Electronic Supporting Information (ESI)

DNA Tetrahedron-Based Molecular Beacon for Tumor-Related mRNA Detection in Living Cells †

Nuli Xie, Jin Huang*, Xiaohai Yang, Yanjing Yang, Ke Quan, He Wang, Le Ying, Min Ou, Kemin Wang*

EXPERIMENTAL SECTION

Chemicals and Materials

All DNA oligonucleotides were synthesized and HPLC purified by Sangon Biotechnology Co., Ltd (Shanghai, China). The sequences of these oligonucleotides are shown in Table S1. β-Estradiol and Tamoxifen were from Sigma Aldrich Chemical Co. Ltd (St. Louis, MO). All aqueous solutions used in the experiments were prepared using ultrapure water(≥18MΩ, Milli-Q water purification system, Millipore). Cell medium RPMI 1640 was obtained from GIBICO (USA). All other reagents were of analytical pure. Human hepatocellular liver carcinoma cell line HepG2 and human hepatocyte cell line HL-7702 was obtained from our lab.

Instrumentation

All fluorescent spectra were measured using F-7000 fluorescence spectrometer (Hitachi, Japan). The AFM characterization of DNA tetrahedron nanostructure was analyzed by Bruker Multimode V8 Scanning Probe Microscopy (USA). Cells was incubated by using a Thermo FORMA 3111 CO_2 incubator (ThermoFisher, USA). The confocal fluorescence imaging studies was performed using an Olympus IX-70 inverted microscope with an Olympus FV 500 confocal scanning system (Japan) with an objective lens (100×). The Flow cytometry analysis was gained from Gallios machine (Beckman Coulter, USA).

Self-assembly and characterization of DNA tetrahedron-base molecular beacon (DTMB)

DTMB were self-assembled according to a well-understood protocol.[1] Four customized oligonucleotide strands (P1, P2, P3 and P4) were respectively diluted with

TM buffer (20 mM Tris, 50 mM $MgCl_2$, pH=8.0) to stock solutions which have a final concentration of 10 μM. The four strands were then mixed in equimolar in TM buffer, heated to 95℃ for 5 minutes and then immediately cooled on ice in 1 minutes, finally stored at 4℃ for at least 4h.

The characterization of synthesized DTMB was performed by Native-PAGE and AFM. PAGE gel analysis of the nanostructure was conducted using a 12.5% non-denaturing polyacrylamide gel in 1×TBE (Tris borate-EDTA) buffer, respectively. After running at 200 V for 10 min, the gels were run at a constant voltage of 80 V for 3h. For the AFM analysis, 20 μL of DTMB sample was deposited onto freshly cleaved mica for 5 min, washed with 30 μL of water for more than 10 times and then dried with compressed air. A MultiMode V8 AFM (Bruker) was used to image the samples under ScanAsyst-Air mode, using a ScanAsyst-Air probe (Bruker).

Fluorescence experiments in vitro

The ability for DTMB to detect TK1 mRNA targets was determined using F-7000 fluorescence spectrometer. FAM fluorescence emission signal was recorded from 510 to 650 nm in 2nm increment, under an excitation wavelength of 488 nm. The concentration of DTMB used for the fluorescence calibration curve assays was 50 nM in 1×PBS (pH=7.4), and treated with target of a series of concentrations (0, 2, 5, 10, 15, 20, 30, 50, 100, 150nM). The fluorescence signal without target was recorded as the background signal. All experiments were repeated at least three times. In single-base mismatch recognition and thermodynamic studies, DTMB of 50 nM and 25 nM were employed respectively. The single-base mismatch recognition study was performed at 37℃. In thermodynamic studies, fluorescence intensity was monitored at temperatures varied from 20 to 60℃, with 5 min incubation before each measurement.

