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Protocols

Biocompatible zwitterionic phosphorylcholine polymers with aggregation-induced emission feature

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ABSTRACT

Two novel zwitterionic phosphorylcholine polymers (**MTP1** and **MTP2**) with aggregation-induced emission (AIE) feature were prepared through reversible addition fragmentation chain transfer polymerization between an AIE monomer with vinyl end group and a zwitterionic phosphorylcholine monomer. The synthesized copolymers were characterized and confirmed by ^{1}H NMR, FT-IR, and X-ray photoelectron spectra. By introduction of the zwitterionic phosphorylcholine component, the synthesized copolymers showed amphiphilic properties and tended to self-assemble into fluorescent polymeric nanoparticles (FPNs) in water. The dynamic light scattering results indicated the size distribution of the **MTP1** FPNs was 345 ± 22 nm, and that of the **MTP2** FPNs was 147 ± 36 nm. The transmission electron microscopy results demonstrated spherical nanoparticle morphology for the FPNs. The high dispersibility of the FPNs in water was proved by the UV–vis absorption study with high transmittance of the solution. Fluorescent spectra of the prepared FPNs revealed bright green fluorescence with high fluorescence quantum yield of 45% for **MTP1** and 34% for **MTP2**. More importantly, the FPNs showed excellent particle stability with low critical micelle concentration of 0.008 mg mL^{-1} for **MTP1** and 0.007 mg mL^{-1} for **MTP2**. The cytotoxicity evaluation confirmed high cytocompatibility of the prepared FPNs at different concentrations, and demonstrated excellent biocompatibility for cell imaging. In virtue of the high-performance **MTP1** and **MTP2** FPNs, including high water dispersion, good particle stability, and excellent cytocompatibility, this work would inspire more researches about high-performance biocompatible fluorescent polymers for biomedical application.

1. Introduction

Zwitterionic polymers, in which the cationic and anionic groups are linearly arrayed on the pendant side chain of the molecular backbone, have attracted great attention due to their unique structures, hydration capacity, and anti-polyelectrolyte behavior [1–3]. Many zwitterionic polymers have been synthesized and explored for various applications, such as improvement of nanoparticle stability, ion exchange, sewage treatment, and biomedical applications [4–6]. Hydrophobic fluorescent molecules have combined with zwitterionic polymers to form amphiphilic fluorescent polymers, and assembled into fluorescent polymeric nanoparticles (FPNs), which could be applied in cell imaging and targeting with high colloidal stability [7–9]. Conventional organic fluorescent molecules suffer from fluorescence quenching when aggregated into nanoparticles due to strong intermolecular π-π stacking interactions [10–12]. To circumvent this issue, a new class of fluorescent dyes named aggregation-induced emission (AIE) dyes emerged with anti-quenching nature, and showed greater advantage for bioimaging [13–16]. Some archetypical AIE fluorophore structures, included tetraphenylethylene [17–20], triphenylethylene [21–26], siloles [27–29], cyano-substituted diarylethlene [30–32], and dis-

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tyrylanthracene [33–38], could be utilized for the construction of AIE-based FPNs for cell imaging [39–44].

Liu et al. reported the fabrication of ultra-bright AIE dots for cellular imaging and cell tracking, using an AIE fluorogen and a mixture of zwitterionic lipid-poly(ethylene glycol) and lipid-PEG-maleimide. Such AIE dots possessed high photostability, excellent cellular retention and biocompatibility [45]. Ji et al. developed a novel zwitterionic copolymer of poly(MPC-co-FPEMA) *via* reversible addition fragmentation transfer (RAFT) polymerization and further covalently connected with tetraphenylethylene, which could self-assemble into spherical micelles with excellent physiological stability and low protein adsorption, and were also demonstrated with high-quality AIE imaging and nonfouling surface [46]. Wang et al. have successfully synthesized a novel zwitterionic conjugated polyelectrolyte containing tetraphenylethylene and was encapsulated with lipid to form nanoparticles, which showed enhanced cell-staining efficiencies, low cytotoxicity, and high photostability [47]. Self-assembly of polymer to form nanoparticles is a promising strategy for various biomedical applicaitons, but such self-assembled system is often unstable in dilute solution below the critical micelle concentration (CMC) [48,49]. Thus, reducing the CMC value is very important for the FPNs to broaden their applications in dilute solution. For this purpose, we have utilized RAFT polymerization to combine AIE monomer and phospholipid monomer to afford zwitterionic fluorescent copolymers, such copolymers tended to self-assembly into FPNs with not only bright fluorescence, high dispersibility, and excellent biocompatibility, but also low CMC value [50,51]. Despite great progress of biocompatible AIE-based FPNs, new strategy to design zwitterionic AIE polymers with lower CMC and better particle stability is still highly demanded for cell imaging.

In this work, two novel zwitterionic phosphorylcholine copolymers (**MTP1** and **MTP2**) with AIE feature were prepared through RAFT polymerization between an AIE monomer with vinyl end group, NSP2E, and a zwitterionic phosphorylcholine monomer, MTP. Due to the introduction of hydrophilic zwitterionic component, such copolymers showed amphiphilic property and would self-assemble into FPNs in water (Scheme 1). The chemical structures of the copolymers were characterized by ^{1}H NMR, FT-IR, and X-ray photoelectron spectra. The morphologies and size distributions of the FPNs were analyzed by transmission electron microscopy and dynamic light scattering. The fluorescent property, particle stability, and biocompatibility of the FPNs were investigated and evaluated for cell imaging application.

