Supplementary Information (SI) for Nanoscale Horizons.
This journal is © The Royal Society of Chemistry 2024

Electronic Supplementary Materials

Circular RNA oligonucleotides: Enzymatic synthesis and scaffolding for construction

Shijie Li[a], Yanxin Chu[a], Xin Guo[b], Chengde Mao[c,*], Shou-Jun Xiao[a,*]

[a] School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210023, China

[b] Bruker (Beijing) Scientific Technology Co. Ltd, China

[c] Department of Chemistry, Purdue University, West Lafayette, Indiana 47907, USA

* E-mails: mao@purdue.edu, sjxiao@nju.edu.cn

S1. Materials and methods

Materials. All RNA strands including 5'-phosphated ones used in this research were purified by HPLC, DNA strands were purified by denaturing polyacrylamide gel electrophoresis (dPAGE) at Sangon Biotech (www.sangon.com) without further purification. T4 DNA ligase, Recombinant DNase I, T7 RNA polymerase and their corresponding buffers were from Takara Biotechnology Co., Ltd. (Beijing, China). RNase R and buffer were purchased from Beyotime Biotech Co., Ltd. (Shanghai, China). ATP, NTPs mix (25 mM), RNase-free ddH_2O, urea, TAE-Mg buffer (40 mM Tris acetate, 1 mM EDTA, 12.5 mM Mg^{2+}, pH 8.0), 1× TBE buffer (89 mM Tris borate, 2 mM EDTA, pH 8.2), 30% acrylamide/bis-acrylamide (29:1) solution, N, N, N', N'-tetramethylethylenediamine (TEMED), ammonium persulfate (APS), magnesium acetate tetrahydrate, sodium chloride, potassium chloride, and magnesium chloride hexahydrate were from Sangon Biotech. RNAclean Kit was purchased from Tiangen Biotech Co., Ltd. (Beijing, China). Mica sheets for Atomic Force Microscopy (AFM) were purchased from Nanjing Zhongjingkeyi Technology Co., Ltd. (Nanjing, China). RNase-free ddH_2O was used for all reactions containing RNA strands.

Preparation and purification of circular RNAs. The circular ssRNA of N nt (cNR) was prepared as follows. A 5'-phosphorylated (abbreviated as 5'-p in section S5) linear ssRNA of N nt (LNR) was mixed with a DNA splint with a molar ratio (1:1.2) in 100 μL of 1× T4 DNA ligation buffer. The mixture was annealed in a PCR thermo cycler programmed at 75 ℃ for 2 min, then from 75 ℃ to 50 ℃ at −2 ℃ min^{-1}, and finally to 16 ℃ at −1 ℃ min^{-1}. The total annealing time was about 50 min, followed by the addition of T4 DNA ligase and incubation at 16 ℃ for 16 h. The mixture was heated to 65 ℃ and kept for 10 min to inactivate the T4 DNA ligase and then cooled down to room temperature. Recombinant DNase I (5 μL) was added for digestion of DNA splints at 37 ℃ for 30 min, further 5 μL RNase R was added to digest linear ssRNA residues at 37 ℃ for 30 min. The circRNA molecules were left intact and then purified by 15 % dPAGE, followed by n-butanol extraction and ethanol precipitation.

Denaturing polyacrylamide gel electrophoresis (dPAGE). The 15 % denaturing PAGE (dPAGE) gel (20 mL) was prepared with a 30% acrylamide/bis-acrylamide (29:1) solution, 7.5 M urea, and 1× TBE buffer. After mixing, 20 μL TEMED and 200 μL 10% APS were added to initiate polymerisation. To analyse circRNAs, a sample solution (5 μL) containing 10 μM LNR was mixed with an equal volume of RNA Loading Dye, (2× TBE-urea), heated to 70 ℃ and kept for 5~8 min, then subjected to 15% dPAGE in 1× TBE buffer at 150 volts for about 1 h. After dyed by GelRed™, the gel was scanned by a Bio-Rad scanner.

Annealing ramps for solution assembly of hybrid c44R:DNA nanostructures. A set of c44R and

DNA strands in stoichiometric ratios for solution assembly were mixed in TAE-Mg buffer to a final concentration of 1.0 μM per strand and to a final volume of 30 μL. The mixture was annealed in a PCR thermo cycler from 75 °C to 16 °C for a total time of about 24 h The annealing ramp is as follows: 75 °C for 3 min, then from 75 °C to 60 °C at −0.2 °C min^{-1}, to 50 °C at −0.04 °C min^{-1}, to 40 °C at -0.02 °C min^{-1}, to 21 °C at −0.04 °C min^{-1}, and finally to 16 °C at −0.05 °C min^{-1}.

Annealing ramps for solution assembly of pure RNA nanostructures. A set of c44R and other RNA strands in stoichiometric ratios were added in TAE-Mg buffer to a final concentration of 1.0 μM per strand and a final volume of 30 μL. The mixture was annealed in a PCR thermo cycler programmed for about 24 h. Detailed procedures are the same as the annealing ramps for the hybrid c44R:DNA assembly.

Preparation and purification of the kissing-loop (KL) RNA strand. The KL RNA strand was transcribed as follows. The dsDNA template (150 ng/μL × 10 μL), NTPs (25 mM × 8 μL), T7 RNA polymerase (3 μL) were mixed in series in 1× transcription buffer (8 mM $MgCl_2$, 2 mM spermidine, 5 mM DTT, 40 mM Tris-HCl, pH 8.0) to a final volume of 50 μL. The transcription reaction was carried out at 37 °C for 4 h. Then Recombinant DNase I (3 μL) was added to digest DNA templates at 37 °C for 30 minutes. The KL RNA strand was purified using the RNAclean Kit from Tiangen Biotech Co., Ltd. (Beijing, China).

Annealing ramps for solution assembly of two-strand DAE-KL nanostructures. Both equimolar c44R and KL RNA strands were mixed in TAE-Mg buffer containing additional 50 mM Na^+ and 50 mM K^+ to a final concentration of 1.0 μM per strand and a final volume of 30 μL. The mixture was annealed in a PCR thermo cycler programmed for about 24 h. The annealing protocol is the same as that for the hybrid c44R:DNA assembly.

AFM imaging. AFM images were captured in the "ScanAsyst mode in fluid" (Dimension FastScan, Bruker) with ScanAsyst-Fluid+ probes (Bruker). The sample preparation procedure was as follows: a 4 μL annealed sample solution was deposited onto a freshly cleaved mica surface and incubated about 4 min for adsorption of DNA nanostructures, then the specimen was washed twice with 70 μL TAE-Mg buffer. Finally, 70 μL TAE-Mg buffer were deposited on the specimen, and 30 μL of the same buffer at the AFM tip. The specimen was imaged in the fluid mode.

S2. Additional dPAGE photos for optimisation of T4 DNA ligation conditions

We screened the following ligation conditions, T4 DNA ligase concentrations, LNR concentrations, APT concentrations, Mg^{2+} concentrations, hairpin loop sizes of nT, temperature and incubation time for a standard ligation condition to synthesize the monomeric c22R molecules efficiently. The standard ligation conditions have been optimised as: 1) the 5'-monophosphated ssRNA concentration is 10 μM, 2) the ligation is carried out with a T4 DNA ligase concentration of 2500 U per nanomolar RNA at 16 °C for 16 h.