Cell culture

HepG2 cells (Human hepatocellular liver carcinoma cell line), and HL-7702 cells (human hepatocyte cell line) were grown in RPMI 1640 medium supplemented with 10% inactivated fetal bovine serum, 100 U/ml 1% penicillin and streptomycin solution. All cells were cultured in a humidified CO_2 incubator containing 5% CO2 at

37°C. For confocal imaging studies, HepG2 and HL-7702 cells were cultured on 35-mm confocal laser culture dishes with the same medium for 24 h.

Growth inhibition assay (MTT)

To investigate the cytotoxicity of DTMB, MTT assay was carried out when the probes existed. HepG-2 cells were dispersed with replicate 96-well microtiter plates at a density of 1×10^6 cells/well. Plates were then maintained at 37°C in 5% CO2 atmosphere for 24 h. Thereafter, the cells were treated with varying concentrations of molecular beacon (0, 50, 100nM) for 24 h and 100 μL MTT solutions were then added to each well for 4 h. After removing the remaining MTT solution, 150 μL DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at 490 nm with a RT 6000 microplate reader.

Confocal Fluorescence Imaging

In comparative experiment of cancer cells and normal cells, we chose HL-7702 as the negative control of HepG-2 cells. All cells were plated on 35-mm confocal laser culture dishes for 24 h. Then the DTMB (the final concentration is 100 nM) was respectively delivered into TK1 mRNA overexpressed HepG2 cancer cells and HL-7702 normal cells in culture medium at 37 °C for 4 h. The cells were examined by confocal laser scanning microscopy (CLSM). The fluorescence emission was collected and imaged with oil immersion objective 100X.

In the experiments for expression levels of TK1 mRNA, one group of HepG2 cells was treated with tamoxifen (10^{-6} mol/L) for 24 h and the other group of HepG2 cells was treated with β-Estradiol (10^{-8}mol/L) for 24 h. One group of HepG2 cells without regulation was served as control. Other steps performed as same as described above.

For the comparative experiment of cellular uptake ability of general MB and DTMB, all of the cells were incubated with 100 nM probes for 2h. After the incubation, the cells were washed three times with PBS (pH 7.4) before imaging.

Nuclease stability

For nuclease stability experiment, we incubated exonuclease III (the final concentration is 1 U/ml) with DTMB and MB. The fluorescence of these samples was

monitored and was collected at 5 min intervals during this time period. Then the fluorescence was measured at appropriate excitation wavelengths.

Flow Cytometry

Samples (5×10^5 cells/mL) were incubated with fluorescently labeled DTMB on dishes for 4 hour at 37 ℃. After treatment, cells were detached from culture dishes using Trypsin-EDTA Solution. The solution containing treated cells was centrifuged (2000 rpm, 4 min) and resuspended in PBS three times. Flow cytometry was performed using Beckman Coulter Gallios machine.

qRT-PCR

Total cellular RNA was extracted from HepG2 cells or L02 cells using Trizol reagent S5 (Sangon Co. Ltd., Shanghai, China) according to the indicated protocol. The cDNA samples were prepared by using the reverse transcription (RT) reaction with AMV First Strand cDNA Synthesis Kit (BBI, Toronto, Canada). qRT-PCR analysis of mRNA was performed with SG Fast qPCR Master Mix (2X) (BBI) on a LightCycler480 Software Setup (Roche). Below is the primers (from 5' to 3 ') used in this experiment. We evaluated all the data with respect to the mRNA expression by normalizing to the expression of GAPDH and using the $2^{-\Delta\Delta Ct}$ method.

TK1 forward: CTCCTACCCACTGGTCTGCTTA

TK1 reverse: CAGGGAGAACAGAAACTCAGCA

GAPDH forward: TGGGTGTGAACCATGAGAAGT

GAPDH reverse: TGAGTCCTTCCACGATACCAA

References

1. R. P. Goodman, A. T. Schaap, C. F. Tardin, C. M. Erben, R. M. Berry, C. F. Schmidt, and A. J. Turberfield , *SCIENCE*, 2006, **310**, 1661

Supporting tables

Table S1. Sequences of Oligonucleotides Used in This Work.

