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Ultrabright and biocompatible luminescent polymeric nanoparticles were prepared via RAFT polymerization of an AIE dye and a zwitterionic monomer.
The development of novel fluorescent nanoprobes with remarkable optical properties, suitable particle size, high water dispersibility and good biocompatibility has recently attracted increasing interest for various biomedical applications. In this work, a novel type of luminescent polymeric nanoparticles based on polymerizable dyes with aggregation induced emission (AIE) properties and a zwitterionic monomer were prepared via reversible addition fragmentation chain transfer polymerization. Due to their amphiphilic properties, these copolymers could facilely self-assemble into AIE dye contained luminescent zwitterionic polymeric nanoparticles, which were characterized by a series of characterization techniques including transmission electronic microscopy, Fourier transform infrared spectroscopy, fluorescence spectroscopy and X-ray photoelectron spectroscopy. Results showed that these luminescent polymeric nanoparticles with diameter at tens of nanometers showed high water dispersibility and strong fluorescence in aqueous solution. To explore their potential for biomedical applications, biocompatibility and cell uptake behavior of these luminescent polymeric nanoparticles were further evaluated. We demonstrated that these polymeric nanoparticles are biocompatible with A549 cells and promising for bioimaging applications. Taken advantage of these merits of AIE dye based zwitterionic polymeric nanoparticles, which could elegantly avoid the aggregation induced quenching of conventional organic dyes and nonbiodegradability of fluorescent inorganic nanoparticles, these ultrabright and biocompatible luminescent polymeric nanoparticles described in this work should be of highly potential for various biomedical applications.

1. Introduction

Over the past few decades, there is an increasing current interest for fabrication of novel fluorescent nanoprobes owing to their superior luminescent properties and multifunctional capability as compared with conventional organic dyes.1 A promising nanoprobe should be of remarkable luminescent properties, good biocompatibility, high water stability and suitable particle size.2-9 Although a number of fluorescent nanoprobes based on inorganic, organic and hybrid components have thus been developed since the first reports of using semiconductor quantum dots for biomedical applications.10-13 Most of currently used nanoprobes are still difficult to fulfill the requirement for biomedical applications due to their inherent limitations. For example, the fluorescent inorganic nanoparticles such as semiconductor quantum dots with tunable luminescent properties, controllable morphology and high photostability are promising for bioimaging applications, however, their accumulation in reticuloendothelial system (RES) and verified toxicity of semiconductor quantum dots from heavy metal compositions made their biomedical applications still under intensive debate.14, 15

To overcome the inherent shortcoming of fluorescent inorganic nanoparticles, fluorescent organic nanoparticles have recently emerged as alternative candidates for biomedical applications due to their relative better biocompatible and biodegradability.16-28 To date, various luminescent polymeric nanoparticles based on conventional organic dyes, conjugated polymers, aggregation induced emission (AIE) dyes and polydopamine have been reported.29-34 A general strategy for preparation of fluorescent organic nanoparticles is incorporation of hydrophobic organic dyes into amphiphilic copolymers, which can be self-assembled into core-shell polymeric nanoparticles in aqueous solution through hydrophobic interactions.35 Upon self-assembly, the hydrophobic dyes were encapsulated in the core of nanoparticles, while the hydrophilic segments were expanded into water, endowing them water dispersibility. However, it is still challengeable to obtain fluorescent organic nanoparticles with strong luminescent intensity due to the notorious aggregation-caused quenching (ACQ) effect of most organic dyes.36 Therefore, development of novel fluorescent polymeric nanoparticles which could overcome the ACQ effect of conventional organic dyes is of great research interest.

AIE is an abnormal optical phenomenon, which suggested some organic dyes could emitted much stronger fluorescence in their solid or aggregated state than in solution.37-43 Taken advantage of the unique AIE properties, luminescent polymeric nanoparticles based on AIE dyes could therefore elegantly avoid the ACQ effect of conventional organic dyes, which provided an novel approach to develop ultrabright and biodegradable luminescent polymeric nanoparticles.44 In recent years, a number of dyes with AIE properties were
synthesized and various strategies for fabrication of AIE dye based luminescent nanoparticles were developed. These novel luminescent nanoparticles have demonstrated to be very promising for biomedical applications for their superior luminescent properties, good biocompatibility, biodegradable potential.

