

## NANOPARTICLES WITH AGGREGATION-INDUCED EMISSION FOR MONITORING LONG TIME CELL MEMBRANE INTERACTIONS

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**Abstract**—We perform the long time monitoring of nanoparticle-cell membrane interaction with high spatial and temporal resolution. The 2, 3-bis(4-(phenyl(4-(1, 2, 2-triphenylvinyl) phenyl)amino)phenyl) fumaronitrile (TPE-TPA-FN) is doped in organically modified silica (ORMOSIL) to be a biocompatible nanoprobe, which displays an aggregation-induced emission (AIE) effect. Photobleaching resistance of this synthesized nanoparticle is tested and compared with its similar counterpart, which proves its superiority and capability of long term fluorescence emission. We utilize the objective-based total internal reflection microscopy combined with the living cell incubation platform to investigate the cell uptake process of this nanoparticle in real time.

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*Received 2 April 2013, Accepted 16 May 2013, Scheduled 4 June 2013*

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## 1. INTRODUCTION

A majority of organic fluorophores that are highly emissive in solution show weak or no light emission in the solid state due to the aggregation formation in the condensed phase [1]. Aggregation formation induces the light emission quenching from the  $\pi$ - $\pi$  stacking in many  $\pi$ -conjugated fluorophores, and this is notoriously known as aggregation-caused quenching (ACQ) [2]. In bio-imaging technology nowadays, fluorophore-doped nanoparticles with their various advantages [2] have emerged as a novel imaging contrast agent. However, the photophysical phenomenon of ACQ obstructs the capability of increasing the fluorophore-doped concentration in nanoparticles as a brighter emitter for bio-imaging. Many approaches have been taken to control this effect, which are usually accompanied by some side effects such as the steric effects and electronic effects [3]. Fortunately, a novel class of fluorophores have been discovered and display the opposite effect; they show no emission in dilute solutions but are brightly fluorescent upon concentration or solidification [4]. The so-called aggregation-induced emission (AIE) feature of this kind of fluorophores has been extensively studied. Based on the understanding of the AIE mechanism, novel nanoparticles have been designed and have shown their versatility in various application areas, including fluorescence imaging, biosensing, organic light-emitting diodes, etc. [5]. Since the AIE effect allows a higher concentration of fluorophores to be loaded in a single nanoprobe, it makes the AIE solutions more emissive and photobleaching-resistant as well [5]. Therefore, in comparison to normal ACQ materials, AIE materials are endowed with diverse benefits in laser-induced *in vivo* biological imaging and investigation, where lower average excitation power and long term observation is demanded. In addition, the cytotoxicity test experiments also support the biocompatibility of this type of materials [6], making it a more desirable bioprobe than the inorganic quantum dots (QDs).

Total internal reflection fluorescence microscopy (TIRFM) is based on the principle of evanescent wave (EW) excited emission [7]. This electromagnetic energy is generated at the interface of two kinds of medium with different refractive indexes. It decays exponentially from the boundary of the medium of lower refractive index. Usually, the penetration depth of EW is of the order of the excitation light wavelength (50 nm  $\sim$  250 nm) [8,9] and varies accompanied with the angle adjustment of total reflection. Because of this thin film excitation, only the fluorophores close enough to the interface could be excited, and the out-of-focus fluorescence is suppressed to a large extent. Therefore, it enables a sub-wavelength imaging

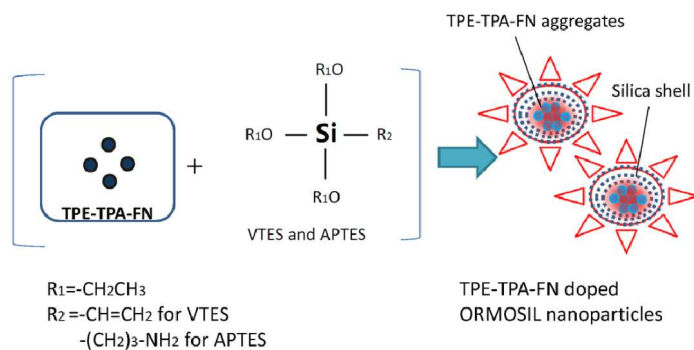
resolution perpendicular to the interface, which conveniently allows for the real-time monitoring of cell surface events and minimizes the photodamage of the cell. In microscopy systems, TIRFM is commonly realized in two types: the prism-based type and the objective-based type [7]. Though comparatively complicated, the objective-based type shows its advantages with sample-top accessibility, higher light collection efficiency and better image quality [10], which makes it more flexible and stable in biological application. In this geometry, an objective lens of high numerical aperture ( $> 1.4$ ) [11] is utilized for both excitation and signal collection. When in combination with a conventional inverted fluorescence microscopy system, it permits the direct observation and manipulation of specimens located on the surface of the coverslip or cell culture dish. With all these specific advantages, TIRFM offers a desirable platform for studying the cell membrane of a living cell in the nanoscale area.