Oligo	Sequence(5'—3')
P1	AGG CAG TTG AGA CGA ACA TTC CTA AGT CTG AAA TTT ATC ACC CGC CAT AGT AGA CGT ATC ACC
P2	CTT GCT ACA CGA TTC AGA CTT AGG AAT GTT CGA CAT GCG AGG GTC CAA TAC CGA CGA TTA CAG
P3	GGT GAT AAA ACG TGT AGC AAG CTG TAA TCG ACT CTA GCG AGT GTC TTT GGC ATA CTT CTC GCG GCT CAC TAC TAT GGC G
P4	FAM-TAG AGA CGG TAT TGG ACC CTC GCA TGA CTC AAC TGC CTG GTG ATA CGA GAG CC-Dabcyl
perfectly matched target	AAG TAT GCC AAA GAC ACT CGC
single-base mismatch target	AAG TAT TCC AAA GAC ACT CGC
molecular beacon	FAM-A GCG AGT GTC TTT GGC ATA CTT CTC GCT-Dabcyl

Supporting Figures

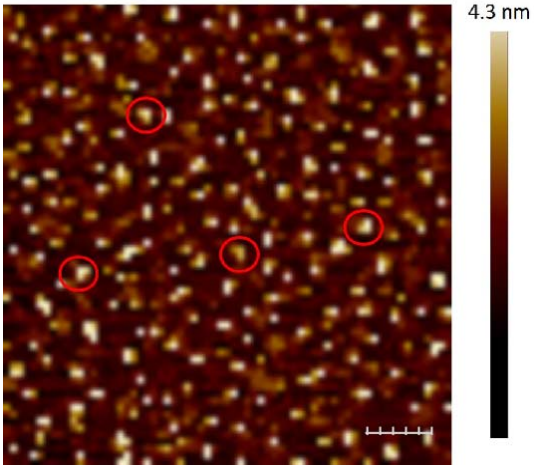

Figure S1. Characterization of DTMB with atomic force microscopy (AFM), Scale bars are 50 nm.

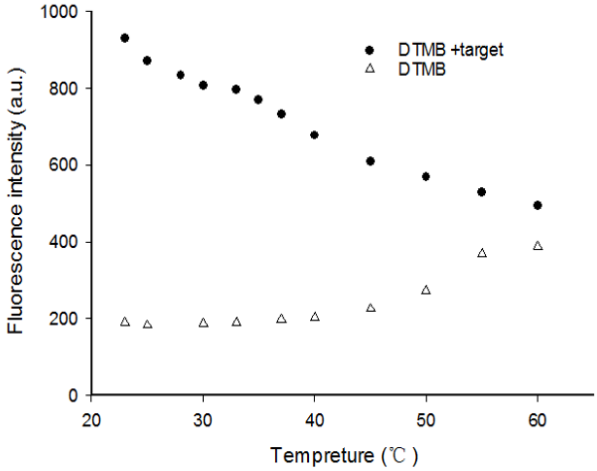

Figure S2. Thermodynamic studies (22℃-60℃) of DTMB with or without target DNA.

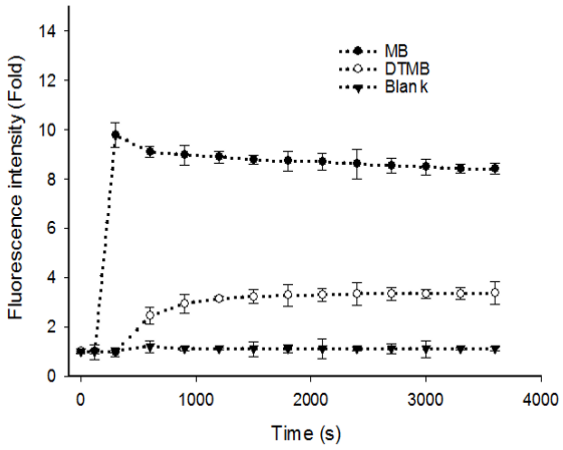

Figure S3. Studies of nuclease (Exo Ⅲ) digestion resistant ability between DTMB and MB.