2. Experimental procedure

2.1. Materials and characterization

4-Vinylphenylboronic acid (97%, TCI), 4-bromobenzaldehyde (99%, Sigma-Aldrich), potassium bicarbonate (99.5%, J&K), benzothiazole-2-yl-acetonitrile (98%, Sigma-Aldrich), 2,2'-azoisobutyronitrile (AIBN, 99%, J&K), palladium(II) chloride (99.9%, J&K), tetrabutylammonium hydroxide (40% in water, J&K), 1,4-dioxane (99.5%, J&K), isopropanol (99.7%, J&K), and 2-methacryloyloxyethyl phosphorylcholine (MTP, 97%, J&K) were used as received. All other agents and solvents were purchased from commercial source and used as received without further purification. Water was purified using a Milli-Q-system (Millipore, Bedford).

Gel permeation chromatography (GPC) analyses of polymers were performed with a Shimadzu LC-20AD pump system comprising of an auto injector, a MZ-Gel SDplus 10.0 mm guard column (50×8.0 mm, 10^2 Å) followed by a MZ-Gel SDplus 5.0 μm bead-size columns (50–10^6 Å, linear) and a Shimadzu RID-10A refractive index detector. ^{1}H NMR spectra were recorded on a Mercury-Plus 400 MHz spectrometer using d_6-DMSO as solvent and tetramethylsilane (TMS) as internal standard. The FT-IR spectra were conducted on a Shimadzu Spectrum 8400 spectrometer (Japan). Typically, 16 scans at a resolution of 1 cm^{-1} were accumulated to obtain one spectrum. The X-ray photoelectron spectra (XPS) were measured using a VGESCALAB 220-IXL spectrometer with an Al K_α X-ray source (1486.6 eV). The size distributions of the FPNs in phosphate buffer solution (PBS) were determined using a zeta Plus apparatus (ZetaPlus, Brookhaven Instruments, Holtsville, NY). Transmission electron microscopy (TEM) images were conducted on a HT7700 microscope (Hitachi, Japan) with the acceleration voltage for the electrons of 100 kV, the TEM samples were prepared by placing a drop of the FPNs dispersion on a carbon-coated copper grid. UV–vis absorption spectra were recorded on UV/Vis/NIR 2600 spectrometer (Shimadzu, Japan). Fluorescence spectra were measured on an F-4600 spectrometer with a slit width of 3 nm for both excitation and emission.

2.2. Preparation of MTP1 and MTP2

The AIE monomer of NSP2E was synthesized according to our previous literature [52]. 4-Cyano-4-(ethylthiocarbonothioylthio) pentanoic acid (CTA) was synthesized as literature showed [53]. To synthesize **MTP1**, as shown in Scheme 1, NSP2E (36.4 mg, 0.10 mmol), MTP (295 mg, 1.0 mmol), CTA (57.9 mg, 0.22 mmol), and AIBN (4.92 mg, 0.03 mmol) were dissolved in 3 mL of dioxane. The solution was introduced into a Schlenk tube and bubbled by nitrogen flow for 30 min. The above mixture was heated with an oil bath and maintained at 70 °C for 12 h. Then stopped the reaction of polymerization, the purified polymer of **MTP1** was obtained *via* precipitation from dioxane to petroleum ether for three times, and then dried under vacuum for further characterization. FT-IR (cm^{-1}): 2958, 2925, 2855, 1722, 1588, 1487, 1450, 1402, 1248, 1159, 1064, 964, 873, 759. GPC (Da): 11058 for M_n, 12168 for M_w, 1.10 for polydispersity index. XPS: C: N: O: S: P = 76.2: 2.8: 18.7: 0.9: 1.4.

The synthetic procedure of **MTP2** was almost the same as that of **MTP1**, whereas the amount of CTA was changed to 28.9 mg. FT-IR (cm^{-1}): 2958, 2925, 2855, 1722, 1588, 1457, 1406, 1377, 1248, 1159, 1064, 964, 873, 759. GPC (Da): 15195 for M_n, 16492 for M_w, 1.09 for polydispersity index. XPS: C: N: O: S: P = 78.3: 2.5: 17.3: 0.7: 1.2.

2.3. Cytotoxicity and cell imaging of MTP1 and MTP2 FPNs

The cytotoxicity of the **MTP1** and **MTP2** FPNs was evaluated by cell counting kit-8 (CCK-8) assay with A549 cells, and the cells were treated with different doses of FPNs samples for 8 and 24 h, respectively. Cell imaging of the **MTP1** and **MTP2** FPNs was measured with a confocal laser scanning microscope (CLSM, Zeiss 710 3-channel, Germany). Specific experimental conditions were adopted according to our previous paper [54]. For cytotoxicity study, A549 cells were cultured in a 96 well plate in 160 μL of respective media containing 10% fetal bovine serum (FBS). Each well plate with attached cells was treated with 10 μL of CCK-8 dye and 100 μL of Dulbecco's modified eagle medium (DMEM) cell culture medium, and was incubated for 2 h at 37 °C. The percent reduction of CCK-8 dye was compared with control cells, which were no exposure to **MTP1** or **MTP2** FPNs, representing 100% CCK-8 reduction. Three replicate wells were used per microplate, and the experiment was repeated for three times. For cell imaging study, A549 cells were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin at 37 °C and 5% CO_2. Then A549 cells were seeded into 96 well plates with 500 μL DMEM media. After growth overnight, the **MTP1** or **MTP2** FPNs