Facile preparation and biological imaging of luminescent polymeric nanoprobes with aggregation-induced emission characteristics through Michael addition reaction

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Abstract:

Water dispersion aggregation-induced emission (AIE) dyes based nanomaterials have recently attracted increasing attention in the biomedical fields because of their unique optical properties, outstanding performance as imaging and therapeutic agents. The methods to conjugate hydrophilic polymers with AIE dyes to solve the hydrophobic nature of AIE dyes and make them widely used in biomedicine, which have been extensively explored and paid great effort previously. Although great advance has been made in the fabrication and biomedical applications of AIE-active polymeric nanoprobe, facile and efficient strategies for fabrication of biodegradable AIE-active nanoprobe are still highly desirable. In this work, amphiphilic biodegradable fluorescent organic nanoparticles (PLL-TPE-O-E FONs) have been fabricated for the first time by conjugation of AIE dye tetraphenylethene acrylate (TPE-O-E) with Poly-L-Lysine (PLL) through a facile one-step Michael addition reaction, which was carried out under rather mild conditions, including air atmosphere, near room temperature and absence of metal catalysts or hazardous reagents. Due to the unique AIE properties, these amphiphilic copolymers tend to self-assemble into highly luminescent water dispersible nanoparticles with size range from 400-600 nm. Laser scanning microscope and cytotoxicity results revealed that PLL-TPE-O-E FONs can be internalized into cytoplasm with negative cytotoxicity, which implied that PLL-TPE-O-E FONs are promising for biological applications.

Key words: aggregation-induced emission; amphiphilic polymers; Poly-L-Lysine; Michael addition reaction; biological imaging

1. Introduction

Over the past few decades, fluorescent nanomaterials have been extensively explored for biomedical applications because of their outstanding optical properties as compared with the small organic dyes.[1, 2] Typically, fluorescent nanomaterials could be mainly classified as fluorescent organic nanoparticles (FONs) and fluorescent inorganic nanoparticles (FINs) [3-6]. These fluorescent nanoparticles have been widely used for biomedical applications because of their high sensitivity, rapid response, non-destructive character and signal stability that can visualize the morphological details of cells [7, 8]. Promising fluorescent nanoparticles for biomedical applications should possess following features, which included desirable optical properties, high water dispersibility, biodegradable potential and good biocompatibility [9-18]. As compared with FINs, FONs showed obvious advantages for bioimaging applications for their designability, biodegradability and biocompatibility. However, it is well known that the fluorescence of FONs based on the conventional organic dyes will largely quenched due to the aggregation-caused quenching (ACQ) effect, which will occur in high concentrations or in solid state [19-22]. This fatal flaw makes us challengeable to prepare ultrabright FONs using the conventional organic dyes. Since Tang et al discovered and proposed the aggregation-induced emission (AIE) in 2001 [20], more and more attention has been attracted by this abnormal fluorescence phenomenon. Opposite to the ACQ effect, AIE active dyes emitted much stronger fluorescent intensity in their aggregate state due to the restriction of intramolecular rotations (RIR) and prohibition of energy dissipation via non-irradiative channels [19]. To date, a large number of new AIE luminogens containing silole, tetraphenylethene (TPE), triphenylamine (TPA), 9,10-distyrylanthracene (DSA), 1,4-distyrylbenzene (DSB) and their derivatives have been designed and explored for different applications [23-29]. However, the inherent hydrophobic features of AIE fluorogens have limited their application in physiological solution, which is of great challenges to modify these AIE-active fluorogens with stable and excellent water dispersibility. Since amphiphilic polymers are generally intended to self-assemble into vesicle/micelle-like uniform structures in aqueous solutions [23, 30-33]. A variety of FONs had been reported by Tang et al and Zhang et al based on AIE-active dyes [28, 34-44], which showed good water dispersibility and biocompatibility.

