Electronic Supplementary Information (ESI)

Functionalization, re-functionalization and rejuvenation of ssDNA nanotemplates

Ming-Yu Lin [a,b], Fu Han Ho[c], Chung Yao Yang[d], J. Andrew Yeh[d], and Yuh-Shyong Yang*[a]

[a] Institute of Molecular Medicine and Bioengineering
National Chiao Tung University.
[b] Instrument Technology Research Center, National Applied Research Laboratories.
[c] Graduate Institute of Applied Science and Technology,National Taiwan University of Science and Technology
[d]Institute of Nanoengineering and Microsystems, National Tsing Hua University

***Author for correspondence:**

E-mail: ysyang@mail.nctu.edu.tw (Yuh-Shyong Yang)
Fax: (+) 886-3-5729288;
Tel: +886-3-5731983;

Experiment section

Materials: PDGF was purchased from Upstate (USA). Bovine serum albumin (BSA), 3'-Aminopropyl triethoxysilane (APTES), Tween 20, sodium cyanoborohydride (NaBH3CN), phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 °C), streptavidin-conjugated peroxidase (Strep-HRP, #S2438 from Sigma, USA), streptavidin-conjugated alkaline phosphatase (Strep-AP, #S2890), TMB (3,3',5,5'-tetramethylbenzidine), dibasic sodium phosphate, citric acid, pNPP (p-nitrophenyl phosphate) and ethanolamine were purchased from Sigma-Aldrich (USA). Glutaraldehyde (25% in aqueous solution) was purchased from Fluka (USA). T4 DNA ligase and Phi29 DNA polymerase were purchased from Epicentre (USA). T4 Gene 32 protein was purchased from Ambion (USA). Chamber slide system (Lab-Tek™), 96-well microtiter plates was purchased from Nalge Nunc International (USA). Hind III restriction enzyme assay was purchased from N.E. Biolabs, Schwalbach/Taunus, Germany. All reagent solutions were prepared and steam sterilized with water (resistance of water = 18.2 MΩcm) purified from an ultra-pure water system (Sartorius, Germany).

DNA sequence design

All the following DNA strands were commercially synthesized (Genedragon, Taiwan) and purified by denaturing gel electrophoresis: 5'-CCGATCTCTCCCACTCTCTCCAACTCACA GGCTACGGCACGTAGAGCATCACCATGATCCTGTGGGTGTGTTGTTGATGGATCG GATCATGGTGAT-3' (PDGF aptamer with the primer binding site and probe sequence underlined), 5'-NH2-TTTTTTAAGCTTAGTTGGAGAGAGTGGGAG-3' , (5'-aminomodified primer with HindIII cutting site underlined), 5'-biotin-GGTGTGTTGTTGATG-3' (15 n.t. biotinylated probe), 5'-GGTGTGTTGTTGATGACGTAA-biotin-3' (21 n.t. biotinylated probe with 6 n.t. extended sequence), 5'-TTACGTCATCAACAACACACC-3' (recovery DNA; sequence complementary to 21 n.t. biotinylated probe). The DNA sequences of PDGF aptamers, 5'-aminomodified primers, and biotinylated probes were reported and modified from previous report.[1]

Circularization of PDGF aptamers

PDGF aptamers were circularized by incubating 20 μL linear form of PDGF aptamers (465 nM), 20 μl of PDGF (600 nM, dissolved in 4 mM HCl with 2 mg/mL BSA), and 148 μL ligation buffer (33 mM Tris-acetate at pH 7.8, 66 mM potassium acetate, and 10 mM magnesium acetate) for 30 min preligation. Ligase mixtures (12 μL of 25 mM ATP and 12 U T4 DNA ligase) was then added into the preligation product for 50 min at room temperature. The ligation reaction was terminated by heating at 95 °C for 5 min.

Surface modification and immobilization of DNA primer on the glass substrate

A SAM of APTES was formed on the glass substrate by silanization. The glass substrate on the chamber slide system (Lab-Tek™, Nalge Nunc International) was first washed with ethanol solution to remove contaminants before it was incubated with 100 μL, 2.0% APTES in ethanol solution for 30 min and then heated at 120 °C for 10 min. Next, the substrate was treated with a 100 μL solution containing 2.5% glutaraldehyde and 4 mM sodium cyanoborohydride for 1.5 h and then washed with water. Finally, 70 μL of 500 nM 5′-aminomodified primers were immobilized on the glass substrate at 4 °C overnight.

Time-dependent synthesis and functionalization of ssDNA nanotemplates

The primer-immobilized glass substrate was incubated with RCA mixture (40 mM Tris-HCl at pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 0.88 mM dNTP, 5 ng T4 gene-32 protein, 10 units Phi 29 DNA polymerase, and 0.23 nM circularized ssDNA) and 1.3 μM 15 n.t. biotinylated probe at room temperature (100 μL final volume). The glass substrate was washed with PBS buffer after RCA reaction and blocked with 150 μL 3% BSA in PBS solution at room temperature for 60 min. Excess BSA was removed with 300 μL washing buffer (0.2 % Tween 20 in PBS buffer, pH7.4) three times. The glass substrates were then

treated with 70 µL Strep-HRP (5 nM) in PBS buffer, pH7.4, #S2438, purchased from Sigma, USA) or Strep-AP (7.2 nM in PBS buffer, pH7.4, #S2890, Sigma, USA), respectively, for 45 min at 25°C to introduce the DNA nanostructures with peroxidase or alkaline phosphatase activity. Following functionalization of ssDNA nanotemplates as described above, any excess reagent, enzyme and DNA were removed by rinsing three times with 300 µL washing buffer.