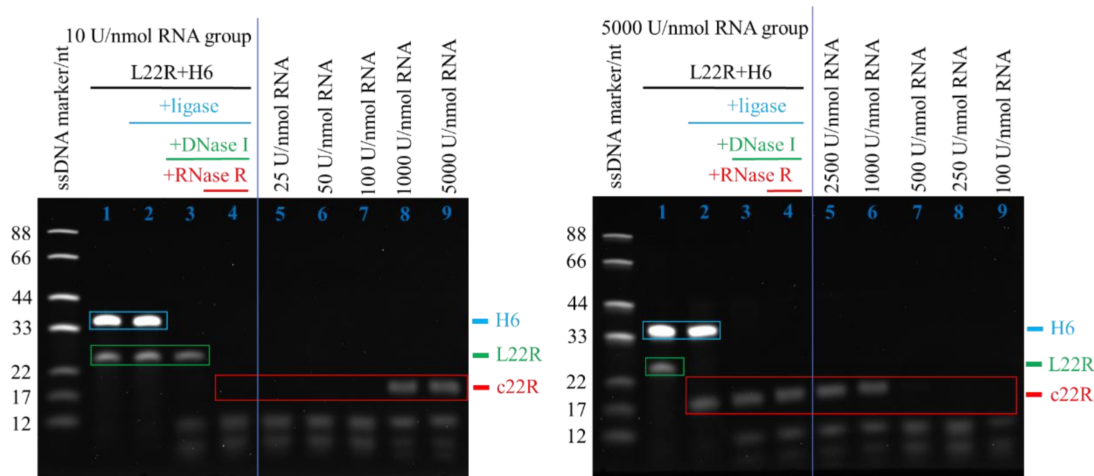


Fig. S1. Effect of T4 DNA ligase concentrations on the ligation efficiency. (ligation conditions: 10 μM RNA, 1 mM ATP, 10 mM Mg^{2+}, 16 °C, 16 h.)

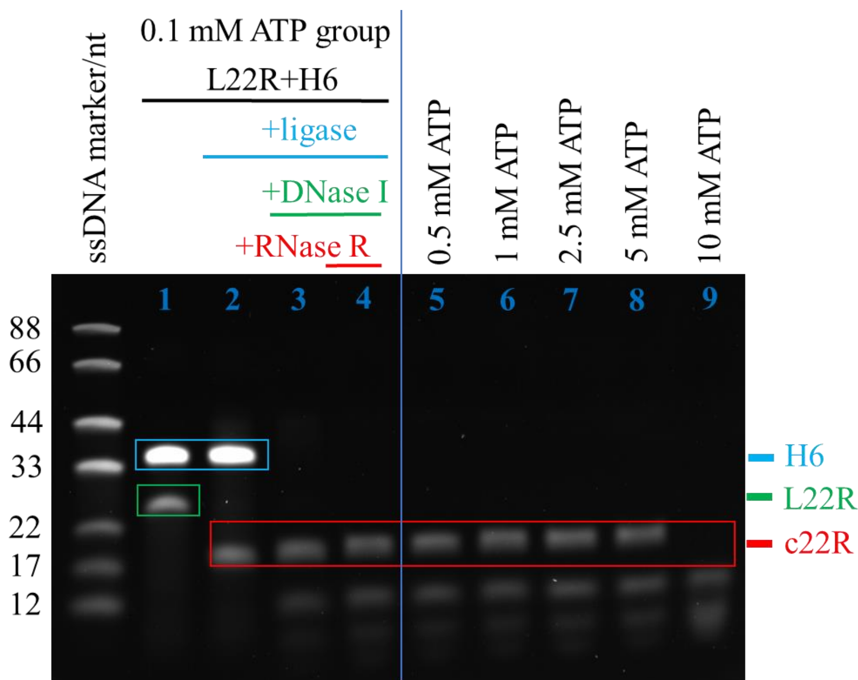


Fig. S2. Effect of ATP concentrations on the ligation efficiency. (ligation conditions: 10 μM RNA, 2500 U per nanomolar RNA, 10 mM Mg^{2+}, 16 °C, 16 h.)

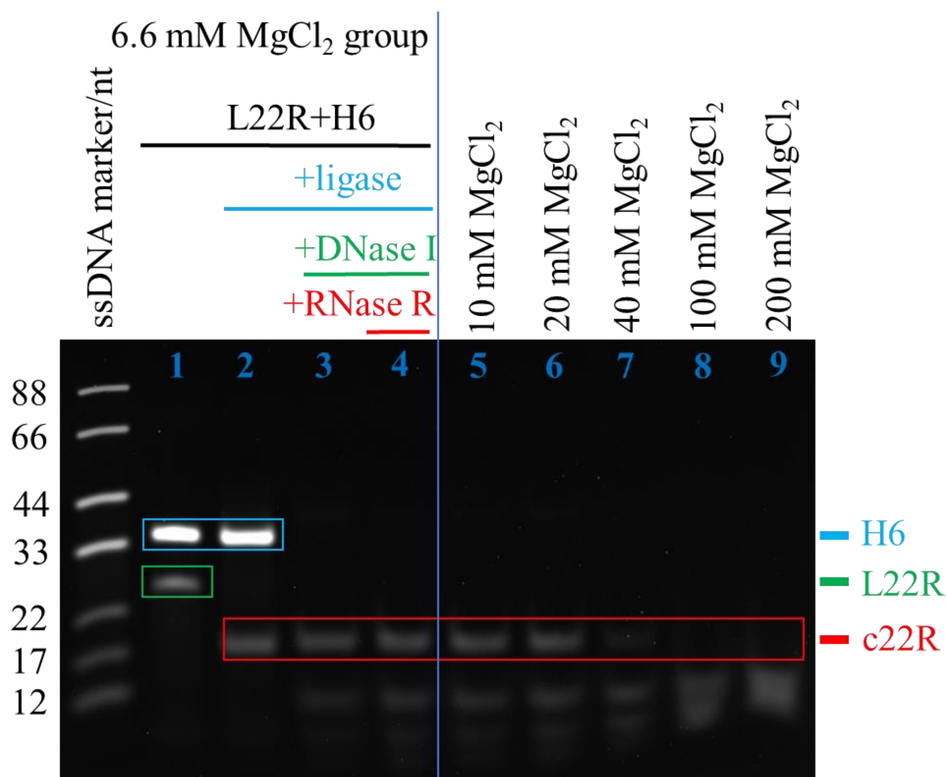


Fig. S3. Effect of $MgCl_2$ concentrations on the ligation efficiency. (ligation conditions: 10 μM RNA, 2500 U per nanomolar RNA, 1 mM ATP, 16 °C, 16 h.)

