In recent years, many efforts have been made for the development of fluorescent probes for detection and imaging of tumor markers in living cells. Cancer is undoubtedly a pervasive and destructive disease in which the body cells begin to grow and divide in an uncontrollable way. The survival rate of cancer patients can be greatly increased if the cancer is detected at the cell level in an early stage. For this purpose, a major target is to identify the expression abnormalities of tumor markers in living cells. In recent years, many efforts have been made for the development of fluorescent probes for detection and imaging of tumor markers in living cells. Although these nanoprobes have performed well in detecting a single type of tumor marker, it is difficult to detect multiple, different types of tumor markers in a single assay. To comprehend the complexity of cancer, researchers try to monitor the alterations of multiple tumor markers in living cells. However, it remains a challenge, as cancer commonly involves multiple types of tumor markers and the expression levels of these markers change dynamically in tumour progression. Moreover, assembling many different types of recognition units in a single probe is hard to achieve. Therefore, simultaneous detection of various different targets is of crucial importance to improve the accuracy of early cancer detection and in obtaining a better understanding in the changes of tumor marker expression levels in living cells.

Herein, we have developed a fluorescence nanoprobe for simultaneous detection and imaging of multiple mRNAs and matrix metalloproteinases (MMPs) in living cells. As a tumor-related marker, mRNA has been widely utilized in the diagnosis and treatment of cancer. The MMPs have been associated with the invasive properties of cancer cells and are expressed at statistically higher levels in tumors. The nanoprobe was prepared using gold nanoparticles (AuNPs) functionalized with a dense shell of synthetic DNA molecular beacons (MBs) and peptides, step-by-step via gold–thiol bond formation, which allows good dispersibility in aqueous systems, and good biocompatibility. Firstly, the AuNPs were modified with DNA MBs before the next assembly. The MB was designed to form a unique stem–loop configuration with recognition sequences and reporter fluorophores. The recognition sequences can identify two specific mRNA transcripts: TK1 mRNA and GALNAc-T mRNA, which are important markers in cancer cells. The ends of the MBs are labelled with reporter fluorophores: Alexa Fluor 405 and Cy5, respectively. Next, the synthetic peptides can be specifically cleaved by the MMPs when the nanoprobe meets the corresponding targets, thereby separating the fluorophores and AuNPs and restoring the fluorescence. The details of the assembly are depicted in Scheme 1.

Large-sized AuNPs with diameter of 20 nm were employed in this study, because they cause stronger surface plasmon resonance (SPR) absorption and assemble more recognition units than small-sized AuNPs. As shown in Figure S1 in the Supporting Information, the AuNPs were typical spherical with clear borders, while the borders of the nanoprobe was ambiguous due to the assembly of recognition units on the surface of the AuNPs. The UV/Vis absorption spectra (Figure S2 in the Supporting Information) further confirmed that the nanoprobe was successfully assembled owing to the red shift of the maximum absorption from 519 nm (for AuNPs) to 525 nm (for the nanoprobe). According to the previous method, each AuNP was calculated to carry 17 ± 1 Alexa Fluor 405 labelled MB1 tar-
targeting TK1 mRNA, 33 ± 1 FITC labelled peptide 1 targeting MMP-2, 38 ± 1 Rb labelled peptide 2 targeting MMP-7 and 18 ± 1 Cy5 labelled MB2 targeting GalNAc-T mRNA.

The ability of the nanoprobe for simultaneous detection of two DNA targets and two MMP targets was evaluated by incubating the nanoprobe with the four corresponding targets. Figure 1 shows that the fluorescence intensity of each dye increased with the increase of its corresponding target concentration, thus demonstrating that the response of nanoprobe toward the targets led to fluorescence recovery.

