Supporting Information (SI)

Sensitive, Simultaneous Quantitation of Two Unlabelled DNA Targets Using a Magnetic Nanoparticle-Enzyme Sandwich Assay

Yue Zhang,† Chalermchai Pilapong,†‡ Yuan Guo,†,* Zhenlian Ling,† Oscar Cespedes,¶ Philip Quirke,‡ and Dejian Zhou†,*

† School of Chemistry and Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds LS2 9JT, UK.
‡ School of Physics and Astronomy, University of Leeds, Leeds, LS2 9JT, United Kingdom
¶ Section of Pathology and Tumour Biology, Leeds Institute of Molecular Medicine, Wellcome Trust Brenner Building, St James’s University Hospital, University of Leeds, Leeds LS9 7TF, UK.
¶ Current address: Laboratory of Physical Chemistry, Molecular and Cellular Biology, Center of Excellence for Molecular Imaging (CEMI), Department of Radiologic Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, 50200 Thailand.

* To whom correspondence should be addressed: y.guo@leeds.ac.uk (YG) or d.zhou@leeds.ac.uk (DZ); Tel: +44-113-3436230; Fax: +44-113-3436565.
Experimental Details

A) Preparation and modification of the MNPs

1) Preparation of citrate-stabilized Fe₃O₄ MNPs

5 mmol FeCl₂·4H₂O and 10 mmol FeCl₃·6H₂O were dissolved in 50 mL deoxygenated H₂O under magnetic stirring. Then 4.3 mL NH₃·H₂O (35%) solution mixed with 50 mL deoxygenated H₂O was added into the above solution dropwisely with N₂ gas bubbling. The mixture was heated at 80 °C for 30 min in an oil bath under vigorous stirring and then for 2 h at 90 °C following the addition of 50 mL of 0.2 M trisodium citrate (see Scheme S1). The obtained Fe₃O₄ magnetic nanoparticles (MNPs) appeared as black precipitate which was separated by centrifugation (4000 g for 3 min) and then washed once with acetone. Finally, the MNPs were dispersed in deionized water (with the volume being adjusted to 30 mL).¹,²

2) Preparation of Fe₃O₄/SiO₂ core/shell MNPs

Fe₃O₄/SiO₂ core/shell MNPs were prepared by using a modified Stöber method.³ In typical preparation, 1 mL of the hydrophilic Fe₃O₄ seeds prepared above was firstly diluted with the mixture of 4 mL deionized water and 20 mL absolute ethanol. Under continuous stirring and N₂ bubbling, 0.43 mL NH₃·H₂O (35%) solution and 0.5 mL of (3-aminopropyl)triethoxysilane (APTES) were consecutively added to the solution under continuous stirring and N₂ gas bubbling. The reaction was carried out for 3 h under N₂ gas bubbling at room temperature (see Scheme S1). Subsequently the obtained Fe₃O₄/SiO₂ core/shell MNPs were separated by centrifuging at 4000 rpm for 5 min and then washed by water twice. Finally, the obtained MNPs were re-dispersed in deionized water (the volume was adjusted to 1 mL).

3) Preparation of NH₂-Functionalsied MNPs (MNP-NH₂)

Amine functionalised Fe₃O₄/SiO₂ core/shell MNPs were prepared as follow:²,³,⁴ 1 mL of the above silica-coated Fe₃O₄/SiO₂ core/shell MNP prepared above was dispersed in 4 mL deionized water and 20 mL absolute ethanol. Then 0.43 mL NH₃·H₂O (35%) solution and 0.5 mL of (3-aminopropyl)triethoxysilane (APTES) were consecutively added to the solution under continuous stirring and N₂ gas bubbling. The reaction was carried out for 3 h at room temperature, leading to APTES to hydrolyse and subsequently condensed on the MNP surface to introduce amine groups (see Scheme S1). Afterwards, the amine functionalized MNPs (NH₂-MNPs) were separated, washed and then re-dispersed in deionized water. Concentration of the MNP suspension was estimated by comparing the suspension’s weight with pure water of the same volume. It should be noted that the density difference of the Fe₃O₄/SiO₂ core/shell MNP in water was ignored.

