A Versatile Platform for Highly sensitive Detection of Protein: DNA enriching magnetic nanoparticles Based Rolling Circle

Amplification Immunoassay

Qingwang Xue,[†] Lei Wang,^{*,‡} and Wei Jiang^{*,†}

[†]School of Chemistry and Chemical Engineering, Shandong University, 250100 Jinan,

P.R. China

[‡]School of Pharmacy, Shandong University, 250012 Jinan, P.R. China

Corresponding author:

Tel: +86 531 88363888; fax: +86 531 88564464. Email: wjiang@sdu.edu.cn;

Tel: +86 531 88382330. Email: wangl-sdu@sdu.edu.cn

EXPEEIMENTAL SECTION

Materials and Reagents. Human immunoglobulin G (IgG), monoclonal antihuman IgG-Biotin conjugate (Clone HP-6017), 3-glycidyloxypropyltrimethoxysilane, and bovine serum albumin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The oligonucleotides with the following sequences were obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (China): biotinylated 35-mer single-stranded c-DNA (5'-GTAACTGTTTAATTCTAGCAC GGACATTTTTTTT-3'-biotin), complementary primer, (5'-TGTCCGTGCTAG AAGGAAACAGTTAC-3'), Circular template: (5'-p-TAG CAC GGA CAT ATA TGA TGG TAC CGC AGT ATG AGT ATC TCC TAT CAC TAC TAA GTG GAA GAA ATG TAA CTG TTT CCT TC-3'). Detection probe: (5'-ATGTAACTGTTT CCTTCTAGCAC-3'). Phi29 DNA polymerase, T4 DNA ligase, and dNTP were obtained from Fermentas (Lithuania). Streptavidin-MNBs (350 nm diameter, 1.343 g mL⁻¹, aqueous suspension containing 0.1% bovine serum albumin (BSA), 0.05% Tween-20, and 10 μ M EDTA at a concentration of 3.324× 10¹¹ beads mL⁻¹) from Bangs Laboratories Inc. (Fishers, IN), Tris (>99.8%) from Amresco Inc. (Solon, OH), and Tween-20 from Sigma (St. Louis, MO) were used in the work. Other chemicals (analytical grade) were obtained from standard reagent suppliers. Microscope cover glasses $(22 \times 22 \text{ mm}^2)$ were purchased from Cole-Parmer (Illinois, USA).

The physiological buffer saline (PBS) consisted of 0.15 M NaCl, 2.4 mM NaH₂PO₄, and 7.6 mM Na₂HPO4 (pH 7.4). PBS-T buffer consisted of 0.15 M NaCl, 7.6 mM Na₂HPO₄, 2.4 mM NaH₂PO₄, and 0.05% Tween-20 (pH 7.4). TE buffer

consisted of 10 mM Tris-HCl and 1.0 mM Na₂EDTA (pH 8.0). TTL buffer consisted of 0.10 M Tris-HCl (pH 8.0), 0.1% Tween-20, and 1.0 M LiCl. TT buffer consisted of 0.25 M Tris-HCl (pH 8.0) and 0.1% Tween-20. TTE buffer consisted of 0.25 M Tris-HCl (pH 8.0), 0.1% Tween-20, and 20 mM Na₂EDTA (pH 8.0). A phosphate buffer (pH 7.0) containing 1% BSA was used as a buffer for blocking.

All the fluorescence measurements were performed on a Hitachi F-4500 spectrofluorimeter (Hitachi, Japan). The excitation wavelength was 495 nm, and the spectra are recorded between 500 and 605 nm. The fluorescence emission intensity was measured at 525 nm.

