Supplementary data

Material and methods.

Material:

All oligonucleotides (details in Table S1) were purified using HPLC and PAGE, and purchased from Anhui General Biology Co., Ltd. (China). T4 DNA ligase (10,000 U/μl), dNTP (25 mM each), and phi 29 DNA polymerase (10 U/μl) were acquired from Beyotime Biotechnology Co., Ltd. (China). BSA (10mg/ml) was obtained from Baiolaibo Technology Co., Ltd. (China). 5-FAM-dUTP (1mM) was acquired from ABP Biosciences Co., Ltd. (USA). The DNA marker, 6×DNA loading buffer, agarose, and gelred nucleic acid dye were purchased from Biomed Gene Technology Co., LTD (China). 50×TAE buffer was purchased from Biomed Gene Technology Co., LTD (China). The sequences of the oligonucleotides are presented in Table S1.

Table S1. The sequences of oligonucleotides used for the preparation of RCA-based hydrogel

Oligo name	Sequences
Design I circular template	5’-P-ATCTGTGTTTGGTTGCTGCTGCCACGTATCACCAGGCAGTGCAGCAGCACACCAAATGACGTTAGAAGTCAGGTTGC-3’
Design I primer	5’-CAACCAAACACAGATGCAACCTGA-3’
Design II circular template	5’-P-GTCAGGTTGCATCTGTGTTTGGTTGCTGCTGCAGCGATACGCGTATCGCTATGGCATATCGTACGATATGCCGCAGCAGCACACCAAATGACGTTAGAA-3’
Design II primer	5’-ACCAAACACAGATGCAACCTGACTTCTAACGTCATTTGGTGTGCTGCTGC-3’
Design III circular template	5’-P-ACGCCGGAAGTCCCGGCCACTGCGTGCTGCTGCAGCGATACGCGTATCGCTATGGCATATCGTACGATATGCCGCAGCAGCACGCTCGGCATATCGCCGTC-3’

Design III primer	5’-CGCAGTGGCCGGGACTTCCGGCGTGACG GCGATATGCCGAGCGTGCTGCTGC-3’

The process of preparing circular templates:

The RCA process is bifurcated into two parts stages. The initial stage involves the circularization of linear templates as shown in the following Table 1. Mix components in one PCR tube. The molar ratio of template and primer was recommended at 1:2.

Table S2. The preparation of a circular DNA template

Component	Amount	Final concentration
12.48 μM template	2.4 μl	0.6 μM
13.02 μM primer	4.61 μl	1.2 μM
T4 DNA ligation buffer	5 μl	1×
Milli-Q water	37.99 μl	–
Total	50 μl	–

Then, this was achieved by subjecting to a temperature cycle of 95 ℃ for 2 minutes, 65 ℃ for 30 minutes, 50 ℃ for 30 minutes, 37 ℃ for 30 minutes and 22 ℃ for 30 minutes. Then 0.5 μl T4 ligase was incorporated into the reaction system and incubated at room temperature for a duration of 3 hours. This facilitated the ligation of the 5’-phosphate and 3’-hydroxyl ends of the template, resulting in the formation of a circular DNA template. Next, place the centrifuge tubes at 4 °C for at least 5 hours.

Synthesis of RCA-based DNA macro hydrogels:

The second step was the rolling circle amplification reaction (RCA). Initially, add the components shown in Table 2 below to a 200 μl tube. The circular template, which had been hybridized with primers, was incubated with a phi 29 DNA polymerase elongation system. Afterward, put the tubes into the PCR thermal cycler at 30 °C for 15 hours. The circular DNA template and RCA products were subjected to analysis by scanning electron microscopy (SEM) and Dynamic light scattering (DLS).

Table S3. The component of phi 29 DNA polymerase elongation system

Component	Amount	Final concentration
circular DNA template	50 μl	0.3 μM
10× phi29 DNA polymerase buffer	10 μl	1×
10 U/μl phi29 DNA polymerase	10 μl	1 U/μL
BSA (10 mg/mL)	0.5 μl	0.05 mg/mL
dNTPs (25 mM)	2 μl	0.5 mM
Milli-Q water	27.5 μl	–
Total volume	100 μl	–

To compare with other approaches reported in the literature that generated macro hydrogels through RCA reaction. RCA-based macro hydrogels synthesized by oscillation were also prepared. Initially, the formation of the circular DNA template using the primer as the linker. Prepare the solution for circular DNA template precursor as shown in the following Table 3. Mix components in one PCR tube. We recommend a molar ratio of template and primer of 1:1.