Poly-L-lysine (PLL) has plentiful active amino groups, which can covalently connect with drugs or be modified for further application. Besides, PLL is also an ideal gene delivery system because of the plentiful positively charged amino groups. And most importantly, PLL is a water-soluble, low-cost and commercial available polymer, which can be easily degraded into digestible lysine. Therefore, the PLL based materials

can also avoid long-time retention in tissue after they were accumulated by tissues through enhanced permeability and retention effect (EPR) [45]. PLL-functionalized nanomaterials have been widely used in cell labeling, DNA electrochemical sensors, and gene or drug delivery [46, 47]. PLL is biodegradable, nontoxic, non-immunogenic and non-inflammatory, which makes it an ideal carrier for systemic drug delivery applications. However, the fabrication of AIE-active polymeric nanoprobe with good water dispersibility, biocompatibility and biodegradable has rarely reported.[48, 49] For example, Zheng et al have described the conjugation of AIE-active dye with chitosan through the covalent reaction between isocyanate group of AIE dye and the amino group of chitosan.[49] Park et al have also developed a covalent method for fabrication of aggregation induced emission enhancement nanoprobe through conjugation between the carboxyl group of AIE dye and the amino groups of water-soluble glycol chitosan.[48] The Michael addition reaction between amino group and ene bond is a simple, fast and efficient method for synthesis of many useful materials (e. g. polyamidoamine dendrimers) in mild reaction conditions.[50, 51] It can be occurred under near room temperature, absent of metal catalysts and air atmosphere. Therefore, it is very useful for fabrication of biomaterials but maintaining their biological active and the luminescent properties. However, to the best of our knowledge, the fabrication of AIE-active luminescent nanoparticles using Michael addition reaction has not been reported thus far.

In this contribution, we reported a rapid and simple method to prepare water dispersible FONs based on AIE-active dyes with biodegradable PLL as a linker through a facile Michael addition reaction (**Scheme 1**). The PLL-functionalized AIE active materials (PLL-TPE-O-E FONs) were high water dispersibility, biodegradable and nontoxic. With plentiful amino groups, these AIE active nanoparticles can be further modified with bioactive molecules, targeting agents and other imaging agents, and present great potential application in future. The AIE dyes as well as the final PLL-TPE-O-E FONs were characterized by a number of techniques to determine their structure and properties in detail. As compared with other fabrication methods, the Michael addition reaction can occur under rather mild conditions (e. g. near room temperature 35 °C, air atmosphere, absent of metal catalysts and in the present of water, neutral pH). Therefore, the Michael addition reaction is specific useful for conjugation of AIE dyes with many other biomacromolecules such as proteins, peptides and synthetic molecules with amino groups but can well keep their structure and bioactivity.

Scheme 1 Reaction scheme for the synthesis of PLL-TPE-O-E FONs through one-step Michael addition reaction between the carbon-carbon double bond of TPE-O-E and amino groups of PLL. The fabrication reaction can occur under rather mild conditions such as low temperature (35°C), air atmosphere, absent of metal catalysts.

2. Materials and methods

2.1. Materials and characterization

Bromotriphenylethylene, tetrakis (triphenylphosphine) palladium, etrabutyl ammonium bromide (TBAB), 4-Carboxybenzeneboronic acid and acryloyl chlorid (99%) purchased from Aladdin (Shanghai, China) were used as received. Anhydrous tetrahydrofuran (THF, J&K Chemical), ethyl acetate (J&K Chemical) and triethylamine (Et_3N , J&K Chemical, 99.5%) were used without further purification. ^1H nuclear magnetic resonance (NMR) spectra were performed on Bruker Avance-400 spectrometer with D_2O or CDCl_3 as solvent and tetramethylsilane (TMS) as reference. The Fourier transform infrared (FT-IR) spectra were carried out by using a Nicolet 380 Fourier transform spectrometer with a resolution of 2 cm^{-1} . Perkin Elmer LAMBDA 35 UV/vis system was used to measure UV-vis absorption spectrum. Transmission electron microscopy (TEM) images were recorded on a Hitachi 7650B microscope operated at 80 kV, and the specimens were made by dropping a drop nanoparticle suspension on a carbon-coated copper grid. Fluorescence spectra (FL) were tested through a PELS-55 spectrometer with a slit width of 3 nm for the

emission of PLL-TPE-O-E FONs in water. The quantum yield of PLL-TPE-O-E FONs in aqueous solution was determined using quinine sulfate as reference dye. The quinine sulfate was dispersed in 0.05 mol L⁻¹ sulfuric acid aqueous solutions (the concentration of quinine sulfate is 1×10^{-3} mol L⁻¹). The adsorption values of PLL-TPE-O-E FONs and quinine sulfate was determined using UV-Vis spectrometer. The fluorescence spectra of PLL-TPE-O-E FONs and quinine sulfate excited at 360 nm were recorded on PEELS-55 spectrometer. The integrated areas based on their fluorescence spectra (wavelength range 380-580 nm) were calculated. The relative QY of PLL-TPE-O-E FONs was calculated using the formula as described in our previous report.[14] Zeta-potential and size distribution of PLL-TPE-O-E FONs in water were measured using a Zeta Plus particle size analyzer (Zeta Plus, Brookhaven Instruments, Holtsville, NY).

