Letter

Simultaneous Imaging of Three Tumor-related mRNAs in Living Cells with a DNA Tetrahedron-based Multicolor Nanoprobe

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Simultaneous Imaging of Three Tumor-related mRNAs in Living Cells with a DNA Tetrahedron-based Multicolor Nanoprobe

Song Wang †, ‡, &, Mengchan Xia†, &, Jie Liu†, Sichun Zhang*, † and Xinrong Zhang †

† Beijing Key Laboratory for Microanalytical Methods and Instrumentation Department of Chemistry, Tsinghua University, Beijing 100084 , P. R. China

‡ National Insititute of metrology, Beijing 100029, P. R. China

Supporting Information Placeholder

ABSTRACT: We constructed a DNA tetrahedron based multicolor nanoprobe, which could simultaneously imaging of three tumor-related mRNAs in living cells through fluorescence restoration caused by competitive chain replacement reaction. The oligonucleotides used to construct the tetrahedron were extended by adding three 21-base recognition sequences modified with different fluorophores (FAM, Cy3 and Cy5) in the 5' end. Three 11-base complimentary sequences modified with quencher (BHQ1 for FAM and BHQ2 for Cy3 and Cy5) were hybridized with the recognition sequences to quench the fluorescence. In the presence of the specific mRNA targets, the recognition sequences hybridized with the targets to form longer duplexes and the fluorescence was restored. Compared with previously reported nanoprobes based on DNA tetrahedron, the multicolor nanoprobe can effectively avoid false positive results.

KEYWORDS: biosensor, nanotechnology, mRNA detection, DNA nanostructures, live-cell imaging

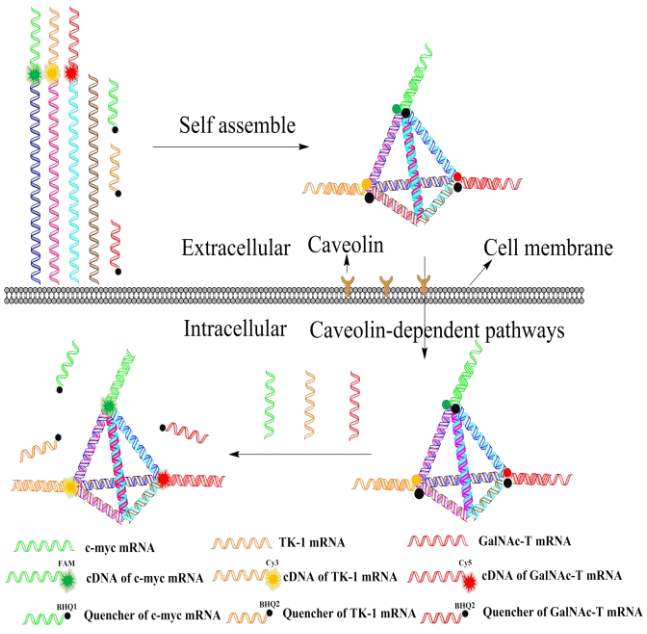
Tumor-related mRNAs are important genetic cancer biomarkers whose expression level correlated with tumor burden and malignant progression.[1] A variety of tumor-related mRNAs are simultaneously expressed in cancer cells, and some of them are also expressed in normal cells just in different amount.[2] Simultaneous visualization of multiple tumor-related mRNAs with multicolor cell imaging methods will promote more accurate research on the gene expression at single cell level, which holds great promise for cancer diagnosis, prognosis and therapy.

Fluorescent probes for mRNAs are mainly hybridization DNA probes such as competitive hybridization probes and molecular beacon probes.[3] These "off-on" probes are easy to prepare and can be used to monitor the change of mRNA level with high sensitivity and selectivity.[4] However, several drawbacks restrict their application in multicolor fluorescence imaging, including the poor transmembrane ability caused by its poly-anion nature,[5] the easily degradation in complex biological medium,[6] and the difficult multiplex detection. In order to solve these problems, several attempts have been made, such as microinjection of several probes simultaneously,[7] transfection with liposomes,[8] and combination with nanomaterials.[9] Unfortunately, these methods have their own limitations. For instance, microinjection is invasive and low throughput. The ratio of modified probes on nanomaterials is difficult to control precisely. Also, most of these nanomaterials suffer from poor dispersion and metabolism ability. New multicolor nanoprobes for intracellular mRNA detection are desired urgently.