Zwitterionic polymers are a very important class of functional polymers which simultaneously contained both cationic and anionic functional groups in polymer pendant-side chains. Due to their unique molecular structures, excellent chemical properties, hydration capacity, and preferable thermal stability, zwitterionic polymers have attracted a great deal of research interest in recent years. Various zwitterionic polymers have been synthesized and explored for various applications in the fields such as petroleum industry, sewage treatment, drug synthesis and biomedical applications. Especially, due to their special anti-polyelectrolyte behavior in solution, zwitterionic polymers are expected more suitable for fabrication of zwitterionic polymers contained copolymers for biomedical applications.

In this contribution, a biocompatible zwitterionic monomer 2-methacryloyloxyethyl phosphorylcholine (named as PhE) was copolymerized with a polymerizable AIE dye (named as MTP) through reversible addition fragmentation chain transfer (RAFT) polymerization. Due to their amphiphilic properties, thus obtained copolymers could self-assemble into core-shell nanoparticles (named as PhE-MTP NPs) which exhibited strong fluorescence and high water dispersibility in aqueous solution. To explore their bioimaging applications, biocompatibility and cell uptake behavior of thus obtained zwitterionic polymeric nanoparticles were further evaluated.

2. Experimental details
2.1 Materials and measurements

All of the chemical agents and solvents were obtained from commercial sources and used as received. PhE was synthesized and characterized in our previous report. UV-visible absorption spectra were recorded on UV/Vis/NIR PerkinElmer 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. On the other hand, fluorescence stability of PhE-MTP NPs in aqueous solution was also recorded on a PE LS-55 spectrometer under time drive model. The excitation wavelength was set at 488 nm with slit width of 15 nm and the emission wavelength was set at 581 nm with slit width of 10 nm. The fluorescence quantum yields (ΦF) of PhE-MTP NPs in aqueous solution were measured using Rhodamine 6G in ethanol as the standard (ΦF = 95%), the absorbance of the solutions was kept around 0.05 to avoid internal filter effect. The X-ray photoelectron spectra (XPS) were performed on a VGESCALAB 220-1XL spectrometer using an Al Kα X-ray source (1486.6 eV). The energy scale was internally calibrated by referencing to the binding energy (Eb) of the C1s peak of a carbon contaminant at 284.6 eV. The Fourier transform infrared spectroscopy (FT-IR) 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. 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 of PhE-MTP NPs in water was determined using a zeta Plus apparatus (ZetaPlus, Brookhaven Instruments, Holtsville, NY). ¹H NMR spectra were measured on a JEOL 400 MHz spectrometer [d₆-DMSO as solvent and tetramethylsilane (TMS) as the internal standard]. 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 SD plus 10.0 mm guard column (50×8.0 mm, 10² Å) followed by a MZ-Gel SD plus 5.0 μm bead-size columns (50×10⁶ Å, linear) and a Shimadzu RID-10A refractive index detector. The system was calibrated with narrow molecular weight distribution polystyrene standards ranging from 200 to 10⁶ g mol⁻¹.

1.2 Preparation of PhE-MTP NPs

CTA (9.7 mg, 0.037 mmol), PhE (50 mg, 0.074 mmol), MTP (200 mg, 0.67 mmol), AIBN (3.0 mg) and DMF (5 mL) were introduced in schlenk tube and purged by nitrogen flow for 30 min. The above mixture was put into an oil bath maintained at 70 °C for 12 h. Then stopped the reaction of polymerization, and dialyzed against tap water for 24 h. The solution in dialysis bag was carried out by freeze-drying to obtain PhE-MTP-20 NPs. For synthesis of PhE-MTP-40 NPs, CTA was changed to 4.85 mg.

![Fig. 1 Synthesis of PhE based luminescent polymeric nanoparticles via RAFT polymerization.](image)

1.3 Cytotoxicity of PhE-MTP NPs

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

The cell viability of PhE-MTP NPs on A549 cells was evaluated by cell counting kit-8 (CCK-8) assay based on our previous reports. Briefly, cells were seeded in 96-well microplates at a density of $5 \times 10^3$ cells mL$^{-1}$ in 160 µL of respective media containing 10% FBS. After 24 h of cell attachment, the cells were incubated with 10, 20, 40, 80, 120 µg mL$^{-1}$ PhE-MTP NPs for 8 and 24 h. 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. Plates were then analyzed with a microplate reader (Victor III, 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 PhE-MTP NPs), 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 ± standard deviation (SD).

