Supporting Information

Non-template synthesized porous carbon nanospheres coated with DNA-cross-linked hydrogel for simultaneous imaging of dual biomarkers in living cells

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Experiment

Reagents and Instruments. 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC,>98%), N-hydroxysuccinimide (NHS,>99%), Tris (hydroxymethyl) aminomethane (Tris-base), 2-Hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (I2959) were purchased from Sigma-Aldrich Co (USA). Sodium chloride (NaCl), 3-aminophenol (3-AP), formaldehyde (HCHO), isopropanol (C_3H_8O), and hydrogen peroxide (H_2O_2) were purchased from Sinopharmm Chemical Reagent Co.,Ltd. Acetone (C_3H_6O) and ammonia were purchased from Yantai Far East Fine Chemical Co., Ltd. (China). 3-Aminopropyltriethoxysilane (APTES) was purchased from Aladdin Biotechnology Co., Ltd. (China). Fluorescein isothiocyanate (FITC) was purchased from Shanghai Macklin Biochemical. Co., Ltd (China). Methylpropenoic acid (MA) were purchased from Shanghai Maclean Biochemical Technology Co., Ltd. RPMI 1640 medium, fetal bovine serum, penicillin/streptomycin were purchased from Thermo Fisher Scientific Co. (USA). Cell Counting Kit-8(CCK-8) was purchased from Shanghai Qihai Futai Biological Technology Co., Ltd. Sequences of the oligonucleotides listed in Table S1 were ordered from Sangon Biotechnology Inc. DL-Dithiothreitol (DTT), N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), adenosine triphosadenine (ATP), cytidine triphosphate (CTP), thymine triphosphate (TTP) and guanosine triphosphate (GTP) were synthesized by Sangon Biotechnology Inc. (Shanghai, China). Human breast cancer cells (MCF-7) and human umbilical vein endothelial cells (HUVEC) were obtained from Jiangsu Keygen Biotech Corp., Ltd (China). All aqueous solution was prepared with ultrapure water produced by Milli-Q.

Transmission electron microscopy (TEM) images were recorded using a scanning electron microsope (H-7650, Hitach, Japan). The UV-vis absorption spectroscopy was recorded by a UV-vis spectrophotometer (UH5300, Hitachi, Japan). The zeta potential were determined by using a laser particle size distribution analyzer (NanoZS, Malvern, United Kingdom). Fluorescence spectra were measured by a fluorescence Spectrophotometer (F-4500, Hitachi, Japan). Confocal fluorescence imaging was performed on a confocal laser scanning microscope (TCS SP5, Leica, Germany). The pH was measured with the type of pHS-3C meter from Shanghai instrument scientific instruments company. Milli-Q machine (USA) was used to get ultrapure water (resistance >18.2 MΩ· cm). CCK-8 assay was recorded by a microplate reader (Bio-tek ELx800, Bio Tek, USA).

Table S1. The sequences of the nucleic acid.

Name	Sequence（5′-3′）
DNA 1	TTTTTTTTTTT, amino at the 5′ terminus, sulfydryl at the 3′ terminus
DNA 2	TTTTCCTTCCTCC, amino at the 5′terminus, black hole quencher 3 (BHQ 3) at the 3′ terminus
DNA 3	CACCTGGGGGAGTATTGCGGAGGAAGG
DNA 4	AATACTCCCCTTTT, Cyanine 5 (Cy5) at the 5′terminus, amino at the 3′ terminus

Synthesis of Porous carbon nanosphere (PCNs). PCNs were synthesized according to previous report[1]. Typically, the resin precursor was prepared as follows : 3-AP (0.1 g, 0.907 mmol), HCHO (0.1 mL, 1.331 mmol), 20 mL of NH_4OH as a catalyst and 10 mL of ethanol were added and reacted at room temperature for 20 min. 20 mL of acetone was added to selectively remove the inside of the solid heterogeneous nanospheres, and then the resin was collected, centrifuged and purified by using distilled water, and carbonized at 800 ℃ for 5 h in N_2 atmosphere to obtain the PCNs.

Synthesis of Oxidized porous carbon nanosphere (O-PCN)[2] and Aminated porous carbon nanosphere (N-PCN)[3]. The PCN (10 mg) was added to 5 mL of H_2O_2 (30%), and after ultrasonication for 4 h, O-PCN was obtained by centrifugal washing. The O-PCN was dispersed in isopropanol and added APTES after sonication. After refluxing at 85 ℃ for 24 h, the N-PCN was obtained by centrifugal washing.