The development of DNA nanotechnology based on Watson-Click base pairing rule brings new opportunities for intracellular mRNA imaging.[10] DNA tetrahedron, which is made up of four DNA oligonucleotides, is one of the most outstanding DNA nanostructures.[11] Benefited from its great biocompatibility,[12] high enzyme resistance,[13] good transmembrane ability[14] and easily multiple modification properties, DNA tetrahedron has been applied in intracellular delivery[15] and sensing.[16] Tay *et al* developed a nature-inspired DNA nanosensor for intracellular mRNA detection by directly conjugating a molecular beacon (MB) to a vertex of DNA tetrahedron.[17] Xie *et al* developed a DNA tetrahedron-based molecular beacon (DTMB) for intracellular mRNA by embedding the MB in one edge of DNA tetrahedron.[18] Recently, Wang *et al* constructed a competition-mediated FRET-switching DNA tetrahedron molecular beacon for mRNA detection.[19] However, most of these studies focused on just one kind of mRNA, which may lead to false positive results. The DNA tetrahedron can be easily modified with different components. Therefore, there is a great opportunity to develop a multicolor nanoprobe based on DNA tetrahedron for multiplex tumor-related mRNAs.

Herein, we constructed a DNA tetrahedron based multicolor nanoprobe, which could simultaneously detect three tumor-related mRNAs (Scheme 1). Three of the oligonucleotides used to construct the tetrahedron were extended by adding three 21-base recognition sequences modified with different fluorophores (FAM, Cy3 and Cy5). The nanoprobe was generated by annealing seven oligonucleotides (Table S1) according to the previous reports.[16a, 17-18] In the absence of the

Scheme 1. Simultaneous detection of three tumor related mRNAs inside cells with a DNA tetrahedron-based nanoprobe.



mRNA targets, three 11-base complimentary sequences modified with quencher (BHQ1 for FAM and BHQ2 for Cy3 and Cy5) were hybridized with the recognition sequences and quenched the fluorescence. After internalization, the recognition sequences hybridized with the targets to form longer duplexes. The 11-base quenching sequences released and the fluorescence restored. Simultaneous visualization of three tumor-related mRNAs could be successfully realized.

The formation of DNA tetrahedron nanoprobe was verified by atomic force microscope (Figure S1) and agarose gel electrophoresis. Figure S2a shows that the migration speed decreased with the increase of DNA strands. Similar phenomenon was observed when we add quenching sequences to the DNA tetrahedron (Figure S2b), indicating the successful synthesis of nanoprobe. The change of fluorescence intensity was also monitored. As shown in Figure S3, the fluorescence intensities of the three dyes decrease significantly when the quenching sequences were added, proving the hybridization between the DNA tetrahedron and the quenching sequences.

In order to demonstrate the feasibility of mRNA sensing with the presented nanoprobe, we studied the fluorescence restoration ability after adding the target DNAs. Figure S4 indicates that fluorescence intensities of FAM, Cy3 and Cy5 increase 3.7-fold, 4.4-fold, and 3.6-fold in the presence of three DNA targets, respectively. Remarkably, our nanoprobe had high sequence specificity. The existence of one DNA target had little impact on the fluorescence signal of others. The fluorescence restoration of one dye in the presence of three targets was comparable to that in the presence of the specific target. These results demonstrated that the nanoprobe can be employed to detect multiple DNA targets simultaneously.