1.4 Confocal microscopic imaging of cells using PhE-MTP NPs

A549 cells were cultured in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U mL$^{-1}$ penicillin, and 100 µg mL$^{-1}$ of streptomycin. Cell culture was maintained at 37 °C in a humidified condition of 95% air and 5% CO$_2$ in culture medium. Culture medium was changed every three days for maintaining the exponential growth of the cells. On the day prior to treatment, cells were seeded in a glass bottom dish with a density of $1 \times 10^5$ cells per dish. On the day of treatment, the cells were incubated with PhE-MTP NPs at a final concentration of 10 µg mL$^{-1}$ for 3 h at 37 °C. Afterward, the cells were washed three times with PBS to remove the PhE-MTP NPs and then fixed with 4% paraformaldehyde for 10 min at room temperature. Cell images were taken with a confocal laser scanning microscope (CLSM) Zesis 710 3-channel (Zesis, Germany) with the excitation wavelength of 543 nm.

3. Results and discussion
3.1 Characterization of PhE-MTP NPs

Thus obtained PhE-MTP NPs were first characterized by $^1$H NMR spectra (Fig. S1). Many characteristic signals of PhE and MTP were observed through $^1$H NMR spectra, implying the successful copolymerization of PhE and MTP. The number average molecular weight ($M_n$) of PhE-MTP was also determined by GPC, which showed that the $M_n$ values of PhE-MTP-20 and PhE-MTP-40 are 41900 and 79800 Da with narrow polydispersity index (PDI = 1.16 and 1.19 for PhE-MTP-20 and PhE-MTP-40), respectively (Fig. S2). The GPC results further confirmed successful synthesis of PhE-MTP. Due to the amphiphilic properties of PhE-MTP, which could self-assemble in aqueous solution. As evidenced by TEM images, many spherical nanoparticles with diameter at tens of nanometers were observed (Fig. 2). No significant difference was found between the diameter of PhE-MTP-20 NPs (Fig. 2A) and PhE-MTP-40 NPs (Fig. 2B). Furthermore, the hydrodynamic size of PhE-MTP NPs was also determined by dynamic light scattering (DLS). Results showed that the size distribution of PhE-MTP-20 NPs and PhE-MTP-40 NPs in water is 132.4 ± 58.9 and 136.3 ± 59.5 nm, respectively (Fig. S3). As compared the hydrodynamic size of PhE-MTP NPs, the size of PhE-MTP NPs characterized by TEM is somewhat smaller, which might be due to the shrinkage of micelle for TEM observation. As compared with our previous reports, the size of PhE-MTP NPs is relative small. The possible reason may be ascribed to the unique zwitterionic properties of MTP, which could given better water dispersibility.

The FT-IR spectra of MTP, PhE-MTP-20 NPs, PhE-MTP-40 NPs were shown in Fig. 3A. It can be seen that a series of absorption peaks located at 954, 1058, 1254, and 1705 cm$^{-1}$ were observed, indicating that the functional groups such as $\text{N}(\text{CH}_3)_3$, POCH$_2$ and C=O were existed in MTP. On the other hand, C=C stretching vibration located at 1636 cm$^{-1}$ was also observed in MTP. After formation of PhE-MTP NPs, the absorption located at 1058 cm$^{-1}$ can still be observed. However, the intensity located at 1636 cm$^{-1}$ is significantly decreased. These results suggested that MTP was successfully copolymerized with PhE. On the other hand, intensity of absorption peaks at 2977 and 2922 cm$^{-1}$ assigned to alkyl chain of PhE were obviously enhanced, further confirming the successful incorporation of PhE into PhE-MTP copolymers.

The chemical compositions of MTP, PhE-MTP-20 NPs and PhE-MTP-40 NPs were characterized by XPS. As shown in Fig. 3B, the elements including Carbon (C), Nitrogen (N), Oxygen (O), Phosphorus (P) were detected by survey curve of XPS spectra ranged from 0-1200 eV. The existence element P...
in samples of PhE-MTP NPs further confirming the successful copolymerization of PhE and MTP.

