Electronic Supplementary Material (ESI) for ChemComm.
This journal is © The Royal Society of Chemistry 2019

Electronic Supplementary Information

For

A triple-amplification strategy based on the formation of peroxidase-like two-dimensional DNA/Fe_3O_4 networks initiated by hybridization chain reaction for highly sensitive detection of microRNA

Sheng Tang[a,*], Yana Li[a], Anni Zhu[a], Yao Yao[a], Jun Sun[a], Fenfen Zheng[a], Zixia Lin[b] and Wei Shen[a,*]

[a]School of Environmental and Chemical Engineering, Jiangsu University of Science and Technology, Zhenjiang 212003, Jiangsu Province, PR China

[b]Testing Center, Yangzhou University, Yangzhou 225000, Jiangsu University, PR China

Corresponding authors' e-mail addresses: tangsheng.nju@gmail.com (S. Tang); shenweivv@126.com (W. Shen)

EXPERIMENTAL SECTION

Materials

The DNA probes, synthetic miRNAs, and all other oligonucleotides were custom-made by Sangon Biotechnology Co., Ltd. (Shanghai, China) and their respective sequences are listed in Table S1. Anhydrous sodium acetate, glacial acetic acid, sodium hydroxide, ferric chloride and tris (hydroxymethyl) aminomethane were purchased from China National Pharmaceutical Group Corporation (Shanghai, China). Disodium phosphate dodecahydrate, sodium dihydrogen phosphate dehydrate were obtained from Rich Joint Chemical Co., Ltd. (Shanghai, China). Hydrogen peroxide was from China Sun Specialty Products Co., Ltd. (Jiangsu, China). TMB and ferrous chloride tetrahydrate was acquired from Energy Chemical (Shanghai, China). All solutions were prepared using nuclease free ultrapure water with an electrical resistance of 18 MΩ·cm.

The HCR system was designed for *let-7a* detection, and the sequences were listed in Table S1. It should be noted that in the sequences of hairpin DNA probes, the sequences used to hybridize with ssDNA are highlighted in yellow, sticky ends are underlined and

loops are italicized. The sequence of ssDNA used to hybridize with hairpin DNA probes is highlighted in blue. *Let-7a* is the target miRNA. *Let- 7b*, *let- 7c* and miRNA-21 are the interfering miRNAs with mismatched bases used to evaluate the selectivity of the proposed assay for *let-7a*.

Table S1. All oligonucleotides and their respective sequences

Oligonucleotides	Sequence (from 5' to 3')
H1	TTTTTTTTTTTTTTTAGTAGGTTGTATAGTT***CAAAGT***AA CTATACAACCTACTACCTCA
H2	ACTTTGAACTATACAACCTACT***TGAGGT***AGTAGGTTG TATAGTTTTTTTTTTTTTTTT
ssDNA	NH_2-$(CH_2)_6$-AAAAAAAAAAAAAAAAA
Let- 7a	UGAGGUAGUAGGUUGUAUAGUU
Let- 7b	UGAGGUAGUAGGUUGUGUGGUU
Let- 7c	UGAGGUAGUAGGUUGUAUGGUU
miR-21	UAGCUUAUCAGACUGAUGUUGA

Apparatus

All UV-vis absorption experiments were conducted on a Shimadzu UV-2550 UV-vis spectrophotometer. All pH measurements were performed with a pH-3c digital pH-meter (Shanghai Lei Ci Device Works, Shanghai, China) with a combined glass-calomel electrode. The composition and the valence states of materials were examined by X-ray photoelectron spectroscopy (XPS, Thermo ESCALAB 250XI). The X-ray diffraction (XRD) spectra of both native Fe_3O_4 and DNA/Fe_3O_4 networks were obtained by Bruker D8 ADVANCE Powder X-ray diffractometer (Karlsruhe, Germany). All mixing processes involving the DNA chains and Fe_3O_4 nanosheets, solution preparation were conducted on a Vortex-6 Kylin-Bell (Qilinbeier Instrument Manufacturing Co., Ltd. Beijing, China). The stabilization of the Fe_3O_4 nanosheets suspension was processed by centrifuging at a speed of 10000 RPM for 1 min by a H1850 Table Top High Speed Centrifuge (Changsha Xiangyi Centrifuge Instrument, Changsha, Hunan, China). Fourier-transform infrared (FT-IR) spectra were recorded

using a Nicolet 380 spectrophotometer in the range of 4000-400 cm^{-1} (Thermo Fisher Scientific, Shanghai, China). Serum samples were provided by Nanjing Synthgene Medical Technology Co., Ltd.

Synthesis of the Magnetic Fe_3O_4 Nanosheets

$FeCl_2 \cdot 4H_2O$ (8.946 g, 0.045 mol) and $FeCl_3$ (2.433 g, 0.015 mol) were dissolved in a beaker containing pure water and transferred into a three-necked round bottom flask. The total amount of water was 100 mL. With N_2 gas purging, NaOH (2.5 M) was added dropwise until the pH of the solution was within 10 to 11 (Control the drop rate of NaOH slow). Then the reaction mixture was stirred in a 60 °C water bath for 4 hours. The mixture was then poured into a reaction kettle and incubated at 60 °C for 36 hours. The supernatant was removed by centrifugation, and the solid was washed with water until the pH of the mixture reached 7. The final Fe_3O_4 product was dried at 60 °C in a vacuum oven for 48 hours, milled, collected, and stored in a sealed bottle.

Fe_3O_4 Nanosheets Modification

The modification of the Fe_3O_4 nanosheets followed a surface modification process reported in a previous study.[1] Briefly, 500 μL of 600 nM single-stranded DNA (ssDNA) (20 bases) was deposited on the surface of 0.5 mg of the Fe_3O_4 nanosheets (100 μL) at room temperature and the mixture was incubated in saturated phosphate buffer for 2 hours. The supernatant was then separated out by centrifugation. Finally, the sample (Fe_3O_4/ssDNA) was rinsed with saturated phosphate buffer and stored at room temperature for later use.