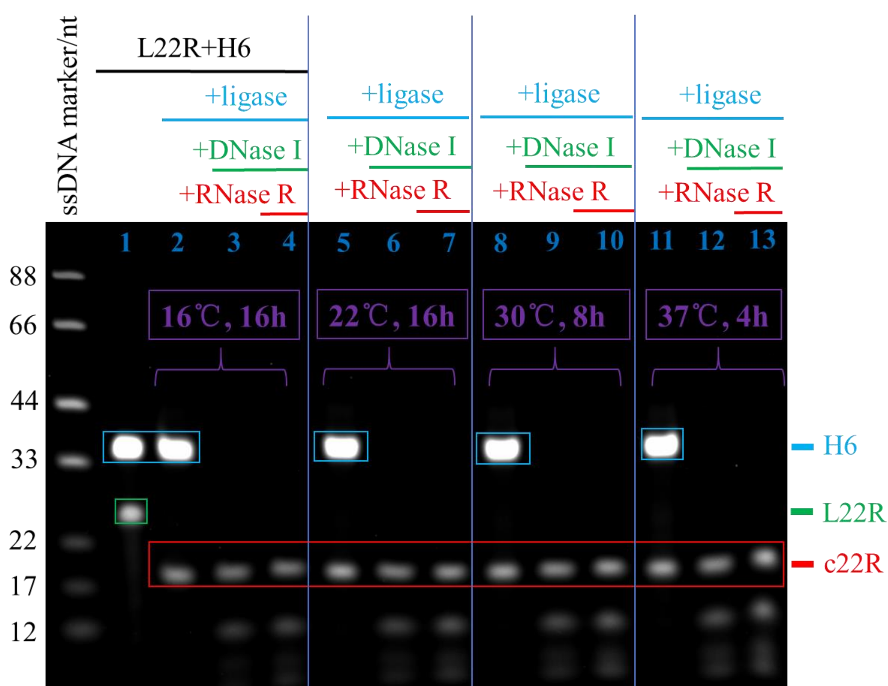


Fig. S4. Effect of temperature and incubation time on the ligation efficiency. (ligation conditions: 10 μM RNA, 2500 U per nanomolar RNA, 1 mM ATP, 10 mM Mg^{2+}.)

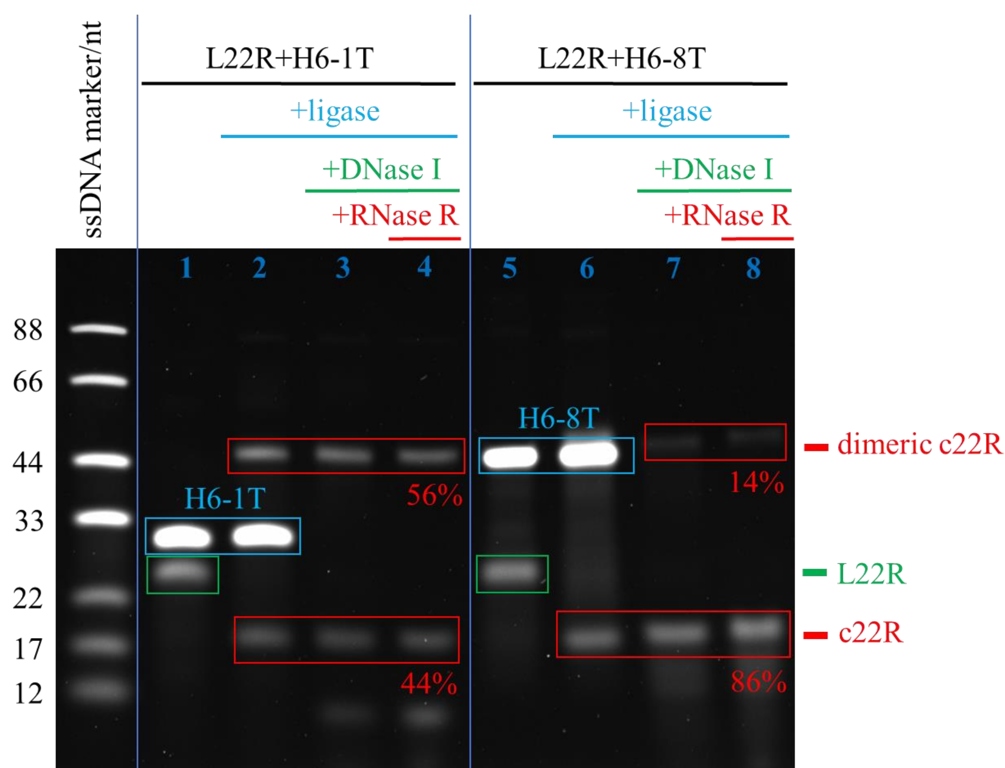


Fig. S5. Impact of H6's hairpin loop sizes (H6-1T and H6-8T represent the H6's hairpin loop sizes at 1T and 8T, respectively) on cyclisation of L22R. (ligation conditions: 10 μM RNA, 2500 U per nanomolar RNA, 1 mM ATP, 10 mM Mg^{2+}, 16 °C, 16 h.)

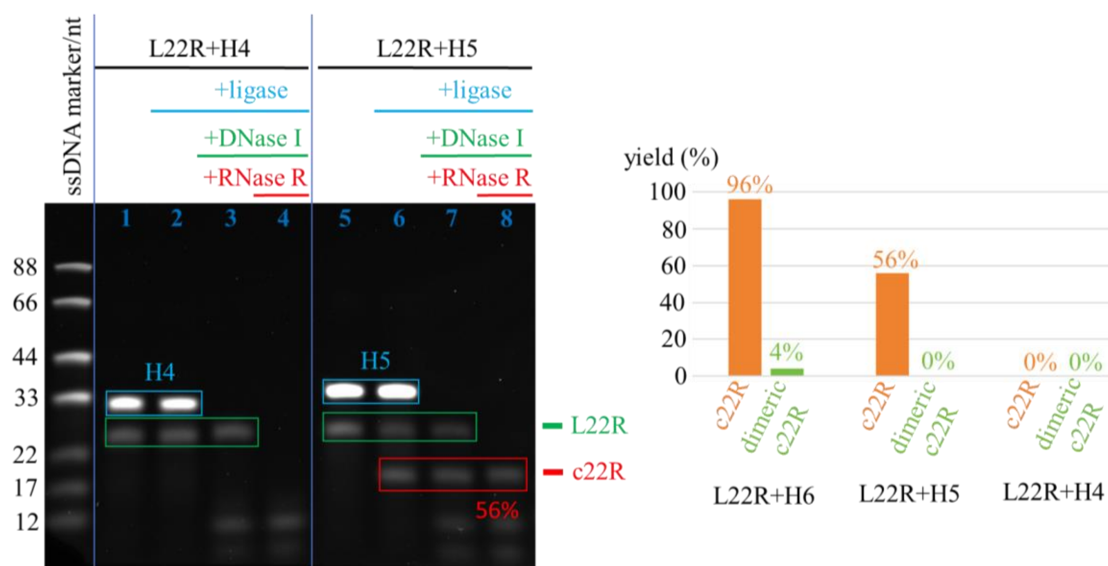


Fig. S6. Left panel: Effect of splinting base pair numbers complementary to L22R's 5′ and 3′ ends each by 4 (H4) and 5 (H5) nt on cyclisation of L22R. The c22R yield is indicated under the c22R bands. Right panel: Histogram depicting the monomeric and dimeric c22R yields against H6, H5,

and H4. (ligation conditions: 10 μM RNA, 2500 U per nanomolar RNA, 1 mM ATP, 10 mM Mg^{2+}, 16 °C, 16 h.)

