

Facile Fabrication of Aggregation-induced Emission Based Red Fluorescent Organic Nanoparticles for Cell Imaging*

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Abstract A cyano-substituted diarylethylene derivative aggregation-induced emission (AIE) dye with two amino end-groups and 4,4'-(hexafluoroisopropylidene)diphthalic anhydride were facilely incorporated into red fluorescent organic nanoparticles (FONs) *via* room temperature anhydride ring-opening polymerization under an air atmosphere. These obtained **RO-HFDA** FONs were characterized by a series of techniques including gel permeation chromatography, Fourier transform infrared spectroscopy, size distribution and zeta potential measurements, UV-Vis absorption spectrum, fluorescent spectroscopy and transmission electron microscopy. Biocompatibility evaluation and cell uptake behavior of **RO-HFDA** FONs were further investigated to explore their potential biomedical application. We demonstrated that such FONs showed high water dispersibility, stable uniform spherical morphology (150–200 nm), broad excitation band (350–605 nm), intense red fluorescence (627 nm) and excellent biocompatibility, making them promising for cell imaging applications.

Keywords: Cyano-substituted diarylethylene derivative; Aggregation-induced emission; Anhydride ring-opening polymerization; Red fluorescent organic nanoparticles; Cell imaging.

INTRODUCTION

Fluorescent organic nanoparticles (FONs) have attracted increasing interest for their high potential in various sensing, imaging and biomedical applications^[1–3]. Compared with the conventional fluorescent inorganic nanoparticles including carbon dots, quantum dots and fluorescent silicon dots, FONs have recently received more and more attentions due to their many advantages such as low toxicity, biocompatibility, biodegradability and adjustable functionalities^[4–7]. However, owing to the inherent hydrophobic features, most organic fluorophores are insoluble in physiological solution, thus need modification of hydrophilic groups before practical applications. Although the introduction of hydrophilic groups could improve the water dispersibility of the organic dyes, the hydrophobic component of the amphiphilic product still easy to self-assemble as cores in the physiological solution at the expense of the fluorescence intensity^[8]. The reason for this is when organic dyes are concentrated in a limited space like nanoparticle, luminescence quenching may emerge simultaneously due to the notorious aggregation-caused fluorescent quenching (ACQ) effect^[9]. Therefore, to overcome this issue, unique aggregation induced emission (AIE) materials with anti-ACQ effect appeared, which emit stronger fluorescence in the aggregation state^[10–12]. Henceforth, various AIE dyes such as siloles^[13–16],

* This work was financially supported by the National Natural Science Foundation of China (Nos. 21134004, 21201108 and 51363016), and the National 973 Project (No. 2011CB935700), China Postdoctoral Science Foundation (Nos. 2012M520243, 2013T60100).

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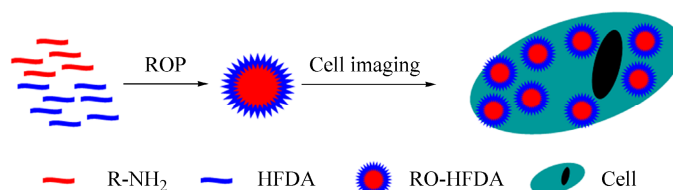
Received October 9, 2013; Revised November 17, 2013; Accepted November 19, 2013

doi: 10.1007/s10118-014-1461-6

tetraphenylethene^[17–22], cyano-substituted diarylethene^[23–25], triphenylethene^[26–31] and distyrylanthracene^[32–37] derivatives have been reported and widely explored for potential biomedical applications thanks to their attractive AIE feature, biocompatibility and biodegradability.