HCR-Initiated DNA/Fe_3O_4 Networks Formation.

The HCR process was similar to a previously reported process.[2] Generally, the hairpin DNA probes (H1 and H2) were respectively heated to 60 °C for 5 min, and then were naturally cooled down to room temperature for 1 hour before use. In a typical HCR process, samples containing the target miRNA (*let-7a*) were mixed with 500 nM H1 and 500 nM H2 (total volume, 200 μL) in Tris-HAc/Ac buffer (10 mM Tris, 500 mM

HAc/NaAc, pH = 6.8) at 25 °C for 2 hours. The HCR-treated sample (200 μL) was added to the Fe_3O_4/ssDNA nanosheets, and the final mixture was further incubated at 25 °C for 1 hour. The supernatant was then separated out by centrifugation, and the product was washed with the Tris-HAc/Ac buffer. Finally, 100 μL Tris-HAc/Ac buffer was added to the synthetic product, and the DNA/Fe_3O_4 networks were separated by a magnet for several seconds.

Preparation of Serum Samples

Pretreatment of serum sample was performed according to the recommended procedure from producer. Generally, it follows as: first, 200 μL serum was taken into a centrifugal tube, adding 1 mL volume of TRIzon Reagent. The mixture was mixed by shaking for 30 seconds. Then leave it at room temperature for 5 minutes so that the protein nucleic acid complex was completely separated. Then, 200 μL of chloroform was added in the mixture and the latter was shaken for 15 seconds vigorously. After a centrifugation at 12000 rpm for 15 minutes, the sample was divided into three layers: the red organic phase, the intermediate layer and the colorless aqueous phase. The upper colorless aqueous phase was transferred to a new 1.5 mL centrifugal tube. Then, 300 μL of anhydrous ethanol was added, and the solution was transferred into the adsorption column RM (Spin Columns RM) loaded into the collection tube. The adsorption column RM was discarded and the effluent was retained after centrifugation at 12,000 rpm for 30 seconds. Later, 600 μL of anhydrous ethanol was added into the effluent and mixed well. The solution and precipitate obtained in the previous step were transferred into the adsorption column RS (Spin Columns RS) loaded in the collection tube. After a centrifugation at 12,000 rpm for 30 seconds, the waste was discarded from the collection tube. The adsorption column RS was put back into the collection tube. Then, 700 μL Buffer RWT was added and the resulted solution was centrifuged at 12,000 rpm for 30 seconds. The waste was discarded again. Later, 500 μL Buffer RW2 was added and the resulted solution was centrifuge at 12,000 rpm for 30 seconds, and the waste was discarded. The adsorption column RS was kept at room temperature for a few minutes to dry thoroughly. Then, put it in a new RNase free centrifuge tube and add 50

μL Rnase-free water. Finally, it was placed at room temperature for 1 min, then centrifuged at 12,000 rpm for 1 min, and RNA solution was collected. The final volume of the collected RNA solution was *ca.* 50 μL.

Catalyzed Colorimetric Reaction

The catalytic oxidation of TMB was performed according to a published procedure.[3] In brief, in 0.1 M NaAc/HAc buffer, 5 μL of 0.1 M TMB and 10 μL of 5.0 M H_2O_2 were added to the DNA/Fe_3O_4 networks, making up a total volume of 1.0 mL. The reaction was monitored by the UV-vis spectrometer and the change in absorbance was recorded at 650 nm.

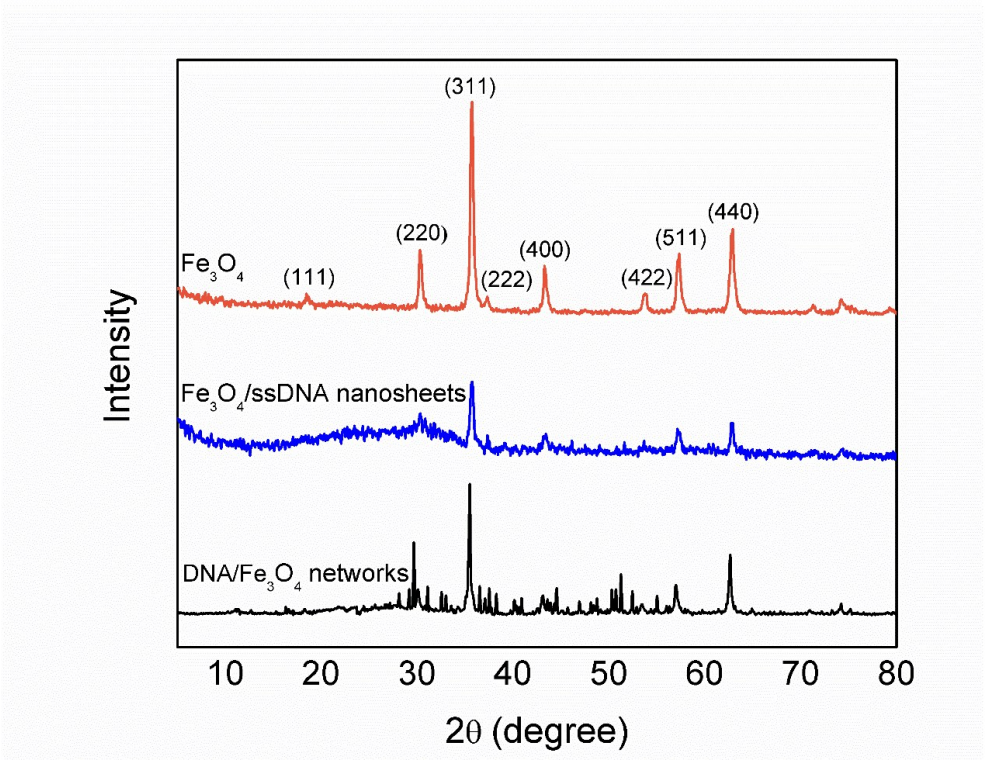

Fig. S1. XRD spectra of Fe_3O_4, Fe_3O_4/ssDNA nanosheets and DNA/Fe_3O_4 networks.

