Supporting Information

Hierarchical Self-assembly of Cholesterol-DNA Nanorods

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Materials and Methods

Cholesterol-DNA block copolymer and DNA strand preparation. All the DNA block copolymers (DBC) and DNA strands were directly purchased from Integrated DNA Technologies (IDT). For Cholesterol-DNA, cholesterol was conjugated to the 3' end of DNA strand, and a triethylene glycol (TEG) linker was required between these 2 parts. The internal hexa-ethyleneglycol spacer was added between DNA part and TEG linker, and multiple sets of internal spacers were able to be incorporated. The synthesis of Cholesterol-DNA required High Performance Liquid Chromatography (HPLC) purification, thus the influence of impurity could be excluded during assemblies. For DNA strands, they were ordered with the "DNA oligo" service and provided as single strands. Only standard desalting was ordered for single strand DNA. All DBCs and DNA strands were dissolved in Deionized water (DI water) with the final concentration of 100 μM according to IDT's instruction. The DBCs and DNA strands' concentration was all 20 μM in reaction solution unless specifically indicated.

DNA sequence design. The initially tested DBC **18B** has a DNA part with randomly assigned 18 bases. When designing this sequence, all hairpin, self- and hetero-dimer structures under physiological conditions were avoided which was confirmed using OligoAnalyzer on IDT's website. **12B**'s DNA part was designed by deleting 6 bases from **18B**'s DNA part's 5' end, and **24B**'s DNA part was designed by adding 6 bases to **18B**'s DNA part's 5' end. Still, all potential secondary structures under physiological conditions were avoided for these two strands. **PolyT** was designed by switching all bases of **18B**'s DNA part to thymine. It was used as a control group in this paper because it would not have any interactions between DNA strands. The sole DNA parts of all DBCs and the complementary strand of **18B**'s DNA part were also designed and prepared.

Annealing process. Many amphiphilic DBCs, including Cholesterol-DNA, need to undergo an annealing process for successful assembly. The annealing process for most trials in this paper was:

Step 1: Stay at 37 °C for 30 minutes;

Step 2: From 37 to 29 °C, decrease 0.1 °C for every 10 minutes;

Step 3: Stay at 29 °C forever.

This annealing process was adapted from previous research.[1-3] We tested different initial annealing temperature and discovered that 37 °C was the most appropriate one for our system. We also tested the influence of the time of staying at the final 29 °C stage and found no noticeable difference between all groups. Unless specifically indicated, this annealing process was used by all trials in this paper.

Tuning the salt concentration and pH. For salt concentration, sodium ion was chosen to tune the ion strength in the buffer for the ease of experiment. The 4 levels of sodium ion concentration were referred to the buffer constitution of phosphate-buffered saline (PBS). To be detailed, Low (Comparable to 0.1× PBS) had 15 mM Na^+, Medium (Comparable to 1/2 1× PBS) had 70 mM Na^+, High (Comparable to 1× PBS) had 150 mM Na^+, and Very high (Comparable to 2× PBS) had 270 mM Na^+. For both salt concentration and pH control, NaAc-HAc buffers with different Na^+ concentration and pH were prepared. As for magnesium concentration, the 4 levels chosen were referred to the general practice of DNA nanostructure assembly. $MgCl_2$ solution with different concentration was prepared for controlling Mg^{2+} concentration in the reaction buffer. The reaction buffer constitution was tuned by mixing NaAc-HAc buffer, $MgCl_2$ buffer and Cholesterol-DNA solution with a certain ratio that would produce the desired condition.

Sodium Acetate was purchased from LabChem Inc. Glacial acetic acid was purchased from

Macron Fine Chemicals. Magnesium chloride was purchased from EMD Millipore Corporation.

Transmission electron microscopy (TEM) imaging. For the sample preparation for TEM imaging, 3 μL sample was deposited on the surface charged carbon film coated copper EM grids for 30 seconds, then a filter paper was used to remove the excess liquid on the grid. 8 μL 1% uranyl formate (UF) solution was used for negative staining, and excess liquid was also removed with a filter paper after 20 seconds. The samples were imaged using a Hitachi HT-7700 120 kV W (Tungsten) TEM with AMT CCD camera. Note that the number of nanostructures shown on the images does not represent the actual yield of them, and the brightness of the figures solely depend on the staining and imaging technique.

To prepare the 1% UF solution, 10mg UF powder was dissolved in 1 mL DI water and heated to the point when no changes further appeared. Then 1 μL of 5 M NaOH was added to the solution and mixed well. The solution was further filtered using a 0.2 μm syringe filter with cellulose acetate membrane and the filtrate was collected. Copper EM grids were charged using Pelco easiGlow Glow Discharge Cleaning System. UF powder was purchased from Electron Microscopy Sciences. 5 M NaOH was purchased from Fisher Scientific. EM grids were purchased from Electron Microscopy Sciences, and the model of grids was CF400-CU. 0.2 μm syringe filter was purchased from VWR International.

Atomic force microscopy (AFM) imaging. Topographic AFM images were captured by peak force tapping mode (PFT) experiments on a Multimode VIII system (Bruker Corporation, Santa, Barbara, CA) in liquid. The samples were prepared by deposition of a 2 μL sample onto freshly cleaved mica. And then the liquid cell was filled with around 80 μL 1× buffer with. Commercial silicon nitride cantilevers with integrated sharpened tips (Bruker, SNL-10) were used.

Testing the stability of nanorods. We used **18B1S** in the buffer with pH=4.25, low salt concentration and 1 mM Mg^{2+} to do the annealing, then placed the product solution under room temperature for different time to see whether the black gaps would disappear. Samples were collected on day 0, 1, 3, 7 and 15, and imaged under TEM.

Polyacrylamide gel electrophoresis (PAGE). Nondenaturing polyacrylamide gel was prepared according to the standard protocol provided by Thermo Scientific. A 20% native polyacrylamide gel was prepared with 1X TBE and 10mM Mg^{2+} for the separation of small DNA fragments. 100ng sample was loaded in each well. 6 μL DNA ladder was loaded for reference. The electrophoresis was carried out with 120V for 180 minutes, and stained with 1× SYBR gold for 10 minutes, followed by washing with DI water. The gel was imaged using Bio-Rad Gel Doc EZ Imager.

The polyacrylamide solution, ammonium persulfate, TEMED and electrophoresis instruments were purchased from Bio-Rad Laboratories. The DNA ladder used was GeneRuler Ultra Low Range DNA Ladder purchased from Thermo Scientific. The 1× TBE was prepared by mixing 27g tris, 13.75g boric acid, and 1.85g EDTA in 500mL DI water and diluted to the desired concentration. Tris was purchased from Sigma-Aldrich Co. Boric acid was purchased from Spectrum chemical MFG. Corp. EDTA was purchased from Avantor Performance Materials. The concentration of Mg^{2+} was controlled using 1M $MgCl_2$ solution.