Table S4. The preparation of a circular DNA precursor

Component	Amount	Final concentration
100 μM template	2 μl	10 μM
100 μM primer	2 μl	10 μM
NaCl solution (800 mM)	2 μl	80mM
Milli-Q water	14 μl	–
Total	20 μl	

Secondly, put PCR tubes into a PCR thermal cycler, and set the annealing program using the following cycling conditions: the template was denatured by cycling at 95 ℃ for 2 minutes, 65 ℃ for 2 min; 60 ℃ for 6 min; a ramp from 60 to 20 ℃ at −0.5 ℃/30 seconds for 80 cycles, held at 20 ℃ for 30 seconds and held at 4 ℃ for 10 minutes. This program will hybridize the template and primer, resulting in the cyclization of the template to obtain the circular DNA template precursor. Then prepare a solution of T4 DNA ligase to close the nick of the cyclization product to obtain a circular DNA template for the RCA process. Add components as shown in the following Table 4 into a 1.5 mL tube, and vortex the mixture. Divide 140 μL of mixed solution into 7 PCR tubes. Then put the tubes into the PCR thermal cycler at 16 °C for 8 hours. After the ligation, heat the sample in a thermal incubator at 65 °C for 10 minutes to inactivate the enzyme.

Table S5. The preparation of a circular DNA template

Component	Amount	Final concentration

10 μM circular template precursor	4 μl	0.29 μM
10× T4 DNA ligase buffer	14 μl	1×
1000 U/μL T4 DNA ligase	0.01 μl	0.07 U/μL
Milli-Q water	122 μl	–

The oscillation system was subsequently prepared, as outlined in Table 5. The samples were then incubated at a constant temperature of 37 °C within a shaker operating at 450rpm/min to yield macro hydrogels. For the formation of these hydrogels, the DNA strand must be as long as possible, typically necessitating a duration exceeding 24 hours. After the RCA reaction, put the tubes into a thermal incubator at 65 °C for 10 minutes to inactivate phi29 DNA polymerase.

Table S6. RCA for physical entanglement of DNA chains

Component	Amount	Final concentration
0.29 μM circular template	17.2 μl	50 nM
10× phi29 DNA polymerase buffer	10 μl	1×
10 U/μl phi29 DNA polymerase	2 μl	0.2 U/μl
BSA (10 mg/mL)	2 μl	0.2 mg/ml
NaCl solution (800 mM)	10 μl	80 mM
dNTPs (25 mM)	4 μl	1 mM
Milli-Q water	54.8 μl	–
Total volume	100 μl	–

Synthesis of RCA-based DNA nanoparticles:

This aligns with the methodology used to prepare circular DNA template of macro hydrogels: 0.6 μM of the phosphorylated linear template was amalgamated with 1.2 μM of primer and annealed in a 50 μl volume of 1× T4 DNA ligation buffer (comprising 50 mM Tris-HCl, 10 mM MgCl2, 10 mM DTT, and 1 mM ATP, pH 7.5). The initial stage involves the circularization of linear templates as shown in the following Table 6.

Table S7. The preparation of a circular DNA template

Component	Amount	Final concentration
12.48 μM template	2.4 μl	0.6 μM
13.02 μM primer	4.61 μl	1.2 μM
T4 DNA ligation buffer	5 μl	1×
Milli-Q water	37.99 μl	–
Total	50 μl	–

This was accomplished by subjecting the sample to a temperature cycle of 95 ℃ for 2 minutes, 65 ℃ for 30 minutes, 50 ℃ for 30 minutes, cooling to 37 ℃ for 30 minutes, and finally, lowering the temperature to 22 ℃ for an additional 30 minutes. Then 0.5 μl T4 ligase was incorporated into the reaction system and incubated at room temperature for a duration of 3 hours. The circular DNA template was successfully synthesized.

The circular DNA template was subsequently incubated with phi29 DNA polymerase,

dNTP, and BSA in 1× phi 29 DNA polymerase buffer for 10 minutes, 1 hour, 3 hours, and 24 hours by RCA reaction. The components of the phi 29 DNA polymerase elongation system as shown in the following Table 7.

Table S8. The component of phi 29 DNA polymerase elongation system

Component	Amount	Final concentration
circular DNA template	50 μl	0.6 μM
10× phi29 DNA polymerase buffer	10 μl	1×
10 U/μl phi29 DNA polymerase	10 μl	1 U/μL
BSA (10 mg/mL)	0.5 μl	0.05 mg/mL
dNTPs (25 mM)	2 μl	0.5 mM
Milli-Q water	27.5 μl	–
Total volume	100 μl	–

Agarose gel electrophoresis:

The RCA products were subjected to analysis by 1.0% agarose gel, employing electrophoresis at 100 V and a temperature of 25 °C for a duration of 1 hour. The electrophoresis was conducted in Tris-acetate-EDTA (TAE, pH 8.3). Subsequently, the gel underwent staining for a period of 30 minutes utilizing Gelred. Finally, the agarose gel was imaged under UV irradiation utilizing the Gel Image System (Tanon-1600, China).

SEM characterization of RCA products:

The morphologies of RCA products were observed by SEM. Silicon wafers used in this assay were fully washed with 75% ethyl alcohol and pure water. The RCA products and macro hydrogels underwent dehydration via gradients of ethanol, specifically at concentrations of 35%, 50%, 75%, 95%, and 100%. The RCA products were cast on silicon wafers, and Au was sputtered after drying by the ion sputtering instrument (Gressington 108Auto, UK). The SEM samples were observed under the voltage of 3 kV by field emission scanning electron microscope (ZEISS GeminiSEM 300, Germany).

