One-step synthesis, self-assembly and bioimaging applications of adenosine triphosphate containing amphiphilics with aggregation-induced emission feature

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Abstract

Amphiphilic molecules with aggregation-induced emission (AIE) characteristics have attracted intensive interest for biological imaging applications for their self-assembly into nanostructures and obvious enhanced fluorescence intensity in aqueous solution. Although many AIE-active fluorescent organic nanoparticles (FONs) have been fabricated recently, the direct linkage of hydrophilic small molecules and hydrophobic AIE dyes has rarely been reported. In this work, we reported a one-pot strategy for preparation of adenosine triphosphate (ATP) containing molecules that conjugated the amino group of ATP and aldehyde-terminated AIE dye (PhCHO) based on mercaptoacetic acid locking imine (MALI) reaction. These AIE-active ATP-PhCHO showed amphiphilic properties and could self-assemble into micelles, which displayed high water dispersibility, strong yellow fluorescence, good biocompatibility and biological imaging capability. These advantages make ATP-PhCHO FONs promising for biomedical applications.

Key words: biological imaging, aggregation-induced emission, self-assembly, biocompatibility

1. Introduction

Fluorescent organic nanoparticles (FONs) have received a great deal of attention for various biomedical applications since the fluorescent based imaging technique is highly simple, sensitive and low background noise.[1-8] The general route for the fabrication of FONs is synthesis of luminescent amphiphilics.[7, 9-17] As compared with fluorescent inorganic nanoparticles, FONs possess relative low toxicity, well designability of chemical structure/fluorescent properties and better biodegradability. However, most of FONs based on conventional organic dyes have some intrinsic drawbacks.[5, 6, 18-21] For example, the self-assembly procedure will result in aggregation of hydrophobic dyes in the core of FONs. This will lead to the decrease or quenching of fluorescence intensity because of the well-known aggregation caused fluorescence quenching (ACQ) effects of conventional organic dyes. Contrast with ACQ, the aggregation-induced emission (AIE) phenomenon was also found in some organic dyes, which emit more efficiency in their aggregation or solid state.[15, 22-28] The unique AIE feature makes the possible fabrication of ultra-bright FONs using AIE-active dyes.[23, 24, 29-31] Since the first report by Tang's group in 2001, AIE-active dyes have attracted extensive attention for biomedical applications.[32] Over the past several years, a large number of AIE-active dyes with different chemical structural characteristics have been discovered and utilized for fabrication of AIE-active FONs.[30, 31, 33-36] However, to the best of authors' knowledge, the fabrication of AIE-active amphiphilics through one-pot multicomponent reaction has not been demonstrated.

As we known, most researchers use biomacromolecules such as polyethylene glycol (PEG), dextran (Dex), polyethylenimine (PEI) etc. that possess high molecular weight as the hydrophilic parts to fabricate AIE-active FONs via traditional stepwise methods, which are laborious, time-consuming. While thus prepared AIE-active FONs may difficult to penetrate into the cytomembrane once they are applied to bio-imaging. Luckily, we herein first utilize ATP, a small biomolecule that as a direct source of life activity energy, to fabricate AIE-active ATP-PhCHO FONs via a novel 'one pot' mercaptoacetic acid locking imine (MALI) reaction, which was used to conjugate the amino group of ATP and aldehyde group of PhCHO using mercaptoacetic acid as the "lock" (**Scheme 1**). The 'one pot' strategy avoided complex separation and purification of intermediate products, which showed distinct advantages, including simplicity, high efficiency, universality and atom-economy. Besides, ATP, as a universal energy storage molecule in all living organisms, plays an important role in regulation of cellular metabolism along with the fast degradation.[27-29] that will make the AIE-active ATP-PhCHO

FONs easier to enter into cytomembrane, suggesting that ATP-PhCHO FONs were more promising for biological imaging applications. In a word, we hope thus ‘one pot’ MALI reaction that utilize small biomolecules in living organisms will bring a new spring to fabricate more multifunction AIE-active FONs for various biomedical applications.

