One-pot preparation of cross-linked amphiphilic fluorescent polymer based on aggregation induced emission dyes

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ABSTRACT

Facile one-pot preparation of cross-linked amphiphilic fluorescent polymer based on aggregation induced emission (AIE) dyes and 2-isocyanatoethyl methacrylate (IM) has been developed. This was carried out first by free radical polymerization between AIE monomer (PhE) and IM, and then polyethyleneimine (PEI) was introduced to obtain the cross-linked fluorescent polymer. The resulted cross-linked amphiphilic polymer was prone to self-assemble into stable nanoparticles in aqueous solution with surplus amino groups on the surface which made them highly water dispersible and can be further functionalized. The as-prepared fluorescent polymer nanoparticles (PhE-IM-PEI FPNs) were fully characterized by a series of techniques including ¹H NMR spectrum, X-ray photoelectron spectroscopy, Fourier transform infrared spectroscopy, transmission electron microscopy, dynamic light scattering, UV–vis absorption spectrum, and fluorescence spectra. Such FPNs demonstrated intense orange fluorescence with a high quantum yield of about 40%. Biocompatibility evaluation and cell uptake behavior of the nanoparticles were further investigated to explore their potential biomedical applications; the demonstrated excellent biocompatibility made them promising for cell imaging.

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1. Introduction

Fluorescent polymeric nanoparticles (FPNs), which mean fluorophore-labeled polymeric systems, have attracted great interest for sensing, bioimaging and biomedical applications, due to their simple operation, fluorescent characteristics and prompt response [1–8]. Compared with their small-molecule counterparts, FPNs are much more promising for biological applications because of water dispersibility, their biocompatibility, and facile synthesis strategy with functional groups for modification, etc. [9–13]. Furthermore, the good permeability of the cell membrane or nucleus, which is closely related to many diseases, makes these nanoparticles a desirable system for drug delivery and cell imaging [14–17]. However, for most small-molecule fluorophores, hydrophobic planar structures will induce strong intermolecular π–π interactions, resulting in fluorescence quenching and photo-bleaching when aggregated in aqueous solution, which is known as aggregation-caused quenching (ACQ) [18–20]. FPNs prepared utilizing these fluorophores would also encounter the same problem, which would severely limit their real biomedical applications. In order to solve this ACQ problem, another type of unique organic dyes was developed, which can emit much stronger luminescence in their aggregation states. These organic dyes were first reported by Tang et al. in 2001 and were called aggregation induced emission (AIE) dyes [19,21]. More importantly, a lot of AIE fluorogens such as siloles, tetraphenylethene, triphenylethene, cyano-substituted diarylethene, and distyrylanthracene derivatives have already been synthesized and extensively investigated for chemosensors and bioimaging applications [22–28].

Recently, many kinds of AIE dyes based FPNs have been rapidly developed and have received much attention owing to their facile processability, good solubility, and high emission efficiency in the aggregated states, etc. [25,29–33]. By now, two major strategies for constructing AIE dye based FPNs have been developed including a physical method and a chemical method. The physical method is mainly to encapsulate the AIE dyes with biocompatible amphiphilic polymers to afford FPNs [34–36]; however, dye leakage or surface coating detachment is the main obstacle in this non-covalent system. On the other hand, the chemical method to prepare FPNs comprises Schiff-base reaction, reversible addition

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fragmentation chain transfer (RAFT) polymerization, emulsion polymerization, ring-opening polymerization, and cross-linked polymerization [33,37–40]. Despite many impressive advances in fabricating AIE based macromolecules, more versatile and robust strategies are still highly demanded. As most of aforementioned AIE based fluorescent polymers are linear polymers, which are not stable in dilute solution below the critical micelle concentration, and will limit their real biomedical application [41,42]. In this case, cross-linked polymeric nanoparticles have been expected more stable than those non-crosslinked ones. However, the related construction methodology of cross-linked FPNs based on AIE dyes is still limited [29,43–45].

In this work, a facile strategy to prepare AIE dyes based cross-linked FPNs (PhE-IM-PEI FPNs) was developed by one-pot method for the first time. Firstly, free radical polymerization between AIE monomer (PhE) and 2-isocyanoethyl methacrylate (IM) was conducted, and then polyethyleneimine (PEI) was introduced to obtain the cross-linked fluorescent polymer. Such a PhE-IM-PEI amphiphilic polymer was prone to self-assemble into nanoparticles with great stability because of the cross-linked structures and high water dispersibility due to the surplus amino groups covered on the surface. The fluorescence property, morphology, and stability of the FPNs were further characterized by fluorescence spectrum, transmission electron microscopy, and dynamic light scattering. Finally, the biocompatibility and cell uptake behavior of PhE-IM-PEI FPNs were determined to evaluate their potential cell imaging applications (Scheme 1).