Re-functionalization of ssDNA nanotemplates

To replace the enzyme activity originally introduced into the ssDNA nanotemplate with another enzyme activity, varied concentrations of the second streptavidin conjugated enzymes were used to compete with streptavidin-biotin binding sites. Strep-AP (0, 7, 35, 700 nM in PBS buffer, pH7.4 respectively) or Strep-HRP (0, 3.5, 17.5, 350 nM in PBS buffer, pH7.4 respectively) were mixed with HRP or AP functionalized ssDNA nanotemplates, respectively. The remaining enzyme activities with ssDNA nanotemplates were measured after the three thorough wash steps following re-functionalization. To confirm that enzyme activities came from the functionalized ssDNA, the DNA nanotemplates were digested with 20 units restriction enzyme, HindIII (in 100 µL solution containing 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM Dithiothreitol) at 37 °C for 50 min.

Rejuvenation of activated ssDNA nanotemplates

To be able to remove the biotinylated probes and to restore ssDNA nanotemplates, a different set of biotinylated probes and recovery DNA were designed for the functionalization and rejuvenation of ssDNA nanotemplates (Fig. 2). The ssDNA nanotemplates were synthesized and functionalized according the procedures described above except that 21 n.t. biotinylated probes(1.3 µM), recovery DNA (Fig. S7), respectively, were used to replace the 15 n.t. biotinylated probes. Similar procedures, as described above, were used to activate the ssDNA nanotemplated with enzymatic activities using 70 µL Strep-PO (4.8 nM in PBS buffer, pH7.4). To remove the enzyme activity together with 21 n.t. biotinylated probes, different concentration of recovery DNA (0, 0.65, 1.95, 3.25 µM), were added sequentially to compete the base-paring binding sites of probes, respectively, on the ssDNA nanotemplates at 37 °C for 60 min.

Determination of enzyme activities

The functionalized DNA nanotemplates were washed thoroughly (three times) with 300 µL washing buffer. HRP activity on the DNA nanotemplate was determined by incubating the glass substrate with 150 µL of 0.1 µg/µL 3,3',5,5'-tetramethylbenzidine (TMB) colorimetric reagent in phosphate citrate buffer (51.4 mM dibasic sodium phosphate, 24.3 mM citric acid, pH5.0) at 25 °C for 5 min and 100 µL was transferred to 96-well microtiter plates to measure the absorbance at 650 nm by using the Victor Multilabel Counter (Perkin-Elmer Life Sciences,

Inc.; USA). To determine the specific activity of Strep-HRP at 25 °C, free form of Strep-HRP (0, 0.5, 1, 2 ng in 20 µl buffer solution) were incubated in 180 µl of TMB substrate. The increase of absorbance at 650 nm was recorded within 5 mins. The enzyme activities were presented as unit (U), which is defined as absorbance changes per minute. The extinction coefficient for TMB is 39000 M^{-1} cm^{-1} at 650 nm. In our study, the specific activity of Strep-HRP was 3464 U/mg.

To evaluate the activity of AP, the colorimetric reaction was developed by adding p-nitrophenyl phosphate (pNPP) reagents from Alkaline Phosphatase Yellow Liquid Substrate System for ELISA (Sigma) and incubated for 10 min at 25 °C. Absorbance at 405 nm was measured using a spectrophotometer (Victor Multilabel Counter). To determine the specific activity of Strep-AP at 25 °C, free form of Strep-AP (0, 0.2, 0.4, 0.8, 1.6, 2, 4 ng in 20 µl buffer solution) were incubated in 180 µl of pNPP substrate. The increase of absorbance at 405 nm was recorded within 10 mins. The extinction coefficient for pNPP is 18000 M^{-1} cm^{-1} at 405 nm. In our study the specific activity of Strep-AP was 1016 U/mg.

Prediction of ssDNA secondary structures

Figure S1 shows one of the predicted secondary structures of the 384-n.t. ssDNA nanotemplates (corresponding to 4 RCA cycles) by UNAFOLD.[2] Three periodically identical double-hairpin units are shown and each unit contains two hairpin structures and one adapter-binding site. The double-hairpin structure of the ssDNA template first appeared after the synthesis of 192 n.t. (the second RCA cycle) and occurred repeatedly following the elongation of the ssDNA nanotemplate (Table S1). Other 2D structures were also predicted by UNAFOLD, especially in the early stages of nanotemplate formation, but most predicted structures contained the double hairpin (Table S2). After 6 RCA cycles, all the predicted 2D structures had the repeated double-hairpin structure (Table S1-S2).

Analyses of the ssDNA nanostructure by atomic force microscopy (AFM)

Following RCA, the glass substrate was thoroughly cleaned with water as preparation for AFM observation. All images were captured and analyzed by using the semi-contact mode with a Bioscope AFM (Veeco, Inc.; Woodbury, NY, USA) and image software (Nanoscope (R) III v5.31R1, Veeco, Inc.; Woodbury, NY, USA). The height resolution was less than 0.1 nm with a piezo tube translator. The radius of the AFM tip (PPP-RT-NCHRTM; Nanosensors, Neuchatel, Switzerland) was less than 7 nm with a force constant of 42 N/m.