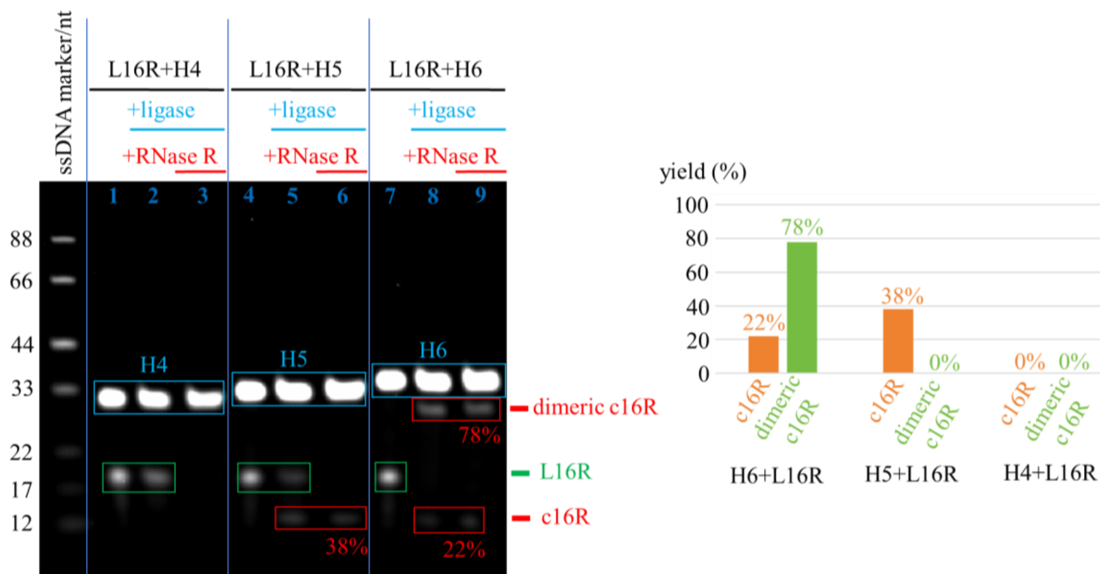


Fig. S7. Left panel: Effect of splinting base pair numbers complementary to L16R's 5′ and 3′ ends each by 4 (H4), 5 (H5) and 6 (H6) nt on cyclisation of L16R. The yields of c16R and dimeric c16R are indicated under the corresponding bands. Right panel: Histogram depicting the monomeric and dimeric c16R yields against H6, H5, and H4. (ligation conditions: 10 μM RNA, 2500 U per nanomolar RNA, 1 mM ATP, 10 mM Mg^{2+}, 16 °C, 16 h.)

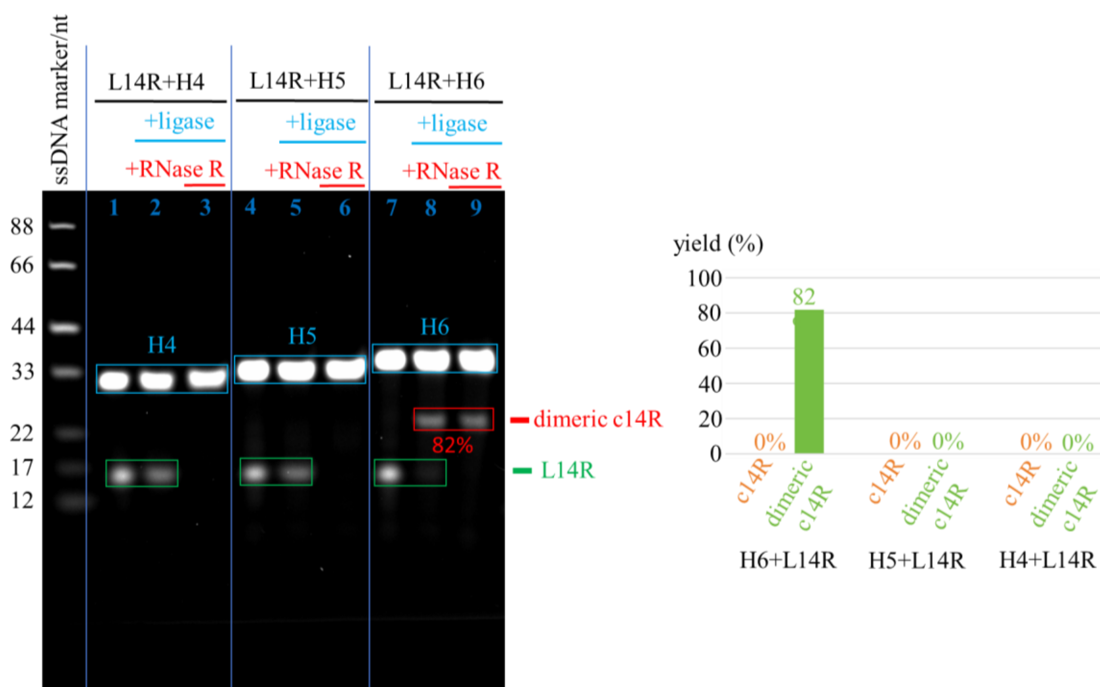


Fig. S8. Left panel: Effect of splinting base pair numbers complementary to L14R's 5′ and 3′ ends each by 4 (H4), 5 (H5), and 6 (H6) nt on cyclisation of L14R. The yields of dimeric c14R is indicated under the dimeric c14R bands. Right panel: Histogram depicting the monomeric and

dimeric c14R yields against H6, H5, and H4. (ligation conditions: 10 μM RNA, 2500 U per nanomolar RNA, 1 mM ATP, 10 mM Mg^{2+}, 16 °C, 16 h.)

S3. Additional AFM images and sectional profiling diagrams

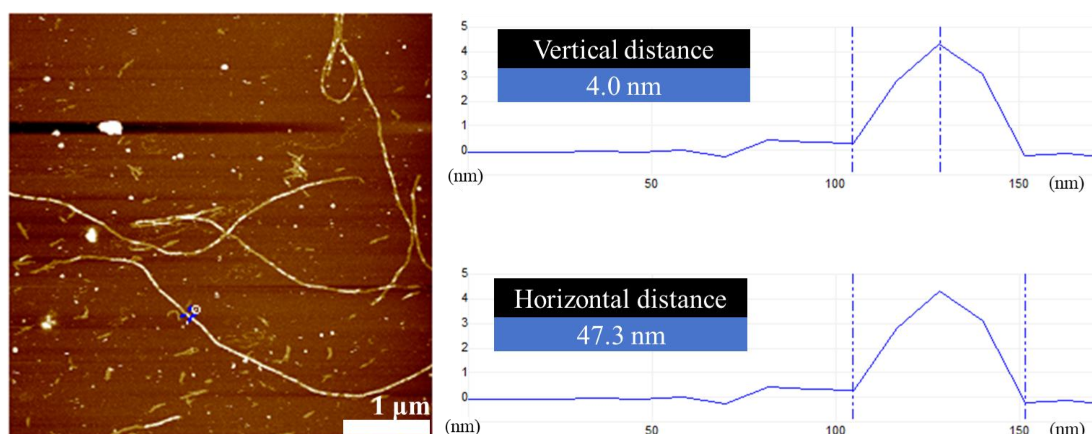


Fig. S9. The hybrid c44R:DNA DAE-$E_{21/5}$ nanotubes' height and width measurements are 4.0 nm and 47.3 nm, respectively. It is generally believed that the nanotubes measured are squashed, thus, the measured width is half of the nanotube perimeter. In the main content, we characterised nucleic acid nanotubes with their corresponding diameter parameters, thus the nanotube diameter here is (47.3 nm × 2)/π = 30.1 nm.