2. Experimental

2.1. Materials and methods

Phosphoryl chloride, N,N-dimethylformamide, 2-(4-bromophenyl)acetoniitrile, tetrabutyl ammonium bromide, tetrakis(triphenylphosphine) palladium(0), Aliquat 336, 1,2-dichloroethane, 4-vinylphenylboronic acid, terabutyl ammonium hydroxide, 2-isocyanoethyl methacrylate, and polyethyleneimine were purchased from J&K Scientific Ltd. and used as received. All other agents and solvents were purchased from commercial sources and used directly without further purification. Ultra-pure water was used in the experiments.

Gel permeation chromatography (GPC) analyses were performed using DMF as the eluent. TheGPC system was a Shimadzu LC-20AD pump system comprising of an auto injector, a MZ-Gel SDplus 10.0 mm guard column (50 mm × 8.0 mm, 105 Å) followed by a MZ-Gel SDplus 5.0 mm 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. UV–vis 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. 1H NMR spectra were measured on a JEOL 400 MHz spectrometer [D2O, CDCl3 or d6-DMSO] as solvent and tetramethylsilane (TMS) as the internal standard]. The X-ray photoelectron spectra (XPS) were performed on a VG ESCALAB 220-IXL 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. 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-IM-PEI FPNs in water and phosphate buffer solution (PBS) was determined using a zeta plus apparatus (ZetaPlus, Brookhaven Instruments, Holtsville, NY).

2.2. Preparation of PhE-IM-PEI FPNs

The AIE monomer PhE was prepared according to the literature methods [43,45]. For synthesis of PhE-IM-PEI FPNs, PhE (34 mg, 0.050 mmol), IM (78 mg, 0.50 mmol), ABN (5.0 mg), and ethyl acetate (6 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 80 °C for 12 h. PEI (300 mg, 0.50 mmol) was added into the above mixture and stirred for another 2 h at room temperature. Afterwards, the reaction was stopped and dialyzed against tap water for 24 h and ethanol for 6 h using 7000 Da MW cutoff dialysis membranes. Finally, this solution in the dialysis bag was freeze–dried to obtain the product (Scheme 2).

2.3. Cytotoxicity of PhE-IM-PEI FPNs

Cell morphology was used to examine the effects of PhE-IM-PEI FPNs to A549 cells. Briefly, cells were seeded in 6-well microplates at a density of 1 × 105 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 PhE-IM-PEI FPNs 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 ×10.

The cell viability of PhE-IM-PEI FPNs 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 × 104 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-IM-PEI FPNs 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 Dulbecco’s modified Eagle’s medium (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 PhE-IM-PEI FPNs), 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).

2.4. Confocal microscopic imaging of cells using PhE-IM-PEI FPNs

A549 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 μg mL−1 of streptomycin. Cell culture was maintained at 37 °C in a humidified condition of 95% air and 5% CO2 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 × 104 cells per dish. On the day of treatment, the cells were incubated with PhE-IM-PEI FPNs at a final concentration of 20 μg mL−1 for 3 h at 37 °C. Afterward, the cells were washed three times with PBS to remove the PhE-IM-PEI FPNs and then fixed with 4% paraformaldehyde for
Scheme 1. Schematic demonstration of the preparation of PhE-IM-PEI FPNs.

Scheme 2. Synthetic route of PhE-IM-PEI.

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 405 nm.

3. Results and discussion

3.1. Characterization of PhE-IM-PEI FPNs

The AIE fluorogen PhE was prepared following the synthetic route described in our previous report. Its structure was characterized and confirmed by standard spectroscopic methods [43,45]. For preparation of PhE-IM-PEI FPNs, PhE with alkenyl end group was first reacted with IM through free radical polymerization to obtain a linear hydrophobic polymer with PhE and IM pendants. Then PhE-IM was facely reacted with PEI under air atmosphere to afford the resulting cross-linked polymer. The feed molar ratio of PhE, IM, and PEI was designed as 1:10:10, respectively, so the designed degree of polymerization (DP) was 10. After the resulting PhE-IM-PEI copolymers were purified, the number average molecular weight ($M_n$) values of PhE-IM-PEI were determined by GPC and showed as 65,842 Da with a polydispersity index (PDI) of 1.08. Thus the DP of the copolymers could be calculated as 8, approximately. Such a PhE-IM-PEI amphiphilic polymer was prone to self-assemble into nanoparticles with great stability because of their cross-linked structure and high water dispersibility due to the surplus amino groups on the surface. As compared with other AIE based fluorescent polymer nanoparticles, AIE dyes were covalently cross-linked with IM and PEI to afford FPNs, which are more stable and can avoid the leakage of dyes.

