Hydrazone chemistry assisted DNAzyme for analysis of double targets

Anzhi Sheng,[a] Lihong Su,[a] Mohammed Jalalah,[b] M.S. Al-Assiri,[b] Farid A. Harraz*[bc] and Juan Zhang*[a]

[a]*Center for Molecular Recognition and Biosensing, School of Life Sciences, Shanghai University, Shanghai 200444, China*

[b]*Promising Centre for Sensors and Electronic Devices (PCSED), Advanced Materials and Nano-Research Centre, Najran University, P.O. Box: 1988, Najran 11001, Saudi Arabia*

[c]*Nanomaterials and Nanotechnology Department, Central Metallurgical Research and Development Institute (CMRDI), P.O. 87 Helwan, Cairo 11421, Egypt*

Experimental section

Materials and reagents

All oligonucleotides (as listed in Table S1 in Supporting Information) were synthesized and purified by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). N-hydroxysuccinimide (NHS) and polyacrylamide were bought from Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). 4-Hydrazinobenzoic acid and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) were purchased from Sinopharm Chemical Reagent Beijing Co., Ltd (Beijing, China). Lipopolysaccharide (LPS) from *Pseudomonas aeruginosa* 10 and 5-hydroxymethylfurfural (HMF) were purchased from Sigma-Aldrich (Shanghai, China). Streptavidin coated magnetic beads (1 μm) were purchased from New England biolabs Inc. (Beijing, China). Purified water (Nongfu Spring, Nongfu Spring Incorporated Company, China), full-fat sterilization milk (DELUXI, Inner Mongolia Mengniu Dairy Co. Ltd, China), grapefruit juice (Wei Chuan, Hangzhou Weiquan Food Co. Ltd, China), and green tea (Nongfu Spring, Nongfu Spring Incorporated Company, China) were purchased from Shanghai education supermarket. All reagents were prepared with Milli-Q water (18.2 MΩ cm^{-1}) obtained through a Milli-Q purification system (Millipore Corp, Milford, MA, America). All other chemicals were of analytical reagent grade.

Preparation of hydrazine modified partial enzyme strand I (ESI)

20 μL 4-hydrazinobenzoic in ethanol (500 μM) was added into 10 μL mixed solution containing EDC (0.195 mg) and NHS (0.705 mg), followed by reaction for 30 min at 25 °C. After that, 20 μL amino modified partial enzyme strand (50 μM) were added and incubated for 3 h at 25 °C. The mixture was separated by using a gel column (illustra MicroSpin G-25 column), to give ESI. The formed ESI was further characterized by using Thermo LTQ liquid chromatography / mass spectrometry (LC/MS) (Thermo Fisher Scientific Inc, USA).

Construction of hydrazone chemistry assisted DNAzyme

4.8 μL aldehyde group modified partial enzyme strand (ESII) (0.6 μM) was mixed with 7.2 μL ESI (0.9 μM) at 37 °C for 30 min, to give the whole enzyme strand (WES) which was identified through gel electrophoresis and LC-MS. Then, the obtained WES (12 μL) was mixed with 4 μL SS (0.5 μM) in the Tris-HCl buffer (pH 7.4) containing 200 mM sodium chloride and 20 mM magnesium sulfate at 37 °C for 1 h. The fluorescence intensity of mixture was measured by using a F4500 fluorometer (Hitachi, Japan) with 480 nm of excitation wavelength.

Quantitative analysis of LPS

4.8 μL LPS aptamer (LPSA) (0.6 μM) was mixed with 4.8 μL signal probe strand (PS) (0.6 μM). The mixture was heated at 95 °C for 5 min and naturally cooled to room temperature, to give the hybridized strand (LPSA/PS). Then, 9.6 μL LPSA/PS was added into 70.4 μL magnetic beads (MB) (0.5 mg/mL) and the mixture was incubated at 37 °C for 30 min followed by magnetic separation, to give LPSA/PS modified MB (LPSA/PS/MB). Subsequently, 4.8 μL ESII (0.6 μM), 7.2 μL ESI (0.9 μM), 4 μL substrate strand (SS) (0.5 μM) and 10 μL LPS with different concentrations (8000 ng/mL, 2000 ng/mL, 800 ng/mL, 200 ng/mL, 80 ng/mL, 20 ng/mL, 8 ng/mL, 2 ng/mL, 0.8 ng/mL, 0.2 ng/mL, 0.08 ng/mL and 0.02 ng/mL) were simultaneously added into 54 μL LPSA/PS/MB and further incubated at 37 °C for 1 h . After magnetic separation, the fluorescence intensity of the supernatant obtained was analyzed.