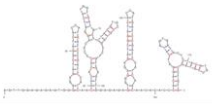
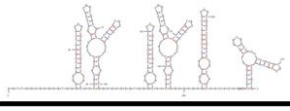
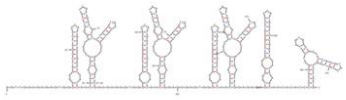
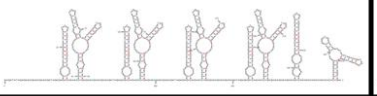
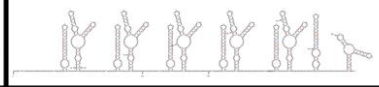
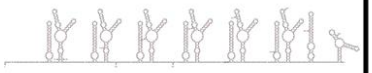
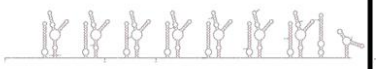
The AFM images and topographical analyses of the covalently immobilized ssDNA primers on the glass substrate are shown in Figure S1. No surface structures similar to an 18-mer primer was observed on the glass substrate when only the APTES SAM layer was formed (Fig. S1A). Three images of the DNA primers (a, b, and c) were randomly selected for further analysis (Figs. S1B and S1C). All three ssDNA primers had a globular shape, with

diameter 22.3–45.7 nm and height 7.1-8.1 nm. Although consistent with previously reported measurements, the results indicated a limitation regarding the use of an AFM tip of about 20 nm to observe a small molecule of size about 9 nm (18-mer primer, assuming ~0.2 nm/base for the ssDNA).[3,4] Noteworthy variations in AFM images could be observed following 90 min of RCA and four of the significantly expanding ssDNA nanotemplates were selected for 3D and topographical analysis (Fig. S2). Unlike the AFM images reported for dsDNA,[3,4] which showed a strand-like linear form, the periodic ssDNA nanostructures demonstrated an extended globular form with a diameter of 50.6–124.4 nm and a height of 9.0-22.2 (Fig. S2). The ssDNA globular structure expanded further and following 120 min of RCA, the globular and dumbbell forms of ssDNA nanotemplates were notably visualized by AFM. Among the 6 ssDNA nanotemplates selected for 3D imaging and topographical analysis, 2 were globular with a diameter of 217.5–245.8 nm and height of 14.7–27.1 nm, and the other 4 nanotemplates were dumbbell shaped with a length of up to 506.6 nm (Fig. S3). It is interesting to note that for the dumbbell-shaped ssDNA, the height at the connecting part of the 2 dumbbell-head structures was about 5.8–7.4 nm, which was consistent with the height of the predicted two-hairpin structure (Fig. S3), i.e., 7.48 nm (22 n.t. assuming 0.34 nm/base for dsDNA[3,4]) and 5.44 nm (16 n.t.), respectively. The immobilized globular and dumbbell-shaped ssDNA observed in this study was similar to previous reported structures of ssDNA synthesized by RCA in a free aqueous environment.[5]

Table S1: Predicted ssDNA structures following 1 to10 RCA cycles. Most of the ssDNA has periodic double-hairpin structure as predicted by the UNAFOLD[2] program. The conserved periodic structure has begun to appear since the second RCA cycle. The proportion of double-hairpin structures among the total predicted secondary structures increases with RCA cycles.

RCA cycle numbers	1	2	3	4	5	6	7	8	9	10
Sequence length	96	192	288	384	480	576	672	768	864	960
Periodic double-hairpin structure (A)	0	4	14	15	24	16	11	11	1	2
Total predicted structures (B)	5	15	36	26	28	16	11	11	1	2
Percentage of double-hairpin structures (A/B * 100 %)	0	26.7%	38.9%	57.7%	85.7%	100%	100%	100%	100%	100%

Table S2: Structures of ssDNA predicted by UNAFOLD[2]. One of the ssDNA structures from 1 to 10 RCA cycles is shown. The secondary structures shown are predicted based on the lowest free energy of the reaction. The parameters for UNAFOLD were set corresponding to the RCA reaction: 25 °C, 80 mM Na^+, 20 mM Mg^{2+} in a linear form. The repeating identical double-hairpin structures appear after 2 RCA cycles.

