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Robust Colloidal Nanoparticles of Pyrrolopyrrole Cyanines J-Aggregates with Bright Near Infrared Fluorescence in Aqueous Media: From Spectral Tailoring to Bioimaging Application

Cangjie Yang¹, Xiaochen Wang¹, Mingfeng Wang*, Keming Xu, Chenjie Xu

Abstract: We report a new class of colloidal nanoparticles called JAGG containing near-infrared fluorescent J-aggregates of pyrrolopyrrole cyanines (PPcys) stabilized by amphiphilic block copolymers in aqueous media. The formation of such JAGG can be tuned by the chemical structure of PPcys, the concentration of the chromophores inside the polymeric nanoparticles and ultrasonic treatment. These JAGG nanoparticles exhibit a narrow emission band at 773 nm, a fluorescence quantum yield comparable to that of indocyanine green, and significantly enhanced photostability that is ideal for long-term bioimaging.

Introduction

Fluorescence imaging in vivo in the near-infrared (NIR) window is important to enable deep tissue penetration with high optical contrast.¹ To date there are only two clinically approved NIR fluorophores, indocyanine green (ICG) and methylene blue (MB), both of which are small molecules that can be rapidly excreted during circulation in physiological systems.² In contrast to small molecular imaging probes, fluorescent nanoparticles (NPs) with sizes in the range of 10-100 nm usually show prolonged intracellular retention and enhanced permeation retention (EPR) effect,³ making them suitable for long term cell tracking⁴ and tumor targeting.⁵ One common strategy to prepare fluorescent colloidal NPs is to encapsulate the molecular fluorophores in a polymer matrix. However, the significant fluorescence (FL) quenching caused by the intermolecular aggregation of most organic fluorophores within NPs remains a challenge for bioimaging, with an exception of a group of special chromophores with strong emission in aggregated state [⁶] and a series of fluorescent dyes covalently tethered with polymers.⁷,⁸

Since the first discovery in 1936 by Jelley and Scheibe,⁹ J-aggregates (also called Scheibe aggregates), which exhibit strongly red-shifted light absorption and a nearly resonant fluorescence, have attracted considerable attention for various applications.¹⁰ Such a resonant fluorescence band of J-aggregates with quite narrow bandwidth is highly desirable for simultaneous detection of multiple targets to minimize the spectral overlapping and interference. However, the bioimaging application of existing J-aggregates of dyes is limited by relatively low FL quantum yield, short emission wavelength and poor solubility in aqueous media. For instance, J-aggregates of ICG associated by van der Waals forces in water have been observed.¹¹,¹² But the colloidal stability of these aggregates without stabilization by surfactants was susceptible to changes of environmental factors such as concentration, ionic strength, and temperature. Recently, Zheng and coworkers¹³ reported that porphyrin J-aggregates formed in synthetic liposomes (also called porphysomes) showed dual photothermal and fluorescent properties for cancer imaging applications. Würthner and coworkers¹⁴ reported the J-type aggregation of H-bonding perylene bisimide derivatives with far-red fluorescence and high quantum yield, close to unity. But such self-assembly took place in nonpolar solvents which are not suitable for bioimaging.

Scheme 1 Schematic diagram of encapsulation of the PPcys derivatives into H-type and J-type aggregated nanoparticles (HAGG and JAGG): (a) molecular structures of PC, PCBF, PCBF and the routes to formation of JAGG and HAGG nanoparticles; (b-c) schematic representation of J-aggregate (b) and H-aggregate (c); (d) chemical structure of the amphiphilic block copolymer PCL-b-POEGMA.