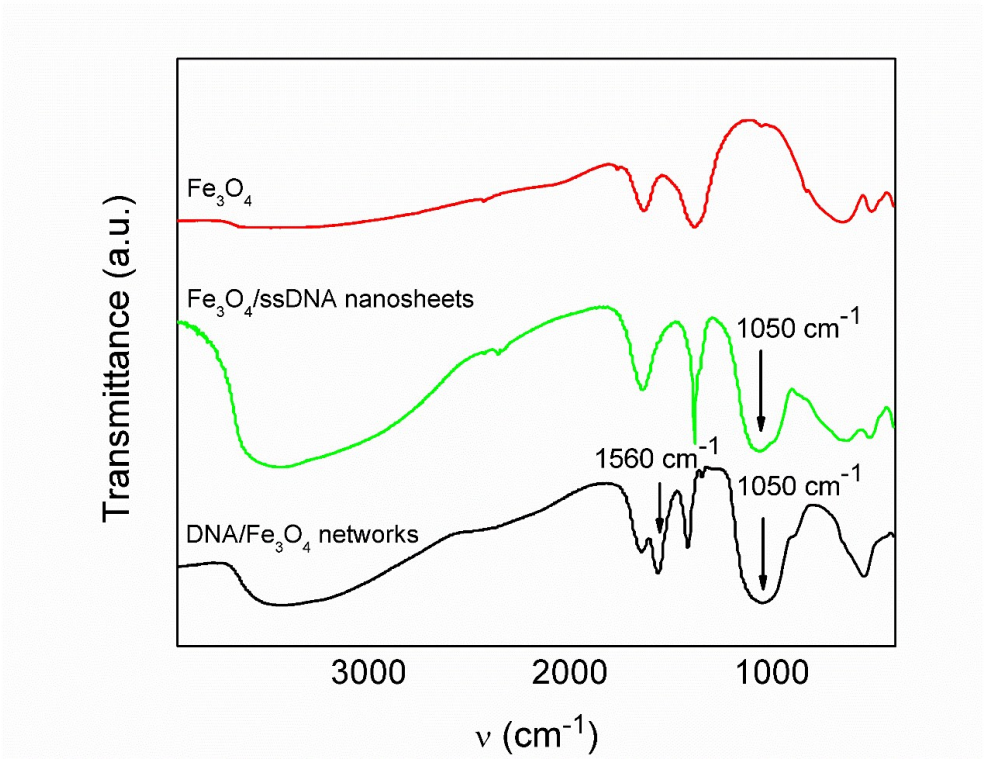

Fig. S2. FT-IR spectra of Fe_3O_4, Fe_3O_4/ssDNA nanosheets, and DNA/Fe_3O_4 networks.

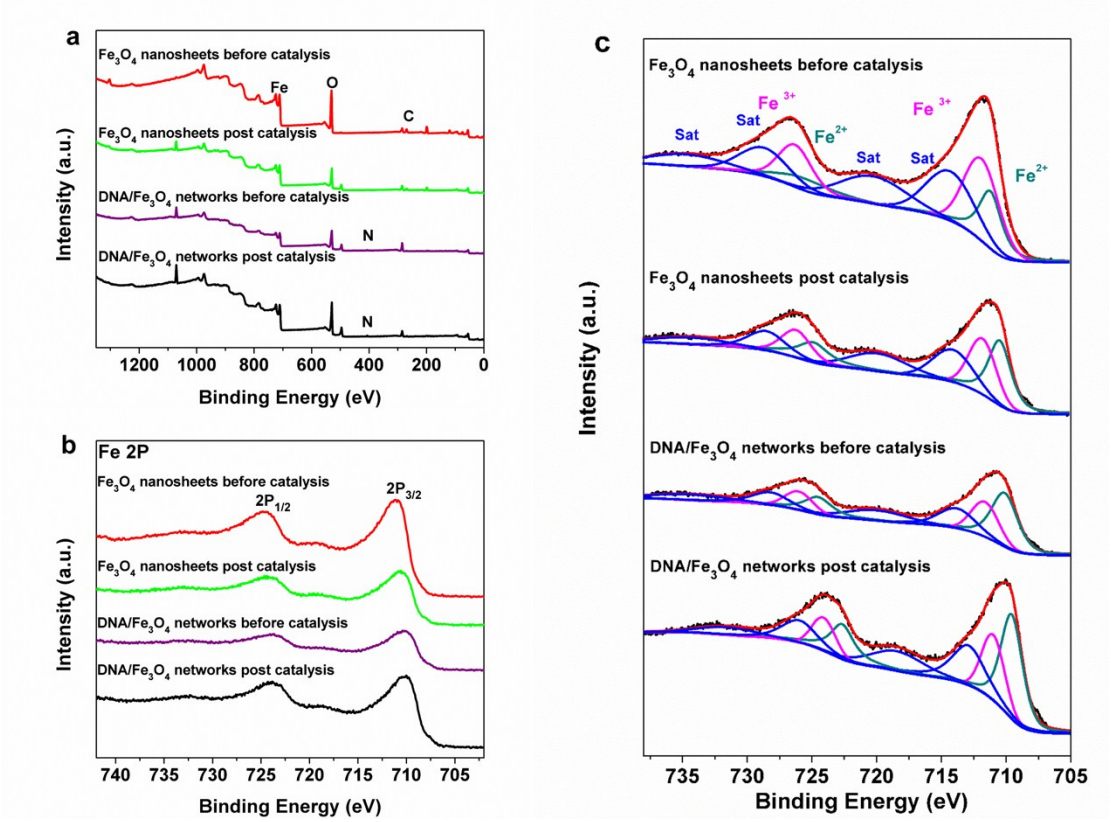

Fig. S3. (a) XPS survey scans of Fe_3O_4 nanosheets and DNA/Fe_3O_4 networks before and post catalysis. (b) The Fe 2p spectra of Fe_3O_4 nanosheets and DNA/Fe_3O_4 networks before and post catalysis. (c) Fe 2p of these materials. The black lines represent experimental results, and the pink, green and red lines correspond to Gaussian fits arising from Fe^{3+}, Fe^{2+} and the sum, respectively.