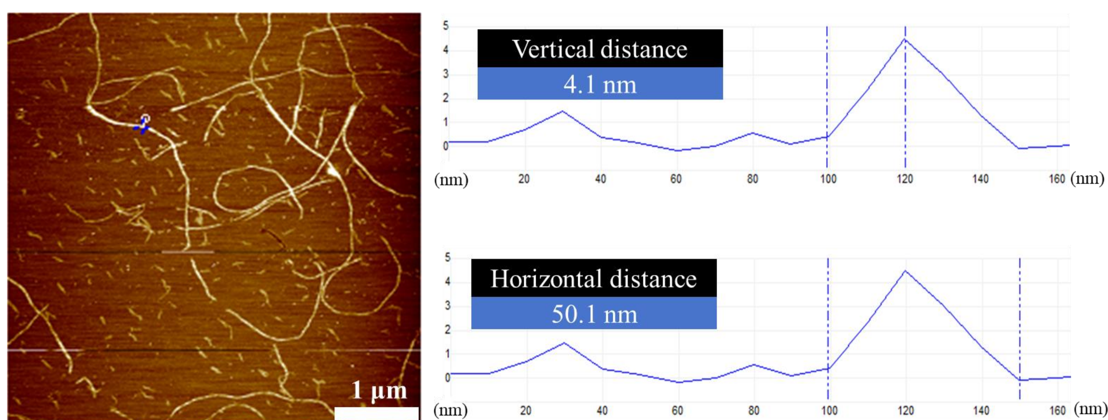


Fig. S10. The hybrid c44R:DNA DAE-$E_{20/4}$ nanotubes' height and width measurements are 4.1 nm and 50.1 nm, respectively. As described in the Fig. S9 caption, we converted the measured width to the diameter of 31.9 nm.

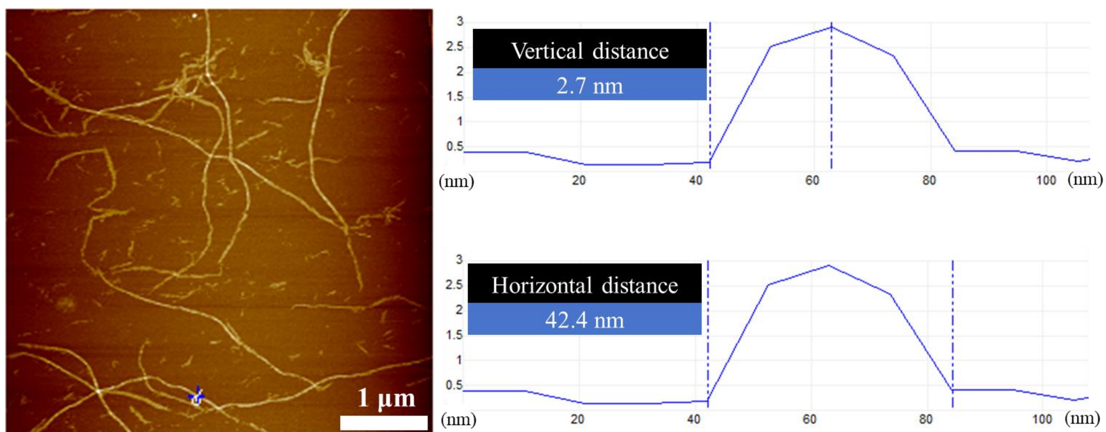


Fig. S11. The hybrid c44R:DNA nanotubes' height and width measurements for DAE-$E_{22/6}$ are 2.7 nm and 42.4 nm, respectively. Thus, the tube diameter is 27.0 nm.

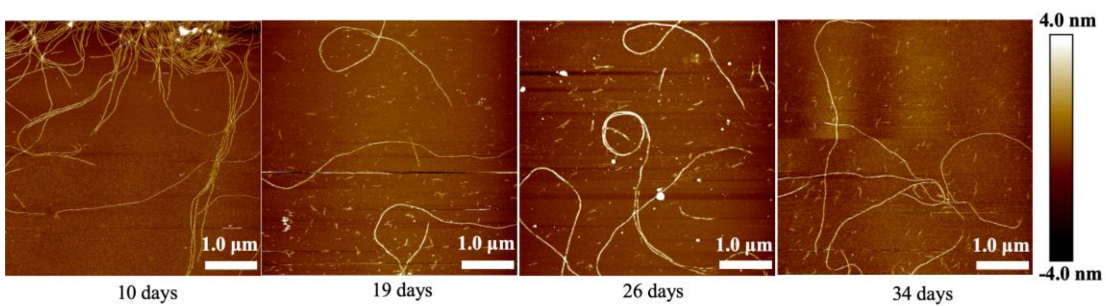


Fig. S12. AFM images of hybrid c44R:DNA nanotubes with different storage times.

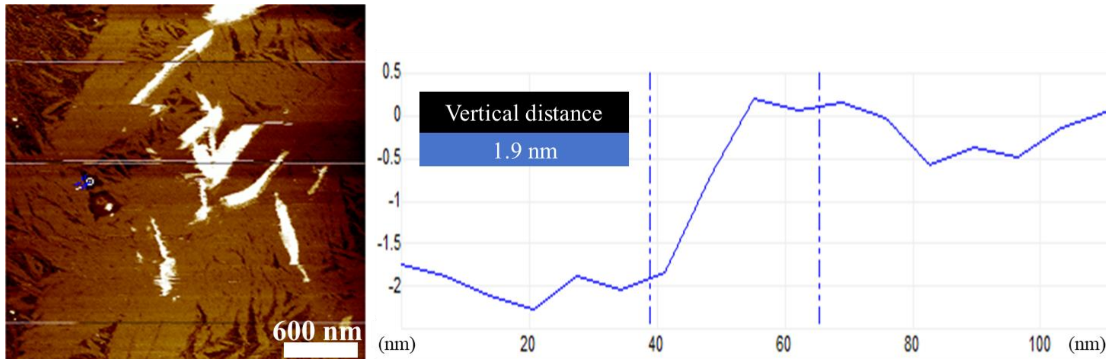


Fig. S13. The height of hybrid c44R:DNA DAE-$O_{27/5}$ nanogrids is 1.9 nm.

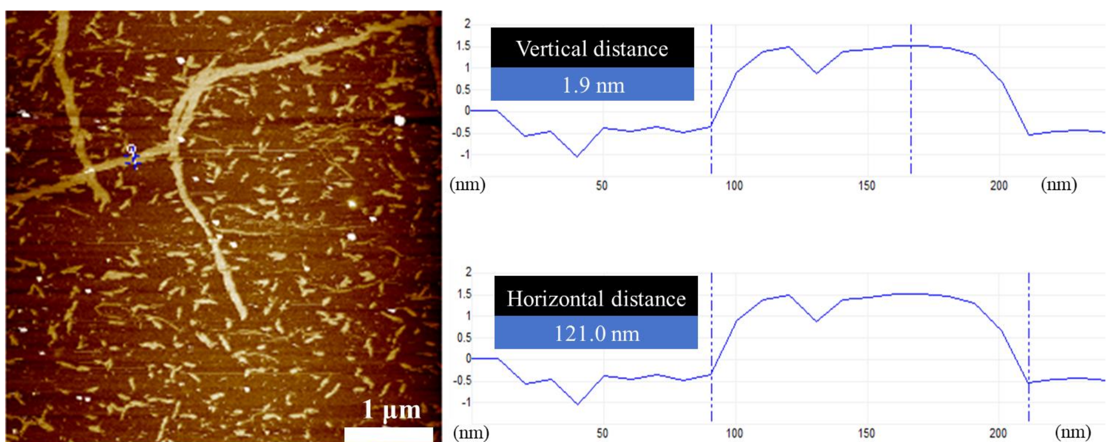


Fig. S14. The hybrid c44R:DNA DAE-$O_{26/4}$ nanostructures' height and width measurements are 1.9 nm and 121.0 nm, respectively.