Herein, we present colloidal NPs of J-aggregates (denoted as JAGG) in water with bright NIR fluorescence and narrow bandwidth for bioimaging applications. The key component of the JAGG is a brightly fluorescent molecule, PCBF (as shown in Scheme 1), a pyrrolopyrrole cyanine (PPcy) derivative. PPCys containing a pyrrolopyrrole unit as the central element of a cyanine-type chromophore with emission in the NIR region up to 1000 nm and higher quantum yields than those of other organic NIR dyes were previously reported by Ficsher and coworkers.¹⁵
To render the hydrophobic dye PCBF dispersible in water, a synthetic amphiphilic block copolymer PCL-b-POEGMA (M<sub>n</sub> = 31,100; M<sub>w</sub>/M<sub>n</sub> = 1.37) was employed to encapsulate PCBF into the hydrophobic core of NPs via a process of nanoprecipitation (Scheme 1).<sup>7c</sup> Both H-aggregation and J-aggregation of PCBF occur in the corresponding NPs. These NPs exhibited bright NIR fluorescence, which is mainly contributed by J-aggregating components, and robust photostability that is important for bioimaging application.

Results and Discussion

The synthetic route to PCBF is illustrated in Scheme 2, using a modified method reported by Fischer and coworkers.<sup>13a</sup> 3,6-Bis(4-octyldodecylxyloxyphenyl)-2,5-dihydropyrrolo[3,4-c]pyrrole-1,4-dione (Compound 3, Scheme 2) was prepared by using a condensation of disopropyl succinate and 4-octyldodecylbenzonitrile in the solution of potassium tert-butoxide and 2-methyl-2-butanol, where octyldodecyl branch group can provide DPP with a relatively good solubility. Subsequently, condensations of C-H acidic methylene groups from 2-cyanomethylpyridine at the carbonyl group of 3 proceeded in toluene with an excess of phosphoryl chloride to give compound PC, respectively. In order to stiffen the chromophores to activate NIR emission, trifluoroborane etherate was added to the dichloromethane solution of PC at the presence of Huning base to give PCBF with a quantitative yield.

The UV-Vis-NIR absorption and FL emission spectra of PCBF in CHCl<sub>3</sub> (Figure 1a) show all the intrinsic features of PPcy. The absorption maximum of monomeric PCBF appears at 700 nm (absorption coefficient: ε = 1.55×10<sup>5</sup> cm<sup>-1</sup>M<sup>-1</sup>, even higher than that of porphyria<sup>14</sup>) while the FL emission spectrum exhibits a maximum at 715 nm. Thus PCBF in CHCl<sub>3</sub> shows a Stokes shift of 15 nm and a quite high FL quantum yield of 0.69 using ICG in water (QY~0.01) as a standard. The emission band is relatively broad with full-width-at-half-maximum (FWHM) value of 6910 cm<sup>-1</sup> than that of absorption band (3849 cm<sup>-1</sup>) (Figure 1a). The optical properties of PCBF observed here are consistent with what was reported by Fischer and coworkers.<sup>13</sup>

In order to prepare colloidal NPs of PCBF in water, a mixture of PCBF and PCL-b-POEGMA codissolved in THF was rapidly injected into large excess water under ultrasonication, followed by evaporation of the organic solvent, resulting in colloidal stable NPs. Our previous work has demonstrated that the size of nanoparticles prepared by this nanoprecipitation method can be facilely tuned from 50 to 400 nm by varying the weight ratio of fluorophore/PCL-b-POEGMA.<sup>7c</sup> In this work, a dispersion of NPs based on PCBF was obtained at a weight ratio of PCBF/PCL-b-POEGMA at 3.5 and a concentration of PCBF at 0.4 mM. As shown in Figure 1c, roseberry-like spherical NPs
with an average diameter of 30.0 ± 2.5 nm were observed in the transmission electron microscopy (TEM) image of JAGG NPs (Figure 1c). Beneficial from the higher electron density of PCBF than the PCL-b-POEGMA matrices, dye aggregation inside the NPs gave a clear contrast of PCBF aggregates as darker dots in the grey matrices of the polymeric NPs. The hydrodynamic diameter of JAGG NPs measured by dynamic light scattering (DLS) is 47.5 nm (Figure 1d), which is larger than that from TEM result due to the hydration of POEGMA corona.