Quantitative analysis of HMF

7.2 μL ESI (0.9 μM), 4.8 μL ESII (0.6 μM), 4 μL SS (0.5 μM) and 10 μL HMF at different concentrations (0.01 μM, 0.03 μM, 0.05 μM, 0.07 μM, 0.1 μM, 0.2 μM, 0.3 μM, 0.5 μM, 0.7 μM, 1 μM, 3 μM, and 5 μM) were simultaneously added into 54 μL LPSA/PS/MB and further incubated at 37 °C for 1 h. After magnetic separation, the supernatant was measured by using the fluorometer.

Comprehensive analysis of LPS and HMF

7.2 μL ESI (0.9 μM), 4.8 μL ESII (0.6 μM) and 4 μL SS (0.5 μM) were mixed with different combinations of LPS and HMF including 20 μL LPS (2 ng/mL), 20 μL HMF (0.5 μM), as well as the mixture of 10 μL LPS (4 ng/mL) and 10 μL HMF (1 μM), followed by the addition of 44 μL LPSA/PS/MB. the mixture was further incubated at 37 °C for 1 h, followed by magnetic separation. The fluorescence intensity of the supernatant was tested by using the fluorometer.

In order to rapidly analyze HMF and LPS in food products, the obtained fluorescence intensity has been further normalized and analysed. It has been reported that HMF concentration is generally more than 65.9 mg/kg (about 0.5 μM)[1] and LPS concentration is usually more than 1 EU/mL (about 2 ng/mL) in food product detected by Limulus Amoebocyte Lysate test.[2, 3] Herein, 0.5 μM for HMF and 2 ng/mL for LPS have been utilized and the corresponding FAM and Cy3 fluorescence responses have been separately compared with those with 2 μg/mL LPS. The obtained normalized fluorescence intensities (0.35 for Cy3 and 0.45 for FAM) have been defined as the threshold values.

Investigation for versatility of the established method and its application for qualitative analysis of hydrazine

7.2 μL ESI (0.9 μM), 4.8 μL ESII (0.6 μM), 4 μL SS (0.5 μM) and 10 μL hydrazine hydrate (3 μM) were simultaneously added into 54 μL LPSA/PS/MB and further incubated at 37 °C for 1 h. After magnetic separation, the supernatant was measured by using the fluorometer.

Sequences for DNA strands

Table S1 Sequences for DNA strands in this work

Name	Sequences
LPSA	5'-Biotin-CTTCTGCCCGCCTCCTTCCTAGCCGGATGGCTGAGGTAGGGATCCTCAAGGGTCAGCCCCCCAGGAGACGAGATAGGCGGACACT-3'
PS	5'-FAM-TTGAGGATCCCTACCTGTC-3'
ESII	5'-CHO-AAATAGTTGGTCGCTGGGGGGCTGACC-3'
ESII'	5'-AAATAGTTGGTCGCTGGGGGGCTGACC-3'
ESI	5'-CATCTCTTCTCCGAGCCGGTCG-NHNH$_2$-3'
ESI'	5'-CATCTCTTCTCCGAGCCGGTCG-3'
FES	5'-CATCTCTTCTCCGAGCCGGTCGAAATAGTTGGTCGCTGGGGGGCTGACC-3'
SS	5'-Cy3-CCACCATCACCAACTAT(A)rGGAAGAGATGTTTGGTGG-BHQ2-3'

The formation of hydrazone chemistry assisted DNAzyme and its catalyzed capability

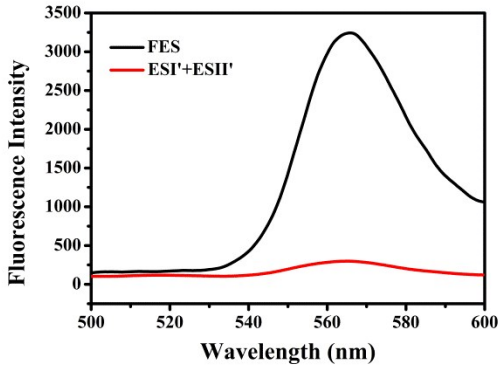

Figure S1 Fluorescence spectra with full-length enzyme strand (FES) and the mixture of the unmodified split enzyme strand I' (ESI') and enzyme strand II' (ESII').