In this paper, we use the organically modified silica (ORMOSIL) nanoparticles to encapsulate the AIE-active 2, 3-bis(4-(phenyl(4-(1, 2, 2-triphenylvinyl)phenyl)amino) phenyl) fumaronitrile (TPE-TPA-FN) aggregates based on the techniques developed from our former works [12–14]. This kind of nanoparticle displays aggregation enhanced fluorescence as desired. Photobleaching resistance of this material has been tested both *in vitro* and in living cells, which proves its beneficial effect of long term bright emission. To take advantage of its excellent features, we combine it with the TIRFM system to enable the monitoring of the interaction between the nanoparticle and the cell membrane at a located region. Real time images are achieved and discussed to unravel its potentiality in emerging biological applications.

## 2. MATERIALS AND METHODS

### 2.1. Synthesis of the AIE Nanoparticles

Figure 1 shows the synthesis procedure of the TPE-TPA-FN doped ORMOSIL nanoparticles in the nonpolar core of Aerosol-OT/DMF/water micelles. Briefly, the micelles were prepared by dissolving a certain amount of Aerosol-OT and 1-butanol in 10 ml of DI water through vigorous magnetic stirring. 200  $\mu$ l of TPE-TPA-FN in DMF were then added to the solution under magnetic stirring. Half an hour later, 100  $\mu$ l of neat VTES were added to the micellar system, and the resulting solution was stirred for about 1 hour. Next, ORMOSIL nanoparticles were precipitated by adding 10  $\mu$ l of APTES and stirred for another 20 h at room temperature. After the formation of the nanoparticles, surfactant Aerosol-OT, cosurfactant 1-butanol, residual VTES and APTES were removed by dialyzing the solution against DI



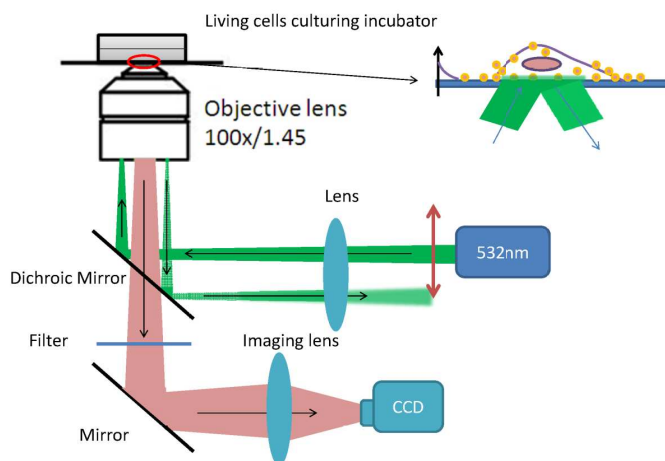
**Figure 1.** Schematic illustration of the AIE nanoparticles used in this study.

water in a 12–14 kDa cutoff cellulose membrane for 50 h. The dialyzed solution was then filtered through a 0.45  $\mu\text{m}$  cutoff membrane filter to be used in later experiments.

In the control group for the bleaching test, the TPE-TPA-FN was replaced by Nile Red, a type of dye which would bear the ACQ effect, and encapsulated in the same way. A detailed method can be referred to Ref. [14].

