

Tetraphenylethene End-capped Polyethylenimine Fluorescent Nanoparticles for Cell Imaging*

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Abstract Water soluble tetraphenylethene-based (TPE) aggregation-induced emission fluorescent organic nanoparticles (FONs) were facilely prepared *via* Schiff base condensation with polyethylenimine (PEI) and subsequent reduction. The obtained TPE-PEI FONs were characterized by a series of techniques including ¹H-NMR, ¹³C-NMR, gel permeation chromatography, UV absorption spectra, fluorescence spectra, Fourier transform infrared spectroscopy, size distribution and zeta potential measurement, and transmission electron microscopy. Biocompatibility evaluation and cell imaging of TPE-PEI FONs were further explored. We demonstrated that such FONs showed intense fluorescence, spherical morphology and excellent biocompatibility, making them very suitable for cell imaging application.

Keywords: Aggregation-induced emission; Polyethylenimine; Schiff base condensation; Fluorescent organic nanoparticles; Cell imaging.

INTRODUCTION

Aggregation-induced emission (AIE) materials have attracted more and more research interest because of their extraordinary anti-aggregation-caused quenching effect^[1–3]. A wide variety of AIE unit based conjugated molecules such as siloles^[4, 5], cyano-substituted diarylethylene^[6–10], tetraphenylethene^[11–20], triphenylethene^[21–26] and distyrylanthracene derivatives^[27–30] have been developed and utilized for bioimaging and chemosensor applications. Compared with the current used optical bioprobes including quantum dots (QDs)^[31–34], fluorescent proteins^[35] and other fluorescent inorganic probes^[36–39], fluorescent organic nanoparticles (FONs) have recently received increasing attentions for cell imaging due to the biodegradation potential and flexible synthetic approaches based on small organic molecules^[40–44]. In this case, ionic luminophores have often been used for cell imaging because of their good water miscibility. However, their inherent charges can perturb membrane potential and even kill the cell at high concentrations.

An alternative solution is to introducing non-ionic AIE derivatives into FONs to fabricate biocompatible probes. Previously, we reported on water soluble and biocompatible fluorescent organic nanoparticles based on surfactant and synthetic polymers modified AIE material for cell imaging application^[45, 46]. However, it is still difficult to control the size and morphology of such AIE organic nanoparticles. A alternative strategy to solve

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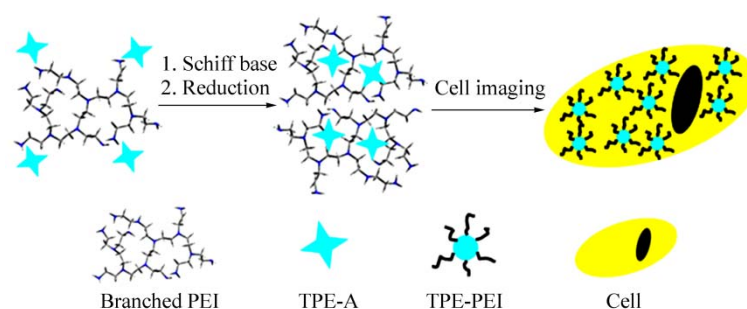
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this problem emerged through the combination of AIE luminophores and silica nanoparticles, the results demonstrated that uniformly sized, surface-charged and colloidally stable FONs could be prepared and utilized for cell imaging^[47–49]. Nevertheless, the previous silica FONs often suffered from non-biodegradable nature, which would limited their practical bioimaging applications due to the potential toxicity to living organisms.

Polyethylenimine (PEI) is a synthetic polymer with repeating unit composed of the amine group and two carbon aliphatic ethyl spacers. Particularly, branched PEI contains primary, secondary and tertiary amino groups, which finds many applications in detergents, adhesives, water treatment agents, and cosmetics. PEI is still a polymeric transfection agent and extremely appealing for building siRNA carriers^[50–53].

In this study, we utilized the Schiff base condensation of hydrophobic 4-(tetraphenylethene)benzaldehyde (TPE-A) and hydrophilic branched PEI to form a tetraphenylethene-based (TPE) intermediate, which was then reduced by sodium borohydride to afford stable product with carbon-nitrogen single bond (TPE-PEI). The obtained TPE-PEI showed amphiphilic property and obvious AIE performance, which could form strong fluorescent emission TPE-core micelles in water circumstance. Then the resulting FONs were utilized for cell imaging. The TPE-PEI FONs preparation process is shown in Scheme 1. The surface morphology of the synthesized FONs was characterized, which exhibit colloidally stable and spherical morphology. The biocompatibility of such AIE FONs and cell imaging characterization were further investigated to study the potential biomedical applications.



