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One-step preparation of branched PEG functionalized AIE-active luminescent polymeric nanoprobes

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The synthesis of amphiphilic aggregation-induced emission (AIE) dyes based organic nanoparticles has recently attracted increasing attention in the biomedical fields. These AIE dyes based nanoparticles could effectively overcome the aggregation caused quenching effect of conventional organic dyes, making them promising candidates for fabrication of ultrabright organic luminescent nanomaterials. In this work, AIE-active luminescent polymeric nanoparticles (4-NH_2-PEG-TPE-E LPNs) were facilely fabricated through Michael addition reaction between tetraphenylethene acrylate (TPE-E) and 4-arm-poly(ethylene glycol)-amine (4-NH_2-PEG) in rather mild ambient. The 4-NH_2-PEG can not only endow these AIE-active LPNs good water dispersibility, but also provide functional groups for further conjugation reaction. The size, morphology and luminescent properties of 4-NH_2-PEG-TPE-E LPNs were characterized by a series of techniques in detail. Results suggested that these AIE-active LPNs showed spherical morphology with diameter about 100–200 nm. The obtained 4-NH_2-PEG-TPE-E LPNs display high water dispersibility and strong fluorescence intensity because of their self assembly and AIE properties of TPE-E. Biological evaluation results demonstrated that 4-NH_2-PEG-TPE-E LPNs showed negative toxicity toward cancer cells and good fluorescent imaging performance. All of these features make 4-NH_2-PEG-TPE-E LPNs promising candidates for biological imaging and therapeutic applications.

aggregation-induced emission, branched PEG, Michael addition reaction, biological imaging

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1 Introduction

The biomedical applications of luminescent polymeric nanoprobes (LPNs) have attracted great research attention recently [1–7]. As compared with luminescent inorganic nanoprobes such as semiconductor quantum dots, carbon nanodots, luminescent silica nanoparticles, metal nanoclusters and Ln ions doped luminescent nanomaterials, LPNs should be better candidates for biomedical applications because of their bespoke structure, tunable optical properties, ease surface functionalization, disirable biocompatibility, biodegradability, multifunctional potential, etc. [8–25]. Taken advantage of the self assembly of amphipihlic polymers, a large number of LPNs with well designable structure and tunable properties can be fabricated via amalgamation of luminscent agents into these polymers [4,26]. However, most of these LPNs based on conventional organic dyes will encounter the dilemma of encapsulation hydrophobic dyes in the core of LPNs and significant decrease of

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their fluorescent intensity. It is well known that the fluorescent intensity of conventional organic dyes will largely decrease in high concentration or aggregated state, which was also called the aggregation caused quenching (ACQ) effect [27]. Therefore, it is of great challenge to fabricate ultrabright LPNs using conventional organic dyes. Since the first discovery of aggregation-induced emission (AIE) phenomenon by Tang *et al*. in 2001, AIE fluorogens (AIEgens) have raised increasing interest in biomedical applications because of their anti-aggregation-caused quenching (ACQ) effect [28,29]. Significantly different from conventional organic dyes, AIE dyes based nanoparticles could emit strong luminescence when aggregated but very weak in solution owing to the restricted intramolecular rotations (RIRs). The fascinating features of AIEgens make them new promising candidates for biomedical imaging and organic light-emitting diode etc. [30–36]. Various strategies have been developed for fabrication of water dispersible AIE dyes based nanoparticles [31]. And a number of AIE-active LPNs have recently been fabricated through surfactant assisted self assembly, formation of Schiff base, reversible addition-fragmentation chaintransfer (RAFT) polymerization and ring-opening reaction [37–45]. Although much progress has been made in fabrication of AIE dyes based LPNs, many of these strategies still have some shortcomings, which include stringent reaction conditions, complex experimental procedure and long reaction time. Therefore, it is still necessary to develop facile and efficient strategies for fabrication of novel AIE-active LPNs with outstanding properties.