The $^1$H NMR spectra of PhE-IM-PEI FPNs in $d_6$-DMSO were conducted and shown in Fig. 1A. The chemical shifts of alkenyl end group in PhE were around 5.2, 5.7 and 6.7 ppm, which were disappeared in PhE-IM-PEI due to the polymerization. While the peaks of aromatic group around 7.7 could still be observed after polymerization of PhE. Amplified $^1$H NMR spectrum of PhE-IM-PEI from 6.5 to 8.5 ppm was shown in Fig. 1B, and the peaks of aromatic hydrogens were obviously broaden in the as-prepared copolymers, as the original monomer of PhE showed multiple sharp peaks in the
same region. The broad peaks around 2.5 indicated the successful attachment of PEI.

Successful syntheses of PhE-IM-PEI were also confirmed by FTIR spectra. As shown in Fig. 2A, two characteristic peaks located at 2920 and 2847 cm⁻¹ were observed in the sample of PhE, which evidenced the stretching vibration of CH₃ and CH₂ groups. While one characteristic peak located at 1750 cm⁻¹ could be clearly observed in IM, which were ascribed to the stretching vibration of C=O band. And the broad peak at 3286 cm⁻¹ was the stretching vibration of N–H band for PEI. After the radical copolymerization of PhE, IM, and PEI, the resulting cross-linked copolymers PhE-IM-PEI were formed. The broad and strong absorption at 3286 cm⁻¹ verified the successful attachment of PEI, and the obvious stretching vibration band of C–N at 1036 cm⁻¹ also demonstrated that there were many PEI being linked on the polymer. Furthermore, the peak located at 1750 cm⁻¹ could be ascribed to the C=O stretching vibration band, while another peak located at 1115 cm⁻¹ could be assigned to the stretching vibration band of C–O. All these results confirmed the successful formation of PhE-IM-PEI.

To further characterize the chemical information about the as-prepared PhE-IM-PEI FPNs, elemental composition of these FPNs has been determined by XPS studies. The XPS result indicated the presence of carbon as major component along with other three minor components of nitrogen and oxygen, respectively. The overall wt.% of elements present in FPNs was calculated as C:N:O:S ~ 77:18:4:1. The raw XPS data of the FPNs showed four signals corresponding to carbon, nitrogen, oxygen, and sulfur (Fig. 2B). The C 1s XPS spectrum of PhE-IM-PEI showed peak at ~285 eV, and the N 1s spectrum showed peak centered at ~398.5 eV, while the O 1s spectrum and S 2p demonstrated peaks at ~531.6 eV and ~163.5 eV, respectively (Fig. S1). All of the above results verified that IM and PEI were successfully incorporated into the PhE-IM-PEI FPNs.

The obtained amphiphilic PhE-IM-PEI polymers were tended to self-assemble into nanoparticles when they were dispersed in aqueous solution. The surfaces of these nanoparticles were covered with hydrophilic amino groups, while the conjugated aromatic groups were aggregated into the hydrophobic cores. Therefore, the resulting FPNs are expected to be highly dispersive in aqueous environment. At the same time, along with the aggregation of AIE components into the cores, these obtained FPNs are expected to emit intense fluorescence. Herein, the UV absorption spectra and fluorescence spectra of PhE-IM-PEI FPNs were studied to determine the water dispersibility and fluorescent properties. The UV spectra were shown in Fig. 3A, and it could be found that there were two absorption peaks located at 328 and 429 nm. The spectra were also found starting to increase from the very beginning, indicating the existence of nanoparticles in the solution, which is ascribed to the Mie effect [23]. The PhE-IM-PEI FPNs showed strong orange fluorescence in water, which was attributed to the aggregation of the AIE components. The fluorescence spectra of PhE-IM-PEI FPNs in water were shown in Fig. 3B. The maximum emission wavelength was located at 580 nm, while the fluorescence excitation wavelength was around 438 nm. Meanwhile, fluorescence quantum yield (Φ_F) of PhE-IM-PEI FGNs were estimated using quinine sulfate as the standard, and the absorbance of the solutions was kept around 0.05 to avoid internal filter effect. We demonstrated that the Φ_F of PhE-IM-PEI FGNs in PBS is about 40%.

The transmission electron microscopy (TEM) images were utilized to confirm the formation of the PhE-IM-PEI FPNs. Fig. 4A demonstrated that many spherical nanoparticles with diameters ranged from 20 to 80 nm could be clearly identified, which gave us direct evidence that the resulting amphiphilic cross-linked copolymers were self-assembled into nanoparticles in aqueous solution. Meanwhile, the size distribution of PhE-IM-PEI FPNs in phosphate buffer solution (PBS) was also determined using a zeta-Plus particle size analyzer, showing that the size distribution was 199.2 ± 27.1 nm, with a polydispersity index (PDI) of 0.051 for PhE-IM-PEI FPNs (Fig. 4B). As compared with the above size distribution, the size characterized by TEM was somewhat smaller due to the drying-causing shrinkage of the amphiphilic copolymers. Moreover, the leakage study of the dye from PhE-IM-PEI FPNs was conducted with dialysis against PBS solution or THF for three days using 7000 Da Mw cut-off dialysis membranes, the results showed that no dye leakage occurred, which was due to the covalent connection of the AIE dye with the polymer.