(A) 1 cycle	(B) 2 cycles	(C) 3 cycles
		
(D) 4 cycles	**(E) 5 cycles**	**(F) 6 cycles**
		
(G) 7 cycles	**(H) 8 cycles**	**(I) 9 cycles**
		
(J) 10 cycles		
		

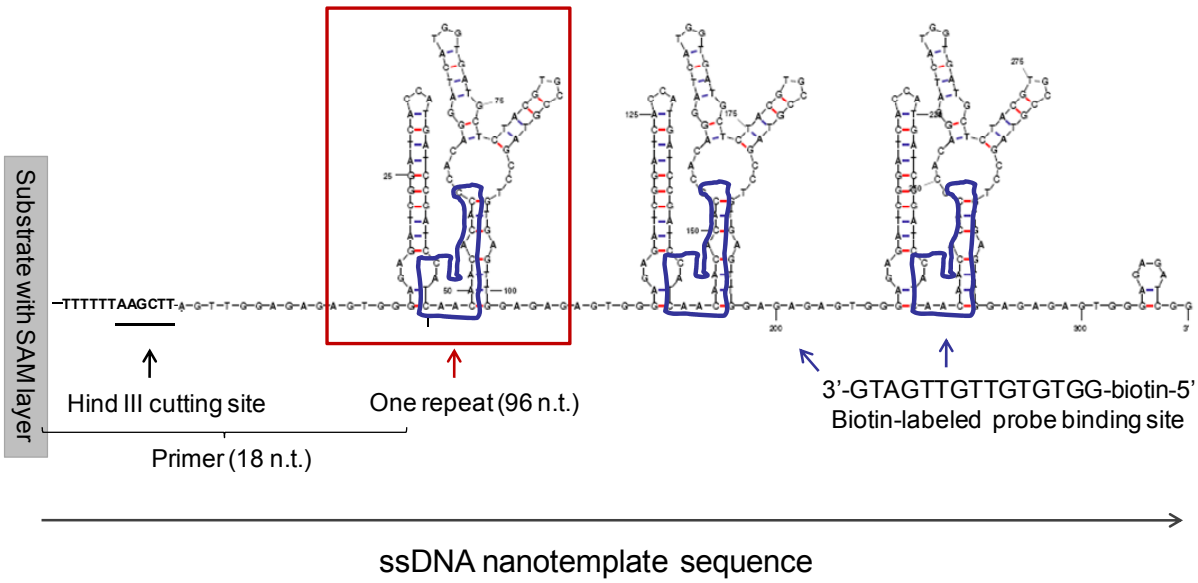


Fig. S1 Secondary structure of a 384-n.t. ssDNA nanotemplate predicted by UNAFOLD[2]. There is an 18-mer primer sequence starting at the 5'-terminal following by 96 n.t. repeats. Each repeat of the nanotemplate contains 96 n.t., which is double-hairpin structure complementary to the circular ssDNA template, and one is marked in a red box. The sequence of the binding site for biotin probe is highlighted as indicated.

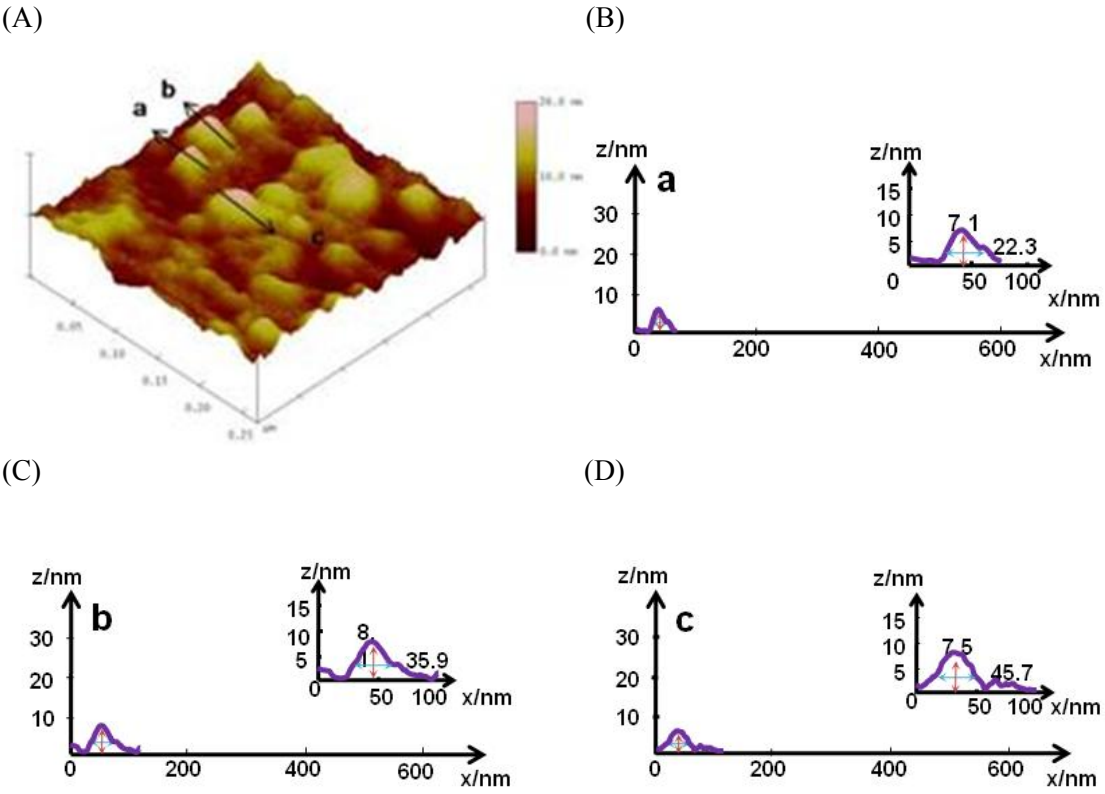


Fig. S2 AFM images and topographical analysis of the DNA primers covalently immobilized on the glass substrate. (A) Two-dimensional (2D) image of the glass surface following APTES silanization. (B) 2D image of the DNA primer (18 n.t., 500 nM) before RCA. Three primers (a, b, c) were randomly selected for further topographical analysis and their Three-dimensional (3D) images are shown in (C). (D) Cross sections of the selected nanotemplates are shown in profiles a to c, respectively.