Construction of PCNs-SPDP-DNA 1 complex (DP). 10 μL SPDP (3.2 mM) and 200 μL of water were added to 0.05 mg of N-PCN. After ultrasonic dispersion, they were incubated at 25 ℃. After 2 h, 10 μL of DTT (10 mM) was added thereto, and the mixture was incubated at 25 ℃ for 1 h. The incubation product was centrifuged (10000 r/min, 5 min) and the supernatant was removed, and washed with deionized water twice. And then, add 100 μL of FITC (0.01 mM) to sonicate. Then added 16 μL thiol and amino-modified DNA 1 (10^{-5} M) and incubate at 25 ℃ for 6 h to obtain PCNs-SPDP-DNA 1 complex (DP).

Preparation of PMA-DNA 2 and PMA-DNA 4. 100 μL of EDC (0.1 M) and 50 μL of NHS (0.1 M) were added to 1.0 mL of MA (11.7 mM) and incubated at 25 ℃ for 2 h. The activated MA was then mixed with DNA2 and DNA 4, shaken for 3 minutes, and then placed at 4 ℃ for 12 h. MA-DNA 2 and MA-DNA4 complex were obtained. At room temperature, 50 μL MA-DNA 2, 4 μL of the initiator I2959 (1%), 20 μL of buffer (100 mM Tris-HCl, 500 mM NaCl), and 119.85 μL of water were mixed together. In a similar way, 50 μL MA-DNA 4, 10 μL of the initiator I2959 (1%), 100 μL of buffer (100 mM Tris-HCl, 500 mM NaCl), and 346.5 μL of water were mixed. The two solutions were irradiated with UV light for 30 min, respectively.

Synthesis of DNA-cross-linked hydrogel coated PCNs (DHP) probe. At room temperature, 100 μL of PMA-DNA 2 solution, 19.58 μL of PMA-DNA 4 solution, and 20 μL of ATP (10^{-5} M) DNA 3 were mixed together and kept in a water bath at 95℃ for 5 min. Then placed in an ice water bath to obtain DHP probe.

Cell culture. The MCF-7 cells, HUVEC cells were cultured in a 1640 medium at 37 °C under 5% CO_2, completed with 1% penicillin-streptomycin and 10% FBS. The medium was replaced every day, and the cells were digested with trypsin before further used.

CCK-8 Assay. The CCK-8 assay was adopted to study the cytotoxicity of the N-PCN and probe. Briefly, MCF-7 cells (8×10^3 cells/well) were dispersed within replicate 96-well microtiter plates to a total volume of 200 μL well-1 and were incubated at 37 °C in a 5% CO_2 incubator for 24 h. Then th original medium was removed, and the MCF-7 cells were incubated with fresh medium containing nanoprobe and N-PCN (20 μg/mL) for 1, 2, 3, 4, 5 and 6 h. Ultimately, the cells were washed with 1 × PBS for three times and 10 μL CCK-8 solutions were added into each well. After incubation for 1−2 h, the absorbance was measured at 450 nm with a bioTek microplate reader.

Cells uptake and imaging. The cellular uptake behavior of PCNs nanoprobe in MCF-7 cells were investigated by the confocal laser scanning microscopy (CLSM). A density of 1.0×10^5 MCF-7 cells per well were seeded into 16-well plate and incubated with RPMI 1640 medium in an atmosphere containing 5% CO_2 at 37 °C for 24 h. The mixture washed three times after incubation for different time. The FITC and Cy5 signal were recorded by fluorescence microscopy at excitation of 488 nm and 633 nm, the emitted light was collected.

Results and Discussion

The loading amount of FITC (W_F) in the DHP. In order to evaluate the loading amount of FTIC in DHP, the fluorescence responses to different concentrations of FITC was measured. The loading amount of FITC was determined by the Fluorescence spectroscope at 520 nm (Fig. S1A). The fluorescence intensity increased linearly with FITC concentration over the range from 0 μM to 100 μM, with a correlation coefficient R^2 of 0.9920. The linear regression equation is Y = 27.57X + 435.76 (Y is the relative fluorescence intensity; X represents the concentration of FITC, M; n = 3). We use 0.1 mg PCNs to load the FITC. The fluorescence intensity of FITC before loading is 2989, the amount of FITC (W_b) is 92.6 μM. The fluorescence intensity of FITC after loading is 860, the amount of FITC (W_a) is 15.4 μM. (Fig. S1B) According to the equation: $W_F = (W_b - W_a)/ 0.1$, the loading amount of FITC is 10.07 μg/mg.