Sample preparation for PAGE analysis. For the experimental group, 20 μM **18B**'s DNA part was annealed in the solution with pH=3.1, low salt concentration and 1mM Mg^{2+}. This was the lowest pH condition we employed in this paper. After annealing, the solution was diluted, adjusted to neutral pH with 5M NaOH, and mixed with equal amount of its complementary strand. The mixture was vortexed for 30 minutes under room temperature to ensure the complete hybridization. For the control group, 20 μM of **18B**'s DNA part was directly diluted into the same concentration and mixed with equal amount of

its complementary strand without annealing. The concentration of double strands was measured using Thermo Scientific Nanodrop 2000 Spectrophotometer.

Annealing 18B with the complementary strand of its DNA part. 20 μM **18B** and its DNA part's complementary strand were mixed together to do the normal annealing in the solution with pH=3.6, low salt concentration and 1mM Mg^{2+}. The assembly product of 20 μM **18B** annealed under same condition without the complementary strand was chosen as the control group.

Collecting samples of 18B1S at different annealing point. 18B1S was used to do the normal annealing in the solution with pH=4.25, low salt concentration and 1mM Mg^{2+} because this group produced nanorods with clear black gaps under TEM. Samples at 0, 5, 10, 20, 30, 40, 240, 440, 640 and 840 minutes after annealing starts were collected and imaged under TEM. 0 to 40-minute groups were samples at 37°C. The 240-minute sample was collected at 35°C. The 440-minute sample was collected at 33°C. The 640-minute sample was collected at 31°C. The 840-minute sample was collected at 29°C.

Using 18B1S to undergo annealing process with different total time. For all groups in this trial, 20 μM **18B1S** and the solution with pH=4.25, low salt concentration and 1mM Mg^{2+} were used. The basic steps of the annealing process used by each group were the same as the original one aforementioned; However, the time length of each step was different, so the total annealing time varies: 0, 10, 20, 30, 40, 50, 60, 120, 360, and 720 minutes. The samples were collected after the annealing process was done and imaged under TEM.

Measuring the size of nanostructures using ImageJ. The width of the nanorods was analyzed using ImageJ and plotted to exhibit the trend. Three experimental groups were used for comparison: **18B**, **18B1S**, and **18B2S** annealed in the solution with pH=3.6, low salt concentration and 1mM Mg^{2+}. 20 data points were selected for each group and their average width was calculated and plotted. Standard deviation of each group was also calculated and plotted as error bars.

Measuring the CMC of DNA-cholesterol DBC. 18B1S was assembled at concentrations of 10μM, 7.5μM, 5μM, 2.5μM, 1μM, 0.5μM and 0.1uM. Then the reaction solution was incubated on the TEM grid overnight to ensure the complete adhesion. The assembly of all groups was checked using TEM imaging. The strand concentration was considered below CMC if no well-defined structure could be found.

Tables and Figures

Table S1. All DBCs and DNA strands.

Name	Sequence (5' to 3')
18B	GGT AGT AAT AGG AGA ATG-TEG linker-cholesterol
12B	AAT AGG AGA ATG-TEG linker-cholesterol
24B	AGT GAG GGT AGT AAT AGG AGA ATG-TEG linker-cholesterol
PolyT	TTT TTT TTT TTT TTT TTT-TEG linker-cholesterol
18B1S	GGT AGT AAT AGG AGA ATG-Internal spacer-TEG linker-cholesterol
18B2S	GGT AGT AAT AGG AGA ATG-Internal spacer-Internal spacer-TEG linker-cholesterol
18B complementary DNA	CAT TCT CCT ATT ACT ACC
18B-high GA	GGA AGG AAG AGG AGA AGG-TEG linker-cholesterol
18B-low GA	TGT AGT ATT ATG AGT ATG-TEG linker-cholesterol

Note: TEG linker = triethylene glycol linker. Internal spacer = hexa-ethyleneglycol spacer.

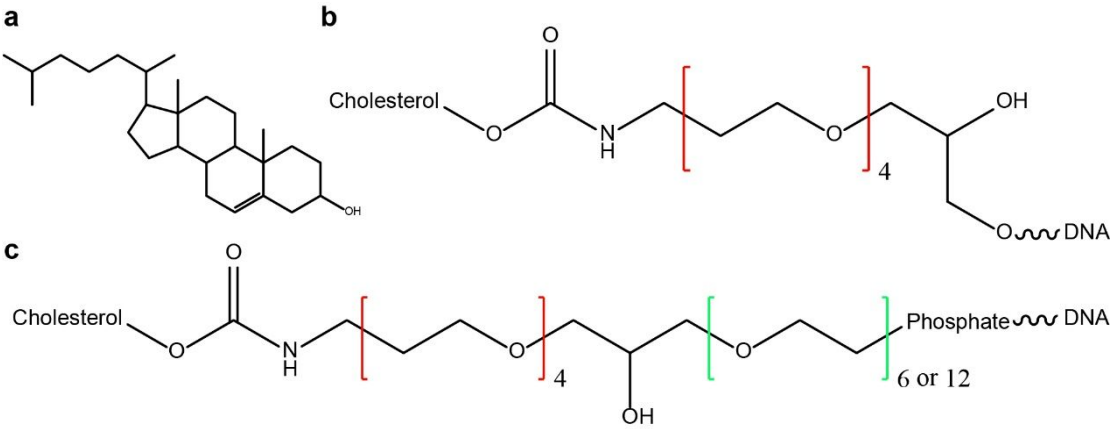

Figure S1. Composition of Cholesterol-DNA. (a) The molecular structure of cholesterol. (b) The structure of Cholesterol-DNA with only triethylene glycol linker. (c) The structure of Cholesterol-DNA with both triethylene glycol linker and additional hexa-ethyleneglycol spacer. Triethylene glycol linker is indicated between red square brackets, and hexa-ethyleneglycol internal spacer is indicated between green square brackets. Cholesterol and internal linkers are conjugated to 3' end of the DNA strand.