Scheme 1 Schematic showing the process of TPE-PEI FONs preparation and their utilization for cell imaging

EXPERIMENTAL

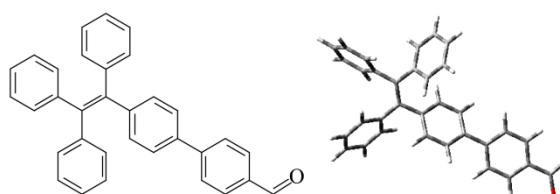
Materials and Characterization

Benzophenone, *n*-butyl lithium (*n*-BuLi) in hexane (2.4 mol/L), 4-formylphenylboronic acid, tetrabutyl ammonium bromide (TBAB), tetrakis(triphenylphosphine) palladium(0) [Pd(PPh₃)₄], diphenylmethane, 4-bromophenyl phenylmethanone and *p*-toluenesulfonic acid were purchased from Alfa Aesar and used as received. All other agents and solvents were purchased from commercial sources and used directly without further purification. Tetrahydrofuran (THF) was distilled from sodium/benzophenone. Ultra-pure water was used in the experiments. ¹H-NMR and ¹³C-NMR spectra were measured on a JEOL 400 MHz spectrometer with CDCl₃ as solvent and tetramethylsilane (TMS) as the internal standard. UV-Visible absorption spectra were recorded on a UV/Vis/NIR Perkin-Elmer lambda750 spectrometer (Waltham, MA, USA) using quartz cuvettes of 1 cm path length. Fluorescence spectra were measured on a PE LS-55 spectrometer with a slit width of 3 nm for both excitation and emission. The FTIR spectra were obtained in a transmission mode on a Perkin-Elmer Spectrum 100 spectrometer (Waltham, MA, USA). Typically, 8 scans at a resolution of 1 cm^{−1} were accumulated to obtain one spectrum. The size distribution and zeta potential measurement of TPE-PEI FONs in phosphate buffer solution (PBS) were determined using a zeta Plus apparatus (ZetaPlus, Brookhaven Instruments, Holtsville, NY). Transmission electron microscopy (TEM) images were recorded on a JEM-1200EX microscope operated at 100 kV, the TEM specimens were made by placing a drop of the nanoparticles suspension on a carbon-coated copper grid. The size distribution and zeta potential measurements of TPE-PEI FONs in

phosphate buffer solution were determined using a zeta Plus apparatus (ZetaPlus, Brookhaven Instruments, Holtsville, NY). Fluorescence quantum yields, measured in THF, THF/water mixture and water using 9,10-diphenylanthracene as standard, excited at 365 nm. Gel permeation chromatography (GPC) analyses of polymers were performed using DMF as the eluent. The GPC system was a Shimadzu LC-20AD pump system comprising of an auto injector, a MZ-Gel SDplus 10.0 mm guard column (50 mm $\times$ 8.0 mm, 10 nm) followed by a MZ-Gel SDplus 5.0 μ m bead-size columns (5–10⁵ nm, linear) and a Shimadzu RID-10A refractive index detector. The system was calibrated with narrow molecular weight distribution polystyrene standards ranging from 200 g·mol⁻¹ to 10⁶ g·mol⁻¹.

Preparation of TPE-PEI

The intermediates TPE-A were prepared according to the literature methods^[30]. The chemical structure of TPE-A is presented in Scheme 2.



Scheme 2 The chemical structure (left) and the optimization geometry (right) of TPE-A

Branched PEI (M_w = 600, 0.040 g) was dissolved in 10 mL of ultra-pure water. TPE-A (0.10 g) in 10 mL THF was introduced to the PEI solution at room temperature. The mixture was allowed to react for 10 min under stirring. Then sodium borohydride (0.050 g) was added into the mixture, the reaction was carried out for 0.5 h to obtain TPE-PEI. And then the mixture was evaporated to completely remove the organic agent (THF) on a rotary evaporator at 40 °C. To remove the excess sodium borohydride, the TPE-PEI water dispersion was treated by repeated centrifugal washing process for three times.