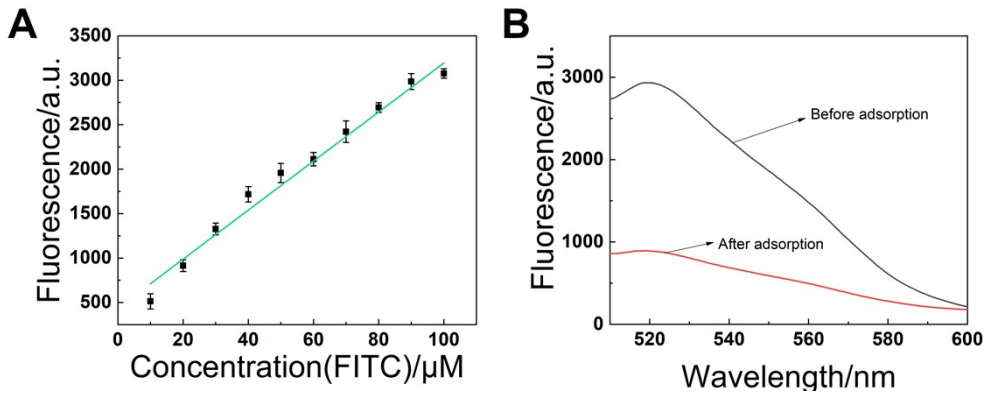

Fig. S1 Fluorescence intensities of FITC before adsorption (curve a) and after adsorption (curve b) with DHP.

The detection feasibility of strategy. The feasibility of the developed strategy for targets substance is validated. To confirm the obtained fluorescence spectra in the present nanoprobe was depend on amount of ATP and GSH, control experiments were done. The results were shown in Fig. S2. The fluorescence signals variation of Cy5 and FITC were respectively monitored before

and after addition of the targets ATP and GSH. The fluorescence intensity of Cy5 increased around 7.98-fold in the presence of 0.3 μM ATP, similarly, the fluorescence intensity of FITC increased around 3.49-fold in the presence of 0.5 μM GSH, which proved the detection strategy is feasible.

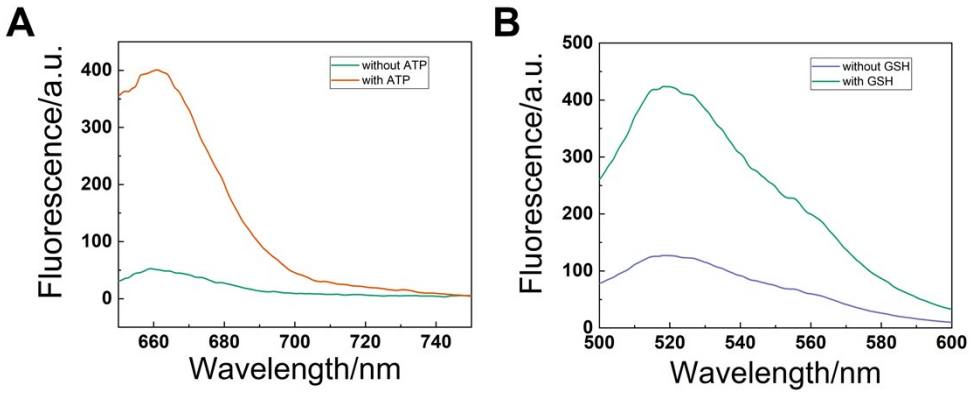

Fig. S2 Fluorescence intensities of DHP in the presence of 0.3 μ M ATP, 0.5 μM GSH and blank.

The optimization of condition. To obtain the best performance of fluorescent intensity assay, confirming pH is necessary. As shown in the Fig. S3A, the fluorescent intensity was influenced by pH. When the pH of 6.5 and 7.4 were reaction condition, the fluorescent intensity had obvious performance. What' s more, because the pH of 6.5 is important characteristic in cancer cells, the nanoprobe is suitable for sensing target ATP and GSH in cancer cells. In addition, Fig. S3B showed that the intensities of FITC decreased rapidly upon the loading time of FITC from 1 h to 8 h, and then when the time exceeded 6 h, the fluorescent intensity tended to constant. Thus, the loading time of FITC of 6 h was used all through the assays. What is more, Fig. S3C showed that the intensities of Cy5 decreased rapidly upon the loading time of Cy5 from 0 min to 80 min, and then when the time exceeded 30 min, the fluorescent intensity tended to constant. Thus, the construction time of DHP of 30 min was used all through the assays. Besides, Fig. S3D showed