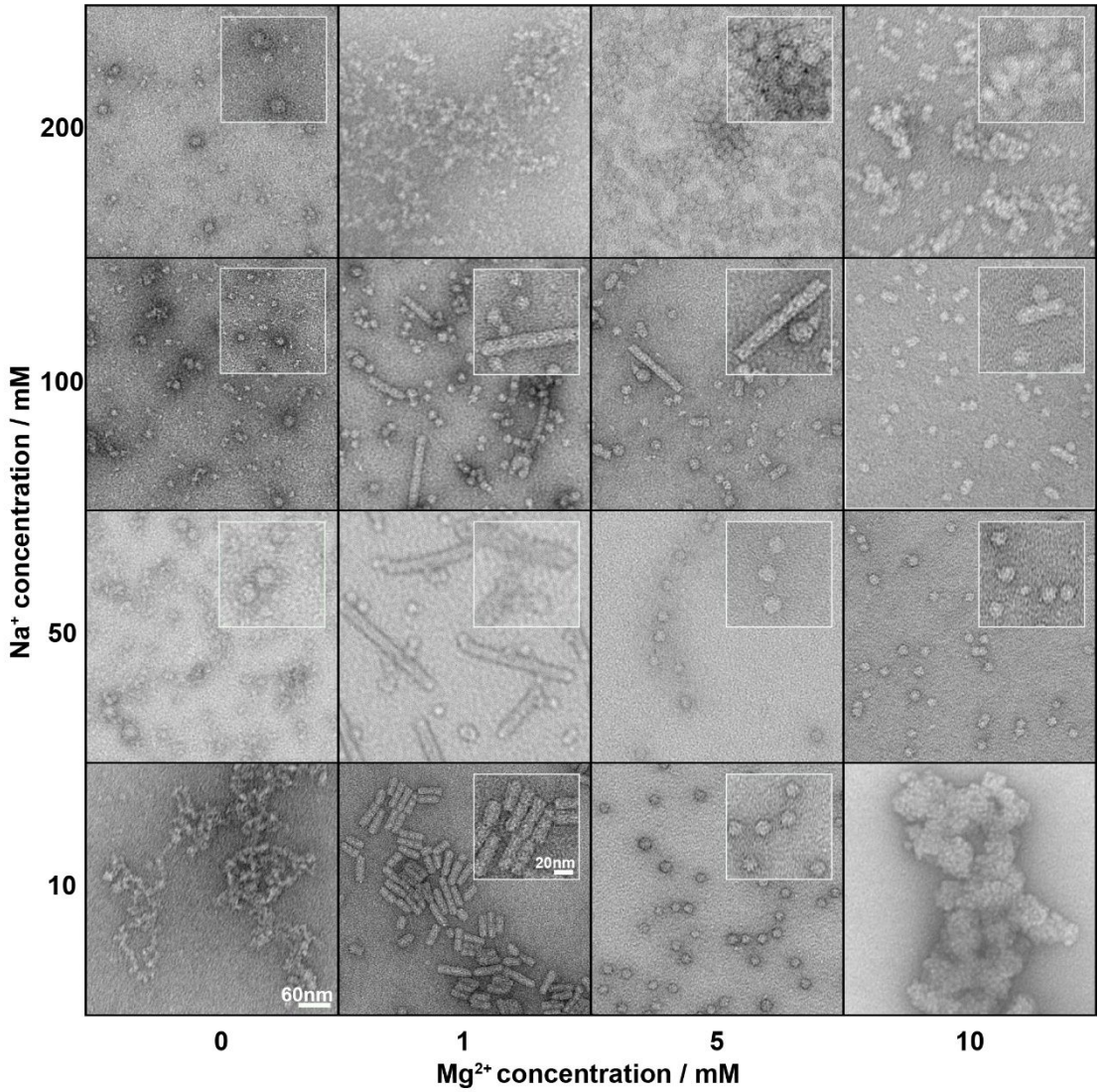

Figure S2. TEM images of **18B** annealed with different salt concentration. The rationale for choosing Na^+ and Mg^{2+} concentration can be found in the "Materials and methods" section. Large images are captured with the magnification of 25k times under TEM. They all share the same scale bar as shown in the lower left. Inset zoomed-in images were captured with the magnification of 50k times, and they share the same scale bar as shown in the lower middle. Groups without well-defined product do not include a zoomed-in image.

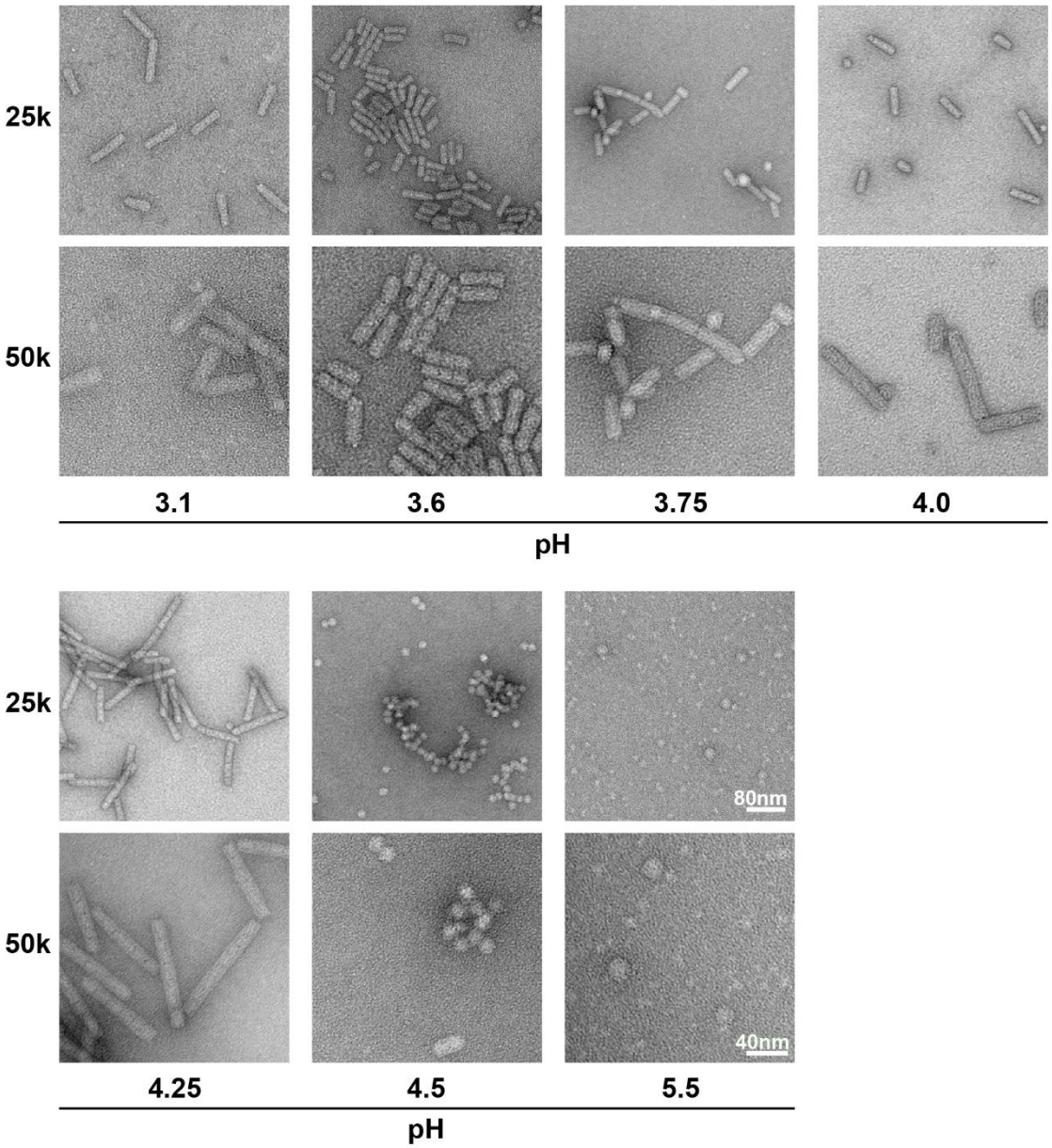

Figure S3. TEM images of the annealed products of **18B** under different pH. These groups are all tested in the solution with 10mM Na^+ and 1mM Mg^{2+}. The left column indicates the magnification rates (25k and 50k). Images with the same magnification share the same scale bar.