Moreover, the fluorescence intensity is correlated positively with the concentrations of the targets in a concentration-dependent manner. Selectivity is a key property for application in living cells, because the activity of each target is influenced by the other three targets co-existing in living cells. Figure S4 in the Supporting Information shows that every MB specifically bound to its own DNA target and generated a high fluorescence signal. By comparison, the signals did not obviously change in the presence of the other three targets or its single-base mismatched DNA target, which were of comparable magnitude to background fluorescence. Similar results were obtained when the nanoprobe was incubated with the two MMP targets. These results indicated that the nanoprobe was capable of simultaneously monitoring four different targets without mutual interference, suggesting that each recognition unit of the nanoprobe retained high sequence specificity. Next, the kinetic studies of the nanoprobe toward four different targets were investigated. Figure 2 shows that the nanoprobe responded rapidly to the perfectly matched DNA targets within 5–10 min and it responded to the MMP targets within 20–30 min. The matched response time for the two types of targets makes the nanoprobe highly attractive for simultaneous monitoring of two kinds of tumour makers in living cells.

The cytotoxicity of the nanoprobe was tested with the MTT assay in the human breast cancer cell line, MCF-7. Figure S5 in the Supporting Information indicates that the cell viabilities were all more than 95% when incubated with free AuNPs as well as the nanoprobe for 3, 6, 12 and 24 h. The results reveal that the nanoprobe shows almost no cytotoxicity or side effects in living cells. This confirms that the nanoprobe can be used for intracellular multimarker diagnosis.

The internalization pathways of the nanoprobe in living cells were studied using different endocytosis inhibitors. As shown in Figure 3, the fluorescence intensities of all four signal channels did not show obvious change for EIPA-treated cells and chlorpromazine-treated cells, indicating that the endocytosis of the nanoprobe is not mediated by macropinocytosis or clathrin. Remarkably, after the cells were treated with filipin

Scheme 1. Schematic illustration of the designed nanoprobe for detection and visualization of intracellular mRNAs and MMPs.

Figure 1. The fluorescence intensity of the nanoprobe (1 nM) in the presence of various concentrations of: a) TK1 mRNA target, b) GalNAc-T mRNA target, c) MMP-2, and d) MMP-7 measured with different excitation wavelengths.

Figure 2. Kinetics of the nanoprobe. The nanoprobe (1 nM) was incubated with: a) TK1 mRNA target, b) GalNAc-T mRNA target, c) MMP-2, and d) MMP-7. The mRNA target concentrations are 200 nM and the MMP activities are 0.4 μg mL⁻¹.

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and dynasore, all of the four fluorescence signals decreased sharply, suggesting that the caveolae-mediated pathway mainly contributes to cellular uptake of the nanoprobe.

To evaluate the ability of the nanoprobe to simultaneously detect multiple mRNAs and MMPs, two different cell lines were firstly chosen: normal immortalized human mammary epithelial cell line (MCF-10A) and MCF-7 cells. After MCF-7 cells were incubated with the nanoprobe (1 nM), strong blue fluorescence signal for TK1 mRNA, green fluorescence signal for MMP-2, yellow fluorescence signal for MMP-7 and red fluorescence signal for GalNAc-T mRNA were observed with confocal laser scanning microscopy (CLSM; Figure 4). When MCF-10A cells were incubated with the nanoprobe under the same conditions, all of the four fluorescence signals were very low. Interestingly, strong green and yellow signals for MMPs were observed when the MCF-10A cells cultured with the MCF-7 medium were incubated with the nanoprobe, indicating that the content of MMPs increased when MCF-10A cells were influenced by cancer cell culture medium. Next, human hepatocellular liver carcinoma cell line (HepG2) and human hepatocyte cell line (HL-7702) were also chosen to evaluate the nanoprobe. Figure 4 shows that when HepG2 cells were incubated with the nanoprobe, strong blue, green, yellow and red fluorescence signals for the four tumour markers were observed, which was similar with the MCF-7 cells. Notably, after HL-7702 cells were incubated with the nanoprobe under the same conditions, the blue, green and yellow signals were faint and the red signal was strong, indicating GalNAc-T mRNA was also overexpressed in HL-7702 cells, which is consistent with the previous study. When HepG2 medium cultured HL-7702 cells were incubated with the nanoprobe, only the blue fluorescence signal was faint, while the green, yellow and red fluorescent signals were strong. The results reveal that multiplexed detection of intracellular mRNAs or MMPs could avoid false positive results produced by identifying one mRNA or MMP. Furthermore, these results suggest that the nanoprobe was capable of distinguishing tumour cells and normal cells even if one tumour maker of normal cells was overexpressed or the normal cells were influenced by the environment.