that the fluorescent intensity rapidly increased upon the reaction temperature from 37 ℃ to 45 ℃, and then when the reaction temperature is 37 ℃, the fluorescent intensity is constant. Thus, the reaction temperature of 37 ℃ was used all through the assays. Furthermore, Fig. S3E showed that the fluorescent intensity increased rapidly upon the ATP treated time from 0 min to 250 min, and then when the ATP treated time exceeded 120 min, the fluorescent ratio tended to constant. Thus, the ATP treated time of 120 min was used all through the assays. Similarly, Fig. S3E showed that the fluorescent intensity increased rapidly upon the GSH treated time from 0 min to 240 min, and then when the GSH treated time exceeded 160 min, the fluorescent ratio tended to constant. Thus, the GSH treated time of 160 min was used all through the assays.

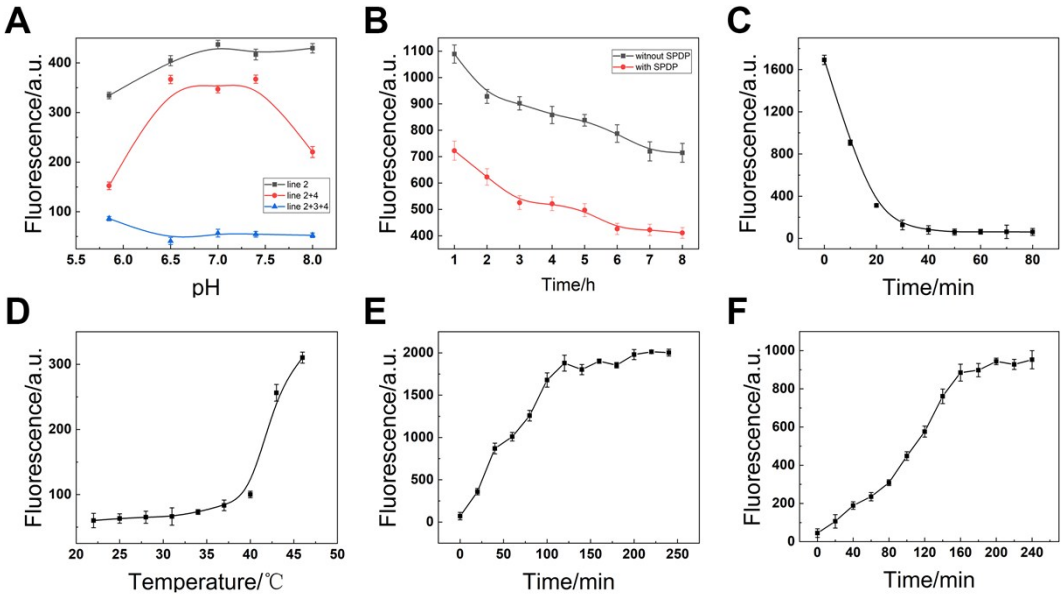

Fig. S3 The optimization of reaction condition. (A) The optimization of pH in presence of targets. (B) The optimization of FITC loading time (C) The optimization of construction time of DHP. (D) The optimization of reaction temperature in presence of targets. (E) The optimization of ATP treated time. (F) The optimization of GSH treated time.

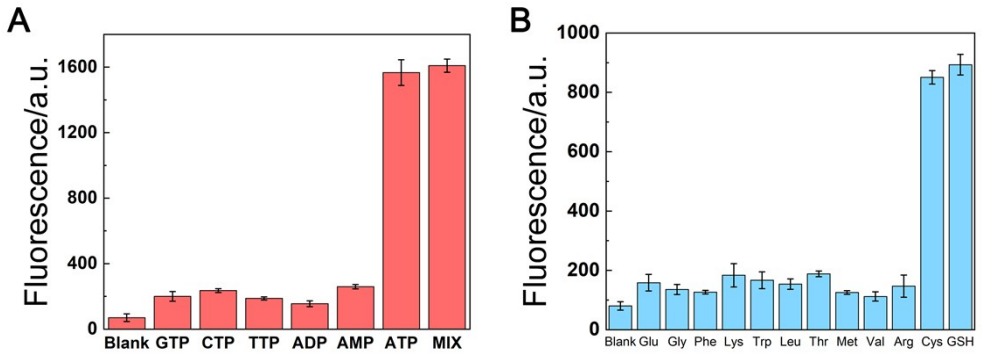

Fig. S4 (A) The intensity of fluorescence to diverse bioanalytes in PBS. (The concentration of ATP was 1.0 μM, the concentration of non-targets were 1.0 mM). (B) The intensity of fluorescence to diverse bioanalytes in PBS. (The concentration of GSH was 1.0 μM, the concentration of non-targets were 1.0 mM).