Magnetic nanoparticle probe preparation. For the magnetic probe preparation, Six microliters of streptavidin-MNB suspension $(3.324 \times 10^{11}\text{beads/mL})$ was washed five times with 400 µL of TTL buffer to remove surfactants. Then, 6 µL of TTL buffer was added. An amount of 1 µL of the MNB suspension was diluted with 30 µL of PBS buffer. Bio-c-DNA and monoclonal antihuman IgG-Biotin conjugate were conjugated to the streptavidin-MNBs by adding 10µL of bio-c-DNA solution $(1.0 \times 10^{-5} \text{ mol/L})$ and 10µL monoclonal antihuman IgG-biotin conjugate $(1.0 \times 10^{-7} \text{ mol/L})$ and incubating for 2 h at room temperature. The unreacted biotin-c-DNA and biotin-Ab washed away with 400 µL PBS-T obtaining complex suspension. The complexs were washed twice with 400 µL of TT buffer and twice with 400 µL of TTE buffer, respectively. After the MNBs were magnetically separated, 40 µL of PBS buffer was added. Subsequently, 10 µL of $1.0 \times 10^{-5} \text{ mol/L}$ complementary DNA primer was added to the vessel, and the suspension was incubated for 6 h at room temperature, followed by washing the resulting p-DNA-c-DNA-MNBs-Ab four times with 400 μ L of PBS. Then, 50 μ L of PBS was added for subsequent experiments. All washing steps in this work were performed under a magnetic field.

Substrate Preparation and Antigen Coating. The epoxy-functionalized glass surfaces were prepared according to the modification described in the literature. The freshly prepared substrate surface was coated with 50 μ L human IgG solutions of various concentrations. The substrate was immediately placed in a sealed Petridish at 37 °C for 5 h.¹ After that, the substrate was washed three times with PBS-T washing buffer to remove unbound human IgG and impurities.

Blocking. We used a phosphate buffer containing 1% BSA as a blocking buffer. To the substrate of antigen coating was added 50 μ L of blocking, and then, the substrate was incubated overnight. After that, the substrate was washed three times with PBS-T washing buffer.

Binding of p-DNA-c-DNA-MNBs-Ab to the Antigen on Substrate. An amount of $50 \ \mu$ L of the p-DNA-c-DNA-MNBs-Ab suspension obtained above was added into the well with the substrate coating human IgG. The well was placed in a constant-humidity chamber, and the solution was incubated for 2 h to bind p-DNA-c-DNA-MNBs-Ab to antigen on the substrate through the immunoreaction. After the incubation, the redundant MNB were separated magnetically from the substrate using a neodymium-boron (Nd-B) magnet that produced an inhomogeneous magnetic field on the top of the sample cell for 5 min. Then, the substrate of the cell was gently rinsed eight times with 200 μ L of PBS-T by drawing these solutions out

along the wall of the cell.

Release of DNA primer from the MNBs on the Substrate and Rolling Circle Amplification. To release DNA primer from the MNBs on the substrate through dehybridization, 50 μ L of 2 × SSC buffer was added to the well with MNBs on the substrate and then heated for 10 min at 95 °C. Subsequently, DNA primer was removed quickly by a magnetic separation and collected and transferred into a microcentrifuge tube. To the separated DNA primer solution was added 4 µL of circular template oligonucleotide (25.4 µM) followed by heating at 90 °C for 5min and incubating at 37 °C for 30 min. Then 0.5 μ L of T4 DNA ligase, 10 μ L of 10× ligase buffer (400 mM Tris-HCl, pH 7.8, 100 mM MgCl₂, 100 mM DTT, 5 mMATP) was added and incubated at 37°C for 1 h. After ligation, T4 DNA ligase was inactivated by heating the reaction mixture at 65 °C for 10 min. Then, RCA reaction was performed by mixing with 10μ L $10\times$ reaction buffer [330mM Tris-acetate, 100mM Mg(Ac)₂, 660mM Potassium Acetate (KAc), 1% Tween 20 and 10mM DTT (pH 7.9), 0.5 µL (10 u/µL) phi29 DNA polymerase (Fermentas), 20 µL 10mM dNTPs mixture and continued for 80 min at 37 °C. The RCA reaction was terminated by heating at 65 °C for 10 min to inactivate the phi29 DNA polymerase. After the reaction mixture cooled to room temperature, 10 μ L the detection probe (2 μ M) was added and hybridized to the RCA products.