PEG is a hydrophilic polymer, which has been extensively utilized for biomedical applications for its water dispersibility, non-immunogenicity and biocompatibility. It has been demonstrated that PEGylated nanomaterials could not only possess enhanced water dispersibility and biocompatibility, but also influence the pharmacokinetic behavior of nanomaterials [46]. The possible reasons can be attributed to the surface PEGylation, which can significantly decrease the interaction between nanomaterials and plasma proteins, and therefore avoid the accumulation of nanomaterials in reticuloendothelial system and largely prolong the blood circulation time [47,48]. Over the past few decades, surface PEGyaltion of a large number of nanomaterials with different compositions, size and shape has been investigated and examined for different purposes [49–53]. The preparation of PEGylated AIE dyes based nanoparticles have also been done by some groups in recent years. For example, Tang *et al*. [54,55] have prepared the AIE dyes based PEGylated LPNs via self assembly of PEG contained amphiphilic polymers and AIE dyes. Zhang *et al*. [56,57] have also fabricated some PEGylated AIE dyes based LPNs through covalent conjugation and controlled living polymerization. As compared with the linear PEG, branched PEG possesses different topologic structure and high density of functional groups, which could provide more active sites for further conjugation reaction and also different pharmacokinetic behavior of PEGylated materials [46]. However, to the best of our knowledge, the preparation of PEGylated AIE-active nanoprobes using branched PEG has been seldom reported.

In this work, we developed a facile and efficient strategy for fabrication of branched PEG functionalized AIE-active LPNs through one-step Michael addition reaction between the ene bond of AIE dye and amino groups of aminated branched PEG. The Michael addition reaction can occur under rather mild conditions such as low temperature, short reaction time and absent metal catalysts. Because of their amphiphilic properties, these AIE-active copolymers modified with branched PEG will self assembly into 100–200 nm spherical nanoparticles in aqueous solution, in which the AIE dye (TPE-E) was aggregated in the core, while the hydrophilic PEG was covered on their surface. Therefore, the obtained 4-NH_2-PEG-TPE-E LPNs can emit strong blue-green fluorescence and good dispersibility in pure aqueous solution. Cytocompatibiity and cell uptake evaluation results indicated that 4-NH_2-PEG-TPE-E LPNs can be internalized into cells and exhibited almost nontoxicity toward HepG2 cells. Considering these advantages, these branched PEG modified AIE-active LPNs should hold great potential for different biomedical applications.

2 Experimental

2.1 Materials and characterization

Bromotriphenylethylene, tetrakis palladium (triphenylphosphine), etrabutyl ammonium bromide (TBAB), 4-carboxybenzeneboronic acid, methyl alcohol, 4-arm-poly(ethylene glycol)-amine (4-PEG-NH_2; M_w: 6000), and acryloyl chlorid (99%) purchased from Aladdin (China) were used as received. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolim assays (MTS; Promega, USA), anhydrous tetrahydrofuran (THF; J&K Chemical, China), ethyl acetate (J&K Chemical, China) and triethylamine (Et_3N; 99.5%; J&K Chemical, China) were used without further purification. Penicillin-streptomycin, fetal bovine serum (FBS) and trypsin-EDTA solution were purchased from Sigma-Aldrich (USA).

The UV-Vis absorption spectrum of 4-NH_2-PEG-TPE-E LPNs was measured using a Perkin Elmer LAMBDA 35 UV-Vis system (USA). Photoluminescence (PL) spectra were recorded on a PELS-55 spectrometer (Perkin-Elmer, USA). The morphology and size of the samples was examined by transmission electron microscopy (TEM), and the specimens were made by dropping a drop nanoparticle suspension on a carbon-coated copper grid. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were measured on a Bruker Avance-400 spectrometer (Germany) using D_2O or $CDCl_3$ as solvent and tetramethylsilane (TMS) as internal standard. The Fourier transform infrared (FTIR) spectra

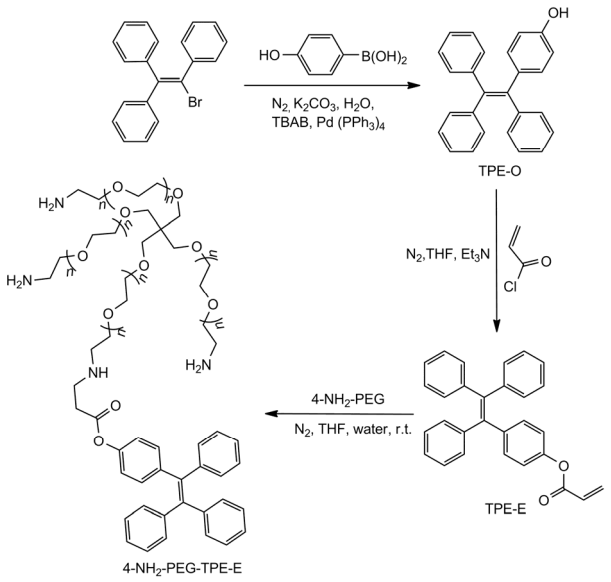
were carried out by using a Nicolet 380 Fourier transform spectrometer (USA) with a resolution of 2 cm^{-1}. The hydrodynamic size distribution of 4-NH_2-PEG-TPE-E LPNs in phosphate buffer solution (PBS) was characterized by using a Zeta Plus apparatus (ZetaPlus, Brookhaven Instruments, USA).