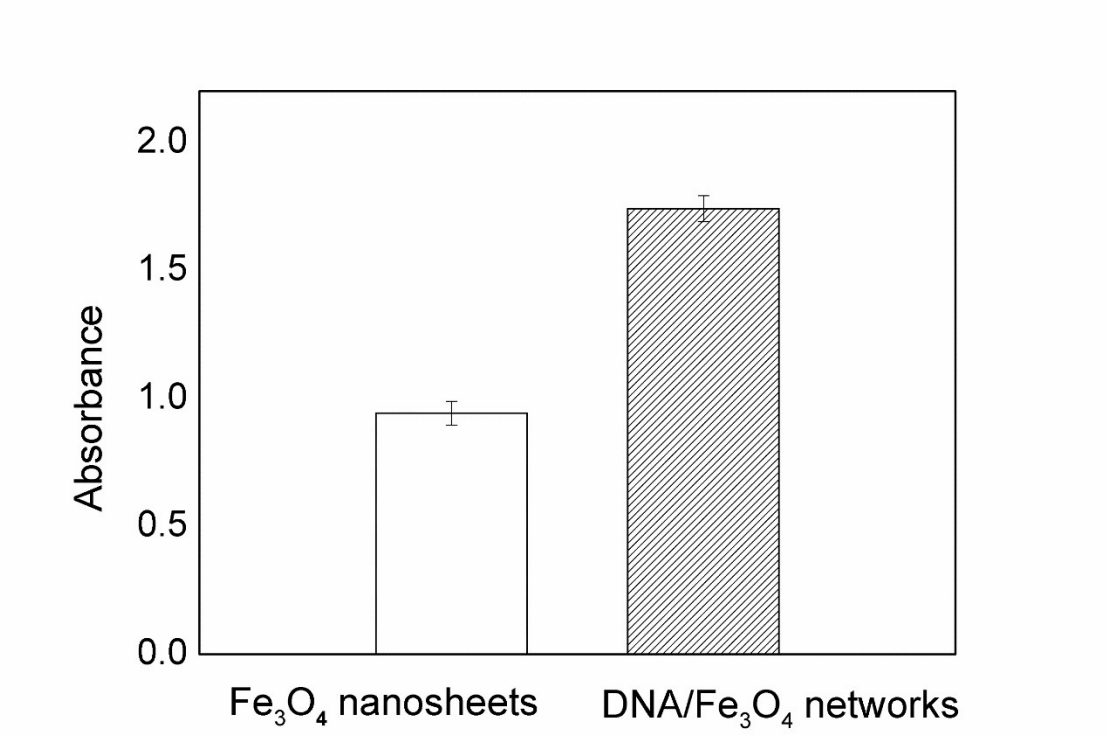

Fig. S4. Comparison of catalytic ability of Fe_3O_4 nanosheets and DNA/Fe_3O_4 networks. Conditions: 0.5 mg Fe_3O_4 nanosheets or DNA/ Fe_3O_4 networks (0.5 mg Fe_3O_4, 500 nM H1, 500 nM H2, 500 nM ssDNA, 100 fM *let-7a*); catalytic reaction time, 3 min; pH, 4.0.

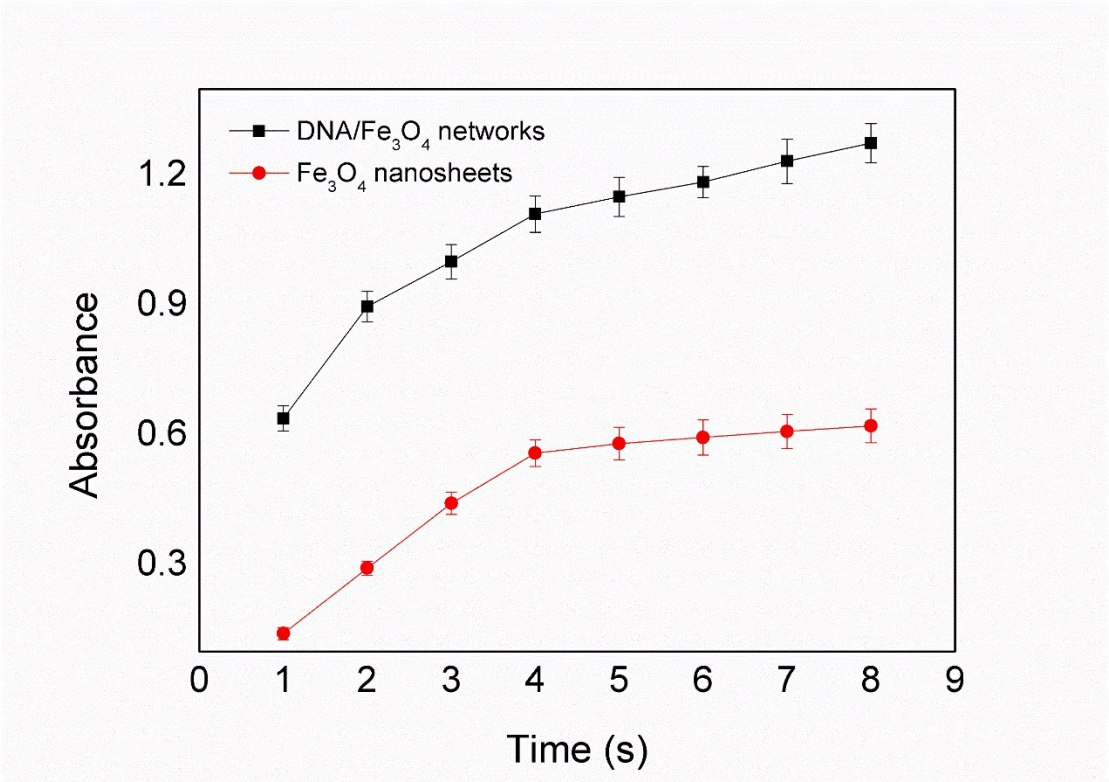

Fig. S5. Optimization of magnetic separation time of DNA/Fe_3O_4 networks and Fe_3O_4 nanosheets from 1 s to 8 s.