In striking contrast to the optical absorption of monomeric PCBF in CHCl₃, the JAGG NPs in water exhibit two new absorption bands as shown in Figure 1b. The blue-shifted band versus the monomeric band (M-band) appears as a typical signal of the formation of H-type and/or dimer aggregation (both are included as H-band here for simplification). The maximum of the H-aggregate absorption band (H-band) is centered at 647 nm. As compared to the M-band, another narrow and intense band in the absorption transition shows a 61 nm red-shift to 768 nm (Figure 1b), which is a spectral signature of ordered J-aggregates. Furthermore, the FWHM of J-band is narrower (2235 cm⁻¹) than that of PCBF in CHCl₃ (3849 cm⁻¹). This spectral-narrowing phenomenon is a known property of coherently coupled J-aggregates. It has been predicted that the absorption spectral width of the aggregate is narrower than that of the monomer by a factor N₁², where N is the number of monomers in direct communication with each other. The average J-aggregation number N calculated from the spectral narrowing of JAGG NPs is 7.4.

A control experiment was also conducted to monitor the formation of PCBF aggregates by varying the volume ratio of water/THF mixture in the absence of PCL-b-POEGMA. As shown in Figure S3a, with the gradual increase water fraction (f_w, by volume) from 0 to 40%, there is no significant change in the absorption spectra. Further increase of f_w to 50% leaded to the appearance of J-band, accompanied with a transition from transparent solution to suspension of aggregates which precipitated after standing overnight, implying the poor colloidal stability in the absence of the polymeric surfactant. The DLS histogram (Figure S3b) of the formed aggregated shows an average diameter of 10 μm, significantly larger than the size of the JAGG NPs stabilized by PCL-b-POEGMA. When f_w was increased to 60%, the J-band became stronger and sharper. The absence of H-band in the absorption spectra of the neat PCBF aggregates suggests the preferable formation of J-aggregation under the present experimental condition. The presence of the relatively high fraction of good solvent (i.e. THF) in the THF/water mixture might enable PCBF molecules to self-assemble to reach the more thermodynamically stable state, i.e. J-aggregation. In contrast, the aforementioned JAGG NPs were prepared by a rapid injection of a small amount of PCBF/THF solution (1 mL) into water (10 mL). The flash precipitation process may not allow all of the PCBF molecules to form J-aggregation immediately, resulting in the mixture of H-aggregates, J-aggregates and some PCBF monomers in JAGG NPs. In other words, the JAGG NPs described above represent a kinetically trapped or instantly frozen state in the nanoprecipitation process.
and FL emission intensity of ICG aqueous solution decreased quickly, accompanied by the color change of ICG solution from green to colorless (Figure 2a & 2b). In contrast, only negligible decrease of absorbance could be observed in JAGG solution under the same condition of irradiation (Figure 2c). After 55 min of continuous irradiation, the decrease of the FL intensity at 771 nm of JAGG solution was ~12% (Figure 2d), much lower than that (97%) at 805 nm of ICG solution, indicating relatively high photostability of JAGG NPs. The excellent photostability could be attributed to the intrinsic structural stability of PPcY[13a] and its isolation from unfavorable influences of the environment.[6b, 18]

Hence, these JAGG NPs with such a large size are preferable for long-term tracking due to the increase of circulation half-life and excellent photostability compared to water-soluble dyes.[18b, 19]

![Figure 2](image)

**Figure 2** The evolution of UV-Vis-NIR absorption spectra (a) and FL emission spectra (b) of ICG solution in water under continuous irradiation by a 770 nm laser for ~1h at a power density of 0.22 W/cm². UV-Vis-NIR absorption spectra (c) and FL emission spectra (d) of PCBF based JAGG dispersion under continuous irradiation by a 770 nm laser for ~1h at a power density of 0.22 W/cm². (e) NIR absorbance ratio A/A₀ of ICG solution and JAGG dispersion plotted as a function of irradiation time. A₀ is the initial absorption maximum and A is the absorption maximum of the sample at different time points after illumination. (f) NIR fluorescence intensity ratio A/A₀ of ICG solution and JAGG dispersion plotted as a function of irradiation time. F₀ is the initial fluorescence maximum and F is the fluorescence maximum of the sample at different time points after illumination.

