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Ultrabright organic dots with aggregation-induced emission characteristics for cell tracking

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ABSTRACT

Noninvasive fluorescence cell tracking provides critical information on the physiological displacement and translocation of actively migrating cells, which deepens our understanding of biomedical engineering, oncological research, stem cell transplantation and therapies. Non-viral fluorescent protein transfection based cell tracing has been widely used but with issues related to cell type-dependent expression, lagged readout, immunogenicity and mutagenesis. Alternative cell tracking methods are therefore desired to attain reliable, stable, and efficient labeling over a long time. In this work, we have successfully developed ultra-bright organic dots with aggregation-induced emission (AIE dots) and demonstrated their capabilities for cellular imaging and cell tracking. The AIE dots possess high fluorescence, super photostability, and excellent cellular retention and biocompatibility. As compared to commonly used pMAX-GFP plasmid labeling approach, the organic AIE dots showed excellent cell labeling on all tested human cell lines and superior tracing performance, which opens up new opportunities in the cell-based immunotherapies and other related biological researches.

1. Introduction

During the past decades, fluorescence imaging has been extensively utilized in visualizing various biological species and progresses [1–3], such as cell organelles [4,5], programmed cell death [6], and cell lineage commitment [7]. As compared to other imaging modalities, including positron emission tomography [8,9], magnetic resonance imaging [10–12], single photon emission computing tomography [13], fluorescence imaging is capable of producing high resolution images at sub-cellular levels, making the study of cell–cell interaction possible to provide unique insights in immunology and oncology [14–18]. Continuous non-invasive cell tracking by fluorescence over a long period of time is pivotal to extract critical spatiotemporal cellular information of physiological displacement, translocation and the fate of cancer and stem cells. This information facilitates the understanding of cancer or stem cell development and intervention, providing insights for basic oncological researches and development of preclinical cell based therapies [19–22].

Since its initial inception as a potent cell labeling agent, engineered expression of green fluorescent protein (GFP) and its variants has dominated the field of cell transplantation and tracking [23–27]. This approach capitalizes on the cells' innate machinery to produce proteins and requires the reporter gene to be transfected into the cells and subsequently translated into fluorescent proteins [27–29]. Although viral transduction by integration of GFP gene into the cell genome can result in stable GFP expression for long term tracing, it suffers from high cost and safety issues due to the introduction of random insertional mutation at integration sites [30–32]. Consequently, non-viral plasmid transfection using a wide range of biomaterials has been explored to circumvent the safety

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issues by intentionally avoiding the genomic integration but expressing the GFP plasmid directly from the cytoplasm [30–33]. While this works well for short-lived experiments in the time scale of days, the plasmid is quickly lost with a correlated drop in fluorescence. In addition, the non-viral method presents low and cell type dependent transfection efficiencies [34]. Primary cell lines, mesenchymal stem cells are often refractory to non-viral transfection [31]. Moreover, all protein expression starts with a convoluted and time-consuming transcriptional, translational and post-translationally regulated process and is subject to ubiquitination and proteasomal degradation. These can result in an inconsistent and sometimes even cyclical net amount of fluorescent signal even when actual intracellular plasmid concentration is high [35,36]. Therefore, new cell tracking methods that are able to attain reliable, stable and efficient cell labeling over a long time is highly desirable.

Direct cell labeling by organic or inorganic nanomaterials for tracing has drawn great attention recently, which is fairly straightforward and does not involve genetic modification of the cells [14–20]. However, currently available fluorescent probes suffer from serious drawbacks and short tracking time. For example, quantum dots-based cell trackers contain toxic heavy metals, while small organic molecules suffer from small Stokes shifts, rapid photobleaching and cytoplasm leaking upon cell proliferation [37–39]. Researchers recently showed that organic nanoparticles (NPs) possess longer cellular retention time and lower exocytosis rate as compared to their discrete molecular counterparts [39,40]. However, the notorious aggregation-caused quenching (ACQ) effects [41] of conventional organic molecules often lead to weakened or even annihilated fluorescence in NPs. We have recently reported a series of unique organic fluorogens with aggregation-induced emission (AIE) characteristics, which are highly emissive when aggregated [42–46]. Based on these AIE fluorogens, we and others have successfully fabricated organic AIE dots with high fluorescence, excellent photostability and good biocompatibility [47–52], revealing their great potentials to overcome the obstacles possessed by the currently used cell trackers for cell tracking.

Herein, we report the fabrication of surface functionalized green emissive AIE dots for long-term cell tracing using an AIE fluorogen 4,7-bis[4-(1,2,2-triphenylvinyl)phenyl]benzo-2,1,3-thiadiazole (BTPEBT) as an example. A mixture of lipid-poly(ethylene glycol) (lipid-PEG) and lipid-PEG-maleimide was chosen as the encapsulation matrix to endow BTPEBT into AIE dots with surface functionality. A cell penetrating peptide derived from HIV-1 transactivator of transcription protein (Tat) was further conjugated to the dot surface to yield AIE-Tat dots with high cellular internalization efficiency. We firstly evaluated the size, morphology, brightness, colloidal stability and single particle fluorescence of the formed AIE-Tat dots. The cellular imaging capability of the AIE-Tat dots in HEK 293T cell was then studied by varying AIE-Tat dot concentrations. The labeling performance of the AIE-Tat dots towards different human cell lines was subsequently compared with the classical calcium phosphate mediated GFP transfection method under similar experimental conditions. Finally, the AIE-Tat dots were used to trace the activity of HEK 293T cells using GFP transfection method as a control. The high brightness of AIE dots and their excellent cell labeling rate will offer new opportunities to the areas of cancer research, real-time monitoring of stem cell transplantation and other cell-based therapies.