Dynamic light scattering (DLS):

The quantification of circular DNA template and RCA products was achieved by comparing their absorbances at 405 nm against a calibration curve derived from a standard serial dilution of DNA hydrogel in PBS. The size distribution of the prepared RCA products was characterized using the DLS (MalvernPanalytical Mastersizer 3000, UK).

We have designed three distinct scaffolds for the RCA monomer structure. In design I, the template sequence incorporates a complementary base sequence to TC01 aptamer, along with 36 nt arbitrary free sequences. These free sequences were randomly selected and verified using the Mfold program to ensure they did not form hairpin or internal duplex structures. In design II and design III, the template sequence incorporates a complementary base sequence to TC01 aptamer and Tgg2 aptamer, along with

palindromic complementary sequences where with long dsDNA on the backbone and stem of branched aptamer structures protruding and aligning alternatively on the sides. The design of palindromic sequences enhances the binding affinity of bases to drugs, offering benefits in drug loading, as exemplified by doxorubicin. This approach can be employed for multichannel cellular imaging and traceable targeted drug delivery.

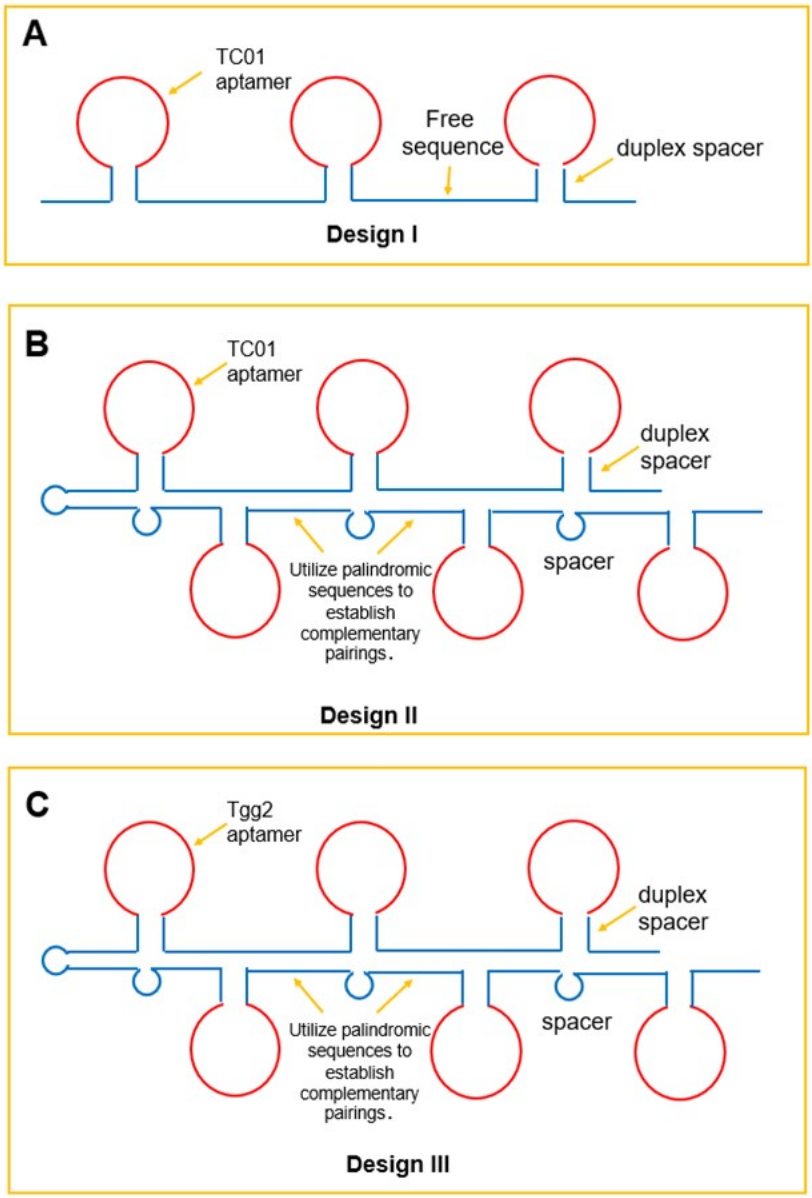

Figure S1. Schematic representations of three RCA designs. (A) The primary architecture of Design I encompasses the TC01 aptamer and a 36 nt free sequence. (B)

The primary architecture of Design II encompasses the TC01 aptamer and the 18 nt palindromic sequence. With palindromic complementary design, and long ssDNA on the backbone and stem of branched aptamer structures protruding and aligning alternatively on the sides. (C) The primary architecture of Design III encompasses the Tgg2 aptamer and the 18 nt palindromic sequence. With palindromic complementary design, and long ssDNA on the backbone and stem of branched aptamer structures protruding and aligning alternatively on the sides.