2.2 Synthesis of 4-NH_2-PEG-TPE-E LPNs

4-NH_2-PEG-TPE-E LPNs were prepared through direct Michael addition reaction between the amino group of 4-NH_2-PEG and the ene bond of TPE-E (Scheme 1). Detailed procedures for the synthesis of tetraphenyl ethylene-acrylate (TPE-E) were described in the supplementary information. The final products (4-NH_2-PEG-TPE-E LPNs) were synthesized by one-step Michael addition reaction. In brief, the mixture of TPE-E (30 mg) dissolved in 6 mL THF and 4-NH_2-PEG (500 mg) dissolved in 4 mL methyl alcohol were stirred at room temperature for 8 h (monitored by TLC until no TPE-E was rest). Then, the polymer solution was washed with water and extracted with ethyl acetate. After dried through anhydrous Na_2SO_4, the organic agent was removed on a rotary evaporator at 50 °C under vacuum to obtain pure 4-NH_2-PEG-TPE-E LPNs. The chemical structure of 4-NH_2-PEG-TPE-E LPNs was determined by ^{1}H NMR (D_2O as solvent) and FTIR, and the optical properties were measured by UV-Vis spectroscopy and fluorescent spectroscopy.

2.3 Cell viability evaluation of 4-NH_2-PEG-TPE-E LPNs

The cell viability of 4-NH_2-PEG-TPE-E LPNs toward HepG2 cells was evaluated by cell counting kit-8 (CCK-8) assay [58,59]. Briefly, cells were put into 96-well microplates at a density of 5×10^4 cells mL^{-1} in 160 μL of respective media containing 10% FBS. After HepG2 cells were seeded in 96-well tissue culture plates at a density of 4×10^4 cells $well^{-1}$ for 24 h, the medium was replaced by medium containing various concentrations of 4-NH_2-PEG-TPE-E LPNs (0, 10, 20, 40, 80, 120 μg mL^{-1}), and then the cells were incubated for 8 and 24 h, respectively. Then nanoparticles were removed and cells were washed with PBS three times. 10 μL of CCK-8 dye and 100 μL of DMEM cell culture medium were added to each well and incubated for 2 h at 37 °C. Afterward, plates were analyzed using a microplate reader (VictorIII, Perkin-Elmer, USA). Measurements of formazan dye absorbance were carried out at 450 nm, with the reference wavelength at 620 nm. The values were proportional to the number of live cells. The percent reduction of CCK-8 dye was compared to controls (cells not be exposed to hydrophilic LONs), which represented 100% CCK-8 reduction. Three replicate wells were used per microplate, and the experiment was operated for three times. Cell survival was expressed as absorbance relative to that of untreated controls. Results are presented as mean±standard deviation (SD).



Schemes 1 The synthesis of TPE dyes and preparation of 4-NH_2-PEG-TPE-E LPNs through Michael addition reaction between the double bond of TPE-E and the amino groups of 4-NH_2-PEG.

2.4 Cellular uptake of 4-NH_2-PEG-TPE-E LPNs

HepG2 cells were cultured in DMEM medium chamber containing 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C in a humidified environment containing 5% CO_2. After cells attachment, the medium was removed and the cells were washed three times with PBS buffer. Then 20 μg mL^{-1} of 4-NH_2-PEG-TPE-E LPNs dispersing in DMEM medium without FBS was added into the chamber. After incubation for 3 h, cells were washed three times with PBS buffer to remove fluorescence nanoparticles that did not enter cells. Finally, cell images were obtained using a confocal laser scanning microscope (CLSM) Zesis 710 3-channel (Zesis, Germany) with the excitation wavelength of 405 nm.