Hereafter, some various strategies for fabricating AIE based FONs have been developed including bare AIE nano-assembly, non-covalent strategy and covalent strategy. Thus AIE based FONs exhibited intense luminescence, uniform size, high water dispersibility and excellent biocompatibility, making them promising for cell imaging applications^[38–47]. However, the bare AIE nano-assembly and non-covalent strategies have encountered one major problem that the fluorophores can leak out of the particles with time^[8]. While another problem occurs in the covalent strategy including the previous reported Schiff-base reaction^[44, 46], reversible addition fragmentation chain transfer polymerization^[24, 48] and emulsion polymerization^[45], that is the cumbersome synthetic conditions such as high temperature, initiator, chain transfer agent and inert gas. Most recently, a novel covalent strategy named anhydride ring-opening polymerization (ROP) has been reported by our group^[25], which represented facile preparation of AIE based FONs by one-step synthesis under an air atmosphere at room temperature, based on an AIE dye and a dianhydride. Thus ROP strategy has been proved with greater advantage in the facile condition including one-step synthesis, room temperature, under an air atmosphere and catalyst free. Despite many impressive advances in fabrication of AIE based FONs, red/near-infrared (> 600 nm) FONs are highly desirable for biological applications due to their low optical absorption and autofluorescence of biological media^[38, 39].

Herein, a facile method of preparing AIE based red luminescent FONs was developed *via* room temperature anhydride ROP based on a cyano-substituted diarylethene derivative AIE dye (**R-NH₂**) with two amino end-groups and 4,4'-(hexafluoroisopropylidene)diphthalic anhydride (HFDA) under an air atmosphere (Scheme 1). Such obtained amphiphilic copolymers (**RO-HFDA**) are tended to self-assemble into polymeric nanoparticles with the hydrophobic aromatic groups as cores, while the hydrophilic carboxyl groups covered onto the surfaces as shells, which would result in high dispersibility in aqueous solution. These obtained FONs were characterized by a series of techniques including gel permeation chromatography (GPC), Fourier transform infrared spectroscopy (FTIR), size distribution and zeta potential measurements, UV-Vis absorption spectrum (UV), fluorescent spectroscopy and transmission electron microscopy (TEM). Owing to the AIE feature of **R-NH₂**, the resulting FONs emitted strong red fluorescence in aqueous environment. Finally, the biocompatibility and cell uptake behavior of **RO-HFDA** FONs were further studied to evaluate their potential for cell imaging applications.



Scheme 1 Preparation of **RO-HFDA** FONs *via* room temperature anhydride ROP under an air atmosphere based on an AIE dye (**R-NH₂**) for cell imaging applications

EXPERIMENTAL

Materials and Characterization

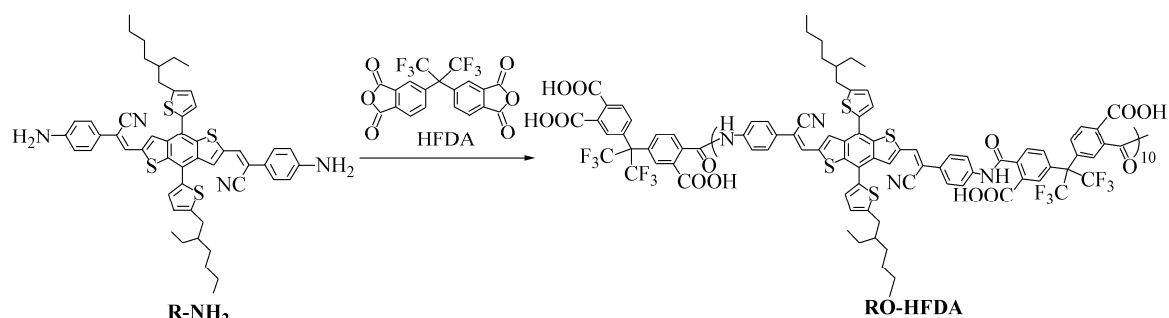
Thiophene, 3-(bromomethyl)heptane, thiophene-2-carboxylic acid, thionyl chloride, dimethylamine, *n*-butyllithium, stannic chloride, 2-(4-aminophenyl)acetonitrile, tetrabutylammonium hydroxide (0.8 mol/L in methanol), *N,N*-dimethylacetamide (DMAc) and 4,4'-(hexafluoroisopropylidene)diphthalic anhydride (HFDA) purchased from Alfa Aesar were used as received. All other agents and solvents were purchased from commercial sources and used directly without further purification.

