Simultaneous detection of multiple targets involved in the PI3K/AKT pathway for investigating cellular migration and invasion with a multicolor fluorescent nanoprobe†

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We develop a multicolor fluorescent nanoprobe for assessing cellular migration and invasion by simultaneously imaging miRNA-221, PTEN mRNA and MMP-9 involved in the PI3K/AKT pathway which can regulate cellular mobility and invasiveness.

Cancer in humans is a highly fatal disease with poor prognosis and the most life-threatening aspect of cancer is tumor metastasis which requires cellular migratory and invasive capacities.1 Cell motility is regarded as the predominant regulator of tumor invasion.2 There has been increasing interest in studying cell migration and invasion because this is pivotal to establish effective strategies for cancer diagnosis, prognosis and treatment promoting control in cancer metastasis.3 Many migration/invasion assays such as wound healing and transwell invasive assays have been established to evaluate migration and invasion; however, these approaches are labor intense and time consuming to manage.4 Hence, it is necessary to develop a quicker, simpler and more accurate approach to assess cellular migration and invasion. The migration and invasion of cancer cells were reported to be regulated through a network of signaling pathways which can be cooperatively influenced by microRNAs (miRNAs), messenger RNAs (mRNAs) and matrix metalloproteinases (MMPs).5 It was reported that miR-221 promoted cellular migration and invasion via directly repressing PTEN and TIMP3 expression leading to the aberrant activation of the PI3K/AKT pathway and MMP-9 expression.6

The current widely used approaches to analyze genes and proteinases in signaling pathways are based on traditional techniques such as northern blotting, real-time reverse transcription-PCR (RT-PCR), microarray hybridization, western blotting and immunohistochemistry.7 However, these approaches require substantial time, many steps and a large number of cell samples. Moreover, the cell lysate or immobilization process used in these methods makes it impossible to study the dynamic changes and natural situation of these molecules. Thus, it is important to develop a noninvasive method for detecting multiple molecules in signaling pathways. And we anticipate that cellular migration and invasion could be assessed by identifying the expressions of genes and proteinases involved in signaling pathways. Fluorescence imaging analysis could have great potential in simultaneously monitoring genes and proteinases involved in signaling pathways of living cells and providing useful information about cellular migration and invasion.

Here, we construct a multicolor fluorescent nanoprobe for investigating cellular migration and invasion through detecting miRNA, mRNA and MMP involved in the PI3K/AKT pathway of living cells (Scheme 1). The nanoprobe was composed of gold nanoparticles (AuNPs), MB1, MB2 and MMP-9. The MB1, MB2 and PTEN mRNA were connected via peptide bonds. The MB1 was connected with the oligonucleotide complementary to the miR-221 target sequence. The AuNPs were conjugated with the MB1 via a disulfide linker. The MB2 and PTEN mRNA were connected via a disulfide linker.
nanoparticles (AuNPs) anchored with a dense layer of oligonucleotide molecular beacons (MBs) and a peptide, modified step-by-step via Au–S bond formation.6 Firstly, the AuNPs were assembled with MBs folding into a hairpin conformation.9 The MBs could respond specifically to miR-221 and PTEN mRNA targets. Next, the synthetic peptide was assembled on the surface of AuNPs. The peptide could be specifically cleaved by MMP-9.10 In the absence of the targets, the fluorescence of three fluorophores linked with the MBs and the peptide was efficiently quenched by AuNPs. In the presence of the corresponding targets, the MBs were forced to unwind and the peptide was cleaved by MMP, thus leading to fluorescence recovery.

The nanoprobe was prepared using 20 nm AuNPs, because large-sized AuNPs can accommodate more recognition units and cause higher surface plasmon resonance (SPR) absorption than small-sized AuNPs. The morphologies of AuNPs and the nanoprobe were characterized by transmission electron microscopy (TEM) (Fig. S1, ESI†). The results showed that they had a spherical morphology. The UV/Vis spectra (Fig. S2, ESI†) showed that the AuNPs were successfully functionalized owing to the red shift of the peak from 519 to 524 nm. According to a well-established assay,11 each AuNP was determined to carry 19 ± 1 Cy5 labeled MB1 targeting miR-221, 20 ± 1 Alexa Fluor 488 labeled MB2 targeting PTEN mRNA, 82 ± 1 RhB labeled peptide targeting MMP-9 (Fig. S3, ESI†).

The capability of the nanoprobe to recognize the two DNA targets and MMP target was evaluated. The nanoprobe was incubated with the complementary miR-221 target, PTEN mRNA target and MMP-9 target, respectively. The fluorescence of each dye was intensified with the addition of respective target in a concentration-dependent manner (Fig. 1), suggesting that the fluorescence recovery should be attributed to the response of the nanoprobe towards the targets.

Selectivity is an important aspect for simultaneously monitoring different targets in living cells. Fig. S4 (ESI†) shows that every MB could generate notable fluorescence upon meeting its own DNA target. However, the fluorescence signals were faint in the presence of single-base mismatched DNA target or the other two targets and comparable to the background fluorescence. Similar results were found when the nanoprobe was incubated with the MMP target. These results confirmed that the nanoprobe could sense different targets with high specificity.

A dynamics study of the nanoprobe toward three different targets was performed. The nanoprobe could respond to DNA targets within 15–20 min and MMP-9 within 20–30 min (Fig. S5, ESI†). The matched response time for DNA and MMP targets makes the nanoprobe hold great promise for simultaneously monitoring different biomolecules in living cells. Further experiments showed that the nanoprobe had good stability and reproducibility (Fig. S6 and S7, ESI†).