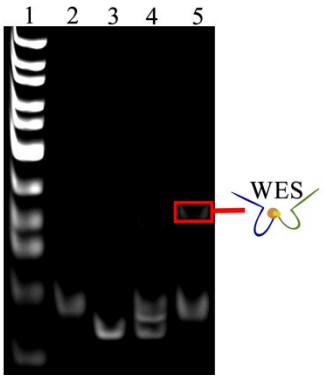

Figure S2 Electrophoretogram for polyacrylamide gel electrophoresis. From left to right: 1: DNA marker; 2: ESII'; 3: ESI'; 4: ESI' + ESII'; 5: ESI + ESII.

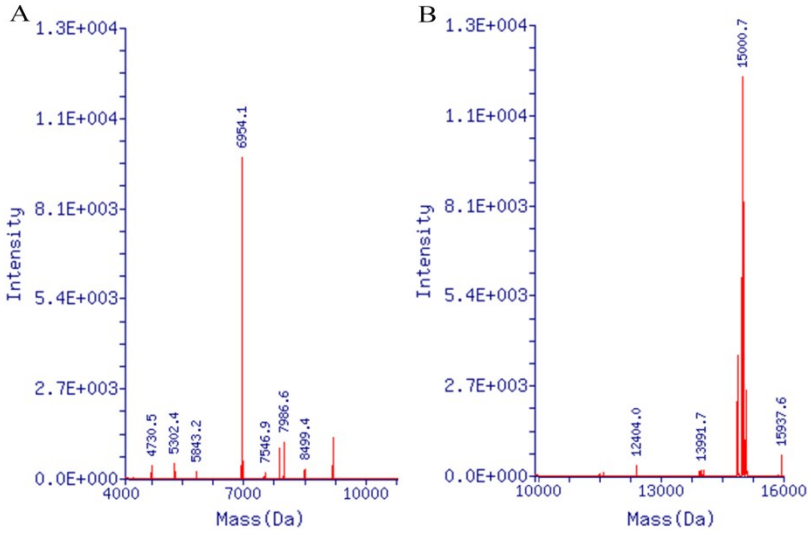

Figure S3 Mass spectra of (A) ESI and (B) WES.

Optimization of experimental conditions

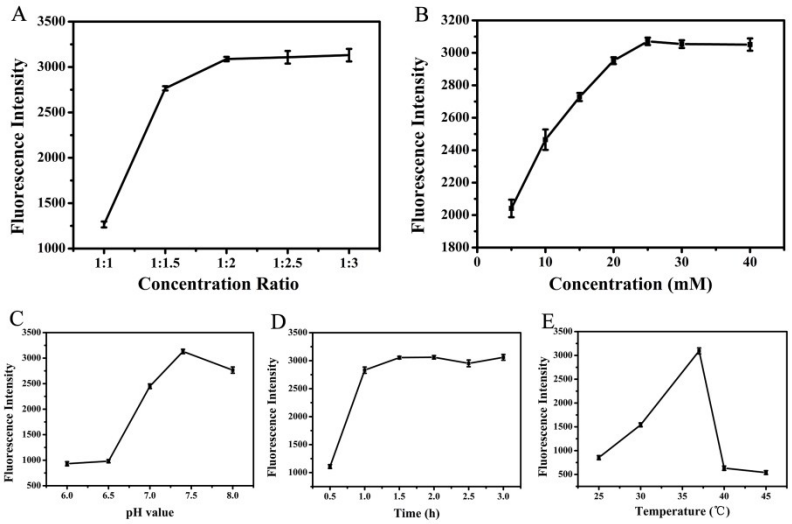

Figure S4 (A) Fluorescence intensity versus the ratio between ESII and ESI. (B) Fluorescence intensity versus Mg^{2+} concentration. (C) Fluorescence intensity versus the pH values of hydrazone reaction between ESII and ESI. (D) Fluorescence intensity versus time of hydrazone reaction between ESII and ESI. (E) Fluorescence intensity versus temperature of hydrazone reaction between ESII and ESI.