3 Results and discussion

The tetraphenyl ethylene-acrylate (TPE-E) was synthesized through two steps. The successful synthesis of TPE-E was confirmed by ^{1}H NMR spectroscopy (Figure S1, Supporting Information online). The presence of a series of peaks between 7.39–7.02 ppm due to the protons of aromatic ring, and two typical peaks at 6.25 and 5.84 ppm can be ascribed to protons of carbon-carbon double bonds (C=C). These results indicated successful formation of TPE-E. The chemical structure of the final products (4-NH_2-PEG-TPE-E LPNs) was also confirmed by ^{1}H NMR spectrum. As shown in Figure 1(A), typical peaks of –C=C were replaced by a characteristic singlet at around δ=2.60 ppm owing to meth-

ylene group of the side chain connected with benzyl (–CH_2–CH_2–COO–). Furthermore, the characteristic peaks of 4-NH_2-PEG chain (3.40–3.74 and 2.85 ppm) were also observed in the sample of 4-NH_2-PEG-TPE-E. All of these results indicate that TPE-E was successfully conjugated with 4-NH_2-PEG through Michael addition reaction. The ^{13}C NMR is displayed in the Figure S2. It can be seen that the signals from δ 131.9–122.1 ppm were observed, indicating the presence of benzene. On the other hand, the strong quaternary carbon of 4-NH_2-PEG signals at δ 70.4, 70.1 and 76.5 ppm were also found. The characteristic peak of –C–NH– at δ 45.2 ppm was detected. All these suggested that 4-NH_2-PEG has been conjugated to the TPE-E successfully. To further comfirm the successful fabrication of these branched PEG functionalized AIE-active nanoparticles, the chemical structure information of TPE-E and 4-NH_2-PEG-TPE-E was further determined by FTIR spectra. As shown in Figure 1(B), four peaks at 1668–1390 cm^{-1} were observed, which can be attributed to the stretching vibration of aromatic rings of TPE-E (marked with arrows). On the other hand, strong bending vibration peak of –C=O at about 1740 cm^{-1} and the characteristic peak of –C–O– at 1027 cm^{-1} were also observed in the samples of TPE-E. Furthermore, the typical peak of –C=C– was found at 966 cm^{-1}. All of these results confirmed the successful synthesis of TPE-E. However, in the FTIR spectrum of 4-NH_2-PEG-TPE-E, the characteristic peak of C–N stretch was observed at 1348 cm^{-1}. And the stretching vibration bond at 1105 cm^{-1} was also significantly enhanced, implying that more C–O–C existed in the sample of 4-NH_2-PEG-TPE-E. Moreover, a strong peak at 2879 cm^{-1} emerged in the sample of 4-NH_2-PEG-TPE-E, which can be assigned to the –CH_2 and free NH^{3+} groups of 4-NH_2-PEG. The comparison of FTIR spectra clearly demonstrated that 4-NH_2-PEG was indeed conjugated with TPE-E through Michael addition reaction. More importantly, due to the existence of free amino groups, many functional components (e.g. targeting agents, drugs and other imaging agents) can also be further linked onto these AIE-active LPNs. Therefore, the free amino groups should be useful for fabrication of multifunctional AIE-active nanotheranostic systems.

It is well known that PEG is a hydrophilic polymer, which has been extensively utilized for fabrication of PEGylated nanomaterials for biomedical applications [56,57]. In this work, the amphiphilic polymers can be obtained after successful conjugation of hydrophobic TPE with hydrophilic PEG. These branched PEG functionalized AIE copolymers tend to self assemble into nanoparticles in pure aqueous solution. The morphology and size of these AIE-active assemblies were examined by TEM characterization. As shown in Figure 2, spherical assemblies with size ranged from 100–200 nm can be clearly observed. The successful formation of spherical nanoparticles is likely due to the encapsulation of hydrophobic AIE dye in the core of nanoparticles, while the hydrophilic PEG was coated on their surface to endow them good water dispersibility. Furthermore, the molar ratio of TPE-E to 4-NH_2-PEG was calculated to be about 2:1 based on the ^{1}H NMR characterization. Therefore, there are still a large number of free amino groups on the surface of the 4-NH_2-PEG-TPE-E LPNs. And these amino groups are of great importance for the further conjugation reaction and fabrication of multifunctional AIE-active nanotheranostic systems.