¹H-NMR spectra were measured on a JEOL 400 MHz spectrometer (d₆-DMSO as solvent and tetramethylsilane (TMS) as the internal standard), and the scanning time is 4 h. 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 the spectrum. UV-Visible absorption spectrum was 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. 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 measurement of **RO-HFDA** FONs in phosphate buffer solution (PBS) were determined using a zeta Plus apparatus (ZetaPlus, Brookhaven Instruments, Holtsville, NY). GPC analysis of the copolymers was 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 ($50\text{ mm} \times 8.0\text{ mm}$, 10 nm) followed by a MZ-Gel SDplus 5.0 μm bead-size columns ($50\text{--}10^5\text{ nm}$, linear) and a Shimadzu RID-10A refractive index detector. The system was calibrated with narrow molecular weight distribution polystyrene standards ranging from 200 to $10^6\text{ g}\cdot\text{mol}^{-1}$.

Preparation of **RO-HFDA** FONs

The synthetic route of **RO-HFDA** is showed in Scheme 2. **R-NH₂** was synthesized according to our previous literature^[49], and the AIE characteristic of **R-NH₂** has also been determined in this literature.



Scheme 2 Synthetic route of **RO-HFDA**

In the synthesis of **RO-HFDA**, **R-NH₂** (26 mg, 0.03 mmol) and HFDA (15 mg, 0.033 mmol) were dissolved in 10 mL DMAc. The above mixture was stirred under an air atmosphere at room temperature for 2 h, and then the reaction of polymerization was stopped and dialyzed against tap water for 24 h and ethanol for 6 h using 7000 Da M_w cutoff dialysis membranes. Finally, this solution in dialysis bag was carried out by freeze-drying to obtain **RO-HFDA** FONs.

Cytotoxicity of **RO-HFDA** FONs

Cell morphology was observed to examine the effects of **RO-HFDA** FONs to A549 cells. Briefly, cells were seeded in 6-well microplates at a density of $1 \times 10^5\text{ cells mL}^{-1}$ 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 **RO-HFDA** FONs 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 **RO-HFDA** FONs on A549 cells was evaluated by cell counting kit-8 (CCK-8) assay. Briefly, cells were seeded in 96-well microplates at a density of $5 \times 10^4\text{ cells mL}^{-1}$ 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 $\mu\text{g}\cdot\text{mL}^{-1}$ **RO-HFDA** FONs 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 (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 **RO-HFDA** 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 **RO-HFDA** FONs

A549 cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% heat-inactivated FBS, 2 mmol·L⁻¹ glutamine, 100 U·mL⁻¹ penicillin, and 100 μ g·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 1×10^5 cells per dish. On the day of treatment, the cells were incubated with **RO-HFDA** FONs at a final concentration of 10 μ g·mL⁻¹ for 3 h at 37 °C. Afterward, the cells were washed three times with PBS to remove the **RO-HFDA** FONs 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.

RESULTS AND DISCUSSION

Characterization of **RO-HFDA** FONs

To prepare **RO-HFDA** FONs, the hydrophobic AIE dye (**R-NH₂**) was copolymerized with 4,4'-(hexafluoroisopropylidene)diphthalic anhydride (HFDA) *via* room temperature ROP^[50] under an air atmosphere for 2 h, based on the feed molar ratio (HFDA:**R-NH₂**) of 1.1, to obtain **RO-HFDA** with the designed degree of polymerization as 10. The number average molecular weight (M_n) of the copolymers was determined by GPC. Result showed that the M_n for **RO-HFDA** was 12410 Da. Then ¹H-NMR spectrum of **RO-HFDA** was conducted and is shown in Fig. 1(a), the emergences of carboxyl peak at $\delta = 10.8$ and alkyl peaks around $\delta = 1.0$ suggested the successful synthesis of **RO-HFDA**, the degree of polymerization calculated from the ¹H-NMR spectrum is approximately equal to 10. Furthermore, the **RO-HFDA** copolymer was confirmed by FTIR. As shown in Fig. 1(b), two characteristic peaks located at 2924 and 2854 cm⁻¹ were observed in the sample of **R-NH₂**, evidencing the stretching vibration of CH₂ and CH₃ groups. The N—H stretching vibration of **R-NH₂** located at 3373 cm⁻¹ was also found. Meanwhile, a series of peaks distributed between 1450–1600 cm⁻¹ can be ascribed to the stretching vibration of their aromatic rings. After anhydride ROP, strong bending vibration of O—H band of