The stability studies of DHP probe. In order to confirm the stability of DHP nanoprobe, DHP stored from 1 day to 7 day were evaluated by fluorescence spectroscopy assay. 400 nM and 1.0 μM ATP and DHP were incubated at 37 ℃ for 2 h, the fluorescent intensities are constant at 392 and 1598. Furthermore, 100 nM and 800 nM GSH and DHP were incubated at 37 ℃ for 2.5 h, the fluorescent intensities are constant at 134 and 791 (Fig. S5). These results indicate that the nanoprobe has high stability and easy to store.

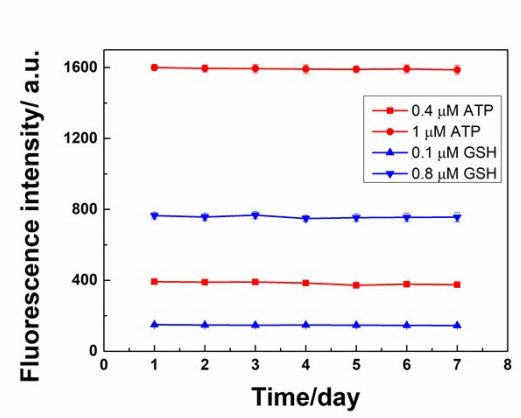

Fig. S5 The stability of DHP nanoprobe.

Table S2 Comparisons of the proposed approach with other probes for the detection of ATP.

NO.	Materials	Method	Detection range (nmol/L)	LOD (nmol/L)	Ref.
1	CuNCs-Al^{3+}/ZIF-90	fluorescence	1.0×10^3-2.0×10^6	670	4
2	Thioflavin-T-sulphated-b-cyclodextrin supramolecular	fluorescence	2.0×10^3-1.5×10^5	1.3×10^3	5
3	Concatenated DNA Circuit	fluorescence	1.0×10^3-2.0×10^6	170	6
4	polymer probes	fluorescence	1.0×10^4-2.0×10^6	1.0×10^4	7
5	This work	fluorescence	10-1.5×10^3	5.73	

Table S3 Comparisons of the proposed approach with other probes for the detection of GSH.

NO.	Materials	Method	Detection range (μmol/L)	LOD (μmol/L)	Ref.
1	G-triplexes	fluorescence	0.1-20	0.05	8
2	BSA@AuNCs-MnO_2	fluorescence	2-200	1.0	9
3	TCF-imidazo[1,5-α]pyridine	fluorescence	2-30	0.097	10
4	Gold nanoclusters	fluorescence	19-480	4.7	11
5	This work	fluorescence	0.05-1.0	0.0182	