Due to the self assembly of 4-NH_2-PEG-TPE-E LPNs in pure aqueous solution, the AIE dye (TPE-E) will aggregate in the core of these polymeric nanoparticles and therefore emit strong fluorescence for the unique AIE properties of TPE-E (inset (a) in Figure 3). On the other hand, the 4-NH_2-PEG-TPE-E suspension is also very stable in water because hydrophilic PEG was covered on the surface of 4-NH_2-PEG-TPE-E LPNs (inset (b) in Figure 3). No precipitation was observed after 4-NH_2-PEG-TPE-E LPNs were dispersed in water for more than 48 h. The excellent water dispersibility of 4-NH_2-PEG-TPE-E LPNs is very important for the biomedical applications. Figure 3 shows UV-Vis absorption spectrum of 4-NH_2-PEG-TPE-E in water. There is a maximum absorption peak at 274 nm due to the π-π* electronic transition of the TPE-based triazoles. Besides, the absorption band raised from 800 nm because of the light scattering of 4-NH_2-PEG-TPE-E LPNs. All of these results confirmed that 4-NH_2-PEG-TPE-E can self assemble into nanoparticles, which exhibited high water dispersibility and

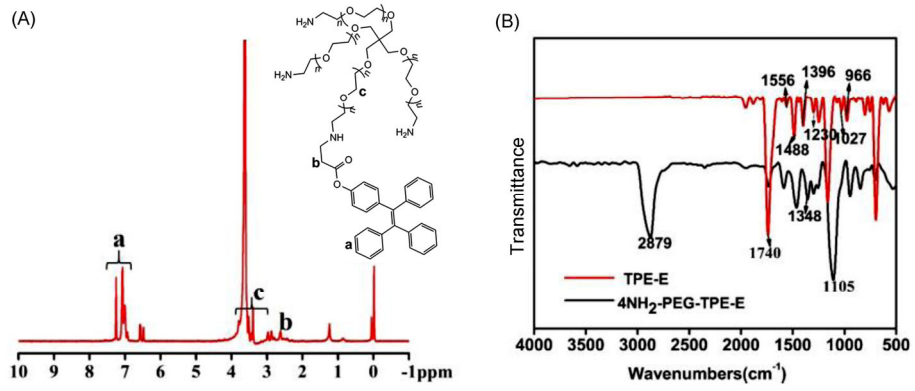


Figure 1 (A) ^{1}H NMR spectrum of 4-NH_2-PEG-TPE-E; (B) FTIR spectra of TPE-E and 4-NH_2-PEG-TPE-E.

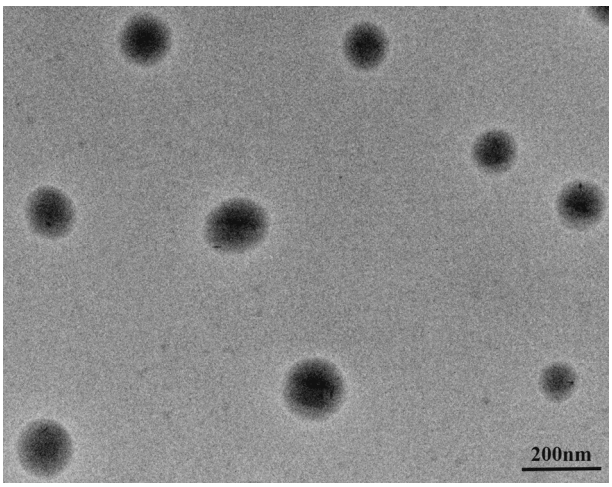


Figure 2 Representative TEM image of 4-NH_2-PEG-TPE-E LPNs. The scale bar=200 nm. It can be seen that these amphiphilic AIE dye contained copolymers can self assemble into spherical nanoparticles with size about 100–200 nm.

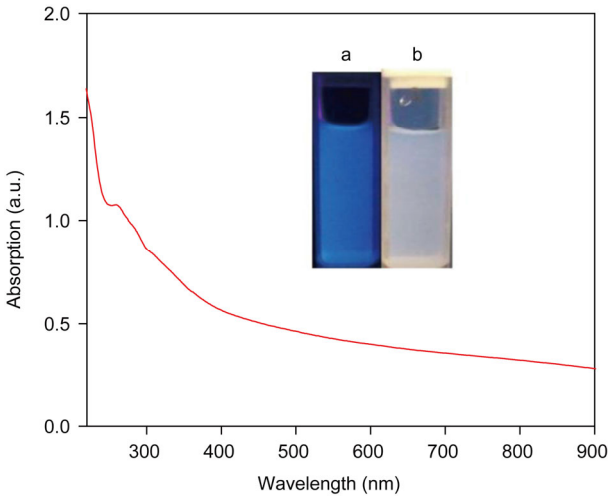


Figure 3 UV-Vis spectrum of 4-NH_2-PEG-TPE-E LPNs. The insets are the photographs of water suspension of 4-NH_2-PEG-TPE-E LPNs. Inset: (a) the suspension was irradiated by UV lamp at λ=365 nm; (b) water suspension of 4-NH_2-PEG-TPE-E LPNs did not irradiate by UV lamp (color online).