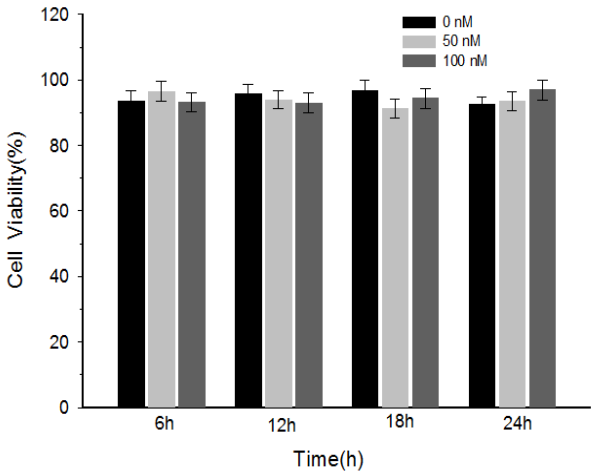

Figure S4. MTT assay: HepG2 cells were incubated with different concentrations (0, 50 and 100 nM) of the DTMB for 6 h, 12 h, 18 h and 24 h.

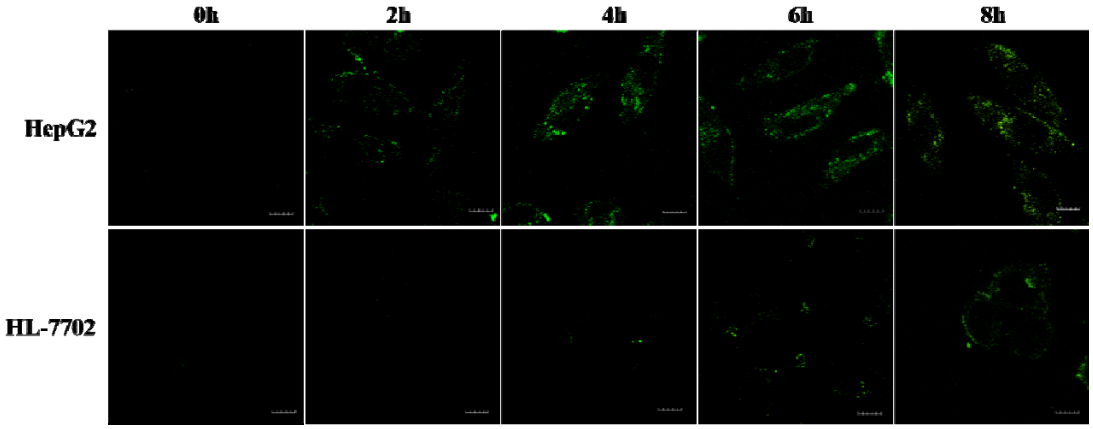

Figure S5. Optimization of incubation time for DTMB with living cells. HL-7702 and HepG2 cells were incubated with 100nM probes for different time point at 37℃ for confocal microscopy. Scale bar = 10 μm.

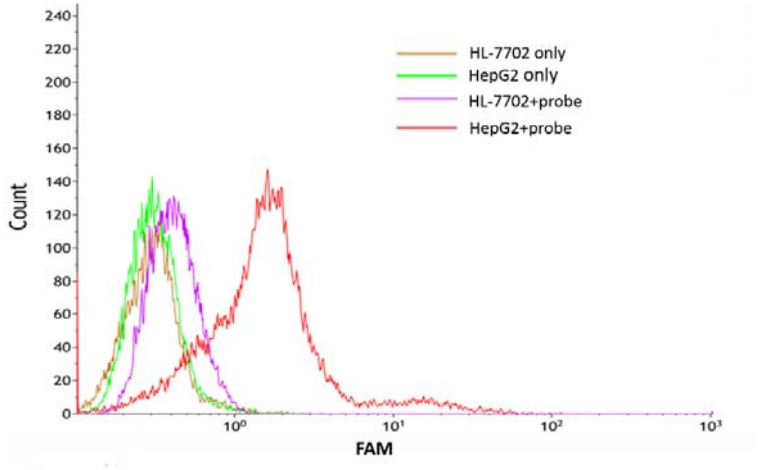

Figure S6. Flow cytometry analysis of the DTMB incubated with HepG2 cells and HL-7702 cells, respectively.