Cytotoxicity of TPE-PEI

Cell morphology was observed to examine the effects of TPE-PEI FONs to A549 cells^[54]. Briefly, cells were seeded in 6-well microplates at a density of 1×10^5 cells·mL⁻¹ in 2 mL of respective media containing 10% fetal bovine serum (FBS). After cell attachment, plates were washed with PBS and cells were treated with complete cell culture medium, or different concentrations of TPE-PEI FONs prepared in 10% FBS containing media for 8 h and 24 h. Then all samples were washed with PBS three times to remove the uninternalized FONs. The morphology of cells was observed by using an optical microscopy (Leica, Germany), the overall magnification was $\times 100$.

The cell viability of TPE-PEI FONs on A549 cells was evaluated by cell counting kit-8 (CCK-8) assay based on our previous reports^[55]. Briefly, cells were seeded in 96-well microplates at a density of 5×10^4 cells·mL⁻¹ in 160 μ L of respective media containing 10% FBS. After 24 h of cell attachment, the cells were incubated with 5, 10, 20, 40, 60 μ g·mL⁻¹ TPE-PEI for 8 h and 24 h. Then nanoparticles were removed and cells were washed with PBS three times. 10 μ L of CCK-8 dye and 100 μ L of DMEM cell culture medium were added to each well and incubated for 2 h at 37 °C. Plates were then analyzed with a microplate reader (VictorIII, Perkin-Elmer). 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 exposure to TPE-PEI FONs), which represented 100% CCK-8 reduction. Three replicate wells were used per microplate, and the experiment was repeated three times. Cell survival was expressed as absorbance relative to that of untreated controls. Results are presented as (mean $\pm$ standard deviation (SD)).

Confocal Microscopic Imaging of Cells Using TPE-PEI

The cell uptake of TPE-PEI FONs was evaluated by confocal microscopic imaging according to our previous reports^[56]. On the day prior to treatment, cells were seeded in a glass bottom dish with a density of 1×10^5 cells per dish. On the day of treatment, the cells were incubated with TPE-PEI FONs at a final concentration of $10 \mu\text{g}\cdot\text{mL}^{-1}$ for 3 h at 37 °C. Afterward, the cells were washed three times with PBS to remove the TPE-PEI FONs and then fixed with 4% paraformaldehyde for 10 min at room temperature. Cell images were taken with a confocal laser scanning microscope (CLSM) Zeiss 710 3-channel (Zeiss, Germany) with the excitation wavelengths of 405 nm.

RESULTS AND DISCUSSION

Characterization of TPE-PEI

The AIE fluorogen TPE-A was prepared following our previous report^[30]. The AIE property of TPE-A is possible ascribed to the restricted intramolecular rotation of tetraphenylethene group as proposed by Tang *et al.*^[1]. The optimization of TPE-A was obtained by a Gaussian 03 software at the B3LYP/6-31G level, which demonstrated the twist structure of TPE-A (Scheme 2, right)^[57]. This AIE fluorogen was introduced into the branched PEI by Schiff base condensation and subsequent reduction of sodium borohydride to afford TPE-PEI. ¹H-NMR spectra recorded for TPE-A, PEI and TPE-PEI are shown in Fig. 1(a), it could be found that the