Scheme 1 Schematic showing the preparation of AIE-active ATP (ATP-PhCHO) FONs.

2. Experimental procedures

2.1 Materials and characterization

Adenosine 5'-triphosphate disodium salt, trihydrate ($\text{Na}_2\text{ATP} \cdot 3\text{H}_2\text{O}$) was purchased from Sangon Biotech (Shanghai, China) Co., Ltd. Mercapto acetic acid, Phenothiazine, sodium hydride, 1-Bromohexadecane, N,N-dimethylformamide, phosphoryl chloride, anhydrous THF, Dichloromethane (DCM) were all purchased from Aladdin company (Shanghai, China). All chemicals were of analytical grade and were used as received without any further purification.

^1H NMR spectra were recorded on Bruker Avance-400 spectrometer with D_2O and CDCl_3 as the solvent. The synthetic molecules were characterized by Fourier transform infrared spectroscopy (FT-IR) using KBr pellets, The Fourier transform infrared (FT-IR) spectra were supplied from Nicolet5700 (Thermo Nicolet corporation). Transmission electron microscopy (TEM) images were recorded on a Hitachi 7650B microscope operated at 80 kV, the TEM specimens were got by putting a drop of the nanoparticle ethanol suspension on a carbon-coated copper grid. The fluorescence data was obtained from the Fluorescence spectrophotometer (FSP, model: C11367-11), which purchased from Hamamatsu (Japan). The absolute fluorescent quantum yields were analyzed using the fluoroSENS instrument (Gilden Photonics). The

2.2 Fabrication of AIE-active ATP amphiphilics via a ‘one pot’ method

The PhCHO was synthesized according to our previous work.[37] The luminescent AIE-active ATP amphiphilics containing an AIE dye (PhCHO) and water soluble ATP were prepared via a facile and atom-economical one-pot ‘MALI’ reaction.[38] As shown in **Scheme 2**, typically, PhCHO (270 mg, 0.60 mmol), Na₂ATP·3H₂O (453 mg, 0.75 mmol) were charged into a dry schlenk flask with a suitable proportion between THF and water, the mixture was stirred for 2 h. Then mercaptoacetic acid (386 mg, 4.2 mmol) was added into the schlenk flask and reacted for next 2 h. The mixture was poured into saturated brine and then extracted with ethylacetate for 3 times. The organic layer was concentrated using a rotary evaporator, then the resulting product was dried under vacuum at 45 °C.

Scheme 2 Schematic showing the synthetic of amphiphilic AIE-active ATP-PhCHO.

2.3 Cytotoxicity evaluation of ATP-PhCHO FONs

The cell viability of AIE-active ATP-PhCHO FONs on HeLa cells and PC12 cells was examined by cell counting kit-8 (CCK-8) and MTS assay, respectively.[39-42] The HeLa cells were seeded into 96-well flat culture plates with the density of 5×10^4 cells per well and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified incubator for 24 h. After culturing overnight, the cells were washed with FBS-free Dulbecco's modified Eagle medium (DMEM) and incubated with a specific concentration of AIE-active ATP-PhCHO FONs in a FBS-free culture medium at 37 °C for 12 and 24 h. The cells were then washed with phosphate buffered saline (PBS) three times. 10 mL of CCK-8 dye and 100 mL of DMEM cell culture medium were added to each well and incubated for another 4 h at 37°C. The plates were shaken for 10 min, and the absorbance was measured by microplate reader (Victor III, Perkin-Elmer) at 450 nm. The percentage of cell viability was calculated relative to negative control (media alone). Three replicates were performed for each sample and the mean values were used as the final data.