Gel electrophoresis results of the HCR system

Agarose gel electrophoresis was carried out to demonstrate the practicability of *let-7a*-initiated HCR process. H1 and H2 monomers were separately heated at 55 ºC for 8 min and then cooled down to room temperature for 1 h before use. Different concentrations of *let-7a* were incubated with 300 nM H1 and H2 for 2 h. The 2% agarose gels containing GoldView (1 μL per 10 mL of gel volume) were prepared by using TAE buffer (pH 8.5). Agarose gels were run at 80 V for 60 min and visualized under UV light.

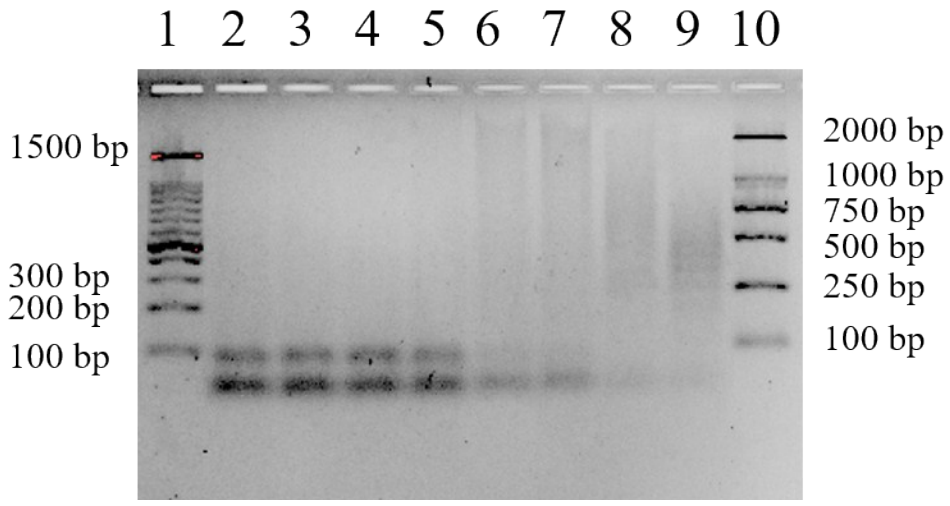

Fig. S6. Gel electrophoresis of the HCR products initiated by different concentrations of *let-7a* target containing 300 nM H1 and H2. Lane 1 and 10, DNA ladder markers; lane 2-9, 0, 0.01, 0.1, 1, 10, 20, 50, 100 nM, respectively, of *let-7a*.

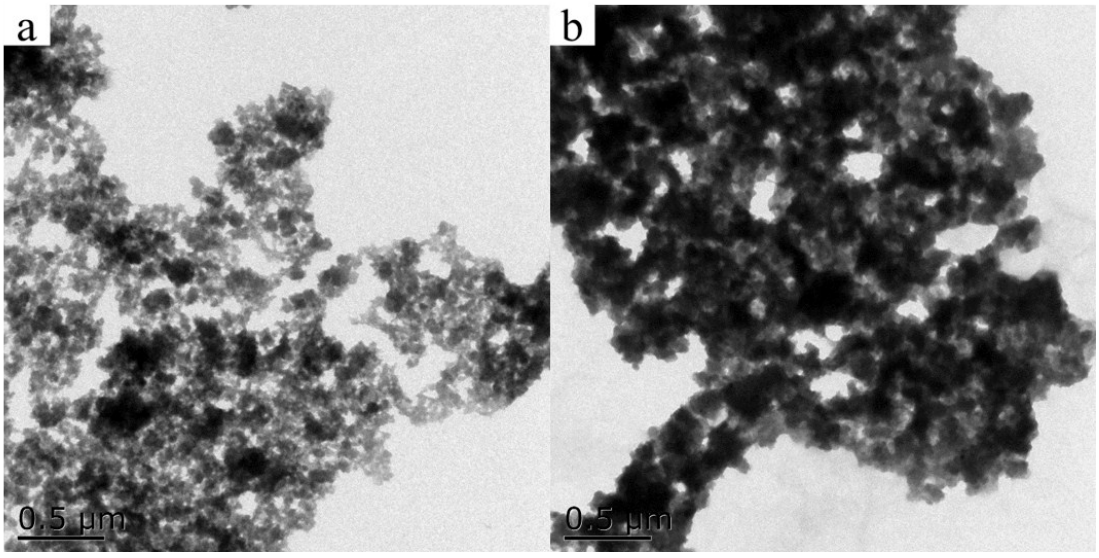

Fig. S7. TEM images of 2D DNA/Fe_3O_4 networks initiated by different concentration of target, *let-*

7a. (a) Conditions: 0.5 mg Fe_3O_4 nanosheets, 500 nM H1, 500 nM H2, 500 nM ssDNA, 1 nM *let-7a*. (b) Conditions: 0.5 mg Fe_3O_4 nanosheet, 500 nM H1, 500 nM H2, 500 nM ssDNA, 10 nM *let-7a*.

Optimization of the ratio of DNA single strands

Since the DNA single strands (ssDNA) modified on the surface of the magnetic Fe_3O_4 nanosheets were paired with the tail chain of H1 and H2. The magnetic Fe_3O_4 nanosheets and the dendritic DNA double-stranded structure are thus conjugated. In order to achieve the optimal reaction conditions, the amount of ssDNA was investigated, and the results were depicted in Fig. S8. It was found that n (ssDNA): n (H2) = 3 is optimal for the proposed assay.