## 2.2. TIRFM for Living Cell Investigation

Figure 2 gives the representative configuration of our imaging system. The Olympus IX71 inverted microscope with an optical fiber-based



**Figure 2.** Configuration of TIRFM system.

TIRFM accessory is used for light excitation. A 532 nm single mode laser (MSL 30 mW, CNI) is coupled and aligned into the laser optical path, where a power density of  $200 \mu\text{W}/\text{mm}^2$  is achieved. The fiber light exit point is fixed to the conjugate plane of the back focal plane of the high numerical aperture TIRF objective lens (Olympus PLAPO100XOTIRFM, N.A. 1.45). Then the system enables the simple operation switching between TIRF and wide-field (WF) illumination through the adjusting of relative position between the laser path and the optical central axis of the system. To achieve TIR illumination, a droplet of match oil ( $n = 1.518$ ) is smeared between the objective lens and the bottom of a special dish. The evanescent wave illumination is obtained at the interface between the dish bottom and the cell culture environment when the laser pathway is tilted enough to surpass the critical angle ( $\sim$ normally  $62^\circ$  under this circumstance). Real-time images are collected by the same objective lens and then captured by the color CCD camera (Pro-microscan, OPLENIC). To separate the fluorescence emission from excitation background, a dichroic mirror and a high pass filter (U-MNIG, Olympus) are used.

Under the experiment for monitoring the nanoparticles-cell membrane interaction process in TIRF, a  $\text{CO}_2$  incubation system for microscopes (Olympus MIU-IBC) is mounted on the stage of the inverted microscope and the live images are captured by the EMCCD camera (iXon3 885, Andor) with fast speed and high contrast.

### 2.3. Cell Culture

HeLa cells were cultivated in Dulbecco's minimum essential media (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin, and 1% amphotericin B at  $37^\circ$  in the humidified atmosphere under 5%  $\text{CO}_2$ . The cells were seeded in special 6 cm dishes, of which the bottom has been replaced by a coverslip with thickness of  $\sim 0.17 \text{ mm}$  to enable observation under the TIRF objective lens. After growth for 24 h, the diluted TPE-TPA-FN doped ORMOSIL nanoparticles solution was added to a final concentration of  $12.5 \mu\text{g}/\text{ml}$  in the culture medium. The same concentration of Nile red doped ORMOSIL nanoparticles solution was added in the control experiment, and all samples were incubated for another 2 h. Afterwards, the cells were gently washed thrice by phosphate buffered saline (PBS,  $\text{pH} = 7.4$ ,  $10 \text{ mM}$ ) and imaged by microscopy under 532 nm light excitation.

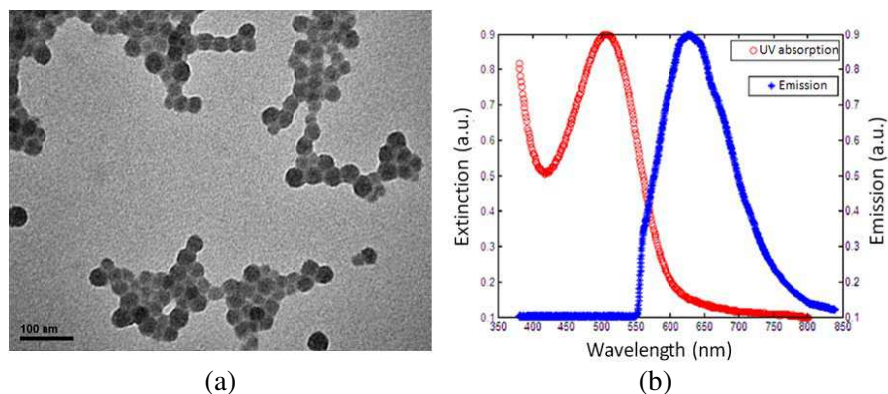
When conducting the real-time TIRF experiment, the cells were washed after 5 min incubation and quickly transferred to the small incubator mounted on the microscope. Here the cells could be kept in a stable  $37^\circ$  environment throughout the experiments by three heater

modules in combination with heating of the objective lens, which was in direct thermal contact with the bottom of the dish. Tubes for the supply of CO<sub>2</sub> were also available to control the CO<sub>2</sub> level for long time experiments.

### 3. RESULT AND DISCUSSION

#### 3.1. Characterization of the AIE Nanoparticles

The TEM image (Fig. 3(a)) shows the uniform spherical shape and an average size of about 20 nm for these nanoparticles. The silica encapsulation provides a stable optical transparent protection for the organic dyes and could be functionalized further for specific biological applications [14]. Fig. 3(b) shows the UV absorption and Fluorescence spectra (532 nm excited) of the nanoparticles. Excitonic and emission peaks are located at 510 nm and 630 nm respectively. The nanoparticles dispersed in the aqueous solution give a pink color and does not show any significant discolor or sediment for months, indicating its chemical stability.