4) Preparation of Capture-DNA immobilized MNP (Cap-MNP)

5 mg NH₂-MNP and 2.5 mg SM(PEG)₁₂ were mixed and incubated in Buffer C (PBS buffer containing 1mM EDTA, pH...
= 7.2) at room temperature for 1 h, leading to the MNP surface being functionalised with maleimide groups. Thereafter, the MNPs were washed by Buffer C twice, and then 5 nmol of thiolated capture-DNA in 1 mL Buffer C was added into the above MNPs and incubated for 1 h at room temperature. The capture-DNA is covalently conjugated to the MNP surface via Michael addition of the thiolate to the MNP surface maleimide groups (see Scheme S1). The MNPs were subsequently washed twice by Buffer C. It should be noted that all of the original and washing supernatants were collected and combined for UV measurement at 260 nm to determine the amount of free-unbound capture-DNA, allowing the estimation of the capture-DNA conjugation efficiency on the MNP. The capture-DNA loaded MNPs were then treated with 5 μL of 2-mercaptoethanol in 1 mL Buffer A (PBS plus 1 mg/mL BSA) to cap any unreacted maleimide groups and to block the MNP surface to reduce non-specific absorption of HRP-NAV. Typically, the capture-DNA surface loading on the MNP is around 0.5 nmol/mg of MNP.

B) General procedures for DNA detection using HRP based assay

The capture DNA (cDNA) was covalently conjugated to an amine modified MNP surface via a PEG containing hetero-functional cross linker SM(PEG)₁₂ as described in the SI. The PEGylated cross-linker is used here because of its well-know ability of resisting non-specific adsorption¹⁷,¹⁹, allowing greatly reduced assay background and hence achieving higher sensitivity. The amount of cDNA loaded on the MNP was estimated by using our previously established method.¹⁹ The signal DNA (sDNA) was linked to neutravidin- (NAV-) HRP or ALP conjugate via the strong, specific biotin-NAV interaction at 1 to 1 molar ratio. NAV-En conjugate was selected here because it can offer significantly reduced non-specific adsorption compared to the avidin- or streptavidin- enzyme conjugate. A certain amount of MNP-cDNA was mixed with 5 pmol HRP-sDNA and various amounts of target DNA. The final volume of each sample is 500 μL in PBS buffer (containing 1 mg/mL BSA). After 1 h incubation at room temperature, all samples were washed by PBS buffer once, by PBS buffer (containing 0.1% Tween-20) twice, by PBS buffer once again to remove any unbound target DNA and HRP-sDNA. Then the MNP sandwiches were dispersed in 380 μL PBS, the enzymatic amplification was initiated by the addition of 50 μL amplex red (0.2 mM) and 50 μL H₂O₂ (0.2 mM). After a fixed period for enzymatic amplification, 20 μL N₃Na (1 M) was added into each sample to terminate the enzymatic assay and the UV-vis absorption spectra of all supernatants were recorded.

For SNP discrimination of experiment, the detection procedures were effectively same as those described above. Specifically, MNP-cDNA3 (20 μg) and 5 pmol HRP-sDNA3 are mixed with the full complementary T-DNA3, or their SNP targets (T-DNA4 or T-DNA5) at identical concentrations (100 pM) in 500 μL PBS buffer, and after incubation and washing procedures, only 10 min of enzymatic amplification time was conducted.

C) Limit of detection challenges

To evaluate the limit of detection of this proposed sensor using colorimetric readout, the assay procedures were basically the same as those described above, except where 10 μg MNP-cDNA3 and 0.8 pmol HRP-sDNA3 were used to detect different amounts of T-DNA3 in 200 μL PBS buffer. After incubation and washing procedure, resulting MNP-dsDNA-En assemblies were dispersed in 200 μL PBS buffer containing amplex red and H₂O₂ (5 μM each). Subsequently, real-time fluorescence change was monitored on an Envision plate reader using BODIPY TMR FP 531 as excitation filter and Cy3 595 as emission filter.