To ascertain the broad applicability of the method for synthesizing macro hydrogels via a cold treatment step, we conducted an additional 4 ℃ coldly incubated process lasting 5 hours on the circular DNA template of design II and design III. Following 15 hours of RCA reaction, the control sample, which did not undergo any additional cold treatment steps, exhibited a clarified liquid. SEM results indicated that the control was primarily composed of DNA nanoparticles, which were uniformly dispersed within the RCA system. However, following the additional cold treatment step, the RCA products manifested as macro hydrogels.

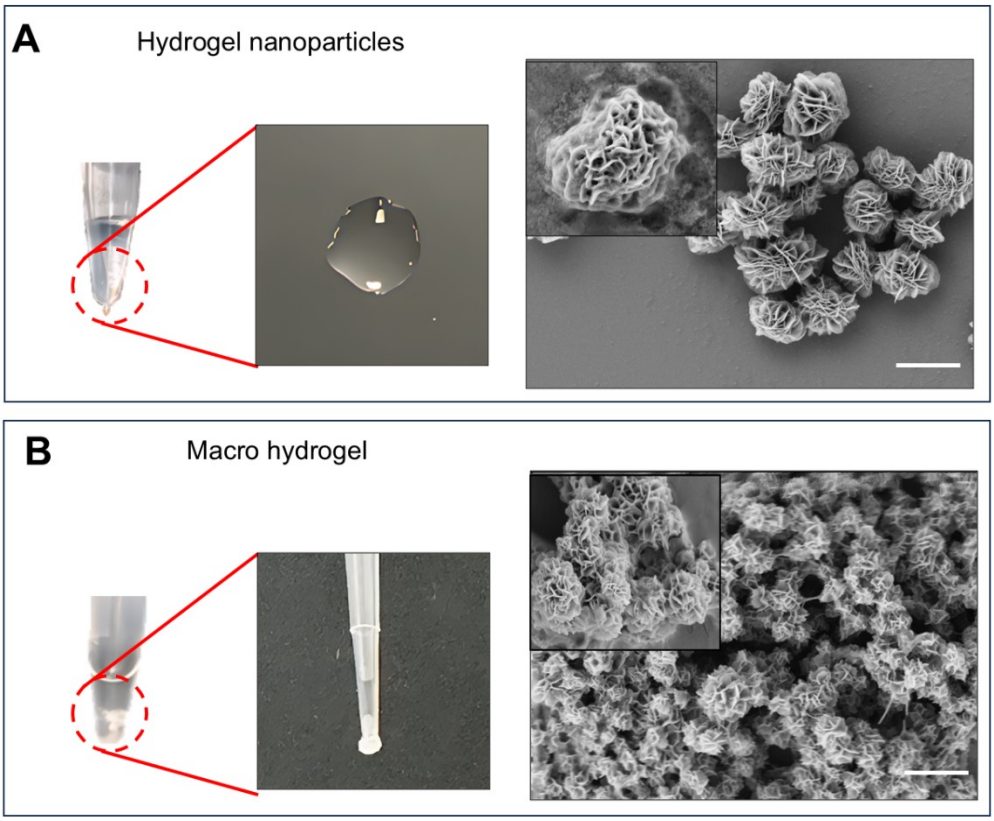

Figure S2. The effect of additional cold treatment step on the products of RCA. (A) The RCA products of design II without additional cold treatment of the circular template chain are presented. After an identical RCA reaction time, the control group without additional cold treatment remained in a clear state. SEM image revealed there were nanoparticles with a particle size of 1 μm in the sample. (B) The RCA products of design II with the additional cold treatment of the circular template chain. We generated a viscous solution through additional cold treatment before the RCA reaction. Due to a small degree of viscosity elasticity deformation, the macro hydrogels hung onto the pipette head, and the SEM image showed the wound chain with spherical particles attached to the surface. Scale bar = 1 μm.