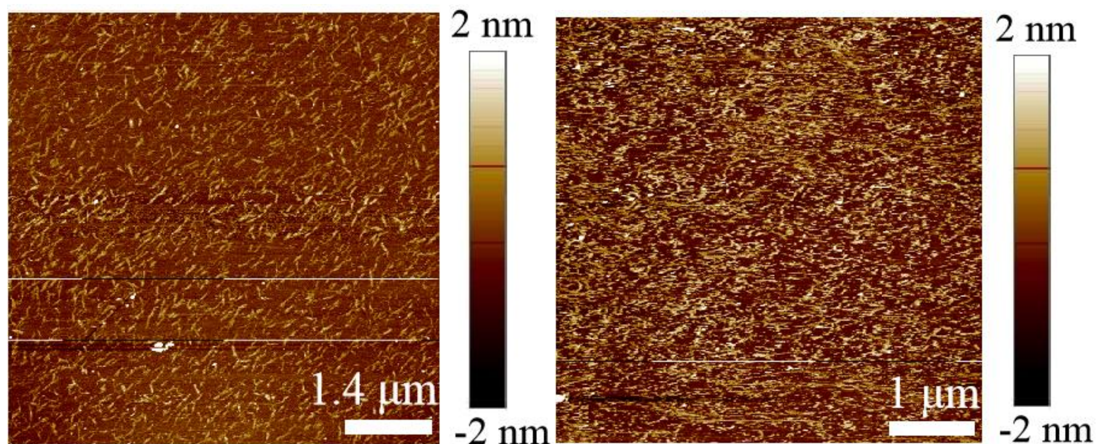


Fig. S15. AFM images of hybrid c44R:DNA DAE-$O_{28/4}$ (left) and DAE-$O_{29/5}$ (right) nanostructures.

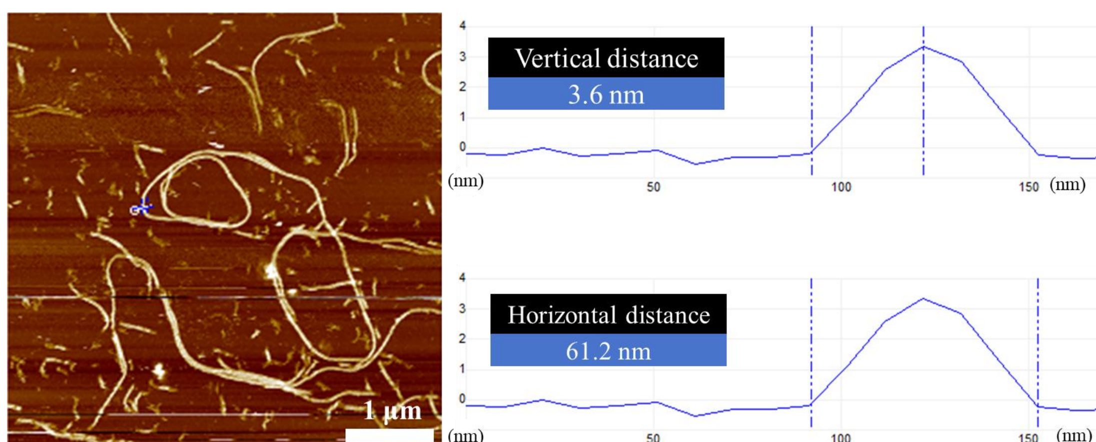


Fig. S16. The pure c44R:RNA DAE-$E_{22/6}$ nanotubes' height and width measurements are 3.6 nm and 61.2 nm, respectively. The tube diameter converted from the width is 39.0 nm.

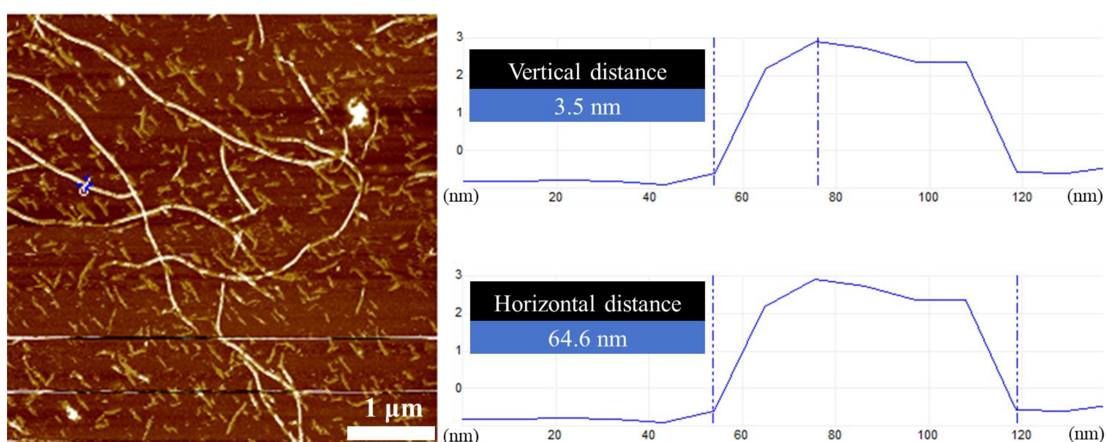


Fig. S17. The pure c44R:RNA DAE-$E_{22/4}$ nanotubes' height and width measurements are 3.5 nm and 64.6 nm, respectively. The tube diameter is 41.1 nm.

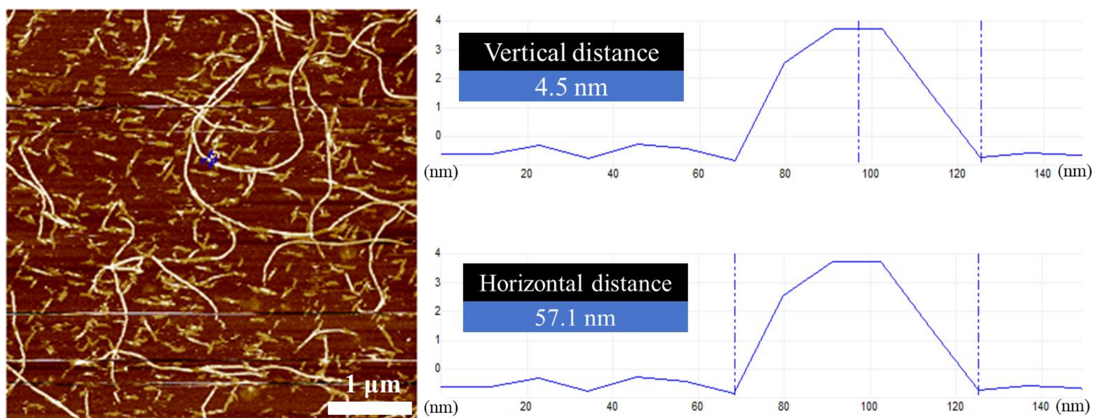


Fig. S18. The pure c44R:RNA DAE-$E_{23/5}$ nanotubes' height and width measurements are 4.5 nm and 57.1 nm, respectively. The tube diameter is 36.4 nm.