remarkable AIE properties. The hydrodynamic size of 4-NH_2-PEG-TPE-E LPNs in PBS was determined to be 212.5±12.4 nm with 9 olydispersity index of 0.05. The value is much close to the size by TEM characterization. These results implied that 4-NH_2-PEG-TPE-E LPNs possess excellent water dispersibility and solution stability.

The PL property of 4-NH_2-PEG-TPE-E LPNs in water was further determined by PL spectroscopy. As shown in Figure 4(a), the maximum emission wavelength was located at 433 nm using 365 nm as the exitation wavelength. And the maximum excitation wavelength was located at 293 nm when the emission wavelength was set at 433 nm. On the other hand, when 4-NH_2-PEG-TPE-E LPNs were excited by different wavelength (from 290 to 336 nm), the emission wavelength did not change. The broad range of excitation wavelength was also reported by Zhang *et al*. [57]. Because of the independent and narrow emission wavelength and the broad excitation wavelength, various fluorescent materials can be exhibited by one excitation wavelength at the same time, and then emitted different fluorescence, which has great applied potential in fluorescence labeling. Quantum yield (QY) is a very important parameter of luminescent materials. In this work, the QY of 4-NH_2-PEG-TPE-E LPNs was examined using quinine sulfate as the reference dye. We demonstrated that the QY of 4-NH_2-PEG-TPE-E LPNs is as high as 26.4%. More importantly, the fluorescence intensity of 4-NH_2-PEG-TPE-E LPNs had almost no decrease after 4-NH_2-PEG-TPE-E LPNs were irradiated by a UV lamp at 365 nm for 1 h (Figure 4(b)). Considering the unique AIE properties, high QY and good photostability, these PEGylated AIE-active polymeric nanoparticles should be promising candidates for different biomedical applications.

As a critical issue in fluorescence imaging of cells, the cytotoxicity of 4-NH_2-PEG-TPE-E LPNs was evaluated using CCK-8 assay to determine the metabolic viability of HepG2 cells. After incubation with different concentrations of 4-NH_2-PEG-TPE-E LPNs for 8 and 24 h, the cell viability values remain above 98% (Figure S3). No significant difference was observed in cell proliferation when the con-

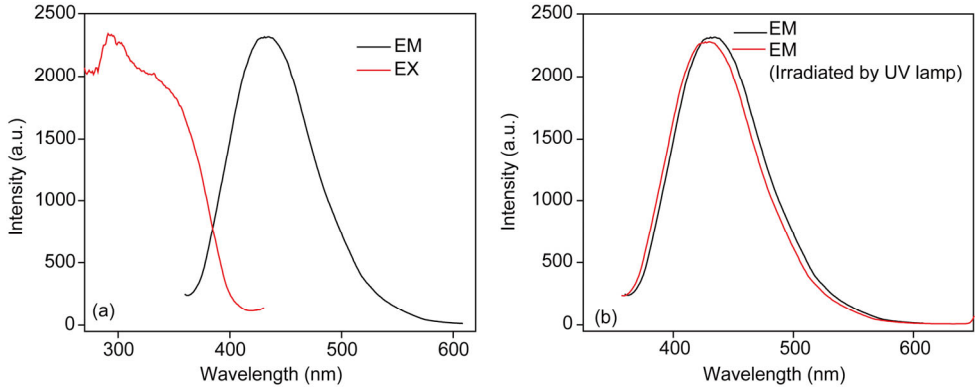


Figure 4 Fluorescence excitation (Ex) and emission (Em) spectra of 4-NH_2-PEG-TPE-E LPNs (a) and 4-NH_2-PEG-TPE-E LPNs irradiated by a UV lamp at 365 nm (b). λ_{Ex}=293 nm, λ_{Em}=433 nm (color online).