The effect of the concentration of PCBF and the weight ratio of PCBF/PCL-b-POEGMA on the spectral characteristics were studied. We gradually decreased the feeding amount of PCBF when the amount of surfactant was kept constant. The concentration-dependent UV-Vis-NIR spectroscopy revealed that the J-aggregation in JAGG NPs was enhanced with the increase of the concentration of PCBF from 0.04 to 0.4 mM, accompanied by a gradually sharpening J-band and increasing ratio between J-band and H-band absorbance (Figure 3a & 3c). This result, as expected, indicates that formation of the J-aggregate was favoured by increased concentration of PCBF. 

With the increase of J-aggregate proportion, a slight 4 nm red-shift in the absorption from 764 to 768 nm was observed (Figure 3c). A shoulder peak at longer wavelength can be found in the J-aggregate absorption band of the JAGG dispersions at 0.04, 0.08 and 0.16 mM, respectively. Interestingly, the J-band in the absorption spectrum of the NPs at 0.24 mM exhibits two overlapping peaks at 766 and 771 nm, respectively (Figure 3c). This is a clear indication of the two overlapping bands of J-aggregation presumably due to two populations of J-aggregates with different geometrical configurations.[30] Evidently, with the increase of PCBF concentrations from 0.04 to 0.4 mM, the intensity of the emission band with λ_{max,em} at 773 nm gradually increased and the bandwidth turned narrower (Figure 3b), which is attributed to the increasing proportion of J-aggregation and configuration corresponding to the longer absorption wavelength.

![Figure 3](image)

**Figure 3** Concentration-dependent absorption spectra (a) and FL emission spectra (b) of PCBF based JAGG NPs upon the encapsulation of PCL-b-POEGMA. (c) Concentration-dependent normalized absorption spectra and zoom-in J-band variation of PCBF based JAGG NPs. C_{PCBF} corresponds to concentration of PCBF.

We envisioned that such unusual optical property of the J-aggregate of PCBF was encoded in its molecular structure: a planar conjugated core stiffened by difluoroborane group. To confirm this presumption, PC, the precursor of PCBF, was encapsulated by PCL-b-POEGMA to give colloidal dispersion as...
The optical properties of several kinds of J-aggregates based on supramolecular assembly have been reported to be tunable by changing the environmental temperature. In contrast, as shown in Figure S10, only a slight change around J-band was observed in the UV-Vis-NIR absorption spectra of JAGG dispersion (0.4 mM) with the gradually increased temperature from 25 to 60 °C.

We previously reported that ultrasoundation played a role to induce the colloidal crystallization of an indigo derivative. Here, the colloidal NPs of PCBFB were also treated with ultrasoundation. Upon the ultrasoundation treatment for 200 mins, the absorbance of J-band at 774 nm gradually increased, accompanied with the decrease of H-band absorbance (Figure 5a). The change can be observed with naked eyes, as the bluish solution of JAGG NPs turned green upon ultrasoundation (Figure 5a). These results clearly indicate the transition from H-aggregate to J-aggregate. Moreover, the J-band in the absorption spectra exhibits a red-shift of 6 nm (from 768 to 774 nm) upon ultrasoundation for 200 mins while the FL emission spectrum shows a λmax,em of 774 nm and negligible Stokes shift (Figure 5b & 5c). The FL quantum yield of JAGG NPs after ultrasoundation for 200 mins was increased to 0.025, consistent with the increase of J-aggregate population. It is noted that there was no obvious change of the morphology and the size of JAGG after the ultrasoundation (Figure 5d & Figure S11).