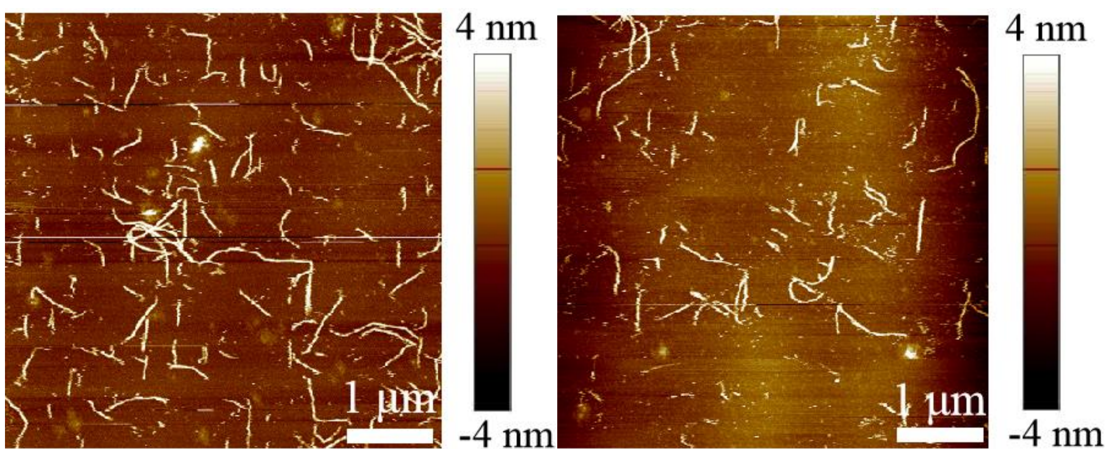


Fig. S19. AFM images of pure c44R:RNA DAE-$E_{23/7}$ (left) and DAE-$E_{24/8}$ (right) nanostructures.

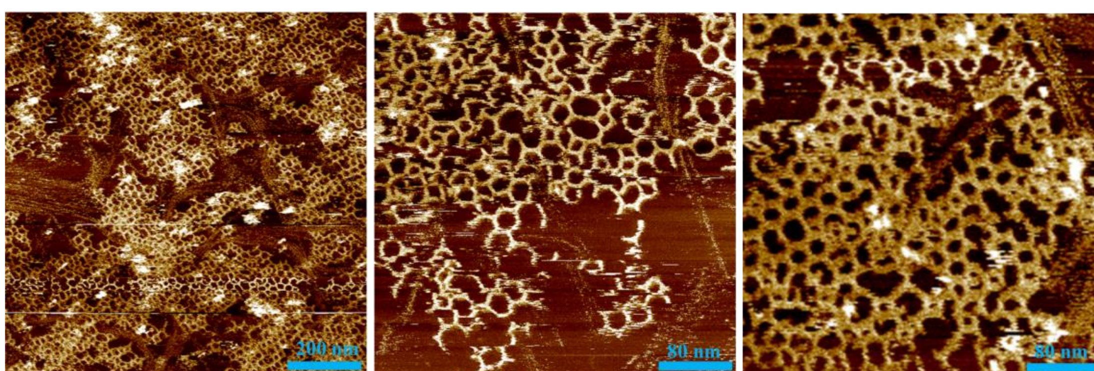


Fig. S20. Additional pure c44R:RNA DAE-KL hexagonal nanogrids.

S4. Nucleic acid sequences used in this work

All DNA and RNA strands sequences are listed below.

RNA sequences:

L22R (miR-16): 5'-pUAGCAGCACGUAAAUAUUGGCG-3'

L16R: 5’-pUAGCAGAAUAUUGGCG-3’

L14R: 5’-pUAGCAGUAUUGGCG-3’

L44R: 5’-pGCCAGAACGGCUGUGGGCUAAACAGUAACCGAAGCACCAACGCU-3’

DNA sequences:

H6 for splinting L22R, L16R, and L14R:

CTAGTTTTCTAGCTGCTACGCCAACGTGTTTTCACG

S6 for splinting L22R: CTGCTACGCCAA

S10 for splinting L22R: CGTGCTGCTACGCCAATATT

H5 for splinting L22R, L16R, and L14R:

CTAGTTTTCTAGTGCTACGCCACGTGTTTTCACG

H4 for splinting L22R, L16R, and L14R:

CTAGTTTTCTAGGCTACGCCCGTGTTTTCACG

H6-1T for splinting L22R: CTAGTCTAGCTGCTACGCCAACGTGTCACG

H6-8T for splinting L22R:

CTAGTTTTTTTTCTAGCTGCTACGCCAACGTGTTTTTTTTCACG

H6 for splinting L44R:

CTAGTTTTCTAGTCTGGCAGCGTTCGTGTTTTCACG

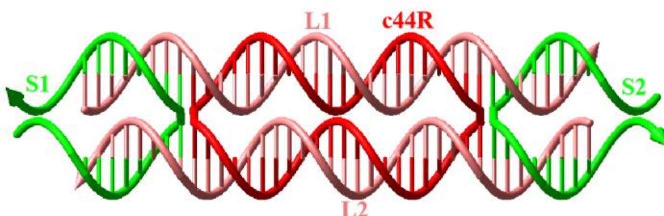
S6 for splinting L44R: TCTGGCAGCGTT

S10 for splinting L44R: CCGTTCTGGCAGCGTTGGTG

The same c44R sequence is used in all hybrid RNA:DNA and pure RNA:RNA assemblies.

c44R: GCCAGAACGGCUGUGGGCUAAACAGUAACCGAAGCACCAACGCU

Design model of the hybrid c44R:DNA DAE-E one-tile assembling system composed of c44R, two main helper strands L1 and L2 and two auxiliary helper strands S1 and S2:



Helper strand sequences of the hybrid c44R:DNA DAE-$E_{20/4}$ one-tile assembling system:

L1: GTCAGTGGACAGCCGTTCTGGCAGCGTTGGACGAAACT

L2: GGATGACCTGCTTCGGTTACTGTTTAGCCCTGCTCTAC

S1: GTCCGTAGAGCACCACTGACGAGC

S2: GGACAGTTTCGTGGTCATCCGCTC

Helper strand sequences of the hybrid RNA:DNA DAE-$E_{21/5}$ one-tile assembling system:

L1: GTCAGTGGACAGCCGTTCTGGCAGCGTTGGACGAAACT

L2: GGATGACCTGCTTCGGTTACTGTTTAGCCCTGCTCTAC

S1: GTCTGGTAGAGCACCACTGACAGGTA

S2: CAGACAGTTTCGTGGTCATCCTACCT

Helper strand sequences of the hybrid RNA:DNA DAE-$E_{22/6}$ one-tile assembling system:

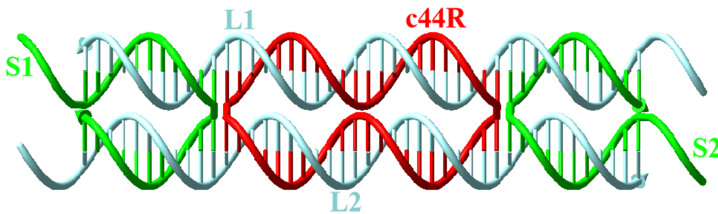
L1: GTCAGTGGACAGCCGTTCTGGCAGCGTTGGACGAAACT