2.3 Confocal microscopic imaging

Confocal microscopic imaging of HeLa cells using AIE-active ATP-PhCHO FONs were conducted with a confocal laser scanning microscope (CLSM) Zeiss 710 3-channel (Zeiss, Germany), with the excitation wavelength was set as 405 nm. HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2mM glutamine, 100U mL^{-1} penicillin, and 100 $\mu\text{g mL}^{-1}$ of streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. For imaging experiments, exponentially growing cells (at a density of 1×10^5 cells per dish) were seeded in 24-well plate. Cells were cultured with AIE-active ATP-PhCHO FONs with an eventual concentration of 40 $\mu\text{g mL}^{-1}$ at 37°C in a 5% CO_2 atmosphere for 24 h before they were exposed to reagents. Later, the cells were washed three times with PBS to remove the AIE-active ATP-PhCHO FONs then fixed with 4% paraformaldehyde for 10 min at room temperature.

3. Results and discussion

The successful synthesis of ATP-PhCHO was determined by ^1H NMR spectra. As compared with ^1H NMR spectra of $\text{Na}_2\text{ATP} \cdot 3\text{H}_2\text{O}$ and PhCHO, the peaks with chemical shift at 6.0 and 10.0 ppm in ATP-PhCHO were disappeared. These peaks could be ascribed to the ^1H NMR signals of $-\text{NH}_2$ of $\text{Na}_2\text{ATP} \cdot 3\text{H}_2\text{O}$ and $-\text{CHO}$ of PhCHO, respectively. Moreover, the peaks between 8.5-6.5 ppm were also found in ATP-PhCHO, indicating that aromatic rings were introduced ATP-PhCHO. Besides, the detailed chemical shift position of H from ATP-PhCHO in D_2O was marked in the spectra (Fig. S1). From Fig. S1, we could conclude that the ATP-PhCHO has been successfully synthesized. Therefore, we could deduce that PhCHO has reacted with ATP successfully via MALI reaction based on ^1H NMR spectra.

Fig. 1 ^1H NMR spectra of $\text{Na}_2\text{ATP}\cdot 3\text{H}_2\text{O}$ in D_2O , PhCHO in CDCl_3 and ATP-PhCHO in D_2O . Inset figure is the amplified ^1H NMR spectrum of ATP-PhCHO .

The molecular structure of ATP and ATP-PhCHO was further verified by FT-IR spectra. As shown in **Fig. 2**, the new stretching vibration peaks at 2858 and 2929 cm^{-1} ascribed to the $-\text{CH}_2$ and $-\text{CH}_3$ groups of alkyl chain were clearly observed in ATP-PhCHO . This indicated that PhCHO was successfully introduced in ATP-PhCHO because $\text{Na}_2\text{ATP}\cdot 3\text{H}_2\text{O}$ is absent of alkyl chain signals. On the other hand, a series of peaks distributed from 1560 to 1450 cm^{-1} can be ascribed to the stretching vibration of aromatic rings. Moreover, the new stretching vibration peak located at 1691 cm^{-1} can be assigned to $-\text{N-C=O}$. Meanwhile, a new absorption peak at 1283 cm^{-1} attributed to $-\text{C-S}$ was also appeared. More importantly, the strong peak at 1191 cm^{-1} assigned to the $-\text{P=O}$ stretching vibration in PPi backbone, indicating the presence of ATP. Therefore, based on the FT-IR spectra, we can confirm successful synthesis of amphiphilic ATP-PhCHO .

Fig. 2 FT-IR spectra of $\text{Na}_2\text{ATP}\cdot 3\text{H}_2\text{O}$ and ATP-PhCHO.