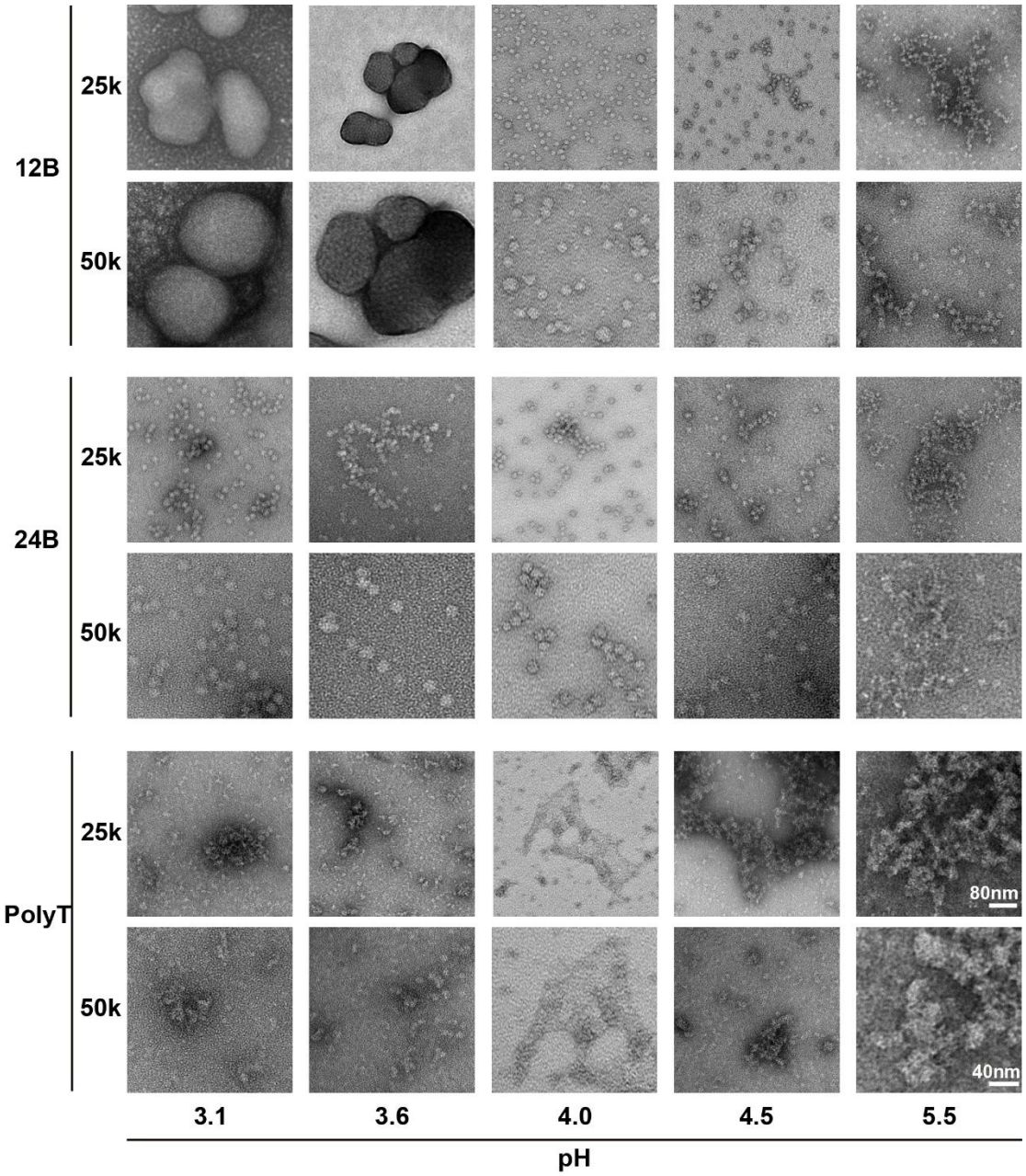

Figure S4. TEM images of the assembly products of **12B**, **24B** and **PolyT** under different pH. These groups are all tested in the solution with 10mM Na^{+} and 1mM Mg^{2+}. “25k” and “50k” indicate the magnification rates of TEM setup when capturing the image. Images with the same magnification share the same scale bar.

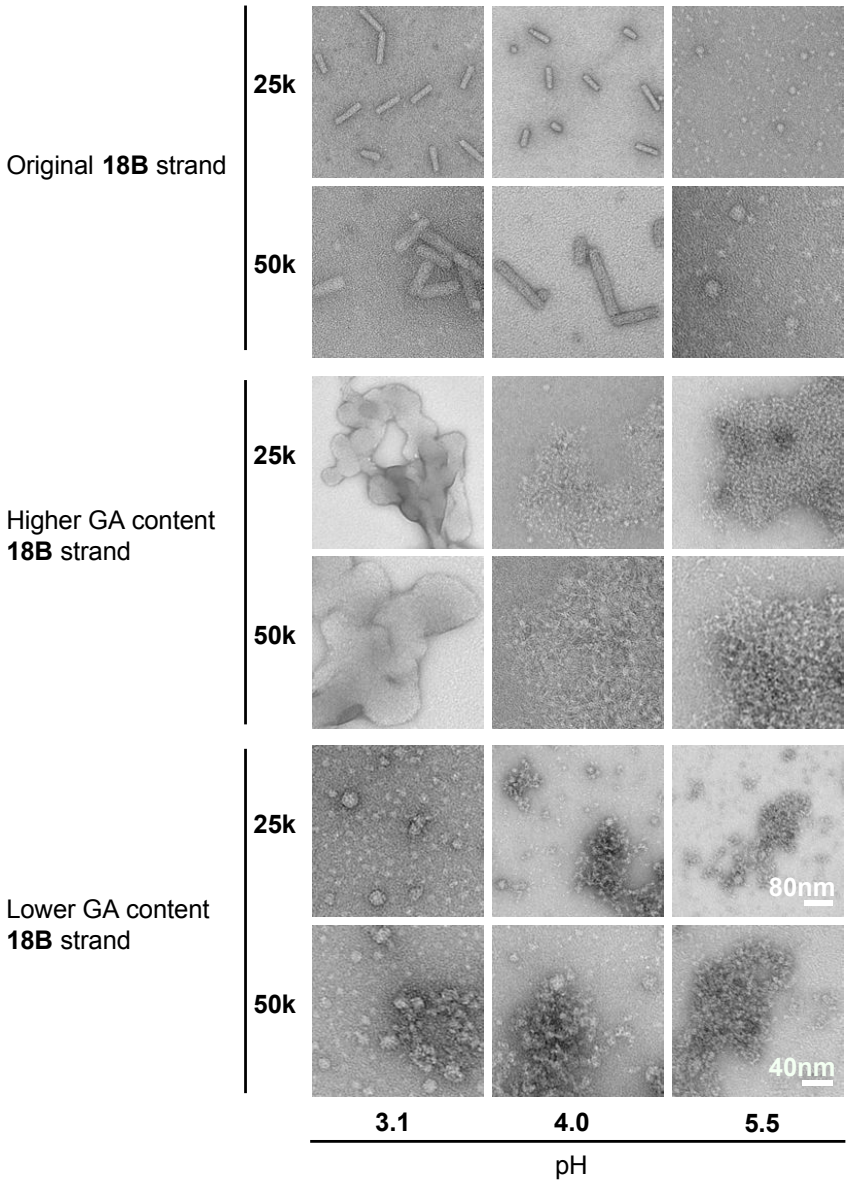

Figure S5. TEM images of the assembly products of **18B** with 100% GA and **18B** with 56% GA, in comparison to the original **18B** with 78% GA. The samples are all assembled in the buffer with 10mM Na^+ and 1mM Mg^{2+}. "25k" and "50k" indicate the magnification of TEM setup when capturing the images. Images with the same magnification share the same scale bar.