L2: GGATGACCTGCTTCGGTTACTGTTTAGCCCTGCTCTAC

S1: GTCTGCGTAGAGCACCACTGACAAGGTA

S2: GCAGACAGTTTCGTGGTCATCCTACCTT

Design model of the hybrid c44R:DNA DAE-O one-tile assembling system composed of c44R, two main helper strands L1 and L2 and two auxiliary helper strands S1 and S2::



Helper strand sequences of the hybrid RNA:DNA DAE-$O_{26/4}$ one-tile assembling system:

L1: GCAGGACGTCAGTGGACAGCCGTTCTGGCAGCGTTGGACGAGCTAACT

L2: CGTCGGATGACAGTCTGCTTCGGTTACTGTTTAGCCCTGCTCTACTGC

S1: CTGCGCAGTAGAGCACCACTGACGTC

S2: GACGAGTTAGCTCGTGACTGTCATCC

Helper strand sequences of the hybrid RNA:DNA DAE-$O_{27/5}$ one-tile assembling system:

L1: GCTAGGACGTCAGTGGACAGCCGTTCTGGCAGCGTTGGACGAGCTAACT

L2: CAGTCGGATGACAGTCTGCTTCGGTTACTGTTTAGCCCTGCTCTACTGC

S1: CTAGCGCAGTAGAGCACCACTGACGTC

S2: GACTGAGTTAGCTCGTGACTGTCATCC

Helper strand sequences of the hybrid RNA:DNA DAE-$O_{28/6}$ one-tile assembling system:

L1: GCAGACGACGTCAGTGGACAGCCGTTCTGGCAGCGTTGGACGAGCTAACT

L2: CCTCAGGGATGACAGTCTGCTTCGGTTACTGTTTAGCCCTGCTCTACTGC

S1: GTCTGCGCAGTAGAGCACCACTGACGTC

S2: CTGAGGAGTTAGCTCGTGACTGTCATCC

Helper strand sequences of the hybrid RNA:DNA DAE-$O_{29/5}$ one-tile assembling system:

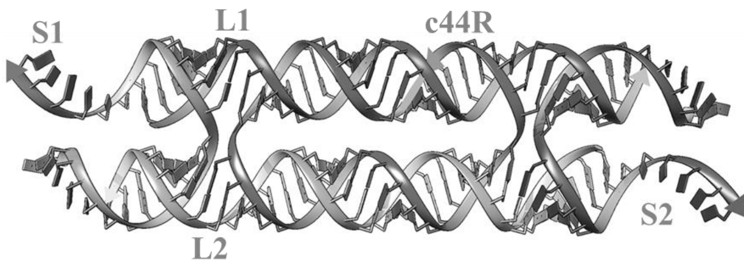
L1: GCTAGGACGATCAGTGGACAGCCGTTCTGGCAGCGTTGGACGAGCTCAACT

L2: CAGTCGGATGACGAGTCTGCTTCGGTTACTGTTTAGCCCTGCTCTATCTGC

S1: CTAGCGCAGATAGAGCACCACTGATCGTC

S2: GACTGAGTTGAGCTCGTGACTCGTCATCC

Design model of the pure RNA:RNA DAE-E one-tile assembling system:



Helper strand sequences of the pure RNA:RNA DAE-$E_{22/4}$ one-tile assembling system:

L1: GUCAGUAGGACAGCCGUUCUGGCAGCGUUGGACGACAACU

L2: GGAUUGACCUGCUUCGGUUACUGUUUAGCCCUGCUUCUAC

S1: GUCUGUAGAAGCACCUACUGACAGGU

S2: AGACAGUUGUCGUGGUCAAUCCACCU

Helper strand sequences of the pure RNA:RNA DAE-$E_{22/6}$ one-tile assembling system:

L1: GUCAGUGGACAGCCGUUCUGGCAGCGUUGGACGAAACU

L2: GGAUGACCUGCUUCGGUUACUGUUUAGCCCUGCUCUAC

S1: GUCUGCGUAGAGCACCACUGACAAGGUA

S2: GCAGACAGUUUCGUGGUCAUCCUACCUU

Helper strand sequences of the pure RNA:RNA DAE-$E_{23/5}$ one-tile assembling system:

L1: GUCAGUAGGACAGCCGUUCUGGCAGCGUUGGACGACAACU

L2: GGAUUGACCUGCUUCGGUUACUGUUUAGCCCUGCUUCUAC

S1: GUCUGGUAGAAGCACCUACUGACAAGGU

S2: CAGACAGUUGUCGUGGUCAAUCCACCUU

Helper strand sequences of the pure RNA:RNA DAE-$E_{23/7}$ one-tile assembling system:

L1: GUCAGUGGACAGCCGUUCUGGCAGCGUUGGACGAAACU

L2: GGAUGACCUGCUUCGGUUACUGUUUAGCCCUGCUCUAC

S1: GUCUGCCGUAGAGCACCACUGACCAAGGUA

S2: GGCAGACAGUUUCGUGGUCAUCCUACCUUG

Helper strand sequences of the pure RNA:RNA DAE-$E_{24/8}$ one-tile assembling system:

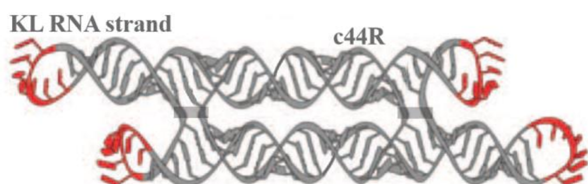
L1: GUCAGUGGACAGCCGUUCUGGCAGCGUUGGACGAAACU

L2: GGAUGACCUGCUUCGGUUACUGUUUAGCCCUGCUCUAC

S1: GGUCUGCCGUAGAGCACCACUGACACAAGGUA

S2: GGCAGACCAGUUUCGUGGUCAUCCUACCUUGU

Design model of the pure RNA:RNA DAE-KL one-tile assembling system composed of c44R and a transcribed KL strand:



Double-stranded DNA template for preparing the KL RNA strand:

5'-TTCTAATACGACTCACTATAGGCAGCGTTGGGCTCGGGAGCGTCCACTGCTCCTGAGC
GCTCCAGTGGACGGAGCTGCTTCGGTTACTGTTTAGCCCCACGGGCTCCGAGACGTGGA
GTCCGTGTCCGCACGTCTCGCGGAACAGCCGTTCT-3'.

5'-AGAACGGCTGTTCCGCGAGACGTGCGGACACGGACTCCACGTCTCGGAGCCCGTGGG
GCTAAACAGTAACCGAAGCAGCTCCGTCCACTGGAGCGCTCAGGAGCAGTGGACGCTCC
CGAGCCCAACGCTGCCTATAGTGAGTCGTATTAGAA-3'.

KL RNA strand:

GGCAGCGUUGGGCUCGGGAGCGUCCACUGCUCCUGAGCGCUCCAGUGGACGGAGCUG
CUUCGGUUACUGUUUAGCCCCACGGGCUCCGAGACGUGGAGUCCGUGUCCGCACGUC
UCGCGGAACAGCCGUUCU