The optical properties of JAGG NPs described above, meeting most requirements of a FL imaging probe, encouraged us to examine their potential for in vivo imaging. Firstly, the cytoxicity of JAGG NPs against mouse fibroblast cells (L929) was studied by PrestoBlue assays. As summarized in Figure 6a, there was no obvious change of the metabolic viability of L929 cells after incubation with JAGG for 72 h at a series of concentrations of 0.01, 0.02, 0.05, 0.1, 0.2 mM, respectively,
indicating the low cytotoxicity of JAGG NPs. In in vivo experiment, subcutaneous and intramuscular injections of JAGG dispersions (50 µL aliquots, 1 mg/mL) into the left flank (position II in Figure 6b) and left leg (position III in Figure 6b) of a nude mouse, respectively, were administered. The mouse was imaged in a fluorescence mode (excitation filter, 605-640 nm, and emission filter, 740-800 nm). The FL signals collected from both injected regions were clearly observed and the FL intensity emanating from deeper tissue (ROI (III) = 9.856 × 10^10, ROI stands for regions of interest) is comparable with that of the near-skin fluorescence (ROI (I) = 1.029 × 10^9) (Figure S12). As a control, the HAGG dispersion of PCBFB was subcutaneously and intramuscularly injected to right flank (position IV) and right leg (position V), respectively (Figure 6b). Obviously, the weak fluorescence signals (ROI (IV) = 5.340 × 10^8; ROI (V) = 5.492 × 10^8) observed here were only slightly stronger than that of the blank (ROI (I) = 1.571 × 10^8), due to the quite low FL quantum yield of the PCBFB NPs.

Figure 6 (a) Metabolic viability of L929 cancer cells after incubation with JAGG NPs at different concentrations. (b) In vivo fluorescence image of JAGG and HAGG NPs (50 µL of 1 mg/mL) injected subcutaneously and intramuscularly on each flank of a mouse. Position I: blank control; II: JAGG NPs subcutaneous; III: JAGG NPs intramuscular; IV: HAGG NPs subcutaneous; V: HAGG NPs intramuscular.

Conclusions

In summary, we have presented a novel type of colloidal NPs based on the J-aggregation of PPcy dyes stabilized with a polymeric surfactant in water. The packing configuration of PPcy in NPs is determined by factors including the chemical structure, the concentration of the dye and the ultrasonication treatment. The outstanding spectroscopic properties of JAGG NPs, such as large light-absorption coefficient, relatively high FL quantum yield, as well as narrow emission band, enable them to be promising candidates for in vivo imaging in the near infrared range. These JAGG NPs may also be useful for multimodal imaging (fluorescence and photoacoustic) and therapeutic (photothermal therapy) applications.23

Experimental Section

Materials

10×phosphate buffer saline (PBS) buffer with pH = 7.4 (ultrapure grade) is a commercial product of 1st BASE Singapore. MilliQ water (18.2 MQ) was used to prepare the buffer solution from the 10×PBS stock buffer. 1×PBS consists of NaCl (137 mM), KCl (2.7 mM), Na2HPO4 (10 mM) and KH2PO4 (1.8 mM). Chlorofor-D (99%) was purchased from Cambridge Isotope Laboratories, Inc. All other chemicals and reagents were purchased from Aldrich or Merck and used as received unless other specified. Block copolymers PCL-b-POEGMA was obtained according to our previous work.74

