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Pure DNA Hydrogel with Stable Catalytic Ability Produced by One-Step Rolling Circle Amplification

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1. Reagents and instruments

DNA sequences listed in Table S1 were purchased from Shanghai Sangon Biotech, China. T4 ligase and low molecular weight DNA ladder were purchased from New England Biolabs, USA. Phi 29 polymerase was purchased from Thermo Fisher, USA. dNTPs and 5000 bp DNA ladder were purchased from Takara, China. $ABTS^{2-}$, H_2O_2, glucose and glucose oxidase were purchased from Shanghai Sangon Biotech. 30% acrylamide-bisacrylamide stock solution was purchased from Bioson Corporation, China. Ammonium persulfate (APS) was purchased from Amresco, USA. GelRed (×10,000) was obtained from Biotium, USA. N,N,N',N'-Tetramethylethylenediamine (TEMED) and other comon reagents were purchased from Aladdin, China.

UV absorbance was measured on BioTek Cytation 3. Circular dichroism spectra were recorded by Chirascan (Applied Photophysics Ltd). RCA reactions proceeded on Eppendorf ThermoMixer®C. PAGE was run on Bio-Rad miniprotern®Tetra system and imaged by Tannon 3500. The morphologies of RCA products were observed by scanning electron microscopy on TESCAN MIRA 3.

Table S1. DNA sequences used in this work

Template T1	5'-phosphorylated-CTTCTTCCCTAACCCTAACCCTAACCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCATCTCT-3'
Template T2	5'-phosphorylated-CTTCTTCCCACCCACCCACCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCATCTCT-3'
primer	5'-CAAGAAGAGAGATG-3'
G4-T1	5'-GGGTTAGGGTTAGGGTTAGGG-3'

G4-T2	5’-GGGTGGGTGGGTGGG-3’
Non G4 template	5’-phosphoraylated-CTTCTTGGGGGAGGGGGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCATCTCT-3’

2. Circular Dichroism (CD) measurement

CD spectra (220–300 nm) were recorded using an Applied Photophysics Chirascan quantitative CD spectropolarimeter with a 200 μL quartz cell (0.5 mm). G4-T1 and G4-T2 (10 μM) were prepared in 20 mM phosphate buffer (pH 6.3) that contained none, 200 mM NaCl, or 20 mM KCl, respectively.

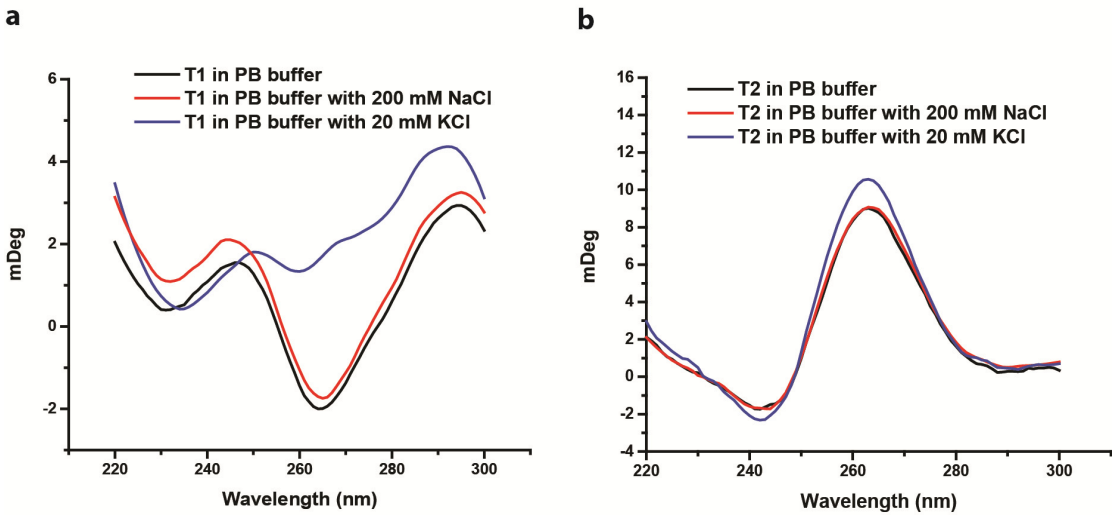

Fig. S1 Circular dichroism characterization of the sequences G4-T1 (a) and G4-T2 (b) under different conditions.