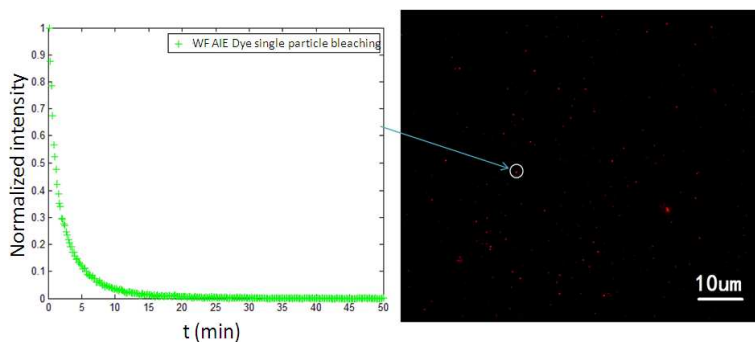
**Figure 3.** (a) TEM picture, (b) the UV absorption and fluorescence emission spectrum of the TPE-TPA-FN doped ORMOSIL nanoparticles.

#### 3.2. Photobleaching Resistance of AIE Nanoparticles

We test the photobleaching resistance of this synthesized AIE nanoparticle in this section, in order to to envision its feature of long-term monitoring for biological application.

### 3.2.1. Single Particle Bleaching Test

The diluted dye solution was dried and dispersed on a cleared coverslip and then illuminated persistently by the 532 nm laser in WF. The intensity image stack was achieved by the color CCD camera every 10 seconds. The result is shown in Fig. 4. The fluorescence intensity of the single isolated nanoparticle decreases along with the illumination lasting time, which is displayed as the imaging spot fading in the image series. The single nanoparticle fluorescence bleaching characteristic could be calculated through the post-process with the image stack. We focus on one particle here, of which the intensity is the accumulation of all its corresponding image pixels. The decay curve illustrates the bleaching process with respect to time. It shows a photobleaching time of minute scale, which is comparably longer than most organic fluorophores.



**Figure 4.** Photobleaching test of single AIE nanoparticle.

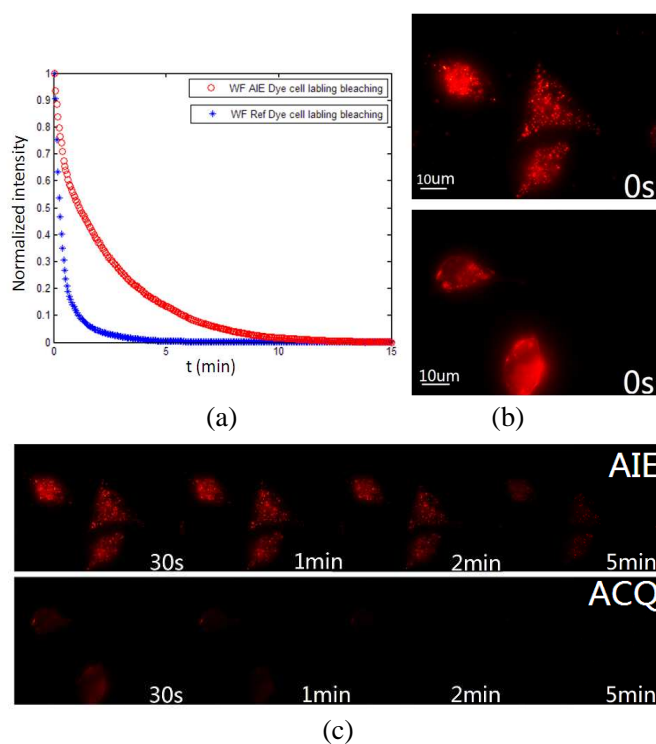
This single particle test demonstrates the long time emission capability of this kind of materials. In addition, it is predicted that if a higher concentration of the aggregates is encapsulated in by the silica, the photobleaching resistance of this single nanoparticle could be strengthened. It provides an alternative probe for the high sensitive fluorescence microscopy techniques, such as single particle tracking (SPT), which allows an improved temporal and spatial resolution for the cell-particle interaction detection [9].