Because of its amphiphilic properties, ATP-PhCHO was prone to self-assemble and form nanoparticles, which displayed high water dispersity and strong yellow fluorescence in pure aqueous solution. The size and morphology of ATP-PhCHO assemblies was characterized by TEM. As shown in (Fig. S2), well-defined aggregates were clearly observed with uniform diameter in about 100 nm and the size distribution of the vesicles is narrow, evidencing the amphiphilic ATP-PhCHO was self-assembled into nanostructures. Moreover, thus prepared ATP-PhCHO FONs presented well stability in H_2O and PBS. As shown in (Fig. S3), after they were placed 60 min, the FONs were still stable in water and PBS, even the time was up to 90 min. No obvious aggregates were found by optical photographs from natural light and UV light irradiation. The optical properties ATP-PhCHO FONs were evaluated by UV and fluorescent spectroscopy. Two absorption peaks were distinctly observed in the UV-Vis spectrum of ATP-PhCHO (Fig. 3A). The absorption peak at 205 nm is attributed to the $\pi \rightarrow \pi^*$ transition of $\text{C}=\text{C}$ of aromatic rings E2 band absorption. While the absorption peak at 260 nm is assigned as the band II absorption of aromatic rings, which were conjugated with -SR group. This clearly implied that MALI reaction was successfully occurred. PL spectroscopy of ATP-PhCHO FONs in water was implemented to quantitatively confirm their excitation and emission wavelengths. As shown in Fig. 3B, the maximum emission wavelength of ATP-PhCHO FONs was located at 538 nm, while their maximum excitation wavelength was located at 405 nm. Due to the aggregation of PhCHO in the nanoparticles, ATP-PhCHO FONs formed by covalent bond also showed strong yellow fluorescence in water (right cuvette in Fig. 3B), which was a typical AIE property. As shown in Fig. S4,

the fluorescence intensity of PhCHO in the solutions with different ratios of THF/H₂O are significant difference. It could be observed that fluorescence intensity of PhCHO was first decreased until reached the lowest value when the ratio of THF/H₂O was 50:50, then increased with the increase of water fractions (**Fig. S4B**). Afterwards, the fluorescence intensity was enhanced dramatically when the fraction of water was further increased. The above results indicated that PhCHO was an AIE-active dye. On the other hand, the AIE characteristic of ATP-PhCHO FONs was also evaluated using the similar method. As displayed by **Fig. S5**, the ATP-PhCHO FONs also exhibited AIE feature. The AIE feature made ATP-PhCHO FONs intensive emitting in pure aqueous solution and promising for biological imaging application. Besides, the fluorescence stability of ATP-PhCHO FONs has also examined. After the ATP-PhCHO suspension was continuously irradiated using UV lamp at 365 nm for 2 h, the maximum emission fluorescent intensity values were decreased from 8983.7 to 8257.8, suggesting ATP-PhCHO FONs possess excellent fluorescence stability (**Fig. S6**). The quantum yield was also determined before and after irradiation 2 h. As shown in **Fig. S7**, the quantum yield was obtained 2.19%, 2.09%, respectively before and after irradiation 2 h via calculated by integration. So we concluded that thus prepared ATP-PhCHO FONs exhibited favorable photostability.

Fig. 3 (A) UV-Vis spectrum of AIE-active ATP-PhCHO FONs; (B) fluorescence excitation (Ex) and emission (Em) spectra of ATP-PhCHO FONs. The insets are the photographs of ATP-PhCHO FONs water suspension with and without UV lamp irradiation.

It is well known that the CMC problem often limits the application of nanoparticles in dilute solutions. To overcome the issue of CMC, the change of the maximum fluorescence emission intensity under different aggregated states of ATP-PhCHO FONs in water was used to track the CMC value. As shown in **Fig. S8**, the FONs show weak fluorescence in the aqueous solution when the concentration is

low. While increasing the concentration of ATP-PhCHO FONs to above its CMC, the fluorescence is enhanced significantly due to the hydrophobic PhCHO core aggregated in the micelles. Therefore, the intensity of the fluorescence emission vs. the logarithm of the concentration of ATP-PhCHO FONs was plotted to determine the CMC by the tangent method, which showed an ultra-low CMC value of ATP-PhCHO FONs as $0.0046 \text{ mg mL}^{-1}$. Considering the molecular weight of ATP-PhCHO was low and the particle size from TEM, the resulting ATP-PhCHO FONs are greatly beneficial to cell imaging. However, the surface charge will affect particles uptake by the cells, so we should study the Zeta potential of the formed ATP-PhCHO FONs before being utilized against cells. As displayed by **Fig. S9**, the Zeta potential of optimized ATP-PhCHO FONs was noticed to be -50 mV , which also suggested that the negative ATP have successfully conjugated with PhCHO and endow the final ATP-PhCHO FONs dispersity in aqueous solution. This amount of zeta potential indicated monodispersity and stability of the synthesized nanoparticles due to the electrostatic repulsion between particles in the solution, which demonstrating thus prepared ATP-PhCHO FONs was promising for biological imaging application.

