Carrier-free, water dispersible and highly luminescent dye nanoparticles for targeted cell imaging†

Xiaojun Diao, Wei Li, Jia Yu, Xiaojing Wang, Xiujuan Zhang, Yinlong Yang, Feifei An, Zhuang Liu and Xiaohong Zhang

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We develop a new strategy of using surface functionalized small molecule organic dye nanoparticles (NPs) for targeted cell imaging. Organic dye (2-tert-butyl-9,10-di(naphthalen-2-yl)anthracene, TBADN) was fabricated into NPs and this was followed by surface modification with an amphiphatic surfactant poly(maleic anhydride-alt-1-octadecene)-polyethylene glycol (C18PMH-PEG) through hydrophilic interactions to achieve good water dispersibility and bio-environmental stability. It should be noted that no additional inert materials were added as carriers, thus the dye-loading capacity of the resulting TBADN NPs is obviously higher than those of previously reported carrier-based structures. This would lead to much larger absorption and then much higher brightness. The resulting TBADN NPs possess comparable, if not higher, brightness than CdSe/ZnS quantum dots under the same conditions, with favorable biocompatibility. Significantly, TBADN NPs are readily conjugated with folic acid, and successfully applied in targeted cell imaging. These results show that water dispersible and highly stable organic NPs would be a promising new class of fluorescent probe for bioapplications in cellular imaging and labeling. This strategy may be straightforwardly extended to other organic dyes to achieve water dispersible NPs for cell imaging and drug delivery.

Introduction

Fluorescent cellular imaging provides a powerful means to study various biochemical processes in living cells. As a result, versatile fluorescent probes have been developed in the past few years.1–4 Organic dyes have been most widely applied in biological imaging in past years, however, the problems of their intrinsic hydrophobic property and instability in bio-environments, place great restrictions on their utilization in long-term imaging. Therefore, a number of strategies to develop more stable fluorescent probes have been pursued. Various dye-nanocarriers such as silica nanoparticles, carbon-based nanostructures and polymer micelles, are emerging, where dyes are covalently linked or physically entrapped to form a matrix structure.5–6 Recently, dye molecules have also been reported to be co-processed with some surfactants to form dye nanosuspensions as probes. Those carriers or suspensions can protect dyes from external chemicals and help encapsulated dyes achieve good bio-environmental stability and excellent dispersibility in water.7 However, all those structures have very limited dye-loading content and thus limited brightness, since the brightness of the probes depends not only on the quantum yield (QY), but also on the absorptivity of the dyes (proportional to the number of dye molecules).8 On the other hand, a small number of dye molecules that enter into the cellular interiors may be easily photobleached in the imaging process. Semiconducting quantum dots (QDs) represent another kind of luminescent probe, they possess excellent properties with high brightness and photostability. However, the problem of inherent cytotoxicity to biological systems still remains a critical concern for long-term imaging.9–13

Therefore, exploring alternative strategies for the design of excellent fluorescent probes with high brightness, good biocompatibility, excellent water dispersibility and bio-environmental stability, is highly desirable. Herein, we report a new and general strategy of using surface-functionalized dye nanoparticles (NPs) as cellular probes. We choose 2-tert-butyl-9,10-di(naphthalen-2-yl)anthracene (TBADN) as a model organic dye to demonstrate the effectiveness of this strategy. TBADN is an important functional molecule which has high luminescence and is widely used in organic optoelectronics.14,15 The dye has two anthryl groups attached at the opposite (9,10)-positions and one butyl group on the anthracene backbone as shown in Scheme 1. The two anthryl groups can enhance the rigidity of this molecule and

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*Functional Nano & Soft Materials Laboratory (FUNSOM), Jiangsu Key Laboratory for Carbon-Based Functional Materials & Devices, Soochow University, Suzhou, Jiangsu 215123, China. E-mail: xzh@jyu.edu.cn; Fax: +86 512 65882846; Tel: +86 512 65889835