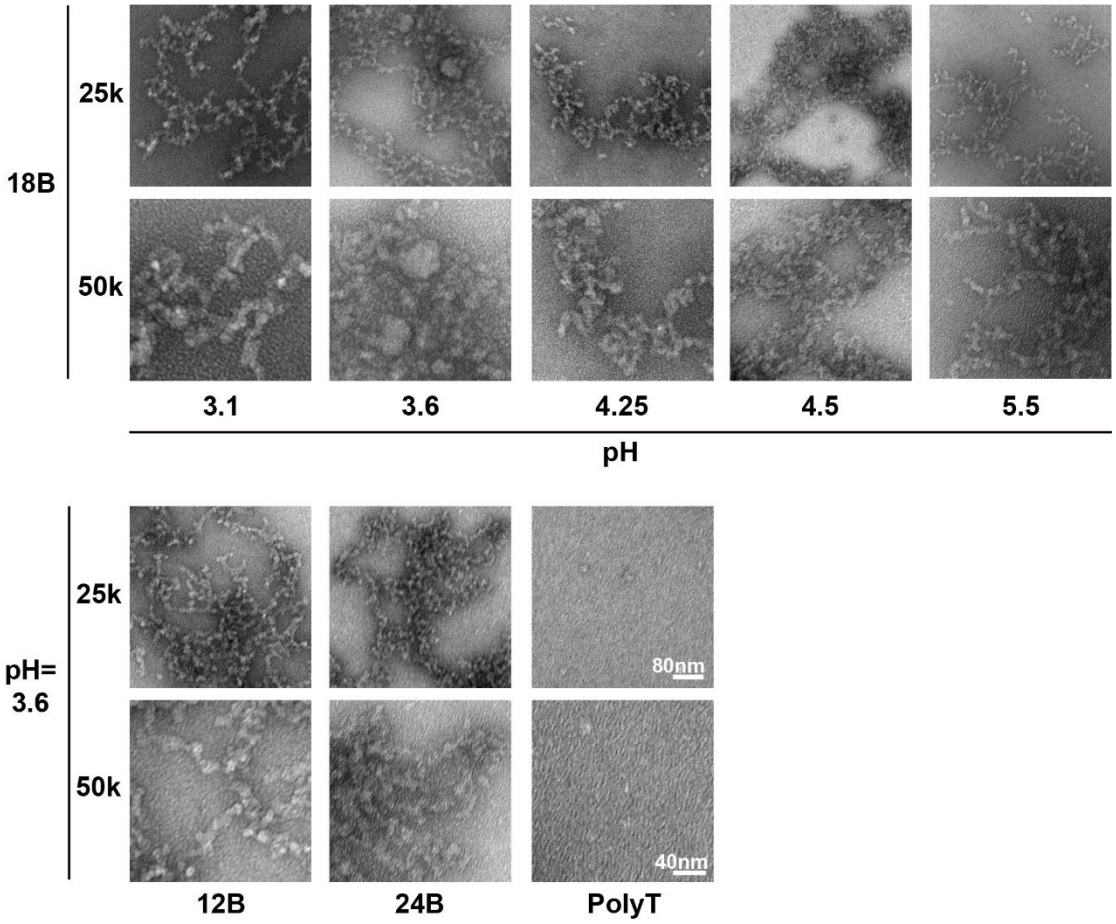

Figure S6. TEM images of the assemblies of sole DNA strands. The upper five groups showed assembly result of **18B** at different pH, with 10mM Na^+ and 1mM Mg^{2+}. The lower three groups compare **12B**, **24B** and **PolyT**, annealed at pH=3.6, with 10mM Na^+ and 1mM Mg^{2+}. "25k" and "50k" indicate the magnification of the image. Images with the same magnification share the same scale bar.