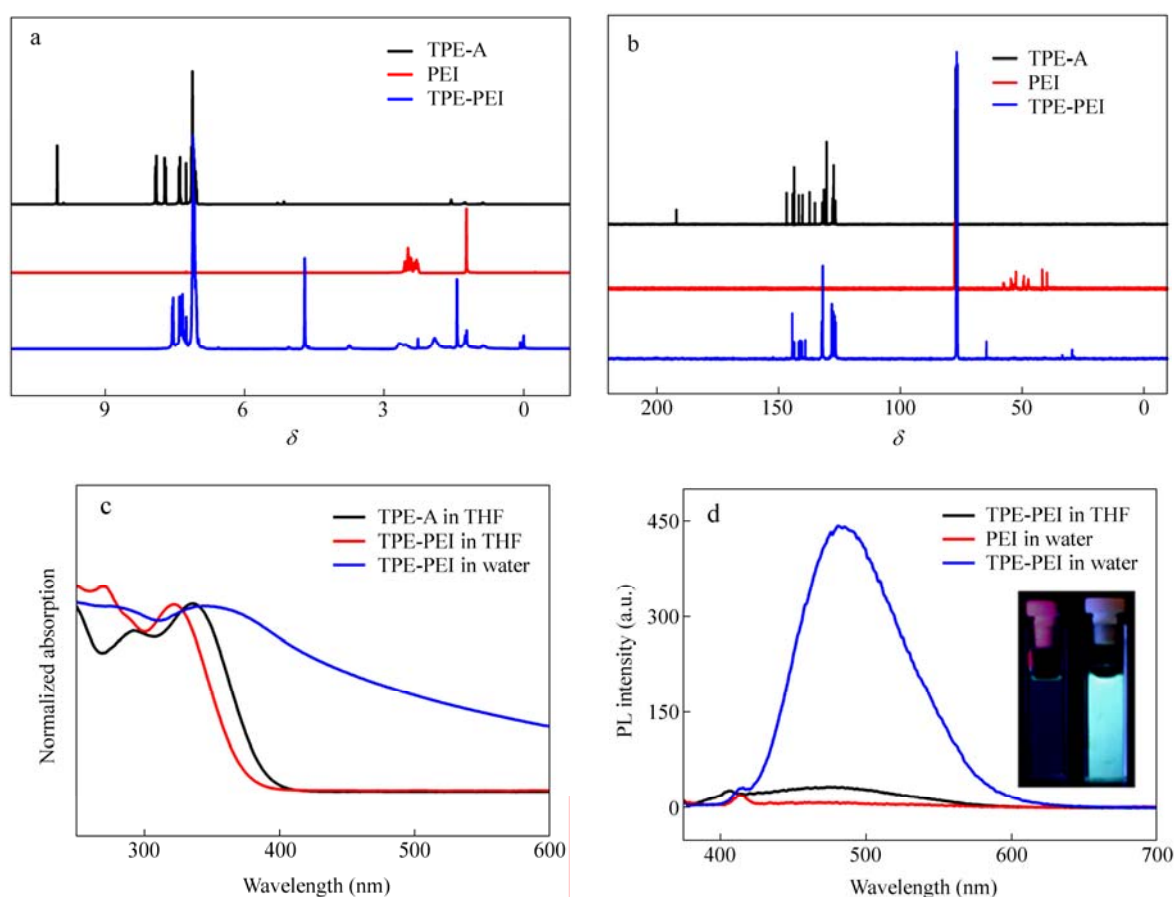
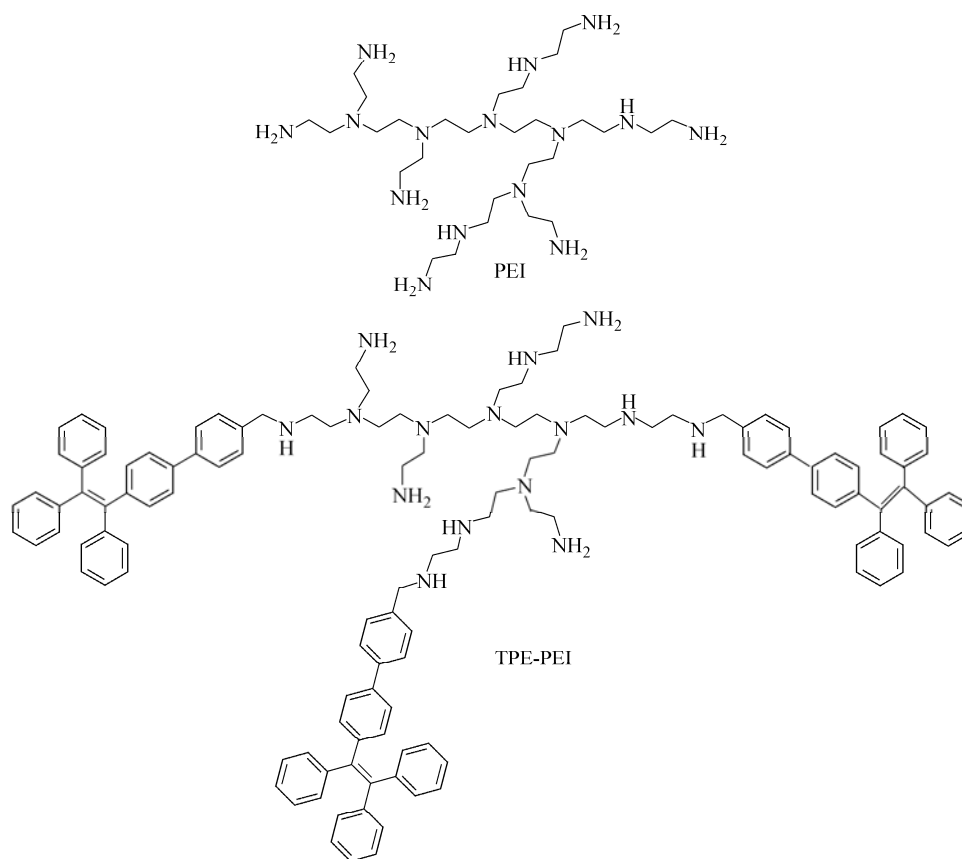