2. Materials and methods

2.1. Materials

1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG_{2000}) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG_{2000}-Mal) were purchased from Avanti Polar Lipids, Inc. Qtracker® 655 cell labeling kit was purchased from Life Technologies, Invitrogen, Singapore. Fluoromount® aqueous mounting medium, Dulbecco's Modified Eagle Medium (DMEM), tetrahydrofuran (THF), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), penicillin-streptomycin solution, fetal bovine serum (FBS) and trypsin-EDTA solution were purchased from Sigma-Aldrich. Cell penetrating peptide derived from transactivator of transcription proteins, HIV-1 Tat (49–57) with C-terminus modified with cysteine (RKKRRQRRRC), was a commercial product customized by GenicBio, China. Milli-Q water was supplied by Milli-Q Plus System (Millipore Corporation, Breford, USA).

2.2. Characterization

NMR spectra were measured on a Bruker AV 500 NMR spectrometer (DRX 500, 500 MHz). UV-vis spectra were recorded on a Shimadzu UV-1700 spectrometer. Photoluminescence (PL) spectra were recorded on a Perkin–Elmer LS 55 spectrofluorometer. Average particle size and size distribution of the samples were measured by laser light scattering (LLS) with a particle size analyser (90 Plus, Brookhaven Instruments Co., USA) at a fixed angle of 90° at room temperature. The morphology of the samples was studied by high resolution transmission electron microscopy (HR-TEM, JEM-2010F, JEOL, Japan).

2.3. Synthesis of AIE-Tat dots

BTPEBT was synthesized according to literature [48]. To synthesize AIE dots, a THF solution (1 mL) containing BTPEBT (0.5 mg) and DSPE-PEG_{2000} (0.5 mg) and DSPE-PEG_{2000}-Mal (0.5 mg) was poured into water (10 mL) under sonication using a microtip probe sonicator at 12 W output (XL2000, Misonix Incorporated, NY). The mixture was further placed in dark in fume hood for THF evaporation at 600 rpm overnight. The AIE dots (1.8 mL) were further mixed and reacted with HIV1-Tat peptide (3×10^{-5} M). After reaction for 4 h at room temperature, the solution was dialyzed against MilliQ water using a membrane with molecular cutoff of 12 KDa for 2 days to eliminate the excess peptide. The AIE dot suspension was further purified by filtering through a 0.2 μm syringe driven filter. The Tat-AIE dots were collected for further use.

2.4. Calculation of Tat-AIE dot concentration

Freeze-drying of the AIE-Tat dot stock solution (2 mL) yielded 0.34 mg of powders. As the Tat-AIE dots are stable in water, the density of the dot suspension could be estimated as ~1 g/cm^3. As the average size of Tat-AIE dots determined from HR-TEM is ~30 nm, the concentration of the Tat-AIE dots in stock can be calculated from the following equation:Total number of AIE-Tat dots in 2 mL of suspension

$$\frac{\text{Total Volume of Tat}-\text{AIE dots}}{\text{Average Volume of Each dot}} = \frac{\frac{0.34\times10^{-3}\text{g}}{1\text{g/mL}}}{\frac{4}{3}\pi\times(15\times10^{-7})^3\text{mL}} = 2.41\times10^{13}$$

Finally, the concentration of Tat-AIE dots in stock solution was calculated as following:

$$[\text{AIE}-\text{Tat dots}] = \frac{\frac{2.3\times10^{13}}{6.02\times10^{23}\,\text{mol}^{-1}}}{2\times10^{-3}\text{L}} = 20\ \text{nM}$$

2.5. Single particle fluorescence imaging

Fluorescence imaging of individual AIE-Tat dot was performed with a custom-built Wide-Field Microscope (WFM) based on a Nikon ECLIPSE Ti–U inverted microscope frame. Light from a CW multi-line Ar ion laser (Melles Griot, CA, USA) was fiber-coupled to a Nikon TIRF attachment and focused on the back aperture of a high NA objective (Nikon TIRF Apo 100×, NA = 1.49, oil immersion). Immersion oil (n_D = 1.4790, Cargille, USA) was added between the high NA objective and the cover slip for index matching. The luminescence was collected by the same objective. After passing through the dichroic mirror and the emission filter, the luminescence was directed onto an iXonEM+897 EMCCD camera (512 × 512 pixels, 150 nm per pixel resolution, Andor Technology, Northern Ireland) connected to the side port of the microscope. A filter set consisting of a z488/10× excitation filter, Z488RDC dichroic mirror, and HQ500LP emission filter was used for imaging. The camera was connected to a computer furbished with camera-dedicated software to control the imaging parameters, and for data acquisition. The samples were prepared by depositing a droplet of NP solutions on a glass cover slide, waiting for 30 s to allow particle adsorption to the substrate, removing excess of liquid and drying under N_2. Fluorescence intensity time-traces were obtained by acquiring 1000 consecutive frames at a rate of 10 frames per second (100 ms exposure time, 100s time-traces) and extracting the number of counts per particle at each frame. The images were analyzed using Andor Solis (ver. 4.14.30001.0, Andor Technology, Northern Ireland) and NIS Elements Ar 4.10.00 (Nikon, Japan) software. The fluorescence intensity time-traces were analyzed by subtracting the background and integrating all the remaining photon counts using a customized analysis software written in LabView (National Instruments).