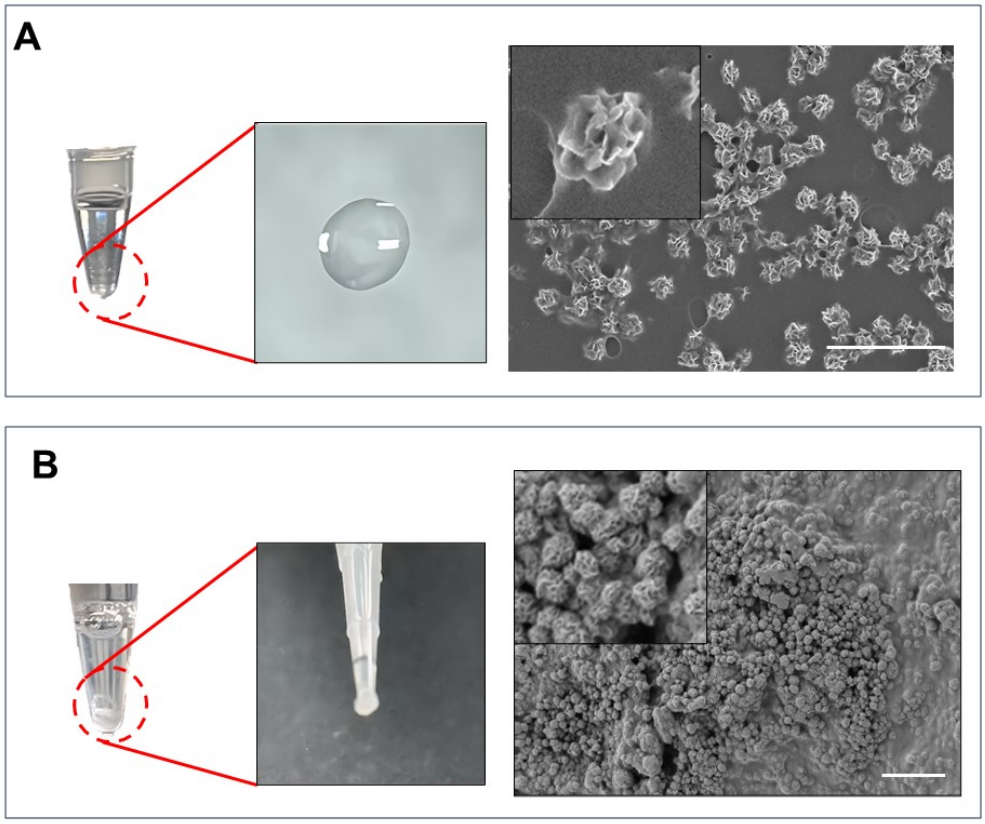

Figure S3. The effect of additional cold treatment on the products of RCA. (A) The RCA products of design III with the additional cold treatment of the circular template chain. We generated a viscous solution through additional cold treatment before the RCA reaction. Due to a small degree of viscosity elasticity deformation, the macro hydrogel hung onto the pipette head, and the SEM image can see the wound chain with spherical particles attached to the surface (B). The RCA products of design III without additional cold treatment of the circular template chain are presented. After an identical RCA reaction time, the control group without additional cold treatment remained in a clear state. SEM image reveals nanoparticles with a particle size of 1 μm. (scale bar = 10 μm).

In addition, we carried out 1 hour, 3 hours, 5 hours, and 7 hours cold treatment steps on the circular DNA template of Design II followed by RCA reaction for 15 hours. The particle size distribution of the design II circular template demonstrated an escalating trend in aggregation degree as the cold treatment time increased. In the RCA products, the groups coldly incubated for 1 hour and 3 hours yielded transparent liquids, suggesting that the RCA products were DNA nanoparticles. Conversely, macro hydrogels were derived from the groups coldly incubated for 5 hours and 7 hours, aligning with Design I. To ascertain whether the cold treatment step contributed to the aggregation of the circular DNA template, we altered the temperature from 4 ℃ to 22 ℃. Our findings revealed that irrespective of the duration for which the circular DNA template was exposed to 22 ℃, the resultant RCA products consistently manifested as DNA hydrogel nanoparticles. This observation elucidated the mechanism underlying macro hydrogels synthesis.