The number of 0.5 mg Fe_3O_4 nanosheets is *ca.* 3×10^{13}. The number of 0.3 nmol ssDNA is *ca.* 6×10^{14}. Thus, each Fe_3O_4 nanosheet was modified by *ca.* 20 ssDNA.

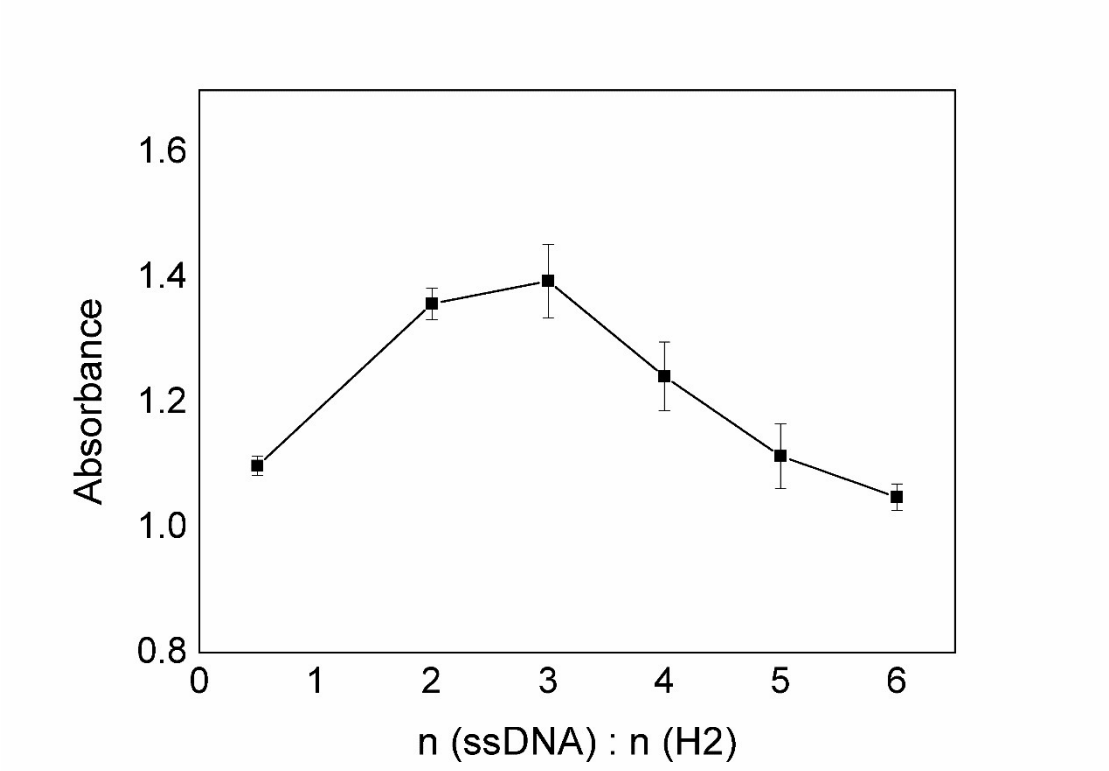

Fig. S8. Optimization of the ratio of DNA single strands on Fe_3O_4 nanosheets and H2. Conditions: 0.1 nmol H1, 0.1 nmol H2, 2 fmol *let-7a*, 0.5 mg Fe_3O_4 nanosheets; catalytic reaction time, 5 min; pH, 4.0. n (ssDNA): 0.05 nmol, 0.2 nmol, 0.3 nmol, 0.4 nmol, 0.5 nmol, 0.6 nmol.

Optimization of the Catalytic Oxidation of TMB

Since the pH value of the solution and reaction time have profound impact on the oxidation TMB. In order to achieve the optimal reaction conditions, pH value and

reaction time were investigated, and the results were depicted in Fig. S9. It was found that pH 4.0 is optimal for the proposed assay. Yan et al. suggested that Fe^{2+} ions in the Fe_3O_4 magnetic nanoparticles (Fe_3O_4 MNPs) may play a dominant role in the peroxidase-like catalytic activity of Fe_3O_4 MNPs.[3] The iron ions could be leached from the Fe_3O_4 MNPs in an acidic reaction solution.[4] This may be because Fe^{2+} ions leached more when the pH value of the solution is 4.0. Thus, pH 4.0 was chose for the following experiments.

We also looked into the dependence of the absorbance of the assay on the reaction time. As demonstrated in Fig. S9, after 20 min, the absorbance leveled off, indicating that the reaction is almost completed. However, a complete reaction is not sensitive for various concentrations of *let-7a*, which limits the detection linearity range. Moreover, the reaction time is too long for practical purposes. Our experiments showed that a period of 5 min reaction is sufficient. Thus, 5 min was adopted as the proper reaction time in this work.