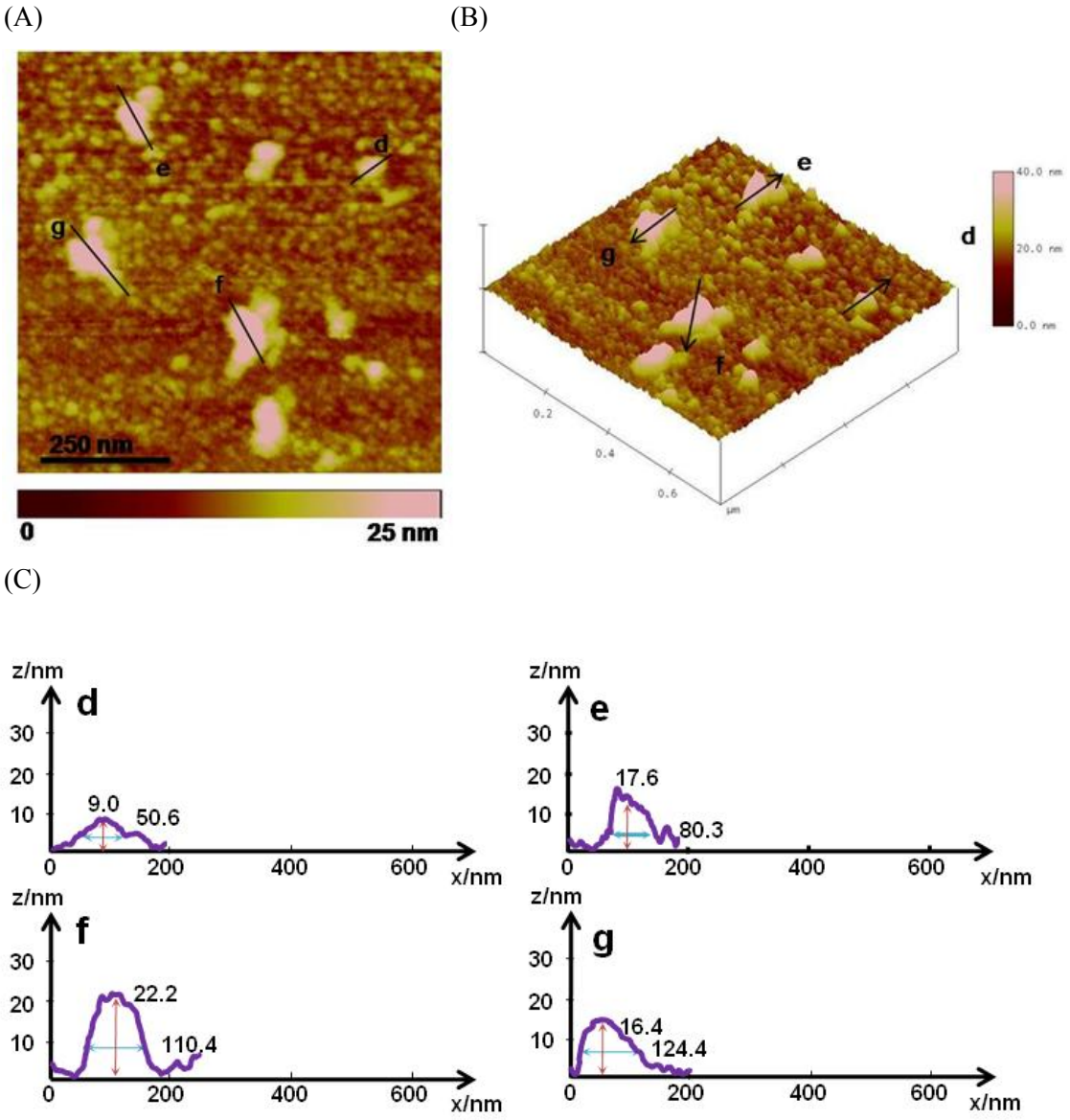
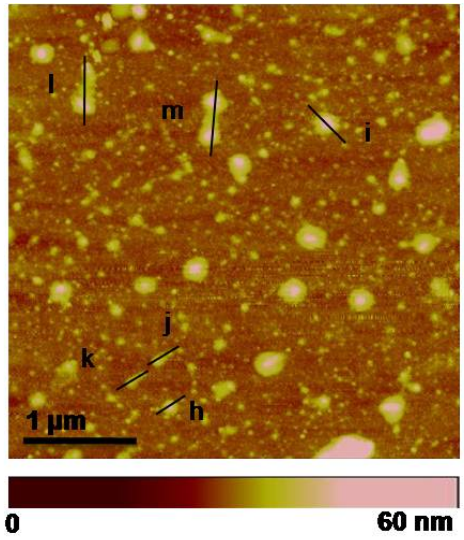
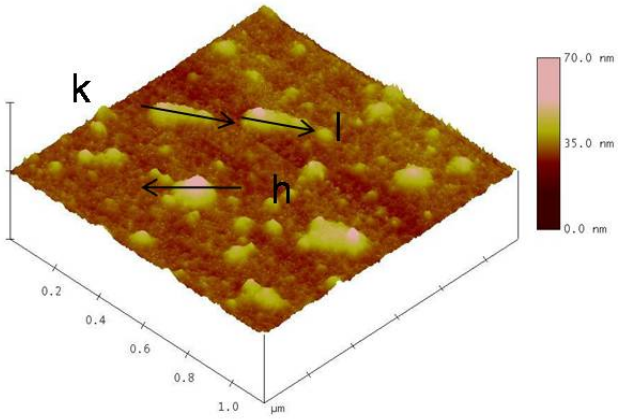