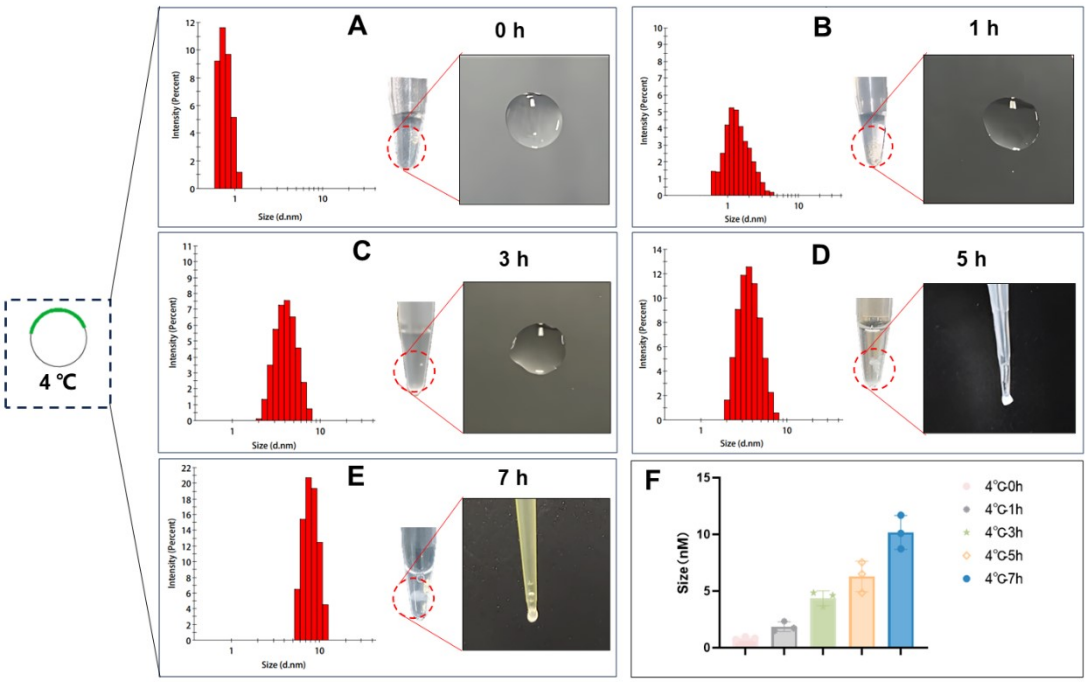

Figure S4. The effect of cold treatment time on the particle size of the Design II circular template and the formation of DNA macro hydrogels. (A) The circular DNA template was not coldly incubated at 4 ℃, resulting in a particle size of approximately 0.84 nm. The clarified liquid indicated that the RCA products were hydrogel nanoparticles after 15 hours RCA reaction. (B) The circular DNA template was coldly incubated at 4 ℃ for 1 hour, yielding a particle size of around 1.85 nm. The clarified liquid indicated that the RCA products were hydrogel nanoparticles after 15 hours of RCA reaction. (C) The circular DNA template was coldly incubated at 4 ℃ for 3 hours, leading to a particle size of approximately 4.36 nm. The clarified liquid indicated that the RCA products were hydrogel nanoparticles after 15 hours of RCA reaction. (D) The circular DNA template was coldly incubated at 4 ℃ for 5 hours, resulting in a particle size of around 6.29 nm. After 15 hours of RCA reaction, the macro hydrogels were obtained, which hung onto the pipette head. (E) The circular DNA template was coldly incubated at 4 ℃ for 7 hours. The particle size of the circular

DNA template was around 10.17 nm, and the macro hydrogels were obtained after 15 hours of RCA reaction. (F) The particle size of the circular DNA template increased as cold treatment time was increased from 1 hour to 7 hours.

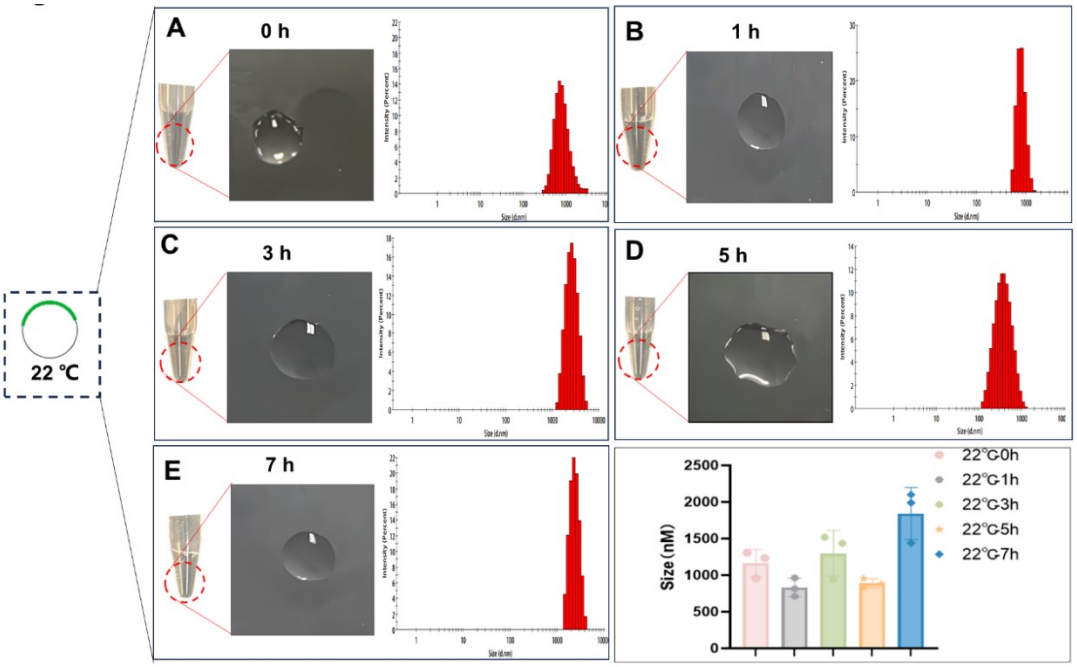

Figure S5. The effect of temperature on the particle size distribution of Design I circular DNA template and the formation of macro hydrogels. (A) The circular DNA template was used immediately for the elongation step. The RCA reaction was conducted for 15 hours. The resultant sample was clear. The particle size distribution was detected by DLS. (B) The circular DNA template was standing at 22 C for 1 hour. After the RCA reaction for 15 hours, the product remained clear, and the particle size was determined by DLS. (C) The circular DNA template was standing at 22 ℃ for 3 hours. After the RCA reaction for 15 hours, the product was still clear, and the particle size was detected by DLS. (D) The circular DNA template was standing at 22 ℃ for 5 hours. Following the RCA reaction for another 15 hours, the product remained clear,

and the particle size was measured by DLS. (E) The circular DNA template was standing at 22 ℃ for 7 hours. After the RCA reaction for 15 hours, the product was in a clear state, and the particle size was detected by DLS. (F) The particle size change of the cyclized template chain after an extra cold treatment time of 0,1,3,5,7 hours for 22 ℃. The nanoparticles exhibited a uniform size of approximately 1μm, with no formation of macro hydrogels observed.