Fig. 1 (a) ¹H-NMR spectra recorded for TPE-A, PEI and TPE-PEI; (b) ¹³C-NMR spectra recorded for TPE-A, PEI and TPE-PEI; (c) UV absorption spectra of TPE-A and TPE-PEI in THF, and TPE-PEI in water; (d) PL spectra of TPE-PEI in THF, and PEI and TPE-PEI in water (Inset: Fluorescence image of TPE-PEI in THF (left) and water (right) taken at 365 nm UV light)

aldehyde peak was disappeared in TPE-PEI, meanwhile, the methylene group on the benzene ring emerged at 4.7 of chemical shift, suggesting successful reaction of TPE-A and PEI and subsequent reduction. The ^{13}C -NMR spectra of TPE-A, PEI and TPE-PEI were also conducted and shown in Fig. 1(b), obvious disappearance of aldehyde peak at $\delta=192$ of chemical shift could be found. From the ^1H -NMR spectrum of TPE-PEI, the ratio of TPE and PEI could be calculated as 3:1. In this case, the structure of PEI and the speculated structure of TPE-PEI have been presented in Scheme 3. The molecular weight of TPE-PEI has been calculated from gel permeation chromatography (GPC), result showed that the M_n value of TPE-PEI was 2100 Da. The ratio of TPE and PEI calculated from the GPC result also approximately suggested 3:1, which was consistent with the ^1H -NMR result. UV absorption spectra of TPE-A and TPE-PEI in THF, and TPE-PEI in water were shown in Fig. 1(c). The maximum absorption wavelength of TPE-A in THF located at 336 nm, while the maximum absorption wavelength of TPE-PEI in THF was observed with some blue-shift to 322 nm. The maximum absorption peak of TPE-PEI in water had obvious red-shift to 348 nm. It is noteworthy that the entire spectrum of TPE-PEI started to increase from 600 nm in absorption due to Mie effect, indicating the formation of nanoparticles in the solution. The AIE feature was well maintained in TPE-PEI, which is shown in Fig. 1(d). The fluorescence intensity increased from 35 a.u. in THF to 440 a.u. in water with an approximate 13-fold increasement, which evidenced obvious AIE characteristic. A control experiment without TPE-A was carried out to further prove the fluorescence came from the TPE-A, instead of PEI (Fig. 1d, the curve of “PEI in water”). The inset fluorescence image also gave a direct evidence of the AIE performance of TPE-PEI.



Scheme 3 The structure of PEI and speculated structure of TPE-PEI

The AIE characteristic of TPE-PEI was also determined in THF-water mixtures (Figs. 2a and 2b). As increasing the water fraction in the mixed solvent system could change its existing form from a well-dispersed

state in the pure THF to aggregated nanoparticles in the mixtures with high water content, the fluorescence intensity showed a significant increase. The fluorescence quantum yields of TPE-PEI nanoparticles in water and TPE-A in 99% water fraction of THF-water mixture were determined as 30% and 39% respectively, while the fluorescence quantum yield of TPE-PEI and TPE-A in pure THF were calculated as 0.21% and 0.24% respectively, further confirming the AIE feature was well maintained in TPE-PEI. The PL spectrum of TPE-PEI in water under excitation of 405 nm UV light was also conducted (Fig. 2c), which showed slightly weaker of PL intensity than that of 365 nm ones. After modification with PEI, the TPE-PEI was readily dispersed in water. Meanwhile, the stability of the FONs was characterized by zeta potential measurement which showed zeta-potential of (34.7 ± 1.8) mV in phosphate buffer solution (PBS), suggesting the successful formation of stable hydrophilic FONs. FTIR spectra (Fig. 2d) showed that the characteristic peaks located at 3340 cm^{-1} and 1060 cm^{-1} corresponding to the N—H and C—N stretching vibration band respectively were observed significantly increased in TPE-PEI, simultaneously, the characteristic peaks located at 2960 cm^{-1} and 2880 cm^{-1} corresponding to the stretching vibration CH_2 band were also significantly enhanced in TPE-PEI. More importantly, the —C=O stretching vibration (1700 cm^{-1}) of TPE-A was disappeared in the spectrum of TPE-PEI, further demonstrating successful reaction of TPE-A and PEI.