D) General procedures for DNA detection using ALP

For single DNA detection using ALP as signal amplifier, MNP-cDNA2 (20 μg) were mixed with 5 pmol ALP-sDNA2 and T-DNA2 of various concentrations. The final volume of each sample is 500 μL in Tris buffer (containing 1 mg/mL BSA). Because the phosphate could inhibit the enzymatic ability of ALP,²⁰ Tris buffer was employed instead of PBS buffer in preparing samples and washing procedures. Then the MNP sandwiches were dispersed in 430 μL Tris buffer, and then each was mixed with 50 μL FDP (0.2 mM) initiating the enzymatic
amplification assay. After 1 h, following the addition of 20 μL PBS buffer to stop the enzymatic reaction, the UV spectra of supernatants of all the samples were recorded.

**E) Simultaneous quantitation of two different DNA targets**

MNPs-cDNA1 and MNP-cDNA2 (15 μg each) were mixed with 15 μL ALP-sDNA1 (0.25 μM) and 15 μL HRP-sDNA2 (0.25 μM) to quantitate two different target DNA strands, T-DNA1 and T-DNA2. All assays and washing steps were carried out in Tris buffer, because PBS can efficiently inhibit the ALP activity. After sandwich formation and washing, the resulting MNP sandwiches were dispersed in 330 μL Tris buffer, and then each sample was added a mixture of 50 μL FDP (0.2 mM), 50 μL Amplex red (0.2 mM) and 50 μL H2O2 (0.2 mM) to initiate the enzymatic amplifications. After 1 h, 20 μL PBS mixed with 20 μL N3Na (1 M) were added simultaneously to stop both enzymatic reactions, the resulting UV spectra of supernatants were then recorded.

![Typical UV-vis assay curves showing the catalytic activities of the HRP-NAV (A571 time trace) and ALP (A485 time trace) in different buffers and their inhibition by NaN3 and PBS phosphate. The rapid decreases of the absorbance observed in the assay curves following the addition of 20 μL PBS / NaN3 are due to dilution, after which the absorbance became effectively constant, due to enzyme activity inhibition.](image-url)

**Fig. S1.** Typical UV-vis assay curves showing the catalytic activities of the HRP-NAV (A571 time trace) and ALP (A485 time trace) in different buffers and their inhibition by NaN3 and PBS phosphate. The rapid decreases of the absorbance observed in the assay curves following the addition of 20 μL PBS / NaN3 are due to dilution, after which the absorbance became effectively constant, due to enzyme activity inhibition.
Fig. S2. (A) Powder X-ray diffraction (XRD) spectra of core only MNP, with the expected diffraction major diffraction peaks for the Fe₃O₄. (B) Typical IR spectra of the MNP and MNP-NH₂. (C) Size distributions of the hydrodynamic diameters for core only MNP and MNP-NH₂, respectively. (D) Magnetization of MNP-NH₂ as a function of the applied magnetic field measured with a vibrating sample magnetometer (VSM).

Fig. S3. Representative photographs of the MNP-NH₂ dispersed in water on being placed onto a biomag separation device (A) and after waited for 1 min (B).
Fig. S4. (A) Effect of cDNA-MNP surface passivation by 2-mercaptoethanol and 1 mg/mL BSA on the assay signal for T-DNA detection, the signal to background (S/B) ratio increased from ~4.4 to ~62 after MNP surface passivation. (B) Absorbance histogram of samples showing effect of the MNP-cDNA1 amount in detecting 200 pM T-DNA1. **Black**: the MNP-cDNA background only without T-DNA1; **green**: signal from MNP-cDNA1 and T-DNA1; and **orange**: net T-DNA1 signal (green – black) was found to be highest at ~20 µg MNP-cDNA. (C) Effect of temperature on the assay sensitivity in T-DNA1 detection, where 24 °C gives the maximum sensitivity. (D) Effect of cDNA loading on MNP on specific assay sensitivity for T-DNA detection, where 0.5 nmol/mg MNP was found to be optimum for the sensor.
Fig. S5. (A) Fluorescence time traces of the assay in detecting different concentrations of T-DNA3 using a fluorescence plate reader, the data are fitted to linear relationships. (B) Comparison of the corresponding slopes of the time traces for different T-DNA concentrations.