Measurement of fluorescent spectra. To the RCA amplification product was added 10 μ l 8 μ M of a highly fluorescent DNA probe, SYBR Green I (Invitrogen) followed by the incubation to assemble cascade fluorescent DNA nanotags for 10 min at room

temperature. Because the free (unbound) probe does not emit fluorescence, measurement can be performed without a wash step. The fluorescent spectra were measured using a spectrofluorophotometer. The excitation wavelength was 495 nm, and the spectra are recorded between 500 and 605nm. The slit widths were set at 10 nm. The fluorescence emission intensity was measured at 525 nm.

Conjugation of p-DNA-c-DNA-MNBs-Ab to Ag Captured on the Substrate, Release of primer from MNBs probe on the Substrate

In order to investigate the p-DNA-c-DNA-MNBs-Ab conjugated onto the substrate, p-DNA was labeled by FAM. The fluorescence image of the substrate with the FAM-p-DNA-c-DNA-MNBs-Ab fabricated from 1.0×10^{-12} mol/L Ag is shown in Fig S1A. Appearance of the bright spots on the substrate indicated that the p-DNA-c-DNA-MNBs-Ab detection probe was specifically bound to antigen captured on the substrate through the immunoreaction. In order to know whether the p-DNA on the surface of **MNBs** attached on the substrate are released, the FAM-p-DNA-c-DNA-MNBs-Ab attached on the substrate fabricated from 1.0×10^{-12} mol/L Ag was heated at 95 °C for 10 min and then FAM-p-DNA was released and removed quickly from the MNB using a magnetic separation. Disappearance of the bright spots on the substrate shown in Fig S1B indicated that the FAM-p-DNA was released from the substrate completely.



Figure S1. Epifluorescence images of the substrate with FAM-p-DNA-c-DNA-MNBs-Ab designed using 1.0×10^{-12} mol/L human IgG (A) before and (B) after dehybridization by heating for 10 min at 95 °C. Scale bar, 30 pixel.

Amplification with mutilabel p-DNA-c-DNA-MNB -Ab

The high available surface-to-volume ratio of the streptavidin magnetic bead provides more binding sites. We attached multiple primers DNA (p-DNA) to the streptavidin magnetic bead surfaces to improve the sensitivity. c-DNA and Ab at a 100/1 c-DNA/Ab molar ratio was reacted with the streptavidin magnetic bead.^{1,2} The c-DNA has a sequence with 9 thymines nucleotides at the biotin end acting as a spacer to reduce the steric hindrance between c-DNA and the surface-confined streptavidin of MNB interaction. Through the irreversible interaction between streptavidin and biotin (Ka= 10^{15} / mol L⁻¹) with rapid binding kinetics and strong affinity, c-DNA combines to the 350nm diameter streptavidin-MNB and produces the resultant c-DNA-MNB-Ab. The complementary primer DNA (p-DNA) with c-DNA added c-DNA-MNB-Ab was to the above, obtaining the resulting p-DNA-c-DNA-MNBs-Ab as detection probe. In this method, the primary amplification factor of the final detection fluorescence intensity is correlated with the number of p-DNA on one MNB.

To determine the average number of p-DNA bound onto the surface of per magnetic bead, p-DNA is replaced by FAM-p-DNA. The average amount of FAM-p-DNA bound onto the surface of per magnetic beads was determined by measuring the fluorescence intensity of the standard solutions of FAM-p-DNA solution added and the solution containing FAM-p-DNA released after magnetic separation. The concentration of the solution containing FAM-p-DNA released from the FAM-p-DNA-c-DNA-MNBs-Ab was determined using the calibration curve of FAM-p-DNA, which was obtained by measuring the fluorescence intensity of the standard solutions of FAM-p-DNA. Based on this analysis, we worked out the average number of p-DNA per magnetic bead to be ~5000, implying an amplification factor of ~5000 for DNA amplification. According to our previous reports,³ the average biotin binding sites on the surface of one streptavidin-MNB is ~30 000 based on the data provided in the manufacturer's instructions of MagPrep streptavidin beads (Bangs Laboratories Inc., Fishers, IN). Although the measured value was different from that offered by the manufacture's instructions, the large amplification factor was achieved. Magnetic beads of diameter 350 nm provided a very high number of labels on the surface, and gave better sensitivities and detection limits.