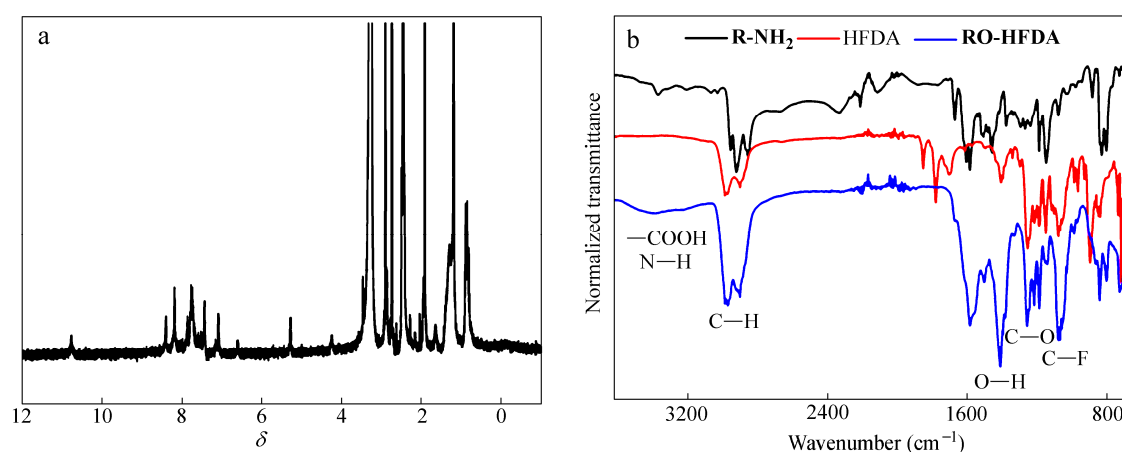


Fig. 1 (a) ¹H-NMR spectrum of **RO-HFDA** dissolved in d₆-DMSO and (b) FTIR spectra of **R-NH₂**, HFDA and **RO-HFDA**

carboxylic groups located at 1410 cm^{-1} was observed for **RO-HFDA** FONs^[51]. On the other hand, a characteristic peak centered at 1250 cm^{-1} was also observed, evidencing that the stretching vibration of carboxylic groups in the copolymers, and another characteristic peak located at 1073 cm^{-1} was also observed, suggesting that C—F band was introduced into the copolymers, meanwhile, the strong stretching vibrations of —COOH and —NH₂ also appeared in the spectrum of **RO-HFDA** FONs located from 3250 cm^{-1} to 3400 cm^{-1} , all confirming the successful formation of **RO-HFDA** copolymers. Owing to the formation of hydrophilic carboxyl groups in **RO-HFDA**, such obtained copolymers showed amphiphilic properties. When dispersed in aqueous solutions, these copolymers are tended to self-assemble into polymeric nanoparticles with the hydrophobic aromatic groups as cores, and the hydrophilic carboxyl groups covered onto the surfaces as shells. As the AIE fluorophore **R-NH₂** and other hydrophobic groups were aggregated into the nanoparticle cores, thus **RO-HFDA** FONs are expected to possess strong fluorescence and high dispersibility in aqueous solutions.