Fig. S3 AFM analyses of ssDNA nanotemplates after 90 min of RCA. (A) 2D images of 4 ssDNA nanotemplates (d, e, f, g) randomly selected for further topographical analysis and their 3D images are shown in (B). (C) Cross sections of the selected nanotemplates are shown in profiles d to g, respectively.

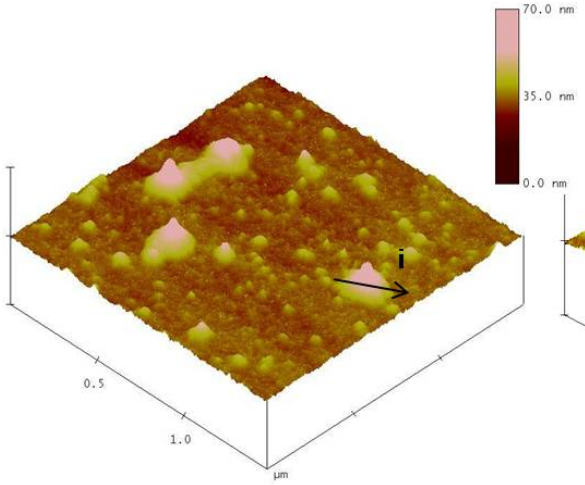
(A)



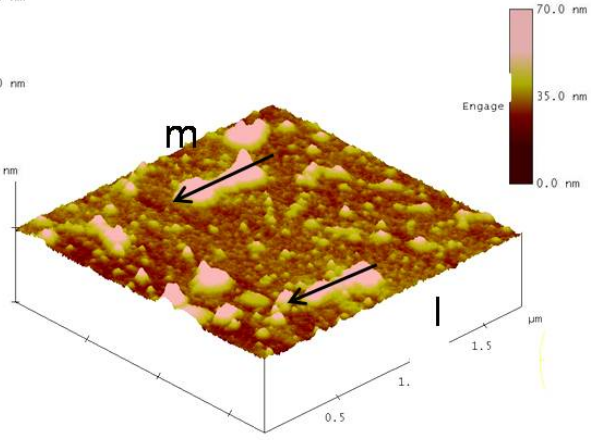
(B)



(C)



(D)



(E)

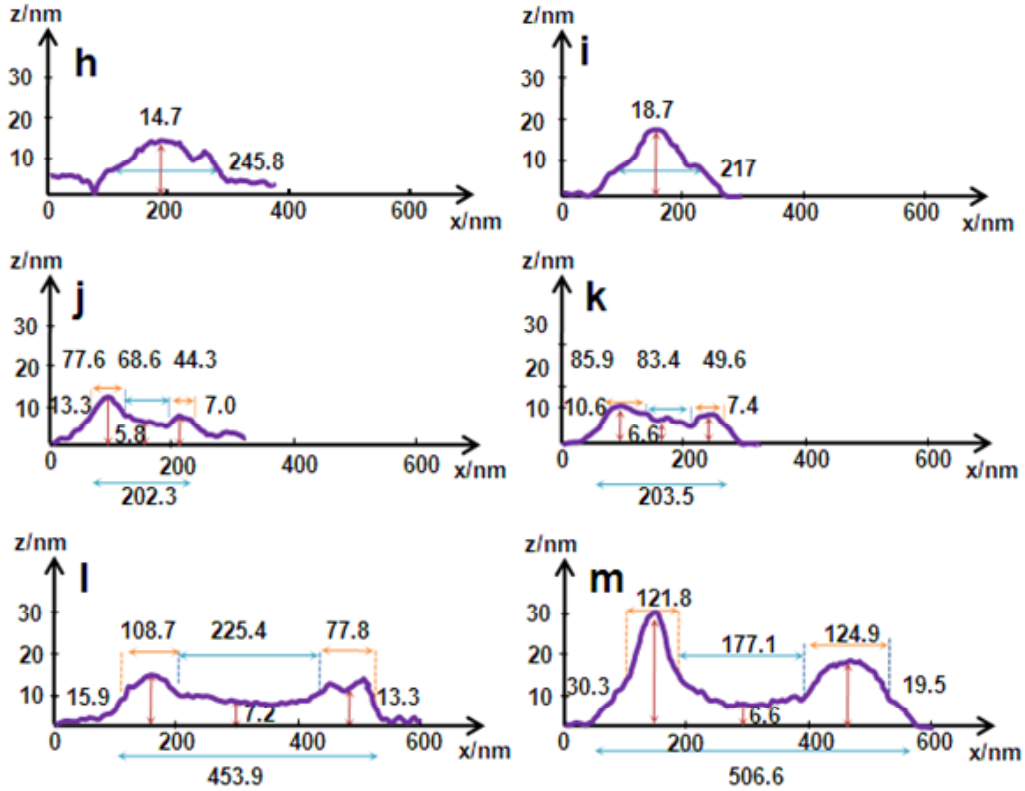


Fig. S4 AFM analysis of extended ssDNA nanotemplates after 120 min RCA. (A) 2D image of the ssDNA nanotemplates. Six images of ssDNA nanotemplates (h, i, j, k, l, m) were randomly selected for further topographical analysis and their 3D images are shown in (B-D). (E) Cross sections of the selected nanotemplates are shown in profiles h to m, respectively.