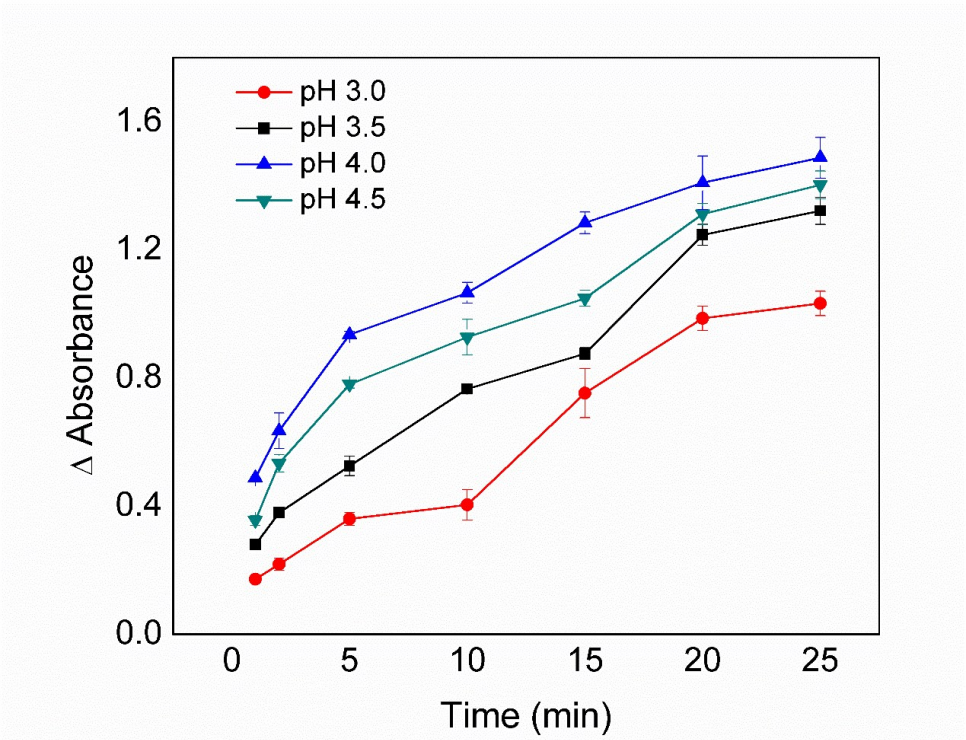

Fig. S9. Effect of pH and reaction time on the oxidation of TMB. Conditions: 500 nM ssDNA, 0.5 mg Fe_3O_4, 500 nM H1 and 500 nM H2, 5.0 nM *let-7a*, 995 μL of 0.1 M NaAc/HAc buffer, 5.0 μL of 0.1 M TMB and 10 μL of 5.0 M H_2O_2. Error bars indicate the standard deviations (n = 3).

Table S2. Detection of *let-7a* in miRNAs isolated from serum samples

Sample	Spiked *let-7a* (fM)	This method (RSD%, n = 3)	Relative recovery (%)	qPCR (fM)
Healthy people Sample 1	Blank	65.15 (2.53)	-	62.33 (2.10)
Healthy people Sample 2	Blank	70.48 (2.73)	-	68.19 (3.26)
	Blank + 0.5	70.93 (4.26)	90.0	
	Blank + 5	75.76 (3.28）	105.6	
	Blank + 50	119.85 (1.66)	98.7	
Cancer patient Sample 3	Blank	32.58 (4.57)	-	31.27 (1.29)
Cancer patient Sample 4	Blank	25.36 (3.55)	-	23.64 (2.42)
	Blank + 0.5	25.87 (2.41)	102.0	
	Blank + 5	30.69 (3.62)	106.6	
	Blank + 50	76.73 (2.53)	102.7	

[a]Relative recovery = $(Concentration_{total}-Concentration_{blank})/Concentration_{spiked}$

Table S3. Detection of *let-7a* in miRNAs isolated from serum samples

Sample	Spiked *let-7a* (fM)	This method (RSD%, n = 3)	Relative recovery (%)
Healthy people	Blank	59.95 (3.76)	
Sample 5	Blank + 500	562.34 (4.21)	100.5
	Blank + 5000	5054.8 (4.55)	99.9
Cancer patient	Blank	20.21 (3.62)	
Sample 6	Blank + 50000	50547.97 (4.32)	101.0
	Blank + 500000	505469.89 (2.57)	101.1

[a]Relative recovery = $(Concentration_{total}-Concentration_{blank})/Concentration_{spiked}$