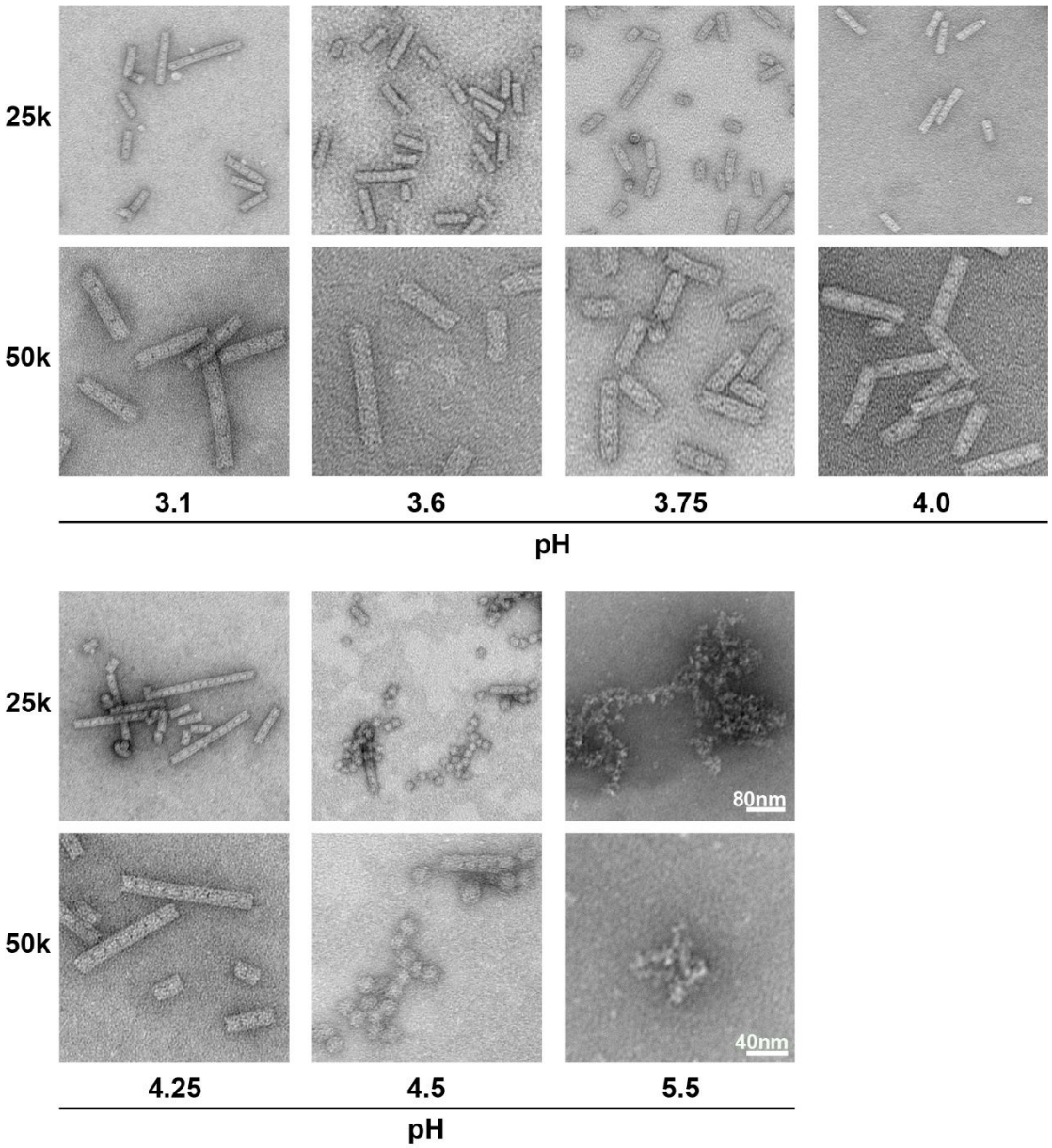

Figure S7. TEM images of the product of **18B1S** under different pH. These groups are all tested in the solution with low salt concentration and 1mM Mg^{2+}. The left column indicates the magnification of TEM setup when capturing the images. Images with the same magnification share the same scale bar.

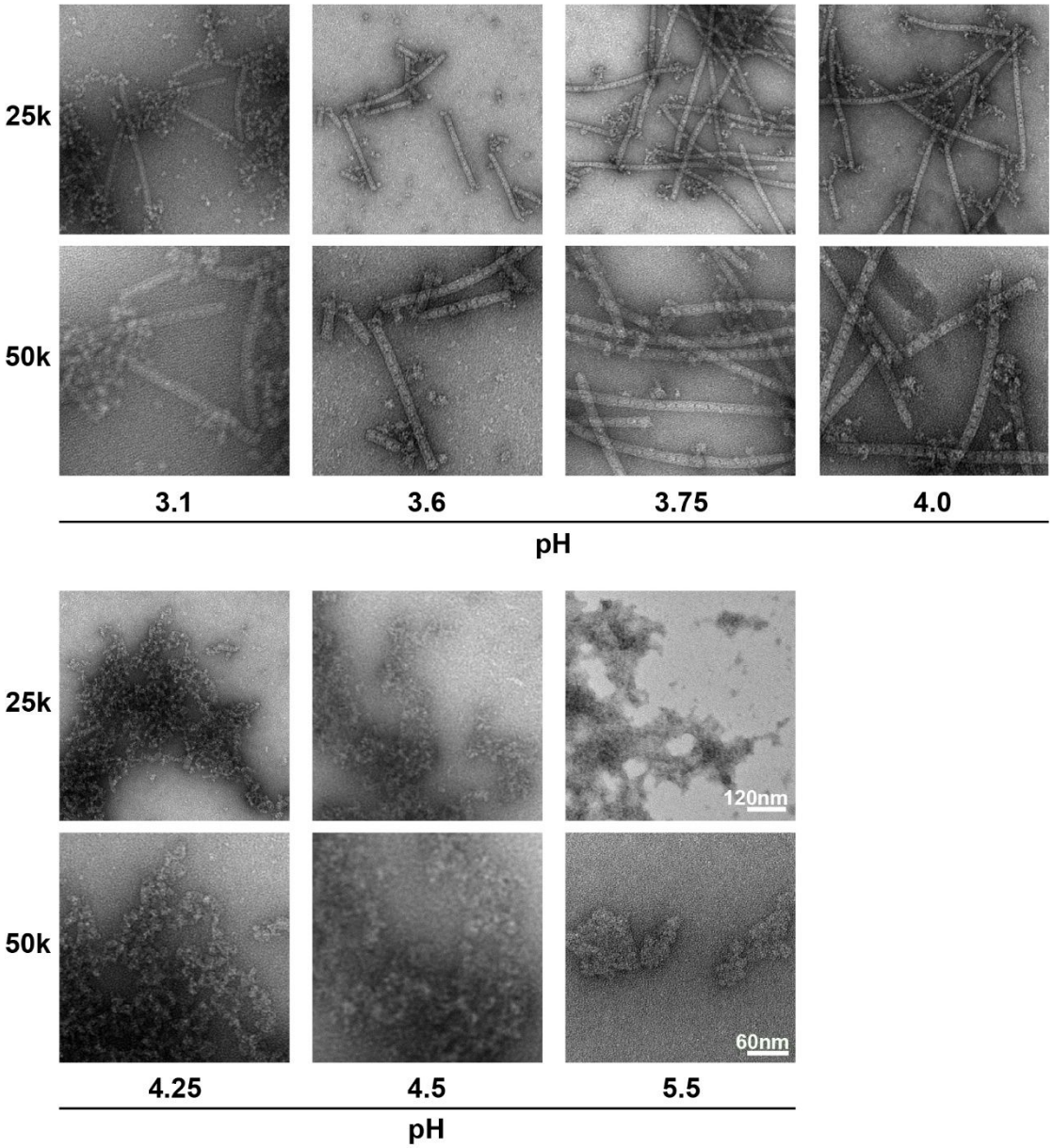

Figure S8. TEM images of the product of **18B2S** under different pH. These groups are all tested in the solution with low salt concentration and 1mM Mg^{2+}. The left column indicates the magnification of TEM setup when capturing the image. Images with the same magnification share the same scale bar. Note that for these groups, the nanorods are too long to be completely included in this grid. To check the whole length of these nanorods, please refer to Figure S6.