3. Polyacrylamide gel electrophoresis characterization

G4-T1 and G4-T2 were prepared in a concentration of 10 μM in phosphate buffer with none, 200 mM NaCl, or 20 mM KCl respectively. 15% Native PAGE was implemented in 100 mM Tris-HCl (pH 7.0) as eletrophoresis buffer. GelRed was used after eletrophoresis for DNA staining.

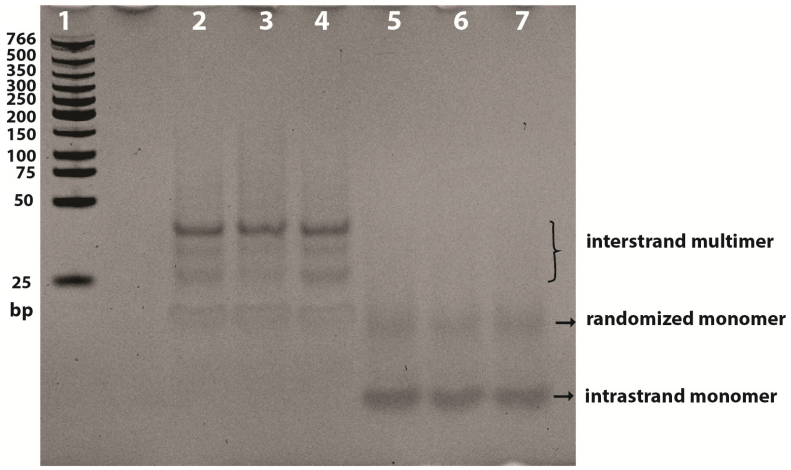

Fig. S2 Native polyacrylamide gel electropheresis (15%) characterization of G4-T1 and G4-T2 sequences in the presence of cations. Lane 1 was low molecular weight DNA marker. Lane 2, 3, 4 were G4-T2 in phosphate buffer without additional cations, with 200 mM Na^+, and with 20 mM K^+, respectively. Lane 5, 6, 7 were G4-T1 in phosphate buffer without additional cations, with 200 mM Na^+, and with 20 mM K^+ respectively.

4. Optimization of reaction time and temperature of RCA process

A higher yield of the amplification reaction is expected for getting a firmer DNA hydrogel, therefore the reaction temperature and time were optimized with respect to maximization of the reaction yields, which were evaluated by agarose gel electrophoresis.

(1) The optimization for reacting temperature: Three RCA reactions were run in parallel according to the method described in the 4th part of ESI. All the reaction conditions were kept identical except for their reaction temperature, which were set at 20, 30, and 40 °C, respectively. The agarose analysis of the products of the three RCA reactions was shown in Fig. S3a. The product of the RCA reaction at 30 °C showed a darker band compared with the other two reactions. It indicates that the reaction temperature at 30 °C is optimal for achieving a higher amplification yield.

(2) The optimization for reacting time: similarly, five RCA reactions were run in parallel under identical conditions except for the reacting time, which were set at 1, 4, 8, 12, and 24 hours, respectively. The agarose analysis of the RCA products produced under different reacting time was shown in Fig. S3b. As the reaction time increased, the quantity and molecular weight of RCA products increased. The result demonstrates that a longer reaction time is necessary to realize a high reaction yield. As a result, the optimized conditions for a RCA reaction were determined to be 30 °C reacting temperature and 24 hours reacting time.