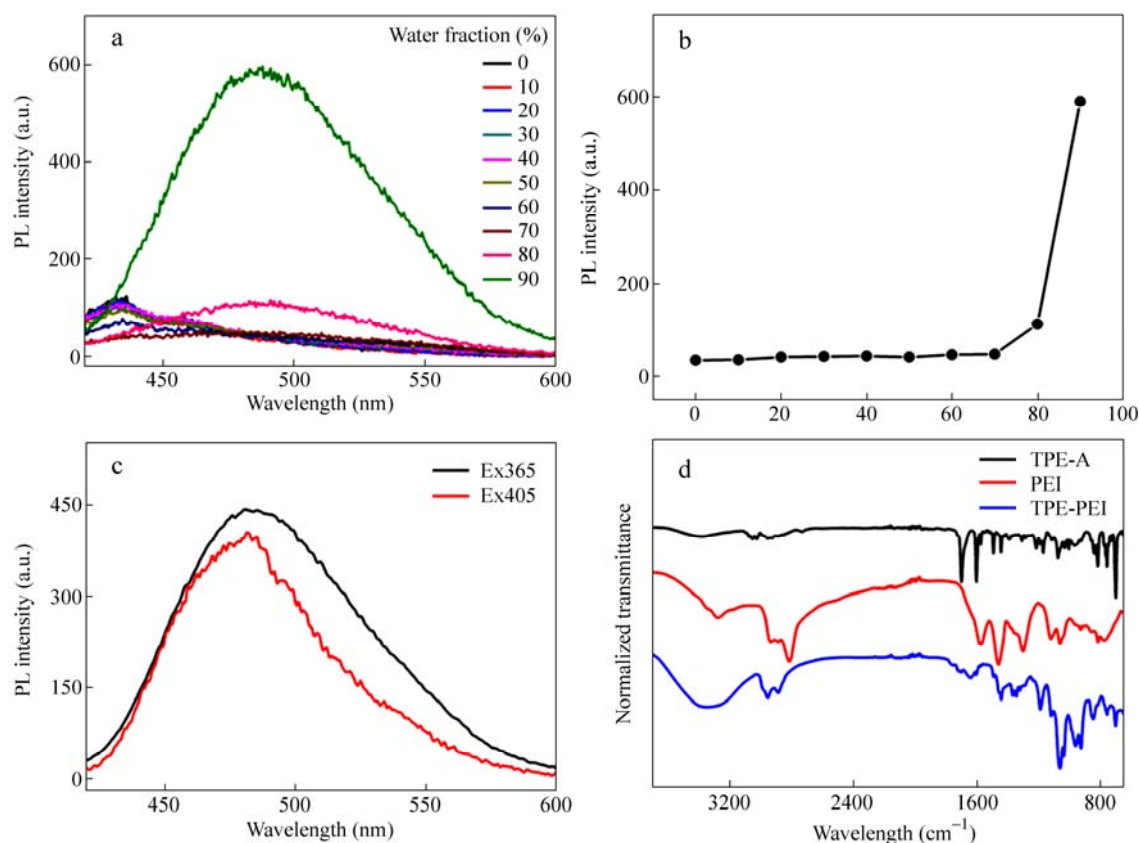


Fig. 2 The AIE characteristic of TPE-PEI ($\lambda_{\text{Ex}} = 365\text{ nm}$): (a) PL spectra of TPE-PEI in THF-water mixtures with different water fractions; (b) the changes in PL peak intensity (486 nm) of the compound in different water fraction mixtures; (c) The FL spectra of TPE-PEI in water under excitations of 365nm and 405 nm UV light and (d) FTIR spectra of TPE-A, PEI and TPE-PEI