Characterization

The samples were dissolved with chloroform-d for 1H NMR and 13C NMR measurements on a Bruker AV300 MHz NMR spectrometer. Emission spectra of solutions were measured by fluorescence spectrophotometry on a Horiba Fluolog 3 spectrophotofluorometer at 25 °C. The fluorescence quantum yield of these materials were measured using indocyanine green (ICG) solution in water as the standard (ΦF = 0.01). UV-Vis-NIR spectra of the samples were measured on a SHIMADZU UV-2450 spectrophotometer. The photostability of ICG solution and JAGG dispersion subjected to the continuous irradiation by a 770 nm laser (power density ~ 0.22 W/cm2) over different periods was monitored by the UV-Vis spectrometer and fluorometer. TEM measurements were performed with a TEM Carl Zeiss Libra 120 Plus at an acceleration voltage of 120 kV. A 5 µL droplet of diluted samples was directly dropped onto a copper grid (300 mesh) coated with a carbon film, followed by drying at room temperature. The size distribution of resulting nanoparticles were determined by dynamic light scattering (DLS) using a BI-200SM (Brookhaven, USA) with angle detection at 90°. Ultrasonic treatment of JAGG dispersion was performed in an ultrasonic cleaner (SB-120 DTN, power: 120 W) bath. Temperature-dependent variation on absorption spectra of JAGG dispersions was monitored by the UV-Vis spectrometer from 25 to 60 °C. Each temperature point was kept for 1 h to treat the sample.

Synthesis of Compound 1

Triphenylphosphine (26 g, 0.1 mol) was dissolved in dichloromethane (100 mL). Then, bromine (16 g, 0.1 mol) was added dropwise at 0 °C. After completion of adding bromine, the reaction mixture was stirred half an hour at room temperature. 2-octyl-1-dodecanol (30 g, 0.1 mol) was added slowly. The reaction mixture was stirred at room temperature overnight. Then the reaction mixture was poured into water (200 mL) and extracted with dichloromethane (3 × 50 mL). The extracts were combined and washed with water and saturate sodium bicarbonate solution then dried over anhydrous magnesium sulfate. After filtration, filtrate was concentrated to 100 mL under reduced pressure. Petroleum ether (200 mL) was added to the mixture and stirred. Resulting triphenylphosphine oxide precipitate was filtered off and filtrate was concentrated to 100
ml. Added another 100 mL petroleum ether and filtered. Repeated this operation till no precipitate observed, and the filtrate was evaporated to dryness. The product 2-octyl-1-dodecyl bromide was obtained in a yield of 92% and used in subsequent reactions without further purification.

**Synthesis of Compound 2**

4-Cyanophenol (1.19 g, 10 mmol) and potassium carbonate (5.52 g, 40 mmol) were dissolved in 60 mL acetone. After being purged for 15 min, the mixture was heated to 100 °C for half an hour. Then, 2-octyl-1-dodecyl bromide (4 g, 11 mmol) was added to the mixture by syringe. The reaction mixture was stirred at 100 °C for 30 h. After cooled to room temperature, the reaction mixture was added to 100 mL of water and extracted with ethyl acetate. The extracts were combined and washed with water then dried over anhydrous magnesium sulfate. After removing the solvent, the crude product was purified by flash chromatography (petroleum ether/chloroform/hexane from 10:0 to 10:1) to yield target product as a yellow viscous liquid in a yield of 90%. \(^1^H\) NMR (300 MHz, CDCl\(_3\)): 8.58 (2H, d), 6.97 (2H, d), 3.88 (2H, d), 1.81 (1H, m), 1.25-1.50 (32H, broad), 0.90 (6H, t).

**Synthesis of Compound 3**

Potassium tert-butoxide (1.68 g, 15 mmol) was added to 2-methyl-2-butanol 15 mL, and the mixture was heated to reflux. When the alkali is dissolved, Compound 2 (4 g, 10 mmol) was added in one portion. Then disopropyl succinate (1.01 g, 5 mmol) was added over three hours with a dropping funnel. After heated for another three hours at 110 °C, the mixture was cooled down and slowly added to a mixture of 100 mL ethanol (containing 2 mL concentrated hydrochloric acid). The red precipitate is filtered and washed with ethanol. The solid is digested in boiling ethanol, filtered and washed with ethanol. This procedure is repeated until the filtrate is clear. Drying in vacuum yields 2.1 g (24 %) orange solid. \(^1^H\) NMR (300 MHz, CDCl\(_3\)): 8.42 (2H, s), 8.26 (4H, d), 7.08 (4H, d), 3.96 (4H, d), 1.84 (2H, m), 1.25-1.50 (64H, broad), 0.90 (12H, m).