In the reaction system, the amount of ESII and ESI will influence the formation of enzyme strand and eventual signal output. As shown in Figure S1 (A), along with the increasing ratio of ESII and ESI, the fluorescence intensity of supernatant gradually increases and reach a plateau. So 1：1.5 has been selected as optimal ratio between ESII and ESI. Moreover, Mg^{2+} concentration, pH value and time have obvious effects on the efficiency of hydrazone reaction. The highest fluorescence intensity of the supernatant can be observed with Mg^{2+} concentration of 20 mM, pH value of 7.4 and reaction time of 1 hour, respectively. Therefore, for the whole experiment, the hydrozine reaction was carried out at pH 7.4 for 1 hour, and the cleavage of the substrate chain was Carried out with Mg^{2+} concentration of 20 mM.

Investigation for versatility of the established method and its application for qualitative analysis of hydrazine

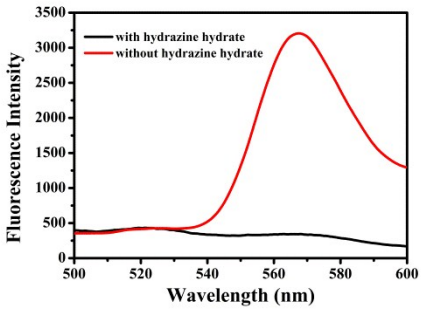

Figure S5 Fluorescence spectra obtained with or without hydrazine hydrate.

In order to investigate the versatility of the established method, hydrazine has been detected by using the established method and the corresponding results have been exhibited in Figure S5. The weak Cy3 fluorescence peak can be observed in the presence of hydrazine hydrate. It can be explained for the reason that reaction between hydrazine hydrate and aldehyde group in the molecular structure of ESII, hinders the linkage of ESII with ESI and the corresponding formation of WES. So, hydrazine hydrate inhibits the cleavage of SS strand so as to prevent the recovery of fluorescence. The result well suggests that our established method can be applied for the analysis of hydrazine hydrate.

The analysis of real sample

Table S2 LPS concentrations detected by our method and the comparison with the given concentrations in soft drink samples.

Sample	LPS detected (ng/mL)	Standard concentration (ng/mL)	Recovery ratio (%)	Relative error (%)
Purified	825.81	800	103.23	2.5
water	77.60	80	97.00	1.4
	0.74	0.8	92.10	3.1
Milk	832.72	800	104.09	3.7
	76.35	80	95.44	1.1
	0.84	0.8	104.87	2.2
Grapefruit	842.57	800	105.32	1.4
juice	78.99	80	98.74	1.0
	0.88	0.8	109.53	1.3
Green	807.84	800	100.98	1.2
tea	72.42	80	90.53	2.2
	0.89	0.8	111.51	1.0

Table S3 HMF concentrations detected by our method and the comparison with the given concentrations in soft drink samples.

Sample	HMF detected (μM)	Standard concentration (μM)	Recovery (%)	Relative error (%)
Purified	0.072	0.07	102.65	1.3
water	0.183	0.2	91.28	4.0
	0.738	0.7	105.42	3.8
Milk	0.071	0.07	101.03	4.9
	0.183	0.2	91.63	3.5
	0.749	0.7	106.95	5.4
Grapefruit	0.069	0.07	99.24	3.6
juice	0.182	0.2	90.80	3.5

	0.774	0.7	110.52	6.2
Green	0.076	0.07	108.18	1.9
tea	0.198	0.2	98.98	2.0
	0.773	0.7	110.47	6.5

References:

1. S. Kowalski, M. Lukasiewicz, A. Duda-Chodak and G. Zięć, *Pol. J. Food Nutr. Sci.*, 2013, **63**, 207-225.
2. T. M. Wassenaar and K. Zimmermann, *Eur. J. Microbiol. Immunol.*, 2018, **8**, 63-69.
3. J. H. van den Berg, S. G. Quaak, J. H. Beijnen, W. E. Hennink, G. Storm, T. N. Schumacher, J. B. Haanen and B. Nuijen, *Int. J. Pharm.*, 2010, **390**, 32-36.