2.2 Synthesis of TPE-O-E

TPE-O-E was performed through two steps. In the first step, the synthetic route of TPE-OH was carried out according to Zhang et al [52, 53] with little changes as following: Bromotriphenylethylene (1.675 g, 10 mmol) and 4-Carboxybenzenboronic acid (2.25 g, 15 mmol) were dissolved in the 60 mL tetrahydrofuran (THF), then, 0.32 g TBAB and 2 M potassium carbonate aqueous solution (18 mL) were mixed with the solution. After the mixture was stirred at 55 °C under nitrogen for about 0.5 h, Pd (PPh₃)₄ (0.010 g, 8.70×10^{-3} mmol) was added in the mixture and then heated to 90 °C for 24 h. The process was monitored by thin layer chromatographic (TLC). After extracted from water and ethyl acetate, the organic layer was dried over anhydrous sodium sulfate (Na₂SO₄). After evaporated under vacuum, the clued product was purified on silica gel with a mixture of n-hexane and CH₂Cl₂ (volume ratio 2:1) to product pure compound TPE-OH. The product was confirmed by ¹H NMR (CDCl₃ as solvent) and FT-IR.

In the second step, 417.6 mg TPE-OH (1.2 mmol) was reacted with 350 mg acryloyl chloride (3.6 mmol) at a 1:3 molar ratio in 10 mL absolute THF, and then triethylamine (1 mL) was added dropwise. The reaction was stirred in ice-bath under nitrogen until TPE-OH completely reacted (the process was about 6 h and monitored by TLC). After filtered under vacuum, the solution was extracted with saturated sodium bicarbonate three times to remove unreacted chemical agents. The organic phase was collected and the solvent was removed under vacuum. The forming white product was dried under vacuum to acquire TPE-O-E.

2.3 Synthesis of PLL-TPE-O-E FONs

The mixture of TPE-O-E (50 mg) dissolved in 6 mL THF and PLL (200 mg) dissolved in 4 mL water

were stirred at 35 °C under nitrogen for 8 h until TPE-O-E was completely reacted (monitored by TLC). Thereafter, the polymer solution was washed with water and extracted with ethyl acetate. Finally, the milky organic phase was separated followed purified by dialysis to remove unreacted TPE-O-E. After dried through anhydrous Na₂SO₄, organic solvents were removed on a rotary evaporator at 50 °C under vacuum to obtain pure PLL-TPE-O-E. The PLL-TPE-O-E FONs were characterized by ¹H NMR, FT-IR, UV-Vis and FL.

2.4 Confocal microscopic imaging of cells using PLL-TPE-O-E

The cell uptake behavior of PLL-TPE-O-E FONs was conducted with a confocal laser scanning microscope (CLSM) Zeiss 710 3-channel (Zeiss, Germany) at 405 nm excitation wavelength. After HeLa cells were cultured in culture dish overnight with a density of 1×10^5 cells, cells were treated with PLL-TPE-O-E (20 µg mL⁻¹) for 3 h. Then cells were washed three times with phosphate buffered saline (PBS) to completely remove fluorescence nanoparticles that did not enter into cells. The cell uptake of PLL-TPE-O-E FONs was analyzed using CLSM and digital monochromatic images were acquired using Leica Confocal Software.

2.5 Cytotoxicity of PLL-TPE-O-E FONs

The toxicity effects of PLL-TPE-O-E on HeLa cells were further determined to evaluate their potential for biomedical applications [54-57]. Briefly, HeLa cells were seeded in 96-well tissue culture plates at a density of 5×10^4 cells/well in a final volume of 200 µl. After culturing for 24 h, cells were treated with 10, 20, 40, 80, 120 µg mL⁻¹ for 8 and 24 h respectively. Every sample was repeated three times. Then cells were washed with PBS for three times. Finally, HeLa cells were incubated in serum-free medium mixed with 20% MTS solution for 2 h at 37 °C. The absorbance was determined at 490 nm using a microplate reader (Sunrise, TECAN, Mannedorf, Switzerland). Cell viability rate was calculated as percentage compared to the control cells: (absorbance of the treated wells) / (absorbance of the control wells) × 100 %.