The verification of RCA reaction

To verify the amplification of the RCA reaction, the agarose gel electrophoresis experiment was performed. The RCA products were investigated by gel electrophoresis. It is observed that the RCA products in lane 1-3 reaction show extremely low mobility in Figure S2. The anticipated high molecular weight of RCA product was confirmed in lane 1-3. Compared with lane 1-3, lane 4, 5 displayed no bands in negative control experiment. These results give immediate evidence for the high molecular weight of these products, indicating the proposed method could provide enormous signal amplification in immunoassay.⁴ In addition, the amplification of the RCA reaction was also verified using fluorescent intensity of assembly fluorescent DNA nanotags. It is observed that the RCA products based assembly fluorescent DNA nanotags in Figure S3 were significantly higher than the assembly fluorescent DNA nanotags based on the hybridization products by the released p-DNA and c-DNA.



Figure S2. Agarose gel (0.7%) electrophoresis experiments. The products of RCA reaction (1 h) (indicated by 1-5) were denatured at 95 °C for 5 min and quenched with ice-cooled water for 10 min. The marker was indicated by M. The high molecular weight RCA products are observed in lines 1-3.



Figure S3. Fluorescence emission spectra. The releasing p-DNA initiates RCA. The RCA products assemble the cascade fluorescent DNA nanotags (black). The hybridization products by the released p-DNA and c-DNA assemble the fluorescent DNA nanotags (red). SG (blue).

Time-Dependent Signal Amplification of RCA

To generate more tandem repeat complementary copies of the circular template, a long RCA reaction time is expected to yield enhanced signal amplification. The effect of RCA reaction time on the fluorescent signal was shown in Figure S4. It is clear that the fluorescent intensity response was very slow when the RCA reaction time is at the initial 10 min, indicating a relatively slow rate of RCA reaction. The fluorescent intensity response exhibits a rapid increase with a further increase in the RCA reaction time after 10 min and trends to a constant value at 60 min, indicating the saturation of RCA product due to the exhaustion of RCA substrates or inactivation of the phi 29 DNA polymerase. Thus, 80 min was selected as the optimum time for the RCA reaction in the experiment.



Figure S4. Influence of RCA reaction time on the fluorescent intensity signal responding to 1 pM of human IgG.

Assay Performance of magnetic nanoparticles-RCA immunoassay



Figure S5. Fluorescence emission spectra obtained in magnetic nanoparticles-RCA immunoassay of human IgG with varying concentrations $(1.0 \times 10^{-12}, 5 \times 10^{-13}, 1 \times 10^{-13}, 5 \times 10^{-14}, 1 \times 10^{-14}, 5 \times 10^{-15}, 1 \times 10^{-15}, 5 \times 10^{-16}, 1 \times 10^{-16}, 5 \times 10^{-17}, 1 \times 10^{-17}, and 0 mol/L).$

Notes and references

- 1. V. Mani, B. V. Chikkaveeraiah, V. Patel, J. S. Gutkind and J. F. Rusling, ACS nano, 2009, **3**, 585-594.
- X. Yu, B. Munge, V. Patel, G. Jensen, A. Bhirde, J. D. Gong, S. N. Kim, J. Gillespie, J. S. Gutkind, F. Papadimitrakopoulos and J. F. Rusling, *J Am Chem Soc*, 2006, **128**, 11199-11205.
- Q. Xue, D. Jiang, L. Wang and W. Jiang, *Bioconjug Chem*, 2010, 21, 1987-1993.
- 4. L. Zhou, L. J. Ou, X. Chu, G. L. Shen and R. Q. Yu, *Anal Chem*, 2007, **79**, 7492-7500.