The cytotoxicity of AIE-active ATP-PhCHO FONs against HeLa cells was investigated after 12 and 24 h incubation using a cell counting kit-8 (CCK8) assays. As shown in **Fig. 4**, the cell viability of HeLa cells was slightly reduced as the increase of nanoparticle concentrations or elongation of the incubation time. However the cell viability values were still obviously greater than 90%. Even the concentration of these FONs was up to $120 \text{ }\mu\text{g mL}^{-1}$. This confirmed that tiny AIE-active ATP-PhCHO FONs had good biocompatibility and low cytotoxicity. To further tested toxicity of such FONs on cells, we used PC12 cells to examine the cell viability of AIE-active ATP-PhCHO FONs by MTS assay. As shown in **Fig. S10**, the cell viability of PC12 cells was slightly reduced as the increase of nanoparticle concentrations. Furthermore, even the concentration of these FONs was up to $120 \text{ }\mu\text{g mL}^{-1}$, the cell viability values were still nearly 100%. Both of them confirmed that AIE-active ATP-PhCHO FONs had good biocompatibility and low cytotoxicity, which suggested that ATP-PhCHO FONs were promising for biological imaging applications.

Fig. 4 Cell viability evaluation of ATP-PhCHO FONs. HeLa cells were incubated with different concentrations of ATP-PhCHO FONs for 12 and 24 h.

The cell uptake behavior of ATP-PhCHO FONs was investigated using CLSM. As shown in **Fig. 5A**, HeLa cells were dyed with ATP-PhCHO FONs when cells were excited with 405 nm laser. The ATP-PhCHO FONs stained cytoplasm of HeLa cells with strong yellow fluorescence signals can be clearly observed; however, the fluorescence intensity in the nucleus region is relatively weak. It indicates that such AIE-active ATP-PhCHO FONs can readily enter into the cell membranes and bring about fast fluorescence staining, demonstrating that AIE-active ATP-PhCHO FONs were mainly distributed in cytoplasm but could not enter in cell nucleus. **Fig. 5B** showed the bright field CLSM image of HeLa cells. It can be seen that HeLa cells were well attached on the cell plates with normal morphology, indicating that ATP-PhCHO FONs possess good biocompatibility. **Fig. 5C** shows the merged CLSM image, we can find that fluorescent signals are exactly overlapped with locations of HeLa cells. This implied that ATP-PhCHO FONs can be internalized by HeLa cells through non-specific endocytosis. Therefore, we believe that ATP-PhCHO FONs should be promising candidates for bio-imaging applications and detection a specific protein.[43-46]

Fig. 5 CLSM imaging of HeLa cells incubated with $40\ \mu\text{g mL}^{-1}$ of AIE active ATP FONs for 3 h. (A) bright field, (B) fluorescent imaging under the excitation with a 405 nm laser, (C) merged image of A and B. Scale bar = 20 μm .

4. Conclusions

In summary, AIE-active amphiphilics were fabricated via a one-pot MALI reaction based on the conjugation of PhCHO and ATP. The final ATP-PhCHO FONs showed enhanced hydrophilic, photostability, biodegradability and low cytotoxicity and biological imaging performance. Considered these advantages, we expect that this AIE-active amphiphilics will have a critical application in the fields of biological and biomedical processes, therapeutic agents, and medical diagnostics.

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A simple one-pot multicomponent reaction has been developed for the fabrication of fluorescent organic dyes with aggregation-induced emission feature via direct conjugation of organic dye and adenosine triphosphate