### 3.2.2. Cell Labeling Bleaching Test Compared with ACQ Nanoparticles

Afterwards, we utilize this nanoparticle as an optical probe for cell imaging. Due to the cationic charges introduced by silica

encapsulation, nanoparticles could adhere to the cell membrane by electrostatic bonding. This rapid process occurred in less than 1 minute in our experiments and then until  $\sim 5$ –10 minutes fluorescence was detectable from cytoplasm. By two more hours of cell culturing after adding the AIE nanoparticles, it could be confirmed that the labeling process was fully accomplished. In the control group, we prepared the sample with Nile red doped ORMOSIL nanoparticles solution in the same condition.

After a 2 h incubation, we gently wash both samples with PBS and examine them under our imaging system. Both nanoparticles exhibit their capability of cell labeling. And through a procedure similar to that for the single particle bleaching test, we compare



**Figure 5.** Comparison between AIE nanoparticles and ACQ nanoparticles for cell imaging. (a) Illustrates the bleaching curves, (b) show WF cell labeling images with these two types of nanoparticles, (c) shows the time lapse bleaching processes of these two types of nanoparticles.

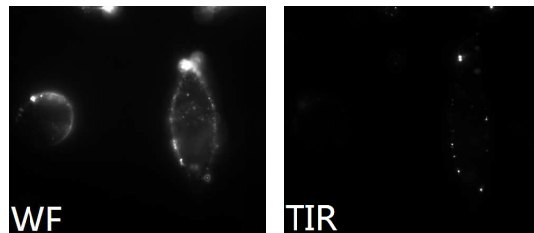


the photobleaching resistance of these two types of bioprobes. As shown in Fig. 5, under the same excitation and collection condition, the imaging intensity decreases rapidly in the control group, while remains detectable by the color CCD camera until 5 minutes after AIE nanoparticles labeling.

### 3.3. Cellular Uptake Investigation with TIRF

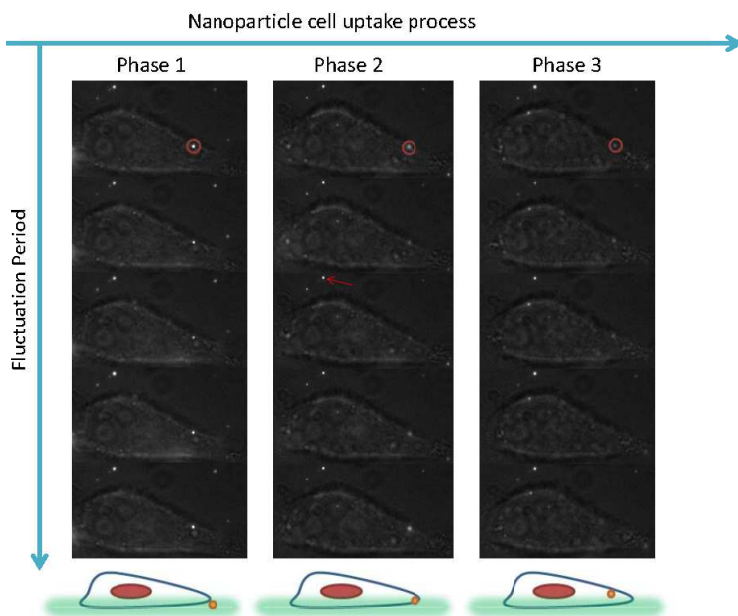
The photobleaching resistance as well as the biocompatibility features allow for the real time observation of the pathway and process of this novel nanoparticle entering into the living cell.

The images captured in WF and TIRFM by the EMCCD camera are compared in Fig. 6. In TIR mode, an image of higher signal to noise ratio and contrast was achieved. Fewer nanoparticles were excited in this mode due to the illumination selectivity by the evanescence wave, and fluorescence from cytoplasm was suppressed.