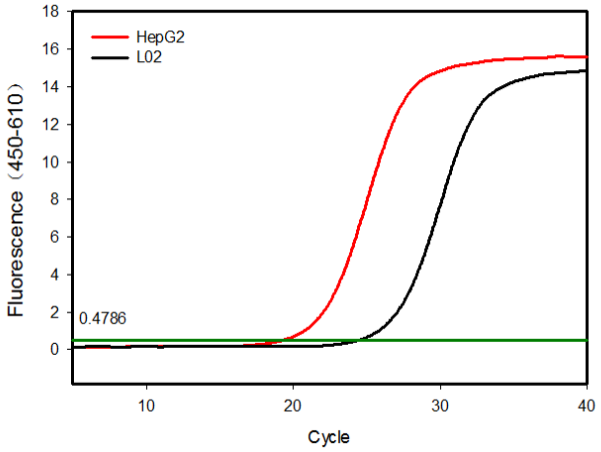

Figure S7. Analysis of Tk1 mRNA expressions in HepG2 and HL-7702 cells by qRT-PCR.

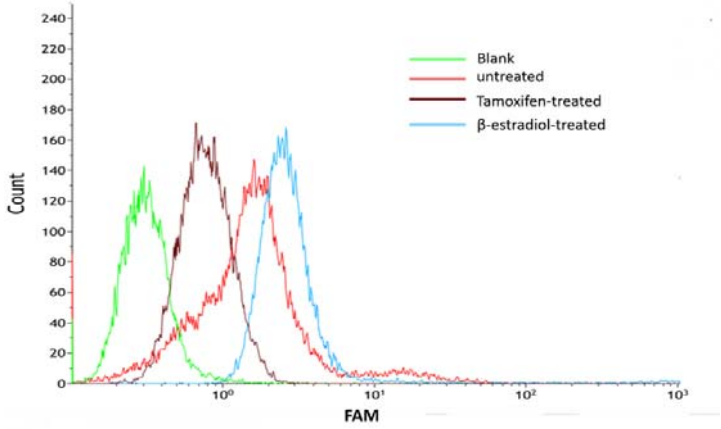

Figure S8. Flow cytometry analysis of the DTMB incubated with different groups of HepG2 cells (untreated, tamoxifen-treated and β-estradiol-treated groups).

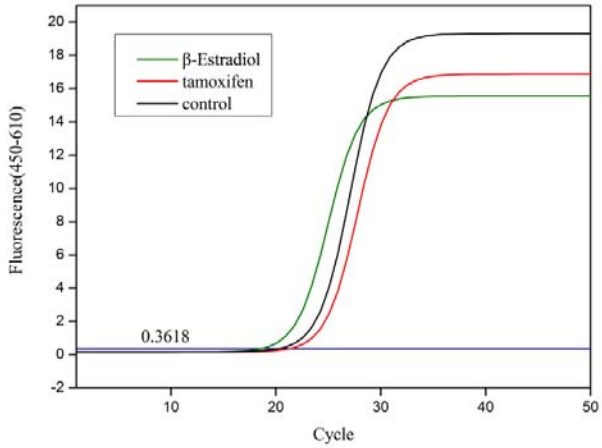

Figure S9. Relative expression levels for Tk1 mRNA in HepG2 cells treated with β-estradiol for up-regulation and tamoxifen for down-regulation by qRT-PCR.