3. Results and Discussion

TPE is one of the typical AIE-active fluorogen, which has been widely utilized for biological imaging application because of ease synthesis and obvious AIE feature [44, 53, 58]. For instance, Tang et al has designed a series of probes based on TPE [59]. In this work, TPE was facilely conjugated with a biodegradable polymer PLL through the Michael addition reaction between the ene bond of TPE-O-E and the amino groups of PLL. The reaction can occur under rather mild environment and without using any toxic metal catalysts. The successful formation of the amphiphilic TPE based FONs was determined by

many characterization techniques. For example, the TPE-O-E FONs were characterized by ^1H NMR spectroscopy. As shown in **Fig. S1**, the peaks with chemical shift around 7.39-7.02 ppm can be ascribed to the protons of benzyl. On the other hand, the peaks with chemical shift at 6.25 and 5.84 ppm can be attributed to the protons of carbon-carbon double bonds (C=C). The ^1H NMR spectrum indicated the successful formation of TPE-O-E. After reacted with PLL through Michael addition reaction, the peaks with chemical shifts at $\delta = 4.5$ ppm, 1.53 ppm and 1.24 ppm was found in the sample of PLL-TPE-O-E, which can be attributed to the main chain protons of PLL. On the other hand, a single peak with chemical shift around $\delta = 3.4$ ppm that due to methylene group of the side chain connected with benzyl ($-\text{CH}_2-\text{COO}-$) was appeared showed in **Fig. 1A**. More importantly, expect from the characteristic signals of benzyl, the typical peaks of C=C were disappeared in the sample of PLL-TPE-O-E. All these manifested PLL was covalently conjugated with TPE-O-E and PLL-TPE-O-E FONs were successfully synthesized. The structure of PLL-TPE-O-E was further confirmed by FT-IR (**Fig. 1B**). The spectrum of TPE-O showed the presence of O-H (ν O-H at 3504 cm^{-1}). Four peaks at $1668\text{-}1390\text{ cm}^{-1}$ due to stretching vibration of various benzyls were also found. After reacted with acryloyl chloride, strong bending vibration of $-\text{C}=\text{O}$ at about 1740 cm^{-1} and the characteristic peaks of $-\text{C}-\text{O}-$ at 1027 cm^{-1} were observed in the samples of TPE-O-E. And the typical peaks of $-\text{CH}=\text{CH}_2$ was found at 966 cm^{-1} . The FT-IR spectrum of PLL-TPE-O-E exhibits PLL absorption features, such as N-H (ν N-H at 3352 and 3519 cm^{-1}). Especially, the characteristic peak of C-N stretching vibration was observed at 1361 cm^{-1} . These results further confirmed that the PLL has been covalently conjugated with TPE-O-E successfully. According to the ^1H NMR spectrum, the molar ratio of NH_2 groups to AIE dye is 4.03:1. Therefore, there still have a number of free NH_2 groups existed in PLL-TPE-O-E FONs. It was noteworthy that the presence of free NH_2 groups has potential usefulness for the further conjugation reaction. Furthermore, amino groups can be used for carrier drugs and genes for therapeutic applications. Therefore, the successful formation of PLL-TPE-O-E FONs with core-shell structure has great potential to be used in biological areas.

Fig. 1 (A) ¹H NMR spectrum of PLL-TPE-O-E. (B) FT-IR spectra of TPE-O-E and PLL-TPE-O-E.