To ascertain that merely extending the RCA reaction time does not facilitate the transformation of nanoparticles into macro hydrogels, we refrained from incorporating an additional cold treatment step before the RCA reaction. Subsequently, we monitored the morphological alterations of RCA products following 10 minutes, 1 hour, 3 hours, and 24 hours of RCA reaction duration. Our findings revealed that irrespective of the RCA reaction time—whether it was 10 minutes, 1 hour, 3 hours, or even exceeding 24 hours—the resultant RCA products consistently manifested as a clear liquid, devoid of any macro hydrogels formation. SEM analyses further elucidated that with the prolongation of the reaction time, there was a proportional increase in the size of DNA nanoparticles commensurate with the length of the RCA reaction. However, upon attaining a specific size threshold, these nanoparticles ceased to enlarge and persisted in their nanoparticle form. Additionally, we investigated the impact of T4 ligase inactivation on RCA products after the circularization of linear templates. Agarose gel electrophoresis results demonstrated that T4 ligase inactivation did not influence the RCA products.

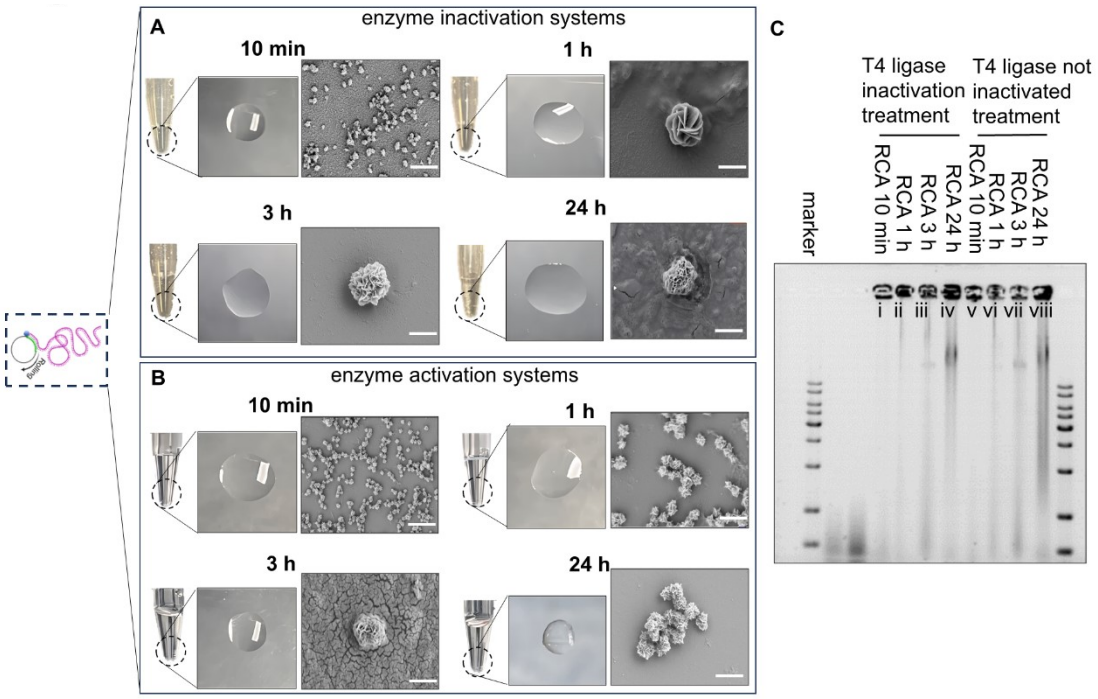

Figure S6. The effect of RCA reaction time on the microstructure of DNA hydrogel particles. (A) Following the inactivation of T4 ligase, RCA reactions were conducted for durations of 10 minutes, 1 hour, 3 hours, and 24 hours. SEM images of DNA nanoparticles from each sample were shown. (B) T4 ligase was not inactivated. RCA reactions were conducted for durations of 10 minutes, 1 hour, 3 hours, and 24 hours. SEM images of DNA nanoparticles from each sample were shown. Scale bars = 1 μm. (C) Agarose gel (1%) electrophoresis of RCA products. The gel included eight lanes, each loaded with different samples: i) RCA products with 10 minutes reaction duration. T4 DNA ligase was inactivated before the elongation step; ii) RCA products with 1 hour reaction duration. T4 DNA ligase was inactivated before the elongation step; iii) RCA products with 3 hours reaction duration. T4 DNA ligase was inactivated before the elongation step; iv) RCA products with 24 hours reaction duration. T4 DNA ligase was inactivated before the elongation step; v) RCA products with 10 minutes reaction

duration. T4 DNA ligase was not inactivated before the elongation step; vi) RCA products with 1 hour reaction duration. T4 DNA ligase was not inactivated before the elongation step; vii) RCA products with 3 hours reaction duration. T4 DNA ligase was not inactivated before the elongation step; viii) RCA products with 24 hours reaction duration. T4 DNA ligase was not inactivated before the elongation step.