**Synthesis of Compound 4**

Compound 3 (0.88 g, 1 mmol) and 2-cyanomethylpyridine or 2-cyanomethylpyridine (0.295 g, 2.5 mmol) were heated to reflux in absolute toluene (20 mL) under nitrogen. Phosphoryl chloride (0.75 mL, 8 mmol) was then added. The reaction was monitored by thin-layer chromatography. As soon as 3 was used up, the reaction mixture was cooled down, quenched by water and alkalized by sodium bicarbonate solution. Water was separated and extracted with chloroform. The combined organic layer was dried over anhydrous sodium sulfate. After filtration, the volatiles were removed under reduced pressure. The crude product was purified by column chromatography on silica, eluting with dichloromethane/petroleum ether (from 1:5 to 1:1) to give Compound 4 as a bluish green solid (173 mg, 16%). \(^1^H\) NMR (300 MHz, CDCl\(_3\)): 13.6 (2H, s), 8.45 (2H, d), 7.65 (8H, m), 7.12 (4H, d), 7.01 (2H, t), 3.97 (4H, d), 1.84 (2H, m), 1.25-1.50 (64H, broad), 0.89 (12H, m).

**Synthesis of Compound 5**

Compound 4 (25 mg, 0.02 mmol) and N,N-diisopropylethylamine (0.2 mL, 1.2 mmol) were dissolved in dichloromethane (2 mL). Trifluoroborane etherate (0.2 mL, 1.6 mmol) was added and the mixture was stirred at room temperature for 2 hours. The reaction mixture was washed with water and dried over anhydrous sodium sulfate. After removing the solvent, the crude product was purified by column chromatography, eluting with dichloromethane to give Compound 5 as a green solid (26 mg, quantitative yield). \(^1^H\) NMR (300 MHz, CDCl\(_3\)): 8.36 (2H, d), 7.88 (2H, t), 7.65 (6H, m), 7.22 (2H, t), 7.08 (4H, d), 3.95 (4H, d), 1.83 (2H, m), 1.25-1.50 (64H, broad), 0.91 (12H, m).

**Synthesis of Compound 7**

5-bromo-2-cyanomethylpyridine was used to replace 2-cyanomethylpyridine as the raw materials. The procedure to synthesize 8 was similar with that of 2 and the similar yield was achieved. \(^1^H\) NMR (300 MHz, CDCl\(_3\)): 8.43 (2H, d), 7.91 (2H, d), 7.64 (4H, d), 7.55 (2H, d), 7.07 (4H, d), 3.95 (4H, d), 1.83 (2H, m), 1.25-1.50 (64H, broad), 0.91 (12H, m).

**Synthesis of Compound 8**

Compound 8 (133.5 mg, 0.1 mmol), phenylboronic acid (36.6 mg, 0.3 mmol) and K\(_2\)CO\(_3\) (1.38g) was dissolved in degassed mixture of toluene (10 mL), water (2 mL) and ethanol (2 mL) under nitrogen atmosphere. It is followed by addition of a catalytic amount (1% m/m) of Pd(PPh\(_3\)). The resulting mixture was heated at 100 °C for 6 h, then cooled down to room temperature. The organic layer was washed by water for three times and concentrated under reduced pressure. The product was purified by column chromatography on silica using dichloromethane/hexane (1:1) as the solvent and dried under vacuum at 80 °C overnight. Finally, we obtained 121.4 mg dark green powder (yield 91.3%). \(^1^H\) NMR (300 MHz, CDCl\(_3\)): 8.61 (2H, s), 8.09 (2H, d), 7.70 (6H, m), 7.49 (10H, m), 7.10 (4H, d), 3.96 (4H, d), 1.84 (2H, m), 1.25-1.50 (64H, broad), 0.90 (12H, m).