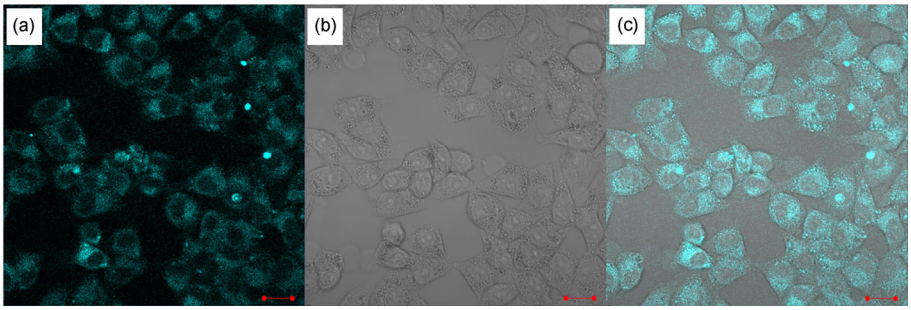


Figure 5 Cell uptake behavior of 4-NH_2-PEG-TPE-E LPNs examined by CLSM. The HepG2 cells were incubated with 20 μg mL^{-1} of 4-NH_2-PEG-TPE-E LPNs for 3 h. (a) Excited with 405 nm laser; (b) bright field; (c) merge image of (a, b). Scale bar=20 μm (color online).

centration was up to 120 μg mL^{-1} for 24 h, indicating low cytotoxicity in the cells. The results indicated that 4-NH_2-PEG-TPE-E LPNs can be applied in cell fluorescence imaging applications. Therefore, the cell uptake behavior of 4-NH_2-PEG-TPE-E LPNs was subsequently to examine the potential application of 4-NH_2-PEG-TPE-E LPNs by CLSM. After cells were incubated with 20 μg mL^{-1} of 4-NH_2-PEG-TPE-E LPNs for 3 h, strong fluorescence signal could be observed at the cell location, and no significant background interference was detected (Figure 5(a)). Compared with cells imaging in bright field (Figure 5(b)), most of the cells was labeled with blue fluorescence shown in sectional confocal image of Figure 5(a, b) (Figure 5(c)). On the other hand, cells still adhered to the cell plates very well and kept their morphology (Figure 5(b)), further confirming the good cytocompatibility of 4-NH_2-PEG-TPE-E LPNs. Moreover, a number of free amino groups existed on the surface of 4-NH_2-PEG-TPE-E LPNs, which can be used for further conjugation reaction with other functional components. Due to the lack of targeting agents on the surface of 4-NH_2-PEG-TPE-E LPNs, these PEGy-lated AIE-active nanoprobes should be internalized by HepG2 cells through nonspecific endocytosis. If the surface of 4-NH_2-PEG-TPE-E LPNs were further linked with the targeting agents, the dosage of 4-NH_2-PEG-TPE-E LPNs can be further reduced. Finally, the concentration for cell imaging is much lower than the biocompatible concentrations (120 μg mL^{-1}). Therefore, we could conclude that the 4-NH_2-PEG-TPE-E LPNs are biocompatible enough for biomedical applications.

4 Conclusions

A very simple, efficient and versatile method was developed to synthesize amphiphilic PEGyalted AIE-active polymeric nanoprobes through a one-step Michael addition reaction, which was reacted between the amino groups of 4-NH_2-PEG and the ene bond of TPE-E. The resulting copolymers could self assemble into 100–200 nm spherical nanoparticles with strong fluorescent intensity and high water dispersibility. More importantly, a number of free amino groups existed on the surface of 4-NH_2-PEG-TPE-E LPNs, which can be utilized for further conjugation reaction. Biological evaluation results demonstrated that 4-NH_2-PEG-TPE-E LPNs can be uptaken by HepG2 cells and exhibit negative toxicity effects. As compared with previous strategies, the one-step Michael addition reaction can occur uder rather mild experimental conditions, such as low temperature, air atmosphere and without using hazardous metal catalysts and reagents. Therefore, taken advantage of the properties of branched PEG and AIE dye, these branched PEG functionalized AIE-active polymeric nanoparticles fabricated from one-step Michael addition reaction should be promising candidates for biological sensor, drug/gene delivery and multifunctional nanotheranostic systems.

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Conflict of interest The authors declare that they have no conflict of interest.

Supporting information The supporting information is available online at http://chem.scichina.com and http://link.springer.com/journal/11426. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.

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