The ability of the nanoprobe to detect relative mRNA and MMP levels in living cells is very significant for estimating the tumour stage and making treatment decisions, because the expression levels of mRNA and MMP in cancer cells change dynamically in different stages of tumour progression. Next, we investigated if the nanoprobe could detect the changes of mRNA and MMP expression levels in living cells. MCF-7 cells were chosen as an example. It has been reported that tamoxifen induces down-regulation of TK1 mRNA expression,\textsuperscript{26} \(\beta\)-estradiol induces up-regulation of TK1 mRNA expression,\textsuperscript{27} and ilomastat can inhibit the expression of MMP-2 and MMP-7.\textsuperscript{28} MCF-7 cells were parallel divided into four groups. Group 1 was treated with tamoxifen to decrease the TK1 mRNA expression, group 2 was treated with \(\beta\)-estradiol to increase the TK1 mRNA expression and group 3 was treated with ilomastat to inhibit the expression of MMP-2 and MMP-7. The untreated group was used as control. Then the four groups of cells were incubated with the nanoprobe (1 nM). As shown in Figure 5, the blue fluorescence signal for TK1 decreased in the tamoxifen-treated MCF-7 cells and increased in the \(\beta\)-estradiol-treated

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**Figure 3.** Intracellular imaging of TK1 mRNA, GalNAc-T mRNA, MMP-2, and MMP-7 of MCF-7 cells treated with EIPA, chlorpromazine, filipin and dynasore. The four channels were recorded with 405 nm excitation, 488 nm excitation, 543 nm excitation, 633 nm excitation, from left to right. Scale bars: 50 \(\mu\)m.

**Figure 4.** Intracellular imaging of TK1 mRNA, GalNAc-T mRNA, MMP-2, and MMP-7 with CLSM. The cells were incubated with the nanoprobe (1 nM) for 4 h at 37 °C. Scale bars: 100 \(\mu\)m.
MCF-7 cells compared with the untreated cells. The other three fluorescence signals did not show obvious change. The green fluorescence signal for MMP-2 and yellow signal for MMP-7 decreased in the ilomastat-treated MCF-7 cells and the other two fluorescence signals for mRNAs were unchanged. This reveals that the nanoprobe was able to detect changes in the tumour marker expression levels in cancer cells.

In summary, we report a fluorescence nanoprobe that can simultaneously monitor and image multiple mRNAs and MMPs in living cells. The nanoprobe consists of gold nanoparticles assembled with a dense shell of molecular beacons and peptides, step-by-step, which target intracellular TK1 mRNA, GaINAc-T mRNA, MMP-2 and MMP-7, respectively. The nanoprobe could signal the presence of target mRNAs and MMPs with the open form of the stem–loop structure of MBs and the cleavage of peptides, leading to the recovery of fluorescence. The nanoprobe exhibits high specificity, rapid response, and good biocompatibility. The cellular internalization results revealed that the cellular uptake of the nanoprobe was primarily through the caveolae-mediated pathway. Confocal imaging experiments displayed that the nanoprobe could discriminate between cancer cells and normal cells. Additionally, it can determine changes in the expression levels of mRNAs and MMPs in living cells, which is beneficial for evaluating the stage of tumour progression and making treatment decisions. We anticipate that this strategy could provide new avenues for monitoring multiple biomarkers in living cells.

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