The kinetic studies was conducted. As can be seen in Figure S5, the fluorescence intensities of nanoprobe restored rapidly within 1 min after adding the target. Fluorescence

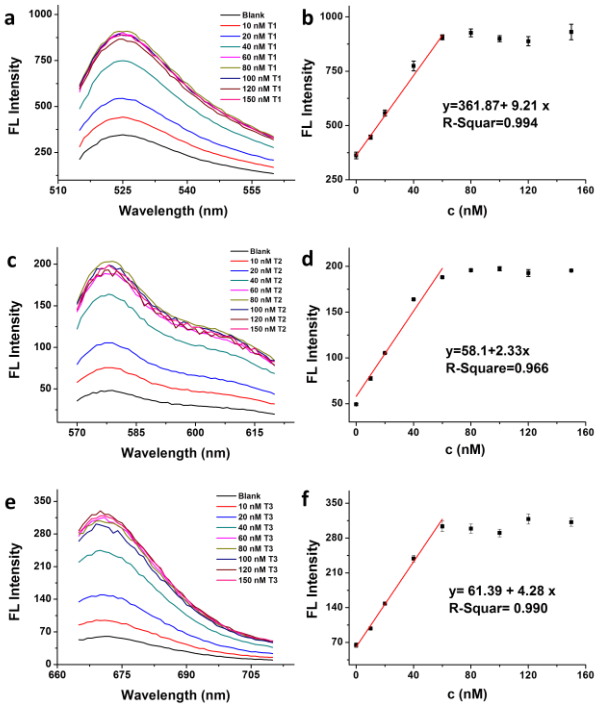


Figure 1. Fluorescence response of DNA tetrahedron-based nanoprobe (100 nM) to varied targets at different concentrations. a, c and e represent the fluorescence spectra after adding different amount of c-myc, TK1 and GalNAc-T targets respectively. b, d and f are the fluorescence intensity to concentration plots of a, c and e, respectively.

intensity changes of the nanoprobe with the increase of the concentration of the DNA targets were also investigated. Figure 1 show that the fluorescence intensity increased linearly with the target concentration varied from 0 nM to 60 nM. The detection limit was calculated 3.1 nM for C-myc mRNA, 1.2 nM for TK1 mRNA, and 3.2 nM for GalNAc-T mRNA.

It has been reported that the formation of tetrahedron structure will significantly improve the enzyme resistance of DNA sequences.[17] In this study, we used a common endonuclease, deoxyribonuclease I (DNase I), to evaluate the nuclease stability. As shown in Figure 2, the fluorescence signals of the nanoprobe treated with DNase I had little increase with time. The fluorescence restoration of the two groups after the addition of DNA targets had no significant difference with each other (Figure 2, insets). In addition, two bands at the same position in agarose gel electrophoresis demonstrated no significant difference between the DNase I treated group and the control group (Figure 2d). These results indicate that the nanoprobe has excellent nuclease stability, which is an important property for live cell studies.

Another important property for live cell studies is the cytotoxicity. In order to study the cytotoxicity of the nanoprobe, we performed the MTT assay in MCF-7 cell line. The cell viability was evaluated by the absorbance of MTT at 490 nm after cells were incubated with nanoprobe for different period of time. Figure S6 indicates low cytotoxicity of the DNA tetrahedron-based nanoprobe in living cells and the potential application of nanoprobe in live-cell imaging.