3.2. Biocompatibility of PhE-IM-PEI FPNs

To evaluate the potential biomedical applications of PhE-IM-PEI FPNs, their biocompatibility to human lung adenocarcinoma epithelial (A549) cells was also determined. The influences of PhE-IM-PEI FPNs to A549 cells were examined by optical microscopy after the cells were incubated with different concentrations of PhE-IM-PEI FPNs for 24 h (Fig. 5). It can be seen that cells still adhered to the cell plate very well after they were incubated with PhE-IM-PEI FPNs. As compared with the control cells (Fig. 5A), no obvious cell morphology change was observed through optical images even when the concentration of PhE-IM-PEI FPNs is as high as 80 μg mL⁻¹ (Fig. 5C). On the other hand, no cell number decrease was found as compared with the control cells and cells incubated with different concentrations of PhE-IM-PEI FPNs (Fig. 5B and C). These optical microscopy observation results indicated that PhE-IM-PEI FPNs are biocompatible with A549 cells. Furthermore, in order to quantitatively evaluate the cytocompatibility of PhE-IM-PEI FPNs, cell viability of the FPNs to A549 cells was determined by cell counting kit-8 (CCK-8) assay. As demonstrated in Fig. 5D, no cell viability decrease was found when the cells were incubated with 10–120 μg mL⁻¹ of PhE-IM-PEI FPNs for 8 h and 24 h, even when the concentration of these FPNs was up to 120 μg mL⁻¹, the cell
3.3. Biological imaging of PhE-IM-PEI FPNs

By taking advantages of the great biocompatibility, hydrophilicity and fluorescence property of PhE-IM-PEI FPNs, their cell uptake behavior was evaluated by confocal laser scanning microscope (CLSM) observation, which was demonstrated in Fig. 6B. Strong orange fluorescence could be observed within the cells after they were only incubated with 10 µg mL⁻¹ of these FPNs. Furthermore, many areas with a relative weak fluorescence intensity were found in the cells, which might be the possible location of cell nucleus (Fig. 6B), suggesting the PhE-IM-PEI FPNs could be facilely uptaken by cells with most of them located at cytoplasm, and these FPNs are considered uptaken by endocytosis of the cells. The cell imaging results of PhE-IM-PEI FPNs also indicated their excellent staining performance. Therefore, we could expect that the PhE-IM-PEI FPNs should be promising candidates for cell imaging with combined advantages, such as stable morphology in dilute physiological solution, intense fluorescence, good water dispersibility, and excellent biocompatibility. Moreover, in this work, IM and PEI were used as alternative monomers for the construction of fluorescent polymer; the highly reactive isocyanatoethyl groups of IM were greatly beneficial to connect with various functional components and the viability value was still greater than 90%. Combination of their unique AIE fluorescence, high water dispersibility, great stability, excellent biocompatibility, and facile one-pot preparation procedure, thus obtained AIE dyes based cross-linked FPNs are expected highly desirable for biomedical applications.
favorable biocompatibility of PEI also endow them potential preponderance for diverse biomedical applications. In short, this work provided a new construction methodology to afford stable fluorescent polymeric nanoparticles for cell imaging.

4. Conclusions

In summary, the AIE dyes based cross-linked amphiphilic fluorescent polymer PhE-IM-PEI was facilely prepared through one-pot method for the first time. Due to the existence of hydrophilic PEI and hydrophobic PhE on PhE-IM-PEI, thus obtained AIE dyes based cross-linked polymers are readily self-assembled into stable FPNs in pure aqueous solution. These FPNs possess numerous excellent properties such as great stability, high water dispersibility, uniform morphology, strong orange fluorescence, and great biocompatibility, making them highly potential for various bioimaging and biomedical applications. Moreover, a large number of amino groups were existed on PhE-IM-PEI FPNs, many other functional components such as drugs, genes, targeting agents and other imaging agents can be further integrated into PhE-IM-PEI FPNs. Therefore, multifunctional theranostic platforms based on PhE-IM-PEI FPNs can be fabricated. Combined all these advantages, such a type of cross-linked PhE-IM-PEI FPNs may open a new door to prepare multifunctional materials useful in biomedicine-related fields.

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Appendix A. Supplementary data

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