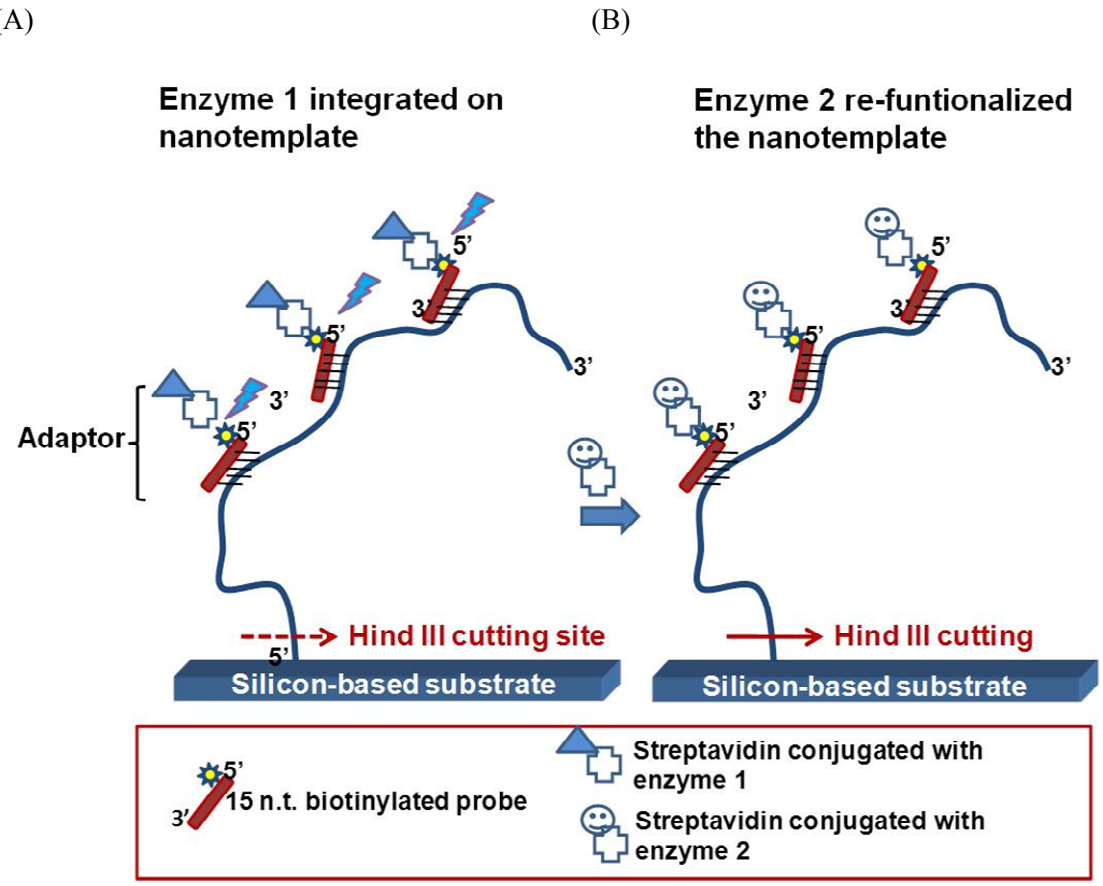


Fig.S5 Functionalization and re-functionalization of immobilized ssDNA nanotemplates. (A) Schematic diagram of self-organized biological functions on ssDNA nanotemplate through interaction between streptavidin conjugated enzyme 1 and biotin labeled DNA probe. (B) Re-functionalization of ssDNA nanotemplate with streptavidin conjugated enzyme 2.