As shown in our previous report, water soluble and biocompatible fluorescent organic nanoparticles could be facilely obtained by modifying an AIE compound An18 with Pluronic F127, however, the morphology of An18-F127 FONs was hard to control^[45]. Compared with the previous report, the morphology of TPE-PEI was

more controllable and stable. As shown in Fig. 3, a lot of small regular organic spheres with diameters ranging from 200 nm to 300 nm were observed. The size distribution of TPE-PEI FONs in PBS was determined using a zeta Plus particle size analyzer. Results showed that the size distribution of TPE-PEI in PBS was (417.6 ± 16.5) nm. The formation of such nanostructures is likely due to the covalent bond connection of TPE and PEI to form spherical TPE-PEI compounds, which render them water dispersible with the redundant amino groups from the starting PEI and the amino groups from the reduction of the Schiff base, which were covered on the surface of the spheres. The results demonstrated that spherical and stable morphology of the FONs could be easily achieved by simple reaction of TPE-A intermediate and branched PEI and subsequent reduction. Meanwhile, this TPE-PEI nanoparticles contains many amino groups in their peripheral structure, which can be utilized to integrate other components including drugs and targeting agents for constructing multifunctional imaging platform. It also provides us a simple common strategy to prepare regular morphology by combining a hydrophobic aldehyde-containing luminescent AIE intermediate and a hydrophilic amino group-containing compound *via* Schiff base condensation.

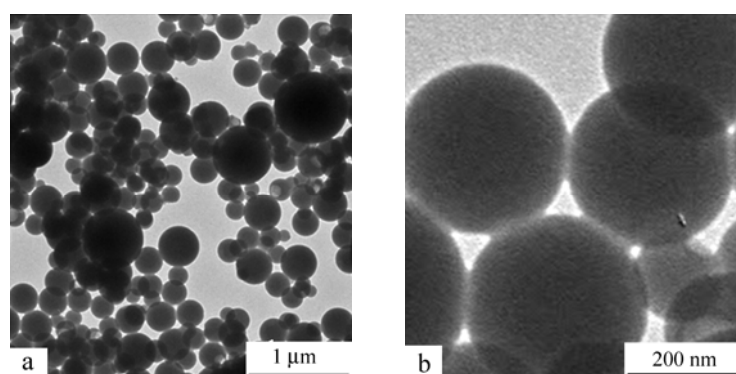


Fig. 3 TEM images of TPE-PEI with different magnifications

Biocompatibility of TPE-PEI

To study its potential biomedical applications, the biocompatibility of TPE-PEI was characterized. Figures 4(a)–4(c) showed the optical microscopy images of A549 cells after their incubation with TPE-PEI for 24 h. Compared with the control experiment (cells without TPE-PEI), all cells still kept their normal morphology even when the concentration of TPE-PEI was as high as $60 \mu\text{g}\cdot\text{mL}^{-1}$. These observations gave us qualitative result that TPE-PEI was biocompatible with cells. Cell viability results from WST assay further demonstrated the excellent biocompatibility of TPE-PEI. As shown in Fig. 4(d), no obvious cell viability decrease was observed *via* cell viability examination after either 8 h or 24 h. Even the concentration of TPE-PEI reached up to $60 \mu\text{g}\cdot\text{mL}^{-1}$, the cell viability was still greater than 90%. All the above results confirmed the excellent biocompatibility and potential biological imaging applications of TPE-PEI.

Cell imaging of TPE-PEI

Figure 5 showed the CLSM images of cells after incubating them with $10 \mu\text{g}\cdot\text{mL}^{-1}$ of TPE-PEI for 3 h. The cell internalized TPE-PEI could be excited by laser with 405 nm excitation wavelength, and emitted bright sky-blue fluorescence. The fluorescent areas were overlapped on the locations of cells (Fig. 5b), indicating that TPE-PEI could be uptaken by cells and accumulated in cells. From CLSM images, many dark areas were also found within the cells, which might be the locations of cell nucleus. For comparison, CLSM images of A549 cells without TPE-PEI were shown in Fig. 5(d), and no cell could be seen under the same condition. Based on the results of CLSM images, it could be concluded that TPE-PEI could translocate into cells and locate at cytoplasm. As many factors, such as particle size, shapes and surface charge, can affect the efficiency and pathway of cellular uptake for polymeric nanoparticles^[58–60], it is difficult to evaluate whether these more spherical nanoparticles have higher cellular uptake for fluorescence imaging than the more random morphology in the

previous reports^[45, 46]. Given these excellent biocompatibility and strong fluorescence, such novel fluorescent nanomaterials should be a promising and alternative bioprobe for bioimaging applications. It is worth mentioning that the above TPE-PEI can also be used as a polymeric transfection agent for building siRNA carriers, which may find potential applications in fluorescent label and gene transfection multifunctional materials.