Detection of T-DNA1 in complex media, ca 10% human serum

Fig. S6. The UV-vis absorption spectra of the assay with different amounts of T-DNA1 (left) and the corresponding calibration curve for T-DNA1 detection in 10% human serum in PBS, data were fitted to a linear function.
Long term storage stability of MNP-cDNA probe

Fig. S7. Investigation of the long storage stability of the MNP-cDNA probes. The graphs in the top panels showing the calibration curves using the MNP-cDNA at various storage period in the detection of the complementary DNA target, and the bottom graph shows the relevant slopes of the calibration curves.

Fig. S8. (A) UV-vis absorption spectra of assay samples showing discrimination of cancer specific SNPs against the wild-type DNA target in KRAS gene using MNP-cDNA3 and HRP-sDNA3 in phosphate buffer with 100 mM NaCl. (B) Comparison of $A_{571}$ values for such different DNA targets.
Table S1. Comparison of the sensing performances of the MNP-enzyme sandwich assay against some recently reported DNA assays. (No. = number of target detected; LOD = limit of detection; S/N = signal to noise ratio)

| Sensing Method                                                        | No. | LOD (pM) | S/N  | Ref. |
|-----------------------------------------------------------------------|-----|----------|------|------|
| MNP-enzyme sandwich assay                                             | 2   | 0.05     | ~177 | This work |
| Photonic crystal hydrogel beads                                       | 2   | 0.66     | ~18  | 5    |
| Electrochemical detection based on bar-coded GNPs                     | 2   | ~4       | ~12  | 6    |
| MNP-dye sandwich assay                                                | 2   | 100      | ~90  | 7    |
| Au particle-on-wire surface-enhanced Raman scattering (SERS)          | 4   | 10       | ~88  | 8    |
| Fluorescence based on Ag@poly(m-phenylenediamine) nanoparticles       | 3   | 250      | ~8.8 | 9    |
| Quantum dot-DNAzyme chemiluminescence resonance energy transfer       | 3   | 10000    | ~14  | 10   |
| Microcantilever based DNA nanomechanical sensing                      | 4   | 1-33     | ?    | 11   |
| Silver particle amplified dual-wavelength surface enhanced Raman scattering | 5   | ~100     | ~13  | 12   |
| Graphene quenched fluorescent DNA nanoprobe                           | 3   | ~100     | ~13  | 13   |
| Graphene-based high-efficiency surface-enhanced Raman scattering      | 2   | 10       | ~22  | 14   |
| Zn(II)-porphyrin/G-quadruplex complex with Exo III-assisted target recycle | 1   | 200      | ~15  | 15   |
| MNP-enzyme probes detected via a personal glucose sensor              | 1   | 40       | ~60  | 16   |
| Electrochemical DNA sensing using a DNA tetrahedron structure         | 1   | 1        | ?    | 17   |
| Gold nanoparticle amplified surface Plasmon resonance sensing         | 1   | 10       | ~8   | 18   |
| Electrochemical sensing via a DNA Supersandwich Assembly              | 1   | 0.1      | ?    | 19   |
| Enzyme-Based Electrochemical DNA Sensing                              | 1   | 0.01     | ~10  | 20   |

Reference

(1) Hui, C.; Shen, C. M.; Yang, T. Z.; Bao, L. H.; Tian, J. F.; Ding, H.; Li, C.; Gao, H. J. J. Phys. Chem. C 2008, 112, 11336; Lei, Z.; Pang, X.; Li, N.; Lin, L.; Li, Y. J. Mater. Process. Technol. 2009, 209, 3218-3225.