**Figure 6.** Cell imaging results with AIE nanoparticles in WF and TIR modes, respectively.

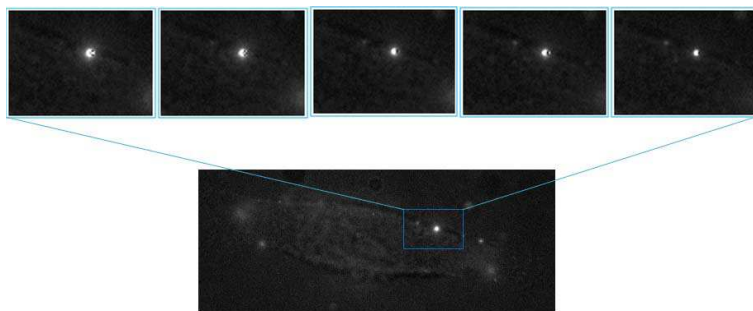
The real time nanoparticles uptake process could be captured by the EMCCD in video mode, and the compressed 40 minutes long observation video could give a vivid display of the entire cell uptake process. Here, only nanoparticles located near the basal membrane area of the cell could be excited and imaged, and dim white light is shined through the transmission mode to give the cell contour in order to confirm that the variation of the nanoparticle image is due to the interaction with the cell. At the beginning of our real time monitoring, we optimize the position of the objective lens to focus on the nanoparticles in the excitation area of the evanescence wave. As displayed in Fig. 7, the particle located at the membrane outline exhibits a periodic fluorescence fluctuation at first (Phase 1), which indicates its contact with the cell. It also moves slightly accompanied by the creep movement of the single cell. Then, this particle becomes



**Figure 7.** Typical AIE nanoparticle movement in cell membrane area (Nanoparticle in the red ring was excited by the evanescence wave and was monitored by TIRFM with the overlap of the cell contour; for reference, the red arrow indicated a fixed nanoparticle keeping focused during all the phases).

dim and displays as an out-of-focusing image while continuing the periodic fluctuation (Phase 2). For reference, some other particles located at some no-cell area (attached to the bottom of the culture dish and no transposition movement occurred during all the observation period) remain focused during the same time interval, which excludes the influence from the instability of imaging condition. Thus, the out-of-focusing image results from the entry of particles and then the gradual departure from the cell membrane's illuminated area. At last, the nanoparticle disappeared immediately from the field of view when it entered into the cell and diffused randomly in cytoplasm (Phase 3).

In Fig. 8, we study the single particle situation and focus on a single fluctuation period (see the inset of Fig. 8 at five different times) to monitor its interaction or diffusion movement on the cell membrane. It is noticeable that the nanoparticle fluorescence lasts longer than that in the former bleaching test experiment, due to the weaker excitation mode in TIR condition.



**Figure 8.** Nanoparticle-cell membrane interaction in details.

Though the TEM image shows that the single nanoparticle is of the size with the diffraction limit, we found that some of the single nanoparticle images captured by the EMCCD camera are not always a perfect spherical shape when it adhered to the cell membrane. It is acknowledged that a fluorescent molecule can be regarded as an electrical dipole [15]. And based on the previous research report, the intensity distribution of the deteriorated images contains information about the molecule or nanoparticle's emission dipole orientation [16]. Thus, the donut shapes could result from the orientation variation of the dye particle on the cell membrane, which indicated both the transitional and rotational diffusions during the cell uptake process of AIE nanoparticles.

#### 4. CONCLUSION

The TPE-TPA-FN doped ORMOSIL nanoparticle has been shown in our study to be a photostable fluorescence organic bioprobe that is suitable for long-term monitoring of the cell-particle interaction. Since the ORMOSIL nanoparticle could be further functionalized with co-conjugated bio-molecules, it is a versatile probe for early diagnosis/therapy of cancer and other diseases [14]. In the last decade, along with the thriving of research in the single molecule and sub-wavelength level, the TIRFM system has been utilized and developed extensively. TIRFM has been used in technical innovations such as smFRET [17], TIR-FCS [10], SPT [9] for single molecule detection and SIM [18], STORM [19] for fluorescence super-resolution microscopy and these reflect its flexibility in the related research. The combination of both advantages of AIE nanoparticles and the TIRFM system provides a fresh optional cell tracing tool and allows for more detailed studies of biological process in this platform.

## ACKNOWLEDGMENT

This work was supported by National Basic Research Program of China (973 Program) No. 2013CB834704, and the National Natural Science Foundation of China (61008052 and 91233208), the Fundamental Research Funds for the Central Universities, the Science and Technology Department of Zhejiang Province.

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