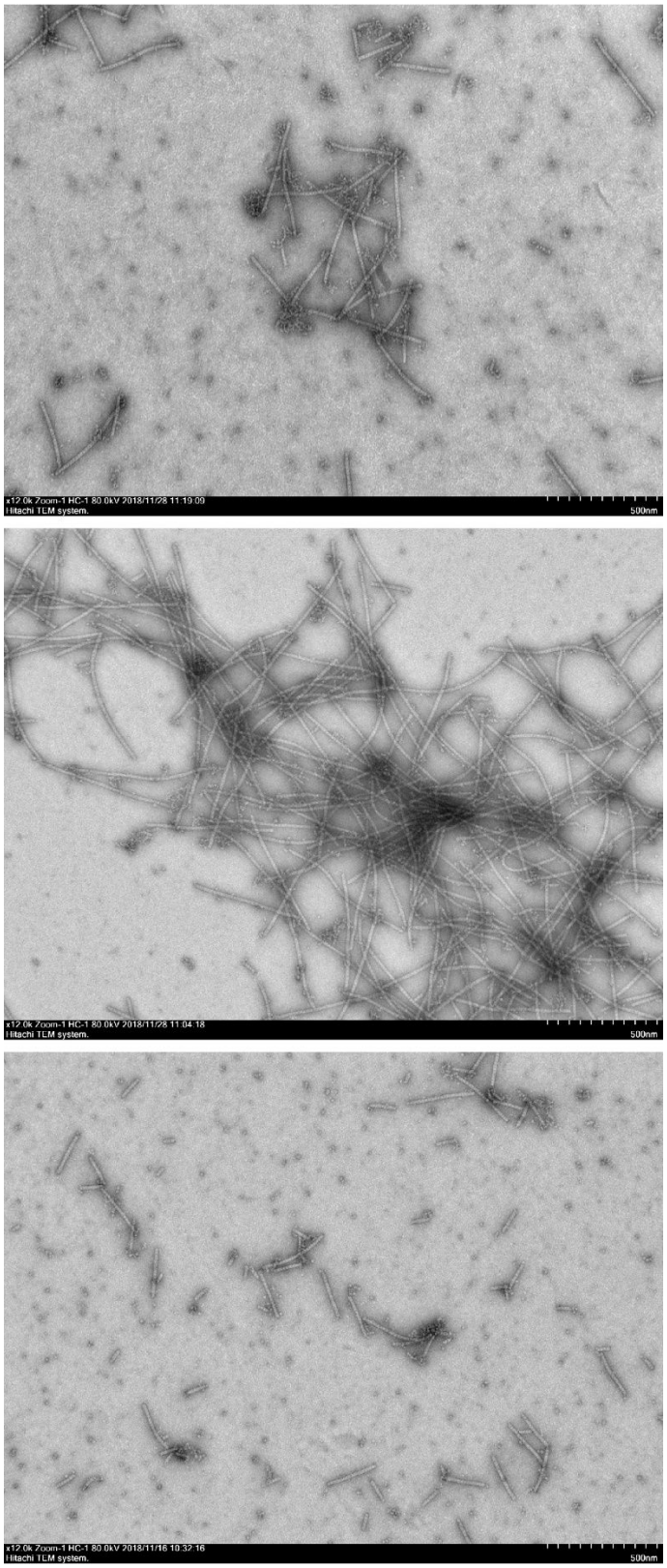

Figure S9. TEM images showing the full size of **18B2S** nanorods. The upper image shows the assembly under pH=3.6. The middle image shows the assembly under pH=3.75. The lower image shows the assembly under pH=4.25. These images are directly captured under TEM. Scale bars are presented at the lower right corner of each image. For experimental details, please refer to Figure S6.

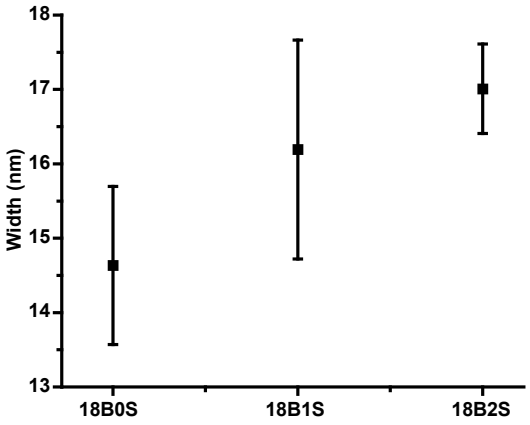

Figure S10. Measurement of nanorod widths. 20 data points were collected for each group and the average value and standard deviation were calculated and illustrated on the plot. With more internal spacers incorporated in the Cholesterol-DNA, the average width of nanorods grows larger with a nearly linear pattern. This result is consistent with our proposed assembly mechanism that cholesterol sits inside as the hydrophobic core while DNA parts point outwards.

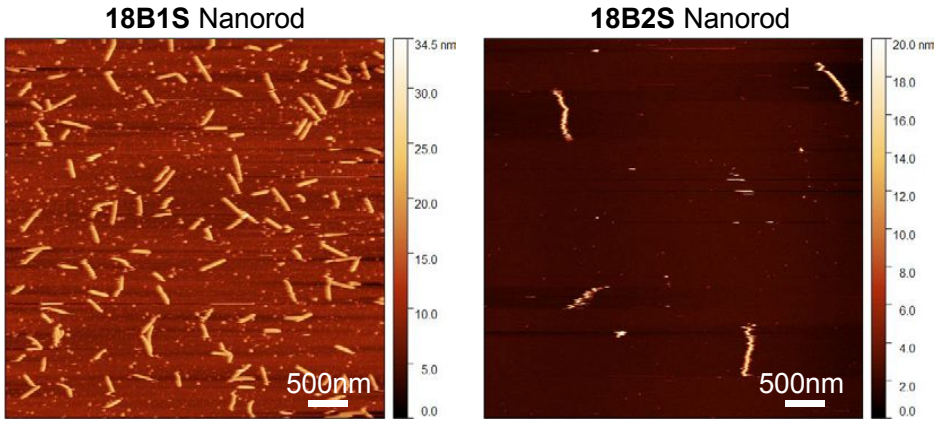

Figure S11. Liquid-phase AFM images of **18B1S** nanorod (pH=4.25, 10mM Na^+ and 1mM Mg^{2+}) and **18B2S** nanorod (pH=3.75, 10mM Na^+ and 1mM Mg^{2+}).