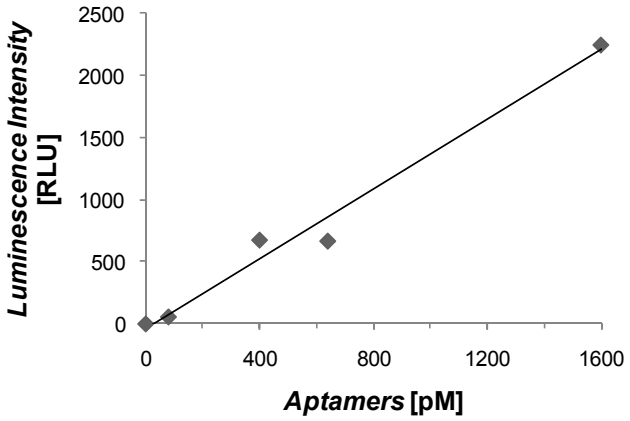


Fig. S6 Aptamer concentration dependent synthesis of ssDNA nanotemplate. Luminescence was catalyzed by peroxidase integrated into the ssDNA nanotemplates. All the procedures were performed on a 96-well plate functionalized with N-oxysuccinimide esters (CORNING; USA). The primers (70 μL of 500 nM 5'-aminomodified DNA in 0.5mM phosphate buffer, pH 8.5) were incubated with N-oxysuccinimide esters-treated microplate at 4 °C overnight. The microplate was washed with PBS buffer after primer immobilization and blocked with 150 μL 2% BSA in PBS solution at room temperature for 60 min. Excess BSA was removed with 300 μL washing buffer (0.2 % Tween 20 in PBS buffer, pH 7.4) three times. Different concentrations of aptamer are added to form circular ssDNA and trigger ssDNA nanotemplates after a 2-h RCA reaction. To prepare the ssDNA nanotemplate, 99.5 μL RCA mixture (40 mM Tris-HCl at pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 0.88 mM dNTP, 5 μg T4 gene-32 protein, 10 units Phi 29 DNA polymerase, and 1.3 μM biotin-labeled probes) and 0.5 μL circularized RCA aptamers (0, 8, 16, 80, 128, 320 nM) were added to the wells. The integration of peroxidase activity into the DNA nanotemplate is described earlier following by rinsing three times with washing buffer. A chemiluminescent substrate (SuperSignal® ELISA Femto Luminol/Enhancer and SuperSignal® ELISA Femto Stable Peroxide; Pierce Biotechnology, Inc.; USA) for peroxidase was added and the intensity of luminescence was determined by the Victor Multilabel Counter (Perkin-Elmer Life Sciences, Inc.; USA).

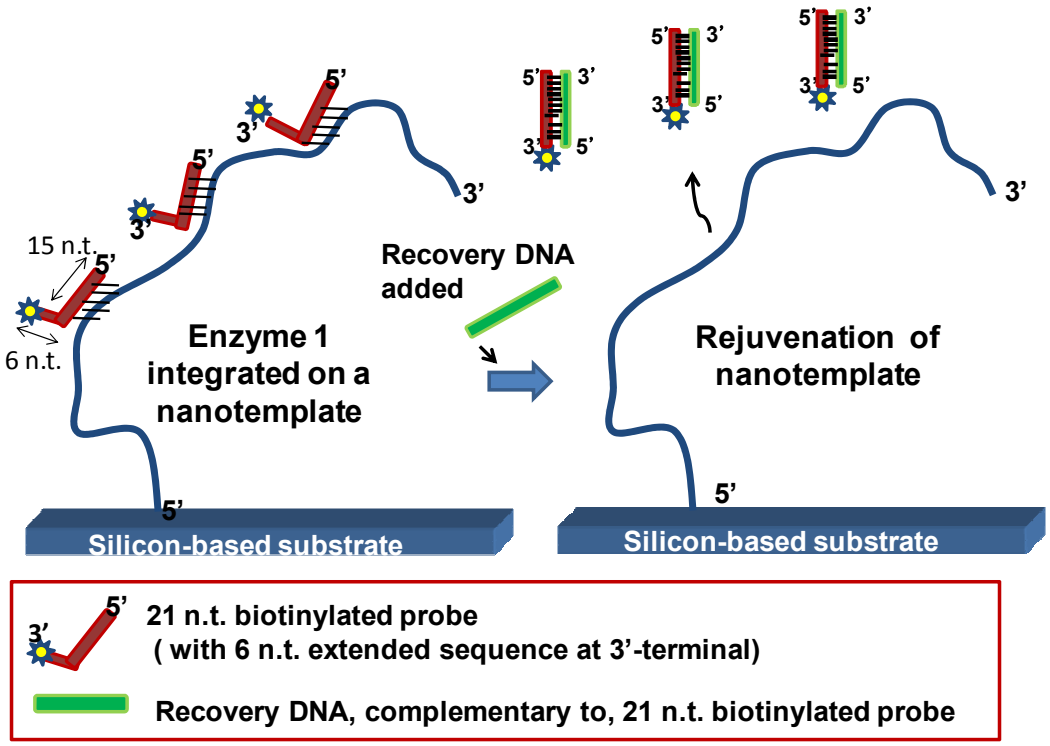


Fig. S7 Rejuvenation of functionalized ssDNA nanotemplate. Schematic diagram to illustrate the recovery of ssDNA nanotemplate. The 21 n.t. 3'-extended probe that composed of 15 n.t. complementary and 6 n.t. 3'-terminal extended sequence with biotin labeling is partially hybridized to the ssDNA nanotemplate. The 21 n.t. recovery DNA is completely complementary to the 21 n.t. 3'-extended probe that can be removed from the DNA nanotemplate.

References

1 Yang, C. W. Fung, E. J. Cho, A. D. Ellington, Anal. Chem. 2007, **79**, 3320.
2 a)N. Markham, M. Zuker, Nucleic Acids Res. 2005, 33, W577; b)M. Zuker, Nucleic Acids Res. 2003, **31**, 3406.
3 H. Hansma, R. Sinsheimer, M. Li, P. Hansma, Nucleic Acids Res. 1992, **20**, 3585.
4 H. Hansma, I. Revenko, K. Kim, D. Laney, Nucleic Acids Res. 1996, **24**, 713.
5 S. Beyer, P. Nickels, F. C. Simmel, Nano Lett. 2005, **5**, 719.