The UV absorption spectrum of **RO-HFDA** FONs dispersed in water indicated that the maximum absorption peak located at 433 nm with an acromion at 525 nm (Fig. 2a). The entire UV spectrum was observed to rise from 800 nm in absorption due to the Mie scattering effect, suggesting the existence of nanoparticles. The inset of Fig. 2(a) showed the transparent red liquid in the cuvette, intuitively demonstrated the high water dispersibility of **RO-HFDA** FONs. It was worth mentioning that no visible precipitation was found even after the above liquid was deposited for more than one month. The size distribution of **RO-HFDA** FONs in phosphate buffer solution (PBS) was tested using a zeta Plus particle size analyzer, indicating that the size distribution of

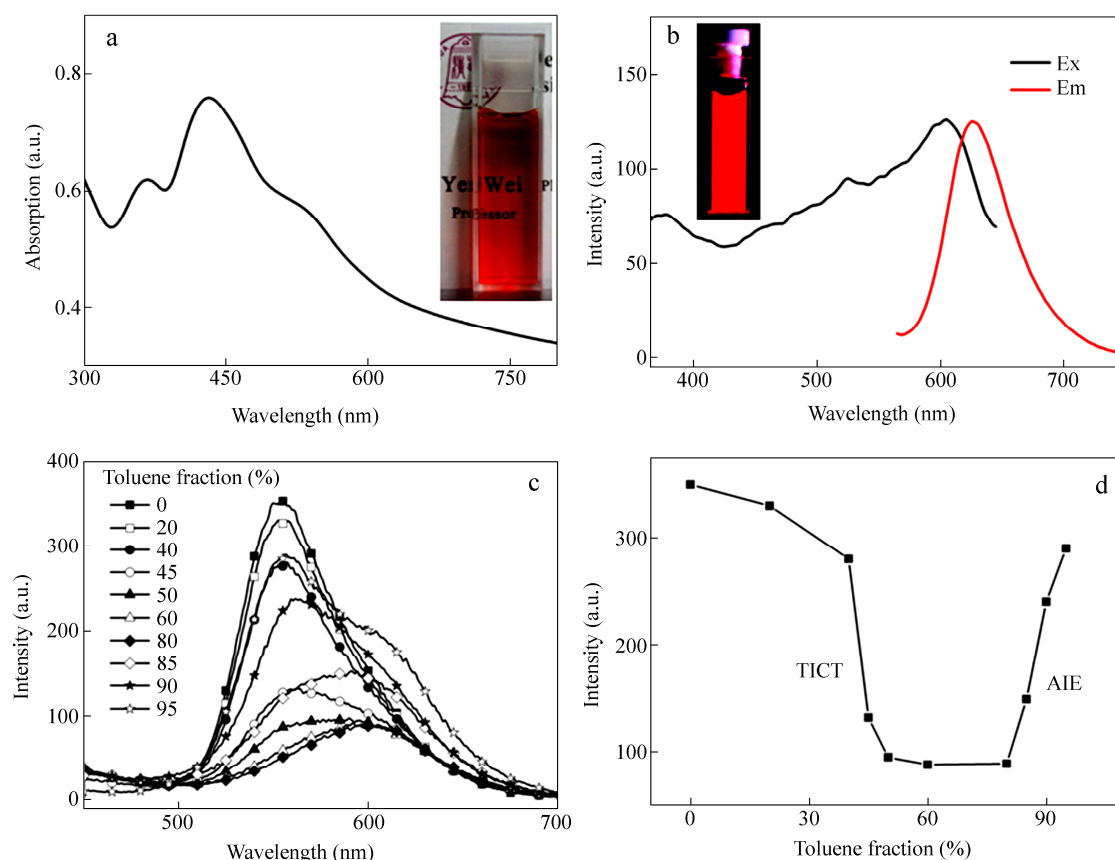


Fig. 2 (a) UV-Vis spectrum of **RO-HFDA** FONs (Inset is the visible image of such FONs in water.) (b) fluorescence excitation (Ex) and emission (Em) spectra of **RO-HFDA** FONs (Inset is the fluorescent image taken at 365 nm UV light), (c) PL spectra of **RO-HFDA** in DMF-toluene mixtures with different toluene fractions and (d) the changes in PL peak intensity of **RO-HFDA** in different toluene fraction mixtures