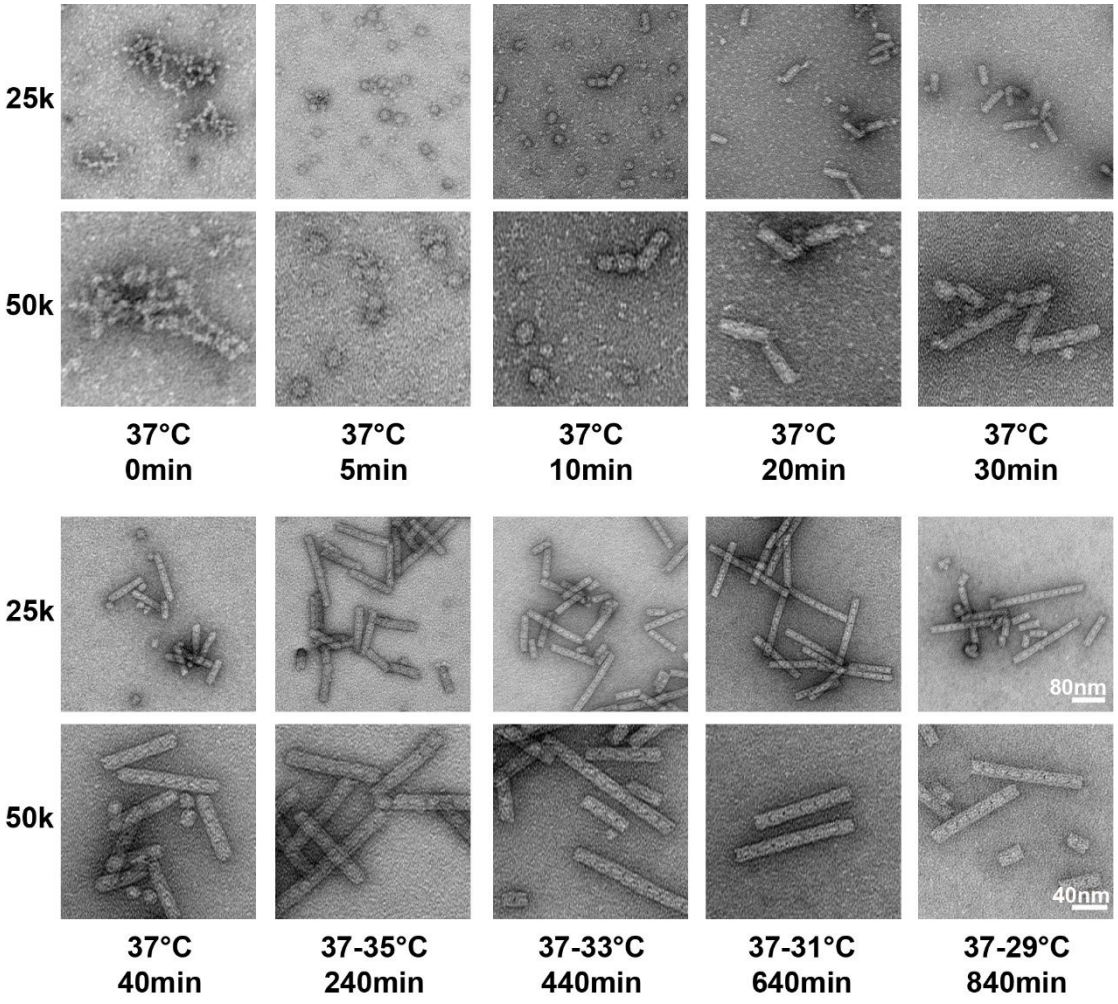

Figure S12. TEM images of the assembly of **18B1S** collected at different annealing time points. These groups are all tested in the solution with pH=4.25, low salt concentration and 1mM Mg^{2+}. The left column indicates the magnification rate of TEM setup when capturing the image. Images with the same magnification share the same scale bar. For the rationale of choosing time points, please check the "Materials and methods" section.

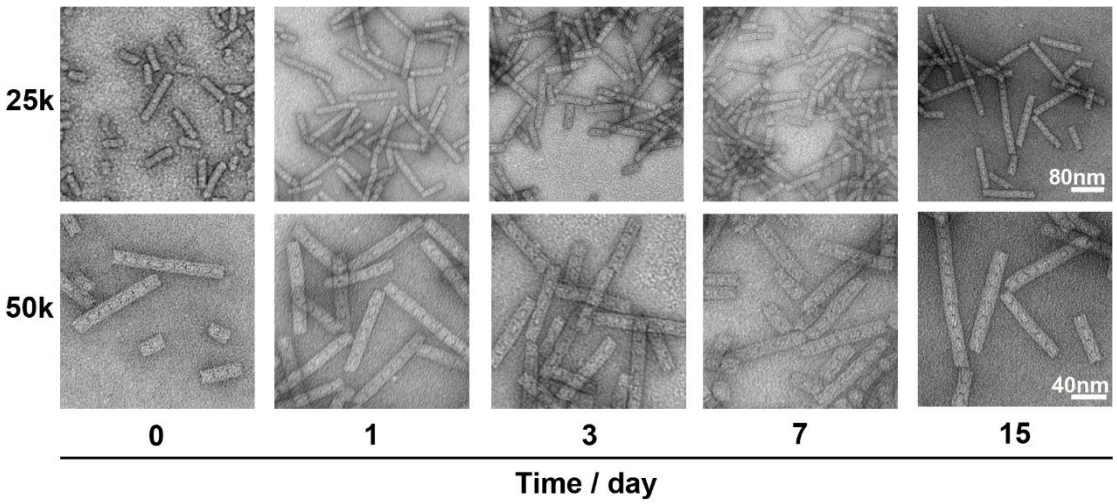

Figure S13. TEM images of preassembled **18B1S** nanorods placed under room temperature for long periods of time. These groups are all tested in the solution with pH=4.25, low salt concentration and 1mM Mg^{2+}. The left column indicates the magnification rate of TEM setup when capturing the image. Images with the same magnification share the same scale bar. After 15 days, the black gaps are still obvious on nanorods.

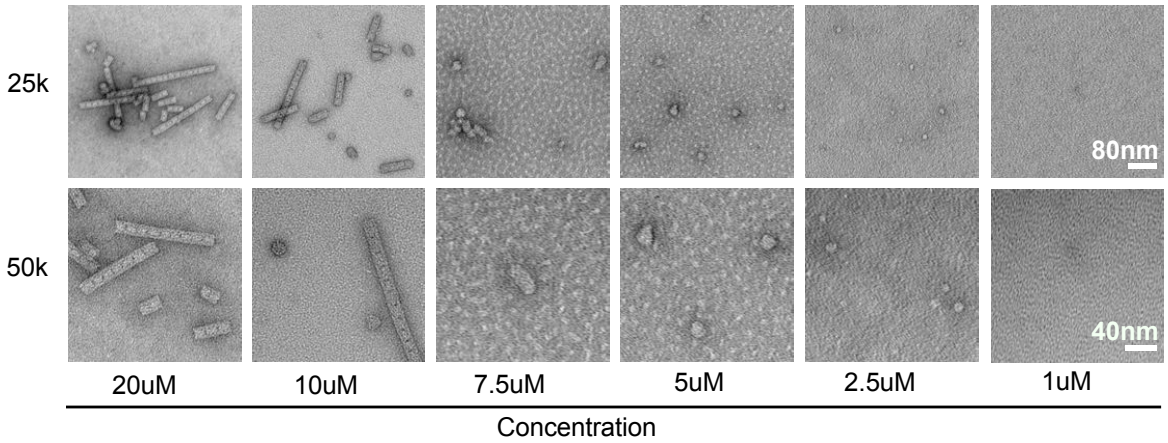

Figure S14. Assembly of **18B1S** at different strand concentrations. For all groups, the reaction solution was incubated on the TEM grid overnight to ensure the complete adhesion. When strand concentration lowered to 1uM, no well-defined structures could be observed under TEM. All groups are assembled in the solution with 10 mM Na^+ and 1mM Mg^{2+}. “25k” and “50k” indicate the magnification of the images. Images with the same magnification share the same scale bar.

References

(1) Wu, F., Zhao, Z., Chen, C., Cao, T., Li, C., Shao, Y., Zhang, Y., Qiu, D., Shi, Q., Fan, Q. H. and Liu, D. (2018) Self-Collapsing of Single Molecular Poly-Propylene Oxide (PPO) in a 3D DNA Network. *Small 14*, 1–4.

(2) Dong, Y., Sun, Y., Wang, L., Wang, D., Zhou, T., Yang, Z., Chen, Z., Wang, Q., Fan, Q. and Liu, D. (2014) Frame-Guided Assembly of Vesicles with Programmed Geometry and Dimensions. *Angew. Chem. Int. Ed. 53*, 2607–2610.

(3) Zhao, Z., Chen, C., Dong, Y., Yang, Z., Fan, Q. H. and Liu, D. (2014) Thermally Triggered Frame-Guided Assembly. *Angew. Chem. Int. Ed. 53*, 13468–13470.