**Nano-organic Photoelectronic Laboratory and Key Laboratory of Photophysical Conversion, Optoelectronic Materials, Technical Institute of Physics and Chemistry, Chinese Academy of Sciences, Beijing 100190, China. E-mail: xzh@ipc.ac.cn; Tel: +86 10 82543510
the butyl group can help to prevent $\pi-\pi$ stacking between molecules as aggregation occurs. Such molecular features effectively decrease the self-quenching effect of aggregates in NPs. TBADN NPs were prepared by a solvent exchanging method which is then followed by surface modifications with amphiphatic surfactant poly(maleic anhydride-alt-1-octadecene)-polyethylene glycol (C18PMH-PEG) to achieve excellent water dispersibility and bio-environmental stability. It should be noted that no additional inert materials were added as carriers and the dye NPs are made of pure aggregations of dye molecules, thus the dye-loading capacity is obviously higher than for other structures, which will lead to much larger absorption and then much higher brightness. Although aggregation of TBADN would induce fluorescence quenching and thus decrease the QY, however, the loss of brightness caused by the QY decrease is much less than the brightness enhancement induced by the increase of the absorption of the pure dye NPs. The resulting TBADN NPs demonstrated comparable, if not higher, brightness than CdSe/ZnS QDs and very low toxicity. They can also be conjugated with folic acid (FA) for targeted cell imaging. Thus it is expected that surface-functionalized dye NPs will become promising fluorescence probes for bioimaging.

Experimental section

Preparation of TBADN NPs

TBADN was purchased from e-Ray Optoelectronics Technology Co., Ltd. High-purity water (resistivity = 18.2 MΩ cm) was produced with a Milli-Q apparatus (Millipore). Tetrahydrofuran (THF) was purchased from Shanghai LingFeng Chemical Reagent Co., Ltd. 0.25 mL of $1 \times 10^{-3}$ M TBADN/THF solution was injected into 5 mL of water solution at 25 °C under vigorous stirring at 1000 rpm. After mixing for 5 min, the sample was left for 12 hours to stabilize.\(^{16}\)

Functionalization of TBADN NPs

2 mg of C18PMH-PEG was dispersed in 10 mL of ultra-pure water by ultrasonication. Then 300 µL of solution was added into 5 mL of TBADN NPs suspension, and ultrasonic treatment was applied for 5 min. After that, the solution was kept for use at room temperature. Poly(maleic anhydride-alt-1-octadecene)-polyethylene glycol-folic acid (C18PMH-PEG-FA) functionalization was executed via the similar approach as that of C18PMH-PEG. C18PMH-PEG and C18PMH-PEG-FA were synthesized according to the literature.\(^{17}\)

Characterization of TBADN NPs

For scanning electron microscope (SEM) research, only a few drops of the sample were dropped onto silicon substrates, then the solvent was left to evaporate. A 2 nm layer of Au was then deposited on the samples to minimize surface charging. After that, they were examined with a SEM (FEI Quanta 200 FEG) operated at an accelerating voltage of 30 kV. The size of the NPs was determined by dynamic light scattering (DLS). Measurements were carried out at 25 °C using a Zetasizer Nano-ZS from Malvern Instruments equipped with a 633 nm He–Ne laser using back-scattering detection. UV-vis absorption spectra were measured using a PerkinElmer Lambda 750 UV/vis/NIR spectrophotometer. Fluorescence spectra were recorded with a (Horiba Jobin Yvon, FluoroMax 4) luminescence spectrometer with the emission and excitation slits of 2 nm. Samples for absorbance and emission experiments were measured in standard 1 cm quartz cells. All measurements were performed at room temperature. Confocal imaging of NPs was carried out using a Leica laser scanning confocal microscope under 405 nm laser excitation, and emission was collected in the range of 425 nm to 530 nm. TBAND NPs and QDs were dropped onto a glass slide by spin-coating and a pure glass slide was used as a control.