these FONs was 236.9 ± 7.1 nm with a polydispersity index of 0.223. Meanwhile, the zeta potential measurements of **RO-HFDA** FONs in PBS were determined and showed zeta-potential of -39.4 ± 2.5 mV. Due to the AIE properties of **R-NH₂** and self-assembly of the amphiphilic copolymers, **RO-HFDA** FONs showed strong red fluorescence when dispersed in pure water (inset of Fig. 2b). The fluorescence excitation and emission spectra of **RO-HFDA** FONs in water are shown in Fig. 2(b). The maximum emission wavelength was located at 627 nm, while the fluorescence excitation spectrum in water exhibited broad excitation band wavelength from 350 to 605 nm. It is noteworthy that the excitation intensity was continued increasing even the fluorescent excitation wavelength exceed 543 nm. Meanwhile, the AIE characteristic of **RO-HFDA** has been conducted (Figs. 2c and 2d). From the changes in PL peak intensity of the compound in different toluene fraction mixtures, two procedures were observed (Fig. 2d). The 0%–60% section indicated obvious twisted intramolecular charge transfer (TICT) effect, while the > 80% procedure demonstrated apparent AIE characteristic. These excellent fluorescent features including broad excitation band, long wavelength excitation and intense red emission are greatly benefited for their potential cell imaging applications.

The TEM images further confirmed the formation of conjugated **RO-HFDA** FONs (Fig. 3). Many spherical nanoparticles with diameters ranged from 150 nm to 200 nm can be clearly identified. As compared with the size distribution in PBS, the size characterized by TEM was somewhat smaller, might be, due to the shrinkage of micelles during the drying process. The TEM images give us direct evidence that the amphiphilic copolymers were self-assembled into nanoparticles in aqueous solutions. In our previous reports, biocompatible AIE FONs were fabricated through covalent strategy including Schiff-base reaction^[44, 46], reversible addition fragmentation chain transfer polymerization^[24, 48] and emulsion polymerization^[45]. Compared with previous synthesized FONs, the FONs fabricated *via* anhydride ROP in this work have many obvious advantages. First, the reaction condition was simple and mild, such as one-step synthesis, room temperature, catalyst free and without protection of inert gas. Second, the fluorescent excitation wavelength (with increasing intensity up to 605 nm) and emission wavelength (627 nm) are much longer, which make them low optical absorption and autofluorescence in biological media. Moreover, many other components including targeting agents and drugs (*e.g.* cisplatin) could be further integrated into the AIE based FONs taking advantage of the introduced reactive carboxyl groups.

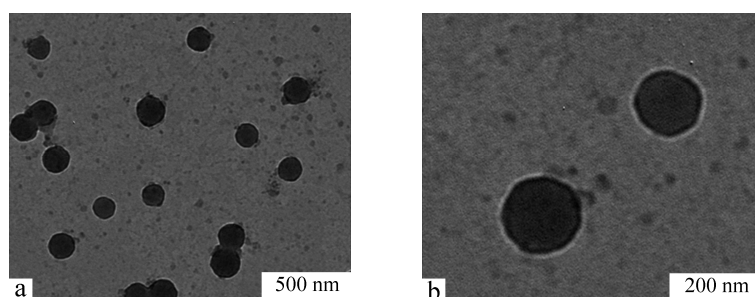


Fig. 3 TEM images of **RO-HFDA** FONs dispersed in water with different magnification