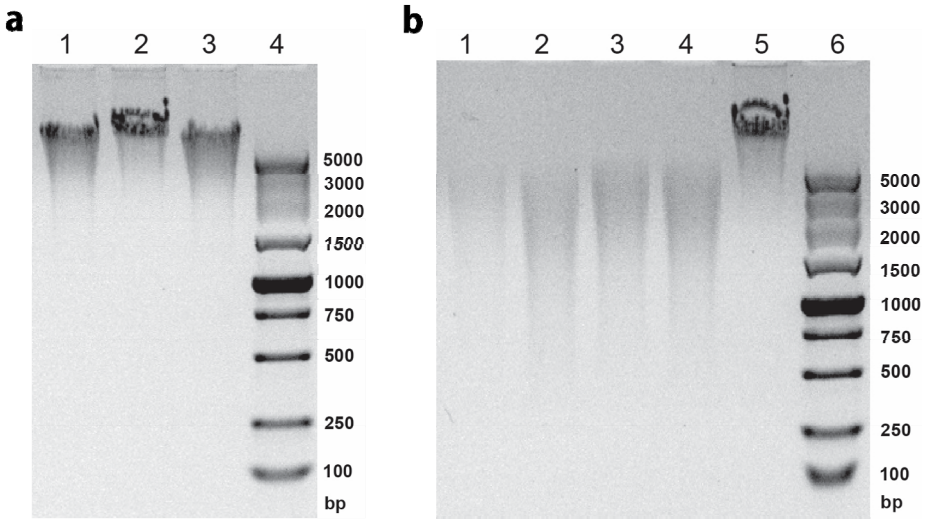

Fig. S3 RCA condition optimization characterized by agarose gel electrophoresis. (a) Incubating temperature optimization. Lane 1, 20 ℃; lane 2, 30 ℃; lane 3, 40 ℃; lane 4, DNA marker. (b) Reaction time optimization. Lane 1, 1 hour; lane 2, 4 hours; lane 3, 8 hours; lane 4, 12 hours; lane 5, 24 hours.

5. RCA process and agarose gel electrophoresis

Template T1 or template T2 (0.3 μM) were firstly incubated with primer (0.6 μM) at 90°C for 10 minutes and allowed to cool down gradually in 1× T4 ligase buffer. Secondly, the template-primer complex was inbubated with 10 U μL^{-1} T4 ligase at 16°C for 16 hours for ligation. After that, the mixture was heated at 65°C for

10min to inactivate the ligase. For a standard RCA reaction, the resultant circlized template (60 nM) and primer were incubated at 30°C for 24 hours in the presence of dNTPs (2 mM each) and 0.2 U/μL Phi 29 DNA polymerase. The RCA products were treated with EDTA (10 mM) to dissolve magnesium pyrophosphate (a byproduct of RCA) thoroughly in order to decrease the viscocity of the samples, thereafter the samples were analyzed by 0.5% agarose gel electrophoresis (Fig S4). It showed that the RCA reaction produced long single-stranded DNAs.

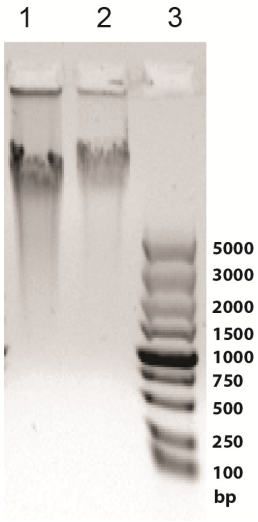

Fig. S4 Agarose gel electrophoresis characterization of the RCA products: Lane 1: RCA-T1; lane 2: RCA-T2; lane 3: DNA marker.

6. The preparation of RCA hydrogel and SEM characterizations

The RCA products were incubated at 4°C for 48 hours which allowed the fully crosslinking of inter-strand G-quadruplex. The morphologies of RCA products RCA-T1 and RCA-T2 were observed by TESCAN MIRA3 scanning electron microscopy (U= 5.0 kV). SEM samples were prepared by physically cutting of hydrogel and spreading on silicon wafers. The samples were carefully dried in a vacuum oven for 8 hours, and were gold-sputtered before the tests.

7. Loading efficiency of hemin in RCA-T2 hydrogel

The loading efficiency was determined by the concentrations of hemin before and after hydrogel loading. First a standard concentration curve of hemin was obtained by measuring the absorbance of hemin at 390 nm as shown in Fig. S5. In the concentration range of 0~50 μM, the absorbance of hemin at 390 nm shows a perfect linear relationship with its concentrations (under the equation of A_{390} = 0.0098× [hemin] + 0.028). For the loading experiment, the DNA hydrogel (20 μL) was soaked in a 20 μL buffer with hemin in an original concentration of 50 μM. The absorbance of the buffer was monitored and recorded until no visible change can be observed. According to the standard curve, the residual hemin left in the buffer was 16.6 μM (according to its absorbance of 0.191±0.008). As a result, the maximal loading efficiency of hemin in the DNA hydrogel is determined to be 21.7 ng/μL.

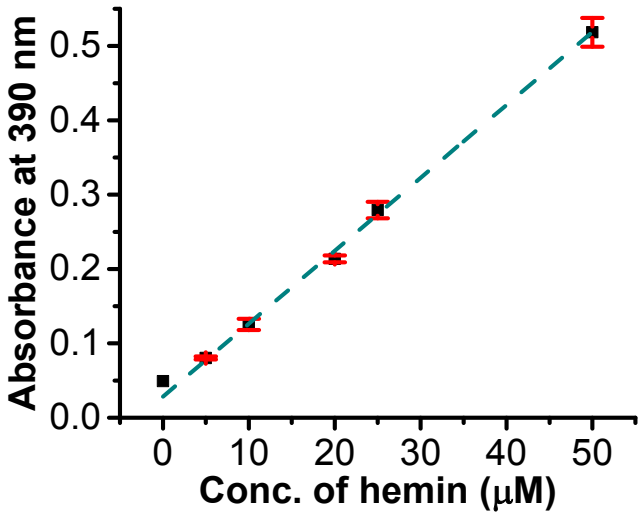

Fig. S5 The standard concentration curve of hemin: a linear relationship between the absorbance of hemin at 390 nm and its concentration.