Biological experiments

Human cervical cell line (HeLa cell) and human nasopharyngeal epidermal carcinoma cell line (KB cell) were obtained from American Type Culture Collection (ATCC). All cell culture related reagents were purchased from Invitrogen. KB and HeLa cells were cultured in FA free Roswell Park Memorial Institute-1640 (RPMI-1640) and normal RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, respectively, the former was considered as a positive group and another as a negative group. The cytotoxicity was measured using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) assay. HeLa cells were grown in a 96-well cell-culture plate at $10^2$–$10^5$ per well and then incubated for 24 h at 37 °C under 5% CO$_2$. After incubating HeLa cells with various concentrations of TBADN NPs and modified TBADN NPs, the standard MTT assay was carried out to determine the cell viabilities relative to the untreated cells. For the FA targeted imaging experiment, both positive and negative cells were incubated with 3 µM C18PMH-PEG-FA functionalized TBADN NPs for 2 h at 37 °C. Confocal imaging of cells was carried out using a Leica laser scanning confocal microscope under 405 nm laser excitation, and emission was collected in the range of 425 nm to 530 nm. All cells were washed twice before imaging.

Determination of the QY

The fluorescence QY was measured using a Perkin–Elmer Lambda 750 UV/vis/NIR Spectrometer and a luminescence spectrometer. We made use of 9,10-diphenylanthracene excited...
at 390 nm (QY = 0.90) as a standard to measure the fluorescence QY of modified TBADN NPs (free molecules were removed from the suspension by centrifugation) in suspension. The QY was calculated according to the following equation:

$$\eta_I = \eta_r \left( \frac{A_I}{A_r} \frac{I_r^2}{I_s^2} \right)$$

where $\eta$, $A$, $I$ and $n$ refer to the QY, absorbance, fluorescence intensity, and refractive index, respectively. The subscripts $s$ and $r$ refer to the sample and the reference, respectively. In our experiments, $A_s = 0.05$, $A_r = 0.05$, $n_s = 1.333$ (H$_2$O, 20 °C), $n_r = 1.4$ (THF, 20 °C).

Results and discussion

Fig. 1a is a SEM image of TBADN NPs, it can be seen that the NPs had uniform shape and are monodisperse. DLS analysis also indicated that the NPs had an average diameter $(D)$ of about 104 nm and a narrow size distribution (Polydispersity Index $(PDI) = 0.157$), consistent with the SEM results. The surfaces of the NPs were then functionalized by amphiphatic surfactant C18PMH-PEG (Fig. S1†), which is often used in surface modification of inorganic NPs. Since the surfaces of organic NPs are quite hydrophobic, it is possible that C18PMH-PEG would be anchored to the surfaces of TBADN NPs through hydrophobic interactions.\(^{17–19}\)

To verify that TBADN NPs were not solubilized by the surfactant, UV-vis absorption and photoluminescence spectra were measured (Fig. 2). No obvious difference was observed between the NPs and the modified NPs (if the NPs were solubilized by the surfactant, the intensities of the spectra would be much changed). Confocal images also revealed that the NPs still retained the particle shape after modification. The diameters of the NPs experienced a little bit of an increase after surface modification, as shown in Fig. 3a. It should be noted that the modified NPs showed great stability in physiological saline without significant change in the diameter (measured by DLS) even after two days. In marked contrast, unmodified TBADN NPs easily aggregated into bunches of large sediments due to the destruction of surface charges by zwitterions in physiological saline (inset of Fig. 3b). DLS characterization of unmodified NPs also indicated a size change in physiological saline within 25 minutes. Thus, it was demonstrated that surface functionalization by C18PMH-PEG can successfully achieve NPs with good water dispersibility and outstanding stability in an ionic environment with unchanged optical properties, which makes targeted cellular imaging possible.

Surface functionalization can also help to improve the photostability of TBADN NPs. It was also found that modified NPs showed much better photostability than unmodified NPs (Fig. S2†), which could be attributed to the protecting effects of anchored surfactants and outer organic molecules around the particles.\(^{20}\) In addition, it is common sense that steady luminescence is favored for imaging and biological tracking. Traditional dye molecules and QDs often suffer from unsteady fluorescence with alleged luminescence blinking every 100 or 1000 seconds.\(^{21}\) However, organic dye NPs are expected to possess much more stable fluorescence because the NPs are composed of a large number of organic dye molecules and are thus endowed with large number of chromophores, which would ensure a steady luminescence supply.\(^{8,22}\)