**Preparation of nanoparticles in the presence of surfactant as stabilizer**

Taking JAGG NPs with 0.4 mM as representative example, PCBF (200 µL×0.01M THF solution) and amphiphilic copolymer PCL-POEGMA (100 µL×6.67 g/L THF solution) were mixed in 200 µL of THF. This mixture was rapidly injected into 5 mL of deionized water under ultrasonication. THF was evaporated by exposure in open air overnight. Similarly, JAGG dispersions with various concentrations were obtained by the similar procedure.
Monitoring the aggregation of PCBF in mixture of THF/water in the absence of surfactant as stabilizer

An aliquot of PCBF (100 µL, 1 mg/mL in THF) was mixed with different amounts of THF (x mL), followed by a quick addition of a known volume of water (3-x mL) and mixed with gentle shaking. Through this process, a series of PDB dispersions were obtained with different water fractions (x = 0%, 10%, 20%, 30%, 40%, 50%, 60% by volume).

Cell culture and cytotoxicity study

L929 mouse fibroblast cells were cultured in folate-free Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum and 1% penicillin streptomycin at 37 °C in a humidified environment containing 5% CO2. Before experiments, the cytotoxicity of JAGG NPs against L929 cancer cells was evaluated by PrestoBlue (PB) assay. Briefly, L929 cells were seeded in 96-well plates (Costar, IL, USA) at an intensity of 5×104 cells mL-1. After 12 h incubation, the cells were exposed to a series of doses of JAGG NPs at 37 °C. After the designated time intervals, the wells were washed twice with 1×PBS buffer and 100 µL of freshly prepared PB solution in culture medium was added into each well. The PB medium solution was carefully removed after 1 h incubation in the incubator. The absorbance of PB at 570 nm and 600 nm was monitored by the microplate reader (Genios Tecan). Cell viability was expressed as the ratio of the percent PB reduction of the cells incubated with JAGG NPs suspension to that of the cells incubated with culture medium only.

In vivo fluorescence imaging

The care and use of laboratory animals were performed according to the approved protocols of the Institutional Animal Care and Use Committee (IACUC) at Nanyang Technological University, Singapore. 50 µL of JAGG solution of PCBFB and HAGG solution of PCBFB (1 mg/mL) were subcutaneously injected to the left or right side of flanks of 8-week-old NCr nude mice (Invivos Pte Ltd), respectively. Next, 50 µL of the same JAGG dispersion of PCBFB and HAGG dispersion of PCBFB were intramuscularly injected to left or right side of rear legs, respectively. The efficacy of dye as imaging agents was then examined using an IVIS SpectrumCT in vivo imaging system (PerkinElmer). The wavelengths of excitation and emission filters used were 605-640 nm, and 740-800 nm, respectively. The auto-fluorescence of the skin was removed using a spectral unmixing software.

Acknowledgements

MW is grateful to the funding support by a start-up grant (M4080992.120) of Nanyang Assistant Professorship from Nanyang Technological University, AcRF Tier 2 (ARC 36/13) from the Ministry of Education, Singapore. XCJ also acknowledges the financial support from NTU-Northwestern Institute for Nanomedicine (M4081502.F40). We thank Dr. Hongwei Duan for access to the fluorometer. We also thank Dr. Quan Liu and Mr. Xiang Li for the assistance in the use of the 770-nm laser.

Keywords: J-aggregates • Near-infrared • Fluorescence • Pyrrolopyrrole Cyanines • Bioimaging


A novel class of colloidal nanoparticles containing near-infrared fluorescent J-aggregates of pyrrolopyrrole cyanines (PPcys) exhibit a narrow emission band at 773 nm, a fluorescence quantum yield comparable to that of indocyanine green, and significantly enhanced photostability that is ideal for long-term bioimaging.

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Robust Colloidal Nanoparticles of Pyrrolopyrrole Cyanines J-Aggregates with Bright Near Infrared Fluorescence in Aqueous Media: From Spectral Tailoring to Bioimaging Application