8. The catalytic assay of hemin/RCA-T2 hydrogel

The RCA-T2 hydrogel (20 μL) was incubated with hemin (50 μM in 20 μL) in binding buffer at 4°C for 48 hours for hemin loading. The hemin loaded hydrogel was rinsed with phosphate buffer prior to the catalytic reaction. The catalytic reaction was performed in a phosphate buffer (20 mM pH 6.3 with 200 mM NaCl and 20 mM KCl) in the presence of $ABTS^{2-}$ (0.8 mM) and H_2O_2 (0.75 mM). To test the temperature stability, the catalytic reactions were carried out at different temperatures. To test the stability after a long-term storage, prior to the catalytic colorimetric reaction, the hemin/RCA-T2 hydrogel was stored at room temperature for different durations.

9. Catalytic assay of RCA-T2-GOx hydrogel

For the preparation of a RCA-T2-GOx hydrogel, GOx (0.2 mg mL^{-1}) was added to the solution of an RCA reaction during process of the enzymatic amplification. As a result, GOx was encapusulated as long as the formation of RCA-T2 hydrogel. The obtained RCA-T2-GOx hydrogel was incubated with hemin (50 μM in 20 μL) followed the same conditions as described above. After being rinsed with phosphate buffer, the catalytic reaction was performed in a phosphate buffer (20 mM pH 6.3 with 200 mM NaCl and 20 mM KCl) in the presence of $ABTS^{2-}$ (0.8 mM) and various concentrations of glucose. To test the temperature stability, the catalytic reactions were carried out at different temperatures. To test the stability after a long-term storage, prior to the catalytic colorimetric reaction, the RCA-T2-GOx hydrogel was stored at room temperature for different durations.

10. The influence of GOx to the RCA reaction

The influence of GOx to the RCA reaction was investigated by analyzing the products amplified in absence and in presence of GOx by agarose electrophoresis. In Fig. S6, Lane 1 shows the products amplified by a RCA reaction in the absence of GOx, and Lane 2 shows the products amplified by a RCA reaction in the presence of 0.2 mg/mL GOx. There are no visible difference can be observed by the two RCA products, indicating that the presence of GOx will not affect the RCA reaction.

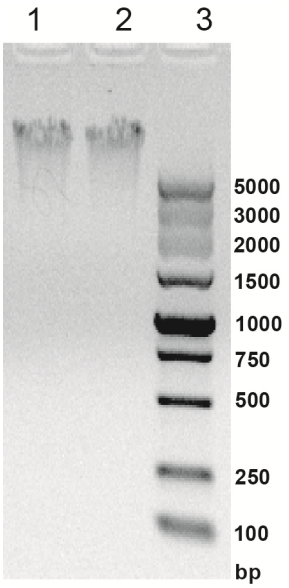

Fig. S6 Agarose gel electrophoresis of RCA products amplified in the absence (Lane 1) and in the presence (Lane 2) of GOx during the RCA process. Lane 3, DNA marker.

11. The selectivity and real sample assay of RCA-T2-GOx hydrogel

To demonstrate the selectivity of the glucose detection assay based upon RCA hydrogels, control experiments were performed to detect the analogues of glucose, such as fructose, galactose, and sucrose. As shown in Figure C2a, although the concentrations of fructose, galactose, and sucrose (10 mM) are 10 times higher than

that of glucose (1 mM), only the sample in the presence of glucose triggered the catalytic reaction inside DNA hydrogel and showed a significant absorbance at 420 nm. The result indicates that the assay based upon the RCA-T2-GOx hydrogel displays a good specificity in glucose detection.

We further examined the potential applications of the RCA-T2-GOx hydrogel in real sample assays. Fetal bovine serum was used to mimic the real sample, and we used the assay based upon RCA-T2-GOx hydrogel to detect the glucose concentration in the serum sample by the *Standard Addition Method*. Different defined amounts of glucose were spiked into diluted serum samples, which were detected by the assays based on hydrogel, resulting in a standard concentration curve (with a linear equation of $y = 0.088 + 4.02\times10^{-4}\ x$, x=*[glucose]*). The standard concentration curve was extrapolated to zero absorbance, and the concentration of glucose in the original serum was determined to be 4.6±0.3 mM. The result is in good agreement with the value (~ 5 mM) measured by a commercial glucometer. Therefore, it demonstrates that the assay based upon integrated RCA hydrogels has good potentials for practical applications.