(2) Lei, Z. L.; Li, Y. L.; Wei, X. Y. J. Solid State Chem. 2008, 181, 480-486; Huang, Y.-F.; Wang, Y.-F.; Yan, X.-P. Environ. Sci. Technol. 2010, 44, 7908-7913.

(3) Stober, W.; Fink, A.; Bohn, E. J. Colloid Interface Sci. 1968, 26, 62-69.

(4) Plueddemann, E. P.; Plenum Press: New York, 1991; Ohmori, M.; Matijevic, E. J. Colloid Interface Sci. 1993, 160, 288-292; Bruce, I. J.; Sen, T. Langmuir 2005, 21, 7029-7035.

(5) Hu, J.; Zhao, X. W.; Zhao, Y. J.; Li, J.; Xu, W. Y.; Wen, Z. Y.; Xu, M.; Gu, Z. Z. J. Mater. Chem. 2009, 19, 5730-5736.

(6) Zhang, D.; Huarng, M. C.; Alocilja, E. C. Biosens. Bioelectron. 2010, 26, 1736-1742.

(7) Liu, H.; Li, S.; Liu, L.; Tian, L.; He, N. Biosens. Bioelectron. 2010, 26, 1442-1448.

(8) Kang, T.; Yoo, S. M.; Yoon, I.; Lee, S. Y.; Kim, B. Nano Lett. 2010, 10, 1189-1193.

(9) Zhang, Y.; Wang, L.; Tian, J.; Li, H.; Luo, Y.; Sun, X. Langmuir 2011, 27, 2170-2175.

(10) Freeman, R.; Liu, X.; Willner, I. J. Am. Chem. Soc. 2011, 133, 11597-11604.
(11) Zhang, J.; Lang, H.; Huber, F.; Bietsch, A.; Grange, W.; Certa, U.; McKendry, R.; Güntherodt, H.-J.; Hegner, M.; Gerber, C. Nat. Nanotechnol. 2006, 1, 214-220.

(12) Faulds, K.; McKenzie, F.; Smith, W. E.; Graham, D. Angew. Chem. Int. Ed. 2007, 46, 1829-1831.

(13) Zhang, M.; Yin, B. C.; Tan, W.; Ye, B. C. Biosens. Bioelectron. 2011, 26, 3260-3265.

(14) He, S.; Liu, K.-K.; Su, S.; Yan, J.; Mao, X.; Wang, D.; He, Y.; Li, L.-J.; Song, S.; Fan, C. Anal. Chem. 2012, 84, 4622-4627.

(15) Zhang, Z.; Sharon, E.; Freeman, R.; Liu, X.; Willner, I. Anal. Chem. 2012, 84, 4789-4797.

(16) Xiang, Y.; Lu, Y. Anal. Chem. 2012, 84, 1975-1980.

(17) Pei, H.; Lu, N.; Wen, Y.; Song, S.; Liu, Y.; Yan, H.; Fan, C. Adv. Mater. 2010, 22, 4754-4758.

(18) He, L.; Musick, M. D.; Nicewarner, S. R.; Salinas, F. G.; Benkovic, S. J.; Natan, M. J.; Keating, C. D. J. Am. Chem. Soc. 2000, 122, 9071-9077.

(19) Xia, F.; White, R. J.; Zuo, X.; Patterson, A.; Xiao, Y.; Kang, D.; Gong, X.; Plaxco, K. W.; Heeger, A. J. J. Am. Chem. Soc. 2010, 132, 14346–14348.

(20) Fan, C.; Plaxco, K. W.; Heeger, A. J. Proc. Natl. Acad. Sci. 2003, 100, 9134-9137.