Cell toxicity of the nanoprobe was measured by MTT assay (Fig. S8, ESI†) in MCF-7 cells (human breast cancer cell line). Cell survival rates were over 95% after treatment with naked AuNPs and the nanoprobe for various times, indicating that the nanoprobe was almost nontoxic.

The multicolor nanoprobe was then used to study cellular mobility and invasiveness by measuring the relative fluorescence of multiple biomarkers in the signaling pathway. miR-221, PTEN mRNA and MMP-9 in less invasive MCF-7 cells were selected as an example. It was reported that anisomycin induced miR-221 and SU11274 reduced miR-221. MCF-7 cells were divided into three groups. One group was treated with anisomycin and another group was treated with SU11274. The untreated group served as control. Then the three groups of MCF-7 cells were incubated with the nanoprobe and imaged using confocal laser scanning microscopy (CLSM). The relative levels of miR-221, PTEN mRNA and MMP-9 were reflected by fluorescence intensities (Fig. 2).

The results showed that the red fluorescence for miR-221 was stronger in the anisomycin-treated cells and weaker in the SU11274-treated cells. The green fluorescence for PTEN mRNA displayed opposite fluorescence changes because miR-221 negatively regulated PTEN mRNA. RT-PCR further demonstrated the relative levels of miR-221 and PTEN mRNA after drug treatment (Fig. S9 and S10, ESI†). The yellow fluorescence for MMP-9 was also observed and the fluorescence changes were similar to those of miR-221 because miR-221 could activate MMP-9.

![Fig. 1](image1.png) Fluorescence recovery of the nanoprobe upon addition of various concentrations of: (a) miR-221 target, (b) PTEN mRNA target and (c) MMP-9 in a concentration-dependent manner. The fluorescence of the nanoprobe was measured with 648 nm, 488 nm and 554 nm excitation wavelengths, respectively.

![Fig. 2](image2.png) Intracellular imaging of different levels of miR-221, PTEN mRNA and MMP-9 under CLSM. The MCF-7 cells were incubated with the nanoprobe (1 nM) for 4 h at 37 °C. The three channels were recorded with 633 nm excitation, 543 nm excitation, and 488 nm excitation, from left to right. Scale bars: 100 μm.
The relative abundance of MMP-9 concurred well with western blot analysis (Fig. S11, ESI†).

To test the effects of miR-221, PTEN mRNA and MMP-9 levels on cellular migration and invasion, wound healing and transwell invasion assays were studied. The cellular migration capability was strongest and the wound was completely closed at 48 h with anisomycin treatment, while the capability was weakest with SU11274 treatment (Fig. 3a and b). Similar to the migration properties, the number of invasive cells was increased with anisomycin treatment and conversely, decreased with SU11274 treatment (Fig. 3c and d). The invasive ability could be further verified by measuring the absorbance at 595 nm (Fig. S12, ESI†). These results implied that the cellular migration and invasion could be influenced by miR-221, PTEN mRNA and MMP-9 and be assessed by the fluorescence changes of these components.

Next, the nanoprobe was applied for analyzing the migration and invasion of normal cells influenced by cancer cells. Two different cell types were chosen: MCF-10A (normal mammary epithelial cells) and MDA-MB-231 cells (highly metastatic breast cancer cells) which were reported to secrete abundant miR-221 via exosomes.12 Furthermore, miRNAs could be internalized by recipient cells via exosomes and regulate genes and proteinases.13 After the MCF-10A cells were incubated with the nanoprobe, the green fluorescence was strong while the red and yellow fluorescence signals were very weak (Fig. 4). Interestingly, after MCF-10A cells were cultured with MDA-MB-231 medium for six days, the red fluorescence and yellow fluorescence were increased, while the green fluorescence was decreased. The results indicated that after being treated with the cancer medium, miR-221 and MMP-9 expressions increased and PTEN mRNA expression decreased. Therefore, based on the imaging results, the migration and invasion of the treated MCF-10A cells were predicted to be enhanced. Then the migration and invasion assays of the treated and untreated MCF-10A cells were further studied. The mobility of the treated MCF-10A cells was enhanced (Fig. 5a and b).

Similar to migration assay, the invasiveness of the treated MCF-10A cells was improved, whereas the untreated MCF-10A cells showed negligible invasiveness (Fig. 5c and d and Fig. S13, ESI†). The migration and invasion of MCF-10A cells were consistent with the CLSM results. This reveals that the multicolor nanoprobe could investigate cellular migration and invasion by detecting the expression changes of miR-221, PTEN mRNA and MMP-9.

In conclusion, we have reported a multicolor fluorescent nanoprobe which can investigate cellular migration and invasion by simultaneously monitoring multiple components involved in the PI3K/AKT pathway. As far as we know, this is the first time that cellular migration and invasion have been assessed using a fluorescent nanoprobe. The nanoprobe consists of AuNPs anchored with recognition units exhibiting high specificity and rapid response. Confocal imaging demonstrated that the nanoprobe could simultaneously detect the expression changes of miR-221, PTEN mRNA and MMP-9 which can cooperatively
influence cellular mobility and invasiveness. The imaging results were further verified by wound healing and transwell invasive assays. In cancer cells culture medium-treated and untreated MCF-10A cells, the cellular migration and invasion were also in accordance with the analysis of CLSM images. The multicolor nanoprobe could provide an easy, noninvasive, instantaneous and intuitive way to evaluate cellular mobility and invasiveness. We expect that the nanoprobe will be a promising tool to investigate cellular migration and invasion and further establish effective targeted therapeutic strategy to limit cancer metastasis.

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Notes and references