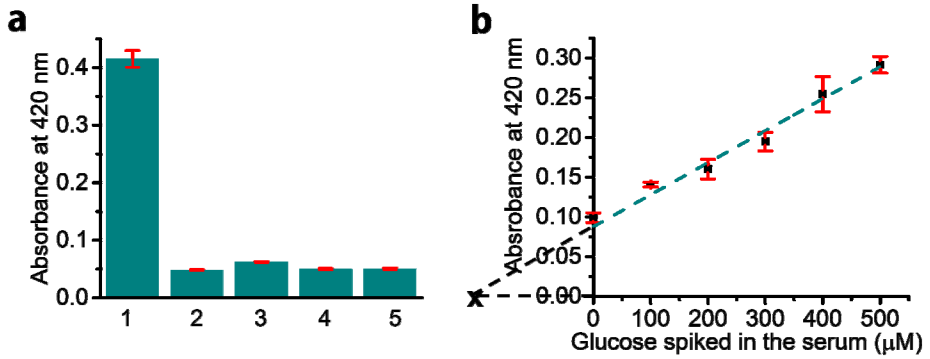

Fig. S7 The selectivity and real sample assay of RCA-T2 hydrogel. (a) Selectivity of RCA-T2 hydrogel. 1, 1 mM glucose; 2, 10 mM fructose; 3, 10 mM galactose; 4, 10 mM sucrose; 5, blank. (b) Real sample assay of RCA-T2 hydrogel. The linear relation

between absorbance at 420 nm and glucose spiked in the serum. The x-intercept of the linear equation corresponds to the actual concentration of glucose in serum.

12. The catalytic assay of G4-T2-hemin complex

G4-T2 DNA (2 μM) was incubated with hemin (2 μM) in a binding buffer (10 mM HEPES pH 7.3, 200 mM NaCl, 20 mM KCl, 1% DMSO, and 0.1% Triton X-100) at room temperature for 30 minutes. After that, the mixture was added to the reaction buffer (20 mM phosphate buffer containing 200 mM NaCl and 20 mM KCl) with other reaction reagents. The final reaction contained G4-T2 (0.4 μM), hemin (0.4 μM), $ABTS^{2-}$ (0.8 mM), and H_2O_2 (0.75 mM). To optimize the assay, different pH values varied from 8.3 to 3.3 (as shown in Fig. S8) were tested. A value of pH 6.3 was determined to be the best condition to obtain an optimized catalytic ability during a 30 minutes reaction. To test the temperature stability, the catalytic reactions were carried out at different temperatures. To test the stability after a long-term storage, prior to the catalytic colorimetric reaction, the hemin/G4-T2 complex was incubated in binding buffer at room temperature for different time-durations.

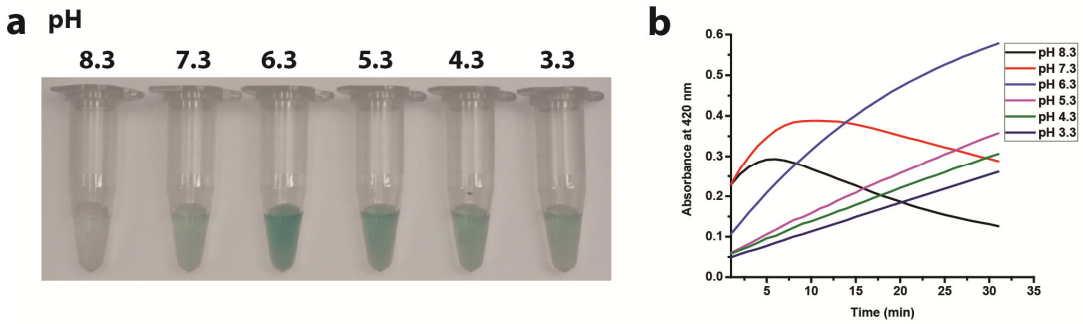

Fig. S8 Catalytic ability of hemin/G4-T2 under different pH values. (a) Catalytic results of hemin/G4-T2 under different pHs after 30 minutes reaction. (b) The reaction kinetics of hemin/G4-T2 towards $ABTS^{2-}$ colorimetric reaction.