![Figure 3](image_url)  
Fig. 3 (A) FT-IR spectra of MTP, PhE-MTP-20 NPs and PhE-MTP-40 NPs. The increase of absorbance intensity at 2977 and 2922 cm\(^{-1}\) and decrease of absorbance intensity at 1636 cm\(^{-1}\) evidenced the successful formation of PhE-MTP NPs. (B) Survey curves of XPS spectra of MTP, PhE-MTP-20 NPs and PhE-MTP-40 NPs. The existence of Phosphorus in samples of PhE-MTP NPs further confirmed the successful formation of PhE-MTP NPs.

More detailed information of XPS spectra of C, N, O and P are shown in Fig. 4. It can be seen that binding energy peaks of C1s in MTP are located at 284.4, 285.7 and 288.2 eV, which can be ascribed to C-C, C-O and C-N, respectively. Upon formation of PhE-MTP NPs, the signal of binding energy peak located at 284.4 eV was obviously enhanced while intensity of binding energy peaks located at 285.7 and 288.2 eV were significantly decreased (Fig. 4A). On the other hand, the binding energy peak of N1s at 401.1 eV could be ascribed to the C-N of MTP (Fig. 4B). However, in the sample of PhE-MTP NPs, another binding energy peak located at 398.0 eV from PhE was emerged, indicating the successful formation of PhE-MTP NPs. Furthermore, two binding energy peaks located at 529.0 and 531.0 eV were observed in the sample of MTP (Fig. 4C), which could be assigned to the P-O and C-O, respectively. After formation of PhE-MTP NPs, the intensity of P-O was decreased while intensity of C-O was enhanced, further implying successful formation of PhE-MTP NPs. Finally, P2p signal was found both in MTP and PhE-MTP NPs, giving direct evidence for formation of PhE-MTP NPs (Fig. 4D). Based on XPS spectra, the element percentages of C, N, O, P in the samples could be calculated. Results showed that element content of C, N, O, P are 59.48, 4.34, 31.2, 4.98 for MTP (Table S1). As compared with MTP, the percentage of P was obviously decreased in the samples of PhE-MTP NPs (1.92 for PhE-MTP-20 NPs and 1.47 for PhE-MTP-40 NPs). The increase intensity of C and decrease of P further confirming successful formation of PhE-MTP NPs.

![Figure 4](image_url)  
Fig. 4 XPS characterization of MTP, PhE-MTP-20 NPs and PhE-MTP-40 NPs. (A) C1s, (B) N1s, (C) O1s and (D) P2p.

Due to the amphiphilic properties, PhE-MTP copolymers are prone to self-assemble into spherical nanoparticles in aqueous solution (Fig. 2). The hydrophobic dye (PhE) was encapsulated in the core of PhE-MTP NPs while hydrophilic segments (MTP) were expended into water, which made these luminescent polymeric nanoparticles excellent water dispersibility. Even PhE-MTP NPs were dispersed in water for more than one week, no obvious precipitation was observed (left cuvette of Fig. 5). On the other hand, due to the aggregation of PhE in the core of nanoparticles, PhE-MTP NPs showed strong yellow fluorescence in water (right cuvette of Fig. 5). The optical properties of PhE-MTP NPs were further investigated by UV-vis spectroscopy and fluorescence spectroscopy. Two absorption peaks located at 326 and 425 nm were observed by UV-vis spectroscopy (Fig. 5A). These peaks should be ascribed to PhE because MTP showed no absorption peaks in these regions. Fluorescence spectra shown in Fig. 5B suggested that when PhE-MTP NPs were excited with 488 nm wavelength, the emission peak of PhE-MTP NPs was located at 581 nm. Excitation spectra of PhE-MTP NPs was obtained using 581 nm as emission wavelength. It can be seen that two peaks located at 340 and 435 nm were found in excitation spectra. Based on excitation spectra, we found that PhE-MTP NPs could be excited by different wavelength ranged from 300-500 nm, the broad excitation characteristics made PhE-MTP NPs promising for biological applications. The fluorescence quantum yields of PhE-MTP-20 NPs and PhE-MTP-40 NPs in water are 9.84% and 9.21% using Rhodamine 6G in ethanol as the standard. The quantum yields of PhE-MTP NPs is much higher than our previous reported AIE dots.58 Furthermore, the fluorescence stability of was PhE-MTP NPs also examined. As shown in Fig. S4, both PhE-MTP-20 NPs and PhE-MTP-40 NPs showed excellent photostability after they were irradiated at 488 nm for 30 nm. Given the excellent fluorescent properties of PhE-MTP NPs, we could expect that PhE-MTP NPs are very suitable for bioimaging applications as compared with the small organic dyes.
NPs were found in the cell membrane areas, leaving a larger dark areas surrounded by yellow fluorescence (Fig. 7B and C). These results are obviously different from many other AIE dye based nanoparticles as described previously, which suggested that AIE dye based nanoparticles were internalized by cells via phagocytosis and mainly distributed in the whole cytoplasm. The significant difference between the PhE-MTP NPs and other AIE dye based nanoparticles is likely due to their different physicochemical properties. It is well known that structure of MTP is very similar with the phospholipids of cell membrane. Therefore, after PhE-MTP NPs were incubated with cells, they were first merged with cell membrane. At the same time, the AIE dye were therefore encapsulated in cell membrane, which made PhE-MTP NPs potential for cell membrane dyeing. On the other hand, due to the flexibility of RAFT polymerization, many other functional monomers could also be introduced into our polymeric system. Thus properties of these luminescent polymeric nanoparticles could be finely tuned by adjusting polymerization parameters. Furthermore, many other functional components such as targeting agents can also be linked onto these polymeric nanoparticles via subsequent coupling reactions. Therefore polymeric nanoparticles with targeting ability could be achieved.