We utilized laser confocal to observe the ability of RCA products from Design I and Design II to target Ramos. In comparison to non-target cell HL-60, Ramos exhibited strong fluorescence. This suggested that the nanoparticles generated from both of the two designs possess the ability to target Ramos. Such advancements hold significant potential for applications in cell culture, cell coating, organoid culture, argeted drug delivery in lymphoma, and tissue engineering.

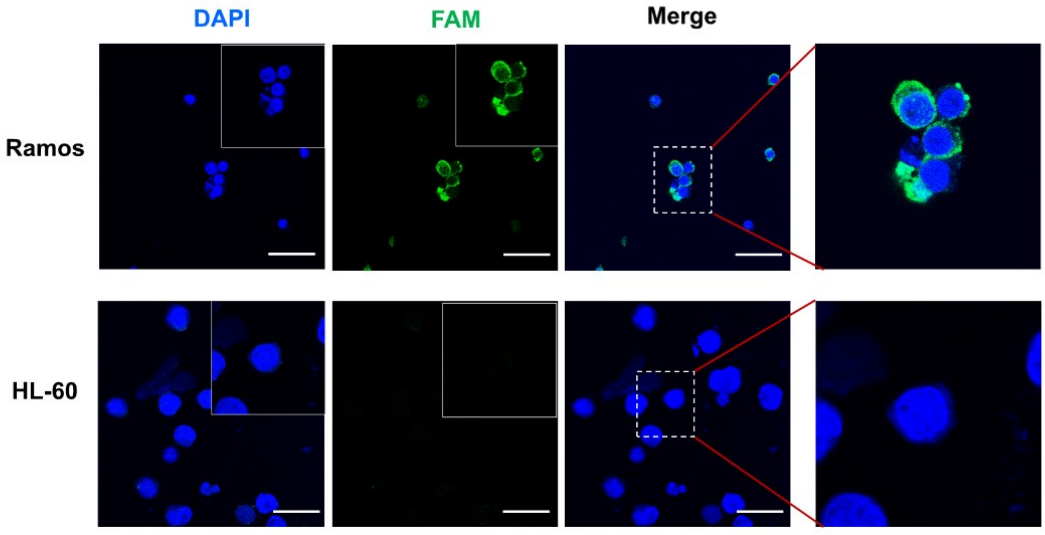

Figure S7. Representative confocal images of target cells (Ramos) and non-target cells (HL-60) after incubation with RCA products from Design I for 12 h.

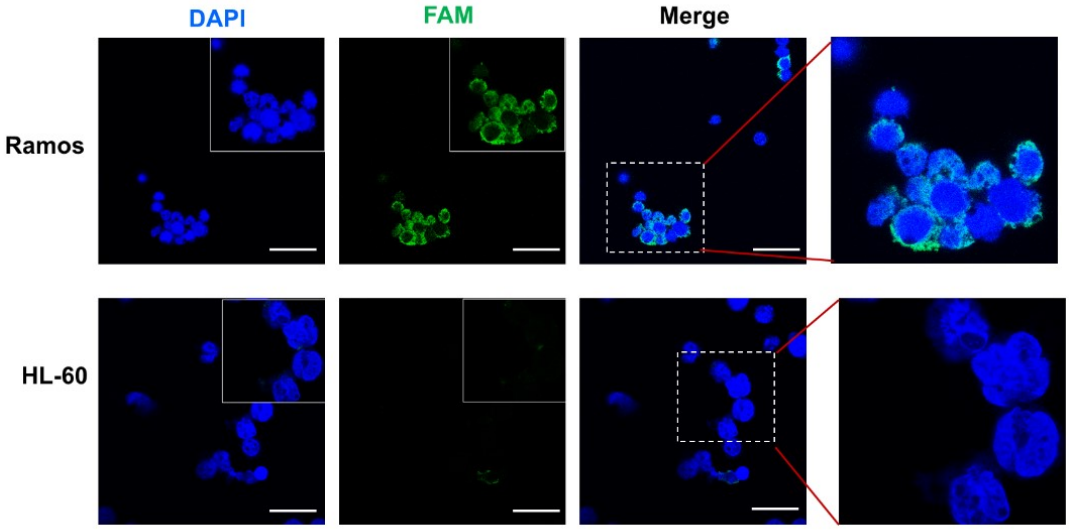

Figure S8. Representative confocal images of target cells (Ramos) and non-target cells (HL-60) after incubation with RCA products from Design II for 12 h.