box). (d) Top view of the hydrophobic domain in (b) from *b* direction showing

anti-parallel V shapes along the *c* direction. The structure is overlapped with cryo-TEM image shown in (c).

Fig. 4. Nanosheet assembled from Nte_4-$(N4BrpeNpe)_3$ (**9**). (a) Chemical structure of Nte_4-$(N4BrpeNpe)_3$ (**9**). (b) Molecular model of sheet **9**. The molecules are packed anti-parallel along *c* direction and parallel along *a* direction. The green and red planes show the opposite chirality of adjacent backbones. (c) Cryo-TEM image of sheet **9** from *b* direction (top view) showing parallel V shapes along the *c* direction. (d) Top view of the hydrophobic domain in (b) from *b* direction showing parallel V shapes along the *c* direction. The structure is overlapped with cryo-TEM image shown in (c).

Fig. 5. DSC measurements for dry sheets**1**, **4**, **7** and **9**.

Fig. 6. WAXS measurements of dry sheets **1**, **4**, **7** and **9** at room temperature. (a) WAXS measurements showing the peaks corresponding to the *c* dimension. The peak at q = 0.4 $Å^{-1}$ is from the Kapton windows. (b) WAXS measurements showing the peaks corresponding to the *a* dimension.