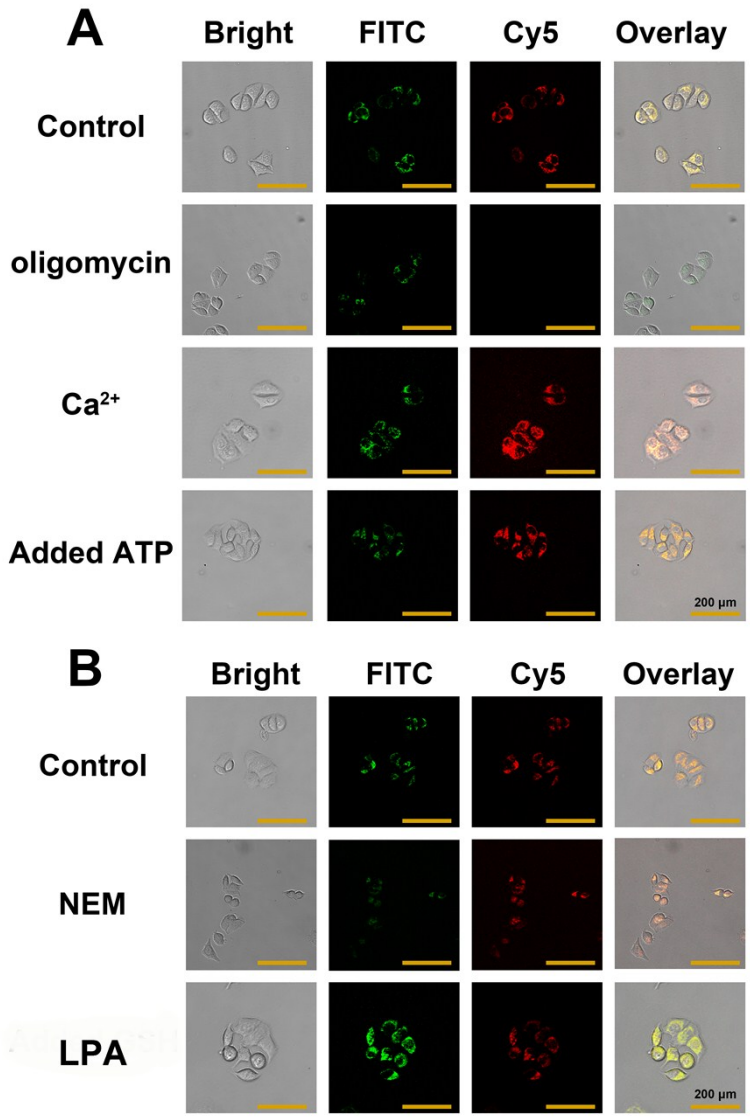

Fig. S6 Confocal microscopy (CLSM) images of MCF-7 cells treated with 0.1 M PBS, 10 μM oligomycin, 5 mM Ca^{2+}, 0.1 μM commercial ATP, 0.5 mM NMM and 0.5 mM LPA followed by incubation with 20 μg/mL DHP for 3 h at 37 °C. Scale bars: 200 μm.

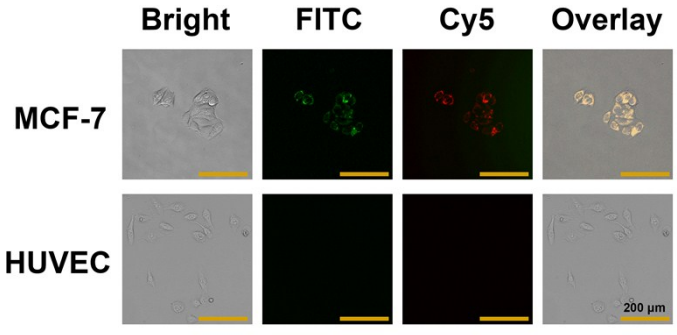

Fig. S7 CLSM images of MCF-7 cells incubation with20 μg/mL DHP for 2 h at 37 °C. Scale bars: 200 μm.

References

1. D. S. Bin, Z. X. Chi, Y. Li, K. Zhang, X. Yang, Y. G. Sun, J. Y. Piao, A. M. Cao and L. J. Wan, *J Am Chem Soc*, 2017, **139**, 13492-13498.
2. H. Wang, X. Li, Z. Ma, D. Wang, L. Wang, J. Zhan, L. She and F. Yang, *Int J Nanomedicine*, 2016, **11**, 1793-1806.
3. D. Li, W. Y. Teoh, J. J. Gooding, C. Selomulya and R. Amal, *Advanced Functional Materials*, 2010, **20**, 1767-1777.
4. X. Gao, J. Liu, X. Zhuang, C. Tian, F. Luan, H. Liu and Y. Xiong, *Sensors and Actuators B: Chemical*, 2020, **308**.
5. V. R. Singh and P. K. Singh, *J Mater Chem B*, 2020, **8**, 1182-1190.
6. Y. Zhou, L. Yang, J. Wei, K. Ma, X. Gong, J. Shang, S. Yu and F. Wang, *Analytical Chemistry*, 2019, **91**, 15229-15234.
7. J. Qiao, C. Chen, D. Shangguan, X. Mu, S. Wang, L. Jiang and L. Qi, *Analytical Chemistry*, 2018, **90**, 12553-12558.
8. R. M. Kong, L. Ma, X. Han, C. Ma, F. Qu and L. Xia, *Spectrochim Acta A Mol Biomol Spectrosc*, 2020, **228**, 117855.
9. Y. Du, H. Liu, J. Liang, D. Zheng, J. Li, S. Lan, M. Wu, A. Zheng and X. Liu, *Talanta*, 2020, **209**, 120524.
10. P. Hou, J. Sun, H. Wang, L. Liu, L. Zou and S. Chen, *Sensors and Actuators B: Chemical*, 2020, **304**, 127244.
11. X. Xie, Z. Peng, X. Hua, Z. Wang, K. Deng, X. Yang and H. Huang, *Biosensors and Bioelectronics*, 2020, **148**, 111829.