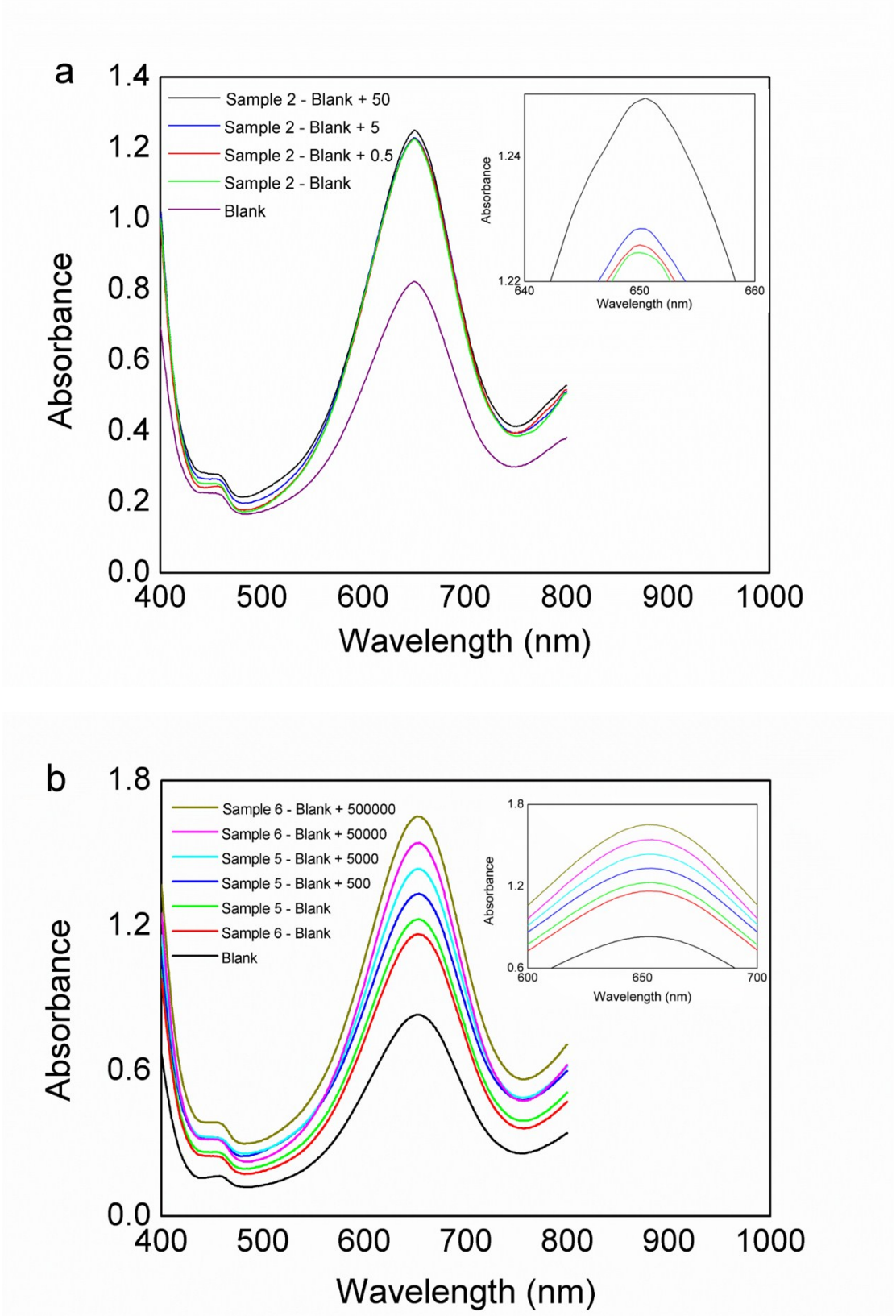

Fig. S10. (a and b) The UV-vis spectra of varied serum samples. The absorbance value of the solution was recorded at 650 nm.

Table S4. A comparison of different HCR strategies for detection of miRNA

Target	Mechanism	Signal transduction	LOD	Linear range	Ref.
Let-7a	Graphene oxide-assisted HCR	Fluorescence	1 pM	1 pM - 5 nM	5
Let-7a	HCR modulated DNA-hosted Ag nanoclusters as signal reporters	Fluorescence	0.78 nM	1.56 - 400 nM	6
MiR-122	Self-assembly of hyperbranched DNA structures through integrating CHA with HCR	Chemiluminescence	0.72 pM	1 pM to 10 nM	7
MiR-21 and miR-10b	HCR-amplified assay using nucleotide-modified AuNPs as signal reporters	Colorimetry	20 fM	-	8
MiR-122b	Probe-conjugated 3D tetrahedral DNA nanostructure modified electrode for HCR	Electrochemistry	10 aM	10 aM – 1 pM	9
Hsa-miR-17-5p	Tetrahedral DNA nanostructure modified electrode using AuNPs as initiator carriers for HCR	Electrochemistry	2 aM	0.1 fM - 0.1 nM	10
MiR-141	Tetrahedral DNA nanostructure modified electrode using metal-catalyst-free click chemical signal amplification via HCR	Photoelectrochemical Sensing	27 aM	0.1 fM - 0.5 nM	11
MiR-141 and miR-21	Multifunctional magnetic NPs probe coupling with HCR	Electrochemistry	0.44 fM, 0.46 fM	1 fM - 1 nM	12
MiR-141	Dual-amplification system based on CHA and HCR	Fluorescence	36 fM	0.75 pM - 20 pM 0.4 nM - 40 nM	13
MiR-21	Planar intercalated copper (II) complex molecule as small molecule enzyme mimic combined with Fe_3O_4 nanozyme	Electrochemistry	33 aM	100 aM - 100 nM	14
MiR-203	Combining bio-barcode probe and HCR	Surface-enhanced Raman scattering	0.15 fM	1 fM - 1 pM	15
MiR-21	Plasmon-enhanced HCR	Fluorescence	0.043 fM	1 fM – 1 pM	16
Let-7a	DNA/Fe_3O_4 networks based on HCR	Colorimetry	13 aM	50 aM – 12 nM	This work

Notes and references for ESI only

1. Z. M. Baccar, D. Caballero, R. A. Eritja, *Electrochim. Acta*, 2012, **74**, 123-129.
2. R. M. Dirks, N. A. Pierce, *P. Natl. Acad. Sci. U S A*., 2004, **101**, 15275-15278.
3. L. L. Gao, J. Zhuang, L. Nie, J. Zhang, *Nat. Nanotechnol.*, 2007, **2**, 577-583.
4. H. Wei, E. Wang, *Anal. Chem.*, 2008, **80**, 2250-2254.
5. L. Yang, C. Liu, W. Ren, Z. Li, *ACS Appl. Mater. Inter.*, 2012, **4**, 6450-6453.
6. X. Qiu, P. Wang, Z. Cao, *Biosens. Bioelectron.*, 2014, **60**, 351-357.
7. S. Bi, S. Yue, Q. Wu, J. Ye, *Chem. Commun.*, 2016, **52**, 5455-5458.
8. M. Rana, M. X. Balcioglu, M. Kovach, *Chem. Commun.*, 2016, **52**, 3524-3527.
9. Z. Ge, M. Lin, P. Wang, H. Pei, *Anal. Chem.*, 2014, **86**, 2124-2130.
10. P. Miao, Y. Tang, J. Yin, *Chem. Commun.*, 2015, **51**, 15629-15632.
11. C. C. Ye, M. Q. Wang, Z. F. Gao, *Anal. Chem.*, 2016, **88**, 11444-11449.
12. Y. H. Yuan, Y. D. Wu, B. Z. Chi, *Biosens. Bioelectron.*, 2017, **15**, 325-331.
13. S. Li, F. Zhang, X. Chen, C. Cai, *Sensor. Actuat. B-Chem.*, 2018, **263**, 87-93.
14. L. Tian, J. Qi, O. Oderinde, *Biosens. Bioelectron.*, 2018, **110**, 110-117.
15. S. J. Ye, Y. Y. Wu, X. M. Zhai, B. Tang, *Anal. Chem*., 2015, **87**, 8242-8249.
16. F. F. Yin, H. Q. Liu, Q. Li, *Anal. Chem*., 2016, **88**, 4600-4604.