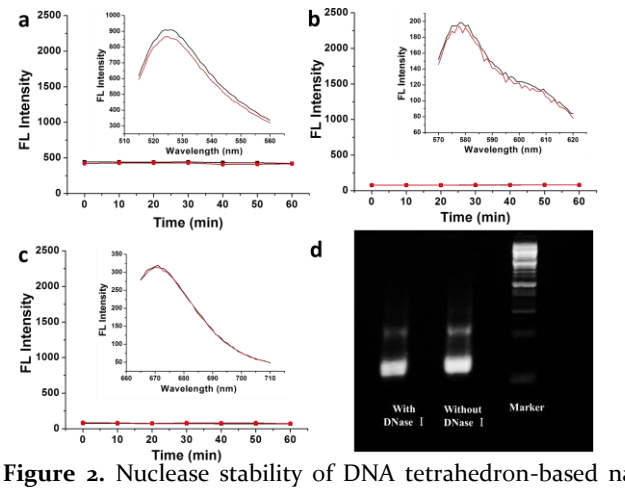


Figure 2. Nuclease stability of DNA tetrahedron-based nanoprobe in the presence or absence of DNase I. Fluorescence change of the nanoprobe without DNase I (black trace) or with DNase I (red trace) during 1 h, measured every 10 min. Insets: fluorescence spectra after adding DNA targets to the nanoprobe treated with DNase I (red curve) and without DNase I (black curve). (a) c-myc target, EX/EM: 488/525, (b) TK1 target: EX/EM: 550/570, (c) GalNAc-T target, EX/EM: 635/670. (d) Agarose gel analysis of the nanoprobe treated with or without DNase 1.

Three mRNA targets C-myc, TK1 and GalNAc-T were chosen to study the application of the nanoprobe in live cells. The C-myc mRNA plays a critical role in breast tumorigenesis and progression.[20] The TK1 mRNA is associated with cell division and proposed to be a marker for tumor growth.[21] The GalNAc-T mRNA is a key factor in the biosynthetic pathway of gangliosides GM2/GD2.[22] All the three mRNAs are overexpressed in MCF-7 cells. Therefore, cancer cell line MCF-7 was selected as the positive cell while normal cell line MCF-10A was used as control in this work. After incubation with nanoprobe, significant difference between two cell lines was observed (Figure 3). Significant green fluorescence for c-myc mRNA, red fluorescence for TK1 mRNA, and purple fluorescence for GalNAc-T mRNA were obseved in MCF-7 cells. However, little fluorescence for the three mRNAs was observed in MCF-10A cells, especially for GalNAc-T mRNA (Figure S7). The merge image of MCF-7 cells is totally different with that of MCF-10A cells, which indicates that the nanoprobe can be used to detect breast cancer.

Cancer cells in different stages of tumor progression express different amount of tumor-related mRNAs. Determination of the relative expression level of tumor-ralated mRNAs is significant for the cancer detection and therpy. Here, we investgated whether the nanoprobe can viualize the different expression level of TK1 mRNA in living cells. Before incubated with the nanoprobe, we modulated the expression level of TK1 mRNA in MCF-7 cells with Tamoxifen and β-estradiol. Tamoxifen was used to inhibit the mRNA expression and β-estradiol was used to upregulate the expression level. After 48 h modulation, MCF-7 cells were inculated with nanoprobe for another 4 h. The confocal imaging results were shown in Figure 4. The fluorescence intensity of Tamoxifen treated cells was significantly lower than the untreated cells. In contrast, the fluorescence

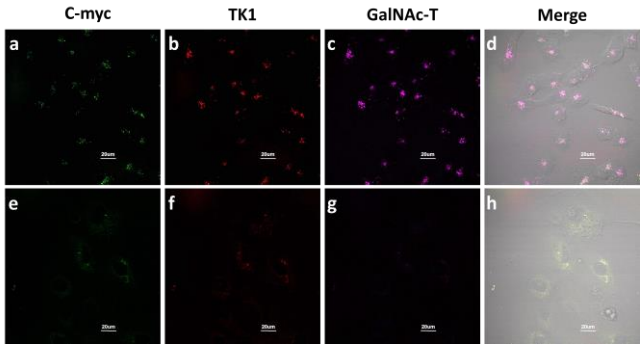


Figure 3. Intracellular imaging of three mRNAs inside MCF-7 cells (a-d) and MCF-10A cells (e-h) using DNA tetrahedron based nanoprobe.