Biocompatibility of **RO-HFDA** FONs

The biocompatibility was evaluated for the potential biomedical applications of **RO-HFDA** FONs^[52–54]. First, the influences of **RO-HFDA** FONs to human lung adenocarcinoma epithelial (A549) cells were examined by optical microscopy after cells were incubated with different concentrations of FONs for 24 h (Figs. 4a–4c). The result showed that there were no significant differences between the control cells and cells incubated with 10 and 80 $\mu\text{g}\cdot\text{mL}^{-1}$ of **RO-HFDA** FONs, indicating that these FONs are biocompatible with cells. To further confirm the cytocompatibility of **RO-HFDA** FONs, cell viability of these FONs to A549 cells was determined by cell counting kit-8 (CCK-8) assay^[55–57]. As shown in Fig. 4(d), no cell viability decrease was observed when cells were incubated with 10–120 $\mu\text{g}\cdot\text{mL}^{-1}$ of these AIE based FONs for 8 h and 24 h. The cell viability value is still higher than 95% even exposed to 120 $\mu\text{g}\cdot\text{mL}^{-1}$ FONs for 24 h, while no significant difference was observed. These results suggested that such FONs were highly potential for biomedical applications.

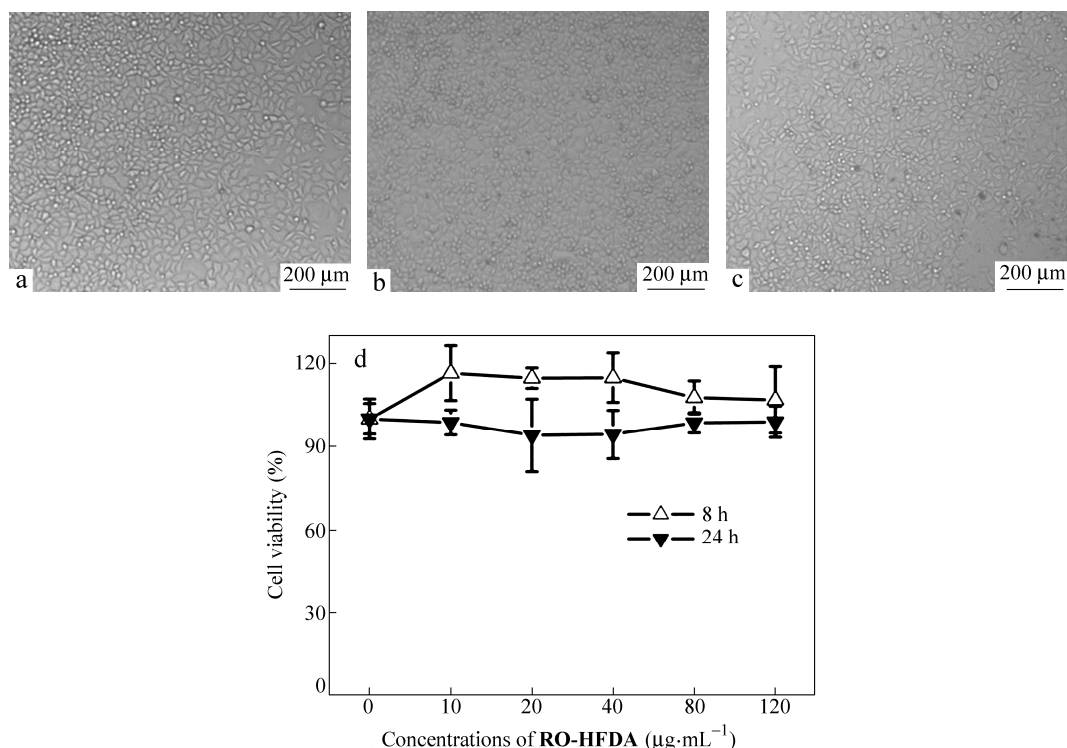


Fig. 4 Optical microscopy images of A549 cells incubated with different concentrations of **RO-HFDA** FONS for 24 h: (a) control cells, (b) 10 $\mu\text{g}\cdot\text{mL}^{-1}$, (c) 80 $\mu\text{g}\cdot\text{mL}^{-1}$; (d) cell viability of **RO-HFDA** FONS with A549 cells for 8 h and 24 h