The UV-vis absorption spectrum of PLL-TPE-O-E in water was showed in **Fig. S2**. There was a maximum absorption peak at 280 nm due to the π - π^* electronic transitions of benzene ring. Absorption band was observed in the visible region because of the scattering peculiarity of particles. Besides, the narrow absorption peak width indicates that the distribution of particle was homogeneous. When irradiated by UV lamp ($\lambda = 365$ nm), PLL-TPE-O-E FONs emit strong blue luminescence in water (left cuvette of insets in **Fig. 2A-a**). Because the hydrophobic TPE was aggregated at the core of nanoparticles in water, and then present the character of AIE properties. With the hydrophilic PLL as corona to improve dispersible stability of the nanoparticles in water, the core-shell structure of PLL-TPE-O-E showed high water dispersibility (right cuvette of insets in **Fig. 2A-b**). Furthermore, the plentiful of amino groups on PLL can be further functionalized with temperature and pH responsive components. So the PLL-TPE-O-E FONs can be potentially applied in bioimaging and therapeutic agent delivery. The photoluminescence (PL) properties of PLL-TPE-O-E FONs in water were further determined by PL spectra (**Fig. 2A**). There were two excitation peaks, which were mainly located at 292 nm and 347 nm, respectively. The broad and multiple excitation was also reported by Zhang et al in previous reports [60-62]. The maximum emission peak was located at 447 nm that can exhibit strong blue fluorescence when these AIE dye based nanoparticles were irradiated by UV lamp. However, when PLL-TPE-O-E FONs were excited by different excitation wavelength (from 285 to 356 nm), the emission wavelength didn't change, which indicated that the emission wavelength of PLL-TPE-O-E FONs is independent of the excitation wavelength. What is

strikingly noticeable is the broad excitation wavelength and narrow emission wavelength. Based on this, various fluorescent materials can be exhibited by one excitation wavelength and present different fluorescence, which has great applied potential in fluorescence labeling. Besides, there was no obvious fluorescence bleaching was observed after these FONs were irradiated by a UV lamp at 365 nm for 1 h (**Fig. 2B**), which suggested the fluorescence was very stable and suitable for long-term biological imaging application. Furthermore, the quantum yield (QY) of PLL-TPE-O-E FONs was also calculated using quinine sulfate as the standard reference dye. We demonstrated that the relative QY of PLL-TPE-O-E FONs was as high as 25.6%. All of these results suggested that PLL-TPE-O-E FONs possess strong luminescence, high photostability and good water dispersibility, making these AIE-active nanoprobe promising for biological imaging applications.

Fig. 2 (A) fluorescence excitation (EX) and emission (EM) spectra of PLL-TPE-O-E ($\lambda_{Em} = 365$ nm, $\lambda_{EX} = 285$ nm, 356 nm). (B) fluorescence emission (EM) spectra of PLL-TPE-O-E FONs before and after these AIE dye based nanoparticles were irradiated by UV lamp for 1 h. Insets were the fluorescent and visible images of such FONs in water.

Due to the amphiphilic properties of PLL-TPE-O-E copolymers, they are expected to self-assemble into nanostructures in aqueous solution. In this work, the size and morphology of PLL-TPE-O-E FONs was first investigated by TEM (**Fig. 3**). The TEM image reveals that PLL-TPE-O-E FONs were spherical with 400-600 nm in diameter. The formation of spherical evidenced that PLL was conjugated with TPE-O-E successfully. Among them, the hydrophobic TPE-O-E was encapsulated in the core of FONs, while the PLL with plentiful of residual amino groups was coated on the surface of these FONs. Furthermore, the hydrodynamic size distribution of PLL-TPE-O-E FONs was demonstrated by dynamic laser scattering (DLS). The results showed that the size of PLL-TPE-O-E FONs 633.59 ± 204.6 nm with relative broad dispersible polydispersity index (PDI = 0.256). As compared with the TEM characterization, the size of PLL-TPE-O-E

FONs is comparable but shows large by DLS characterization. This difference should be ascribed to the shrinkage of nanoparticles during the preparation of samples for TEM characterization.

Fig. 3 TEM image of PEI-TPE-O-E FONs dispersed in water, scale bar = 2 μm .

Biocompatibility of PLL-TPE-O-E FONs is critical for its potential application in bioimaging. MTS was adapted to investigate the cytotoxicity of PLL-TPE-O-E by studying the metabolic viability of HeLa cells. **Fig. 4** showed the cell viability of PLL-TPE-O-E after they were co-cultured with HeLa cells for 8 and 24 h. The concentrations of PLL-TPE-O-E FONs were ranged from 10-120 $\mu\text{g mL}^{-1}$. It can be seen that the cell viability showed no obvious decrease after cells were incubated with different concentrations of PLL-TPE-O-E FONs for upto 24 h. Even the concentrations of PLL-TPE-O-E FONs were as high as 120 $\mu\text{g mL}^{-1}$, the cell viability value remains greater than 95% after 24 h incubation. These results demonstrated that PLL-TPE-O-E FONs have excellent cytocompatibility. On the other hand, there were no obvious morphology change, and the cells showed better transmittance, good adhesion and smaller cytoplasmic that was similar to control group, which means PLL-TPE-O-E can be used for fluorescence imaging.