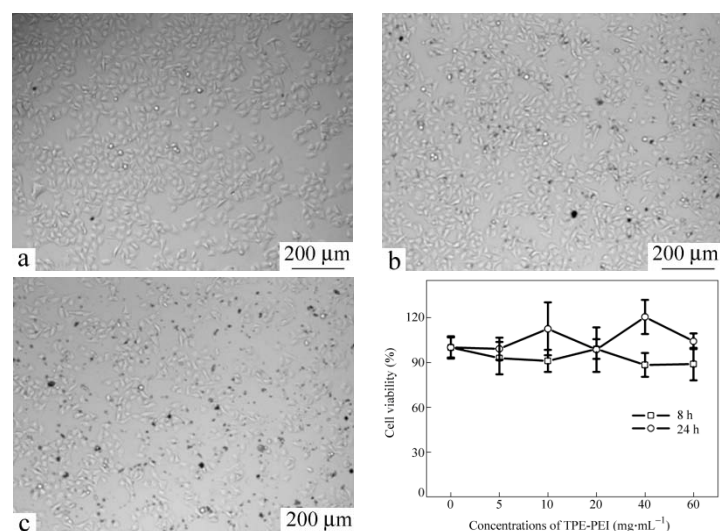


Fig. 4 (a–c) Optical microscopy images of A549 cells incubated with different concentrations of TPE-PEI for 24 h: (a) control cells, (b) 10 µg·mL⁻¹, (c) 60 µg·mL⁻¹; (d) time- and dose-dependent cell viability of TPE-PEI with A549 cells

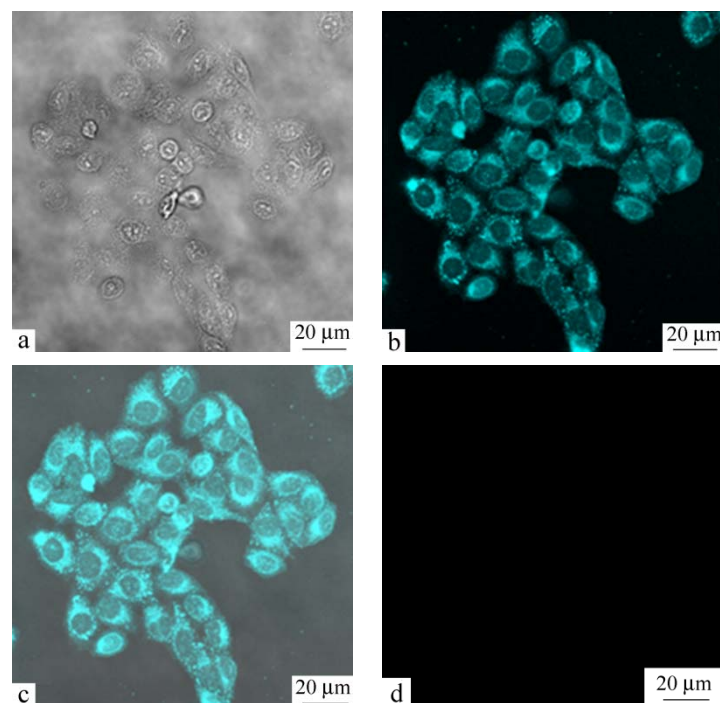


Fig. 5 CLSM images of A549 cells incubated with 50 µg·mL⁻¹ of TPE-PEI for 3 h: (a) bright field, (b) excited with 405 nm laser, (c) merged image of A and B; (d) CLSM images of A549 cells without TPE-PEI, excited with 405 nm laser

CONCLUSIONS

In summary, a facile method for the preparation of tetraphenylethene-based AIE FONs (TPE-PEI) *via* Schiff base condensation and subsequent reduction was reported for the first time. Such water soluble TPE-PEI FONs showed obvious AIE feature and spherical morphology (with diameters about 200–300 nm). Cell imaging application results demonstrated they were highly biocompatible with cells. Given facile preparation procedure, AIE property, spherical morphology and excellent biocompatibility, these TPE-PEI FONs are expected to be novel and promising for cell imaging applications.

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