Cell Imaging Applications of RO-HFDA FONS

Based on the biocompatibility results, cell imaging applications of **RO-HFDA** FONS were further explored^[58–60]. The cell uptake behavior of these FONS was evaluated by Confocal Laser Scanning Microscope (CLSM) observation. As shown in Fig. 5, strong red luminescence could be observed at the cell location after incubation with only 10 $\mu\text{g}\cdot\text{mL}^{-1}$ of **RO-HFDA** FONS. Furthermore, many areas with relative weak fluorescence intensity were found in the cells, indicating the possible location of cell nucleus (Fig. 5b), which suggested that **RO-HFDA** FONS could be facilely uptaken by cells and mainly located at cytoplasm. It is considered that these

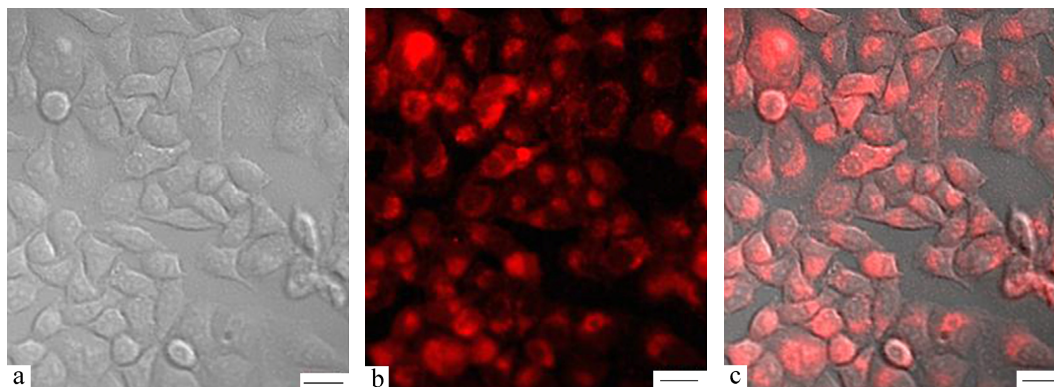


Fig. 5 CLSM images of A549 cells incubated with 10 $\mu\text{g}\cdot\text{mL}^{-1}$ of **RO-HFDA** FONS for 3 h: (a) bright field, (b) excited with 543 nm laser and (c) merged image of A and B (Scale bar = 20 μm)

FONs could not enter the cell nucleus directly as the size of FONs are much larger than the nucleus pore (9 nm × 15 nm). More importantly, thanks to the intense red fluorescence of these FONs, strong signal could be detected. According to the cell viability results, **RO-HFDA** FONs are considered biocompatible enough for bioimaging applications.

CONCLUSIONS

In summary, AIE based **RO-HFDA** FONs were facily prepared through room temperature anhydride ROP based on a cyano-substituted diarylethylene derivative AIE dye (**R-NH₂**) with two amino end-groups and a dianhydride (HFDA) under an air atmosphere. Such obtained amphiphilic copolymers were tended to self-assemble into uniform spherical FONs with diameters ranged from 150 nm to 200 nm. These **RO-HFDA** FONs showed high dispersibility in physiological solution as their surfaces were covered with many hydrophilic carboxyl groups. At the same time, such FONs exhibited excellent fluorescent features including broad excitation band (350–605 nm), strong long wavelength excitation (with increasing intensity up to 605 nm) and intense red emission (627 nm). Biocompatibility evaluation and cell imaging results suggested that these FONs were biocompatible enough for bioimaging applications. More importantly, various AIE based FONs could be facily fabricated *via* room temperature anhydride ROP under an air atmosphere, taking advantage of different AIE based amino end-groups dyes and various multianhydrides, and can be further integrated with other components like drugs and targeting agents into the system utilizing their various functional groups to obtain multifunctional platforms. Therefore, the obtained FONs are expected highly potential for various biomedical applications thanks to their various excellent performances.

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