Fig. 4 Cell viability evaluation through MTS assay. No cell viability decrease was found after cells were incubated with different concentrations of PLL-TPE-O-E FONs for upto 24 h. These results clearly confirmed the good biocompatibility of PLL-TPE-O-E for biomedical applications.

The cellular uptake behavior of PLL-TPE-O-E FONs was investigated by CLSM. As shown in **Fig. 5**, strong luminescent signal was observed at the cell location after incubated with $20 \mu\text{g mL}^{-1}$ of PLL-TPE-O-E FONs for 3 h and no significant background interference was detected. Due to the existence of a number of amino groups on the surface of PLL-TPE-O-E FONs, the PLL-TPE-O-E FONs can be facilely uptake by cells. On the other hand, after the PLL-TPE-O-E FONs were absorbed by cells, the morphology of cells has not significant change. This further evidenced the good biocompatibility of these AIE nanoprobes. Because of large numbers of amine outsides of the PLL-TPE-O-E FONs, this can induce cationic surface charge of nanoparticles. For example, the Zeta potential value of the final PLL-TPE-O-E FONs in PBS is 21.5 ± 2.4 eV. This clearly indicated that PLL could endow the good dispersibility and capability of direct encapsulation of biological active agents such as DNA for disease treatment. The nanoparticles with cationic surface charge can easily interact with anionic cell membrane, and then were internalized through endocytosis. Moreover, we can also find that many areas with relate weak fluorescent signal were surrounded by areas with strong fluorescent signal (**Fig. 5A** and **Fig. 5C**). The areas with weak fluorescence should be the location of cell nucleus. Therefore we speculate that PLL-TPE-O-E FONs are mainly distributed in the cytoplasm. It is well known that the size nucleus pore is less than 30 nm. And the

size of PLL-TPE-O-E FONs is as large as 400-600 nm. Therefore, these AIE-active nanoparticles are difficult to be internalized into cell nucleus directly. More importantly, as indicated by our previous results, there still remain many amino groups on the surface of PLL-TPE-O-E FONs. These free amino groups can be potentially used for subsequently conjugation reactions. For example, these amino groups can directly link with targeting agents such as folate acid, which can specifically recognize with some cancer cells.[63-65] And these targeting agents modified AIE active nanoparticles could display specific internalization capability towards cancer cells. Considered the outstanding optical properties, good colloid stability and desirable biological properties, we can expect that these AIE active nanoparticles should be of great potential for biological imaging and bioactive agents' delivery applications.

Fig. 5 CLSM images of HeLa cells which were incubated with $20\ \mu\text{g mL}^{-1}$ of PEI-TPE-O-E FONs for 3 h. (A) excited with 405 nm laser, (B) bright field and (C) merge image of A and B. Scale bar = 20 μm .

4 Conclusions

In this work, we have developed a novel strategy for synthesis of the amphiphilic biodegradable PLL-functionalized AIE nanoparticles through a facile Michael addition reaction. With AIE dye as the hydrophobic core and PLL as the hydrophilic shell, these amphiphilic polymers tend to self-assemble into AIE-active FONs in aqueous solution and present strong fluorescence. Cell viability evaluation and biological imaging results indicate that PLL-TPE-O-E FONs have less cytotoxicity and high cellular uptake. Furthermore, with large numbers of free active amino groups on PLL, which can be modified with many other functional components such as therapeutic agents (siRNA or drugs) and targeting agents. More importantly, the synthetic method to conjugated polymers with TPE-O-E was rather simple without catalysts and the whole process was carried out in air atmosphere using low temperature. Due to the mild reaction conditions, the Michael addition reaction is specifically for fabrication of biomolecules modified AIE nanoprobe and still kept their structure and biological activity. Therefore, multifunctional amphiphilic FONs can also be easily fabricated for bioimaging applications using the same method, which are

promising for various biomedical applications.

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