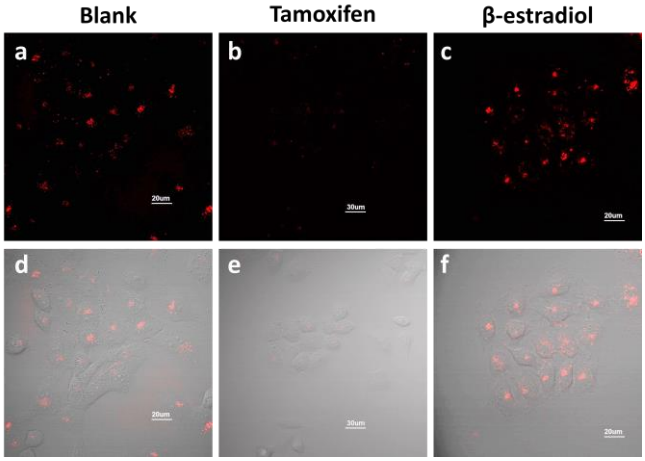


Figure 4. Intracellular fluorescence imaging of the different expression levels of TK1 in MCF-7 cells under drug stimulations. a and d, untreated; b and e, Tamoxifen stimulated; c and f, β-estradiol stimulated.

intensity of β-estradiol treated cells was relatively higher compared with the untreated ones (Figure S8). These results indicate that the nanoprobe can be used to monitor the change of gene expression level in living cells.

In order to further verify that the different fluorescence intensities correctly represent the different mRNA expression level, we extracted the mRNA from cell lysis and conducted reverse transcription-PCR (RT-PCR) experiments. Figure 5a demonstrated that the expression level of three tumor-related mRNAs, especially GalNAc-T mRNA, was significantly higher in cancerous MCF-7 cells than that in normal MCF-10A cells. The expression level of TK1 mRNA in MCF-7 cells

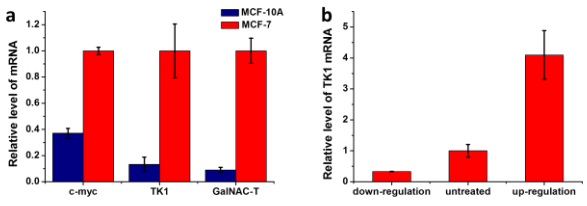


Figure 5. qRT-PCR analysis of the different mRNA expression level in (a) MCF-7 cells and MCF-10A cells. (b) The expression level of TK1 in MCF-7 cells under different stimulation.

under different drug stimulation was shown in Figure 5b. The results confirmed that the Tamoxifen caused down-regulation of TK1 mRNA expression while β-estradiol caused up-regulation of TK1 mRNA expression. The results of RT-PCR experiments are in agreement with those of confocal imaging, which proves that the DNA tetrahedron-based multicolor nanoprobe is suitable to be applied in mRNA detection in living cells.

In summary, we have developed a DNA tetrahedron-based multicolor nanoprobe for simultaneous detection of three different mRNAs by competitive chain replacement reaction. Benefited from the DNA tetrahedron structure, the nanoprobe has good membrane permeability, high enzyme resistance and low cytotoxicity. The competitive chain replacement between the target sequences and quencher sequences enabled high specificity and sensitivity. The nanoprobe could distinguish cancerous cells from normal cells and monitor the change of the intracellular mRNA expression level with good accuracy. We believe that this strategy had potential use in diagnoses and treatment of cancer.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Materials, synthesis and characterization of DNA tetrahedron-based nanoprobe, in vitro fluorescence experiments, nuclease stability, MTT assay, confocal fluorescence imaging and qRT-PCR experiments are included in the Supporting Information.

AUTHOR INFORMATION

Corresponding Author

E-mail: sczhang@tsinghua.edu.cn

Author Contributions

& The two authors contributed equally to this work and should be considered co-first authors.

Notes

The authors declare no competing financial interests.

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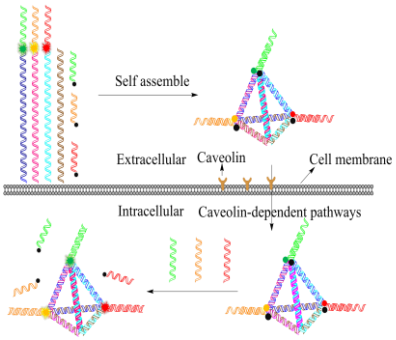
Self assemble
Extracellular
Caveolin
Cell membrane
Intracellular
Caveolin-dependent pathways