The most important requirement for a fluorescence probe is high brightness.\(^{11}\) It is well known that the brightness is determined by the absorption cross-section and the QY. Benefiting from the special molecular structure, TBADN NPs possess QY values up to 15%. Although NPs possess lower QYs than single molecules due to the aggregation-induced self-quenching effect, NPs have larger absorption cross-sections according to the principle that increased size is accompanied by an increase in cross-section.\(^{8}\) Thus it is expected that modified TBADN NPs would exhibit high luminescence (Fig. S3†). A comparison of the confocal images for TBADN NPs and CdSe/ZnS QDs showed...
that TBADN NPs exhibited even higher or at least comparable brightness compared to that of CdSe/ZnS QDs (purchased from WuHan JiaYuan QD CO., LTD, QY higher than 50%), which are commonly used in fluorescent imaging probes.

The cytotoxicity of materials is crucial for their biomedical applications. To evaluate the cytotoxicity of TBADN NPs and modified TBADN NPs, MTT assays were performed on KB and HeLa cell lines. As shown in Fig. 4a, these cell lines still retain high cell viabilities greater than 85% even after 24 h of treatment with the both types of NP with concentrations up to 20 μmol L⁻¹. The above results indicated that the NPs didn’t interfere with the cell physiology and proliferation.

As is well known, targeted cell imaging is highly desirable for early stage cancer diagnosis. Folate receptor (FR) is an attractive cancer cell probe that is over-expressed in many types of cancer cells. To realize FR-mediated targeted cell imaging, we conjugated FA to PEG terminals of C18PMH-PEG polymer and used the obtained C18PMH-PEG-FA to functionalize TBADN NPs. The conjugation of FA onto the terminal of C18PMH-PEG was confirmed by the characteristic absorption peak of FA at ~280 nm (Fig. S4a†). In addition, we also examined the surface charge of the NPs before and after C18PMH-PEG-FA modification by measuring the ζ-potentials, which were −27.33 ± 1.29 mV, −10.90 ± 0.69 mV, and −8.94 ± 0.91 mV for the TBADN NPs, FA-TBADN NPs and C18PMH-PEG-FA (dispersed in water, 0.012 mg mL⁻¹), respectively (Fig. S4b†). All these results suggested that FA was successfully modified onto the surfaces of the NPs. As expected, C18PMH-PEG-FA modified TBADN NPs also showed great water dispersibility and bio-environmental stability (Fig. 4b). KB cells cultured in FA-free medium with high FR expression were used as FR-positive cells incubated with C18PMH-PEG-FA functionalized TBADN NPs. KB cells cultured with C18PMH-PEG functionalized NPs, and observed rather weak fluorescence signals from the blocked cells (Fig. 5e). The above results clearly show highly specific FR targeting by FA modified TBADN NPs.

FR-negative HeLa cells after incubation with either C18PMH-PEG or C18PMH-PEG-FA modified TBADN NPs (Fig. 5b–d). To further demonstrate the specificity of FR-mediated targeting, we blocked the FR receptors on KB cells by adding excessive free FA prior to the cell incubation with C18PMH-PEG-FA modified TBADN NPs, and observed rather weak fluorescence signals from the blocked cells (Fig. 5e). The above results clearly show highly specific FR targeting by FA modified TBADN NPs.

Conclusions

In summary, we report a new strategy of using surface functionalized dye NPs for bioapplications such as targeted cellular imaging. Organic dye (TBADN) was fabricated into pure NPs and this was followed by surface modification with amphipathic surfactant C18PMH-PEG through hydrophobic interactions to achieve good water dispersibility and bio-environmental stability. The resulting functionalized TBADN NPs possess much stronger absorptivity, which contributes much for the brightness enhancement, resulting in similar, if not higher, brightness compared to that of CdSe/ZnS QDs under the same conditions. They also have favorable biocompatibility and can be conjugated with FA for targeted cell imaging, which exhibits significantly enhanced imaging efficacy for detecting specific receptor-positive KB cells with high selectivity. All these results indicate that organic dye NPs obtained by this strategy would be promising fluorescent probes for bioapplications.
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