3.3 Biological images of PhE-MTP NPs

To evaluate their potential biomedical applications, the cellular uptake of PhE-MTP NPs was investigated by CLSM. As shown in Fig. 7, the cell uptake of PhE-MTP NPs were found in the cell membrane areas, leaving a larger dark areas surrounded by yellow fluorescence (Fig. 7B and C). These results are obviously different from many other AIE dye based nanoparticles as described previously, which suggested that AIE dye based nanoparticles were internalized by cells via phagocytosis and mainly distributed in the whole cytoplasm. The significant difference between the PhE-MTP NPs and other AIE dye based nanoparticles is likely due to their different physicochemical properties. It is well known that structure of MTP is very similar with the phospholipids of cell membrane. Therefore, after PhE-MTP NPs were incubated with cells, they were first merged with cell membrane. At the same time, the AIE dye were therefore encapsulated in cell membrane, which made PhE-MTP NPs potential for cell membrane dyeing. On the other hand, due to the flexibility of RAFT polymerization, many other functional monomers could also be introduced into our polymeric system. Thus properties of these luminescent polymeric nanoparticles could be finely tuned by adjusting polymerization parameters. Furthermore, many other functional components such as targeting agents can also be linked onto these polymeric nanoparticles via subsequent coupling reactions. Therefore polymeric nanoparticles with targeting ability could be achieved.

4. Conclusion

In summary, novel luminescent polymeric nanoparticles based on a polymerizable AIE dye (PhE) and a zwitterionic monomer were prepared via RAFT polymerization. The PhE-MTP NPs showed uniform spherical morphology with diameter at tens of nanometers. They can be well dispersed in aqueous solution and emitted strong yellow fluorescence because PhE was aggregated in the core of PhE-MTP NPs. These AIE dye based luminescent polymeric nanoparticles showed excellent biocompatibility with A549 cells and mainly distributed in cell membrane after they were incubated with cells. The cell uptake behavior of PhE-MTP NPs were significant different from many other AIE dye based nanoparticles, which were mainly internalized into cells through phagocytosis. It is possible ascribed to the difference physicochemical properties of PhE-MTP NPs and other AIE dye based nanoparticles. Combination of the remarkable fluorescence, high water dispersibility and excellent biocompatibility, thus AIE dye based zwitterionic luminescent polymeric nanoparticles are expected highly potential for various biomedical applications.

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† Electronic Supplementary Information (ESI) available: [detailed information about X NMR spectra of PhE-MTP NPs, size distribution of PhE-MTP NPs in water, and microscopy observation of cells after they were incubated with different concentrations of PhE-MTP NPs et al]. See DOI: 10.1039/b000000xv

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